Distribution and Microchemical Detection of Phenolic Acids, Flavonoids, and Phenolic Acid Amides in Maize Kernels

Alok Sen,[†] David Bergvinson,[‡] S. Shea Miller,[‡] Jeffrey Atkinson,[§] R. Gary Fulcher,[∥] and J. Thor Arnason[*,†]

Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5, Research Branch, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6, Department of Chemistry, Brock University, St. Catharines, Ontario, Canada L2S 3A1, and Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108

Because of the importance of phenolics in the resistance of maize kernels to pests, microspectrofluorometry was employed as a new method of mapping the location of these substances in maize kernels. Autofluorescence due to phenolic acids was detected mainly in the embryo, aleurone, and pericarp of maize kernel cross sections. Resistant maize types showed higher intensities of phenolic fluorescence but no unusual distributions. Borinic acid reagent enhanced fluorescence due to flavonoids in the aleurone layer, and Ehrlich's reagent revealed phenolic acid amide fluorescence in the embryo and aleurone. The localization of phenolic amines was confirmed by HPLC analysis.

Keywords: *Zea mays; ferulic acid; flavonoids; amines; fluorescence; microspectrofluorometry*

INTRODUCTION

There is considerable evidence that phenolics have an important role in plant defense (Harborne, 1976, 1980; Swain, 1977). In wheat, the presence of hydroxycinnamic acids in the pericarp and aleurone cell walls has been related to protection from phytopathogenic fungi (Fulcher et al., 1972; Fulcher and Wong, 1980). Recent studies on maize also implicate phenolic acids in maize kernels as important resistance factors against the maize weevil, *Sitophilus zeamais*, and the grain borer, *Prostephanus truncatus*, two economically important stored-product pests of maize (Serratos et al., 1987; Classen et al., 1990; Arnason et al., 1992).

Phenolics occur in high concentrations in most cereal brans, and several well-developed methods exist to both localize and identify these compounds within grains or tissues at different stages of the milling process [see Fulcher and Wong (1980), Fulcher (1982), Pussayanawin et al. (1988), and Irving et al. (1989)]. For example, in whole plant tissues, phenolic compounds fluoresce intensely upon near-UV excitation, and these characteristics have been exploited using fluorescence microscopy to localize these compounds (Fulcher et al., 1972; Fulcher and Wong, 1980; Smart and O'Brien, 1979; Fulcher, 1982; Serratos et al., 1987). More recently, microspectrofluorometry has been used to quantify phenolics in milling fractions and flour samples (Pussayanawin et al., 1988, Sen et al., 1991).

The diversity of phenolic substances in cereals is substantial when conjugated or bound forms of phenolic acids or flavonoids are considered. Phenolic acids are abundant in cereal cell walls primarily bound to hemicelluloses in forms such as 2-*O*-[5′-*O*-(*E*)-feruloyl-β-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) (Kato and Nevins, 1985; Fincher and Stone, 1986). Recent interest in these substances is due to their ability to form cross-links in cell walls through the production of dimers such as diferulate or truxillic acids (Eraso and Hartley, 1990). Phenolic acid amides (also known as phenolic amines) including feruloyl- and diferuloylputrescine and related *p*-coumaric acid or spermidine analogues have been detected in maize grain (Martin-Tanguy et al., 1982). Substances such as FAXX and diferulate contribute to mechanical resistance against insects due to the fortification and cross-linking they provide in cereal cell walls, while our recent studies suggest that the phenolic amines may be involved in antibiosis (Arnason et al., 1992).

Table 1. Resistance and Phenolic Levels in Maize Populations Used

accession	type	Dobie index[b]	phenolics (μg/g)
Oaxaca 130	Bolito[a]	1.78	2428
Oxaca 179	Zapalote[a]	14.09	1333
Sinaloa 35	Chapalote[a]	0	2563
Yucatan 7	Nal tel[a]	1.78	2293
Muneng	synthetic	6	2246
Ritchie	hybrid	14.09	1154

[a] Land race. [b] Dobie index of 0 is highly resistant to *Sitophilus zeamais*, and 15 is highly susceptible.

Because of the importance of these substances, further efforts were made in the present study to localize and quantify phenolic acids within a maize kernel on the basis of their autofluorescence characteristics. New quantitative imaging techniques based on microspectrofluorometry have been used to examine maize kernel sections in resistant and susceptible maizes. Specific stains adapted from reactions to visualize phenolics on chromatograms were used to assist in localization of flavonoids and amines, with confirming HPLC evidence from dissected tissue. The present paper provides the first definitive localization of phenolic acid amides in maize kernels.

MATERIALS AND METHODS

Maize Populations. Several maize populations used in the study were multiplied at the International Centre for Wheat and Maize Improvement, El Batan, Mexico, as part of a collaborative study on maize resistance to stored insect pests. Selections (Table 1) included land races and modern synthetics

* Author to whom correspondence should be addressed.

† University of Ottawa.

‡ Agriculture Canada.

§ Brock University.

∥ University of Minnesota.

0021-8561/94/1442-1879$04.50/0 © 1994 American Chemical Society

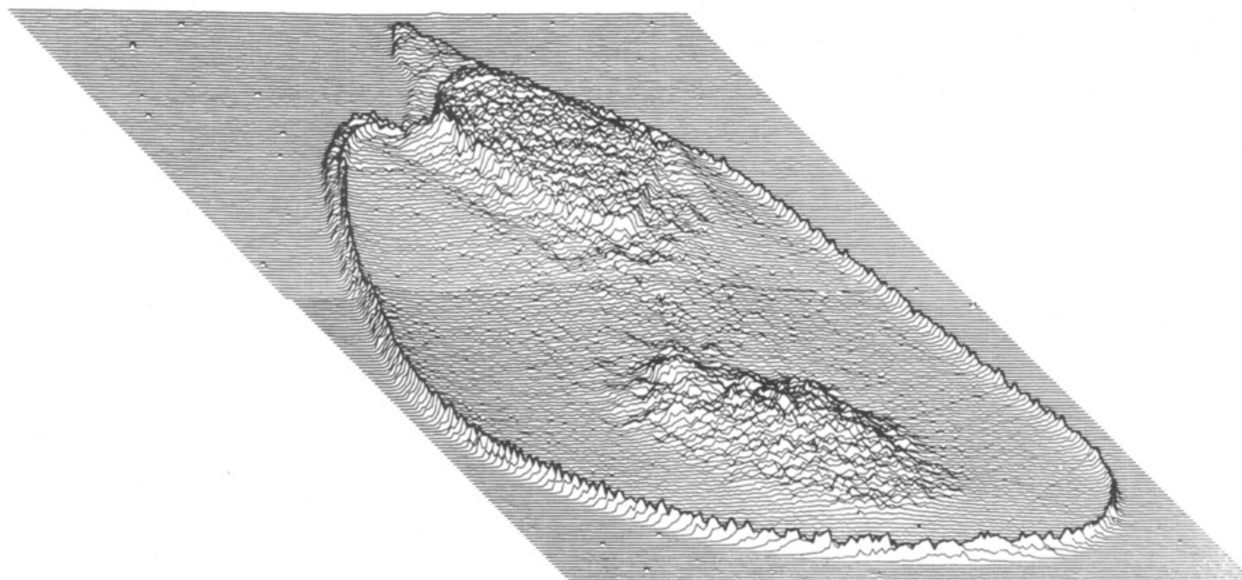

Figure 1. Microspectrofluorometric scan of a cross section of a kernel of Sinaloa 35 showing relative fluorescence intensity due to phenolics on the vertical axis.

and hybrids having various levels of resistance to stored insects as measured by the Dobie index (Classen et al., 1990) and total phenolics (Arnason et al., 1992).

Fluorescence Microscopy. Cryostat sections of imbibed but dry, ungerminated maize kernels were mounted on glass slides and observed either without further treatment or after staining by one of the following procedures: (a) 4-(Dimethylamino)benzaldehyde (DAB) in ethanol containing 1% concentrated HCl (Ehrlich's reagent) was prepared as described (White and Kennedy, 1981) and used as a fluorochrome to detect aminophenol residues. Stained sections were dried at 40–50 °C. (b) Diphenylborinic acid in 80% methanol was used to localize flavonoids in maize kernels as described previously (Serratos et al., 1987).

After staining, sections were observed on a Zeiss Universal microscope equipped with an IIIRS epi-illuminating condensor, an HBO 200-W mercury arc lamp, a 10× or 16× Neofluar objective lens, and a filter combination containing an exciter filter (λ_{max} > 365 nm) and barrier filter (λ_{max} > 418 nm) which is useful for ferulic acid fluorescence. Photomicrographs were obtained with high-speed (ASA 400) 35-mm film.

Microspectrofluorometry. Mature, dry kernels of maize were embedded in polyester resin, which was allowed to harden overnight and later sanded down to provide a uniform and smooth surface. To determine the distribution as well as relative differences in fluorescence intensity in terms of phenolic acids, flavonoids, and amides, the blocks containing maize kernels were cut to reveal internal tissues and scanned using APAMOS software (Zeiss) to generate the intensity profiles. Details of the microspectrophotometer and its use have been given previously (Irving et al., 1989). Briefly, the UMSP80 microspectrofluorometer was equipped with an epi-illuminating condensor, an HBO 100-W mercury arc lamp, and a 365/418-nm fluorescence filter combination (exciter filter g365, barrier filter, LP 420). APAMOS software was used to generate fluorescence intensity profiles. Standardization of the equipment was acheived using uranyl glass (UG1 standard, Carl Zeiss) with the intensity set at 60%. Blocks with a cut maize kernel were placed on the scanning stage and, using the software, four diagonal corners were marked, thereby giving a rectangular field. The field was then scanned with a 20-μm beam at steps of 110 μm. Scans were repeated at 90-μm intervals until the whole field was examined. Four fields were recorded and matched to build up the entire section of a maize kernel cross section.

Phenolic Acid Amides. (*E*)-Feruloylputrescine, (E)-*p*-coumaroylputrescine, (*E*)-diferuloylputrescine, and (*E*)-di-*p*-coumaroylputrescine were synthesized according to methods described previously (Kunesh, 1983) with the slight modification that the phenols were protected as acetates. Analytical and spectral (UV, MS, and ^{1}H NMR) values were identical to literature values. Purity was assessed by HPLC to be greater than 98%. The melting point of *p*-coumaroylputrescine (182–183.5 °C) was in good agreement with the correct value reported by Mizusaki et al. (1971) (179–181 °C).

HPLC Analysis. The combined pericarp–aleurone was removed from corn seed by pearl milling, and the outer abraded tissues were collected for analysis. The embryo and endosperm were hand dissected and ground on a Wiley mill (40 mesh). Extraction of phenolic acid amides from 1 g of each tissue was performed using 20 mL of 80% MeOH with 1% HCl at 4 °C; the extract was mixed in a Polytron for 20 s and then centrifuged at 2000*g* to remove solids. MeOH was removed by rotoevaporation at 35 °C, the pH was adjusted to 3.0 with 1 N HCl, and phenolic acid amides were extracted into ethyl acetate (4 × 30 mL). The extract was taken to dryness and then stored at −20 °C. The residue was taken up in 1 mL of 1 N NaOH, and 10 μL of benzoyl chloride was added; the mixture was incubated at room temperature for 30 min. The reaction was quenched with saturated NaCl, pH was adjusted to 3.0 using 1 N HCl, and then the mixture was extracted into 20 mL of ethyl acetate (three times). The extract was reduced to dryness and taken up in 1 mL of MeOH. For subsequent HPLC analysis of phenolic acid amides, a gradient (Slocum et al., 1989) was used to separate the individual components. HPLC analyses were performed using a Perkin-Elmer LC 480 diode scan array detector and a Perkin-Elmer LC 250 binary pump. The solvent system comprised methanol (A) and water (B) and the gradient was as follows: 50–60% A in 7 min; 65–80% A in 6 min; 80% A for 5 min; 80–100% A in 6 min; 100% A for 5 min; 100–50% A in 4 min.

This 40-min gradient allowed sufficient time for column cleaning and regeneration between injections, and identical retention times were obtained. A C_{18} reversed-phase column (Altex, Spherisorb), 250 × 4.6 mm, 5-μm particle size, was used to separate the compounds. The flow rate was 1 mL/min, and detection was at 254 nm. Excellent separation was achieved by this method; identification of the compounds in the extract was made by comparison of retention times (RT) and on-line spectra with those of authentic synthetic standards and cochromatography with the standards for feruloylputrescine (RT = 21.3 min), diferuloylputrescine (RT = 25.9 min), *p*-coumaroylputrescine (RT = 21.7 min), and di-*p*-coumaroylputrescine (RT = 26.4 min). Quantification of the compounds was based on comparison of peak heights in extracts with a standard curve prepared by injection of different amounts of the standards.

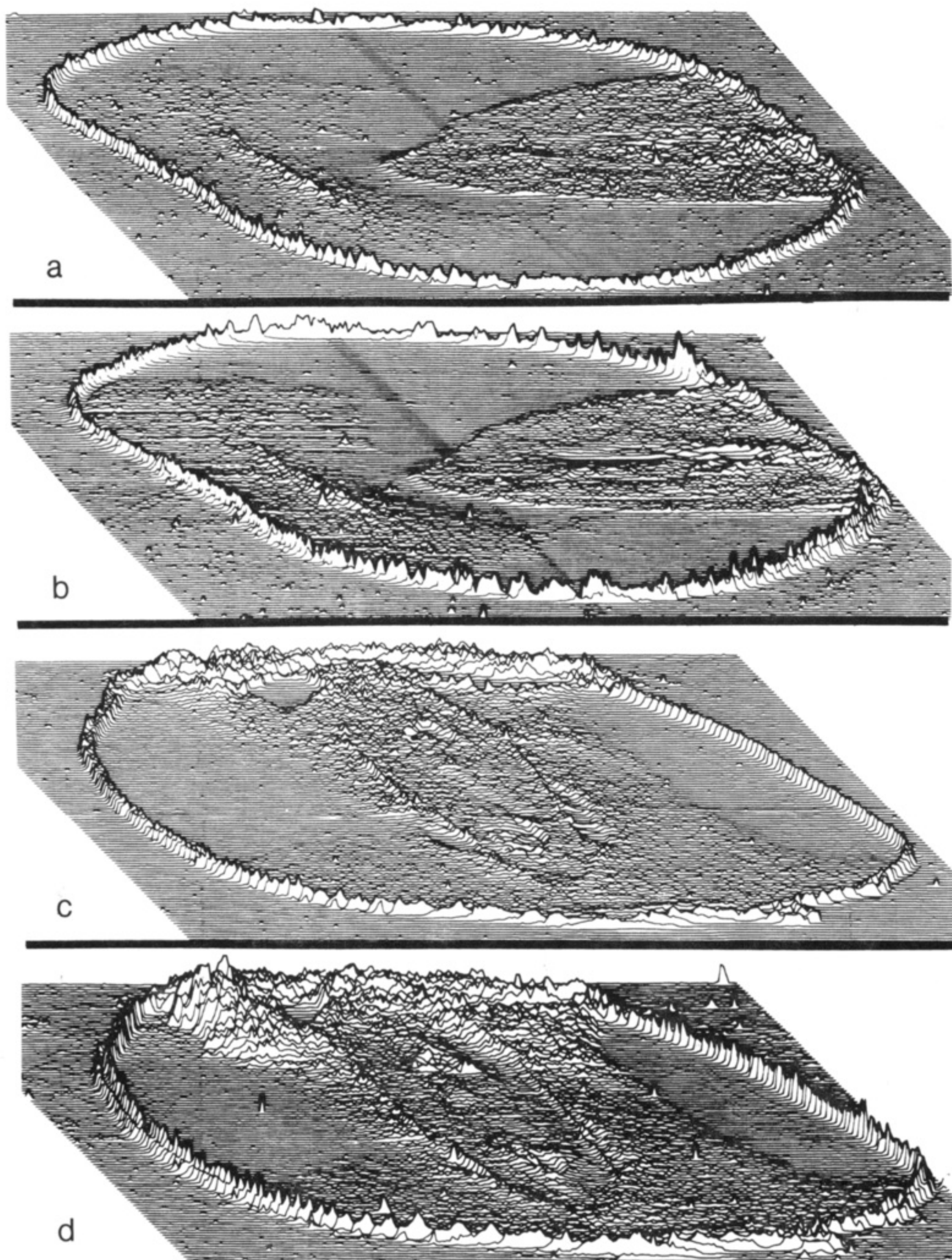

Figure 2. Microspectrofluorometric scan of maize kernel cross sections: Oaxaca 179 showing (a) autofluorescence and (b) fluorescence enhanced by borinic acid reagent for flavonoids; (c) Oaxaca 130 showing autofluorescence; and (d) fluorescence enhanced by borinic acid reagent for flavonoids.

RESULTS AND DISCUSSION

Intensity maps of autofluorescence in maize grain cross sections generated by the multiple scanning technique of microspectrofluorometry (Figures 1 and 2a,c) illustrate that substantial differences in relative fluorescence intensity occur, making it possible to localize and quantify phenolics in individual tissues. This technique provides more information than conventional fluorescence micrographs such as that shown in Figure 3, since the map provides a quantitative assessment of fluorescence intensity as opposed to the more qualitative view of the micrograph. The fluorescence map (Figure 1) of a high phenolic maize kernel (cultivar Sinaloa 35) shows the intense autofluorescence around the periphery of the kernel in the pericarp−aleurone layer. Fluorescence intensity was minimal in the subaleurone regions of the endosperm, while the germ amd central regions of the endosperm show some fluorescence. A linear relationship has been demonstrated between phenolics in maize flour detected by the microspectrofluorometer and phenolic content determined by HPLC analysis (Sen et al., 1991). Thus, the quantitative fluorescence map also should accurately reflect relative amounts of phenolic compounds.

When a high phenolic maize which is resistant to maize weevil, such as Sinaloa 35 (Figure 1) and Oaxaca 130 (Figure 2c), was compared to a low phenolic, susceptible maize such as Oaxaca 179 (Figure 2a) or Muneng and Ritchie (not shown), there were differences

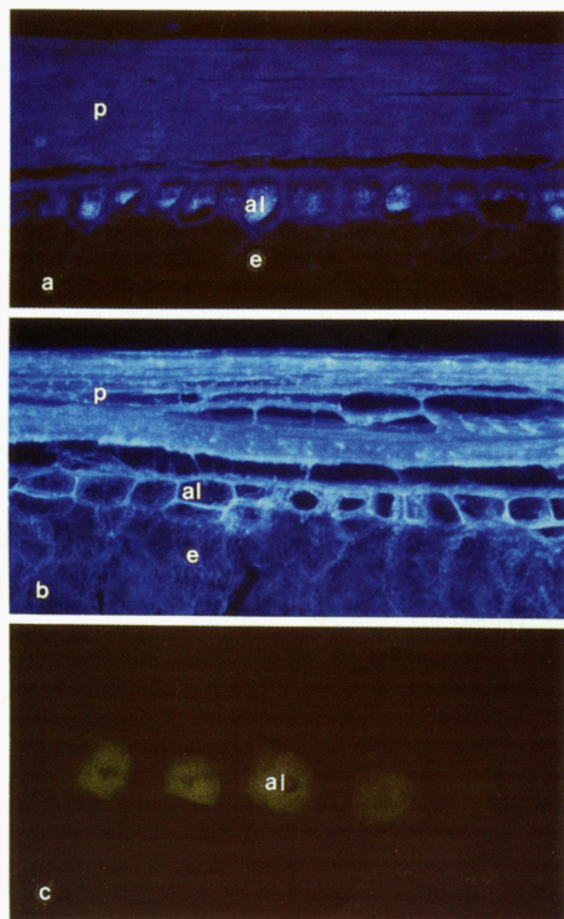

Figure 3. Fluorescence micrographs of cryostat sections of maize kernel [Ritchie (Canadian hybrid)]: (a) autofluorescence; (b) same material stained with borinic acid reagent to enhance flavonoid fluorescence and (c) stained with Ehrlich's reagent to enhance aromatic amines (green fluorescence). Tissues are indicated as aleurone (al), endosperm (e), and pericarp (p).

in the amount of fluorescence but little difference in the distribution of fluorescence due to phenolics. The differences between resistant and susceptible cultivars (Table 1) are most conveniently quantified using the microspectrofluorometer by scanning ground samples of maize placed on a microscope slide (rather than whole cross sections) as described previously (Sen et al., 1991). Except for differences in kernel shape, there was no substantial difference in the tissue distribution of phenolics in land races or modern synthetics or hybrids. Because the microspectrofluorometer was optimized for ferulic acid, which is by far the most abundant phenolic in maize grain (Sen et al., 1991), the autofluorescence measured represents mainly this compound with some contribution from flavonoids.

The microspectrofluorometric technique can be adapted for enhanced detection of specific compounds. Treatment of the blocks containing maize kernels with borinic acid (Figures 2b and 3b) enabled detection of flavonoids in the aleurone layer. Ehrlichs's reagent, used as a fluorochrome to detect aromatic amines in maize kernels, quenched the overall intensity in the kernel, but the phenolic amine fluorescence was detected quantitatively in the aleurone layer as well as the embryo (data not shown).

To confirm the localization, conventional fluorescence microscopy of cryostat sections was also undertaken. Sections treated with borinic acid reagent showed increased fluorescence in the subaleurone as well as the aleurone layers of maize kernels. Ehrlichs's reagent quenched the blue autofluorescence of phenolic acids in the pericarp and endosperm tissues but induced green fluorescence in the aleurone cells (Figure 3) and embryo.

Ehrlich and borinic acid positive structures have been reported in wheat, oats, and barley, where they are present primarily in the aleurone layer (Fulcher and Wong, 1980). Amine residues, however, were not detected in the aleurone layers of sorghum grain (Fulcher et al., 1981).

While there is little doubt that the largest concentrations of flavonoids are located in the aleurone layer, the aromatic amines are less well studied. In fact, the only microanalysis available (Martin-Tanguy et al., 1982) used combined aleurone–pericarp–endosperm samples which were referred to as endosperm samples because of the relatively small mass of the outer tissues. Our data, however, indicate that these outer tissues also have the highest concentration of amines.

To confirm the concentration of amines in the outer layers as suggested by the Ehrlich reagent results in our study, dissection of the combined pericarp–aleurone layer was undertaken, followed by HPLC analysis. The results (Table 2) indicated that diferuloylputrescine and di-*p*-coumaroylputrescine concentrations were significantly higher in the pericarp–aleurone tissues than in the embryo or endosperm. Mean values of both amines were considerably lower in the embryo than in the combined pericarp–aleurone tissue and lowest in the endosperm. Similar trends, though not significant, were observed for the monomers (feruloyl- and *p*-coumaroylputrescine).

The phenolic composition of maize kernels is qualitatively similar to that of other cereal grains and tissues (McDonough et al., 1983). The bulk of phenolics (phenolic acids, flavonoids, and conjugated amines) is concentrated in the pericarp and aleurone layers as well as the germ, with traces in the endosperm. It is interesting to note, however, the precise location of phenolics and consider its significance in terms of insect feeding behavior. To get to the protein- and carbohydrate-rich endosperm, an insect would have to break through the outer layer (bran), where the phenolics are concentrated. In addition, phenolic acids esterified to carbohydrates are involved in dimerization reactions mediated by peroxidase. This process cross-links the cell wall and makes tissues harder, which limits the biodegradability of cell wall polysaccharides (Hartley

Table 2. Microanalysis[a] of Maize Tissues by High-Pressure Liquid Chromatography

tissue	feruloylputrescine (μg/g)	*p*-coumaroylputrescine (μg/g)	diferuloylputrescine (μg/g)	di-*p*-coumaroylputrescine (μg/g)
embryo	6.61[a]	2.28[a]	15.36[b]	3.16[a]
endosperm	1.40[a]	0.42[a]	5.59[a]	0.81[a]
pericarp	2.80[a]	1.64[a]	64.55[c]	12.97[b]

[a] Means of cultivars Oaxaca 179, Sinaloa 35, and Yucatan 7. Means followed by the same letter in a column are not different by lsd test ($P = 0.05$).

and Ford, 1989; Fry, 1983). It is thus not surprising to find cultivars of maize high in phenolic acid content to be resistant to stored-grain pests.

ACKNOWLEDGMENT

A standard of diferuloylputrescine was kindly provided by David Miller (Agriculture Canada). This study was supported by IDRC and NSERC (CRD program).

LITERATURE CITED

Arnason, J. T.; Gale, J.; Conilh de Beyssac, B.; Sen, A.; Miller, S. S.; Philogene, B. J. R.; Lambert, J. D. H.; Fulcher, R. G.; Serratos, A.; Mihm, J. Role of phenolics in resistance of maize grain to the stored products insects, *Sitophilus zeamais* and *Prostephanus truncatus*. *J. Stored Prod.* **1992**, *28*, 229–126.

Classen, D.; Arnason, J. T.; Serratos, J. A.; Lambert, J. D. H.; Nozzolillo, C.; Philogène, B. J. R. Correlation of phenolic acid content of maize to resistance to *Sitophilus zeamais*, the maize weevil in CIMMYT's collections. *J. Chem. Ecol.* **1990**, *16* (2), 301–315.

Eraso, F.; Hartley, R. D. Monomeric and dimeric phenolic constituents of plant cell walls. *J. Sci. Food Agric.* **1990**, *51*, 163–170.

Fincher, G. B.; Stone, B. A. Cell walls and their components in cereal grain technology. *Adv. Cereal Sci. Technol.* **1986**, *8*, 207–295.

Fry, S. C. Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta* **1983**, *157*, 111–123.

Fulcher, R. G. Fluorescence microscopy of cereals. *Food Microstruct.* **1982**, *1*, 167–175.

Fulcher, R. G.; Wong, S. I. Inside cereals, a fluorescence microchemical view. In *Cereals for Food and Beverages: Recent Progress in Chemistry and Technology*; Inglett, G. E., Munck, L., Eds.; Academic Press: New York, 1980; pp 1–26.

Fulcher, R. G.; O'Brien, T. P.; Lee, J. W. Studies on the aleurone layer. Conventional and fluorescence microscopy of the cell wall with emphasis on phenol-carbohydrate complexes in wheat. *Aust. J. Biol. Sci.* **1972**, *25*, 23–34.

Fulcher, R. G.; O'Brien, T. P.; Wong, S. Microchemical detection of niacin, aromatic amine and phytin reserves in cereal bran. *Cereal Chem.* **1981**, *58*, 130–135.

Harborne, J. B. Functions of flavonoids in plants. In *Chemistry and biochemistry of plant pigments*; Goodwin, T. W., Ed.; Academic Press: London, 1976; Vol. I, pp 736–779.

Harborne, J. B. Plant phenolics. In *Encyclopedia of Plant Physiology, New Series Vol. 7. Secondary plant products*; Conn, E. E., Ed.; Academic Press: New York, 1980; pp 269–300.

Hartley, R. D.; Ford, C. W. Phenolic constituents of plant cell walls and wall biodegradibility. In *Plant cell wall polymers*; Lewis, N. G., Plaice, M. G., Eds.; ACS Symposium Series 399; American Chemical Society: Washington, DC, 1989.

Irving, D. W.; Fulcher, R. G.; Bean, M.; Saunders, R. M. Differentiation of wheat based on fluorescence, hardness, and protein. *Cereal Chem.* **1989**, *66* (6), 471–477.

Kato, Y.; Nevins, D. J. Isolation and identification of 2-*O*-(5′-*O*-trans-feruloyl-β-L-arabinofuranosyl)-(1→3)-*O*-β-D xylopyranosyl-(1→4)-D-xylopyranose as a component of *Zea* shoot cell walls. *Carbohydr. Res.* **1985**, *137*, 139–150.

Kunesh, G. An efficient synthesis of some biologically important monoacetylated diamines. *Tetrahedron Lett.* **1983**, *24*, 5211–5214.

Martin-Tanguy, J.; Perdrizet, E.; Prevost, J.; Martin, C. Hydroxycinnamic acid amides in fertile and cytoplasmic male sterile lines of maize. *Phytochemistry* **1982**, *21*, 1939–1945.

McDonough, C.; Beavers, S.; Rooney, L. Factors affecting the free polyphenol contents in cereals. *Cereal Foods World* **1983**, *28*, 559.

Mizusaki, S.; Tanabe, Y.; Noguchi, M.; Tamaki, E. p-coumaroylputrescine, caffeoylputrescine and feruloylputrescine from callus tissue culture of *Nicotina tabacum*. *Phytochemistry* **1971**, *10*, 1347–1350.

Pussayanawin, V.; Wetzel, D. L.; Fulcher, R. G. Fluorescence detection and measurement of ferulic acid in wheat milling fractions by microscopy and HPLC. *J. Agric. Food Chem.* **1988**, *36*, 515–520.

Sen, A.; Miller, S. S.; Arnason, J. T.; Fulcher, R. G. Quantitative determination by HPLC and microspectrofluorimetry of phenolic acids in maize kernels. *Phytochem. Anal.* **1991**, *2*, 225–229.

Serratos, J. A.; Arnason, J. T.; Nozzolillo, C.; Lambert, J. D. H.; Philogene, B. J. R.; Fulcher, R. G.; Davidson, K.; Peacock, L.; Atkinson, J.; Morand, P. The factors contributing to resistance of exotic maize populations to maize weevil *Sitophilus zeamais*. *J. Chem. Ecol.* **1987**, *13*, 751–762.

Slocum, R. D.; Flores, H. E.; Galston, A. W.; Weinsein, L. H. Improved method for HPLC analysis of polyamines, agmatine and aromatic amines in plant tissues. *Plant Physiol.* **1989**, *89*, 512–517.

Smart, M. G.; O'Brien, T. P. Observation on the scutellum. II. Histochemistry and autofluorescence of the cell wall in mature grain and during germination of wheat, barley, oats and ryegrass. *Aust. J. Bot.* **1979**, *27*, 403–411.

Swain, T. Secondary compounds as protective agents. *Annu. Rev. Plant Physiol.* **1977**, *28*, 479–501.

Van Sumere, C. F.; Albrecht, J.; Dedoner, A.; De Pooter, H. Plant proteins and phenolics. In *The chemistry and biochemistry of plant proteins*; Harborne, J. B., Ed.; Academic Press: London, 1973; pp 211–264.

White, C. A.; Kennedy, J. F. Manual and automated spectrophotometric techniques for the detection and assay of carbohydrates and related molecules. *Tech. Carbohydr. Metab.* **1981**, *B312*, 1–64.

Received for review February 8, 1994. Accepted June 8, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, August 1, 1994.