



# An experimental test of ant effects on herbivory and pathogen infection on wild cotton (*Gossypium hirsutum* L.)

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## Abstract

Ant-plant interactions are widespread and have profound effects on plant fitness, food webs, and species diversity. Whereas most experimental work has tested for ant effects on herbivores, few studies have investigated ant effects on plant pathogens, and even less dual effects on herbivores and pathogens. In this work, we tested whether ants protect wild cotton (*Gossypium hirsutum* L.) plants against insect herbivores and fungal pathogens by means of an ant exclusion field experiment. In addition, we measured extrafloral nectar (EFN) traits (volume and concentration) to assess their role in ant attraction, and controlled for cotton direct chemical defenses (phenolics) in testing for ant-mediated defense. Ant exclusion did not have a detectable effect on either insect herbivory or pathogen infection levels on wild cotton leaves, and, accordingly, did not impact plant growth (height) over the growing season. We found that EFN traits were associated with ant recruitment to wild cotton plants, with both nectar amount and concentration being positively correlated with ant abundance on control plants. The above tests accounted for leaf phenolics, a type of direct defense, indicating no independent effect of indirect defense mediated by ants on wild cotton during the study. These results provide limited evidence of biotic defense by ants against insect and pathogens on wild cotton at the study site, but call for further assessments of spatiotemporal variation in wild cotton-ant interactions.

**Keywords** Ant-plant interaction · Wild cotton · Extrafloral nectar · Herbivory · Pathogens

## Introduction

Ants are key ecological players in virtually all terrestrial ecosystems, acting as predators, pollinators or ecosystem engineers (Hölldobler and Wilson 1990; Heil and McKey 2003; Lavelle et al. 2016; Zhong et al. 2021), as well as having notable impacts on ecosystem function (e.g., biogeochemical and nutrient cycles: Sternberg et al. 2007; Swanson et al. 2019). In particular, plants are well known to establish mutualistic interactions with ants (Janzen 1966; Ibarra-Manríquez and Dirzo 1990; Merbach et al. 2007; Heil 2015). In such interactions, plants provide refuge, food or some other resource for ants, whereas ants frequently defend plants against attackers (Bronstein 1998; Rico-Gray and Oliveira 2007) and reduce plant competition against hetero-specific neighboring plants (e.g., Frederickson et al. 2005), with these effects ultimately shaping community structure.

Research has extensively focused on plant traits that mediate ant-plant interactions, including physical structures that provide nesting sites (e.g., domatia, Nishida et al. 2006), as well as plant-based resources such as food

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bodies (e.g., Müllerian bodies; Rickson 1971) and extrafloral nectar (EFN) which are actively collected and consumed by ants (Bentley 1977; Heil and McKey 2003; Heil 2008, 2015). Among these plant traits, EFN has been one of the best studied and is considered an important nutritional resource for ants which mediates ant-plant interactions (Bentley 1977; Heil and McKey 2003; Heil 2015). Several studies have shown that plants actively increase EFN secretions in response to herbivore attack (Heil and Silva Bueno 2007; Pulice and Packer 2008; Rogers et al. 2003), which in turn promotes ant recruitment (reviewed by Turlings and Wäckers 2004; Heil 2015). As a result, these EFN-mediated effects on ants (and other arthropods) end up shaping plant fitness (e.g., reproductive output) and plant-associated interactions (e.g., herbivore and predator composition or diversity; Rudgers and Gardener 2004; Heil 2015). In addition, because plants invest in multiple defenses to more effectively reduce enemy attack, studies separating the effects of direct (e.g., secondary metabolites) from indirect defensive traits such as EFN are particularly valuable (and yet uncommon; e.g., Frederickson et al. 2013) for assessing the independent impacts of ant-mediated plant protection.

Most of studies on ant-plant interactions have focused on the protective effects of ants against insect (Rico-Gray and Oliveira 2007; Rosumek et al. 2009; Fagundes et al. 2017) and vertebrate herbivores (Palmer and Brody 2007; Goheen and Palmer 2010; Martins 2010). In contrast, and despite the prevalence of plant-pathogen interactions, ant protection against plant pathogens has been less studied and the mechanisms by which these protection services take place are less understood (González-Teuber et al. 2014). Indeed, studies have shown that ants frequently glean (e.g., leaf) surfaces to actively remove, for example, bacterial and fungal spores, thus reducing the incidence of plant diseases (Offenberg and Damgaard 2019). Accordingly, studies to date that have looked at ant protection against pathogenic fungi and bacteria in plants have shown that such effects can be as strong as those observed for herbivores (e.g., Heil et al. 1999; Heil et al. 2001; González-Teuber and Heil 2010; González-Teuber et al. 2014; reviewed by Offenberg and Damgaard 2019). Therefore, more studies evaluating predatory ant effects on plant pathogens, as well as their simultaneous effects on multiple attackers (e.g., herbivores vs. pathogens) are needed to close this knowledge gap. Addressing these multiple effects is necessary for a complete understanding of ant-plant mutualisms and their outcomes since plants are frequently attacked by both pathogens and herbivores (Moreira et al. 2018).

Wild cotton, *Gossypium hirsutum* L. (Malvaceae), is a myrmecophilic shrub that is naturally distributed in the Yucatan Peninsula (Mexico), its likely center of origin (Brubaker and Wendel 1994). Populations are found in the coastal scrubland and sand dunes (Wegier et al. 2011;

D'Eeckenbrugge and Lacape 2014; Abdala-Roberts et al. 2018), and to some extent also more inland on edges of tropical dry forest remnants (D'Eeckenbrugge and Lacape 2014). At these sites, wild cotton is attacked by several insect herbivores (mainly leaf-chewing caterpillars, grasshoppers, and beetles) and pathogens (e.g., necrotrophic leaf fungi) (Abdala-Roberts et al. 2019a, b), and is an EFN-bearing species for which more than 20 ant morpho-species have been recorded in coastal and inland forest sites (Vázquez-Barrios et al. 2021; Abdala-Roberts data from this study). Previous work with other cotton species (e.g., *G. thurberi*, Rudgers 2004) found that ants reduce herbivory, but only two studies to date have addressed ant protective effects in *G. hirsutum*. One of these found evidence of ant defense against insect herbivores for several cultivated and wild genotypes of *G. hirsutum* under greenhouse and field conditions in cultivated areas (Llandres et al. 2019), whereas another study conducted in situ found results suggesting a defensive role of ants (e.g., *Camponotus*) against insect herbivory on wild cotton plants (Vázquez-Barrios et al. 2021). In this latter study, however, ant effects were not experimentally tested and work assessing the simultaneous effects of ants on insect herbivory and pathogen infection have not been conducted yet. In this study, we evaluated whether ants provide protection against insect herbivores and fungal pathogens on wild cotton by means of an ant exclusion field experiment. In addition, we measured EFN traits (volume and concentration) to test for their effects on ant recruitment and further accounted for plant chemical direct defenses (phenolics) to test for indirect defense while controlling for potential effects of direct defenses on enemy attack. Overall, this study provides a novel assessment of ant defense in wild cotton plants growing under natural conditions by assessing their dual effects on insect herbivores and pathogens and the role of EFN traits in ant attraction.

## Materials and methods

### Study species

Wild cotton, *G. hirsutum* is a myrmecophilic shrub that grows up to 2 m tall under natural conditions (Oosterhuis and Jernstedt 1999). It is native to Central America, Mexico and the Caribbean Basin (Wendel et al. 1992; Oosterhuis and Jernstedt 1999) and is thought to have originated in southeast Mexico (D'Eeckenbrugge and Lacape 2014). Populations are common along the coasts of the Yucatan Peninsula (SE Mexico), where plants grow in the coastal scrubland or sand dune vegetation (D'Eeckenbrugge and Lacape 2014), and can also be found in inland sites on tropical forest edges (Wegier et al. 2011; Abdala-Roberts et al. 2019b; D'Eeckenbrugge and Lacape 2014). Flowering occurs twice

a year (May–June and December–January) and plants start producing flowers during the first or second year of life.

Wild cotton in the Yucatan Peninsula is attacked mainly by leaf chewers belonging to Lepidoptera, Orthoptera, and Coleoptera, and to a lesser extent, sap feeders such as aphids and other hemipterans (Abdala-Roberts et al. 2019b). In addition, wild cotton is attacked by leaf pathogens, including generalist necrophytic fungi such as *Corynespora cassiicola* (López et al. 2018) and *Alternaria solani* (Abdala-Roberts et al. 2019b) which attack a broad range of plant species (including numerous crops; Rahman et al. 2012; López et al. 2018), whereas cultivated cotton is attacked by several species of specialist fungi (e.g., *Ascochyta gossypii*, *Verticillium albo-atrum*, *Fusarium oxysporum*, *Colletotrichum*) (Horne et al. 1988). At sites in Yucatan, insect damage and pathogen infection on leaves of wild cotton peak during the rainy season (June–July or September–October; Abdala-Roberts et al. 2019a). Insect herbivory at the peak of the rainy season is ca. 20% leaf area removed, on average, though leaf damage levels vary considerably across sites (9–50%; Abdala-Roberts et al. 2019b). In addition, this species is associated with a diverse ant community, tending extrafloral nectaries (Vázquez-Barrios et al. 2021; L. Abdala-Roberts, data from this study). More than 16 species have been identified thus far across natural populations, whereby the identity of the most common species varies across sites (Pérez-Niño, unpublished data). Representative species belong to the following genera: *Camponotus*, *Monomorium*, *Pseudomyrmex*, *Dorymyrmex* and *Crematogaster*. To date, there have been no detailed studies on the protective effects of these ant taxa, though recent work suggests that species of *Camponotus* and *Dorymyrmex* play an important defensive role (Vázquez-Barrios et al. 2021).

Current understanding of chemical defenses in *G. hirsutum* comes mainly from studies with domesticated varieties (reviewed by Hagenbucher et al. 2013), though a few studies with wild populations of other *Gossypium* species have also been conducted (e.g., Rudgers 2004). Wild (and cultivated) *G. hirsutum* produce a number of direct defenses such as pigment glands which contain gossypol and other related terpenes with insecticidal effects (McAuslane et al. 1997; Rudgers 2004; Stipanovic et al. 2006), as well phenolic compounds in leaves which have been shown to provide resistance against chewing insects (Mansour et al. 1997; Nix et al. 2017). In addition, this plant also produces EFN and volatile organic compounds (VOCs) which attract herbivore natural enemies such as ants and parasitoids (McCall et al. 1994; Wäckers and Bezemer 2003; Rudgers 2004). In particular, EFN is produced in nectaries found on the mid-vein (and sometimes also on secondary veins) and is rich in carbohydrates, aminoacids as well as lipids (Wäckers et al. 2001; Rudgers 2004). All these traits, including EFN, are inducible in response to herbivore damage (McCall et al. 1994;

Loughrin et al. 1995; McAuslane et al. 1997; Agrawal and Karbal 2000; Opitz et al. 2008).

## Seed sources and experimental design

In July 2017, we collected seeds from 14 mother plants (“genotypes” hereafter) of wild cotton found in a naturally occurring population located on the northwestern coast of the Yucatan Peninsula (20° 58' 30.2" N, 90° 20' 57" W), near the town of Celestún (Yucatán, Mexico). Distance between plants was at least 2 m. Seeds were germinated in late November 2017 and kept under greenhouse conditions for 3 months. In early March, 40-cm tall plants (8–10 leaves per plant) were transplanted at the Campus de Ciencias Sociales of the Universidad Autónoma de Yucatán (21° 1' 27" N, 89° 33' 15" W), at a site surrounded by secondary deciduous tropical forest. This site is within the natural distribution range of wild cotton in the Yucatan Peninsula (i.e., inland populations, see above). Plants of each genotype ( $N = 10$  plants/genotype) were randomly allocated to eight 4 × 8-m blocks. Distance between plants within each block was ca. 40 cm and distance between blocks was 1.5–2 m. All plants were watered twice a week throughout the experiment. Although common garden experiment such as this provides less realism compared to an exclusion experiment with naturally growing plants, it provides greater control over undesired biotic (e.g., plant features) and abiotic (e.g., microhabitat conditions) variation. In addition, this approach was much more feasible than an experiment with naturally growing plants as plant architecture (cotton plants usually have low hanging branches that touch the ground) and the dense shrubby vegetation makes ant exclusions at the whole-plant level highly complicated, especially for exclusions lasting several months.

In early July, we excluded ants from half of the plants within each block by manually removing all ants and placing a 16-cm diameter by 12-cm high PVC cylinder around the base of the plant. The cylinder was inserted 5 cm deep in the soil and we applied a 5-cm wide strip of bicycle grease (Roshfrans, B.A.T.-3, Mexico) along the upper edge of the cylinder. We reapplied the grease every 2 weeks and removed any ants present on selected plants throughout the study. This method was successful and excluded virtually all ants on ant-excluded plants (excluded =  $0.21 \pm 0.04$  ants; not excluded =  $10.48 \pm 0.73$  ants; mean  $\pm$  SE across six surveys throughout the season). Ant abundance on control plants was within the range observed in natural populations ( $1.63 \pm 0.63$  to  $24.25 \pm 6.93$  ants per plant,  $n = 24$  populations; L. Abdala-Roberts, unpublished data), and ant nests were observed at the experimental site (M. Reyes-Hernández, pers. obs.). Exclusions were maintained until mid-October, covering most of the rainy season which is when cotton growth and insect (including ant and herbivore) activity is greatest.

By the end of the experiment plants had, on average, tripled their height ( $1.33 \pm 0.05$  m) and quadrupled their leaf number ( $42.62 \pm 0.97$  leaves).

## Response variables

### Ant abundance, leaf herbivory and fungal infection

We recorded ant abundance on control (non-excluded) plants throughout the study by individually sampling plants and counting all ants on stems and leaves. Ant observations involved six surveys conducted every 15 days from late July to early October 2017, and specimens were collected for identification. Preliminary assessments indicated that it was not possible to separately analyze abundance for each survey or test for temporal variation in ant abundance due to limited data which prevented model convergence in several cases. We therefore used the mean value across surveys per plant for statistical analyses as an overall measure of ant recruitment and activity throughout the season. Likewise, it was not possible to separately analyze each ant morpho-species except in one case (*Crematogaster* sp., by far the most common; see “Results”) for which results did not change compared to an analysis of all species combined and we therefore opted to include all species in the analysis. Expectedly, patterns pooling all species are likely strongly influenced by this species. Finally, we also counted the number of leaves per plant at the middle and end of the sampling season as a proxy of availability (i.e. number) of nectaries.

In late October 2017, toward the end of the rainy season (once most herbivory had taken place, i.e., measure of cumulative damage), for each plant we visually estimated the amount of area consumed per leaf by chewing insects and necrosis due to fungal infection (mainly *Alternaria* sp. or *Curvularia* sp; E. Herrera, personal observation). In both cases, we selected a 1-m branch or two 0.5-m branches and scored herbivory and infection for each leaf with the following scale: 1 (0% area consumed or infected), 2 (1–15%), 3 (16–30%), 4 (31–45%), 5 (46–60%) and 6 (61–75%), (Abdala-Roberts et al. 2019a). We averaged the scores across leaves to obtain a single (mean) value per plant for statistical analysis. The presence of herbivory and infection on the same leaf was uncommon (M. Reyes-Hernández, personal observation). In addition, during this leaf damage survey we also measured plant height to account for residual variation in response variables due to plant size.

### Extrafloral nectar

We quantified nectar production and concentration by sampling five leaves per plant located close to the apical meristem, where nectar activity is usually highest. Nectar was collected between 6:00 and 8:00 AM throughout a 6-day period.

The amount of nectar was quantified with 5  $\mu$ l micropipettes (Micropipettes Blaubrand® intraMARK, color code white, Germany) and measured in  $\mu$ l, whereas nectar concentration was quantified with a manual refractometer (Atago Master T 0 to 33°Brix, Germany) and measured in °Brix. These measurements were taken only for non-excluded (control) plants and were conducted several times throughout the experiment (every 3–4 weeks from mid-July to late October). We used the mean value (across nectaries and surveys) per plant for statistical analyses. Ant-excluded plants were not considered in these measurements as ants were virtually absent in most cases (see above), precluding an analysis of EFN effects on ant recruitment.

### Phenolics

In early November, we sampled plants to assess the concentration of leaf phenolics and therefore account for effects of direct chemical defenses (Abdala-Roberts et al. 2019a, b; Dixit et al. 2020) when testing for ant-mediated indirect effects on insect herbivore and pathogen attack. To this end, we collected three or four undamaged leaves per plant and samples were immediately stored in a cooler and transported to the laboratory where they were dried at 45 °C. For chemical identification of the polyphenol composition in plant extracts, we used an ultra-performance liquid chromatography coupled with electrospray ionization quadrupole (Thermo Dionex Ultimate 3000 LC) time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) (Bruker Compact™). Chromatographic separation was performed in a Kinetex™ 2.6  $\mu$ m C18 82–102 Å, LC Column 100  $\times$  4.6 mm column using a binary gradient solvent mode consisting of 0.05% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was used: from 10% to 30% B (0–5 min), from 30 to 50% B (5–10 min), from 50% to 100% B (10–12 min), hold 100% B until 14 min, from 100% to 10% B (14–15 min), hold 10% B until 17 min. The injection volume was 3  $\mu$ l, the flow rate was established at 0.4 ml/min and column temperature was controlled at 35 °C. MS analysis was operated in a spectra acquisition range from 50 to 1200 m/z. Negative (–) ESI modes was used under the following specific conditions: gas flow 8 l/min, nebulizer pressure 38 psi, dry gas 7 l/min, and dry temperature 220 °C. Capillary and end plate offset were set to 4500 and 500 V, respectively. MS/MS analysis was performed based on the previously determined accurate mass and RT and fragmented by using different collision energy ramps to cover a range from 15 eV to 50 eV. Individual compounds were identified based on the data obtained from the standard substances or published literature including RT,  $\lambda_{max}$ , ( $[M-H]^-$ ), and major fragment ions. For the quantitative analysis of phenolic compounds, 3  $\mu$ l of each sample was then analyzed using the same column and conditions mentioned in the

previous paragraph, in an UHPLC (Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. Chromatograms were recorded at 330 nm. We identified four groups of phenolic compounds: flavonoids, ellagitannins and gallic acid derivatives (“hydrolysable tannins” hereafter), proanthocyanidins (“condensed tannins” hereafter) and hydroxycinnamic acid precursors to lignins (“lignins” hereafter). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents. We achieved the quantification of these compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and 5 µg/ml. We used concentrations expressed in mg/g tissue on a dry weight basis and summed across groups to obtain a total concentration (i.e., total phenolics) which was used for statistical analyses. By sampling leaves at the end of the season, concentrations measured of these metabolites likely represented constitutive levels plus some unknown level of induction due to enemy attack.

### Statistical analyses

We first ran a general linear mixed model (GLMM) using only control plants (non-excluded) to test for effects of nectar volume and concentration (proxies of nectar quantity and quality, respectively) on mean ant abundance per plant. Then, using all plants, we ran a GLMM testing for the effect of ant exclusion (control vs. excluded) on mean leaf herbivory and pathogen infection. Preliminary analyses of EFN data indicated that mean leaf number did not significantly influence ant abundance on control plants ( $F_{1,53} = 2.85$ ,  $P = 0.10$ ) and we therefore removed it from these models. For the leaf herbivory and infection models, we included plant height and total phenolics as covariates, the latter to account for residual variation in enemy attack due to direct defenses. In addition, we ran a model testing for an effect of ant exclusion on plant height to evaluate whether ant effects impacted plant growth. All models included plant genotype and block as random effects to control for plant-based genotypic and environmental variation, respectively. Significance of random effects was assessed with Wald tests. All responses were normally distributed, except for ant abundance which was log-transformed to achieve normality. We report model least-square means and standard errors (back-transformed for ant abundance) as descriptive statistics. All the above analyses were performed with SAS ver. 9.4 (SAS, 2014) using PROC MIXED. Finally, to visualize results from the model testing for EFN trait effects on ant abundance on control plants we calculated the fixed effects, random effect, and residual contributions to each observed data point using the fitted model (i.e., estimated  $Y$  values)

and then subtracted the genotype and block effect for each observation using the broom package in r ver. 3.6.1 (R Core Team, 2013), i.e., model predicted values after accounting for random effects in the model.

## Results

A total of 2380 ant specimens belonging to seven morpho-species were recorded on wild cotton plants during the experiment. *Crematogaster* sp. (80.00%) was the most common by far, followed by *Dorymyrmex* (11.72%), *Ectatomma ruidum* (5.88%), *Brachymyrmex* sp. (1.05%), *Camponotus planatus* (0.92%), *Pheidole* sp. (0.34%), and *Cephalotes* sp. (0.08%) (Table S1, supplementary material). The analysis of EFN traits for non-excluded plants showed that the mean number of ants was significantly positively associated with the mean volume (slope estimator =  $0.188 \pm 0.077$ ) and concentration (estimator =  $0.131 \pm 0.043$ ) of EFN (Table 1; Fig. 1A, B). Both nectar traits therefore had independent effects on ant recruitment on wild cotton plants.

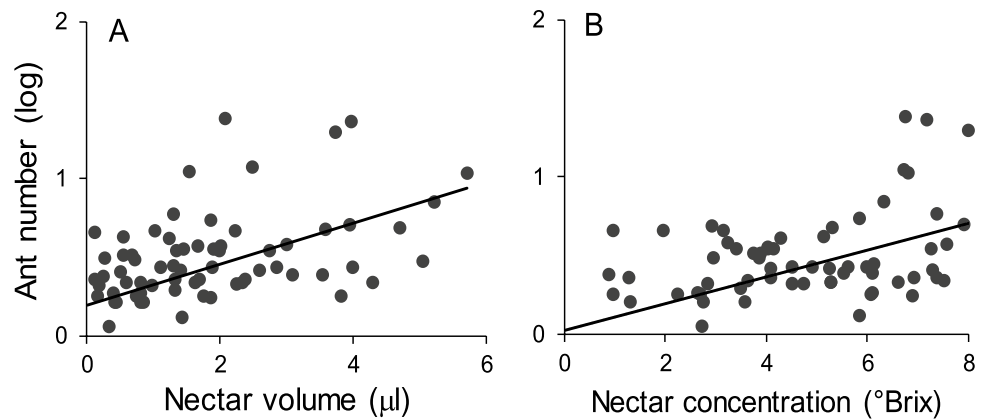
Results indicated no significant effect of ant exclusion on either insect leaf herbivory (control: mean score =  $0.86 \pm 0.09$ ; excluded:  $0.83 \pm 0.08$ ) or pathogen infection (control:  $1.68 \pm 0.21$ ; excluded:  $1.86 \pm 0.21$ ) (Table 2; Fig. 2A, B). In addition, we found no effect of total phenolics on leaf herbivory (slope estimate =  $0.000032 \pm 0.00266$ ) or pathogen infection (estimate =  $-0.00179 \pm 0.00329$ ) (Table 2), as well as no correlation between plant height and leaf herbivory (estimator =  $-0.0564 \pm 0.0573$ ) but a significant negative correlation between height and infection (estimator =  $-0.3249 \pm 0.0734$ ). Finally, we found no effect of ant exclusion on plant height (control:  $1.18 \pm 0.18$ ; excluded:  $1.51 \pm 0.18$  cm) (Table 2; Fig. 2C).

**Table 1** Results from a general linear mixed model (GLMM) testing for effects of extrafloral nectar volume (µl) and concentration (°Brix) on ant abundance for wild cotton (*Gossypium hirsutum*) plants

Predictor	F/Z value	DF	P value
Nectar volume	5.94	1,54	<b>0.018</b>
Nectar concentration	9.24	1,54	<b>0.004</b>
Genotype	0	1,54	0
Block	1.32	1,54	0.093

The model was run only for control (non-excluded) plants and included block and genotype as random effects. Data were log-transformed to achieve normality of residuals. Significant effects ( $P < 0.05$ ) are in bold

**Fig. 1** Associations between ant abundance and **A** extrafloral nectar volume (ml) and **B** concentration (°Brix) on wild cotton (*Gossypium hirsutum*). Predicted relationships are from simple linear regressions (volume:  $R^2=0.27$ ; concentration:  $R^2=0.29$ ) using fitted values from a general linear mixed model after controlling for the effects of plant genotype and block (see “Methods”)



**Table 2** Results from general linear mixed models (GLMMs) testing for effects of ant exclusion on insect leaf damage, pathogen infection, and plant height for wild cotton (*Gossypium hirsutum*)

Predictor	Insect herbivory		Pathogen infection		Plant height	
	F/Z value	P value	F/Z value	P value	F/Z value	P value
Ant exclusion	0.08	0.779	1.43	0.234	3.82	0.053
Total phenolics	0.01	0.989	0.30	0.585	–	–
Plant height	0.97	0.326	19.61	<b>&lt;0.0001</b>	–	–
Genotype	1.45	0.073	1.17	0.122	0.94	0.174
Block	1.33	0.091	1.80	<b>0.036</b>	1.57	0.058

Leaf damage models included plant height and total phenolics (to control for plant size and direct defenses, respectively) as covariates, as well as genotype and block as random effects. Degrees of freedom for main effects in all cases = 1117. Significant effects ( $P < 0.05$ ) are in bold

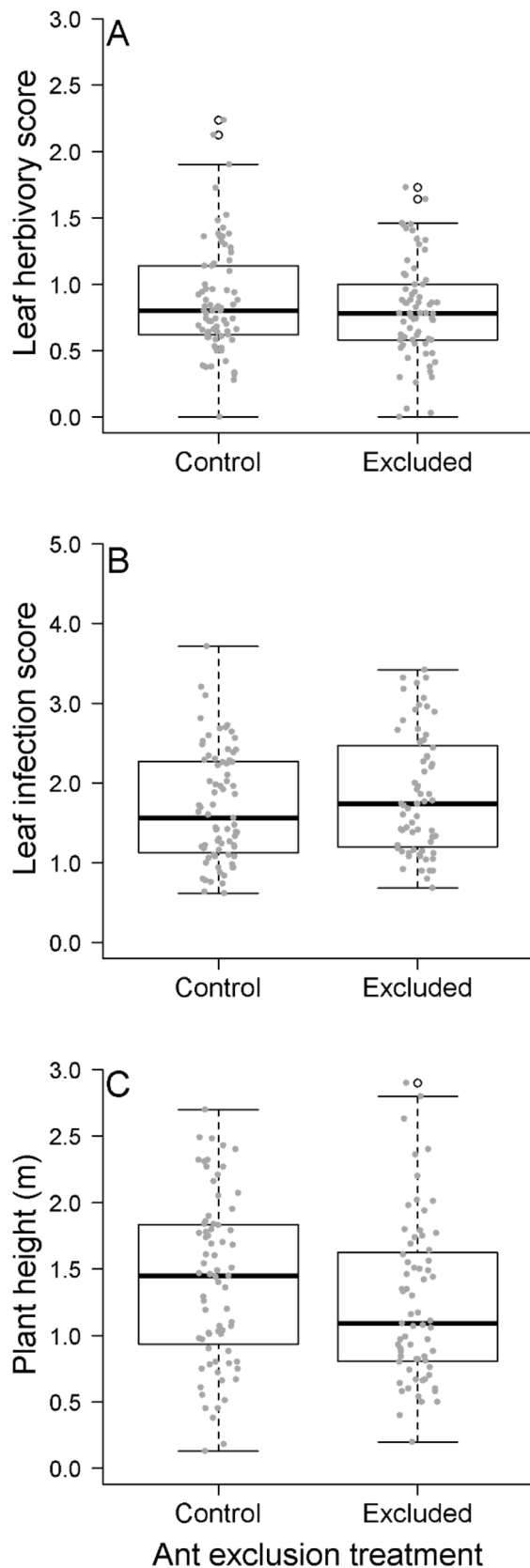
## Discussion

Extrafloral nectar traits were significantly associated with ant recruitment to wild cotton saplings, with both nectar amount and concentration being positively correlated with ant abundance on control plants. However, ant exclusion had no detectable effects on either leaf insect herbivory or pathogen infection levels after controlling for direct defenses, and, accordingly, did not impact plant growth (height) over the growing season. Likewise, the concentration of leaf phenolics was uncorrelated with both types of leaf damage. Accordingly, these results provide no evidence of ant-mediated defense against insect herbivores and pathogens on wild cotton during the sampled growing season and site.

There is a long history of research on the effects of EFN on ant-plant interactions (e.g., Janzen 1966; Bentley 1977; Koptur 1984; reviewed by Heil and McKey 2003; Turlings and Wäckers 2004; Heil 2015). Typically, most studies have measured EFN production, and to some extent also nectar concentration and composition (Ness et al. 2009; Lange et al. 2017; reviewed by Heil 2015). With respect to *Gossypium* species, a recent study found that EFN production correlated with ant abundance on wild and cultivated varieties of *G. hirsutum* (Llandres et al. 2019), and

another study found that induction of EFN increased ant abundance and altered ant species composition in a natural population of wild *G. hirsutum* (Vázquez-Barrios et al. 2021). Likewise, a related study found that the number of active extrafloral nectaries and nectary size correlate with ant abundance in wild populations of *G. thurberi* (Rudgers 2004). To date, however, most studies assessing EFN effects on ants, including cotton, have usually not assessed the effects of multiple EFN traits simultaneously to disentangle their influence on ant recruitment and composition (but see Rudgers 2004; Rudgers and Gardner 2004). In this sense, our joint assessment of EFN amount and concentration (sugars, measured in °Brix) indicated strong independent effects of both traits on ant abundance on wild *G. hirsutum* saplings. This suggests that both nectar traits play an important role in mediating ant-cotton interactions in wild populations of this species. Additional work separating effects of these and other traits (e.g., volatiles; see Rasmann et al. 2014) on ant abundance, composition, and behavior would further understanding of plant trait-mediated controls over associated ant communities on wild cotton.

Counter to expectations, our results indicated no effect of ant exclusion on leaf damage by insect herbivores or pathogenic fungi, suggesting that ants did not confer indirect resistance. A vast number of studies have experimentally



**Fig. 2** Box plots showing effects of ant exclusion on scores of insect leaf damage, pathogen infection, and plant height for wild cotton (*Gossypium hirsutum*) plants. Data points are overlaid in gray, including outliers

tested and found positive effects of ants on EFN-bearing plants involving protection against insect herbivores (reviewed by Heil and McKey 2003; Rico-Gray and Oliveira 2007) as well as plant pathogens (Offenberg and Damgaard 2019). Some of this work also includes investigations on cotton species (e.g., Rudgers and Gardner 2004; Hagenbrucher et al. 2013), all of which have looked at ant effects on insect herbivory. For example, work by Rudgers (2004) showed that experimental reduction of EFN lowered ant abundance which in turn increased insect herbivory on *G. thurberi*. In addition, a recent study by Vázquez-Barrios et al. (2021) found that the induction of EFN in wild *G. hirsutum* plants correlated with increased ant abundance and less herbivory, suggesting ant-mediated defense (though ant abundance was not manipulated). To our knowledge, however, our study is the first to test for effects on pathogens in *Gossypium*. Granted that our study features were fairly robust (e.g., controlled for variation in direct defenses, sampling covered most of the growing season), our failure to detect ant effects is perhaps not surprising in light of previous research showing that ant-plant interactions are labile and strongly context-dependent (Chamberlain and Holland 2009; Chamberlain et al. 2014). For example, the outcome of ant-plant interactions on *G. thurberi* varies across sites, such that ant protective effects are contingent on factors such as ant abundance, composition, and the level of herbivory (Rudgers and Strauss 2004; see also González-Teuber et al. 2014 for an example involving pathogens on an *Acacia* species). In addition, Vázquez-Barrios et al. (2021) found that wild *G. hirsutum* genotypic variability in EFN induction (amount produced) shaped ant composition, with some species being more aggressive and possibly more effective in protecting cotton plants against insect herbivores. In our case, and consistent with previous work at this site (Abdala-Roberts et al. 2019a), herbivory and infection levels were relatively low and rarely exceeded 10% of leaf area damaged which is close to the lower end of herbivory levels observed across populations (9–50%; Abdala-Roberts et al. 2019b). This would result in a narrow margin for ant protective effects to take place combined with low EFN induction and thus low levels of ant recruitment leading to an insignificant effect on leaf damage. In addition, results could also be partly explained by ant species composition as the most abundant species by far (80% of all records) was *Crematogaster* sp., whereas presumably more aggressive species such as *Camponotus planatus* and *Dorymyrmex bicolor* reported previously by Vázquez-Barrios et al. (2021) were much less abundant in our study. Ongoing work indicates that ant composition (and abundance) varies greatly across wild cotton populations (e.g., *Crematogaster* sp. has been observed around half of the populations sampled thus far, with its relative frequency ranging from 17 to 95% of all individuals sampled; L. Abdala-Roberts unpublished), pointing at a potentially key

role of ant species composition and of specific ant species in determining the occurrence and strength of indirect defense across wild cotton populations. Finally, temporal variation in ant visitation and effects are commonplace (Calixto et al. 2021), warranting longer-term exclusion experiments encompassing the flowering phase and comparing effects among seasons or multi-annually.

Another key consideration is undertaking work across multiple sites and habitats known to vary in ant abundance and composition, including coastal populations of *G. hirsutum*. Exclusion experiments can be used to test for variation in the occurrence and strength of ant-based defense across populations and further assess its biotic (ant and herbivore species composition, plant traits; Abdala-Roberts et al. 2019b) and abiotic (e.g., water availability, soil salinity; Quijano-Medina et al. 2021) correlates. In this sense, ongoing surveys of variation in wild cotton-associated ant communities across 12 coastal sites have found more than 16 morpho-species and marked variation in species relative abundances (L. Abdala-Roberts, unpublished). Likewise, while recent experimental work suggests that soil salinity does not affect EFN induction (Quijano-Medina et al. 2021), high in situ variability in soil salinity and water availability levels (T. Quijano-Medina, unpublished) could be strong forces modulating EFN and other traits affecting ant communities. A priori knowledge on spatial differences in ant abundance or composition could inform the selection of contrasting sites for manipulative studies.

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## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest with the content of this article.

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