# Helicoverpa zea (Lepidoptera: Noctuidae) and Spodoptera frugiperda (Lepidoptera: Noctuidae) Responses to Sorghum bicolor (Poales: Poaceae) Tissues from Lowered Lignin Lines

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RESEARCH

# *Helicoverpa zea* (Lepidoptera: Noctuidae) and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) Responses to *Sorghum bicolor* (Poales: Poaceae) Tissues From Lowered Lignin Lines

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**ABSTRACT.** The presence of lignin within biomass impedes the production of liquid fuels. Plants with altered lignin content and composition are more amenable to lignocellulosic conversion to ethanol and other biofuels but may be more susceptible to insect damage where lignin is an important resistance factor. However, reduced lignin lines of switchgrasses still retained insect resistance in prior studies. Therefore, we hypothesized that sorghum lines with lowered lignin content will also retain insect resistance. Sorghum excised leaves and stalk pith *Sorghum bicolor* (L.) Moench (Poales: Poaceae) from near isogenic *brown midrib* (*bmr*) *6* and *12* mutants lines, which have lowered lignin content and increased lignocellulosic ethanol conversion efficiency, were examined for insect resistance relative to wild-type (normal BTx623). Greenhouse and growth chamber grown plant tissues were fed to first-instar larvae of corn earworms, *Helicoverpa zea* (Boddie) and fall armyworms *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), two sorghum major pests. Younger *bmr* leaves had significantly greater feeding damage in some assays than wild-type leaves, but older *bmr6* leaves generally had significantly less damage than wild-type leaves. Caterpillars feeding on the *bmr6* leaves often weighed significantly less than those feeding on wild-type leaves, especially in the *S. frugiperda* assays. Larvae fed the pith from *bmr* stalks had significantly higher mortality compared with those larvae fed on wild-type pith, which suggested that *bmr* pith was more toxic. Thus, reducing lignin content or changing subunit composition of bioenergy grasses does not necessarily increase their susceptibility to insects and may result in increased resistance, which would contribute to sustainable production.

**Key Words:** resistance, agricultural entomology, plant resistance

There is global interest in developing fuels for the transportation sector from renewable resources as a means to reduce dependency on petroleum and other finite fuel sources. One of the impediments to converting biomass to biofuels is the presence of the cell wall polymer lignin, which interferes with the release of sugars from the corresponding cell wall polysaccharides, cellulose, and hemicelluloses during enzymatic saccharification (Dien et al. 2009). Therefore, reducing lignin levels through traditional breeding or genetic engineering is a target for bioenergy feedstock improvement but reducing lignin in cell walls may impair pest resistance where lignin is a major component of resistance.

There are mutants of many plant species that have altered lignin content and subunit composition. The *brown midrib* phenotype has long been associated with reduced lignin content of maize, *Zea mays* L. (referred to as *bm*), and sorghum, *Sorghum bicolor* (L.) Moench (Poales: Poaceae) (referred to as *bmr*) (Sattler et al. 2010). Although *bm* plants have reduced lignin and improved forage digestibility, maize *bm* plants can have increased stalk breakage caused by insect damage or pathogens when compared with wild-type lines (Barriére and Agillier 1993). However, *brown midrib* mutants of other grass species are acceptable agronomically (Pedersen et al. 2005). The sorghum *bmr6* phenotype is due to a nonsense mutation that causes premature truncation of the cinnamyl alcohol dehydrogenase (CAD) open reading frame. The *bmr6* plants have reduced levels of lignin relative to the wild-type and incorporate phenolic aldehydes into the lignin polymer in addition to phenolic alcohols as in wild-type but a lignin relative ratio of two major subunits (syringyl and guaiacyl lignin; S/G ratio) similar to wild-type (Palmer et al. 2008, Saballos et al. 2009, Sattler et al. 2009). The sorghum *bmr12* phenotype is due to a nonsense mutation that causes premature truncation of caffeic *O*-methyl transferase (COMT). The *bmr12* plants have reduced lignin levels, and syringyl subunits within lignin are greatly reduced relative to other subunits compared with wild-type plants (Bout and Vermerris 2003, Palmer et al. 2008). These two *bmr* mutants have increased ethanol conversion efficiency compared with wild-type (Dien et al. 2009). However, the effects of these two mutations on pest resistance have not been examined. On the basis of prior work with switchgrass (Dowd et al. 2013), we hypothesized that the *bmr6* and *bmr12* sorghum lines will retain insect resistance compared with the near isogenic wild-type line.

Here, we report on studies with corn earworms, *Helicoverpa zea* (Boddie) and fall armyworms, *Spodoptera frugiperda*) (J.E. Smith) (both Lepidoptera: Noctuidae) that indicate *bmr* leaves and pith were generally as resistant or more resistant to feeding by these two insects compared with the wild-type near isogenic line. These two species of insects can be serious pests of sorghum and other grasses (Metcalf and Metcalf 1993), and thus impaired insect resistance would impede use of *bmr6* or *bmr12* to improve lignocellulosic biofuels production.

## Materials and Methods

**Insects.** The *H. zea* and *S. frugiperda* were obtained from colonies reared on pinto bean-based diet at 27 ± 1°C, 50 ± 10% relative humidity (RH), and a photoperiod of 14:10 (L:D) h, as described previously (Dowd 1988). First instars without any prior feeding experience were randomly selected and used in all assays.

**Plant Phenolic Bioassays.** The plant phenolics adipic acid (99%), *p*-coumaric acid (≥98.0%), ferulic acid (99%), sinapic acid (≥98%), syringic acid (≥95%), and vanillic acid (97%) were obtained from

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Sigma/Aldrich (St. Louis, MO) (www.sigmaaldrich.com). They were incorporated into warm, still liquid pinto bean diets by blending with a vortex mixer as described previously (Dowd 1988). Phenolic acids were incorporated into diet at concentrations reported previously from near isogenic wild-type, *bmr6,* and *bmr12* RTx430 stalks (Palmer et al. 2008) as follows for compounds listed above in order, in µg/g wet weight: "wild-type" was 67.8, 30.0, 8.8, 0.0, 5.6, and 17.7; "*bmr6"* was 36.1, 84.0 42.8, 13.8, 21.0, and 9.3; and "*bmr12"* was 51.5, 30.1, 21.1, 4.8, 3.2, and 20.7.

Leaf disk diets were prepared by substituting wild-type leaf tissue (12 leaf stage plants; fourth leaf from the top) for pinto beans, wheat germ, and brewer's yeast and a proportional amount of water from the pinto bean diet (Dowd 1987) to provide similar nutritional composition as leaves for examining the effects of added phenolics under nutrient stress conditions. Diet was dispensed between two metal plates spaced 1 mm apart, cut into 4-mm disks when firm and freeze dried (Dowd et al. 2011). Because the wild-type stalks already contained some phenolics at higher levels than the *bmr* lines, only those phenolics that were at a higher concentration in the *bmr* lines were added to the disks, and only those phenolics that had levels at least 10% higher in the *bmr* compared with wild-type plants were used. For the "*bmr6"*-simulated leaf disks, ferulic, vanillic, sinapic, and syringic acids were added at 54.0, 34.0, 13.8, and 16.0 µg/g, respectively. For the "*bmr12*"-simulated disks, only vanillic acid was added at 12.3 µg/g wet weight. For the "wild-type"-simulated disks, no additional phenolic acids were added (only solvent control). Freeze-dried disks were used as described previously (Dowd et al. 2011). The phenolics acids were added in acetone to each dry disk, the acetone was evaporated in a chemical fume hood for 30 min, and the diet disks were rehydrated with sterile distilled water.

Each treatment of the pinto bean diet was cut into pieces sufficient for ad libitum feeding, and each piece was placed in a separate well of a 24-well tissue culture plate (Dowd 1988). An individual larva was added to each well. For leaf diet disk assays, rehydrated sorghum leaf disk materials were placed on a Teflon disk inside a Petri dish containing 3% water agar. Ten first-instar larvae were added to each dish. The surviving larvae were weighed after 5 d (pinto bean diet pieces) or 3 d (sorghum leaf disks).

**Plants.** Wild-type, *bmr6*, and *bmr12* near isogenic plants in the background BTx623 were used in the present experiments. The near isogenic lines containing the *bmr6* and *bmr12* alleles were previously developed by crossing source of the *bmr6* or *bmr12* allele with BTx623 and four generations of backcrossing to BTx623 (Pedersen et al. 2006). When plants were grown in the Peoria greenhouse or a growth room (Peoria only), pots containing a previously reported soil and fertilizer mix, which contained bark mix (Dowd et al. 2007), were used and grown under previously reported conditions (Dowd et al. 2007). Growth conditions in the Peoria, Illinois greenhouse (which has supplemental lighting, heating, and cooling) and growth room were 24 ± 2°C day and 18 ± 2°C night, with a photoperiod of 14:10 (L:D) h at 50 ± 10% RH. The soil mix for plants grown in the Lincoln, Nebraska greenhouse, consisted of soil:peat moss:vermiculite:perlite:sand (4:1:1:1:1), and plants were fertilized approximately every 7 d at 5 mL per pot with 11:15:11 (N:P:K; Ferti-lome Gardener's Special, VPG, Bonham, TX). Temperatures were maintained at 29.5 ± 0.5°C during the day and 26.5 ± 0.5°C during the night with a photoperiod of 16:8 (L:D) h and 50 ± 10% RH. Leaves from plants grown in the greenhouse in Lincoln, NE, were shipped overnight on flaked ice and used the day of arrival. From 10 to 20 plants of each genotype were used in each experiment.

**Plant Tissue Bioassays.** All bioassays were performed in Peoria. Leaf section bioassays were performed using Petri dishes with tight fitting lids as described previously (Dowd et al. 2007). Immature leaves (2nd from top, 12 cm of the leaf including the tip) of five leaf plants and more mature leaves (4th from top, 20 cm of the leaf including the tip) of 10–12 leaf stage plants were used in the bioassays. After 2 d, additional 4 cm long leaf pieces were removed from the initial harvest point of the 10–12 leaf plants to investigate wounding induced factors. Approximately 2 $cm^2$ leaf pieces were used in the assays, which were removed 0–6 cm from the leaf base, except for the additional piece of 4-cm long piece removed 2 d later from previous harvest site. There was sufficient leaf material for ad libitum feeding for the duration of each experiment. Leaf pieces were placed in Petri dishes with tight fitting lids, containing moistened filter paper, and 10 newly hatched first-instar caterpillars without prior feeding experience were added to each dish.

For pith assays, stalks sections were harvested from growth room grown plants after other assays were completed. The plants were post-flowering, at soft dough stage of grain filling, and the stalk were green and turgid. Approximately 1 cm in diameter internodes between the first and second leaf from the top were harvested, which had color differences in the pith amongst the respective lines. Approximately 2-cm-long pith sections were placed in the Petri dishes as described for leaf piece bioassays with 10 first-instar caterpillars.

Feeding damage on leaves was evaluated by determining the total number of 0.25 $mm^2$ or 1 $mm^2$ hole equivalents after 2 d as described previously (Dowd et al. 2007, 2011); caterpillars often molted to the second instar during that period. Because little mortality occurred in leaf assays, survivors were weighed using a Mettler AE104 analytical balance (Mettler Instrument Corp, Hightstown, NJ), which is accurate to 0.01 mg (Dowd et al. 2007, 2011). For the pith assays, insect feeding damage was not determined due to the difficulty of quantifying varying lengths and depths of feeding damage within the pith sections. Only mortality was determined after a 3-day feeding period due to highly variable weights resulting from cannibalism of dead larvae.

**Statistical Analysis.** Statistically significant differences in overall insect mortality for each main effect plant variety treatment were determined by Chi square analysis, and differences in feeding rates and weights of survivors were determined by analysis of variance. SAS Proc Freq was used for Chi square analysis, and SAS Proc GLM was used for the other analyses. Windows Version 8.0 of the SAS software (SAS Institute 1999) was used.

## Results

Consistent with our hypothesis, both first-instar *H. zea*- and *S. frugiperda*-fed sorghum leaves at different plants stage generally did not show statistically significant differences ($P < 0.05$) in feeding damage, mortality (very limited, so data not shown), or survivor weights indicative of reduced resistance for *bmr* compared with wild-type lines after 2 d of feeding on leaves (Table 1, Supp Table S1 [online only]). However, there was one exception; *H. zea*-fed leaves from five-leaf stage *bmr12* plants; leaves from this plant stage had significantly ($P < 0.01$) greater feeding damage ($mm^2$) relative to wild-type leaves in both experiments ($F = 21.08$, $P < 0.001$, df = 1, 19 and $F = 14.06$, $P = 0.001$, df = 1,23) (Table 1). In contrast, there were several cases where the leaves of mutant lines were significantly more resistant to one or both of the insect species, based on significantly lower amounts of leaf damage or lower weights of survivors (Table 1). The leaves from both *bmr6* 5 leaf and 10–12 leaf plants had significantly less ($P < 0.05$ or $P < 0.01$) feeding damage from *S. frugiperda* compared with wild-type leaves in nearly all experiments. The amounts of *S. frugiperda* feeding damage on *bmr12* leaves were not significantly different from wild-type leaves. Similar results were obtained whether plants were grown in the greenhouse or plant growth room, although some variation of some results was noted between the first and second experiments for some components.

Damaging the leaves mechanically did not change relative resistance trends for the *S. frugiperda* that were observed for *bmr* versus wild-type undamaged leaves. Results with the *H. zea* were more variable but significantly ($P < 0.05$ or 0.01) less leaf material was removed from *bmr6* leaves than wild-type leaves in several cases, which indicated *bmr6* leaves were more resistant. For one experiment, in contrast to observations from first assay, when another piece was removed from

**Table 1 Sorghum leaf effects on *H. zea* and *S. frugiperda* larvae**

| | *H. zea* feeding ($mm^2$) | Weight (mg) | *S. frugiperda* feeding ($mm^2$) | Weight (mg) |
|---|---|---|---|---|
| Ten-leaf plant, mature leaf (greenhouse-Lincoln, NE) | | | | |
| Wild-type | 49 ± 2a | 0.23 ± 0.01a | 111 ± 7a | 0.53 ± 0.02a |
| *bmr6* | 53 ± 2a | 0.19 ± 0.01b* | 137 ± 9b | 0.47 ± 0.02b* |
| *bmr12* | 54 ± 2a | 0.19 ± 0.01b* | 126 ± 9ab | 0.48 ± 0.02ab |
| Ten-leaf plant, mature leaf (greenhouse-Peoria, IL) | | | | |
| Wild-type | 29 ± 1a | 0.24 ± 0.01a | 65 ± 2a | 0.69 ± 0.02a |
| *Bmr6* | 25 ± 2a | 0.17 ± 0.01b* | 53 ± 2b | 0.61 ± 0.02b* |
| *Bmr12* | 29 ± 1a | 0.22 ± 0.01a | 63 ± 1a | 0.72 ± 0.03a |
| Five-leaf plant, immature leaf (growth room) experiment 1 | | | | |
| Wild-type | 39 ± 1a | 0.21 ± 0.01a | 68 ± 2a | 0.35 ± 0.01a |
| *bmr6* | 48 ± 2b* | 0.23 ± 0.01a | 54 ± 2b* | 0.31 ± 0.01b* |
| *bmr12* | 50 ± 2b* | 0.23 ± 0.01a | 70 ± 1a | 0.34 ± 0.01a |
| Five-leaf plant, immature leaf (growth room) experiment 2 | | | | |
| Wild-type | 32 ± 1a | 0.36 ± 0.01a | 42 ± 1a | 0.46 ± 0.02a |
| *bmr6* | 29 ± 1b | 0.32 ± 0.02ab | 35 ± 1b | 0.37 ± 0.01b* |
| *bmr12* | 38 ± 1c | 0.31 ± 0.02b | 42 ± 2a | 0.52 ± 0.02a |
| Twelve-leaf plant, mature leaf (growth room) experiment 1 | | | | |
| Wild-type | 48 ± 3a | 0.24 ± 0.01a | 59 ± 3a | 0.37 ± 0.01a |
| *bmr6* | 41 ± 1b | 0.21 ± 0.01b | 44 ± 2b* | 0.42 ± 0.02b |
| *bmr12* | 43 ± 4ab | 0.20 ± 0.01b* | 59 ± 2a | 0.34 ± 0.01c* |
| Twelve-leaf plant, mature leaf (growth room) experiment 2 | | | | |
| Wild-type | 29 ± 1a | 0.26 ± 0.01a | 65 ± 3a | 0.39 ± 0.02a |
| *bmr6* | 25 ± 1b | 0.17 ± 0.01b | 53 ± 2b | 0.30 ± 0.02b* |
| *bmr12* | 29 ± 1a | 0.27 ± 0.01a | 63 ± 3a | 0.37 ± 0.02a |
| Twelve-leaf plant, mature leaf—recut (growth room) experiment 1 | | | | |
| Wild-type | 56 ± 3a | 0.23 ± 0.01a | 71 ± 4a | 0.42 ± 0.02a |
| *bmr6* | 45 ± 3b* | 0.21 ± 0.01ab | 58 ± 3b* | 0.36 ± 0.01b* |
| *bmr12* | 41 ± 3b* | 0.20 ± 0.01b | 61 ± 4ab | 0.38 ± 0.02ab |
| Twelve-leaf plant, mature leaf—recut (growth room) experiment 2 | | | | |
| Wild-type | 29 ± 2a | 0.21 ± 0.01a | 38 ± 2a | 0.48 ± 0.02a |
| *bmr6* | 18 ± 1b | 0.16 ± 0.01b* | 29 ± 2b | 0.33 ± 0.01b* |
| *bmr12* | 29 ± 1a | 0.24 ± 0.01a | 42 ± 2a | 0.55 ± 0.03a |

At least 10 leaves of each line were used. Mean ± standard error values reported are in $mm^2$ (feeding) and mg (weights) after 2 d of feeding. Values in columns for the same experiment followed by different letters are significantly different at $P < 0.05$ by analysis of variance. Values of *bmr* (low lignin) lines in columns for the same experiment followed by a "*" are significantly different from wild-type (normal lignin) values at $P < 0.01$.

**Table 2. Sorghum stalk pith effects on *H. zea* and *S. frugiperda* larvae**

| | *H. zea*, % mortality | *S. frugiperda*, % mortality |
|---|---|---|
| Experiment 1 | | |
| Wild-type | 1.7a | 10.4a |
| *bmr6* | 36.4b* | 27.7b |
| *bmr12* | 29.9b* | 25.5b |
| Experiment 2 | | |
| Wild-type | 6.4a | 2.7a |
| *bmr6* | 47.3b* | 21.2b* |
| *bmr12* | 37.3b * | 34.0b* |

Pith from at least eight plants of each line was used. Values followed by different letters for the same experiment are significantly different at $P < 0.05$ by Chi square analysis. Values of *bmr* (low lignin) lines in columns for the same experiment followed by a "*" are significantly different from wild-type (normal lignin) values at $P < 0.01$.

the same leaf a few days later, *H. zea* feeding damage on *bmr12* leaf pieces was significantly ($P < 0.01$) ($F = 10.14$, $P = 0.004$, df = 1,19) less compared with wild-type leaves, whereas no effect of *S. frugiperda* feeding damage was observed.

After 2 d of feeding, weights of *S. frugiperda* larvae that fed on *bmr6* leaves were generally significantly ($P < 0.05$ or 0.01) lower than larvae that fed on wild-type leaves. Weights of *S. frugiperda* that fed on *bmr12* leaves were generally similar to those that fed on wild-type leaves, although in a few cases, the weights of larvae fed on the *bmr12* leaves were lower than larvae fed on the wild-type leaves. Some of the same trends were observed with *H. zea*, although weights of larvae that fed on *bmr6* leaves were less often significantly lower than larvae fed on wild-type leaves (Table 1). The *H. zea* larvae tended to consume significantly more of the leaf tissue from the 5 leaf *bmr* plants compared with the wild-type leaf tissue but did not weigh significantly more.

The stalk pith was selected because its color was similar to the reddish-brown leaf veins of *bmr* mutants, and more uniformly lignified tissue may provide better insight into the effects of *bmr* mutants on insect susceptibility. *H. zea* and *S. frugiperda* larvae had significantly higher mortality ($P < 0.05$ and $P < 0.01$) when fed the pith from both *bmr* lines when compared with wild-type pith for both experiments (Table 2, Supp Table S2 [online only]). The difference was most dramatic for *H. zea* larvae where very little mortality occurred when they fed on wild-type pith in both experiment, but nearly 50% mortality of larvae occurred when they were fed *bmr6* pith in the second experiment (Table 2).

Larvae fed on pinto bean diet disks containing concentrations of free phenolic acids that corresponded to the levels observed in wild-type, *bmr6,* and *bmr12* plant stalks (Palmer et al. 2008) did not show any significant differences in survivor weights. However, *S. frugiperda* that fed on the artificial pinto bean diets with added phenolic acids simulating those in the *bmr6* and *bmr12* lines were significantly ($P < 0.05$) ($F = 5.73$, $P = 0.020$, df = 1,52; and $F = 5.28$, $P = 0.026$, df = 1,48; respectively) smaller than larvae that fed on the control diet (Table 3, Supp Table S3 [online only]). *S. frugiperda*-larvae-fed diet disks made from wild-type sorghum leaves and supplemented with phenolic acids to simulate concentrations in *bmr* stalks, showed significant differences in weights that depended on the type of supplementation. *S. frugiperda*-larvae-fed "*bmr6"*-type leaf diet disks (containing additional ferulic, vanillic, sinapic, and syringic acids) and "*bmr12"*-type leaf diet disks (containing additional vanillic acid) weighed significantly ($P < 0.01$) less ($F = 14.60$, $P < 0.001$, df 1,24; $F = 13.99$, $P = 0.001$, df = 1,22; respectively) than larvae-fed "normal" leaf diet disks (solvent only, no additional phenolic acids) (Table 3).

## Discussion

Consistent with our hypothesis, no general increased susceptibility of either *bmr6* or *bmr12* leaves to the insect species tested in this study were observed, although in some cases younger *bmr* leaves from five-leaf stage plants had more feeding damage than wild-type leaves. Interestingly, there were several instances where *bmr* leaves and pith were more resistant to these larvae compared with the wild-type tissues based on higher mortality (pith) or lower amounts of feeding (leaves). The enhanced resistance was especially evident for *S. frugiperda*-larvae-fed *bmr6* tissue. Variations between experiments may have been due to environmental factors (temperatures and light intensity in the different growth locations) or subtle differences plant developmental stage. Genotype by environment effects can influence agronomic traits in *brown midrib* lines (Cassler et al. 2003, Palmer et al. 2008), which can also influence insect resistance (Dowd and Johnson 2009). The relative trend for resistance was generally consistent whether the insects fed on undamaged or previously damaged leaves, but we cannot rule out that the relationship may change over time due to resistance factors induced specifically by insect feeding, which can vary depending on the insect species involved (Rodriguez-Saona et al. 2010). However, based on reports in maize (Shen et al. 2000), the time frame we used for the assays should have been sufficient to observe induced responses.

In some cases, feeding damage to leaves was similar between wild-type and *bmr* lines, but postfeeding weights were significantly less for larvae-fed *bmr* leaves compared with larvae-fed wild-type leaves. In other cases, feeding damage on *bmr* leaves was significantly greater than on wild-type leaves, but weights of survivors were not significantly different. Both of these situations suggest that the *bmr* lines were less nutritious than the wild-type, and compensatory feeding was

**Table 3. Altered phenolic diet effects simulating concentrations in sorghum stalks on *H. zea* and *S. frugiperda* larvae**

| | *H. zea*, % mortality | Weight (mg) | *S. frugiperda*, % mortality | Weight (mg) |
|---|---|---|---|---|
| Artificial pinto bean-based diet | | | | |
| Solvent control | 0.0a | 6.3 ± 0.3a | 0.0a | 4.6 ± 0.2a |
| "Wild-type" | 0.0a | 6.7 ± 0.4a | 0.0a | 4.4 ± 0.4a |
| *"bmr6"* | 0.0a | 6.8 ± 0.2a | 0.0a | 3.4 ± 0.3b |
| *"bmr12"* | 0.0a | 6.1 ± 0.4a | 0.0a | 3.5 ± 0.4b |
| Sorghum leaf disk diet | | | | |
| "Wild-type" | 0.0a | 0.21 ± 0.03a | 0.0a | 0.20 ± 0.01a |
| *"bmr6"* | 0.0a | 0.20 ± 0.02a | 0.0a | 0.13 ± 0.01b* |
| *"bmr12"* | 0.0a | 0.15 ± 0.02a | 0.0a | 0.13 ± 0.01b* |

Values are after 3 d for leaf disk diets and 5 d for artificial diet. Weights are means ± standard errors in mg. Values followed by different letters for like studies are significantly different at $P < 0.05$ by Chi square analysis (mortality) or analysis of variance (weights). Values of "*bmr*" diets in columns for the same experiment followed by a "*" are significantly different from "wild-type" diet values at $P < 0.01$. See Materials and Methods for phenolic additions that simulate the phenolic acid compositions found in wild-type, *bmr6,* and *bmr12* stalks (Palmer et al. 2008).

occurring in some cases, but it did not result in greater larval weights. A compensatory feeding response has also been reported when *S frugiperda* were fed diet with increased levels of non-nutritional cellulose (Wheeler and Slansky 1991). In some cases, resistance was due to antibiosis (toxic compounds), which is indicated when reduced feeding results in lower larval weights. This phenomenon was observed in several cases when the larvae fed on *bmr6* compared with wild-type leaves. Lignin can be an important insect resistance factor in plants (Swain 1979), although the complex composition of lignin and interconnected metabolic network involved the synthesis of its precursors makes it difficult to predict how altering lignin concentration or composition of plants will affect insect resistance. Reduced ferulate crosslinking in fescue (*Festuca* sp.) resulted in increased damage by fall armyworms (Buanafina and Fescemyer 2012), which illustrates the importance of ester and ether-linked ferulic acid that are separate from lignin polymers in grass cell walls. However, low lignin lines of switchgrass (*Panicum virgatum* L.) (where lignin levels were reduced from ~7 to 4%) that had higher rates of saccharification and fermentation retained resistance to *S. frugiperda* compared with high lignin lines (Dowd and Johnson 2009, Dowd et al. 2013). Age of plants and tissues can also influence resistance levels (Smith et al. 1994), which we also observed in evaluating feeding on leaves from 5-leafed compared with 10- or 12-leafed plants. Similarly, a significant positive association was observed between degree of insect resistance and lignin levels in younger switchgrass plants but not older plants (Dowd et al. 2013). This study indicated sorghum lines with lowered lignin can result in greater resistant to insects than lines unimpaired in their ability to synthesize lignin.

Unexpectedly, the pith of both *bmr* lines was highly resistant relative to wild-type pith, based on higher rates of mortality observed for larvae-fed *bmr* pith. Previous studies have indicated impairing different steps in monolignol biosynthesis results in reddish brown to tan stem and stalk pigmentation, including the CAD and COMT mutants examined in this study (Mackay et al. 1997, Tsai et al. 1998, Sibout et al. 2005, Zhang et al. 2006). There are differences in pith and midrib (leaf vein) coloration between *bmr6* and *bmr12* (Porter et al. 1978, Saballos et al. 2009), probably because *bmr6* and *bmr12* block different steps of the monolignol biosynthetic pathway. However, the increased mortality of both *H. zea* and *S. frugiperda* on both *bmr* mutant pith types suggests a common chemical or biochemical resistance factor(s), yet to be identified, which is not related to differences in pith color. The greater resistance observed in *bmr* pith and *bmr* leaves at times suggest the similar phenolic compounds or pathway intermediates may accumulate in both tissues due to altered phenylpropanoid metabolisms caused by these mutations. Alternatively, the changes in phenylpropanoid metabolism could induce the expression of unrelated resistance genes not involved in this metabolic pathway. Additionally, the loss of these biosynthetic enzymes in the *bmr6* and *bmr12* lines may have a previously unrecognized role, which is not unprecedented. Transgenic overexpression of a peroxidase altered expression of other, unrelated defensive genes in tomato, *Solanum lycopersicum* L. (Suzuki et al. 2012). In addition, the lignin biosynthetic enzyme Cinnamoyl-CoA reductase (CCR) is also involved in defense signaling in rice, *Oryza sativa* L. (Kawasaki et al. 2006).

The same unknown factors could be present in both *bmr* lines, and these same factors could be responsible for the increased morality, reduced leaf damage, and lower larval weights in the pith and leaf feeding assays, respectively. Considering that monolignol pathway is active in both leaves and stalks, and *bmr6* and *bmr12* both affect this pathway, the accumulation of phenolic compounds resulting from alteration to this pathways may partly play a role in resistance. The studies with diets involving added phenolic acids that simulated levels observed in *bmr* stalks (Table 3) suggest that changes in phenolic composition found in *bmr* mutants may increasing toxicity or interfering with nutrient absorption. No significant differences in insect weights were noted for experiments with nutritionally complete pinto bean based artificial diet was used, but weights of both *H. zea* and *S. frugiperda* were lower when they fed on the simulated "*bmr"* compared with "wild-type" diet made from sorghum leaf material. This information suggests that diet nutrient composition influences the level of resistance conferred by potential resistance molecules. No effect was observed with the nutritionally rich pinto bean diet, but a significant effect was detected with the nutrient poor sorghum leaf diet, which suggests the phenolic acids are interfering with nutrient absorption. *S. frugiperda* caterpillars that fed upon the simulated "*bmr6"* and "*bmr12"* leaf diet disks weighed less than those fed the "wild-type" leaf diet disks. However, the "*bmr12"* diet disks only had additional vanillic acid, whereas the "*bmr6"* leaf diet also had ferulic and syringic acid added. This result suggests that increased levels of vanillic acid may be associated with increased insect resistance in the *bmr* lines. Stem and pith resistance to the stalk borer, *Sesamia nonagrioides* (Lefebvre) in several lines of maize was correlated with *p*-coumaric content but not with several other phenolics, including ferulic, sinapic, syringic, and vanillic acids (Santiago et al. 2005). Syringic and vanillic acid, but not ferulic or *p*-coumaric acid concentration were correlated with pigeon pea, *Cajanus cajan* (L.) Millsp., resistance to the pod borer, *Helicoverpa armigera* (Hübner) (Verulkar and Singh 2000). Why increased vanillic acid and apparently not ferulic or syringic (i.e., expected similar mode of action) affect *S. frugiperda* caterpillars remains to be determined.

This study indicates that sorghum lines *bmr6* and *bmr12*, which are easier to enzymatically saccharify, generally do not reduced levels of insect resistance. The increased resistance of *bmr6* leaves and pith to insects observed suggests the *bmr6* mutant may actually have sufficient enhanced insect resistance, such that it would require fewer insecticide applications in field production. This information suggests that sorghum *bmr* traits, which enhance conversion of lignocellulosic biomass to ethanol, or other biofuels are viable objections for sustainable bioenergy feedstock production. Further evaluations under field conditions are needed to better assess the potential of the *bmr* sorghum lines to be sustainably produced, for which insect resistance is an important component. The toxicity of the pith from the *bmr* lines to caterpillars noted in this study suggests it may promote resistance to stalk boring insects, and field studies are in progress to evaluate this potential. Chemical and molecular analyses of the potential resistance mechanisms within *bmr* pith are in progress. This study represents the first effort to evaluate insect resistance in sorghum lines with potential bioenergy uses. This study also provides incentive to continue investigating insect resistance of these lines under field conditions. Overall, the results of this study and other studies examining effects plants with altered lignin content and composition on insect resistance indicate that changes to this

pathway to augment biofuel production should be evaluated on a case by case basis for both the plant production and the insect resistance.

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## References Cited

**Barriére, Y., and O. Agillier. 1993.** Brown-midrib genes of maize: a review. Agronomie 13: 865–876.

**Bout, S., and W. Vermerris. 2003.** A candidate-gene approach to clone the sorghum brown midrib gene encoding caffeic acid O-methyl transferase. Mol. Genet. Genomics. 269: 205–214.

**Buanafina, M.M.deO., and H. W. Fescemyer. 2012.** Modification of esterified cell wall phenolics increases vulnerability of tall fescue to insect herbivory by the fall armyworm. Planta 236: 513–523.

**Cassler, M. D., J. F. Pedersen, and D. J. Undersander. 2003.** Forage yield and economic losses associated with the brown-midrib trait in sudangrass. Crop Sci. 43: 782–789.

**Dien, B. S., G. Sarath, J. F. Pedersen, S. E. Sattler, H. Chen, D. L. Funnell-Harris, N. N. Nichols, and M. A. Cotta. 2009.** Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolor* L. Moench) lines with reduced lignin contents. Bioenergy Res. 2: 153–164.

**Dowd, P. F. 1987.** A labor-saving method for rearing the driedfruit beetle on pinto bean-based diet. J. Econ. Entomol. 80: 1351–1353.

**Dowd, P. F. 1988.** Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic acid with xenobiotics in *Heliothis zea* (F.) and *Spodoptera frugiperda* (J.E. Smith). Pestic. Biochem. Physiol. 32: 123–134.

**Dowd, P.F, and E. T. Johnson. 2009.** Differential resistance of switchgrass *Panicum virgatum* L. lines to fall armyworms *Spodoptera frugiperda* (J.E.Smith). Genet. Resour. Crop Ev. 56: 1077–1089.

**Dowd, P. F., E. T. Johnson, and T. S. Pinkerton. 2007.** Oral toxicity of β-N-acetyl hexosaminidase to insects. J. Agric. Food Chem. 55: 3421–3428.

**Dowd, P. F., E. T. Johnson, K. E. Vermillion, M. A. Berhow, and D. E. Palmquist. 2011.** Coconut leaf bioactivity toward generalist maize insect pests. Entomol. Exp. Appl. 141: 208–215.

**Dowd, P. F., G. Sarath, R. B. Mitchell, A. J. Saathoff, and K. P. Vogel. 2013.** Insect resistance of a full sib family of tetraploid switchgrass *Panicum virgatum* L. with varying lignin levels. Genet. Resour. Crop Ev. 60: 975–984.

**Kawasaki, T., H. Koita, T. Nakatsubo, K. Hasegawa, K. Wakabayashi, H. Takahashi, K. Urnemura, T. Urnezawa, and K. Shimamoto. 2006.** Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. Proc. Natl Acad. Sci. USA. 103: 230–235.

**Mackay, J. J., D. M. O'Malley, T. Presnel, F. L. Booker, M. M. Campbell, R. W. Whetten, and R. R. Sederoff. 1997.** Inheritance, gene expression and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. Proc. Natl Acad. Sci. USA. 94: 8255–8260.

**Metcalf, R. L., and R. M. Metcalf. 1993.** Destructive and useful insects, fifth edition. McGraw Hill, New York, NY.

**Palmer, N. A., S. E. Sattler, A. J. Saathoff, D. Funnell, J. F. Pedersen, and G. Sarath. 2008.** Genetic background impacts soluble and cell wall-bound aromatics in brown midrib mutants of sorghum. Planta 229: 113–127.

**Pedersen, J. F., K. P. Vogel, and D. L. Funnell. 2005.** Impact of reduced lignin on plant fitness. Crop Sci. 45: 812–819.

**Pedersen, J. F., D. L. Funnell, J. J. Toy, A. L. Oliver, and R. Grant. 2006.** Registration of twelve grain sorghum genetic stocks near-isogenic for the brown midrib genes *bmr-6* and *bmr-12.* Crop Sci. 46: 491–492.

**Porter, K. S., J. D. Axtell, V. L. Lechtenberg, and V. F. Colenbrander. 1978.** Phenotype, fiber composition, and *in vitro* dry matter disappearance of chemically induced brown midrib (*bmr*) mutants of sorghum. Crop Sci. 18: 205–208.

**Rodriguez-Saona, C. R., R. O. Musser, H. Vogel, S. M. Hum-Musser, and J. S. Thaler. 2010.** Molecular, biochemical and organismal analysis of tomato plants simultaneously attacked by herbivores from two feeding guilds. J. Chem. Ecol. 37: 1043–1057.

**Saballos, A., G. Ejeta, E. Sanchez, C. Kang, and W. Vermerris. 2009.** A genome-wide analysis of the cinnamyl alcohol dehydrogenase family in sorghum (*Sorghum bicolor* (L.) Moench) identified SBCAD2 as the brown midrib 6 gene. Genetics 181: 783–785.

**Santiago, R., R. A. Malvar, M. D. Baamonde, P. Revilla, and X. C. Souto. 2005.** Free phenols in maize pith and their relationship with resistance to *Sesamia nonagrioides* (Lepidoptera: Noctuidae) attack. J. Econ. Entomol. 98: 1349–1356.

**SAS Institute. 1999.** SAS/STAT user's guide, version 8th ed. SAS Institute, Cary, NC.

**Sattler, S. E., A. J. Saathoff, E. J. Haas, N. A. Palmer, D. L. Funnell-Harris, G. Sarath, and J. F. Pedersen. 2009.** A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum brown midrib 6 phenotype. Plant Physiol. 150: 584–585.

**Sattler, S. E., D. L. Funnell-Harris, and J. F. Pedersen. 2010.** Brown midrib mutations and their importance to the utilization of maize, sorghum and pearl millet lignocellulosic tissues. Plant Sci. 178: 229–238.

**Shen, B., Z. Sheng, and H. K. Dooner. 2000.** A maize sesquiterpene cyclase gene induced by insect herbivory and volicitin: Characterization of wild-type and mutant alleles. Proc. Natl Acad. Sci. USA. 97: 14807–14812.

**Sibout, R., A. Eudes, G. Mouille, B. Pollet, C. Lapierre, L. Jouanin, and A. Seguin. 2005.** Cinnamyl alcohol dehydrogenase-C and –D are the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis.* Plant Cell 17: 2059–2076.

**Smith, C. M., Z. R. Khan, and M. D. Pathak. 1994.** Techniques for evaluating insect resistance in crop plants. CRC Press, Boca Raton, FL.

**Suzuki, H., P. F. Dowd, E. T. Johnson, S. M. Hum-Musser, and R. O. Mussser. 2012.** Effects of elevated peroxidase levels and corn earworm feeding on gene expression in tomato. J. Chem. Ecol. 38: 1247–1263.

**Swain, T. 1979.** Tannins and lignins, pp. 657–682. *In* G. A. Rosenthal and D. H. Janzen (eds.), Herbivores: their interaction with secondary plant metabolites. Academic Press, New York, NY.

**Tsai, C. J., J. L. Popko, M. R. Mielke, W. J. Hu, G. K. Podila, and V. L. Chiang. 1998.** Suppression of O-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes. Plant Physiol. 117: 102–112.

**Verulkar, S. B. and D. P. Singh. 2000.** Mechanism of resistance to podborer in pigeon pea, pp. 53–78. *In* M. Ali, A. S. Asthana, Y. S. Rathore, S. N. Gurha, S. K. Chaturvedi, and S. Gupta (eds.), Advances in Management of biotic and abiotic streses in pulse crops. Indian Society of Pulses Research and Development, Kanpur, India.

**Wheeler, G. S., and F. Slansky. 1991.** Compensatory response of the fall armyworm (*Spodoptera frugiperda*) when fed water and cellulose-diluted diets. Physiol. Entomol. 16: 361–374.

**Zhang, K. W., Q. Qian, Z. J. Huang, Y. Q. Wang, M. Li, L. L. Hong, D. L. Zeng, M. H. Gu, C. C. Chu, and Z. K. Cheng. 2006.** Gold hull and internode2 encodes a primary multifunctional cinnamyl-alcohol dehydrogenase in rice. Plant Physiol. 140: 972–983.

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