Supporting Information available online

Van Bael et al., Endophytic fungi increase the processing rate of leaves by leaf-cutting ants (Atta). Ecological Entomology

Methods and materials for supporting information

Merremia umbellata is a widespread Neotropical vine (Croat 1978) that grows in open areas, along the edges of forests, gaps and estuaries. It is commonly exploited by leaf-cutting ants in Gamboa (Van Bael, personal observation). In Gamboa, foliar endophyte communities of M. umbellata are dominated by species of the genera Xylaria, Glomerella/Colletotrichum, and Diaporthe; all of which appear to be horizontally transmitted from spore fall in the environment (Van Bael unpubl. data). We screened the most common morphospecies of M. umbellata for their ability to sporulate in laboratory conditions in order to select a species for leaf inoculation experiments and manipulation of endophyte densities within plants. Colleotrichum tropicale (Rojas et al. 2010) was used for leaf inoculation experiments, as it sporulated readily in the laboratory. In a previous study (wet season of 2005), we estimated that C. tropicale was present in ~50% of 42 healthy leaves sampled from forest edges in Gamboa, although we used only conidia morphology and not molecular data to confirm its identity in this collection (Van Bael, unpublished).

Plant inoculations with endophytes

For the single strain experiment, C. tropicale conidia were produced by means of liquid fermentation. For the liquid fermentation, we used 1.5% Molasses Yeast Medium (15g molasses, 2.5g yeast extract, 1 L water). To make the inocula, cultures of endophytic fungi were grown for ten days in Petri dishes with oatmeal agar (18g powdered oatmeal, 12g agar, 600ml H2O) until they colonized the entire Petri dish. The Petri dishes were then flooded with 5 ml of sterile water
and mycelia and conidia were scraped into Erlenmeyer flasks containing 500 ml of 1.5% MYM.

The flasks were put in a shaker at 125 rpm and 23°C for 14 days. Flasks were sealed with foil and parafilm for the initial 7 days and were sealed with cotton (to allow oxygen flow) for the final 7 days.

For inoculations, the contents of the flasks were filtered through a sterile net of nylon stockings to separate the mycelia from the conidia suspension. To concentrate the conidia, the suspensions were centrifuged for 30 seconds, the supernatants were discarded and the conidia pellets were resuspended in sterile water. We added 0.5% Tween 20 to aid in conidia dispersion. Conidia concentrations of $10^7$-$10^8$ conidia/ml were sprayed onto leaves using a Nalgene aerosol spray bottle (Mean conidia concentration was $9.1 \times 10^7$). Conidia concentrations in the final suspensions were determined using a hemacytometer. A control spray was created using sterile water and 0.5% Tween 20. We sprayed *M. umbellata* plants with the conidia suspension and control spray approximately 10 days before providing the leaves to ants.

For the multiple strain experiment, we took advantage of the fact that most sporefall occurs at night in the forest (Gilbert & Reynolds, 2005). As in Bittleston *et al.* (2011), a subset of our plants were taken out of the growth chambers and placed outside 5 nights per week, for at least 10 nights. The plants were sprayed with rainwater and left overnight in fine mesh cages (to protect from insect damage) near the rainforest edge, then returned to the growth chambers early the following morning. Low endophyte plants (*E*_low) remained in the growth chambers overnight. Thus, all plants were maintained in the same growth chamber during daylight growing hours.

*Re-isolations*
We used reisolations of endophytes from leaves to test whether our treatments resulted in significantly greater endophyte abundance and diversity in E\textsubscript{high} relative to E\textsubscript{low} leaves. From most leaves that we offered to ant colonies (see sample sizes in results below), we cut a small (~1 cm\textsuperscript{2}) section of leaf area to assess the abundance and diversity of endophytes in the leaf material we were offering to the ants. These sections were cut into 30 tiny segments (~1.5 mm\textsuperscript{2} each) that we surface sterilized with 70% ethanol (1 min) and 10% commercial bleach (1 min) (Van Bael \textit{et al.}, 2009). Twenty of these leaf segments were plated using sterile forceps on 2% malt extract agar and allowed to incubate for 7 days. For the single strain experiment, we assessed the proportion of leaf pieces (out of 20) with \textit{C. tropicale} growing out of them after 7 days. For the multiple strain experiment, we assessed the proportion of leaf pieces (out of 20) with any fungal endophyte growing out of them after 7 days. In addition we estimated the number of different morphospecies on a particular plate for both the E\textsubscript{high} and E\textsubscript{low} treatment leaves. Morphospecies based on mycelial characteristics are not necessarily reliable in defining species (Arnold \textit{et al.}, 2007), so these data should be interpreted only to assess whether here were indeed multiple strains of endophytes in our forest inoculated plants. These estimates were made as a one-time, rapid \textit{per plate} measurement. We did not isolate the individual strains into pure cultures and we did not attempt to compare morphospecies among plates, only within individual plates. Thus, we do not have detailed species accumulation curves or other standard diversity estimates, but rather a mean number of morphospecies an ant would be likely to encounter while processing a 1 x 2 cm leaf piece on an E\textsubscript{low} versus E\textsubscript{high} plant in our study. The re-isolation data did not pass tests of normality so we used non-parametric Mann-Whitney U tests to compare the E\textsubscript{high} and E\textsubscript{low} re-isolation results.

Surface rinse
Before presenting leaves to ants, all leaves of our experimental plants were rinsed in filtered water to reduce surface microbes (Barthlott & Neinhuis, 1997).

**Fungal growth – analyses of time-lapse photos**

To analyze the photos, we selected the first photo where an “inoculation event” took place – that is, a worker ant brought a piece of their cultivar’s mycelium and placed it on the green leaf material (Figure 1). We scored these events when a mycelium was absent in one photo and then present a minute later in the next photo. We then selected two additional photos per trial in which the focal leaf piece was not blocked by a worker ant, at 1 hr and 3 hrs after the inoculation event. We analyzed four photos per trial, one with the ruler for scale to measure the original substrate area planted, one for the inoculation event, one for 1 hr after inoculation, and one for 3 hrs after inoculation. We used ImageJ to measure the original amount of green leaf area planted, the green leaf area after 1 hr, and the green leaf area after 3 hrs. We subtracted the green area at 1 hr and 3 hrs from the original amount to estimate the amount of fungal growth at each of those times. We also subtracted the fungal growth area at 1hr from the growth at 3 hrs to get a difference in fungal growth over 2 hrs. The precision of these measurements was compromised by two factors. First, they included both new inoculation events by the ants and growth by the ants’ cultivar. Secondly, slight movement of the camera set up or the colony container would change the scale and render the photos incomparable to earlier ones. To be conservative, we discarded data from 6 trials, so that our final analysis used 18 trials to compare the paired data ($E_{high}$ versus $E_{low}$) on 9 colonies. We used a one-tailed, paired t-test (paired by colony) on square-root transformed data to test whether fungal colonization was slower on $E_{high}$ relative to $E_{low}$ leaf material.
Results

Endophyte treatments

For leaves treated with one endophyte strain (*C. tropicale*), the mean ± SE proportion of leaf pieces from the \(E_{\text{high}}\) treatment harboring endophytes was 0.51±0.07, compared to 0.0±0 for leaf pieces from the \(E_{\text{low}}\) treatment (\(n = 12\) \(E_{\text{high}}\) and 10 \(E_{\text{low}}\) leaves, Mann-Whitney \(U = 120, P<0.0001\)). For leaves treated with many endophyte strains, the mean ± SE proportion of leaf pieces with endophytes from the \(E_{\text{high}}\) treatment was 0.71±0.05, while the \(E_{\text{low}}\) treatment showed endophyte growth in 0.14±0.04 leaf pieces (\(n = 17\) \(E_{\text{high}}\) and 16 \(E_{\text{low}}\) leaves, Mann-Whitney \(U = 265\), 1 d.f., \(P<0.0001\)). Among \(E_{\text{high}}\) leaves only, we compared the level of endophyte abundance in leaves with one vs. many strains and found the leaves with many strains had significantly greater proportion of leaf pieces with endophytes (Mann-Whitney \(U = 126\), 1 d.f., \(P=0.036\)). Among \(E_{\text{low}}\) leaves only, we compared the level of endophyte abundance from experimental leaves in the different experiments. We found that the \(E_{\text{low}}\) leaves from the single strain experiment had significantly lower proportion of leaf pieces with endophytes relative to the multiple strain experiment (Table 1, Mann-Whitney \(U = 174\), 1 d.f., \(P<0.0001\)).

For leaves treated with forest spore fall, we isolated an average of 3.7 ± 0.2 different morphospecies of fungi from a given leaf sample (a sample was 20 leaf pieces of ~1.5mm\(^2\) each). The mean number of morphospecies per isolate was 0.25 ± 0.02 for our leaves inoculated with many strains (a value of 1 would indicate that every isolate on the plate was a unique morphospecies).

References for Supporting Information


