Correlation of Kernel (E)-Ferulic Acid Content of Maize with Resistance to *Fusarium graminearum*

Rafik A. Assabgui, Lana M. Reid, Robert I. Hamilton, and J. Thor Arnason

First and fourth authors, research associate and professor, Ottawa-Carleton Institute of Biology, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada; second and third authors, maize breeder and research scientist, Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6.

We wish to thank Alf Bolton for providing the isolate of *Fusarium graminearum*.

Accepted for publication 29 March 1993.

**ABSTRACT**


The relationship between (E)-ferulic acid content in kernels of 19 inbreds of maize (*Zea mays*) chosen to represent a taxonomic and latitudinal series showing variation, and resistance to infection by *Fusarium graminearum* was investigated. A negative, significant correlation (1988: r = -0.68, P = 0.002, and n = 57; 1991: r = -0.71, P = 0.002, and n = 57) was found between the amount of ear rot observed in field trials and the amount of (E)-ferulic acid detected in kernels by high-pressure liquid chromatography (HPLC) analysis. Furthermore, fungal growth in vitro was greatly decreased when pure (E)-ferulic acid was added to artificial media; the effective concentration for 50% inhibition of growth (EC₅₀) was 0.65 mg/g. Based on the results of the present study, conventional breeding programs aimed at attaining maize germ plasm resistant to *F. graminearum* should incorporate genotypes of maize containing high concentrations of kernel (E)-ferulic acid.

*Fusarium graminearum* Schwabe (sexual stage: *Gibberella zeae* (Schwein.) Petch) is the causal organism of a sporadically occurring ear rot of corn (*Zea mays* L.) in cool and humid areas of the world (31). The economic significance of this disease is due to the fact that infected grain may be contaminated with mycotoxins (26). Consumption of contaminated grain by swine can lead to complex reproductive and digestive disorders (26).

Host-plant resistance to *F. graminearum*, and breeding for increased resistance, has been suggested as a feasible means of disease control (16). Phenolic compounds are widespread throughout the plant kingdom, and they or their oxidation products have been implicated in disease resistance (11,13,19,22). Phenolic acids also have been shown to inhibit the in vitro growth and reproduction of a wide array of fungal genera (6,17,20), including *F. oxysporum* Schlechtend.:Fr. f. sp. radicis-lycopersici W.R. Jarvis & Shoemaker (18), *F. o. Schlechtend.:Fr. f. sp. lycopersici* (Sacc.) W.C. Snyder & H.N. Hans. (17), and *F. moniliforme* J. Sheld. (14).

In previous studies by our research group, we suggested a role for flavonoid metabolism in maize-silk resistance to *F. graminearum* (27). The current study examined the possibility of a phenolic acid-based resistance mechanism in maize kernels. The specific objectives of this study were 1) to determine whether a varietal correlation exists between susceptibility of maize artificially inoculated with *F. graminearum* and levels of (E)-ferulic acid in kernels and 2) to determine the ability of (E)-ferulic acid to inhibit the growth of *F. graminearum* in vitro.

**MATERIALS AND METHODS**

Fungal cultures. A highly aggressive strain of *F. graminearum*, AR2C, isolated from infected maize in 1988 by A. Bolton, Agriculture Canada, Ottawa, was used in these studies. The culture was maintained on a potato-sucrose agar (PSA; pH 5.4) (3) at 21°C with a 16:8 (light/dark) photoperiod (light intensity at plate level = 2 W/m²).

Spore suspensions were prepared by spreading sterile distilled water onto 14-day-old cultures of *F. graminearum*. Sterile glass beads were added and agitated to dislodge the spores. The resulting suspension was passed through eight layers of cheesecloth, and the concentration of spores was adjusted to 10⁶ spores per milliliter.

**Plant material.** Nineteen inbreds of maize, chosen to represent a taxonomic and latitudinal series showing variation in resistance to *F. graminearum*, were used in this study. These inbreds were developed from the germ plasm bank at the International Maize and Wheat Improvement Center (CIMMYT), located in El Batan, Mexico (5) (Table 1). This material comprised four broad-based latitudinal gene pools developed by CIMMYT for use in different latitudinal regions. The CIMMYT German maize gene pool (NTR-2) consisted of germ plasm from Mexico, Peru, Bolivia, Pakistan, China, Hungary, and the United States. It was adapted in the temperate regions in latitudes 46°-52° N-S of the equator. The gene pool for the northern temperate region gene pool (NTR-1) consisted of materials originating in the U.S. corn belt and was adapted in latitudes 46°-52° N-S of the equator. The gene pool for the intermediate temperate region gene pool (ITR) consisted of European maize material and was adapted in latitudes 40°-46° N-S of the equator. The gene pool for the southern temperate region gene pool (STR) consisted of germ plasm from the U.S. corn belt and tropical lowlands and highlands and was adapted in latitudes 34°-40° N-S of the equator.

In addition to the latitudinal gene pools, three populations of distinct indigenous races of maize from Mexico, two land races from Argentina, and three CIMMYT maize pools were included (Table 1).

**Determination of phenolic acid content.** The kernel phenolic acid content of the 19 inbreds was determined separately for 1988 and 1991 field trials. At harvest, all visibly noninfected control ears of each inbred within the same row were placed in paper bags and air-dried for 8 wk. Ears from the same row were hulled, and three samples, each consisting of 20 randomly selected kernels, were ground with a Wiley mill equipped with a 1-mm mesh screen. This procedure was repeated for each of the three replicates of the 19 inbreds. Therefore, the kernel phenolic acid content of each inbred replicate represented the mean of three individual phenolic acid-content determinations.

Kernel phenolic acid content was determined by a 4 h base hydrolysis (30). For each analysis, 1 g of maize flour was hydrolyzed in 35 ml of 2 N sodium hydroxide in an Erlenmeyer flask closed with a rubber septum and was purged with nitrogen. The flask was placed on a shaker for 4 h and subsequently acidified to pH 2.0 with 6 N hydrochloric acid. The slurry was centrifuged
at 750 g for 10 min, and the supernatant was decanted into a separatory funnel. The pellet was rinsed with 2 × 20 mL of dH₂O and centrifuged at 750 g for 10 min after each wash. All supernatants were combined and extracted with 4 × 50 mL of high-pressure liquid chromatography (HPLC)-grade ethyl acetate. The pooled organic layers were combined and extracted with 4 × 50 mL of dH₂O. The pooled organic layers from the final extractions, including any emulsion that formed between the organic and aqueous layers, were collected and dehydrated over sodium sulphate (anhydrous). The resultant extract was rød-evaporated to nearly complete dryness in vacuo at 35 C. Final evaporation was carried out under a stream of nitrogen to prevent oxidation and degradation of the samples.

A Beckman HPLC equipped with a model 165 variable wavelength detector, a model 110A solvent metering pump system, and a model 420 system controller programmer (Beckman, Fullertown, CA) was used to quantitate the phenolic acids found in the extracts. Twenty microliters was injected into the system, and the phenolics were quantified with a C-18 reverse-phase ultrasphere 5-μm ODS 4.6-mm × 25-cm column, an isocratic elution consisting of 80% 20 mM citrate buffer (pH 5.4)/20% methanol, at a flow rate of 1 mL/min and detection wavelength = 280 nm, and an online UV scan from 240 to 400 nm of eluted peaks.

**F. graminearum ear rot field trials.** Field evaluations of resistance to ear rot were conducted in 1988 and 1991 at the Plant Research Centre, Agriculture Canada, Ottawa, Ontario. The 19 inbreds were planted in a randomized complete block design with three blocks. Each block consisted of 38 single-row plots, each containing 10 plants, to which the maize inbreds and inoculation treatments (inoculation with sterile distilled water or inoculation with a spore suspension) were randomly assigned.

The date on which 50% of the ears from an inbred had silks protruding at least 1 cm was defined as the mid-silk date for the inbred. At 7-10 days post-mid-silk, all ears of each plant were inoculated with 1 mL of a 10⁷ spores per milliliter conidial suspen-

**TABLE 1. F. graminearum ear rot and kernel (E)-ferulic acid content of 19 maize (Zea mays L.) inbreds**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Amount of seed (E)-ferulic acid (mg/g)</th>
<th>Amount of F. graminearum ear rot (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Northern temperate region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTR-1 3947</td>
<td>1.80 bc</td>
<td>12.23 h-j</td>
</tr>
<tr>
<td>NTR-1 3962</td>
<td>1.59 fg</td>
<td>44.33 a-e</td>
</tr>
<tr>
<td>NTR-2 Canada 4071</td>
<td>1.28 fg</td>
<td>62.18 b-a</td>
</tr>
<tr>
<td>NTR-2 Germany 4042</td>
<td>1.60 bc</td>
<td>18.75 g-c</td>
</tr>
<tr>
<td>NTR-2 Holland 4022</td>
<td>1.43 d-g</td>
<td>47.82 a-hc</td>
</tr>
<tr>
<td>NTR-2 Poland 4064</td>
<td>1.55 c-e</td>
<td>36.32 a-c</td>
</tr>
<tr>
<td>NTR-2 Switzerland 4034</td>
<td>1.63 be</td>
<td>21.60 e-i</td>
</tr>
<tr>
<td><strong>Intermediate temperate region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITR 3862</td>
<td>1.41 c-e</td>
<td>35.90 a-c</td>
</tr>
<tr>
<td>ITR 3878</td>
<td>1.50 c-e</td>
<td>44.25 a-c</td>
</tr>
<tr>
<td><strong>Southern temperate region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STR 3802</td>
<td>1.60 bc</td>
<td>19.37 f-i</td>
</tr>
<tr>
<td>STR 3823</td>
<td>1.37 g</td>
<td>33.80 b-g</td>
</tr>
<tr>
<td><strong>Argentine landraces</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cateto C 2030</td>
<td>1.55 b-e</td>
<td>28.03 d-g</td>
</tr>
<tr>
<td>Cateto E 2051</td>
<td>1.52 c-f</td>
<td>27.07 d-h</td>
</tr>
<tr>
<td>CMMYT maize pools</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POOL 27</td>
<td>1.37 g</td>
<td>51.40 a</td>
</tr>
<tr>
<td>POOL 28</td>
<td>1.58 b-d</td>
<td>34.85 b-f</td>
</tr>
<tr>
<td>POOL 30</td>
<td>1.54 b-d</td>
<td>39.17 d-a</td>
</tr>
<tr>
<td><strong>Mexican landraces</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mexico-5</td>
<td>1.64 be</td>
<td>28.78 c-g</td>
</tr>
<tr>
<td>Oaxaca-179</td>
<td>1.56 c-e</td>
<td>3.17 t</td>
</tr>
<tr>
<td>Puebla-463</td>
<td>1.67 ab</td>
<td>6.20 h</td>
</tr>
</tbody>
</table>

*Amount of seed (E)-ferulic acid is a mean of nine replicates from 1988 field trials and nine replicates from 1991 field trials.

*Amount of Fusarium ear rot is a mean of 30 replicates from 1988 field trials and 30 replicates from 1991 field trials.

*Means in the same column followed by the same letter indicate no significant difference according to Tukey's studentized range test (P = 0.05).

**RESULTS**

**Determination of phenolic acid content.** Analysis of maize kernel extracts revealed peaks with relative retention times corresponding to (E)-ferulic acid, (Z)-ferulic acid, (E)-p-coumaric acid, and (Z)-p-coumaric acid detected in all cultivars of maize. The UV spectra of the peaks corresponded to those of the standard compound. Identity of the phenolic acids was confirmed by GC-MS (gas chromatography-mass spectrometry) of extracts. There was an approximate 3 and 5% conversion of (E)- to (Z)-isomers of ferulic acid standards, even though the sample was protected from UV light and hydrolysis was performed in an atmosphere of nitrogen to prevent oxidation. For the purpose of this study, only the concentrations of the (E)-isomer of ferulic acid and the (E)-isomer of p-coumaric acid were calculated.

Standard curves created from a dilution series (100-6.25 μg) of both (E)-ferulic acid and (E)-p-coumaric acid were linear. Peak heights were measured as absorbance units from the base line to the top of the peak. The response of the detector to different concentrations of the standard compounds was also linear, justifying the use of peak heights and standard curves to calculate the concentrations of both (E)-ferulic acid and (E)-p-coumaric acid in the extracts.

The average amount of kernel (E)-ferulic acid ranged from 1.37 mg/g in inbreds STR 3823 and Pool 27 to 1.80 mg/g in inbred NTR-1 3947 (Table 1).

**F. graminearum-induced ear rot in field trials.** The correlations between the amount of ear rot observed in field trials and the corresponding amount of (E)-ferulic acid, for 1988 and 1991, are shown in Figures 1 and 2, respectively. Silking dates of the plants within the rows varied, leading to a variation in inoculation times of the maize ears. Therefore, percent ear rot and phenolic acid content of the three replicate rows for each cultivar were determined separately and were treated as individual data points.

A negative, highly significant correlation (1988: r = -0.68,
Manipulation of phenolic levels in fungal growth media. The addition of (E)-ferulic acid to PSA had significant effects \( (P < 0.05) \) on the overall growth of *F. graminearum* in vitro at all concentrations tested, causing total inhibition at the highest concentration. Probit analysis of the data resulted in a linear transformation (Fig. 3). Extrapolation from the resultant transformation showed that the effective concentration for 50% inhibition of growth \( \left( EC_{50} \right) \) of the *F. graminearum* culture was induced at an in vitro concentration of 0.647 mg of (E)-ferulic acid per gram.

**DISCUSSION**

Maize inbreds with high kernel concentrations of (E)-ferulic acid were significantly more resistant to infection by *F. graminearum* than were those with low kernel concentrations of (E)-ferulic acid. Although the extracts contained (E)-ferulic acid in the highest concentrations, (Z)-ferulic acid and (Z)-p-coumaric acid also were found in the extracts, and these compounds also may be implicated in resistance.

The inclusion of germ plasm from a wide geographical range did not result in geographical trends with respect to either the amount of ear rot or the amount of kernel (E)-ferulic acid content. This may be due, in part, to the limited number of genotypes used in this study. Further, the use of germ plasm from a wide geographical range may have introduced variation into the disease data, because of the unknown effect of growing conditions at a high latitude on the physiology of plants adapted for low latitudes.

Although the results of two field trials indicated a significant linear relationship between kernel (E)-ferulic acid content and resistance to *F. graminearum*, it is not known whether this rela-
The growth of F. graminearum in vitro was demonstrated by experimentally manipulating the levels of the phenolic acid in fungal growth media. The C_{EC} of (E)-ferulic acid to F. graminearum was calculated to be 0.65 mg/g, well within the range of concentrations of free ferulic acid content inhibiting the growth of F. graminearum in vivo. However, this finding is also significant evidence of a cause-and-effect relationship.

Although free ferulic acid has growth-inhibiting properties, most of the phenolic acids in maize kernels are bound forms. In cell wall cell walls, ferulic and coumaric acids are complexed to hemicellulosic compounds in synthetic sugars. These compounds may be in the cell membrane and have direct toxic effects on fungal growth (1). We are currently investigating what role, if any, these compounds may have in limiting fungal growth.

The presence of phenolic acids, especially (E)-ferulic acid, in a wide geographic range of inbred maize cell wall carbohydrate complexes suggests that the role of these substances is constitutive rather than an induced defense. However, phenolics can accumulate rapidly during host-parasite interactions and can modulate defense suppression through inactivation of fungal enzymes or strengthening of plant structural components (2). The possibility that some forms of phenolics accumulate on infection of maize kernels by F. graminearum also should be investigated.

A negative, highly significant correlation was observed between levels of maize kernel (E)-ferulic acid content and amount of ear rot. However, a positive, highly significant correlation was observed between levels of maize kernel (E)-p-coumaric acid content and amount of ear rot. Such a relationship may be due to the lower conversion of p-coumaric acid to ferulic acid in susceptible plants. The continued proliferation of the fungus within its host plant host also may be due to the potential tolerance of or insensitivity to the defense mechanisms of the plant it invades (35). Such tolerance may be due to the production of mycotoxins. Two mycotoxins, zearalenone (24,25) and deoxynivalenol (32,33), are produced by F. graminearum. It has been reported that production of deoxynivalenol increases as disease severity increases (15), and production of zearalenone is positively correlated with increasing disease severity (7). These studies suggest that deoxynivalenol and zearalenone production would be lower in inbreds resistant to F. graminearum. Further, it has been suggested that these mycotoxins may play a role in overcoming host-plant defenses during infection (34). Therefore, resistance of maize inbreds to F. graminearum may be related to their resistance to fungal mycotoxins or to their ability to detoxify mycotoxins.

Resistance and susceptibility to F. graminearum may be influenced by the heritability of phenolic acids. Heritability of phenolics in maize has been documented (8,21), the heritability of (E)-ferulic acid has been examined, and a maternal effect, with pericarp dominance, has been demonstrated (29).

The demonstration of the correlation between ear rot and (E)-ferulic acid in grain at harvest makes selection easier for the breeder. A newly developed quantitative determination analysis of phenolic acids in maize grain (28) greatly reduces analysis time, making the selection process far more convenient and rapid.

Based on the results of the present study, we suggest that conventional breeding programs aimed at maintaining maize germ plasm resistant to F. graminearum should incorporate inbreds of maize containing high concentrations of kernel (E)-ferulic acid.