# Screening Spring Wheat for Midge Resistance in Relation to Ferulic Acid Content

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The concentration of ferulic acid (FA), the major phenolic acid in the wheat kernel, was found to differ significantly in the mature grain of six wheat cultivars known to have a range of tolerance to the orange wheat blossom midge (*Sitodiplosis mosellana*). Differences in FA content were correlated with floret infestation level of the cultivars. The wheat cultivars ranked similarly in FA content at the four locations where they were tested, despite a significant effect of environment. Ferulic acid was synthesized mainly during the early stages of grain filling but at different rates among cultivars. Ferulic acid was concentrated primarily in the shorts and bran fractions in an insoluble-bound form. A high correlation was obtained between FA contents as determined by GLC, fluorometry, UV, and colorimetry. The colorimetric procedure was modified as a qualitative, simple, and rapid test for identifying midge-resistant wheat and evaluated in several field trials. The method should provide a rapid tool in the preliminary screening of experimental lines in the development of midge-resistant wheat cultivars.

**Keywords:** *Wheat midge; ferulic acid; total phenolics; grain development; screening test for midge resistance; Sitodiplosis mosellana*

## INTRODUCTION

The orange wheat blossom midge (*Sitodiplosis mosellana*) primarily attacks developing wheat kernels, causing severe reduction in the quantity and quality of hard red spring (HRS) wheat grains. It was reported that all Canadian spring wheat cultivars evaluated during a wheat midge outbreak in 1983 were susceptible to the insect (*1*). In 1983, wheat midge caused yield reductions valued at >$30 million in northeastern Saskatchewan. In a subsequent study, 61 spring wheats including Canadian cultivars were susceptible to the insect (*2*). During the past decade, infestations of wheat midge have seriously reduced the yield and quality of HRS wheats in Saskatchewan and Manitoba, the major wheat-producing provinces in Canada. The orange wheat blossom midge is a sporadic pest of wheat in northern Europe (*3*, *4*).

Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) exists primarily in a bound form with arabinoxylans in the outer layers of cereal grains. It constitutes up to 90% of the total phenolic acids in wheat (*5*) and is the only measurable acid in the insoluble-bound fraction, contributing >80% of the total phenolic acids (*6*). The cross-linking of phenolic acids with carbohydrates in the cell walls of cereals is believed to provide a physical barrier against invasive insects and microorganisms. Several reports have linked the increased resistance to mold damage in sorghum (*7*), insect consumption of corn (*8*), and aphid infestation of barley (*9*) to the high level of phenolic acids in those grains. This cross-linking, however, has no contribution to the known differences in molecular structure and nutritive properties of soluble fiber in wheat and rye (*10*). Phenolic acids are also astringent substances when they are unbound, or in free forms, which may also deter consumption by invasive insects and animals (*8*). Phenolic acids provide other benefits to the grains due to their antioxidant properties (*11*) and could be used to predict end-use quality of wheat (*6*, *12*). This indicates a bioactive role of phenolic acids not only during grain development but also through storage and processing. The differences in resistance of wheat genotypes to the midge in relation to phenolic acids are not yet clear, however.

The development of midge-resistant wheat cultivars can be achieved only by a comprehensive breeding program. To screen large plant populations in such programs, a reliable, simple, and rapid method of selection is required. Commonly, the screening method is based on a wheat component that has significant correlation with resistance or susceptibility to the insect. FA content was found to be cultivar-dependent in wheat and to vary significantly among barley cultivars (*13*). On the basis of studies with other crops (*7*−*9*), FA or total phenolic acids may be a potential candidate for screening wheat grains for disease and insect resistance.

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**Table 1. Average Floret Infestation and Seed Damage by Midge in Six Wheat Cultivars Grown at Four Sites (Mean ± SE)[a]**

| cultivar | floret infestation (%) | seed damage (%) |
|---|---|---|
| Katepwa | 21.4 ± 3.08a | 19.9 ± 2.64b |
| Roblin | 23.1 ± 2.02a | 24.1 ± 1.26a |
| Arin | 5.2 ± 1.18c | 6.9 ± 0.87d |
| 94M-011 | 11.1 ± 2.40b | 11.1 ± 2.15c |
| 94M-014 | 9.3 ± 0.95bc | 11.3 ± 1.12c |
| 94M-025 | 5.5 ± 0.73c | 6.8 ± 1.13d |

[a] Means within a column followed by the same letter are not significantly different at $p > 0.05$.

Many methods have been used for the determination of FA including visible spectophotometry (VIS) or colorimetry (*14*), UV spectrophotometry (UV) (*15*), spectrofluorometry (FL) (*12*), gas−liquid chromatography (GLC) (*16*), and high-performance liquid chromatography (HPLC) (*17*). The GLC and HPLC procedures provide accurate quantitative data on FA content but are too time-consuming and costly for routine analysis. In the present study, the GLC procedure was used to evaluate the specificity and precision of the VIS, UV, and FL procedures, as well as their comparative equipment and operating costs and speed of analysis.

The objectives of the present study were to investigate differences in FA content among wheat cultivars known to have a range of resistance to the midge infestation using GLC, FL, UV, and VIS and to study FA distribution within the kernels and its changes during grain development. Due to the urgent need for a screening method in breeding programs aimed at developing midge-resistant wheat, the colorimetric (VIS) method was selected for further modification to provide a simple and rapid screening method based on phenolic acid content. The modified method was evaluated using a large number of experimental wheat lines obtained from several field experiments.

## MATERIALS AND METHODS

**Wheat Cultivars.** Four spring wheat (*Triticum aestivum* L.) cultivars and three homozygous spring wheat breeding lines were selected to study differences in FA content as influenced by cultivar and environment. Arin, a midge-resistant German cultivar (*3*), was selected for comparison with the slightly resistant cultivars Katepwa and AC Foremost and the highly susceptible cultivar Roblin. The three breeding lines, 94M-011, 94M-014, and 94M-025, and those described subsequently (26 homozygous spring wheat breeding lines), were derived primarily from crosses between Arin and Katepwa. The cultivars and the breeding lines showed a wide range in tolerance to midge by floret infestation and seed damage (Table 1), offering an excellent opportunity to evaluate potential prediction tests for FA. The range in floret infestation among cultivars was 4-fold, and seed damage was proportional to floret infestation.

In the initial study, three named cultivars and the three breeding lines were grown at four locations (Kernen, Wynyard, Kelvington, and Jedburgh) in Saskatchewan, Canada, in 1996 to determine the effects of cultivar and environment on FA as measured by GLC and FL and on total phenolic acids as assessed by UV and VIS methods.

The modified VIS procedure was evaluated in a field experiment consisting of 26 homozygous spring wheat breeding lines and six cultivars planted in a four-replicate randomized complete block design (RCBD), giving a total of 128 wheat samples. The test was also conducted on a more diverse population of lines including 15 German spring wheat Plant Introductions from the USDA collection in comparison with 9 commercial cultivars in a four-replicate RCBD at two locations (Saskatoon and Watrous, SK). Finally, the modified procedure was further evaluated on six populations from wheat crosses in the $F_4$ generation grown in 1998, totaling 430 wheat lines.

**Preparation of Wheat Samples.** For phenolic acids extraction studies, mature wheat grains were finely ground on a Udy Cyclone sample mill (Udy Co., Fort Collins, CO) fitted with a 0.5 mm screen.

For the FA distribution experiment, samples of Arin, Roblin, and Katepwa wheat grains were tempered and fractionated on a Bühler mill (Bühler Ltd., Uzwil, Switzerland) into eight streams, three break flours, three reduction flours, shorts, and bran. The break flours represent the inner endosperm, and the reduction flour fractions arise primarily from the outer endosperm layers, whereas shorts and bran originate from aleurone and kernel outer layers.

Changes in FA during the course of grain filling were followed in 1997 and 1998 by sampling wheat spikes on a weekly basis, from 2 to 9 days postanthesis (DPA) to 8 weeks, by which time the grains were thoroughly ripe. Samples of the three wheat cultivars grown in the four-replicate RCBD at Saskatoon were collected on the same day, so that DPA at sampling differed among the cultivars. Four replicates were collected every week. The fresh spikes were sampled for assays of grain moisture content, and the remainder was freeze-dried on the same day to minimize biochemical changes. The grains were removed manually by rubbing the freeze-dried spikes on a rubber mat. The detached grains were thoroughly dried by freeze-drying and ground with a mortar and pestle for FA analysis.

**Phenolic Extraction.** For GLC analysis, 200 mg of ground wheat was placed in a 50-mL glass centrifuge tube and 15 mL of methanol/acetone/water (7:7:6, v/v/v) was added. The mixture was agitated for 15 min using a platform shaker and centrifuged at 1000*g* for 10 min. The supernatant was discarded, and the extraction was repeated once. The residue was digested with 15 mL of 2 N NaOH for 4 h under nitrogen at room temperature to free bound phenolic acids. The tube was shaken every 30 min to ensure the mixture remained homogeneous. After hydrolysis, the pH was adjusted to 2.0 with 6 N HCl, and then 20 mL of diethyl ether/ethyl acetate (1:1, v/v) was added to the hydrolysate and mixed by inverting the tube several times. The mixture was allowed to stand for a few minutes for phase separation before being centrifuged at 1000*g* for 10 min. The organic phase was collected, and the water phase was washed again with 15 mL of solvent. The two organic aliquots were pooled, and 5 g of anhydrous sodium sulfate was added for dehydration. The extract was filtered through glass wool plugged into a 100-mL evaporating flask, and the solvent was evaporated on a rotary evaporator at 45 °C to dryness. In the case of the determination of free, soluble-bound, and insoluble-bound phenolic acids in wheat, the two supernatant portions or methanol/acetone/water extracts were pooled and used for the separation of free and soluble-bound phenolic acids as outlined by Krygier et al. (*16*).

For UV and VIS methods, the samples were digested with NaOH without prior removal of soluble phenolic acids. The sample weights were 30 and 100 mg for UV and VIS, respectively, and the amount of added reagents was adjusted on the basis of the sample weight. Otherwise, the procedure followed was as above. Efforts were made during extraction to minimize the occurrence of FA photoisomerization or conversion of trans to cis forms (i.e., extraction was conducted under dimmed light).

**GLC Analysis.** The dry residue was transferred into a 3.5 mL vial containing 0.1 mL of internal standard (heptadecanoic acid methyl ester) using 100 μL of ethyl acetate. The solvent was evaporated to dryness using nitrogen, and then 200 μL of Tri-Sil/BSA formula D was added and heated at 60 °C for 30 min. The sample was cooled to room temperature before 1 μL was injected on a GLC column. A Hewlett-Packard model 5880A gas chromatograph equipped with a flame ionization detector connected to an HP 3396 series II integrator was used. A WCOT DB5 capillary column of fused silica (10 m × 0.2 mm) was used. The initial column temperature of 185 °C was held for 3 min and then programmed to 220 °C at 5 °C/min,

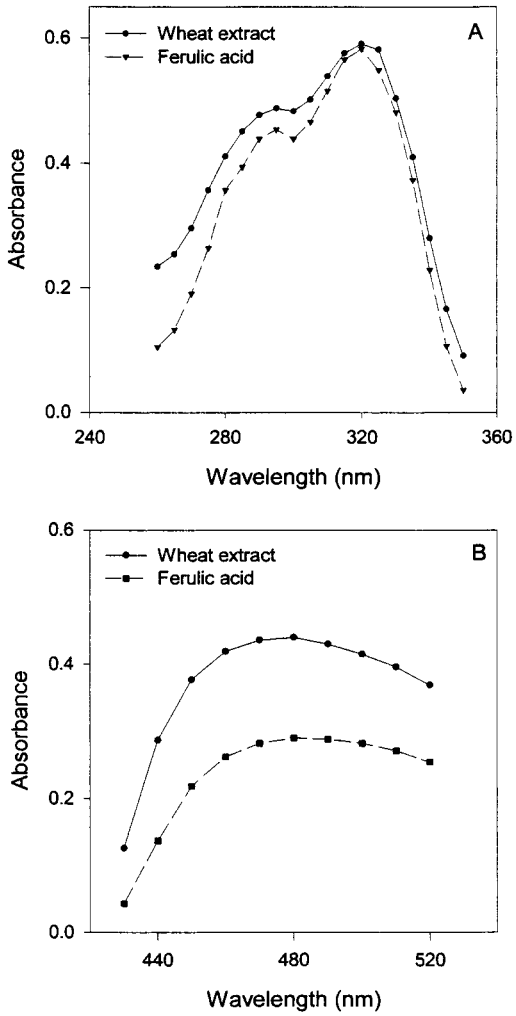


**Figure 1.** Absorbance spectra of FA and wheat extract in the UV region (A) and of their color complexes with titanium tetrachloride reagent (B).

following by a second program to 300 °C at 20 °C/min and final time of 2 min. The injector and detector temperatures were 275 and 300 °C, respectively. A standard mix of phenolic acids (protocatechuic, vanillic, caffeic, syringic, coumaric, ferulic, and sinapic) was used for calibration, identification, and calculation of phenolic acids in wheat samples.

**UV Method.** The dry residue was quantitatively transferred with ethyl acetate into a 5-mL volumetric flask and diluted to the mark. The absorbance was measured at 320 nm against a reagent blank. A stock standard solution of FA in ethyl acetate (10 $\mu$g/mL) was prepared and used to make a series of standard FA solutions (10, 20, 30, 40, and 50 $\mu$g/5 mL). This concentration range had a linear relationship with absorbance and gave a coefficient of determination ($r^2$) of 0.9997 and a regression coefficient of 0.01989. The absorbance spectrum of the extracted phenolic acids from wheat was checked against a standard FA solution. The extracted phenolic acids showed a spectrum similar to that of pure FA (Figure 1A). The maximum UV absorption for both standard FA solution and wheat extract was at 320 nm.

**VIS Method.** The colorimetric method was based on the formation of a colored complex between phenols and titanium (*18*). The dry residue was quantitatively transferred into a 10-mL screw-top glass tube with 100 $\mu$L of ethyl acetate. The solvent was then evaporated to dryness by purging with nitrogen and 0.5 mL of titanium tetrachloride reagent (20%), and 2.5 mL of HCl was added immediately and thoroughly mixed on a Vortex for ~15 s. The solution was heated at 40 °C for ~2 min and again thoroughly mixed. The absorbance was read at 480 nm against a reagent blank. A series of standard FA solutions were prepared by transferring volumes of 0.2, 0.4, 0.6, 0.8, and 1.0 mL of standard FA solution (20 mg/100 mL of ethyl acetate) into a 10-mL screw-top glass tube. The solvent was then evaporated and the reagents added as previously described. The standard solutions gave corresponding FA weights of 40, 80, 120, 160, and 200 $\mu$g. This range (0.0−200.0 $\mu$g) showed a linear relationship against absorbance, with a coefficient of determination ($r^2$) of 0.9993 and a regression coefficient of 0.0025. The absorbance spectrum of the extracted phenolic acids (mainly FA) with titanium reagent was characteristic of FA as shown in Figure 1B.

**Microspectrofluorometric Method.** A UMSP80 microspectrophotometer (Carl Zeiss, Echting, Germany) was used to measure FA in wheat whole meal flours. The instrument was equipped with a UV−vis monochromator, a VIS monochromator, an epi-illuminating condenser, and an XBO 75W/2 high-pressure xenon illuminator. The excitation wavelength was set at 380 nm, and the emission wavelength was set at 445 nm. Each whole meal flour sample was placed on a depression microscope slide and flattened by a quartz microscope cover slip. The sample was mounted on the moving stage of the UMSP80. Using a 40-$\mu$m-diameter measuring spot, mean fluorescence was determined from intensity measurements at 5000 points on the surface of the specimen.

**Screening Test.** Several preliminary trials were conducted using a range of sample weights and saponification temperatures to optimize and accelerate the VIS procedure. The sample weight was optimized on the basis of the formation of a range of color intensities with wheat cultivars ranging in their reaction to midge. The optimum sample weight was found to be ~200 mg. Later, ground samples were replaced with intact grains (6−12 kernels) to further simplify the test for screening purposes. Saponification and extraction times were reduced by heating at 60 °C for 1 h instead of 4 h at room temperature. No significant differences were observed as a result of these modifications. Approximately 200 mg of wheat kernels (~6−12 kernels) was weighed into a 15-mL glass centrifuge tube. Three milliliters of 2 N NaOH solution was added, and the mixture was saponified for 1 h at 60 °C. The kernels were fully submerged in the solution. The tubes were cooled, and 2 mL of 3 N HCl was added and thoroughly mixed. Three milliliters of diethyl ether/ethyl acetate (1:1, v/v) was added and fully mixed by inverting the tube several times. The cap of the tube was kept loose to vent pressure. The mixture was left for a few minutes to allow phase separation before centrifugtion at 1000*g* for 15 min. One milliliter of the organic upper layer was transferred into a 3-mL glass test tube using a Pasteur or transfer pipet, 100 $\mu$L of titanium tetrachloride reagent was added, and the tube contents were vortexed for 2 min. The solution was left for a few minutes to allow color development. The color was ranked using the following scale: 0, very light yellow (blank); 1, light yellowish amber; 2, yellowish amber; 3, light amber; 4, amber; and 5, dark amber. A blank was prepared using 1 mL of solvent and 100 $\mu$L of titanium tetrachloride reagent.

**Data Analysis.** Data were subjected to analysis of variance and correlation using Minitab Statistical software release 12 (Minitab Inc., State College, PA).

## RESULTS AND DISCUSSION

**Variation in FA Content.** FA in mature grains of six wheat cultivars grown at four locations and known to have a range of reactions to the orange wheat blossom midge (Table 1) was determined accurately by GLC and compared with three spectrophotometric methods, FL, UV, and VIS (Table 2). The GLC and FL methods measure the concentration of FA, whereas the UV and VIS methods determine total phenolic acid content. Differences in FA or total phenolic acid content among wheat cultivars were consistent among the four analytical techniques. Values obtained by the three spectrophotometric methods were significantly correlated with

**Table 2. Ferulic Acid and Total Phenolic Acid Contents (Mean ± SD) of Six Wheat Cultivars Grown at Four Locations**

| | ferulic acid (mg/100 g) | | total phenolic acids (mg/100 g) | |
|---|---|---|---|---|
| cultivar | GLC | FL | UV | VIS |
| | | Kernen | | |
| Katepwa | 60.2 ± 0.35 | 49.2 ± 0.52 | 106.7 ± 3.18 | 162.4 ± 0.64 |
| AC Foremost | 62.6 ± 0.64 | 52.8 ± 0.49 | 117.0 ± 1.27 | 181.3 ± 0.71 |
| Arin | 78.3 ± 0.78 | 56.4 ± 0.45 | 138.1 ± 1.84 | 268.7 ± 4.10 |
| 94M-011 | 66.3 ± 0.35 | 49.7 ± 0.67 | 117.4 ± 1.98 | 166.4 ± 5.94 |
| 94M-014 | 73.2 ± 1.27 | 57.7 ± 0.66 | 124.2 ± 1.48 | 195.2 ± 4.24 |
| 94M-025 | 70.7 ± 0.35 | 53.2 ± 0.48 | 132.7 ± 2.76 | 228.0 ± 5.80 |
| | | Wynyard | | |
| Katepwa | 54.2 ± 1.77 | 40.1 ± 0.62 | 102.3 ± 0.28 | 153.8 ± 6.93 |
| AC Foremost | 60.0 ± 1.13 | 45.3 ± 0.56 | 116.6 ± 1.56 | 171.3 ± 2.90 |
| Arin | 67.8 ± 2.26 | 50.5 ± 0.67 | 129.5 ± 3.04 | 229.9 ± 3.75 |
| 94M-011 | 53.5 ± 0.92 | 39.1 ± 0.72 | 115.7 ± 2.83 | 202.1 ± 1.56 |
| 94M-014 | 64.7 ± 0.28 | 48.9 ± 0.66 | 126.1 ± 3.54 | 198.3 ± 1.56 |
| 94M-025 | 63.8 ± 1.48 | 47.3 ± 0.72 | 111.4 ± 1.20 | 226.3 ± 10.54 |
| | | Kelvington | | |
| Katepwa | 59.5 ± 2.83 | 42.7 ± 0.67 | 104.3 ± 0.78 | 165.1 ± 5.16 |
| AC Foremost | 61.6 ± 0.42 | 45.2 ± 0.62 | 118.5 ± 0.71 | 160.9 ± 4.03 |
| Arin | 70.3 ± 0.78 | 47.8 ± 0.72 | 132.4 ± 3.75 | 226.5 ± 3.25 |
| 94M-011 | 63.7 ± 1.27 | 45.9 ± 0.28 | 118.7 ± 0.14 | 191.9 ± 5.02 |
| 94M-014 | 71.3 ± 1.70 | 50.1 ± 0.71 | 128.3 ± 3.04 | 196.7 ± 3.61 |
| 94M-025 | 63.1 ± 3.39 | 48.2 ± 0.78 | 117.8 ± 3.18 | 206.0 ± 4.24 |
| | | Jedburgh | | |
| Katepwa | 54.4 ± 1.48 | 42.0 ± 0.45 | 101.6 ± 0.57 | 176.6 ± 4.88 |
| AC Foremost | 63.2 ± 1.63 | 47.2 ± 0.39 | 120.9 ± 0.64 | 184.2 ± 3.18 |
| Arin | 65.6 ± 2.97 | 47.3 ± 0.62 | 125.1 ± 0.07 | 209.5 ± 3.54 |
| 94M-011 | 54.4 ± 0.99 | 39.3 ± 0.49 | 110.1 ± 1.98 | 160.8 ± 2.21 |
| 94M-014 | 65.7 ± 1.98 | 48.7 ± 0.61 | 123.3 ± 1.20 | 195.7 ± 2.47 |
| 94M-025 | 64.7 ± 0.92 | 48.8 ± 0.63 | 116.6 ± 2.05 | 216.0 ± 5.87 |

those obtained by GLC, but the correlation coefficient for VIS was only 0.70, as compared to 0.85−0.88 for the other two methods (Figure 2). The precision of each method was relatively high, as indicated by the low standard deviations (Table 2) and coefficients of variation of <5%. This suggests that any of the methods could be used to differentiate among wheat cultivars on the basis of their content of FA or total phenolic acids. When consideration was given to the cost of instruments, and analysis time, it was decided to adopt the simple and rapid VIS technique for the screening purposes. The VIS means correctly classified the six cultivars at the four locations and gave a much wider range of values, from 162.4 to 268.7 mg/100 g, than the other three methods.

Analysis of variance of the data in Table 2 showed significant differences in FA contents among wheat cultivars that were consistent by each analytical procedure (Table 3). These differences were consistent with the floret infestation and seed damage data in Table 1. For example, the highly resistant Arin had the highest FA content (70.5 mg/100 g) compared to only 57.0 mg/100 g in the relatively susceptible Katepwa. Two of the experimental lines, 94M-014 and 94M-025, were predicted to be nearly as resistant to midge as Arin, in line with the floret infestation values (5.5−9.3%).

Rankings of wheat cultivars on the basis of FA content were similar at the four locations tested (Table 2), but concentrations of FA varied significantly among the locations (Table 3). Thus, despite the genetic basis for FA content, there is a need to test new genotypes at more than one location or year.

**Distribution of FA.** FA was distributed similarly in the three wheat cultivars with the midge-resistant Arin containing the highest levels of FA in all milling streams (Table 4). The break flours were very low in FA, whereas reduction flour streams were intermediate compared to the bran and short fractions that contained most of the FA, that is, 72.1% of FA and 72.6% of total phenolic acids. Clearly, the concentration of FA increased from the center to the outer layers of the kernel as previously demonstrated by Fulcher (*19*), who reported high concentrations of FA in the seed coat, embryo, and aleurone cell walls and a low concentration in the starchy endosperm of wheat grains.

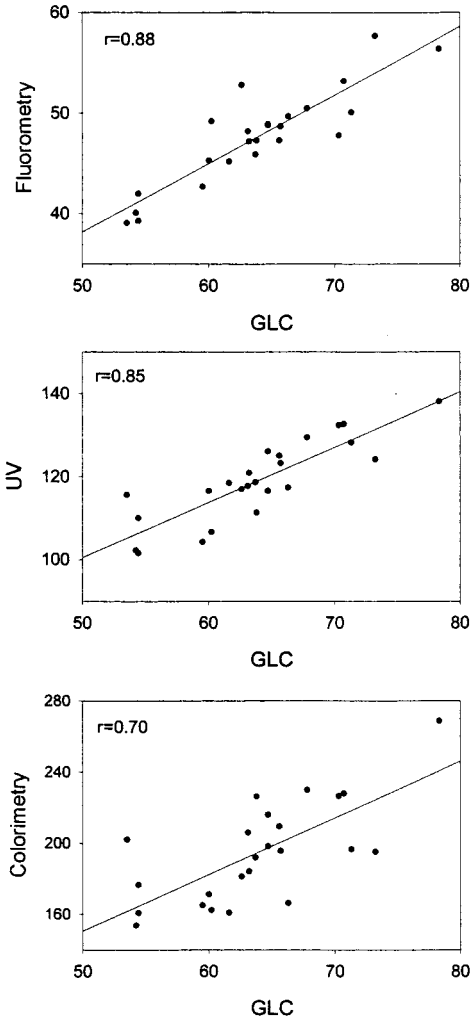


**Figure 2.** Correlation between FA content (milligrams per 100 g) determined by GLC and that determined by fluorometry, UV, and colorimetry.

**Table 3. Average Ferulic Acid and Total Phenolic Acid Contents As Influenced by Wheat Cultivar and Location**

| | ferulic acid (mg/100 g) | total phenolic acids (mg/100 g) | |
|---|---|---|---|
| variable | GLC | UV | VIS |
| cultivar | | | |
| Katepwa | 57.0d | 103.7d | 164.4e |
| AC Foremost | 61.8c | 118.2c | 174.4de |
| Arin | 70.5a | 131.2a | 233.6a |
| 94M-011 | 59.5cd | 115.5c | 180.3d |
| 94M-014 | 68.7a | 125.4b | 196.5c |
| 94M-025 | 65.5b | 119.6c | 219.1b |
| location | | | |
| Kernen | 68.5a | 122.7a | 200.3a |
| Wynyard | 60.6c | 116.9bc | 196.9a |
| Kelvington | 64.9b | 120.0ab | 191.2a |
| Jedburgh | 61.3c | 116.2c | 190.4a |

[a] Within cultivar or location, means within a column followed by the same letter are not significantly different at $p > 0.05$.

**Table 4. Yield of Milling Fractions and Distribution of Ferulic Acid and Total Phenolic Acids in Wheat Kernels**

| flour or fraction | yield (%) | ferulic acid (GLC) mg/100 g | ferulic acid (GLC) % | total phenolic acids (UV) mg/100 g | total phenolic acids (UV) % |
|---|---|---|---|---|---|
| Arin | | | | | |
| 1st break | 12.9 | 4.6 | 0.6 | 10.5 | 0.8 |
| 2nd break | 12.9 | 5.1 | 0.7 | 11.3 | 0.9 |
| third break | 3.4 | 17.2 | 2.3 | 23.1 | 1.8 |
| 1st reduction | 41.4 | 17.5 | 2.4 | 28.9 | 2.2 |
| 2nd reduction | 2.4 | 69.3 | 9.5 | 114.8 | 8.8 |
| 3rd reduction | 1.1 | 90.1 | 12.3 | 169.4 | 12.9 |
| shorts | 3.2 | 205.7 | 28.1 | 378.9 | 28.9 |
| bran | 22.6 | 321.5 | 44.0 | 572.3 | 43.7 |
| Roblin | | | | | |
| 1st break | 10.1 | 4.2 | 0.7 | 11.3 | 1.0 |
| 2nd break | 10.3 | 4.4 | 0.8 | 12.6 | 1.1 |
| 3rd break | 3.2 | 13.9 | 2.4 | 22.9 | 2.1 |
| 1st reduction | 51.4 | 14.4 | 2.5 | 24.1 | 2.2 |
| 2nd reduction | 3.3 | 53.6 | 9.2 | 93.1 | 8.3 |
| 3rd reduction | 1.6 | 64.1 | 11.0 | 97.8 | 8.8 |
| shorts | 4.3 | 173.1 | 29.7 | 320.5 | 28.8 |
| bran | 15.8 | 255.2 | 43.8 | 532.2 | 47.7 |
| Katepwa | | | | | |
| 1st break | 11.5 | 4.4 | 0.7 | 11.3 | 1.0 |
| 2nd break | 10.6 | 4.5 | 0.7 | 11.7 | 1.0 |
| 3rd break | 2.9 | 12.4 | 2.0 | 22.7 | 1.9 |
| 1st reduction | 50.4 | 13.3 | 2.1 | 24.5 | 2.1 |
| 2nd reduction | 2.8 | 62.4 | 10.0 | 111.0 | 9.5 |
| 3rd reduction | 1.0 | 74.9 | 12.1 | 132.9 | 11.4 |
| shorts | 4.2 | 157.7 | 25.4 | 296.7 | 25.5 |
| bran | 16.7 | 291.0 | 46.9 | 553.6 | 47.5 |

Phenolic acids in the three wheats were separated into three categories: soluble-free (SF), soluble-bound (SB), and insoluble-bound (IB) acids as classified by Sosulski et al. (*5*), and individual phenolic acids were determined in each category (Table 5). Arin contained the highest total concentrations of phenolic acids in the three categories compared to Roblin and Katepwa, with FA being the dominant phenolic acid in all cases. The SF fraction in Arin contained more (six) phenolic acids than that of Katepwa (four) and Roblin (three). In contrast, the SB fraction of all three wheat cultivars contained the same five phenolic acids, with FA being the major one. Although the IB fraction contained very few phenolic acids, it contained the great majority of FA in all cultivars. FA was the only measurable acid in the IB fraction in wheat (*6*). It appears that Arin, the midge-resistant cultivar, contained higher concentrations of a variety of phenolic acids in each fraction, but the levels in SF and SB were too low to offer distinct protection mechanisms against insects and microorganisms, as compared to the levels of IB. Because these studies were on mature grain, it was essential to determine the concentrations of FA in the cultivars during grain development when midge infestation occurs.

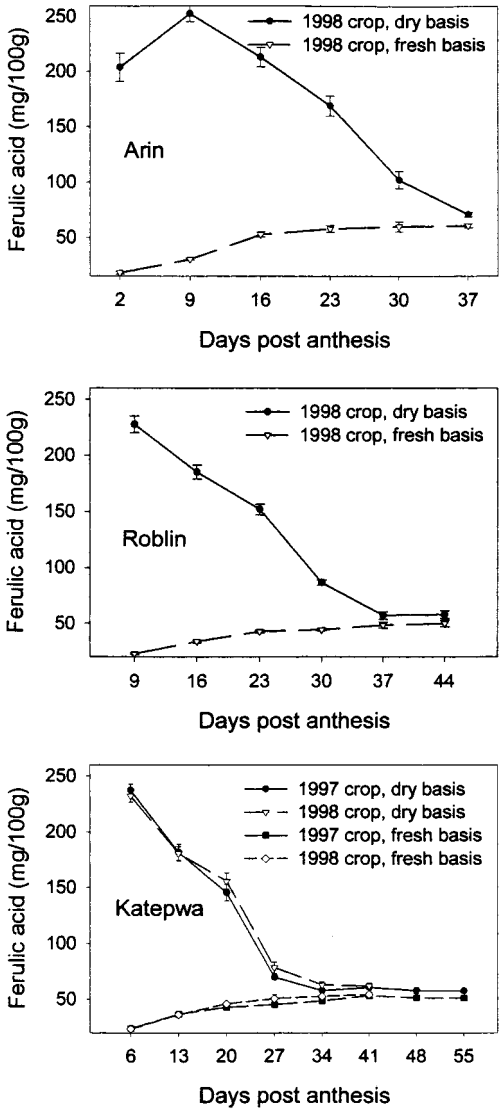


**Figure 3.** Changes in FA content during wheat grain development.

**Changes in FA Content during Grain Development.** On a fresh weight basis, there were progressive increases in FA concentrations during early stages of grain development in each cultivar up to 16 DPA in Arin and up to 23−27 DPA in the other cultivars (Figure 3). After this period, there were only slight increases or a

**Table 5. Soluble-Free, Soluble-Bound, and Insoluble-Bound Phenolic Acids (Milligrams per 100 g) in Wheat Kernels**

| category | protocatechuic | vanillic | caffeic | syringic | coumaric | ferulic | sinapic | total |
|---|---|---|---|---|---|---|---|---|
| | | | | Arin | | | | |
| SF | 0.41 | 0.11 | 0.16 | tr[a] | 0.11 | 0.46 | 0.35 | 1.67 |
| SB | nd[b] | 0.70 | tr | 0.45 | 0.55 | 2.09 | 0.50 | 4.34 |
| IB | nd | nd | nd | nd | 0.44 | 83.26 | 2.78 | 86.48 |
| | | | | Roblin | | | | |
| SF | nd | nd | nd | 0.32 | nd | 0.22 | 0.37 | 0.91 |
| SB | nd | 0.66 | nd | 0.74 | 0.12 | 1.03 | 0.35 | 2.90 |
| IB | nd | nd | nd | nd | nd | 67.86 | nd | 67.86 |
| | | | | Katepwa | | | | |
| SF | 0.27 | tr | 0.15 | tr | tr | 0.40 | 0.31 | 1.19 |
| SB | nd | 0.59 | tr | 0.43 | 0.14 | 1.57 | 0.39 | 3.16 |
| IB | nd | nd | nd | 0.34 | nd | 69.90 | 1.54 | 71.78 |

[a] tr, trace (<0.10). [b] nd, not detected.

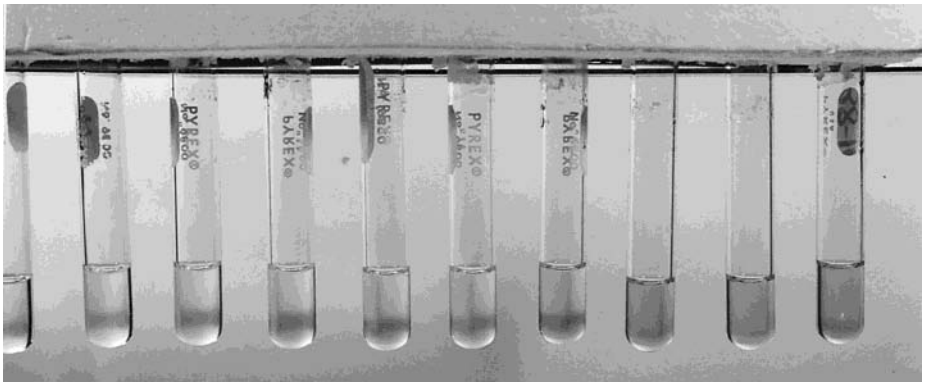

**Figure 4.** Visual color intensity of the developed screening test (modified VIS method) for selected wheat experimental lines ranging from susceptible (left, relatively high seed damage) to resistant (right, relatively low seed damage) to the wheat midge.

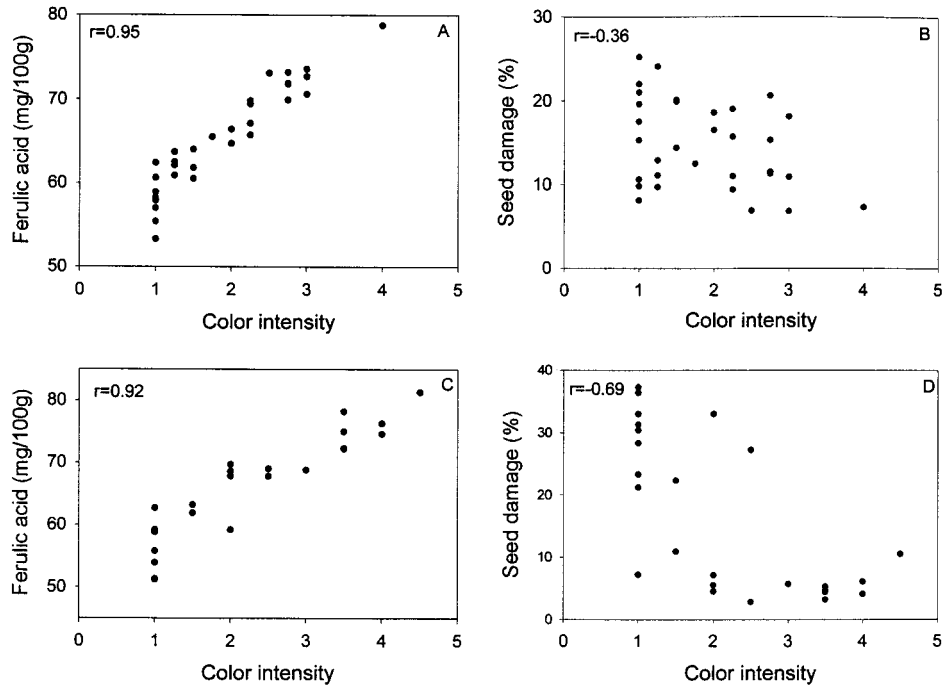


**Figure 5.** Relationships between color intensity of the screening test and FA content and between color intensity and percent seed damage caused by midge of two sets of cultivars [experiment 1, $n = 128$ (A, B); experiment 2, $n = 96$ (C, D)].

steady state as the grain matured. It is apparent that Arin had the highest levels of FA at all stages of grain filling. It was reported that FA concentrations reached a peak concentration at ~27 DPA for cv. Sumai and Roblin (resistant and susceptible to *Fusarium*, respectively) (*20*).

On a dry matter basis, the 2 DPA sample of Arin already contained 200 mg/100 g FA, a high level for deterring midge infection. Thereafter, Arin exhibited the highest concentrations of FA among the three cultivars at each stage of grain development. These results were in agreement with those reported by McCallum and Walker (*21*), who studied changes in bound FA in the wheat cultivar Otane. Midge larvae attack the wheat grain early during grain development, so plant breeding should focus on maximizing the synthesis of FA immediately after anthesis, rather than in mature grain.

**Screening Test for Midge-Resistant Wheat.** The color differences between phenolic acids in wheat generated through the use of titanium tetrachloride reagent enabled us to develop a simple and rapid VIS screening method. The screening method was initially tested on grain from the six cultivars grown at the four locations to investigate the relationship between the screening method and grain damage and with FA content as well. The visual color intensities of the screening method for selected wheat experimental lines ranging from susceptible (left) to resistant (right) to wheat midge are presented in Figure 4. The method can be used to screen up to 96 wheat samples per day as compared with 8 samples using GLC in a modestly equipped laboratory.

The color intensity of the screening method was positively correlated with FA content among wheat cultivars ($r = 0.95^{**}$, $n = 128$, in experiment 1; $r = 0.92^{**}$, $n = 96$, in experiment 2), with Arin producing the highest color intensity (Figure 5A,C). The screening test differentiated among the wheat lines, showing its potential as a preliminary test for screening midge-resistant wheat. Also, the color intensity had a significant correlation ($r = -0.36^{**}$, $n = 128$, in experiment

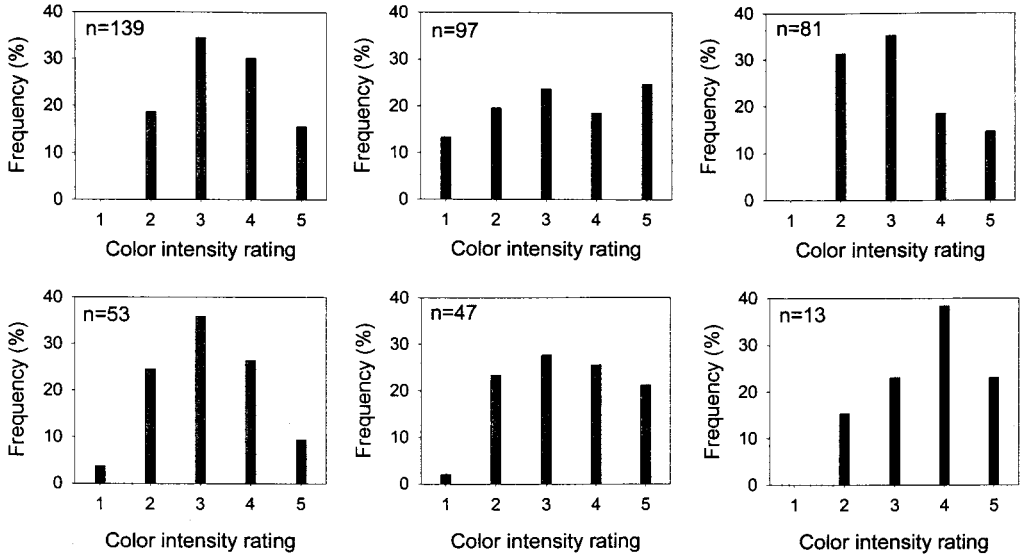


**Figure 6.** Frequency distribution of color intensity of the screening test for six populations of F4 wheat generation.

1; $r = -0.69$**, $n = 96$, in experiment 2) with percent of seed damage (Figure 5B,D). Some experimental lines produced higher color intensities (>4), indicating higher levels of FA and potential for developing midge-resistant wheat cultivars.

In 1998, the screening method was further evaluated using six populations of wheat in the F4 generation. The six populations were selected for rust resistance and plant type prior to harvesting grain samples. Four wheat cultivars (Katepwa, Kenyon, Laura, and Roblin) were included as checks. The distributions of color intensity within each population are presented in Figure 6. Approximately 9−25% of each population was rated with a score of 5 and selected for further evaluation. The average ratings for the wheat checks were 3.2, 4.4, 4.6, and 3.4, respectively, for Katepwa, Kenyon, Laura, and Roblin. On the basis of these results, the colorimetric screening test could provide a simple and rapid tool in the preliminary screening process for the development of midge-resistant cultivars.

**Conclusions.** Ferulic acid, the major phenolic acid in wheat grains, was found to be a significant variable correlated with resistance to wheat midge. Wheat cultivars and experimental lines exhibiting a range of tolerance to wheat midge were found to vary significantly in their content of FA. Several studies have also shown significant correlations between phenolic acid content in grains and their resistance to insects and/or diseases (*8*, *9*, *20*). The mechanism may include a physical barrier formed by the cross-linking between FA and pentosans in the outer layers of grains or the repelling effect of the free phenolic acids.

During the course of this study, a simple and rapid test was developed to aid in identifying midge-resistant wheat cultivars on the basis of phenolic acid content. The screening method would provide a rapid tool in the preliminary screening process in breeding programs aimed at developing midge-resistant wheat cultivars.

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