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Changes in the Phenolic Composition of Maize Silks after Inoculation with *Fusarium graminearum* Schwabe

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27

ABSTRACT

The relationship between hydroxycinnamic acids concentration along time in silk tissues and *Fusarium graminearum* infection progress was investigated in the current research. Significant genotypic differences existed for both the occurrence of *F. graminearum* in the silks and the final ear rot severity ratings. Ferulic acid, *p*-coumaric acid and dimers of ferulic acid esterified to the cell wall components were the main hydroxycinnamic acids quantified in the silk tissues. Ester bound ferulic acid decreased as fungus increase, probably released by fungal hydrolytic enzymes, and this release was faster in susceptible than in resistant genotypes. *p*-Coumaric and diferulates showed slight increases that did not result in delayed *F. graminearum* progression through the silks. The ability of the maize genotypes to slow down ferulic acid liberation through structural features and/or enzyme inhibition could influence the level of kernel infection and thereby the resistance of genotypes to *F. graminearum*.

INTRODUCTION

Fusarium graminearum Schwabe [sexual stage *Gibberella zeae* Schwein (Petch)] is one of the most important plant pathogens infecting several cereal species including maize, wheat or barley in temperate regions (Sutton 1982, Munkvold 2003, Logrieco et al. 2003). In maize (*Zea mays* L.), both the ear and the stalk can be infected (Sutton 1982, Logrieco et al. 2003). Ear rot by *F. graminearum* is a sporadic disease (Sutton 1982) but of major economic importance since this fungus contaminates the grain with mycotoxins (Logrieco et al. 2003, Pestka 2007). Zearalenone and trichothecenes type B toxins such as deoxynivalenol (DON) and its derivates are produced by *F. graminearum* and can result in several disorders and diseases in animals and humans (Pestka 2007, Logrieco et al. 2002).

In maize fields, *F. graminearum* is dispersed from the soil and crop debris in the form of macroconidia, ascospores and hyphal fragments by wind, splashing water or insect vectors (Sutton 1982, Munkvold 2003). Entry into the ear is primarily via the exposed silks but can also occur through kernel wounds created by insects, birds and hail (Munkvold 2003, Koehler 1942). Consequently, infection generally starts in the ear tip and spreads down towards the butt of the cob. Symptoms include the development of a pink or red mycelium on the kernels. In the most severe cases infection reaches the husks and rachis (Sutton 1982, Munkvold 2003). Genotypic differences in resistance have been identified after artificial inoculation of *Fusarium* macroconidia into the silk channel of maize inbreds (Reid et al. 1992b). Miller et al. (2007) followed the progress of *F. graminearum* mycelium down the silks towards the developing kernels and rachis; they observed mycelial growth on the silk surface and inside the epidermal cells of the silk from the second and third days, respectively, after macroconidia attachment. The time it took for mycelium to reach the kernels was

67 different in resistant and susceptible genotypes, suggesting the existence of resistance
68 mechanisms in the silks (Miller et al. 2007).
69 Morphological factors, such as husk coverage, physiological factors, such as
70 flowering time and silk age, and biochemical factors are all believed to influence in the
71 development of *Fusarium* infection in maize (Sutton 1982, Logrieco et al. 2003, Reid
72 et al. 1992c). Changes in silk flavones content were observed after artificial infection
73 with *F. graminearum* (Reid et al. 1992a), but no association between flavones content
74 and resistance was established. Similarly, genotypic differences in the alkane contents
75 of silk wax did not satisfactorily explain differences in resistance to *F. graminearum*
76 (Miller et al. 2003). However, several studies have reported the ability of low
77 molecular weight phenolic compounds, such as phenolic acids, to inhibit *Fusarium*
78 growth (Assabgui et al. 1993, McKeehen et al. 1999, Kasenberg and Traquair 1988,
79 Samapundo et al. 2007) and mycotoxin production (Beekrum et al. 2003, Bakan et al.
80 2003, Boutigny et al. 2009).
81 Besides an antibiotic effect, phenolic acids are important factors for cell wall
82 fortification, cell walls being one of the most important barriers against pathogen
83 attack. In the Poaceae family, cell walls contain significant concentrations of phenolic
84 acids, principally ferulic (FA) and *p*-coumaric (PCA) acids (Ishii 1997). FA is usually
85 esterified to arabinoxylan chains. Ester-bound forms of FA can be covalently coupled
86 by peroxidases to form dehydroferulate dimers (DFA) that cross-link arabinoxylan
87 chains (Ishii 1997, Iiyama et al. 1994). In addition, FA can be a template for the
88 binding and polymerization of lignin and a lignin union link with hemicellulose chains
89 (Iiyama et al. 1994). FA and DFA are suggested to hinder the enzymatic degradation of
90 cell wall polysaccharides thus increasing cell wall strength and limiting cell growth
91 (Matern et al. 1995, de O. Buanafina 2009, Wakabayashi et al. 1997, Bily et al. 2003).

92 PCA is primarily incorporated into cell walls as part of the lignification process, ester
93 bound to monolignols, although PCA can also be esterified to arabinoxylans chains
94 (Hatfield and Marita 2010). An increase in cell wall bound phenolic compounds has
95 been observed after fungal inoculation or elicitation in several crops and tissues (El
96 Modafar et al. 2000, de Ascensao and Dubery 2003, Santiago et al. 2009). In maize,
97 these structural hydroxycinnamic acids have been associated with resistance to
98 *Fusarium* disease in kernels and stalk pith tissues (Assabgui et al. 1993, Bily et al.
99 2003, Santiago et al. 2007). No studies to date have investigated cell wall
100 hydroxycinnamic acids of the silks with respect to resistance to *F. graminearum*.
101 In the current study, hydroxycinnamic acids in silks of maize inbred lines
102 differing in resistance were determined after inoculation with *F. graminearum* or
103 sterile water as control. The main objectives were: (i) to observe whether changes in
104 cell wall hydroxycinnamic acid concentrations occur as silks age, (ii) to determine if
105 any changes are influenced by infection with *F. graminearum* and (iii) to determine the
106 possible relationship between the phenolic acid response in silks and the ear rot
107 resistance.
108

RESULTS

Disease severity ratings and ergosterol concentrations after inoculation. Ears from four Agriculture and Agri-Food Canada (AAFC) maize inbred lines were evaluated at grain harvest for *F. graminearum* ear rot severity. Disease severity ratings in the *F. graminearum* treatment separated the inbreds into two statistically significant groups: CO266 and CO272 with higher ear rot ratings (6.15 and 5.38 respectively) and CO433 and CO441 with lower ratings (2.76 and 2.77 respectively) (Table 1). These ratings concur with previous studies however CO272 is exhibiting more susceptibility than when it was first used many years ago as a source of resistance (Reid et al. 1992b).

In order to characterize the growth of the fungus on the different genotypes, ergosterol from silk tissues was measured at five harvest times (0, 2, 6, 10 and 14 days after inoculation) in the *F. graminearum* treatment. At 0 day none or very low ergosterol was detected while, as expected, ergosterol contents increased with time as the mycelia grew (Table 1). Fungal growth was faster, and ergosterol concentrations were higher, in silks from the more susceptible inbreds CO266 and CO272, while ergosterol increased slower and was lower in silks from CO433 and CO441.

Changes in cell wall hydroxycinnamic acids concentrations after inoculation. Both soluble and cell wall linked hydroxycinnamic acids were extracted from silk tissues recollected at 0, 2, 6, 10 and 14 days after inoculation with *F. graminearum* inoculum or sterile water. Only trace amounts of soluble hydroxycinnamic acids were found in silks at all harvest times and in all genotypes, no quantification was done. The main hydroxycinnamic acids linked to the cell wall were monomers of FA and PCA and dimers of FA (DFA) as shown in Table 2. Three different DFAs were identified and quantified: 5-5′ DFA, 8-5′ DFA (sum of 8-5′-non

cyclic and 8-5′-benzofuran forms), and 8-o-4′ DFA (Table 3). FA was the most prevalent phenol in cell walls of the silk tissue at all harvest times and for both treatments. PCA levels found in silk tissues were much lower than FA levels in both treatments, in accordance with previous studies made with the grain pericarp (Bily et al. 2003). Concentrations of DFAT (sum of the isomers) in the silks tissues, regardless of the treatment, were higher than expected based on quantities found in other tissues (Santiago et al 2006a, Santiago et al.2006b, Barros-Ríos et al. 2011).

In the sterile water control treatment, the highest contents of FA at 0 days were quantified in the inbred lines CO441, CO433 and CO272 (from 5789.7 μg/g to 5133.2 μg/g), and the lowest content in the inbred line CO266 (3965.3 μg/g) (Table 2). Differences were not significant by 14 days. FA concentrations increased with time in all genotypes, although the increment was not significant for the inbred line CO441. Similarly, in the *F. graminearum* treatment, the highest contents of FA at 0 days were found in CO441, CO433 and CO272 (from 5744.9 μg/g to 5144.2 μg/g), and the lowest in CO266 (4142.9 μg/g). Only one inbred, CO272, showed a statistically significant change in FA concentrations with time with FA decreasing after 6 days (Table 2). A similar trend was observed in CO266 where FA tended to decrease after 2 days and in CO443 and CO441 where a similar trend was observed after 10 days. These decreases in FA occurred at the same time that the ergosterol content exceeded 100 μg/g in each inbred (Tables 1 and 2).

In both treatments, PCA concentrations increase slightly with time for all genotypes, with the exception of the water treatment for CO272 and CO433 which exhibited no significant difference over time (Table 2). CO272 had significantly lower PCA concentrations than the others genotypes in the water treatment, while in the *F. graminearum* treatment CO272 had the highest PCA concentrations until day 6, after

159 which significant differences between inbred lines were not apparent in the *Fusarium*
160 treatment.
161 DFAT concentrations increased with time in all genotypes and in both
162 treatments (Table 3). CO266 tended to reach the highest DFAT contents starting at 2
163 days in the water treatment. In the *Fusarium* treatment, results were similar but
164 differences between genotypes were not significant at 2, 10 and 14 days. With respect
165 to the DFA isomers, 8-5′ DFA was the most abundant in all genotypes (average
166 ranging about 50-60 % of DFAT), followed by 8-o-4′ DFA (25-30 %) and at last by 5-
167 5′ DFA (15-20 %) in both treatments (Table 3).
168 Differences between treatments for FA concentration were significant for the
169 inbred lines CO272 and CO441 at 10 days, and inbred lines CO266, CO272 and
170 CO433 at 14 days. In all cases, higher concentrations of FA were found in the water
171 treatment. The largest differences in FA contents were quantified in CO266, at 14 days
172 this inbred had 3435.6 μg/g of FA less than in the water treatment (6171.7 μg/g). In
173 contrast, PCA and DFAT levels tended to be higher in the *F. graminearum* treatment
174 than in the water treatment. CO266 showed significant higher levels of PCA in the *F.*
175 *graminearum* treatment at 6 days (234.4 μg/g), while inbred line CO272 had
176 significantly higher concentrations of PCA from 6 to 14 days (271.4 μg/g to 350.0
177 μg/g). No significant differences between treatments for PCA were found in the inbred
178 lines CO433 and CO441. DFAT contents were significantly higher in the fungal
179 treatment in CO272 at 6 and 14 days, in CO433 at 14 days and in CO441 at 2 days
180 (Table 3). 8-5′ DFA was always involved in the significant differences between
181 treatments related with DFAT.
182

DISCUSSION

Significant genotypic differences existed among four maize inbreds for both the occurrence of *F. graminearum* in the silks as measured by ergosterol and final ear rot severity ratings on the kernels. The inbreds could be classified in two groups: CO266 and CO272 as susceptible (quick fungal development in silks with abundant visible kernel disease symptoms) and CO433 and CO441 as resistant (slow fungal development and lower ear rot symptoms). This suggests that a faster development of the fungus on silk tissue would result in higher ratings of visual symptoms, assuming that resistance mechanisms do not exist in the kernel when they are not present in the silk.

In a search for possible mechanisms of silk resistance, we focused our search on hydroxycinnamic acids because of their structural properties *in planta* and their antibiotic effects *in vitro* (Assabgui et al. 1993, Ishii 1997), and their role in resistance to pests (Bily et al. 2003, Santiago et al. 2006a, Santiago and Malvar 2010). Only trace amounts of soluble forms of hydroxycinnamic acids were observed in the silk tissues. These results were in agreement with the low contents of soluble hydroxycinnamic acids found in pith tissues in a previous study by Santiago et al. (2007). In silk tissues, as well as in pith tissues, most hydroxycinnamic acids were found linked to the cell wall via esterification (Santiago et al. 2007). Cell wall ester bound FA was the major hydroxycinnamic acid quantified in the silks of the genotypes tested in the current study. FA contents in the silks were higher than amounts extracted from the pith and in some cases from grain tissues even in two of the inbreds used in this study, CO441 and CO433 (Assabgui et al 2003, Santiago et al. 2007). FA participates in the cell wall structure by linking to arabinose residues of xylan to form the backbone of hemicellulose, FA is incorporated continually into the cell wall (Hatfield and Marita

208 2010). In silk tissues inoculated with a sterile water control, FA increased as the silks
209 aged, however, after inoculation with *F. graminearum*, FA began to decrease. This
210 decrease began earlier in susceptible genotypes, after 2-6 days from inoculation, than
211 in resistant genotypes, after 10 days. FA decrease under inoculation with *F.*
212 *graminearum* was accompanied by a significant increase of fungal development as
213 measured by ergosterol content in the silks. Lower concentrations of FA in the cell
214 wall of the inoculated silks could be due to cell wall degradation from fungal attack.
215 Degradation of silks tissues by *F. graminearum* was observed in experiments by Miller
216 et al. (2007) and Reid et al. (1992b). Decrease of cell wall linked FA has been also
217 quantified in wheat and barely grains inoculated with *F. graminearum* and *F.*
218 *culmorum*, in comparison with naturally infected grains (Eggert et al. 2010).
219 *F. graminearum* secretes a broad array of enzymes during plant infection
220 among which are xylanases and feruloyl esterases (Walter et al. 2010). FA and/or FA
221 conjugates could be released from cell walls by action of feruloyl esterases and
222 xylanases respectively; these enzymes act synergistically, and the action of xylanases
223 influences the activity of feruloyl esterases (Bartolomé et al. 1995). However, no
224 increase of free FA in silk tissues was observed (data not shown) after cell wall bound
225 FA decreased, and no fungal growth inhibition was observed as would be expected if
226 free FA increased (Assabgui et al. 1993, McKeehen et al. 1999). Trace amounts of FA
227 in the free soluble fraction, and even in the soluble esterconjugate and glycosilate
228 fractions in the inbred CO266 (data not shown) suggests a complex degradation of FA
229 by the fungus after its release (Nazareth and Mavinkurve 1986, Rosazza et al. 1995).
230 Degradation of xylan by xylanases are hypothesised to depend on several
231 factors such as the level of branching of arabinoxylan chains, degree of substitutions of
232 xylan backbone, chain cross-linking, or the presence of xylanase inhibitors (Beaugrand

et al. 2010, Grabber et al.1998, Lagaert et al. 2009). However, simple feruloylation of xylans does not appear to affect maize cell wall degradation by hydrolytic enzymes such as xylanases (Grabber et al. 1998), and important removal of FA have been described after xylanase treatment of wheat bran (Beaugrand et al. 2010). Thus it appears that constitutive contents of monomeric ester bound FA in silks cell walls were not related with resistance to *F. graminearum* cell wall degradation. Other xylan substituents difficult the xylanase activity in maize cell walls such as esterified acetyl groups (Agger et al. 2010). Deacetylation of insoluble corn bran was found to promote the enzymatic degradation of hemicellulose and in a more important degree than deferuloylation (Agger et al. 2010). Moreover, there have been reports in cereals, including maize, of proteinaceous xylanase inhibitors that block fungal xylanases by a competitive inhibitory mechanism (Lagaert et al. 2009, Biely et al. 2008). Thus, hemicelllulose degradation and resulting ester bound FA decreases in silk tissues could be because of the *F. graminearum* infection, and the ability of the maize genotypes to slow down that process through structural features and/or xylanase inhibition could both influence the level of kernel infection.

Concentrations of linked PCA in silks tissues were much lower than FA concentrations. Independent of the treatment, PCA contents increased as the silks aged, however, for the two susceptible inbreds, CO272 and CO266, PCA concentrations increased in the fungal inoculated treatment when compared to the control thus suggesting a possible induction by *F. graminearum.* The PCA increases detected in silk, although significant, were minimal, and not enough to affect fungal development. Cell wall bound PCA, together with FA, is related to cell wall lignification, and PCA is incorporated into cell walls as part of the lignin fraction linked to monolignols (Hatfield and Marita 2010). Increase in lignin concentrations was observed after

Fusarium elicitation in banana (*Musa acuminata*) root tissues (de Ascensao and Dubery 2003), and increase of lignin and cell wall bound PCA concentrations were also detected after *Fusarium* inoculation of roots in date palm (*Phoenix dactylifera*) (El Modafar et al. 2000). The low contents of PCA in silks may indicate a minor significance of lignification in these tissues, especially when compared with PCA concentrations in pith cell walls (Santiago et al. 2007). It is possible that secondary cell walls, where the lignification process mainly takes place, are poorly developed in silks tissues, and most of the PCA observed was ester linked to the sugars of the primary wall. The PCA data together with higher concentrations of FA and DFAT suggest that hemicellulose fractions are more important in silk tissues.

In cell walls, ferulates could be oxidatively coupled to form dehydrodiferultes which cross-link covalently polysaccharides chains (Ishii 1997). Dimerization of ester bound FA monomers in the cell walls of the silk tissues was relatively high in comparison with contents in other maize tissues (Santiago et al. 2007, Santiago et al. 2006b, Barros-Ríos et al. 2011). In addition, contents of DFAT showed an increase during time period that the silks were sampled for both treatments. Thus, polysaccharide cross-linking was related to an increase of the structural strength and decrease in cell wall extensibility (Wakabayashi et al. 1999, Kamisaka et al. 1990). Increase in DFAT in cell walls over time could be produced naturally by cessation of the cell growth. Moreover, silks appear to increase rigidity and to stop elongation after pollination (Sella Kapu and Cosgrove 2010). Polysaccharide cross-linking was also related with cell wall resistance to enzyme degradation, since the access of hydrolytic enzymes to the polysaccharides could be reduced (de O. Buanafina 2009, Grabber et al. 1998, Beaugrand et al. 2010). Significant induction of DFAT accumulation has been observed after inoculation with *F. graminearum*, but it was punctual and did not

have any evident influence on resistance to attack by *F. graminearum*. No relation could be found in silk tissues between DFAT content and resistance of genotypes to *F. graminearum*.

In conclusion, several changes in the cell wall bound hydroxycinnamic acids contents of silk tissues were observed after inoculation with *F. graminearum*: ester bound FA decreased, probably released because of hemicelluloses degradation by hydrolytic enzymes produced by *F. graminearum*, possibly xylanases and feruloyl esterases. Degradation was faster in susceptible than in resistant genotypes. Other cell wall components, PCA and DFAT, did not significantly diminish after inoculation and, in some cases appeared to increase above that of the control. Slight increases in PCA and DFAT contents did not result in delayed *F. graminearum* progression through the silks. In order to establish resistance relationships further studies will be needed in relation to the hemicelullose fraction, as degree of acetylation of arabinoxylan, and occurrence and activity of xylanase inhibitors in the silks tissues.

298 **MATERIALS AND METHODS**

299 **Experimental Design.** Four Agriculture and Agri-Food Canada (AAFC) maize
300 inbred lines were used in this study. The inbreds consisted of CO272, one of AAFC's
301 original sources of moderate resistance to *F. graminearum* silk infection, two inbreds
302 with improved silk (CO433 and CO441), and one susceptible check (CO266). These
303 genotypes were grown in two consecutive years, 2007 and 2008, in a split-split plot
304 design with three replicates, at the Central Experimental Farm in Ottawa, Ontario,
305 Canada. The main plot unit consisted of genotypes, the subplot unit consisted of two
306 inoculation treatments (inoculation with a *F. graminearum* macroconidia suspension
307 and inoculation with sterile water as control), and the sub-subplot unit consisted of six
308 harvest times (0, 2, 6, 10 and 14 days after inoculation for chemical analyses of silk
309 tissues and 70 days after inoculation for evaluation of disease severity on the kernels).
310 Each sub-subplot was a 3.5 m long row, 0.7 m apart from consecutive rows and sown
311 with 15 seeds per row.

312 **Silks Inoculation and Harvest.** Three local DON-producing isolates (DAOM
313 180378, DAOM 194276, and DAOM 212678) of *F. graminearum* obtained from the
314 National AAFC Fungal Culture Collection were used for inoculum production.
315 Preparation followed a modification of the Bilay's liquid medium as described in Reid
316 et al. (1996). Macroconidia of the three isolates were obtained separately and mixed in
317 equal proportions reaching a final concentration of 5×10^5 conidia/ml.

318 Inbreds were inoculated 5-7 days after silk emergence from the ear tip as per
319 Reid et al. (1996). Depending on treatments, 2 ml of *F. graminearum* inoculum or
320 sterile water were injected at a 90º angle into the silk channel of the primary ear using
321 an automatic vaccinator. Ten plants per sub-subplot were inoculated. To harvest silks
322 for chemical analysis, exposed silks at the tip of the ear were discarded, ears were cut

323 in half longitudinally, the husk was carefully peeled off and silks from upper half of
324 the ear were collected. Samples were stored at -20ºC until lyophilized and ground to a
325 fine powder by hand using a mortar and pestle.
326 **Ergosterol Analysis.** Ergosterol, a sterol found in fungal membranes but not in
327 animal or plant cells, was measured in the silks samples inoculated with *F.*
328 *graminearum* in both years as indicator of fungal biomass. Analyses were carried out
329 as per Reid et al. (1999) methodology with slight modifications. 100 mg of silk tissue
330 was placed in culture tubes along with 2 ml of methanol and 0.5 ml of 2M NaOH.
331 Tubes were tightly closed with teflon-lined caps, placed inside capped 1000 ml plastic
332 bottles, and irradiated in a microwave oven (Teka, model MW-219) at 80% power
333 (2450MHz, 800 watts maximum output) for 20 s and, after approximately 5 min, for an
334 additional 20 s. After cooling, samples were neutralized with 1M aqueous HCl and
335 treated with 2 ml of methanol. The samples were partitioned with 3 x 4 ml of pentane,
336 and the extracted top pentane layers were combined and evaporated in a rotary
337 evaporator at 50°C. The extracts were then redissolved in 0.5 ml of methanol HPLC
338 grade and passed through a 13 nylon syringe filter, 0.45-µm pore size, into 2 ml HPLC
339 vials, and stored at -20°C until HPLC analysis.
340 HPLC separation was carried out at room temperature by injection of a 50µL
341 sample onto an ACE C18 column (150 x 4mm i.d., 5µm particle size) at a flow rate of
342 2 ml per minute with acetonitrile-methanol (90%-10%) as the eluent under isocratic
343 conditions. The retention time of ergosterol was approximately 8 min. The peak area at
344 the absorption maximum of 282 nm was used for quantification with an external
345 standard obtained from Sigma (St. Louis, MO, USA).
346 **Extraction and Analysis of Phenolic Fractions.** Phenolic extraction followed
347 the procedure described by Santiago et al. 2007 with some minor modifications. 1 g of

348 freezed-dried sample was extracted in 30 ml of 80% methanol and homogenized for
349 30-60 s with a Polytron mixer (Brinkman Instruments, Westbury, NY, USA). Samples
350 were dark extracted for 1 h and then centrifuged for 10 min at 1000 g. Supernatant
351 was collected and the pellet re-extracted twice with 20 ml of 80% methanol. The
352 pellets contain the insoluble cell wall bound phenolics, and the supernatants contain the
353 soluble phenolics.
354 The supernatants for soluble phenolic acids were combined and concentrated in
355 a Speed Vac (Savant Instruments, Holbrook, NY) to 25 ml. These aqueous solutions
356 were acidified using 6N HCl to a pH of 2.0 before extraction with 30 ml of ethyl-
357 acetate. The ethyl-acetate extract was reduced to dryness in a Speed Vac at medium
358 settings without a radiant cover, and the resulting precipitate was resuspended in 3 ml
359 HPLC grade methanol. This solution was used to determine free phenolic compounds.
360 The remaining aqueous solution was divided into two parts. The fraction for
361 soluble glycoside-bound phenolic determination was hydrolyzed in 15 mL of 2 N HCl
362 for 1 h at 4 °C; whereas alkaline hydrolysis for 3 h in the dark and under a nitrogen
363 atmosphere was used to release the soluble ester-bound phenolic compounds. After
364 both digestions, the pH of the solutions was adjusted to 2.0, and the phenolics were
365 extracted with 30 mL of ethyl acetate. The ethyl acetate extracts were reduced to
366 dryness in a Speed Vac system, and the resulting precipitates were resuspended in 3
367 mL of HPLC methanol.
368 The pellet was digested with 20 ml of 2N NaOH and shaken for 4 h in the dark
369 in a nitrogen atmosphere. Digestion reaction was stopped by adding 6N HCl and the
370 pH was adjusted to 2.0. Samples were centrifuged as above and supernatant was
371 collected. The pellet was washed twice with 10 ml of distilled water, centrifuged, and
372 supernatants collected, mixed and extracted twice with ethyl acetate (50 ml and 35 ml

each). The organic phase was dried using a Speed Vac for 6 h. The extracts were dissolved in 3 ml of HPLC methanol and stored at -20ºC. All standards and samples were filtered through a 20 µm pore poly (tetrafluoroethylene) filter (Chromatographic Specialties, Brockville, ON) before analysis.

Analyses were performed using a Hewlett-Packard ChemStation series 1100 chromatograph with a Waters YMC ODS-AM (Waters, Milford, MA) narrow bore column (100 x 2 mm i.d., 3 µm particle size). Elution conditions with a mobile phase system of acetonitrile (solvent A) and trifluoroacetic acid (0.05%) in water (pH 3.4) (solvent B) were as follows: initial conditions 10:90 (A/B), changing to 30:70 in 3.5 min, then to 32:68 in 6.5 min, then to 100:0 in 4 min, and then to isocratic elution with 100:0 for 4.5 min, finally returning to the initial conditions in 3 min. The mobile phase flow rate was 0.3 ml/min, and the total analysis time was 21.5 min. The sample injection volume was 4 µL, and the elution profiles were monitored online by UV absorbance at 325, 280, and 254 nm. Retention times were compared with those of freshly prepared standard solutions. Standards of the most common phenolics (caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and vanillic acid) were purchased from Sigma (St. Louis, MO). Identities of ferulic acid dimers were confirmed by comparison with the authentic 5-5′ standard and/or retention time and UV spectra previously published (Waldron et al. 1996).

Evaluation of Disease Severity. At normal grain-harvesting time (70 days after inoculation), 10 ears from the corresponding sub-subplot were evaluated for severity of ear rot infection using a visual rating scale where: 1 = no infection, 2 = 1 to 3%, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = >75% of kernels exhibit visible symptoms (Reid et al. 1996).

Statistical Analysis. Combined analyses of variance (ANOVA) for disease severity ratings, ergosterol and phenolic concentrations were computed with the PROC GLM procedure of SAS, version 9.1 (SAS Institute 2002-2003). Years, replications and their interactions were considered random. Comparisons of means among genotypes, harvest times and inoculation treatments were made by Fisher's protected least significant difference (LSD).

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410

411 **LITERATURE CITED**

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555 Table 1. Disease severity ratings at grain harvest for four maize inbred lines silk channel inoculated with *F. graminearum* macroconidia or sterile
556 water (control), and ergosterol contents (μg/g dry weight) in the silk tissues at five harvest times after inoculation. Disease severity visual rating
557 scale was: 1 = no infection, 2 = 1 to 3%, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = >75% of the kernels exhibit visible
558 symptoms[a].

Inbred lines	Pedigree	Disease severity ratings		Ergosterol contents					
		Inoculation treatments		Harvest times (days)					
		Sterile water	*F. graminearum*	0	2	6	10	14	LSD[b] (*P*≤0.05)
CO266	Pioneer 3990	2.01 a	6.15 a	0.00 a	3.76 a	102.97 a	253.26 a	334.00 a	79.53
CO272	BSTEx(CO109xCO106) CO109	1.00 a	5.38 a	0.00 a	4.13 a	8.97 b	159.92 b	342.04 a	87.03
CO433	Pride K127	1.23 a	2.76 b	0.00 a	5.35 a	18.21 b	57.60 c	161.90 b	87.53
CO441	Jacques 7700xCO298	2.07 a	2.77 b	0.23 a	4.37 a	21.66 b	64.45 c	211.49 b	48.80
LSD (*P*≤0.05)		-	2.12	-	-	27.43	97.14	107.47	

559 [a] Means within a column followed by the same lowercase letter are not significantly different (*P*≤0.05).

560 [b] LSD for ergosterol concentration comparison between days.

Table 2. Mean concentrations (μg/g dry weight) of the cell wall monomers ferulic acid (FA) and *p*-coumaric acid (PCA) in silk tissues of four inbred lines at five harvest times after inoculation with *F. graminearum* macroconidia or sterile water[a].

Phenolic compound	Inbred line	Treatment[b]	Harvest times					LSD_1[c]
			0	2	6	10	14	
FA	CO266	W	3965.3 b	5506.2 a	5951.8 b	6304.5 b	**6171.7** a	696.2
		F	4142.9 B	4224.7 A	4121.5 B	3177.5 C	**2736.1** B	-
		LSD_2	-	-	-	-	1498.9	
	CO272	W	5133.2 a	5926.2 a	6480.4 ab	**6872.5** ab	**6987.5** a	390.8
		F	5144.2 A	6162.7 A	6340.0 A	**5247.9** B	**5279.8** A	918.4
		LSD_2	-	-	-	1542.6	688.9	
	CO433	W	5789.7 a	6161.2 a	**7001.0** a	6933.8 ab	**7238.3** a	457.7
		F	5744.9 A	5582.9 A	**5908.9** A	6172.5 A	**5351.3** A	-
		LSD_2	-	-	505.4	-	993.1	
	CO441	W	5291.0 a	5990.0 a	6505.2 ab	**7450.8** a	7738.1 a	-
		F	5385.5 A	5957.2 A	5707.0 A	**6294.6** A	5823.6 A	-
		LSD_2	-	-	-	703.9	-	
	LSD_3	W	858.9	-	555.8	669.7	-	
		F	723.3	-	769.1	738.0	971.1	
PCA	CO266	W	120.8 b	135.8 a	**144.7** a	205.6 a	248.0 a	35.8
		F	135.0 B	142.8 B	**234.4** AB	227.5 A	202.9 A	76.1
		LSD_2	-	-	55.0	-	-	
	CO272	W	192.1 a	185.9 a	**182.2** a	**154.8** b	**143.5** b	-
		F	199.1 A	288.9 A	**271.4** A	**266.5** A	**350.0** A	62.5
		LSD_2	-	-	45.8	100.0	68.7	
	CO433	W	100.3 b	113.6 a	167.7 a	205.8 a	227.6 a	-
		F	97.6 B	97.9 C	174.2 B	219.6 A	205.2 A	52.3
		LSD_2	-	-	-	-	-	
	CO441	W	107.5 b	139.3 a	154.3 a	228.6 a	224.4 a	40.8
		F	108.0 B	134.9 BC	200.3 B	245.7 A	275.2 A	53.2
		LSD_2	-	-	-	-	-	
	LSD_3	W	49.7	-	-	44.3	48.2	
		F	41.5	43.5	64.1	-	-	

[a] Within water treatment, means within a column followed by the same lowercase letter are not significantly different ($P \leq 0.05$). Within *F. graminearum* treatment, means within a column followed by the same uppercase letter are not significantly different ($P \leq 0.05$). Bold is used when differences between treatments for a particular inbred line are significant.

[b] Treatments: W, sterile water treatment, F, *F. graminearum* treatment.

[c] LSD: LSD_1, for comparisons between days, LSD_2, for comparison between treatments, LSD_3, for comparison between inbred lines in each treatment.

Table 3. Mean concentrations (μg/g dry weight) of the cell wall diferulic acid (DFA) and total diferulic acids (DFAT) in silk tissues of four inbred lines at five harvest times after inoculation with *F. graminearum* macroconidia or sterile water[a].

Phenolic compound	Inbred line	Treatment[b]	Harvest times					LSD_1[c]
			0	2	6	10	14	
5-5' DFA	CO266	W	32.2 a	87.9 a	143.5 a	185.6 a	209.4 a	79.9
		F	29.6 A	74.1 A	139.5 A	147.1 A	139.8 AB	49.2
		LSD_2	-	-	-	-	-	
	CO272	W	9.8 a	27.2 b	67.7 b	142.9 a	151.7 a	-
		F	4.6 A	79.6 A	99.5 AB	158.1 A	190.4 A	51.7
		LSD_2	-	-	-	-	-	
	CO433	W	27.2 a	37.3 b	76.4 b	68.6 a	91.8 a	18.4
		F	34.3 A	31.2 A	73.8 B	112.5 A	106.8 B	49.6
		LSD_2	-	-	-	-	-	
	CO441	W	23.1 a	**41.4** b	74.4 b	122.7 a	203.1 a	106.7
		F	24.2 A	**67.9** A	88.8 B	132.0 A	174.0 A	-
		LSD_2	-	25.2	-	-	-	
	LSD_3	W	-	26.7	34.0	-	-	
		F	-	-	46.8	-	57.2	
8-5' DFA	CO266	W	120.7 a	254.9 a	**349.7** a	371.0 a	503.1 a	140.8
		F	103.7 A	203.0 A	**476.8** A	487.3 A	482.9 A	-
		LSD_2	-	-	113.3	-	-	
	CO272	W	38.5 c	85.1 b	**166.6** b	254.5 a	**326.8** b	75.0
		F	39.3 B	185.8 A	**283.7** B	368.5 A	**528.8** A	120.3
		LSD_2	-	-	49.2	-	83.5	
	CO433	W	72.1 bc	120.0 b	147.3 b	197.4 a	**210.8** c	66.2
		F	110.5 A	91.5 A	220.1 B	301.1 A	**299.9** A	118.7
		LSD_2	-	-	-	-	58.4	
	CO441	W	92.5 ab	**140.7** b	218.5 b	334.7 a	388.4 b	183.1
		F	106.2 A	**205.5** A	256.2 B	355.4 A	422.6 A	-
		LSD_2	-	45.7	-	-	-	
	LSD_3	W	43.0	61.4	101.5	-	104.3	
		F	45.7	-	107.3	-	-	
8-o-4' DFA	CO266	W	52.5 a	114.2 a	172.9 a	184.5 a	286.1 a	70.5
		F	49.7 A	84.4 A	188.1 A	216.2 A	342.2 A	136.3
		LSD_2	-	-	-	-	-	
	CO272	W	26.3 b	45.5 b	82.7 b	157.0 a	194.5 b	45.2
		F	23.8 A	103.3 A	127.7 B	162.4 B	200.6 A	42.0
		LSD_2	-	-	-	-	-	
	CO433	W	38.5 ab	58.7 b	75.4 b	112.8 a	142.6 c	37.2
		F	51.0 A	46.6 A	105.6 B	149.2 B	154.4 A	60.6
		LSD_2	-	-	-	-	-	
	CO441	W	36.8 ab	**62.0** b	97.6 b	162.0 a	220.1 b	85.5

		F	43.6 A	**87.2** A	113.1 B	159.2 B	204.8 A	46.6
		LSD_2	-	24.1	-	-	-	
	LSD_3	W	18.7	34.2	47.8	-	41.8	
		F	-	-	57.4	49.2	-	
DFAT	CO266	W	205.4 a	456.9 a	666.0 a	741.1 a	998.7 a	273.6
		F	183.0 A	361.6 A	804.4 A	850.5 A	965.0 A	308.9
		LSD_2	-	-	-	-	-	
	CO272	W	74.7 b	157.8 b	**317.0** b	554.5 a	**673.0** b	137.8
		F	67.7 B	368.7 A	**510.9** B	689.0 A	**919.8** A	204.0
		LSD_2	-	-	95.4	-	178.8	
	CO433	W	137.7 ab	216.0 b	299.1 b	378.8 a	**445.1** c	107.9
		F	195.8 A	169.4 A	399.5 B	562.8 A	**561.1** A	224.2
		LSD_2	-	-	-	-	106.3	
	CO441	W	152.4 a	**244.0** b	390.5 b	619.3 a	811.6 b	371.9
		F	174.0 A	**360.5** A	458.2 B	646.6 A	801.5 A	-
		LSD_2	-	92.4	-	-	-	
	LSD_3	W	67.8	120.3	173.7	-	169.6	
		F	66.4	-	205.1	-	-	

[a] Within water treatment, means within a column followed by the same lowercase letter are not significantly different ($P \leq 0.05$). Within *F. graminearum* treatment, means within a column followed by the same uppercase letter are not significantly different ($P \leq 0.05$). Bold is used when differences between treatments for a particular inbred line are significant.

[b] Treatments: W, sterile water treatment, F, *F. graminearum* treatment.

[c] LSD: LSD_1, for comparisons between days, LSD_2, for comparison between treatments, LSD_3, for comparison between inbred lines in each treatment.