



## Effects of Foliar Endophytic Fungi on the Preference and Performance of the Leaf Beetle *Chelymorpha alternans* in Panama

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### ABSTRACT

Foliar endophytic fungi live inside healthy plant leaves, and in some cases they confer herbivore resistance to the host. All previous studies of endophyte–herbivore interactions have occurred in temperate areas, and many use correlations rather than experiments. In Panama, *Glomerella cingulata* is a common endophyte species found in healthy leaves, and *Chelymorpha alternans* is a common herbivore on *Merremia umbellata*, a tropical vine. We manipulated the abundance of *G. cingulata* in the leaves of *M. umbellata*. We then assessed the effects of high and low endophyte densities on the food choice, development, and reproductive success of the leaf beetle, *C. alternans*. In ‘choice’ experiments, adult females with a history of feeding on wild plants showed no preference when offered food plants with high and low endophyte densities. Further, in ‘no-choice’ experiments, *C. alternans* larvae that were fed high- or low-density endophyte leaves did not differ in development or survivorship. However, when larvae fed on leaves with low endophyte densities became adults, they produced 80 percent more offspring. This suggests high endophyte levels in hosts can have a negative effect on herbivore fecundity. Further experiments are necessary to understand whether the reproductive effects are due to feeding on low-density endophytes in the larval or adult stages, and whether changes in reproductive success are motivated by the adult’s perception of food quality or by physiological constraint due to food quality.

Abstract in Spanish is available at <http://www.blackwell-synergy.com/loi/btp>.

**Key words:** *Colletotrichum gloeosporoides*; endophyte; fecundity; *Glomerella cingulata*; herbivory; horizontal transmission; *Merremia umbellata*; mutualism.

ENDOPHYTES ARE FUNGI that live most of their life cycle within plant tissues without causing any apparent signs of disease (Wilson 1995a). Foliar endophytic fungi are diverse and widespread (Petrini 1986, Arnold & Lutzoni 2007). Although often closely related to pathogens, the effects of foliar endophytes on hosts are generally neutral or beneficial (Carroll 1988, Faeth 2002, Herre *et al.* 2007). The potential benefits of endophytes to their hosts include increased tolerance to heavy metals, increased drought resistance, reduced herbivory, defense against pathogens, or enhanced growth and competitive ability (Saikkonen *et al.* 1998).

Most studies of endophyte-derived host benefits have been carried out in temperate zone grasses, where endophytes can be transmitted vertically (from adult grass to seed) or horizontally (from spores in the environment). In contrast, the endophytes of herbs and woody plants are transmitted horizontally (Arnold & Herre 2003). Symbionts transmitted horizontally are predicted to be less mutualistic, and hence more commensal or parasitic, than those transmitted vertically (Herre 1993, Herre *et al.* 1999). However, experimental and correlational evidence demonstrate that horizontally transmitted foliar endophytes can increase growth (Redman *et al.* 2001), enhance pathogen immunity of hosts (Arnold *et al.* 2003, Mejia *et al.* in press), and decrease herbivory loads (Wilson & Carroll 1994, 1997; Wilson 1995b; Preszler *et al.* 1996; Wilson & Faeth 2001).

Previous work on horizontally transmitted endophytes and herbivores has been limited to correlational studies, with a few ex-

ceptions. In a case where endophytes were manipulated experimentally, the survivorship of leaf-mining insects was affected by some but not all endophytic fungal strains (Faeth & Hammon 1997). In another experiment where *Colletotrichum* spores were applied to milkweed, grasshoppers did not choose or avoid leaves with respect to endophyte density (Devarajan & Suryanarayanan 2006). Thus, the limited evidence that exists is equivocal for plant–fungal mutualisms against herbivores in systems where fungi are horizontally transmitted.

No studies have addressed the effects of horizontally transmitted endophytes on herbivores in a tropical system. There are at least three reasons why endophyte–plant–herbivore interactions may differ between tropical and temperate areas. First, the endophyte density and diversity increase with rainfall (Carroll 1988, Arnold & Lutzoni 2007). Second, tropical plants suffer higher rates of herbivory and disease (Coley & Barone 1996, Leigh 1999); an effect likely most intensely expressed at the seedling stage, where mortality by herbivores or pathogens can reach 100 percent for some seedlings (Clark & Clark 1992). Third, a model by Faeth and Fagan (2002) predicts endophytes should invest in antiherbivore compounds in areas of higher herbivory and soil fertility—the former likely higher in the tropics, the latter likely lower. Thus some, but not all, considerations suggest higher antiherbivore function in tropical endophytes.

Here we test whether foliar endophytic fungal density, experimentally manipulated using species common in wild host plants, affects development, reproductive success, and food choice of a leaf-eating beetle, *Chelymorpha alternans* Boheman (Coleoptera: Chrysomelidae: Cassidinae). This study is novel because it involves

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experimental application of endophytes in a tropical plant, and investigates endophyte effects over the entire life cycle of the insect herbivore.

## METHODS

**STUDY AREA.**—This study was carried out at the Gamboa research station of the Smithsonian Tropical Research Institute (9°07' N, 79°42' W), Republic of Panama. Gamboa is situated on the edge of Soberania National Park, a 22,000 ha forest reserve in an evergreen wet, tropical forest (average of 2131 mm annual rainfall). The plants, fungi, and beetles for this study were collected along edges of secondary growth forest.

**STUDY SPECIES.**—The beetle *C. alternans* is found at 0–1000 m throughout Panama, is common in disturbed habitats along forest edges, rivers, and forest gaps, and is easily cultured. Adults and larvae feed on the family Convolvulaceae ('morning glory') including *Merremia umbellata* (L.) Hallier and numerous *Ipomoea* species. *Merremia umbellata* is a widespread Neotropical vine (Croat 1978) that grows in open areas, forest edges, gaps and estuaries. All endophytes of *M. umbellata* appear to be horizontally transmitted from spore-fall. At least 175 morphospecies of foliar endophytic fungi have been isolated from 30 leaves of *M. umbellata* in Gamboa (S. Van Bael, pers. obs.) and the genera *Xylaria*, *Glomerella/Colletotrichum*, and *Phomopsis* are common. We selected our endophyte study species by screening the most common fungal morphospecies from *M. umbellata* for their ability to sporulate in laboratory conditions. As our focal endophyte, we selected a strain of *Glomerella cingulata* (anamorph *Colletotrichum gloeosporoides*) that was present in five of 12 *M. umbellata* leaves from a 2004 collection, and 15 of 30 *M. umbellata* leaves in 2005. We confirmed this strain's taxonomic affinity with primers ITS4 and ITS5 (White *et al.* 1990) and PCR protocols described in Rehner and Uecker (1994) to amplify a 0.5–0.7 kb region of nrDNA, including both the internal transcribed spacer regions 1 and 2 (ITS1, ITS2), and the highly conserved 5.8s gene. Sequences were submitted to BLAST searches of the GenBank data base.

**EXPERIMENTAL PLANTS.**—In May 2005 we collected one *M. umbellata* plant from each of four locations in Gamboa (at least 0.5 km apart). Greenhouse cuttings over a period of 1 yr generated 16 replicate plants from each of the four originals for a total of 64 plants. Half of the plants from the descendants of each starter plant were designated either low endophyte density ( $E_{low}$ ) or high density ( $E_{high}$ ). As foliar endophyte infections are greatly reduced when water does not touch leaf material (Arnold *et al.* 2003) all plants were kept under a clear plastic tent and watered at the soil level.  $E_{high}$  plants were treated with a spray consisting of *G. cingulata* spores ( $10^6$ – $10^7$  spores/ml), water, and Tween 20 (a detergent).  $E_{low}$  plants received the same solution, but without spores (See methodological details for creation of spore and control sprays in Appendix S1). Plants were sprayed weekly during July–September 2006.  $E_{high}$

plants were kept separate for 24 h during and after the spray treatment to avoid contaminating the  $E_{low}$  plants. Otherwise, all plants were on the same greenhouse tables, so some contamination of  $E_{low}$  plants may have occurred via insect movement from  $E_{high}$  neighbors, or via long-distance dispersal from the surrounding forest.

To determine treatment efficacy, on six different dates we sampled 40 leaves, *ca* 8- to 12-d old, from  $E_{high}$  and  $E_{low}$  plants ( $N = 20$  leaves per treatment). Within 2 h of clipping, we cut a  $20 \times 10$  mm section from the middle lamina of each leaf and further divided it into  $2 \times 2$  mm pieces with a sterile razor blade. We surface-sterilized each piece by immersion in: (1) 70 percent ethanol for 3 min; then (2) 10 percent commercial bleach (0.525% sodium hypochlorite) for 2 min (Appendix S2). We plated the 20 pieces on 2 percent malt extract agar plates and incubated the sealed plates at room temperature for 14 d. Throughout this study, 'percent infection' or 'infection rate' of a leaf is defined as the number of pieces of 20 per leaf ( $\times 100$ ) generating an endophytic fungus.

**FIELD SAMPLING.**—We compared endophyte infection rates in the wild with our greenhouse plants. In September 2006, 60 newly expanding *M. umbellata* leaves were marked on 12 plants at four forest sites. We sampled five leaves from each plant at age of 5, 10, 15, 20, and 30 d. Within 2 h of collection, leaves were prepared and plated following the methods above, and percent infection was assessed after 14 d on plates.

**NO-CHOICE EXPERIMENT: ENDOPHYTE EFFECTS ON PERFORMANCE.**—We established a laboratory colony of *C. alternans* in April 2005, combining offspring from previous laboratory adults and wild adults caught in and around Gamboa. The colony consisted of 55 pairs, with females paired to an unrelated male, kept in separate plastic containers and fed with wild-collected *M. umbellata* three times per week. We removed egg masses from breeding pairs and incubated them in Petri dishes at ambient temperature.

We fed larvae  $E_{high}$  or  $E_{low}$  leaves in Petri dishes the day after they eclosed from eggs. We split each of 32 larval broods to create a paired design. After 3 d we randomly chose six members from each dish, weighed them, and placed them together on leaves of known area on either  $E_{high}$  or  $E_{low}$  plants in the greenhouse. Sibling beetles were assigned to cloned plants (either  $E_{low}$  or  $E_{high}$ ) from one of the original four *M. umbellata* individuals. Larvae fed on leaves that were *ca* 8- to 12-d old in the Petri dishes and greenhouse. On day 6, we brought the larval groups to the lab for weighing and measured leaf area consumed using a scanner and ImageJ software (<http://rsb.info.nih.gov/ij/>). They were returned to their respective plants (on new leaves) until day 10, when they were placed into plastic containers for the prepupal phase. We recorded their weights at pupation, and they were placed in a Petri dish until eclosion. At eclosion, the sex of each individual was assessed using morphological characters (size and shape). Three broods were lost because the larvae disappeared during days 3–6. The final sample size was  $N = 29$  brood comparisons.

To study endophyte effects on adult development, we selected one newly eclosed adult female from each  $E_{low}$  and  $E_{high}$  brood, feeding them their assigned leaves as before in plastic containers.

After 5 d, we added one male to each container to create adult pairs. To reduce possible effects due to male quality, we swapped males among female siblings every 2 d. We recorded the amount of time between eclosion and oviposition, the number of eggs in the first oviposition, and the number of larvae that hatched successfully from those eggs (hatchability). Adult females suffered high rates of mortality in both  $E_{high}$  and  $E_{low}$  food treatments, so that we finished the experiment with only 18 valid comparisons (of the original 29).

**CHOICE EXPERIMENT: ENDOPHYTE EFFECTS OF FOOD PREFERENCE.**—We offered beetles a choice between  $E_{high}$  versus  $E_{low}$  leaves by intertwining the canopies of one plant from each treatment group in mesh cages ( $N = 8$  cages). In each 48-h trial, adult females previously fed on wild-collected *M. umbellata* leaves were placed one to a cage, alternating the placement of the beetle on  $E_{high}$  and  $E_{low}$  plants for each cage. To further force each beetle to choose, after 24 h we moved each beetle to the opposite plant treatment from where it was started. After 48 h we assessed leaf damage by tracing the holes in leaves, scanning the tracings, and measuring leaf damage per treatment with ImageJ software. We repeated this process four times with unique females and unique plants in each trial, for a total of 32 trials. One female died during the trial so we excluded it from the final analysis for a final sample of  $N = 31$ .

**ANALYSIS.**—Each of the response variables from the ‘no-choice’ experiment (larval mass, leaf damage, time to pupation, pupal mass, survivorship, sex ratio, time to first oviposition, number of eggs, number of larvae and hatchability [proportion of eggs with successful larvae]) were tested using paired  $t$ -tests with two tails (SYSTAT 10). Each pair for comparison was a sibling brood. The data were normally distributed (Shapiro-Wilk Test, SYSTAT 10), with the exception of ‘number of eggs’ and ‘hatchability’ for which we used square root and arcsin square root transformations, respectively. All means in Table 1 are reported as nontransformed values. The variable ‘survival to adulthood’ could not be normalized with transformations so we used the nonparametric Wilcoxon signed ranks test (SYSTAT 10). Data from the choice experiment were analyzed by comparing the amount of leaf damage ( $\text{cm}^2$ ) on the two plants in each cage, using the Wilcoxon signed ranks test as data transformations did not normalize the distribution of values.

## RESULTS

**FIELD SAMPLING AND TREATMENT EFFICACY.**—In the wild, foliar endophytic fungi increased from a 33 percent infection rate on 5-d-old leaves, to 89 percent at 30 d (Fig. 1). Greenhouse leaves that were treated with our experimental sprays had infection rates of 44 and 91 percent for  $E_{low}$  and  $E_{high}$  leaves, respectively (Fig. 1). Both  $E_{low}$  and  $E_{high}$  plants were further infected by endophytes from ‘environmental’ spores that were in the greenhouse. As a consequence, beetles fed on leaves with infection rates that were higher ( $E_{high}$ ) and lower ( $E_{low}$ ) than natural densities of endophytes in wild

TABLE 1. Larval and adult performance of *Chelymorpha alternans* when eating *Merremia umbellata* leaves with high ( $E_{high}$ ) and low ( $E_{low}$ ) densities of foliar endophytic fungi.

	$E_{high}$ mean $\pm$ SE	$E_{low}$ mean $\pm$ SE	Paired $t_{df}$	$P^a$
<i>I. Larval performance</i>				
Mass on day 3 ( $\times 10^{-3}$ g)	2.52 $\pm$ 0.13	2.46 $\pm$ 0.11	-0.98 <sub>30</sub>	0.33
Mass on day 6 ( $\times 10^{-3}$ g)	8.35 $\pm$ 0.52	8.18 $\pm$ 0.5	-0.57 <sub>30</sub>	0.57
Leaf area consumed ( $\text{cm}^2$ )	1.69 $\pm$ 0.13	1.73 $\pm$ 0.11	0.39 <sub>30</sub>	0.70
Time to pupation (d)	14.3 $\pm$ 0.12	14.3 $\pm$ 0.11	—	—
Pupal mass (g)	0.055 $\pm$ 0.001	0.054 $\pm$ 0.002	—	—
Survival to adulthood (proportion of larvae)	0.72 $\pm$ 0.05	0.67 $\pm$ 0.05	1.18 <sub>30</sub> <sup>b</sup>	0.24 <sup>b</sup>
Sex ratio (proportion female)	0.51 $\pm$ 0.06	0.51 $\pm$ 0.06	—	—
<i>II. Adult fecundity</i>				
Time to first oviposition (d)	28.9 $\pm$ 1.6	27.4 $\pm$ 1.2	-0.85 <sub>17</sub>	0.40
No. eggs oviposited	26.3 $\pm$ 1.5	32.1 $\pm$ 2.1	1.77 <sub>17</sub>	0.095
No. larvae hatched	9.7 $\pm$ 1.7	17.5 $\pm$ 2.2	3.37 <sub>17</sub>	0.004
Hatchability (proportion)	0.38 $\pm$ 0.07	0.57 $\pm$ 0.07	1.9 <sub>17</sub>	0.073

<sup>a</sup>Bonferroni corrected alpha level is 0.006 to account for eight tests.

<sup>b</sup>Z statistic and  $P$  value from the Wilcoxon signed ranks test.

*M. umbellata* leaves (Fig. 1). We observed no signs of pathogenicity of *G. cingulata* to *M. umbellata* after spraying leaves.

**NO-CHOICE EXPERIMENT: ENDOPHYTE EFFECTS ON PERFORMANCE.**—Beetles fed  $E_{high}$  leaves did not differ in the rate of larval development or pupal mass from those fed  $E_{low}$  leaves (Table 1).

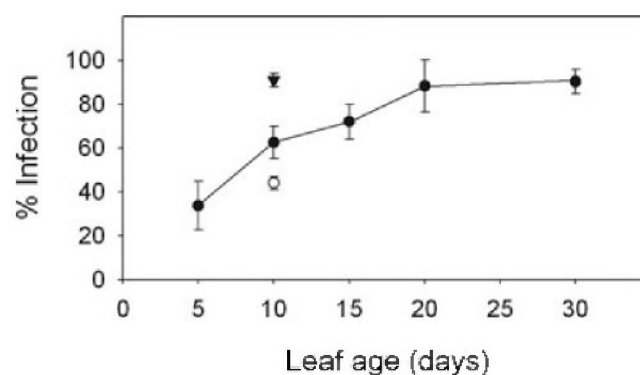


FIGURE 1. The mean ( $\pm$  SE) percent of *Merremia umbellata* leaf pieces infected with foliar endophytic fungi in the field and in the greenhouse. Field samples (full circles) refer to leaves that were collected at four different locations around secondary forest patches in Gamboa, Panama. Low-density (empty circles) and high-density (full triangles) treatments were created by applying a spray with spores or a control spray without spores to greenhouse plants.

Adult females that fed on  $E_{low}$  leaves (as larvae and adults), however, produced more eggs than those that fed on  $E_{high}$  leaves, although this trend was only marginally significant (Table 1). Reproductive success, as measured by 'hatchability' or the proportion of successful larvae from the first oviposition, was greater for 77 percent (14/18) of the females that fed on  $E_{low}$  relative to  $E_{high}$  leaves as larvae and adults. This represented a mean increase of 80 percent in the number of larvae hatched from females feeding on  $E_{low}$  leaves relative to  $E_{high}$  leaves (Table 1).

**CHOICE EXPERIMENT: ENDOPHYTE EFFECTS OF FOOD PREFERENCE.**—We observed no differences in food choice by adult females. Adults that chose between  $E_{high}$  and  $E_{low}$  leaves ate (mean  $\pm$  SE)  $6.8 \pm 1.0$  and  $5.4 \pm 0.95$  cm<sup>2</sup> leaf area, respectively ( $Z = 0.86$ ,  $df = 30$ ,  $P = 0.39$ ).

## DISCUSSION

Previous studies of horizontally transmitted endophyte effects on herbivory have relied mainly on endophyte–insect/presence–absence correlations (*e.g.*, Preszler *et al.* 1996), have been restricted to temperate areas, and have focused on one part of the insect life cycle (*e.g.*, Wilson & Carroll 1997). The present study is the first to investigate experimentally the effects of horizontally transmitted endophytes on larval and adult development and reproductive success of an herbivore. Adult females did not preferentially choose food with respect to endophyte density.

We found that the density of endophytic fungi had no effect on the development or survivorship of *C. alternans* larvae. However, when those larvae became adults, they produced fewer eggs and offspring if they ate food with high endophyte densities.

Insect reproductive success can be affected by host-plant qualities such as plant nutrition and the presence of defensive metabolites (reviewed by Awmack & Leather 2002). Endophytes can change the quality of host-plant tissue by: (1) inducing or increasing intrinsic host defense; or (2) providing defensive components that are extrinsic to the host plant (*e.g.*, alkaloid mycotoxins) (Herre *et al.* 2007). Preliminary data from studies in *Theobroma cacao* (L.) suggest that the presence of horizontally transmitted endophytic fungi may upregulate the production of gene products that are important for defensive pathways (Herre *et al.* 2007) and may increase lignin deposition (S. Maximova & E. A. Herre, pers. comm.). The components of plant quality that change with respect to endophyte density in *M. umbellata* are unknown and require further study.

The fecundity of an individual insect may be shaped by either active choice or passive limitation. For example, active choice occurs when adults modify the number or quality of eggs they oviposit based on the quality of host plants encountered (Hopkins & Ekblom 1999). Passive limitation of fecundity occurs when larval nutrition is sufficiently reduced in quality or quantity to exact physiological constraint or limitation on the number of eggs or successful larvae. In this study, it is unclear whether food quality during the larval period, adult period, or both periods led to effects on reproductive

success. Further, all of the performance variables were measured in no-choice tests for larvae and adults. Future experiments could investigate whether endophytes influence oviposition sites for *C. alternans*.

Several factors must be considered in the interpretation of this experiment. First, we were not able to compare the effect of *G. cingulata*-inoculated tissue with sterile tissue, to test the baseline effect of the fungus on this plant–herbivore interaction. Comparing tissue with low and high densities of fungi, however, more closely approximates the types of leaf tissue that beetles are likely to encounter in the wild, where essentially all leaf tissue contains some endophytic fungi. Second, as in all work with endophytic fungi, observations are limited to the culturability of particular endophytic fungi on any particular growth medium. For example, in this experiment, all statements about percentage infection of endophytic fungi refer to the percentage infection of endophytes that are culturable on malt extract agar. It is possible that other cryptic endophytic fungi existed in the leaf tissues, resulting in treatment infection rates that were not as different as they appear in Figure 1. However, the density of *G. cingulata*, which is dominant in healthy leaves in natural populations, was extremely different between the two treatments. Third, our greenhouse plants had a low diversity of endophytic fungi relative to their counterparts in the wild. Nearly all leaves in the wild host one to two common fungal morphotypes and a large number of apparently rare morphotypes (Van Bael *et al.* 2005), and this diversity may lead to competitive or synergistic interactions among different fungal strains in wild leaves. Differences in reproductive success observed in the greenhouse and laboratory experiments may be less meaningful in wild populations where *C. alternans* is constantly consuming a wide diversity of endophytic fungi. This problem is inherent in experiments where complex communities of organisms require simplification in order to investigate interactions. Nevertheless, we have demonstrated the potential for horizontally transmitted endophytes to reduce herbivore fitness. More complex experiments are needed to show the degree to which this potential is realized in natural populations.

The present study found evidence that the reproductive success of the leaf beetle, *C. alternans*, was modified by an approximately twofold difference in the density of the foliar endophytic fungi, *G. cingulata*, in the tissues of its host plant. The mechanism behind this result requires further study. Were there changes in the nutritional quality of *M. umbellata* with respect to endophyte density? Do defensive metabolites or other defensive characteristics of *M. umbellata* change with respect to endophyte density? If so, why was this not reflected in larval development rates on plant material with differing endophyte densities? Are adults making an active choice to lay more or less eggs or are they constrained by leaf quality? Is this constraint due to larval feeding or adult feeding? In this study, all of the performance variables were measured in no-choice tests for larvae and adults. When offered a choice of oviposition sites, will adults display a tendency toward laying eggs on leaf material with low endophyte densities? Further experiments are necessary to understand the mechanisms behind this plant–fungal–insect interaction.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

APPENDIX S1. Details of spore preparation.

APPENDIX S2. Development of sterilization protocols.

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