

# Reactions of Smooth Bromegrass Clones with Divergent Lignin or Etherified Ferulic Acid Concentration to Three Fungal Pathogens

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## ABSTRACT

Increased digestibility of smooth bromegrass is associated with a reduction in lignin concentration or etherified ferulic acid (EthFA) concentration, either of which may reduce host resistance to fungal diseases. The objective of this study was to determine the relationship of lignin and EthFA concentration with disease reaction in smooth bromegrass. Host clones, divergently selected for lignin and EthFA concentration, were challenged by three pathogenic fungi, one biotroph (*Puccinia coronata* Corda) and two necrotrophs [*Pyrenophora bromi* (Died.) Drechs., anamorph = *Drechslera bromi* (Died.) Shoem., and *Bipolaris sorokiniana* (Sacc.) Shoem.]. Significant positive and negative associations were found between lignin or EthFA and host reaction to *P. bromi* or *B. sorokiniana*. The frequencies of these associations suggested that they arose by chance associations between alleles, rather than tight linkages or pleiotropic (causal) effects. Host reaction to *P. coronata* was consistently and negatively associated with lignin, less so with EthFA. These associations, together with results from other species, suggest that lignin, and perhaps EthFA, may be important components of rust resistance mechanisms in the Poaceae. If these mechanisms are real, they will cause considerable difficulty for breeders attempting to simultaneously improve both rust resistance and forage nutritional value.

CONSIDERABLE EMPHASIS has been placed on improving forage quality of smooth bromegrass herbage, on the basis of the concentration, composition, and digestion of cell wall constituents (Vogel et al., 1996). Breeding for improved digestibility has resulted in large reductions in lignin concentration, but high- and low-lignin populations did not differ in forage yield or lodging potential at several locations (Carpenter and Casler, 1990; Casler and Ehlke, 1986; Ehlke et al., 1986). Phenolic components of the cell wall, such as etherified ferulic acid, which acts as a ferulate bridge between lignin and hemicellulose, also regulate digestibility of smooth bromegrass forage (Casler and Jung, 1999).

The properties of lignin indicate a potential role in resistance to pathogens. An association between preformed lignin and the limitation of fungal growth has been observed for some diseases (Ride, 1983). In addition to preformed lignins, active lignification, by means of increases in the activity of enzymes involved in the lignification pathway, has been observed in the response of plants to pathogen attack (Friend et al., 1973; Southerton and Deverall, 1990). Inhibition of these enzymes increased susceptibility to fungal infection (Moerschbacher et al., 1990; Tiburzy and Reisener, 1990). More-

over, the cell walls of some plants are modified by phenolic deposits in response to infection. Papillae or cell wall apposition formation in epidermal cells of grasses, rich in lignin or lignin-like compounds, have been related to mechanisms for reduced fungal penetration and increased resistance to fungal organisms (Sherwood and Vance, 1976, 1980; Vance and Sherwood, 1975).

Reductions in the concentration of lignin or ferulic acid associated with increased forage digestibility may eliminate or impair the resistance mechanisms of plants to stresses, herbivores, and parasitic organisms. This would be particularly true if selection acts upon the components responsible for these resistance mechanisms and not on the components required for cell wall strength and structure per se (Buxton and Casler, 1993). Lower concentration of neutral and acid detergent fiber, cellulose, and lignin in the stalk and leaf-sheath of maize (*Zea mays* L.) were related to increased feeding by the second-generation European corn borer, *Ostrinia nubilalis* (Hübner), regardless of the selection criterion (Buendgen et al., 1990; Ostrander and Coors, 1997).

Little is known about the direct effects of lignin and ferulic acid on the development of fungal diseases of grasses. Sherwood and Vance (1980), studying epidermal penetration in 12 species of 11 tribes of Poaceae to three different leaf-infecting fungi, found that grasses have both constitutive and inducible resistance mechanisms associated with the epidermis, restricting fungal penetration. Sherwood and Berg (1991), found that lignin was not related to resistance of orchardgrass (*Dactylis glomerata* L.) to purple leaf spot caused by *Stagonospora arenaria* Sacc. In two alfalfa (*Medicago sativa* L.) populations, plants that represented a range of acid detergent lignin (ADL) concentration were inoculated with *Uromyces striatus* J. Schröt., the causal agent of alfalfa rust. Although there were significant differences among clones for infection efficiency, latent period, and sporulation capacity, there was little or no relationship between lignin concentration and components of alfalfa rust resistance (Webb et al., 1996).

Plant pathogens can be conveniently divided into two groups, depending on whether or not they can live in the absence of the living plant. Biotrophs depend on the functional metabolism of their hosts for an adequate supply of nutrients, whereas necrotrophs feed on the breakdown products of host degradation (Heisteruber et al., 1994). Thus, the fundamental difference between necrotrophic and biotrophic parasitism is often reflected in the extent of host cell-wall degradation (Cooper, 1983). Lignin and ferulic acid may have differential effects on disease development, depending on the type of pathogen that causes disease.

**Abbreviations:** EthFA, etherified ferulic acid; KL, Klason lignin; GPD, gametic phase disequilibrium.

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Infection by biotrophic pathogens (obligate parasites) was once considered to result from mechanical penetration, but ultrastructural studies suggest highly localized action by cell-wall degrading enzymes (CWDE), as microfibrils are not distorted around penetration pegs which may be highly convoluted and lacking a cell wall (Cooper, 1983). This type of enzymatic degradation may have similarities to the enzymatic cell wall digestion and breakdown in ruminant livestock (Casler and Vogel, 1999). Biotrophy may also depend on additional controls, such as catabolite repression of CWDE synthesis resulting from the characteristic influx of photosynthates to infected areas (Cooper, 1983).

The objective of this study was to determine the relationship of lignin and etherified ferulic acid (EthFA) concentration of smooth bromegrass clones, divergently selected for lignin or EthFA concentration, with disease resistance to three pathogenic fungi, one biotroph and two necrotrophs.

Genetic relationships (correlations) between two traits (such as lignin concentration and host resistance to a fungus) may be caused by one, or a combination, of three genetic phenomena: gametic phase disequilibrium (GPD), linkage disequilibrium, and pleiotropy. The distinction between the three causes of genetic correlations is of paramount importance to plant breeding. Genetic correlations caused by loci that are in GPD are ephemeral; they can be broken rapidly by either selection, random mating, or both (Hartl and Clark, 1997). Genetic correlations caused by linkage disequilibrium are theoretically ephemeral. However, such a correlation can only be broken by a crossover between the loci controlling the two correlated traits. The problem is further compounded if several such linkage associations exist across the genome and if the linkages are tight, i.e., the loci are close together. Larger population sizes, more frequent recombination, greater selection pressures, and perhaps more time will be required to break or modify an undesirable correlation caused by linkage disequilibrium (Dudley, 1994; Hartl and Clark, 1997). Furthermore, extremely tight linkages between loci controlling two traits may be practically unbreakable because of the low probability of crossover between neighboring loci, giving rise to apparent pleiotropy. A genetic correlation caused by pleiotropy, by definition, cannot be modified by any known breeding method.

## MATERIALS AND METHODS

### Development of Host Germplasm

Smooth bromegrass clones were selected to create repeatable differences in lignin or EthFA concentration, so that potential associations of these two traits with disease resistance could be studied. Divergent selection for lignin and EthFA concentration was practiced in four different smooth bromegrass populations: Alpha, WB19e, Lincoln, and WB88S representing three different levels of domestication. WB88S is a completely wild population from the Altai Mountains of southern Siberia. Lincoln represents the smooth bromegrass germplasm that was originally introduced into the USA, and Alpha and WB19e represent the most elite high-digestibility germplasm available in the USA and Canada (Casler et al., 2000).

Three hundred seedlings of each population were trans-

planted to the field in May 1992. Plants were arranged into 10 blocks of 30 plants each with a spacing of 0.9 m between all adjacent plants. The experiment was located at Arlington, WI, on a Plano silt loam (fine-silty, mixed, mesic, Typic Argiudoll). Plants were fertilized with 56 kg N ha<sup>-1</sup> in late June.

A leaf tissue sample was clipped from each plant in mid-August 1992 at a 9-cm height. Most plants were disease free and those that were heavily diseased were ignored. Tissue samples were placed in paper bags and dried at 60°C. Dried samples were ground through a 1-mm screen of a Wiley-type mill and reground through a 1-mm screen of a cyclone mill. All samples were scanned on a near-infrared reflectance spectrometer (NIRS, Pacific Scientific Model 51A). A stratified random subset of 80 plants, made up of two random plants from each block of each population, was subjected to wet-laboratory analysis. Neutral detergent fiber (NDF) was determined according to the procedure of Van Soest et al. (1991). Klason lignin (KL) was measured as the ash-free residue remaining after cell-wall polysaccharide hydrolysis (Theander et al., 1995). Esterified ferulic acid in the cell wall was determined by 2 M NaOH extraction and high-pressure liquid chromatography (HPLC) analysis (Jung and Shalita-Jones, 1990). The concentration of EthFA was calculated as the difference between total ferulic acid, obtained by 4 M NaOH extraction at 170°C for 2 h, and the esterified fraction (Iiyama et al., 1990). Both NDF and esterified ferulic acid were determined on duplicate samples, while KL was determined on quadruplicate samples. Means over laboratory replicates were used to calibrate the NIRS for prediction of the entire set of 1200 plants.

Some field and laboratory variability was removed from the estimates of plant phenotypes by computing *t*-scores to adjust for differences among block means (Casler, 1992). Plants were selected for divergent KL and EthFA on the basis of adjusted plant values. Plants were identified in the following groups: high lignin-high ferulic acid (HL-HF), high lignin-low ferulic acid (HL-LF), low lignin-high ferulic acid (LL-HF), and low lignin-low ferulic acid (LL-LF). Eighty plants were identified, with five per group within each population. Both KL and EthFA were expressed as a proportion of NDF.

Plants were fertilized again in late April 1993 with 56 kg N ha<sup>-1</sup>. The 80 selected plants were assessed again in mid-May 1993 by the wet-laboratory procedures previously described. On the basis of the mean over the two sampling dates, 32 clones were selected, two from each group (HL-HF, HL-LF, LL-HF, and LL-LF) within each population.

### Preparation of Inoculum

Three fungal pathogens of smooth bromegrass were chosen and pathogenic isolates were collected from foliar lesions on smooth bromegrass plants. The fungi were the causal organisms of brown leafspot [*Pyrenophora bromi* (Died.) Drechs., anamorph = *Drechslera bromi* (Died.) Shoem.], spot blotch [*Cochliobolus sativus* (Ito & Kuribayashi) Dreschs. ex Dastur, anamorph = *Bipolaris sorokiniana* (Sacc.) Shoemaker], and crown rust (*Puccinia coronata* Corda). *Pyrenophora bromi* is a crop-specific, necrotrophic parasite; *B. sorokiniana* is a necrotroph that attacks other grasses; and *P. coronata* is a biotroph that can cause severe infections on smooth bromegrass and numerous other grasses.

The isolate of *D. bromi* was collected at the Arlington Experiment Station. The isolate of *B. sorokiniana* was collected from the University of Wisconsin-Madison campus. Leaves were placed on moist filter paper in petri plates for 2 d to induce sporulation. Single conidia were picked with a needle and placed on potato dextrose agar (PDA) for germination (Carter and Dickson, 1961; Shoemaker, 1962). After 2 or 3 d, agar containing mycelium from each germinated conidium

was transferred to petri plates containing PDA for the *C. sativus* isolates and agar-V8 for the *P. bromi* isolates. The plates were placed under fluorescent light and a temperature range of 21 to 23°C. The *B. sorokiniana* monoconidial isolates produced abundant conidia after 10 d of incubation. The *P. bromi* isolates produced comparatively few conidia after 20 d of incubation.

Urediniospores of *P. coronata* were collected from infected smooth bromegrass leaves at the Arlington Experiment Station. The isolate was maintained with repeated inoculations on 'PL-BDR1' smooth bromegrass, a population developed at the U.S. Regional Pasture Research Lab. (University Park, PA) for resistance to *Pyrenophora bromi* (Died.) Drechs. (Berg et al., 1989).

Conidia of *B. sorokiniana* were scraped from an active colony, suspended in deionized water and strained through cheesecloth. One drop of Tween 20 (polysorbate 20) surfactant was added per 100 mL of suspension. Conidia from four plates (isolates) were pooled and diluted in water to give a concentration of  $5$  to  $6 \times 10^4$  conidia mL<sup>-1</sup>.

Conidia and mycelia of *P. bromi* were scraped from an active colony and suspended in deionized water, agitated in a blender for about 35 s and strained through cheesecloth (Berg et al., 1986). One drop of Tween 20 surfactant per 100 mL of suspension was added. Conidia harvested from about 10 to 15 plates (isolates) were pooled and suspended in water sufficient to give a concentration of  $0.8$  to  $1.0 \times 10^3$  conidia mL<sup>-1</sup>.

Urediniospores of *P. coronata* were collected from infected PL-BDR1 bromegrass plants that had been placed in a dew chamber for 48 h to facilitate germination and infection. The plants were taken out of the dew chamber and placed on greenhouse benches under sodium light supplementation (16-h daylength) and a temperature range of 21 to 28°C. For each inoculation, three spore harvests, spaced 2 d apart, were necessary to obtain inoculum sufficient to run each experiment. The spores were collected with a vacuum pump. To prepare the inoculum, 7- to 10-d-old spores that had been stored at about 4°C, were suspended in deionized water containing 2 drops Tween 20 surfactant per 100 mL and agitated. The spore concentration was approximately of  $2$  to  $4 \times 10^3$  urediniospores mL<sup>-1</sup>.

### Greenhouse Experiments

For all three fungi, plants were sprayed evenly with the mycelia-spore suspension with an air sprayer and about 12.5 mL of inoculum suspension per flat (18 plants), placed in a dew chamber for 48 h, and maintained in a greenhouse until symptoms appeared. All inoculum suspensions were used immediately after preparation. Approximately 10 to 20 single-spore-derived isolates were bulked in approximately equal quantities for each inoculation. Plants inoculated with *B. sorokiniana* were scored 8 d after inoculation, plants inoculated with *P. bromi* were scored 10 d after inoculation and plants inoculated with *P. coronata* were scored 15 to 20 d after inoculation.

The three fungi were inoculated and evaluated in separate greenhouse experiments. The experimental design was a randomized complete block in a split-plot arrangement with four replicates. The four host populations comprised the main plots while the clones within each population were the subplots. Replication was created by vegetatively propagating each smooth bromegrass clone, from propagules with four to eight tillers. Plants were clipped to provide uniform growth prior to inoculation. Plants were inoculated when most tillers were approximately 25 to 30 cm tall. Following the first inoculation and evaluation, plants were clipped to a 7-cm-stubble height,

allowed to recover, and inoculated a second time. Each experiment was repeated in a second run, with two harvests and inoculations, with an new set of clonal propagules. Following the first harvest of the first run for *P. bromi*, the number of replicates was reduced from four to three, because of low spore production.

The youngest fully collared leaf on each of two tillers was collected and preserved on paper towels and covered with transparent tape for a posteriori disease evaluations. Diseased leaf area (DLA) for the *B. sorokiniana* and *D. bromi* experiments was estimated by means of a digital camera and imaging software (Optimas version 6.2). Lesion size (LS) was scored visually on each leaf by means of a scale from 1 to 9, where 1 = no infection, 2 = flecks, 3 = small lesions, 4 = moderately few lesions, 5 = intermediate size lesions (about 2–3 mm long), 6 = moderately large lesions, 7 = large lesions, 8 = very large lesions (>7 mm long) and 9 = leaves completely blighted (Berg et al., 1986). A random sample of 30 leaves were measured for length and width, then scanned by a planimeter to determine their area. A regression calibration was generated to predict leaf blade area from length and width measurements. The number of lesions visible without magnification was counted on each leaf and converted to lesion frequency (LF).

For the experiments inoculated with *P. coronata*, a stereoscope was used to count the number of pustules per leaf on two leaves per plant. Pustule size (PS) was determined for three pustules per leaf and two leaves per plant, by measuring the length and width of the pustules. Leaf area and pustule frequency (PF) were determined as described above. Pustule type (PT) was visually determined by means of a scale from 0 to 4 where 0 = no uredinia, 1 = chlorotic flecks, 2 = small uredinia surrounded by necrosis with limited urediniospore production, 3 = medium size uredinia with moderate sporulation, and 4 = large uredinia and abundant sporulation (Welty and Barker, 1993).

The data were subject to analysis of variance by the split-plot-in-time model (Steel et al., 1996). Single-degree-of-freedom contrasts were calculated to compare means of clones differing in KL but not in EthFA and vice versa (Table 1). Populations and clones were considered fixed effects and all other effects were considered random.

### Field Experiment

The 32 clones were clonally replicated and transplanted to a field trial designed as a randomized complete block with four replicates at Arlington in May 1994. Each propagule consisted of a 100-cm<sup>2</sup> cylinder of sod, 10 cm deep. Plants were spaced 0.9 cm apart. Plants were clipped without harvesting in July and September 1994 and in June 1995. Fertilizer was applied following clipping in June and August 1995 and 1996 at a rate of 56 kg N ha<sup>-1</sup> for each application. Undiseased leaf tissue samples were harvested as described above in early August and late September 1995 and 1996. All samples were dried, ground, and analyzed for KL, EthFA, and NDF by wet-laboratory procedures as previously described.

Severity of brown leaf spot and crown rust, the two leaf diseases prevalent in the field, were scored on three separate occasions. All disease symptoms derived from natural inoculum. Crown rust was scored by the same scale as in the greenhouse. Brown leaf spot was scored on a scale of 0 to 4, where 0 = no infection, 1 = small lesions (<2 mm long), 2 = medium-size lesions (2–3 mm long), 3 = moderately large lesions (4–6 mm long) and 4 = large lesions (>7 mm long). Field data were analyzed as described for greenhouse data.

**Table 1.** Mean differences in etherified ferulic acid (EthFA) concentration and lignin concentration for 12 pairwise comparisons between smooth brome grass clones that differ in lignin or EthFA.†

Population	Characteristics of pairwise clonal comparisons		
	Clone numbers	Difference in EthFA‡	Difference in Lignin‡
		g kg <sup>-1</sup> NDF	
Alpha	5–4	1.07**	–0.3
WB19e	9–12	0.97**	–4.5
Lincoln	19–20	0.98**	–1.9
Lincoln	21–20	0.82**	5.8
WB88S	32–26	0.65**	4.8
Alpha	6–1	–0.28	27.3**
Alpha	7–1	–0.28	24.9**
WB19e	11–15	0.17	46.3**
Lincoln	17–22	0.29	29.3**
Lincoln	18–22	0.25	28.4**
Lincoln	23–22	0.14	26.9**
WB88S	28–31	–0.10	25.5**

\*\* Effect is significantly different from zero at  $P < 0.01$ . All other comparisons had  $P > 0.50$ .

† Adapted from Casler and Jung (1999).

‡ The experiment means were: 3.36 g EthFA kg<sup>-1</sup> NDF and 158.5 g lignin kg<sup>-1</sup> NDF.

## RESULTS AND DISCUSSION

### Background and Host-Plant Characteristics

Genetic associations between two traits are typically measured by phenotypic or genotypic correlation coefficients. Correlation coefficients allow no specific inference about their cause per se and, like all second-order statistics, tend to have relatively high standard errors. The approach used to study genetic correlations in this experiment is unusual in two respects. First, by choosing specific clonal pairs with defined phenotype, measures of disease reaction can be correlated directly to lignin or EthFA without the confounding effects from a correlation between lignin and EthFA per se (Jung and Casler, 1990). Second, the repetition of these comparisons across clonal pairs, both within and among populations, allows for a degree of separation of the three causes: GPD, linkage, and pleiotropy. Consistency of contrast effects, in both sign and magnitude, would indicate pleiotropy, which should be largely independent of genetic background. Inconsistency of contrast effects, in either sign or magnitude, would indicate either GPD or linkage disequilibrium with the distinction depending on the degree of variation.

Analyses of variance revealed significant differences ( $P < 0.05$ ) among clones for all measures of disease reaction in the field and greenhouse. Clone  $\times$  run and clone  $\times$  harvest interactions were usually not significant or, if significant, usually did not involve changes in contrast effects or significance levels. Therefore, means over all harvests, runs, and replicates were used for all data presentations. These results indicate inoculation conditions, in all greenhouse and field trials, were sufficiently uniform to provide precise estimates of clone means, repeatable in both time and space. Least significant differences ( $P < 0.05$ ) were 4 to 36% of the range among clone means, indicating that relatively small differences among clone means could be detected for all variables.

**Table 2.** Mean diseased leaf area, lesion size, and lesion frequency for four populations of smooth brome grass inoculated with conidia of *Pyrenophora bromi* in greenhouse and field trials.

Population	Greenhouse traits†			Field rating§
	Diseased leaf area	Lesion size‡	Lesion frequency	
	%		cm <sup>-2</sup>	
Alpha	11.2	4.0	2.5	1.7
WB19e	10.8	4.3	2.6	1.5
Lincoln	15.0	5.0	2.3	2.1
WB88S	17.7	5.2	3.3	2.2
LSD(0.05)	3.0	0.3	0.3	0.2

† Values are means over eight clones and 13 total replicates across four inoculations.

‡ 1 = no infection, 2 = flecks, 3 = small lesions, 4 = moderately few lesions, 5 = intermediate size lesions (about 2–3 mm long), 6 = moderately large lesions, 7 = large lesions, 8 = very large lesions about (>7 mm long), and 9 = leaves completely blighted.

§ 0 = no lesions to 4 = large lesions (>7 mm long). Values are means of four replicates and three ratings.

Smooth brome grass clones selected for divergent EthFA differed by 0.65 to 1.07 g EthFA kg<sup>-1</sup> NDF, 17 to 29% of the mean (all  $P < 0.01$ ), but did not differ in lignin concentration, with differences of –4.5 to 5.8 g KL kg<sup>-1</sup> NDF (all  $P > 0.50$ ) (Table 1). Smooth brome grass clones selected for divergent KL differed by 24.9 to 46.3 g KL kg<sup>-1</sup> NDF, 18 to 33% of the mean (all  $P < 0.01$ ), but did not differ in EthFA concentration, with differences of –0.28 to 0.29 g EthFA kg<sup>-1</sup> NDF (all  $P > 0.50$ ) (Table 1). Therefore, the pairwise clonal comparisons described in Table 1 allowed a direct test of statistically independent effects of KL or EthFA on disease reaction.

Data for all contrasts are presented as contrast effects and significance levels. For all contrast effects, positive values indicates a positive correlation between the defining component (KL or EthFA) and the measure of disease reaction. Negative values indicate negative correlations.

### *Pyrenophora bromi* and *Bipolaris sorokiniana* (Necrotrophs)

For *P. bromi*, Lincoln and WB88S, the two populations that have not undergone breeding and selection, had the highest diseased leaf area, lesion size, and field reaction, and WB88S had the highest lesion frequency (Table 2). Across the four populations, 11 of 20 divergent-EthFA contrasts were significant for *P. bromi* reaction, with five negative and six positive values (Table 3). Positive and negative values were observed for all three measures of disease reaction in the greenhouse, but only positive values were observed for the field rating of disease reaction. The magnitude of positive and negative divergent-EthFA effects was generally similar. Fifteen of 28 divergent-lignin contrasts were significant for *P. bromi* reaction, with six negative and nine positive values. Low-lignin clones generally had more frequent *P. bromi* lesions, but were consistently lower in field reaction.

Alpha and Lincoln had the greatest diseased leaf area and Alpha had the largest lesions of *B. sorokiniana* (Table 4). Two of four divergent-EthFA contrasts and

**Table 3. Mean differences for various measures of host reaction to *Pyrenophora bromi*, measured in greenhouse and field trials, for 12 pairwise comparisons between smooth brome grass clones that differ in etherified ferulic acid (EthFA) or lignin concentration.**

Comparison/ Population	Clone numbers	Greenhouse traits†			Field rating‡
		Diseased leaf area	Lesion size‡	Lesion frequency	
		%		cm <sup>-2</sup>	
<b>High-EthFA minus low-EthFA</b>					
Alpha	5–4	5.6	1.0**	-0.4	-0.2
WB19e	9–12	8.4**	-0.5	-0.3	0.5**
Lincoln	19–20	-8.2**	-0.3	-1.2**	0.4**
Lincoln	21–20	-1.4	-1.2**	-0.7*	-0.1
WB88S	32–26	0.5	-0.7*	1.0**	1.0**
<b>High-lignin minus low-lignin</b>					
Alpha	6–1	1.0	-0.2	-0.7**	0.4**
Alpha	7–1	-3.2	-0.1	-1.5**	0.3*
WB19e	11–15	-0.6	0.1	-0.7*	0.2
Lincoln	17–22	-0.5	-0.7	1.2**	0.5**
Lincoln	18–22	-8.4**	-2.0**	-0.8**	0.8**
Lincoln	23–22	-3.3	-0.6	-0.3	1.4**
WB88S	28–31	7.6**	1.0**	0.3	2.1**
Experiment mean		13.6	4.6	2.7	1.9

\* Comparison between high-EthFA vs. low-EthFA or high-lignin vs. low-lignin clone was significant at  $P < 0.05$ .

\*\* Comparison between high-EthFA vs. low-EthFA or high-lignin vs. low-lignin clone was significant at  $P < 0.01$ .

† Values are means of 13 total replicates across four inoculations.

‡ See Table 2 for rating scales. Values are means of four replicates and three ratings.

four of eight divergent-lignin contrasts were significant for *B. sorokiniana* (Table 5). Significant positive and negative values were similar in magnitude and equally frequent for both selection criteria and measures of disease reaction.

Taken as a whole, the associations of *P. bromi* and *B. sorokiniana* greenhouse disease reactions with lignin or EthFA appeared to be due largely to GPD, with the possibility of some linkage disequilibrium. Contrast effects were highly variable. Neither their sign nor magnitude was specifically associated with any of the four smooth brome grass populations. Contrast effects in greenhouse trials also appeared to be more or less randomly and symmetrically distributed, with approximately 25% significant positive values, 50% nonsignificant values, and 25% significant negative values. If the loci controlling lignin or EthFA and reaction to *P. bromi* or *B. sorokiniana* were linked, we would expect to see a higher frequency of negative values, because millennia of natural selection would tend to favor phenotypes with

**Table 4. Mean diseased leaf area and lesion size for four populations of smooth brome grass inoculated with conidia of *Bipolaris sorokiniana* in greenhouse trials.**

Population	Diseased leaf area	Lesion size‡
	%	
Alpha	13.3	3.8
WB19e	12.6	3.6
Lincoln	13.4	3.5
WB88S	10.1	3.5
LSD(0.05)	2.7	0.2

† Values are means over eight clones and 13 total replicates across four inoculations.

‡ See Table 2 for rating scales.

**Table 5. Mean differences for various measures of host reaction to *Bipolaris sorokiniana*, measured in greenhouse trials, for 12 pairwise comparisons between smooth brome grass clones that differ in etherified ferulic acid (EthFA) or lignin concentration.**

Comparison/ Population	Clone numbers	Host reaction to <i>Bipolaris sorokiniana</i>	
		Diseased leaf area	Lesion size‡
		%	
<b>High-EthFA minus low-EthFA</b>			
Alpha	5–4	-6.0*	0.0
WB19e	9–12	-4.5	-1.2**
Lincoln	19–20	-3.2	-0.1
Lincoln	21–20	-0.5	-0.2
WB88S	32–26	7.0**	0.7**
<b>High-lignin minus low-lignin</b>			
Alpha	6–1	1.3	-0.5*
Alpha	7–1	-0.2	-1.3**
WB19e	11–15	-7.5**	-0.7**
Lincoln	17–22	1.3	0.5*
Lincoln	18–22	1.4	0.3
Lincoln	23–22	3.0	0.7**
WB88S	28–31	9.7**	0.5*
Experiment mean		12.4	3.6

\* Comparison between high-EthFA vs. low-EthFA or high-lignin vs. low-lignin clone was significant at  $P < 0.05$ .

\*\* Comparison between high-EthFA vs. low-EthFA or high-lignin vs. low-lignin clone was significant at  $P < 0.01$ .

† Values are means of 13 total replicates across four inoculations.

‡ See Table 2 for rating scale.

high lignin, high EthFA, and low disease reaction. Thus, loci controlling resistance to *P. bromi* and *B. sorokiniana* are likely in different linkage groups than the preponderance of loci controlling lignin and EthFA in these smooth brome grass populations. Breeding for decreased lignin or EthFA, combined with increased resistance to *P. bromi* or *B. sorokiniana* should be a relatively simple process, but will require specific selection pressure for each trait to be improved. Without specific selection pressure for low disease reaction, chance associations of these loci can lead to significant increases in disease susceptibility.

The consistent positive association between lignin concentration and field reaction to *P. bromi* suggested the possibility of tight linkage or pleiotropy. Long-term natural selection for high lignin concentration and resistance to *P. bromi* would tend to create consistent negative associations between these traits if loci for the two traits are linked. Because lignin has so many important functions in herbaceous plants (Buendgen et al., 1990; Buxton and Casler, 1993; Casler and Jung, 1999; Ostrander and Coors, 1997), it is unlikely that natural selection would act to reduce lignin concentration, unless a reduction in lignin concentration resulted in increased EthFA (Jung and Casler, 1990). Loci that confer high lignin may also regulate resistance to *P. bromi*, although a mechanism for this is unknown. While puzzling, the positive association between lignin concentration and field reaction to *P. bromi* does not appear to complicate the breeding process.

Brown leaf spot (caused by *P. bromi*) is considered the most serious disease of smooth brome grass (Casler and Carlson, 1995). Differences between the improved (Alpha and WB19e) and unimproved (Lincoln and

**Table 6.** Mean diseased leaf area, lesion size, and lesion frequency for four populations of smooth bromegrass inoculated with conidia of *Puccinia coronata* in greenhouse and field trials.

Population	Greenhouse traits†				Field reaction§
	Pustule frequency	Pustule size	Sporulating area	Pustule type‡	
	cm <sup>-2</sup>	mm <sup>2</sup>	%		
Alpha	40.8	0.06	0.77	1.2	0.8
WB19e	10.9	0.10	0.90	2.2	1.3
Lincoln	24.1	0.13	2.06	2.6	1.8
WB88S	24.5	0.06	0.06	1.4	0.7
LSD(0.05)	2.5	0.01	0.31	0.2	0.2

† Values are means over eight clones and 13 total replicates across four inoculations.

‡ Units are on a scale of 0 = no uredinia, 1 = chlorotic flecks, 2 = small uredinia surrounded by necrosis with limited urediniospore production, 3 = medium size uredinia with moderate sporulation, and 4 = large uredinia and abundant sporulation.

§ Same scale as pustule type rating in greenhouse. Values are means of four replicates and three ratings.

WB88S) populations confirm previous reports that selection has improved resistance to this fungus (Casler and Drolsom, 1995; Casler et al., 2000). Conversely, the relative infrequency with which *B. sorokiniana* causes serious disease on smooth bromegrass (Braverman, 1986; Vogel et al., 1996) suggests little selection pressure for this disease, probably explaining the relatively small differences among populations.

Both *P. bromi* and *B. sorokiniana* are necrotrophic fungi. *Pyrenophora bromi* penetrates the cells directly, the epidermal and mesophyll cell walls collapse and turn brown after penetration and the fungus establishes itself in the intracellular space, able to feed on the products that result from the rupture of the cells (Chamberlain and Allison, 1945). *Bipolaris sorokiniana* penetrates leaf tissue directly through the cuticle at the junction of lateral walls of epidermal cells or by stomata (Couch, 1976; Mower and Millar, 1963). Apparently lignin and EthFA of smooth bromegrass are not important factors limiting the penetration of these fungi into cells. This result is largely similar to that of Sherwood (1996) for lignin concentration of smooth bromegrass populations that were selected for *P. bromi* resistance, although Sherwood found differences in vascular bundle architecture between *P. bromi*-resistant and susceptible lines, suggesting that some aspects of cell wall structure may be a factor in resistance to this fungus. Selection for divergent cell-wall concentration in smooth bromegrass did not lead to consistent changes in reaction to *B. sorokiniana*, while correlations between reaction scores to five major alfalfa diseases and lignin or cell-wall concentration were generally not significant (Fonseca et al., 1999).

### *Puccinia coronata* (Biotroph)

Lincoln and WB19e appeared to be most susceptible to *P. coronata*, indicated by their relatively high means for pustule size, pustule type, and field reaction (Table 6). Alpha and WB88S appeared fairly resistant to *P. coronata* despite relatively high pustule frequency and moderate sporulating area for Alpha. Thirteen of 25 divergent-EthFA contrasts were significant for *P. coro-*

**Table 7.** Mean differences for various host reactions to *Puccinia coronata*, measured in greenhouse and field trials, for 12 pairwise comparisons between smooth bromegrass clones that differ in etherified ferulic acid (EthFA) or lignin concentration.

Comparison/ Population	Clone numbers	Greenhouse traits†				Field reaction§
		Pustule frequency	Pustule size	Sporulating area	Pustule type‡	
		cm <sup>-2</sup>	mm <sup>2</sup>	%		
<b>High-EthFA minus low-EthFA</b>						
Alpha	5-4	-0.1	-0.01	-0.03	-0.2	0.3**
WB19e	9-12	-4.4**	0.03**	-0.71	-0.3*	0.1
Lincoln	19-20	-5.3**	-0.04**	-1.84	-0.3*	0.2
Lincoln	21-20	-1.9**	0.02*	-0.29	0.6**	1.5**
WB88S	32-26	-0.1	-0.07**	-0.92	-0.7**	-0.1
<b>High-lignin minus low-lignin</b>						
Alpha	6-1	-13.4**	-0.14**	-2.44	-3.2**	-0.5**
Alpha	7-1	-13.4**	-0.14**	-2.44	-3.2**	-0.5**
WB19e	11-15	3.4**	0.11**	-0.56	2.5**	0.1
Lincoln	17-22	-6.1**	-0.08**	-2.83*	-1.4**	-2.0**
Lincoln	18-22	-3.1**	0.00	-1.61	-0.3*	-1.7**
Lincoln	23-22	-1.8**	-0.02*	-1.14	-0.5**	-0.3
WB88S	28-31	-2.1**	-0.03**	-0.25	-0.9**	-0.2
Experiment mean		5.2	0.09	1.13	1.9	1.2

\* Comparison between high-EthFA vs. low-EthFA or high-lignin vs. low-lignin clone was significant at  $P < 0.05$ .

\*\* Comparison between high-EthFA vs. low-EthFA or high-lignin vs. low-lignin clone was significant at  $P < 0.01$ .

† Values are means over two clones and 13 total replicates across four inoculations.

‡ See Table 6 for rating scale.

§ Values are means of four replicates and three ratings.

*nata*, with eight negative and five positive values (Table 7). Negative values were generally of greater magnitude than positive values for host reaction to *P. coronata* in the greenhouse. Only positive values were observed for field reaction to *P. coronata*. Twenty-five of 35 divergent-lignin contrasts were significant for *P. coronata*, with 22 negative and three positive values. Negative divergent-lignin effects were prevalent for both greenhouse and field measures of *P. coronata* reaction.

The results for *P. coronata* clearly supported the conclusion that pleiotropy or linkage disequilibrium are the cause of negative genetic correlations between *P. coronata* reaction and lignin. Linkage disequilibrium cannot be ruled out if resistance to *P. coronata* and high lignin both confer evolutionary fitness to smooth bromegrass. Millennia of natural selection could concentrate alleles for *P. coronata* resistance and high lignin into common linkage blocks that, without several generations of random mating and selection, would appear to be pleiotropic. This association was clearly with lignin concentration per se, although EthFA may play a minor role in regulating resistance to *P. coronata*. Most of the significant EthFA contrast effects were negative, suggesting some level of genetic relationship, but more likely due to linkage than to pleiotropy.

*Puccinia coronata* penetrates leaf tissue through stomata; once in the substomatal chamber the fungus is thought to penetrate the mesophyll cells by means of enzymatic degradation that is restricted to the point of contact between the appressorium and the cell wall (Cooper, 1983). Increased lignin concentration may reduce the efficiency and/or effectiveness of cellulolytic enzymes from *P. coronata*, much as it does in ruminant livestock (Van Soest, 1994). Lignin is thought to inhibit

cell wall degradation in ruminants by acting as a physical barrier limiting enzymatic access to polysaccharides (Cowling, 1975; Van Soest, 1973) or by covalent cross-linkages between lignin and arabinose units of xylan chains (Jung and Deet, 1993). The presence of several negative associations between EthFA and *P. coronata* reaction suggests that covalent cross-linkages involving ferulic acid bridges between lignin and hemicellulose may be associated with inhibition of *P. coronata* penetration.

A positive association between *P. coronata* reaction and water-soluble carbohydrate (WSC) concentration, which is positively and closely associated with in vitro dry matter digestibility (IVDMD), has been observed in three studies of perennial grasses (Buxton and Casler, 1993; Cagas, 1979; Cagas and Lukas, 1988). A 37% increase in WSC concentration because of selection resulted in a 128% increase in infection of perennial ryegrass (*Lolium perenne* L.) by *P. coronata* (Breese and Davies, 1970). Selection for resistance to *P. coronata* in meadow fescue (*Festuca pratensis* Huds.) resulted in reduced WSC concentration (Cagas and Lukas, 1988), while *P. coronata* infection in rust-susceptible and high-WSC populations reduced their WSC concentration (Cagas, 1979). Quantitative trait loci for *P. coronata* resistance and WSC concentration have been mapped in very close proximity on the perennial ryegrass genome (Turner et al., 2001), suggesting that alleles for *P. coronata* resistance and low WSC might be tightly linked or pleiotropic.

Infection by rust fungi decreases forage nutritional value by decreasing WSC and IVDMD (Cagas, 1979; Edwards et al., 1981) and increasing lignification and cell-wall development in host plants (Nicholson and Hammerschmidt, 1992; Ride, 1978; Vance et al., 1980). Thus, resistance to fungal diseases can protect host plants from these losses in forage nutritional value (Karn et al., 1989; Lenssen et al., 1991). This relationship may obscure the true relationship between disease resistance and forage nutritional value if forage nutritional value traits, such as lignin and WSC, are measured on infected plant tissue. In such cases, plants may appear low in forage nutritional value because of genes controlling lignin or WSC or because of genes for susceptibility to the disease organism. The studies cited above, as well as this study, appear to have avoided this problem by sampling disease-free tissue.

A consistent negative relationship was found between lignin concentration and *P. coronata* reaction in smooth brome grass. Results from other grasses suggest that this positive association between rust reaction and forage nutritional value may transcend species boundaries. Furthermore, there appears to be two or three distinct mechanisms by which rust resistance may be enhanced at the expense of forage quality: reduced WSC concentration, increased lignification, and (possibly) increased ferulate cross linking. If these are indeed genetic mechanisms for rust resistance in the Poaceae, forage grass breeders must find alternative mechanisms for rust resistance. Ironically, breeding for increased rust resistance by such a mechanism would have the same effect as

rust infection per se—a reduction in forage nutritional value (Cagas and Lukas, 1988). Furthermore, breeding for increased forage nutritional value by one of these mechanisms could strip plants of their inherent rust resistance (Breese and Davies, 1970).

The current literature on this subject is insufficient to permit definitive conclusions to be drawn about the relationship between *P. coronata* resistance and forage nutritional value. More research is required to determine if lignin, ferulic acid, and WSC are mechanistically involved in rust resistance in the Poaceae and if there are alternative mechanisms of rust resistance that may not involve a sacrifice in forage nutritional value. The large number of rust resistance loci available in the Poaceae suggests that numerous resistance mechanisms may be involved. Additional reports are also needed regarding the forage nutritional value status of genetic lines differing in rust resistance and vice versa.

Finally, the observation that lignin and EthFA were related to host resistance for a biotrophic fungus, but not for two necrotrophic fungi, was somewhat surprising. The capability of these two necrotrophic fungi to degrade cell walls enzymatically appears to be physiologically independent of cell-wall chemistry and structure. This suggests that necrotrophic fungi contain a more diverse array of hydrolytic enzymes than do rumen microflora, perhaps giving them the ability to cleave ether and/or ester linkages between phenolic residues and polysaccharide chains. Conversely, the biotrophic fungus showed distinct similarities to rumen microflora, for which cell-wall degradation is severely restricted by lignin and EthFA concentration (Casler and Jung, 1999). Greater a priori lignification and/or ferulic cross-linking of cell walls appears to limit appressorium development or effectiveness in this biotrophic fungus.

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