Original article

Underground herbivory and the costs of constitutive defense in tobacco

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\textbf{Abstract}

Nicotine is both a constitutive and induced defense in cultivated tobacco (\textit{Nicotiana tabacum}). Nicotine is thought primarily to defend against above-ground herbivory; however, below-ground herbivores like the nematode \textit{Meloidogyne incognita} can also damage plants. We evaluated the costs and benefits of constitutive nicotine production in four near-isogenic lines of \textit{N. tabacum} differing in nicotine content. We exposed the four lines to levels of nematode infection below that found to induce nicotine synthesis, and measured nematode density and each line's response to nematode presence. Nematode density did not differ among lines and was not related to leaf nicotine content in any of the lines, suggesting that constitutive nicotine content did not affect nematode survival or reproduction. Most measures of plant performance were unaffected by nematodes; however, nematode infection decreased flowering in the high nicotine line relative to the other lines. Lines with less constitutive nicotine did not incur similar costs, suggesting a tradeoff between nicotine production and tolerance of low levels of herbivory. A cost of nicotine production is also suggested by the fact that flowering was inversely correlated with leaf nicotine content in all four lines. Although nicotine conferred no resistance to nematodes, high nicotine content reduced the plant's tolerance of low levels of nematode infection and was correlated with reduced flowering. In examining the costs and benefits of a constitutive plant defense, this work complements and extends previous research addressing the relationship between plant tolerance and induced defenses.

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1. Introduction

Plants in a variety of ecosystems produce secondary compounds that confer resistance to herbivores (Buschmann et al., 2005; Rohde et al., 2004). Such compounds are important in reducing some forms of herbivory; however, their production may also exact an energetic cost to the plant. The 'cost-benefit ratio' of such compounds has been a source of

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doi:10.1016/j.actao.2006.09.004
debate, with a range of fitness costs reported (Koricheva, 2002). A related question is whether plants with higher constitutive levels of a given defensive compound are less tolerant of herbivore damage. One instance of such ‘resistance-tolerance’ trade-offs occurs in Brassica rapa, where plants selected for high glucosinolate concentrations were less tolerant of clipping damage than were low glucosinolate plants (Stowe, 1998). Although numerous other examples of tradeoffs exist (reviewed in Leimu and Koricheva, 2006), a recent meta-analysis of published literature found a significant negative relationship between resistance and tolerance only in wild plants, and only in those few studies that assessed resistance via a specific chemical or mechanical resistance trait (Leimu and Koricheva, 2006). Resistance and tolerance were positively correlated in agricultural plants, however, suggesting that selective breeding can create genotypes capable of utilizing both defensive strategies simultaneously.

The alkaloid nicotine is both a constitutive and induced defense in cultivated (Nicotiana tabacum) and wild (e.g., N. sylvestris and N. attenuata) tobacco. Nicotine synthesis is thought to be induced primarily by leaf damage; the compound is synthesized in the plant’s roots and transported to the shoots, where it can be lethal to herbivores (Karban and Baldwin, 1997). Nicotine levels are particularly high in cultivated tobacco, where it accounts for more than 95% of the total alkaloid content and can constitute up to 14% of the plant’s dry weight (Sisson and Severson, 1990).

Ecological research into plant-herbivore interactions in tobacco has focused on folivores; however, root-knot nematodes (Meloidogyne sp.) are a major threat to cultivated tobacco (Hanounik et al., 1975). Nicotine may mediate interactions between tobacco and root-knot nematodes. High levels of infection by Meloidogyne sp. reduce tobacco growth and induce nicotine synthesis (Hanounik and Osborne, 1975, 1977; Hanounik et al., 1975; Rich and Barker, 1984). Immersion of Meloidogyne incognita juveniles in a nicotine solution suppressed juvenile motility and adult gall formation, and high (10 individuals cm−3 soil) M. incognita densities induced nicotine synthesis in both a nematode-resistant and -susceptible tobacco cultivar (Davis and Rich, 1987). Nicotine synthesis in these plants significantly suppressed M. incognita root galling, and the number of root galls per plant decreased as nicotine concentrations increased, confirming that nicotine induction can act as a defense mechanism against nematodes.

The above research establishes that high densities of plant-parasitic nematodes can induce nicotine synthesis, and that induced nicotine can decrease root galling by these nematodes. However, tobacco cultivars can also vary in their constitutive (i.e. pre-existing) levels of nicotine. Much less is known about the relative cost and benefits of constitutive nicotine in tobacco, largely because experimental designs with nematode densities high enough to induce nicotine synthesis (e.g. Davis and Rich, 1987) unavoidably confound the role played by pre-existing defenses. In order to explicitly address the role played by constitutive nicotine in herbivore defense, it is necessary to both: (1) find near-identical tobacco cultivars that vary in their levels of constitutive nicotine; and (2) apply herbivores at a density below that known to induce nicotine synthesis. Experiments meeting both of these criteria would be impracticable in most systems; however, the wealth of information available concerning the interaction between Meloidogyne sp. nematodes and tobacco provides a unique opportunity for such work.

Our work builds on previous research by employing near-isogenic lines of a tobacco cultivar differing in constitutive nicotine content (detailed below) to assess the effect of constitutive nicotine on M. incognita, and to explore how interactions between constitutive nicotine and nematode infection affect plant performance. We used published research to determine the threshold nematode density necessary to induce nicotine synthesis (Hanounik and Osborne, 1975, 1977); by applying nematodes at a density below this threshold level, we isolated the effect of constitutive nicotine levels on nematode infection. We report (1) the effect of constitutive nicotine content on nematode survival and reproduction, and (2) the effect of nematode infection and constitutive nicotine content on plant performance both between and within near-isogenic tobacco lines.

2. Materials and methods

Plants from four near-isogenic lines of Nicotiana tabacum L. Solanaceae were grown from seed. Tobacco lines LAFC 53, LMAFC 34, and MAFC 5 are near-isogenic with the nematode-resistant tobacco cultivar NC 95. They differ consistently in nicotine content, containing reported mean dry weights of 0.20% (‘very low’ nicotine content), 1.08% (‘low’), 1.97% (‘medium’), and 3.20% (‘high’) respectively (Chaplin, 1975, 1986; Chaplin and Burk, 1984). The possibility of additional differences between near-isogenic lines due to linked alleles can never be completely discounted. Lines used in this study were near-isogenic rather than isogenic, i.e., these lines are likely to differ at other loci. Furthermore, even if these lines differed only in one locus responsible for alkaloid production, pleiotropic consequences of nicotine production on other traits are possible. For example, the use of nitrogen to produce nicotine may reduce available nitrogen for other plant functions. However, other methods of experimentally manipulating defense compounds, such as the use of transgenic plants, have methodological concerns such as the possibility of interrupting functional genes. Manipulation of factors such as fertilizer level or herbivore damage alter a wide range of plant traits other than production of secondary compounds, and so are not useful to isolate effects of variation in secondary compounds on interactions. As a result, there is no single ideal way to manipulate secondary compounds and be assured that no other traits also vary. Our use of near-isogenic lines in this study thus takes one of several possible approaches to experimentally addressing how constitutive nicotine mediates tobacco’s interactions with M. incognita.

Seedlings were potted individually in pots containing ~3600 cm³ of a 1:1 sand: potting soil mix (Metro Mix 360, Scotts Sierra Horticulture Product Company). The design was a 4 × 2 factorial with the four nicotine lines and two herbivore treatments (nematode addition or control), with 30 plants per treatment. Plants were arranged in 30 blocks in a complete randomized block design in a greenhouse; each block contained eight plants, one of each treatment combination. Plants were watered daily and maintained under natural light. When
aphids were detected, all plants were treated with Safer® soap.

Two weeks after potting, we added *Meloidogyne incognita* nematodes to plants assigned to the nematode treatment. We determined the nematode density in our treatments by examining published research showing nicotine induction as a function of nematode density. *Hanounik and Osborne* (1975) found no nicotine induction at *M. incognita* densities below 1.33 cm⁻³ and 1 cm⁻³ in NC-95 and McNair 30 cultivars, respectively, and *Hanounik and Osborne* (1977) found no nicotine induction below 2.6 cm⁻³ in either cultivar. Based on this information, we chose to apply nematodes at an average density of one nematode cm⁻³ soil by inoculating each plant with 3 ml of a water suspension containing ~3600 *M. incognita* eggs and second-stage juveniles (extracted from infected ‘Rutgers’ tomato plants). This density is a commonly used ‘treatment threshold’ for nematodes and is typical of ambient nematode densities in tobacco fields (E. Lewis, personal observation). Control plants were inoculated with water.

In order to confirm that our experimental density of nematodes did not induce nicotine synthesis, we randomly selected six blocks for alkaloid analysis 2 weeks after inoculation (harvest 1). While nicotine induction by nematodes affects both the root and leaf nicotine content (*Hanounik and Osborne, 1975, 1977*), work by *Zacheo et al.* (1974) specifically examined the effect of *M. incognita* densities ranging from 10 to 10,000/plant on both root and leaf nicotine levels in tobacco. They found that leaf nicotine levels responded to lower nematode densities than did root nicotine levels, suggesting that leaf nicotine is more sensitive than root nicotine to low levels of nematode infection (*Zacheo et al., 1974*) and leading us to examine leaf nicotine content in our experiment. The first full leaf from each plant was removed with a clean razor, immediately put on ice and then frozen at −20 °C. The plants from which leaves were collected were removed from the study. Leaves were freeze-dried for 24 h at approximately −80 °C, with 0 mtorr of vacuum pressure. Total alkaloid content of the leaves was then determined using established procedures (Davis, 1976). Briefly, alkaloids were extracted from leaf tissue with 5% acetic acid. Colorimetric determination was conducted on a Technicon auto-analyzer. Color development was accomplished by the action of cyanogen bromide on the alkaloids in a sulfanilic buffer. In order to test whether nicotine levels in the plant lines and different treatments varied over time, leaves from the remaining plants were analyzed at the end of the experiment (harvest 2).

Plants were harvested by block 7–10 weeks after nematode infection. This length of time was necessary in order to carefully wash, separate, and remove the sand/soil planting mix from the fine root hairs of each tobacco plant; since each block contained plants in each of the treatments, this should not have systematically biased the results. We counted the number of *M. incognita* eggs per plant after extracting them from roots and surrounding soil using a 0.5% sodium hypochlorite solution (Barker, 1985). We measured the number of flowers per plant to assess reproductive fitness. We chose this measurement of reproductive fitness over the number of fruits per plant because *M. incognita* populations decline once the plants set fruit and senesce (Johnson, 1998). Waiting for fruit set would thus have made it impossible to accurately assess nematode densities; since testing for line-specific differences in nematode densities was an integral part of our experiment, we chose to use flowers as our measure of reproductive fitness. For each plant, we also counted the healthy, chlorotic, and senescent leaves as well as the aboveground and root biomass. The effect of nematode parasitism was measured using the number of healthy leaves, combined number of senescent and chlorotic leaves, number of flowers, and aboveground biomass as response variables.

We analyzed between-line differences using the MANOVA GLM procedure in SAS v. 8.2 because of the likelihood of correlated responses. Because we cannot exclude the possibility that the near-isogenic lines differed in some ways other than in their nicotine content, we chose to treat each of the four near-isogenic lines (‘very low’, ‘low’, ‘medium’, and ‘high’) constitutive nicotine content as categorical variables rather than a single continuous population varying only in nicotine content. We conducted individual ANOVAs when the MANOVA showed significant effects of line, block, nematode treatment, or the line × treatment interaction. The mean number of healthy leaves was square-root transformed to meet assumptions of normality; other data were normal without transformation.

Because individual plants within each of the four near-isogenic lines differed in terms of nicotine content, nematode density, and the various plant-level response variables, we used JMP (SAS, 2004) to separately analyze each line for within-line differences in nematode infection and plant fitness. Preliminary inspection of the data revealed a single outlying individual in each of the four lines; removing these individuals left between 37 and 44 individuals per line for the following analyses. We used linear regression to assess the correlation between plant nicotine content (harvest 2) and the number of *M. incognita* eggs per plant in each of the four lines. We similarly assessed the within-line correlation between plant nicotine content (harvest 2) and the number of flowers per plant. Finally, we used ANOVA to analyze the within-line effects of treatment and block on the number of healthy leaves, combined number of senescent and chlorotic leaves, number of flowers, aboveground biomass, and plant nicotine content (harvest 2). We performed a Bonferroni correction on the results to control for multiple comparisons.

### 3. Results and discussion

Nicotine levels in the four near-isogenic lines varied significantly, and in the expected order (Table 1). However, infection by *Meloidogyne incognita* did not affect leaf nicotine content at any point. There was no effect of treatment on nicotine content in either the between-line analysis ($F_{1,34} = 0.93, P = 0.34$) or in any of the four within-line analyses (all $P > 0.10$). In addition, the tobacco lines did not differ in final root weight (Table 2). In a study measuring both root and leaf nicotine content of tobacco in response to low levels of nematode infection, *Zacheo et al.* (1974) found that leaf nicotine levels were more sensitive to nematode presence than were root nicotine levels. Since higher levels of nematode infection clearly induce changes in both root and leaf nicotine, the lack of either between-line or within-line responses in leaf nicotine content
to the low density of nematodes applied in this experiment implies that induction of nicotine synthesis in response to nematode infection did not occur.

The constitutive nicotine present in the four lines of \textit{N. tabacum} had no apparent effect on root-knot nematodes. Despite varying in overall leaf nicotine content, all four lines yielded similar numbers of nematode eggs (Tables 2 and 3). There was also no significant within-line relationship between plant nicotine content and the number of \textit{M. incognita} eggs per sample in any of the four lines (all \( P > 0.10 \)). There is thus no evidence that either between-line or within-line differences in constitutive nicotine content affected nematode population growth. This was surprising because we expected constitutive nicotine to harm \textit{M. incognita}. Although high concentrations of nicotine can harm nematodes (Davis and Rich, 1987), this compound is traditionally considered to defend primarily against aboveground herbivores (Karban and Baldwin, 1997) and may not affect nematodes at low concentrations. Our results suggest that this may be the case; because constitutive nicotine does affect \textit{M. incognita}, nicotine induction is likely required to suppress nematode population growth.

A MANOVA revealed that overall plant performance was affected by plant line (Wilks’ lambda = 0.70, \( F_{12,376} = 4.62, P < 0.001 \)). Univariate analyses revealed that the low and high nicotine lines had more healthy leaves than the very low and medium lines, while the very low line had more chlorotic and senescent leaves than other lines. In addition, the high nicotine line had higher aboveground biomass than other lines (Table 3). However, there was a significant inverse correlation between leaf nicotine content and flower number within each of the four lines (Fig. 1). This correlation suggests that increased investment in constitutive nicotine utilized resources that would otherwise have been available for reproduction.

Nematodes did not affect overall plant performance (Wilks’ lambda = 0.97, \( F_{14,142} = 1.21, P = 0.31 \)). Across all lines, nematodes did not affect aboveground biomass, the number of healthy leaves, or the number of chlorotic plus senescent leaves (Tables 3, 4). This is consistent with previous studies of the NC 95 tobacco cultivar, which showed that nematode densities under 1.33 nematodes cm\(^{-2}\) had no effect on growth or leaf production (Hanounik and Osborne, 1977; Hanounik et al., 1975). There were also no significant (\( P < 0.05 \)) within-line effects of nematode presence, although there was a marginally significant effect of treatment on flower production within the high nicotine line only (\( F_{1,23} = 7.11, \text{Bonferroni-corrected } P = 0.058 \)).

Although there was no significant main effect of nematodes on plant performance across lines, there was a significant line \( \times \) nematode treatment interaction (Wilks’ lambda = 0.84, \( F_{12,376} = 2.12, P = 0.02 \)). Nematode infection only affected flower production in the high nicotine line, where it reduced flowering relative to the control (Fig. 2). Flower production in the other three lines was not significantly affected by nematode infection. Because some plants in the blocks we harvested first had not yet ceased flowering,

<table>
<thead>
<tr>
<th>Table 1 – Results of univariate ANOVAs showing the effect of tobacco line and nematode infection on the nicotine content (% leaf dry weight) in \textit{Nicotiana tabacum} after 2 weeks (harvest 1) and at the end of the experiment (38 days, harvest 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effect</strong></td>
</tr>
<tr>
<td>Line</td>
</tr>
<tr>
<td>Nematode</td>
</tr>
<tr>
<td>Line ( \times ) nematode</td>
</tr>
<tr>
<td>Block</td>
</tr>
<tr>
<td>Error</td>
</tr>
</tbody>
</table>

***\( P < 0.001 \).

<table>
<thead>
<tr>
<th>Table 2 – Effect of tobacco line on the root biomass and number of \textit{Meloidogyne incognita} eggs in \textit{Nicotiana tabacum}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effect</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Line</td>
</tr>
<tr>
<td>Block</td>
</tr>
<tr>
<td>Error</td>
</tr>
</tbody>
</table>

*\( P < 0.05 \); **\( P < 0.01 \).
it is possible that this response may reflect a nematode-induced delay in flowering phenology in the high-nicotine line rather than lowered flower production. Since each treatment–line combination was represented in each block, however, each treatment–line combination should be similarly affected. In addition, Rich and Barker (1984) showed that N. tabacum flowering delay in response to infection by M. incognita was associated with reduced flower production. Even if a delay in flower production led to no change in overall flower number, such delays can lead to increased risks of abbreviated fruit set due to cold or other seasonally varying factors. As a result, both reduced and/or delayed flowering in the high-nicotine line constitute potential costs to plant fitness.

Nematode infection only reduced flowering in the high-nicotine line, suggesting a tradeoff between constitutive defense and tolerance of low levels of herbivore damage. The fact that we found substantial numbers of eggs and juvenile nematode means that nematodes were feeding on the plants; the lack of any between- or within-line effects on nematode abundance indicates that the varying concentrations of constitutive nicotine did not affect nematode population growth. Nematodes did not reduce biomass in any line (Table 4), indicating that the cost of constitutive nicotine with nematode infection was reflected solely in flower production. The mechanistic basis for our results is unclear: one possible explanation for this finding is that, unbeknownst to us, the near-isogenic lines used in this study may have differed in traits other than nicotine content. Regardless of the mechanism, a cost of nematode infection occurred only in the high-nicotine line, documenting a slight but significant cost of plant defense.

Our finding that increased investment in constitutive nicotine was inversely correlated with flower production agrees with the results of several previous studies demonstrating trade-offs between constitutive defense and plant performance (Siemens et al., 2002; Strauss et al., 1999). In the between-line analysis, our results assessing flower production appear to differ with the positive correlation between resistance and tolerance found by Leimu and Koricheva (2006) in agricultural plants. While our between-line results for flower production argue for a negative relationship between these two processes, the fact that the very-low-nicotine line had more chlorotic and senescent leaves while the high-nicotine line had more aboveground biomass than the other lines suggests a positive correlation between resistance and tolerance. One solution to this apparent contradiction may lie in the fact that the relationship between resistance and tolerance can differ as a function of the metric used to assess tolerance (Stowe, 1998); however, a meta-analysis found no overall effect of tolerance metrics on the tolerance–resistance relationship in agricultural plants (Leimu and Koricheva, 2006).

The existence of trade-offs between plant resistance to and tolerance of herbivory has been of interest to researchers studying plant-herbivore interactions in a variety of systems (Molis et al., 2006; Strauss and Agrawal, 1999). Our work adds to a growing body of literature documenting such trade-offs (Buschmann et al., 2005; Rohde et al., 2004; Stowe, 1998).

### Table 3 – Mean aboveground biomass, healthy leaves, chlorotic and senescent leaves, nicotine content (harvest 2), and number of M. incognita eggs of near-isogenic lines of Nicotiana tabacum in the presence and absence (control) of the nematode Meloidogyne incognita

<table>
<thead>
<tr>
<th>Line</th>
<th>Treatment</th>
<th>g aboveground biomass (SE)</th>
<th>No. of healthy leaves (SE)</th>
<th>Chlorotic + senescent leaves (SE)</th>
<th>Nicotine % leaf dry weight (SE)</th>
<th>M. incognita eggs (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very low</td>
<td>Control</td>
<td>237 (9.7)</td>
<td>8.6 (1.00)</td>
<td>9.1 (0.47)</td>
<td>0.0495 (0.0288)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Nematode</td>
<td>244 (11.1)</td>
<td>7.6 (0.83)</td>
<td>9.7 (0.38)</td>
<td>0.0598 (0.0042)</td>
<td>39982 (6687)</td>
</tr>
<tr>
<td>Low</td>
<td>Control</td>
<td>242 (11.9)</td>
<td>10.2 (1.06)</td>
<td>8.7 (0.66)</td>
<td>0.1042 (0.0104)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Nematode</td>
<td>235 (13.8)</td>
<td>11.2 (1.03)</td>
<td>8.6 (0.63)</td>
<td>0.1074 (0.0122)</td>
<td>41611 (7562)</td>
</tr>
<tr>
<td>Medium</td>
<td>Control</td>
<td>225 (11.9)</td>
<td>8.0 (0.82)</td>
<td>7.7 (0.41)</td>
<td>0.1708 (0.0240)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Nematode</td>
<td>250 (10.7)</td>
<td>8.3 (1.12)</td>
<td>8.8 (0.41)</td>
<td>0.1556 (0.0153)</td>
<td>38955 (5981)</td>
</tr>
<tr>
<td>High</td>
<td>Control</td>
<td>269 (10.3)</td>
<td>10.6 (0.70)</td>
<td>7.8 (0.45)</td>
<td>0.3424 (0.0288)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Nematode</td>
<td>264 (8.4)</td>
<td>11.3 (0.69)</td>
<td>8.8 (0.44)</td>
<td>0.3360 (0.0272)</td>
<td>40549 (5721)</td>
</tr>
</tbody>
</table>

‘Line’ refers to nicotine content; furthermore, ‘very low’ refers to line LAFC 53, ‘low’ refers to line LMAFC 34, ‘medium’ refers to line MAFC 5, and ‘high’ refers to line NC 95.

### Table 4 – Results of univariate ANOVAs showing the effect of tobacco line and nematode infection on the aboveground biomass, number of healthy leaves produced, number of senescent and chlorotic leaves, and number of flowers in Nicotiana tabacum

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>Aboveground biomass: F</th>
<th>Healthy leaves: F</th>
<th>Senescent and chlorotic leaves: F</th>
<th>Flowers: F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line</td>
<td>3</td>
<td>5.32**</td>
<td>7.23***</td>
<td>3.98**</td>
<td>0.66</td>
</tr>
<tr>
<td>Nematode</td>
<td>1</td>
<td>0.17</td>
<td>0.04</td>
<td>4.06*</td>
<td>0.01</td>
</tr>
<tr>
<td>Line × nematode</td>
<td>3</td>
<td>2.04</td>
<td>1.39</td>
<td>0.39</td>
<td>3.75*</td>
</tr>
<tr>
<td>Block</td>
<td>23</td>
<td>9.29***</td>
<td>2.85***</td>
<td>3.77***</td>
<td>2.79***</td>
</tr>
<tr>
<td>Error</td>
<td>145</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001.
however, most previous research has addressed the relationship between plant tolerance and induced defense. Future research might use protocols similar to ours with near-isogenic lines of a nematode-susceptible cultivar to further examine the trade-off between constitutive defense and plant tolerance. Although we found no evidence that constitutive nicotine confers resistance to low nematode densities, increased investment in constitutive nicotine reduced the plant’s ability to tolerate low levels of nematode infection and was inversely correlated with flower production.

Acknowledgements

We thank W. Mays, D. Wiley, and the Virginia Tech greenhouse staff for their help throughout this project. M. Tilson conducted alkaloid analyses. M. Anderson, N. Bachmann, M. Denby, M. Gittinger, and D. Sharp helped with data collection, and T. McCoy and E. Perez provided technical assistance. Comments by H. Kaya, A. Agrawal, several anonymous reviewers, and A. Gutierrez greatly improved the manuscript. This research was funded by an REU supplement to NSF-DEB 0211480 (SEG and LSA).

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