

## RESEARCH PAPER

# Effects of amount and recurrence of leaf herbivory on the induction of direct and indirect defences in wild cotton

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## Keywords

Extrafloral nectar; induced defences; phenolic compounds; recurrent herbivory; wild cotton.

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## ABSTRACT

- The induction of defences in response to herbivory is a key mechanism of plant resistance. While a number of studies have investigated the time course and magnitude of plant induction in response to a single event of herbivory, few have looked at the effects of recurrent herbivory. Furthermore, studies measuring the effects of the total amount and recurrence of herbivory on both direct and indirect plant defences are lacking. To address this gap, here we asked whether insect leaf herbivory induced changes in the amount and concentration of extrafloral nectar (an indirect defence) and concentration of leaf phenolic compounds (a direct defence) in wild cotton (*Gossypium hirsutum*).
- We conducted a greenhouse experiment where we tested single event or recurrent herbivory effects on defence induction by applying mechanical leaf damage and caterpillar (*Spodoptera frugiperda*) regurgitant.
- Single events of 25% and 50% leaf damage did not significantly influence extrafloral nectar production or concentration. Extrafloral nectar traits did, however, increase significantly relative to controls when plants were exposed to recurrent herbivory (two episodes of 25% damage). In contrast, phenolic compounds increased significantly in response to single events of leaf damage but not to recurrent damage. In addition, we found that local induction of extrafloral nectar production was stronger than systemic induction, whereas the reverse pattern was observed for phenolics.
- Together, these results reveal seemingly inverse patterns of induction of direct and indirect defences in response to herbivory in wild cotton.

## INTRODUCTION

The induction of plant defences in response to herbivory is a widespread phenomenon (Karban & Baldwin 1997; Agrawal 2007; Karban 2011), and includes traits associated with either direct or indirect resistance. Direct defences are chemical and physical traits that deter herbivores or reduce their consumption or survival (Agrawal 2007; Carmona *et al.* 2011). Indirect defences, on the other hand, provide shelter, rewards or information on herbivore presence to natural enemies of herbivores, which in turn reduce herbivory (Turlings & Wäckers 2006; Kessler & Heil 2011). Within this context, a great deal of research has focused on understanding how plant induced defences vary in space and time as a function of features such as the type and amount of herbivory, particularly in the case of direct defences (Underwood *et al.* 2005; Karban 2011). Likewise, aspects such as the time course characterised by the speed and rate of decay of induced responses to herbivory, as well as their underlying biochemical mechanisms have also been well studied for direct defences (Karban 2011; Kant *et al.* 2015) and to some extent also indirect defences (Heil 2015; Turlings & Erb 2018).

The amount and frequency of herbivory are considered key drivers of variation in plant induced defences (Karban & Baldwin 1997; Karban 2011; Underwood 2012). Increasing amounts of damage frequently correlate positively with the magnitude of plant induction (*e.g.* Baldwin & Schmelz 1996; Heil *et al.* 2001; reviewed by Karban & Baldwin 1997), although induced responses are frequently also characterised by thresholds and non-linearity (*e.g.* Underwood 2000, 2010), suggesting constraints or limits to induction (Baldwin & Schmelz 1996). Likewise, plant–herbivore interactions vary in how they unfold throughout the growing season (Poelman *et al.* 2008). The presence of episodic (sequential) attacks is common and may determine the strength and overall magnitude of plant induced responses (Underwood 2012). For example, numerous studies have reported that levels of resistance after multiple attacks by the same herbivore can be higher (Agrawal 1998; Poelman *et al.* 2008; Underwood 2012) and faster (Baldwin & Schmelz 1996) than resistance levels after a single attack. These patterns comprise so-called plant ‘memory’ responses (*sensu* Karban & Niiho 1995) and have been reported primarily for direct defences (see Underwood 2012). At the same time, however, other studies have found that the magnitude of induced

responses to recurrent herbivory may not exceed those to a single episode of damage (e.g. Karban & Niiho 1995; Underwood 2012), and that subsequent induction can be slower than responses to initial attack (e.g. Underwood 2012). These latter two scenarios may occur when attacks are close together in time and subsequent responses take place before initial responses have subsided, leading to conditions such as depletion of resources which constrain subsequent induction. To date, however, few studies have evaluated the effects of recurrent herbivory on plant defence induction controlling for the total amount of cumulative damage (but see Karban & Niiho 1995; Underwood 2012), and this has made it difficult to tease apart the effects of magnitude *versus* recurrence of herbivory events.

Plant induction of direct and indirect defences is predicted to vary depending on the costs and benefits of each defence type under different ecological settings (Rudgers 2004; Kergunteuil *et al.* 2019). In particular, variation in the magnitude and recurrence of herbivore attack may be especially important and determine the magnitude of induction of one type of defence over another (Karbon 2011; Heil 2015). Unfortunately, most studies addressing the effects of recurrent herbivory have not measured both direct and indirect defences. Accordingly, little is known about the effects of episodic herbivory on the induction of indirect defences (but see Agrawal 1998) or changes in the relative strength of induction of direct *versus* indirect defences. For example, an increasing level of herbivory may or may not lead to parallel increases in the induction of direct and indirect defences, depending on, for example, relative costs of each defence type (Ballhorn *et al.* 2008; Rasmann *et al.* 2010) or the ecological context (Pellissier *et al.* 2016). Similarly, induction of direct defences may not be cost-effective in response to episodic attacks and result in the depletion of resources allocated to induction, particularly when costly defence traits are involved (Underwood 2012). This may in turn give way to increased allocation to presumably less costly induced indirect defences such as extrafloral nectar (Heil 2015), as an alternative strategy under recurrent attacks. To date, tests of these predictions are not yet available.

Another related aspect concerns comparisons of plant local (*i.e.* occurring at the site of damage) *versus* systemic (at distant undamaged sites within a damaged plant) induced direct and indirect defence traits. For example, the induction of indirect defences such as extrafloral nectar is frequently site-specific, leading to strong local induction in comparison to relatively weaker (or absent) systemic induction (reviewed by Heil 2015). Likewise, a number of other studies have documented that local induction of direct defences to herbivory is stronger than systemic induction (Karbon & Baldwin 1997; Karban 2011; Moreira *et al.* 2018), although in some cases the latter has been shown to equal or even exceed local induction (Karbon 2011). However, studies simultaneously assessing local *versus* systemic responses for both induced direct and indirect defences are less common, and to our knowledge none are available involving recurrent herbivory.

Wild cotton, *Gossypium hirsutum* L. (*Malvaceae*) produces both direct and indirect defences. Here we asked whether induction of phenolic compounds (a direct defence) and extrafloral nectar (an indirect defence) varied depending on the amount and recurrence of leaf herbivory. To this end, we

conducted a greenhouse experiment where we simulated insect leaf herbivory by means of mechanical damage and application of caterpillar (*Spodoptera frugiperda*) regurgitant. We applied two levels of leaf damage, 25% and 50% of damaged leaves, to test for effects of different magnitudes of herbivory on induction, and a third treatment involving two events of 25% leaf damage applied over a 1-week period to test for effects of recurrent herbivory. We sought to answer the following: (i) do patterns of leaf defence induction of phenolics and extrafloral nectar vary depending on the amount and recurrence (number of events) of insect leaf herbivory; (ii) do effects of amount and recurrence of insect leaf herbivory vary depending on the type of defensive trait (*i.e.* extrafloral nectar *versus* phenolics); and (iii) does the relative strength of local *versus* systemic induction vary depending on the type of defence trait? We predicted that an increasing amount of (single event) herbivory would lead to concomitant increases in both direct and indirect defences, given limited overlap in the time course of induction of each type of trait (presumably limiting costs due to simultaneous induction of each; see Underwood 2012), as well as due to a presumably low cost of nectar production (Heil 2015) which might preclude defensive trade-offs. In addition, we expected that recurrent damage would lead to greater induction of indirect relative to direct defences, again because the former are presumably less costly (Heil 2015) and would therefore be prioritised over more costly direct defences under a scenario of repeated induction. Likewise, the occurrence of subsequent damage of equal (or greater) intensity than initial damage would imply that induced direct defences failed to reduce subsequent herbivory, which might favour a greater relative induction of (energetically less costly) extrafloral nectar as a substitute defence strategy. Finally, we expected local induction to be equally strong or stronger than systemic induction for phenolics, whereas for extrafloral nectar we expected local induction to be stronger than systemic induction.

## MATERIAL AND METHODS

### Study species

*Gossypium hirsutum* is a perennial shrub that grows up to 2-m tall under natural conditions (Oosterhuis & Jernstedt 1999). It is native to Central America, Mexico and the Caribbean Basin (Wendel *et al.* 1992; Oosterhuis & Jernstedt 1999) and is thought to have originated in southeast Mexico (D'Eeckenbrugge & Lacape 2014). Wild populations are common in the Yucatan Peninsula (Mexico) and are found in the coastal scrubland or sand dune vegetation (D'Eeckenbrugge & Lacape 2014). Flowering occurs twice a year, from May to June and from December to January. Throughout its distribution, wild *G. hirsutum* is attacked by a diverse community of insect herbivores, among which the most important are leaf chewers belonging to Lepidoptera, Orthoptera and Coleoptera (Abdala-Roberts *et al.* 2019a). Other insect guilds, such as sap feeders (Hemiptera), are less common (Abdala-Roberts *et al.* 2019a). Insect leaf damage, particularly by beetles and caterpillars, peaks during the rainy season (June–September). In addition, ants frequently tend extrafloral nectaries, and recent population surveys indicate that a total of ca. 30 species are associated

with wild cotton across sites (L. Abdala-Roberts, unpublished data).

*Gossypium hirsutum* produces a diverse arsenal of anti-herbivore defences, direct defences by terpenoids (e.g. stored in so-called gossypol glands), phenolic compounds and glandular trichomes, as well as traits involved in indirect defence such as volatile organic compounds and extrafloral nectar (McCall *et al.* 1994; Loughrin *et al.* 1995; McAuslane & Alborn 1997; Agrawal & Karban 2000; Opitz *et al.* 2008). With respect to phenolics, of special interest in the present study, both wild and cultivated *G. hirsutum* synthesise these secondary metabolites and a few previous studies have shown that they confer resistance against insect herbivores in this species (Mansour *et al.* 1997; Nix *et al.* 2017). Likewise, our recent work with wild *G. hirsutum* has shown that this species produces extrafloral nectar which fosters ant and parasitoid recruitment (Abdala-Roberts *et al.* 2019a, 2019b). Nectaries are found on the ventral side of the leaves, on the midvein near the base of the leaf blade. To date most work on the induction of direct and indirect defences in *G. hirsutum* has considered domesticated varieties, and wild populations have been largely neglected (for studies with wild individuals of other cotton species see e.g. Wäckers *et al.* 2001 – *G. herbaceum*; Rudgers 2004 – *G. thurberi*).

### Seed source, experimental design and induction treatment

In July 2017, we collected seeds from ten plants (*i.e.* maternal lines or ‘genotypes’ hereafter) of wild cotton from 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). Previous molecular work based on samples taken in this area indicated that these populations represent wild *G. hirsutum* (see D’Eeckenbrugge & Lacape 2014). Distance among sampled individuals was at least 5 m to increase variation among neighbouring plants.

In December 2017 we germinated seeds and kept seedlings under greenhouse conditions (70% mean relative humidity, 22 °C/35 °C minimum/maximum mean temperature during the experiment) for 3 months at the Campus de Ciencias Sociales of the Universidad Autónoma de Yucatán (21°1′27″ N, 89°33′15″ W). In September 2018, once plants were ca. 50-cm tall and had, on average, 20 leaves, we randomly assigned individuals of each genotype to one of the following treatments: (i) 25% of damaged leaves, (ii) 50% of damaged leaves, (iii) two events of 25% of damaged leaves (25% + 25%), or (iv) control (undamaged) (N = 132, 12–13 plants/genotype, 3–4 plants/genotype/treatment). Leaf damage consisted in using scissors to remove 25% or 50% of the leaf blade area of two thirds of the leaves per plant, avoiding young leaves that were not fully expanded (usually 1–2 per plant). This represented approximately 16% and 32%, respectively, of total leaf area removed per plant. Immediately after removing leaf tissue, we punctured the central portion of the remaining leaf tissue with an awl and exposed it to oral secretions from third instar larvae of *S. frugiperda*. This was done by gently squeezing the head and poking the abdomen of the caterpillar (Turlings *et al.* 1993), and we used one or two caterpillars per plant, depending on the number of leaves to be induced (one caterpillar for plants with ca. 4–6 leaves to be induced, two caterpillars for plants with ca. 6–8). Caterpillars were initially fed a wheat germ-based artificial

diet and kept on cotton leaves for ca. 12 h prior to treatment application. The treatment of recurrent damage consisted of applying 25% leaf damage and caterpillar oral secretion twice, once concomitantly to plants from the single event damage treatments, and again a week later removing another 25% of the (original) blade area of the same leaves, resulting in cumulative 50% of area removed per leaf. Control plants were separated (>5 m) from damaged plants at the time of treatment application to avoid plant–plant airborne communication which could induce plant defences. Recent field surveys indicated a mean value of 23% of leaf tissue removed or damaged by insects per plant in wild cotton populations (range: 9.4–53.2 across populations; Abdala-Roberts *et al.* 2019a). Treatment levels thus fell within the natural range of herbivory for this species, with the 25% leaf damage treatment representing a scenario of low herbivory (*i.e.* 16% total leaf area removed) and 50% leaf damage representing a moderate level of herbivory (*i.e.* 32% total leaf area removed), in the latter case involving either a single event or repeated herbivory. We chose a period of 1 week between damage applications for the recurrent herbivory treatment since this is a similar time frame to that observed in the field for natural events of intense herbivory by leaf-chewing insects (caterpillars or katyids) during the rainy season (L. Abdala-Roberts, personal observation).

Previous studies have demonstrated that exogenous application of caterpillar oral secretions or regurgitant combined with mechanical damage provides an effective proxy of natural damage in several cultivated species (e.g. maize – Turlings *et al.* 1993; Alborn *et al.* 1997; tobacco – McCloud & Baldwin 1997; Halitschke *et al.* 2001), including wild cotton (Chappuis & Egger 2016; Abdala-Roberts *et al.* 2019b). In this way, our approach allowed us to precisely control the amount of leaf damage (relative to natural feeding) while achieving realistic levels of defence induction.

### Measurements

We quantified extrafloral nectar production and concentration (proxies of nectar quantity and quality, respectively) 24 h after applying leaf damage. In the case of plants subjected to recurrent damage, this measurement was conducted 24 h after the second application of damage. Measurements were conducted between 06:00 and 08:00 h. For each plant, we sampled two fully expanded leaves that were close to the apical meristem (where nectaries are most active). In the case of damaged plants, one sampled leaf was damaged and the other undamaged to distinguish between local and systemic induction, respectively. Previous work with wild cotton has shown that peak induction is reached 24 h after leaf damage and starts decaying 48 h after damage (M. Reyes-Hernández, unpublished data). Nectar amount (expressed in  $\mu\text{l}$ ) was quantified using 5- $\mu\text{l}$  micropipettes with 1  $\mu\text{l}$  divisions (Micropipettes Blaubrand® intraMARK, colour code white; Merck, Darmstadt, Germany), whereas nectar concentration (expressed in °Brix) was measured with a manual refractometer (Atago Master T 0 to 33 °Brix, Germany). In addition, 1 week after treatment application (second event in the case of plants under the recurrent treatment level) we collected two fully expanded leaves per plant (proximal to the apical meristem) to quantify phenolic compounds. Previous work with wild cotton has shown that statistically significant induction of these

compounds to ca. 20% leaf area damage is detected a week after damage application, and also that subsequent new leaves (produced 2–3 weeks after damage) exhibit significant levels of induction (Abdala-Roberts *et al.* 2019b). These results indicate a longer time frame of induction (several days to weeks) for phenolics than for nectar. Again, for damaged plants we collected one damaged and one undamaged leaf to distinguish between local and systemic induction. For half of the control plants we sampled nectar (24 h post-treatment) and collected leaves for phenolics (1 week post-treatment) at the same time as plants from the single event 25% and 50% damage levels. The other half of the control plants were sampled at the same time as plants subjected to the second application of damage, *i.e.* 24 h and 1 week after the second application for nectar and phenolics, respectively. This matched the time of sampling between control plants and plant subjected to recurrent damage and accounted for background temporal changes in induction under undamaged conditions (Underwood 2012). However, preliminary analyses indicated no significant differences between these two groups of controls for any of the response variables measured. We therefore treated them indistinctly as a single control group in the statistical analyses. There was a positive correlation between nectar concentration and total phenolics ( $r = 0.17$ ,  $P = 0.01$ ), but no correlation between nectar production and phenolics ( $r = 0.01$ ,  $P = 0.89$ ). Nectar production and concentration were significantly positively correlated ( $r = 0.50$ ,  $P < 0.0001$ ).

#### Chemical analyses of phenolic compounds

Upon collection, leaves were stored in a cooler and then transported to the laboratory where they were immediately dried at 45 °C. We extracted phenolic compounds using 20 mg dry plant tissue (pool of leaves per individual) with 1 ml 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira *et al.* 2014). We then transferred the extracts to chromatography vials to perform phenolic profiling. For chemical identification of the polyphenol composition in plant extracts we used an ultra-performance liquid chromatograph coupled with electrospray ionization quadrupole (Thermo Dionex Ultimate 3000 LC; Dionex, Sunnyvale, CA, USA) time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) (Bruker Compact™; Bruker, Billerica, MA, USA). Chromatographic separation was performed in a Kinetex™ (Phenomenex, Aschaffenburg, Germany) 2.6 µm C18 82–102 Å, LC column 100 × 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 for 14 min, from 100% to 10% B (14–15 min), hold 10% B for 17 min. The injection volume was 3 µl, the flow rate was established at 0.4 ml·min<sup>-1</sup> 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 mode was used under the following specific conditions: gas flow 8 l·min<sup>-1</sup>, nebuliser pressure 38 psi, dry gas 7 l·min<sup>-1</sup>, 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 using different collision energy ramps to cover a range from 15

to 50 eV. Individual compounds were identified based on data obtained from the standard substances or published literature, including RT, λ<sub>max</sub>, ([M–H]–), and major fragment ions. For the quantitative analysis of phenolic compounds, 3 µl of each sample was then analysed using the same column and conditions mentioned in the previous paragraph, in an UHPLC (Nexera LC-30AD; Shimadzu, Kyoto, Japan) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. Chromatograms were recorded at 330 nm. 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 phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1.0, 2.0 and 5.0 µg·ml<sup>-1</sup>. We expressed phenolic compound concentrations in mg·g<sup>-1</sup> tissue on a dry weight basis.

#### Statistical analyses

We ran general linear mixed models to test for effects of leaf damage treatment (fixed, four levels) and plant genotype (random, ten levels) on the amount and concentration of extrafloral nectar, and the concentration of flavonoids, lignins, condensed tannins and total phenolics. Models also included as a random factor the effect of plant to account for multiple samples (*i.e.* leaves) taken from each individual. A significant effect of damage may result from varying scenarios of differences between one or more treatment levels, each case providing specific information about the effects of the magnitude and recurrence of leaf damage. A difference between controls and both single 25% and single 50% damage combined with a difference between the latter two would indicate that the amount of herbivory influences the magnitude of defence induction. A difference between the 25 + 25% treatment and controls indicates a total or overall effect on induction after repeated herbivory (Underwood 2012). In addition, a difference between single 25% damage and 25% + 25% damage indicates a change in the plant's ability to respond to subsequent attack, as the level of induction after subsequent attack is a function of the response to new damage as well induced levels remaining from the previous attack (Underwood 2012). Finally, both single 50% damage and 25% + 25% damage involved the same final amount of damage, and therefore a difference between these treatment levels would imply an effect of recurrent damage on induction after accounting for the overall (final) amount of damage inflicted (Karban & Niiho 1995).

We also evaluated differences between local *versus* systemic induction of plant defences by running general linear mixed models testing for an effect of leaf induction (fixed, three levels: control, damaged, undamaged) and genotype (random) on each response variable. These models also included the effect of plant (random) and genotype (random). We tested separately for the effects on induction of leaf damage treatment (magnitude and recurrence, above models) and distance to damage site (local *versus* systemic) because controls would appear twice (once for each factor) if these effects were tested simultaneously in the same model.

The statistical significance of the genotype effect in all models was assessed using the likelihood ratio test, where the difference in –2-times the log-likelihood of models including *versus*

excluding the random effect is distributed as one-tailed  $\chi^2$  test with one degree of freedom (Littell *et al.* 2006). All analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC, USA) using PROC MIXED. Data were normally distributed in all cases. Least-square means and standard errors are reported as descriptive statistics. We report results from *P*-corrected multiple comparisons to test for differences between treatment-level means.

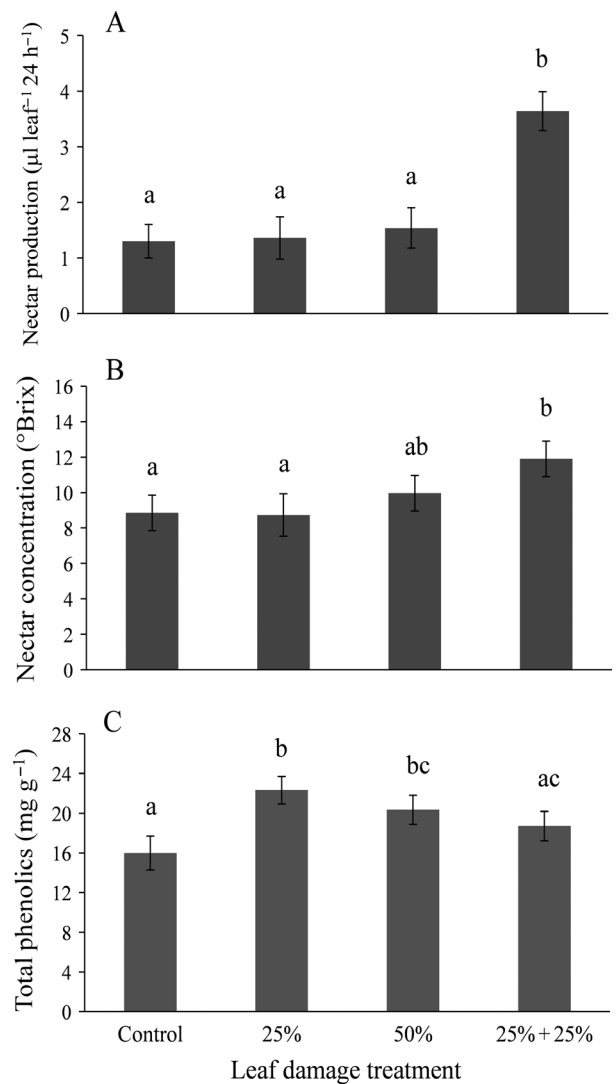
## RESULTS

### Extrafloral nectar

There was a significant effect of leaf damage on both extrafloral nectar production and concentration (Table 1). Plants subjected to recurrent herbivory (*i.e.* two events of 25% leaf damage) exhibited a significantly greater production (2.8-fold) and concentration (1.3-fold) of extrafloral nectar than controls (Fig. 1a, b). In contrast, mean values for single event 25% and 50% leaf damage did not significantly differ relative to controls, and did not differ themselves (Fig. 1a, b). Plants subjected to recurrent damage also produced significantly more extrafloral nectar compared to plants from both single event damage levels (Fig. 1a), whereas for extrafloral nectar concentration they differed from single event 25% but not 50% leaf damage (Fig. 1b). On the other hand, models testing for differences between local *versus* systemic induction indicated a significant effect for extrafloral nectar production but not concentration (Table 2, Fig. 2a, b). Extrafloral nectar production was significantly higher (2.1-fold) for damaged leaves from treated plants relative to controls (*i.e.* local induction effect), whereas undamaged leaves from treated plants did not differ from controls (*i.e.* systemic induction effect; Fig. 2a). Damaged leaves produced significantly (1.5-fold) more extrafloral nectar than undamaged leaves from treated plants (Fig. 2a).

### Leaf phenolics

There was a significant effect of leaf damage on the concentration of total phenolics (Table 1). Plants subjected to single event 25% and 50% damage exhibited a significantly greater mean value (1.4- and 1.3-fold, respectively) than controls and did not differ themselves (Fig. 1c). In contrast, plants subjected to recurrent leaf damage did not differ significantly from controls (Fig. 1c) and exhibited a significantly lower value relative to single event 25% damage plants but did not differ from single event 50% damage plants (Fig. 1c). Analyses by group of phenolic compounds indicated a significant treatment effect



**Fig. 1.** Effect of leaf damage (control, single event damage [25% or 50% leaf area removal] or multi-event damage [25% applied twice over a week]) on wild cotton (*Gossypium hirsutum*) extrafloral nectar production in  $\mu\text{l}$  (A) and concentration in  $^{\circ}\text{Brix}$  (B) and total leaf phenolics in  $\text{mg}\cdot\text{g}^{-1}$  (C). Values are model least-square means and SE accounting for effects of plant genotype and individual plant.

for condensed tannins and a marginally significant effect for flavonoids, but no effect on lignins (Table S1, supplementary material). On the other hand, models testing for local *versus*

**Table 1.** Results from general linear mixed models testing for the effect of leaf damage treatment (control, single event damage [25% or 50% leaf area removal] or multi-event damage [25% applied twice over a week]) and plant genotype on wild cotton (*Gossypium hirsutum*) extrafloral nectar production ( $\mu\text{l}$ ) and concentration ( $^{\circ}\text{Brix}$ ) and total leaf phenolics ( $\text{mg}\cdot\text{g}^{-1}$ ). Values shown are either *F*- (for treatment effect) or chi-square (for genotype effect) tests, degrees of freedom, and *P*-values. All models also included the effect of plant (random) to account for repeated samples per plant (statistics not shown). Significant effects ( $P < 0.05$ ) are in bold.

Source	Nectar production			Nectar concentration			Total phenolics		
	<i>F</i> / $\chi^2$	df	<i>P</i> -value	<i>F</i> / $\chi^2$	df	<i>P</i> -value	<i>F</i> / $\chi^2$	df	<i>P</i> -value
Leaf damage	10.39	3, 122	<b>&lt;0.0001</b>	2.74	3, 122	<b>0.046</b>	4.54	3, 87	<b>0.005</b>
Genotype	0.02	1	0.887	8.71	1	<b>0.003</b>	11.11	1	<b>0.0008</b>

**Table 2.** Results from general linear mixed models testing for the effect of distance to damage site, *i.e.* local versus systemic, on wild cotton (*Gossypium hirsutum*) induction of extrafloral nectar production ( $\mu\text{l}$ ) and concentration ( $^{\circ}\text{Brix}$ ) and total leaf phenolics ( $\text{mg}\cdot\text{g}^{-1}$ ). These models tested for differences between leaves from control (undamaged) plants, undamaged leaves from damaged plants and damaged leaves (across all levels) from damaged plants. Undamaged leaves from treated plants represent trait values due to systemic induction, whereas damaged leaves represent values for local induction. Values shown are *F*-values, degrees of freedom and *P*-values. All models also included the effect of plant genotype and plant (both random, statistics not shown). Significant effects ( $P < 0.05$ ) are in bold.

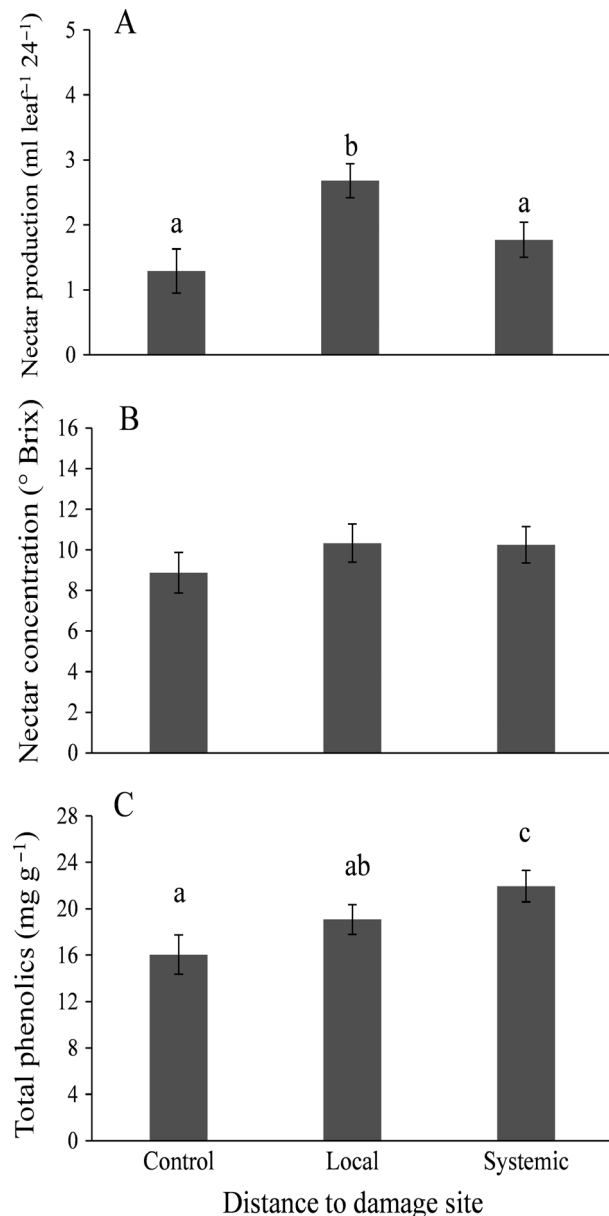
Response	Distance to damage site		
	<i>F</i>	df	<i>P</i> -value
Nectar production	7.79	2, 121	<b>0.0007</b>
Nectar concentration	1.11	2, 121	0.332
Total phenolics	6.73	2, 86	<b>0.001</b>

systemic induction indicated a significant effect of distance to damage site for total phenolics (Table 2). Undamaged leaves from treated plants exhibited a significantly larger mean value (1.4-fold) than controls (*i.e.* systemic induction effect), whereas damaged leaves from treated plants did not differ from controls (*i.e.* no local induction effect; Fig. 2c). Undamaged leaves of treated plants had a significantly larger (1.2-fold) mean value relative to damaged leaves (Fig. 2c). Analyses by group of phenolic compounds indicated a significant effect for flavonoids and condensed tannins, but not for lignins (Table S2).

## DISCUSSION

Our findings showed different patterns of induction for leaf phenolics and extrafloral nectar in wild cotton. Extrafloral nectar amount and concentration were significantly induced only after recurrent leaf damage, whereas total phenolics were significantly induced by single events of low and moderate leaf damage, but not after recurrent herbivory. In the case of phenolics, induction due to single event low damage did not differ from single event moderate damage, suggesting the intensity of herbivory did not lead to concomitant increases in the strength of induction of these compounds. On the other hand, we found evidence of local but not systemic induction of extrafloral nectar production, whereas leaf phenolics were significantly induced systemically but not locally. Overall, these results suggest important differences in the *modus operandi* of induction of these direct and indirect defence traits following herbivory in wild cotton.

Single events of low (25% damaged leaves) and moderate (50% damaged leaves) damage did not significantly induce extrafloral nectar production or alter nectar concentration in wild cotton. This result is surprising considering that previous work has reported positive effects of increasing herbivory intensity on extrafloral nectar induction (reviewed by Agrawal & Rutter 1998; Rico-Gray & Oliveira 2007; Heil 2015), and a prior study with *G. hirsutum* found significant nectar induction in response to single bouts of *S. littoralis* herbivory (Wäckers *et al.* 2001). However, it should be noted that the authors of



**Fig. 2.** Effect of distance to damage site (local versus systemic) on wild cotton (*Gossypium hirsutum*) extrafloral nectar production in  $\mu\text{l}$  (A), concentration in  $^{\circ}\text{Brix}$  (B) and total leaf phenolics in  $\text{mg}\cdot\text{g}^{-1}$  (C). Shown are model least-square means and SE for control (undamaged) plants, undamaged leaves from damaged plants and damaged leaves from damaged plants.

this latter study used domesticated cotton and their plants were considerably younger than ours at the time of treatment application. In addition, we used oral secretions from *S. frugiperda* rather than *S. littoralis* which could have resulted in different patterns of induction. Moreover, work with other *Gossypium* species (*e.g.* *G. herbaceum*) reported that herbivore elicitors are not necessary to induce a full response in extrafloral nectar production (Wäckers & Wunderlin 1999). This suggests that caterpillar elicitors may have not been a crucial difference limiting comparison among studies in patterns of extrafloral nectar induction, and rather other factors such as ontogenetic variation (*e.g.* Koch *et al.* 2016) or the pattern/magnitude of

damage applied may have been more important in explaining differences. Independently, our results suggest that low to moderate single events of leaf damage are not sufficient to produce significant induction of extrafloral nectar, and that presumably a higher amount of damage is necessary to induce this trait in young adult plants of wild *G. hirsutum*. Comparisons of the strength of extrafloral nectar induction between seedlings and adults, as well as between multiple inducing herbivore species (including native caterpillars), would provide a valuable way forward to better understand this induced response in wild cotton.

We did, however, find evidence of increased extrafloral nectar production and concentration in response to repeated leaf damage. In one of the few available studies testing the effects of recurrent herbivory on plant indirect defences, Agrawal (1998) reported that subsequent mechanical damage (24 h after initial damage) resulted in a greater recruitment of ants relative to a single event of damage in the tropical shrub *Cecropia obtusifolia* (*Urticaceae*). However, this result was not explained by an induction of plant-based food rewards to ants, but potentially by the release of volatile compounds (Agrawal 1998). In our case, subsequent damage was applied 1 week after initial induction, and nectar induction due to the first application of damage would have decayed 48 h after leaf damage (M. Reyes-Hernández, unpublished data). Thus, the response to subsequent damage likely occurred after the response to initial damage had fully decayed (e.g. Baldwin & Schmelz 1996), and in any case, presumably did not build on this previous response as there was no significant difference between single events of either 25% or 50% leaf damage relative to controls. This response to recurrent damage cannot be attributed either to differences in the total (i.e. cumulative) amount of herbivory inflicted, as plants subjected to recurrent damage experienced the same final amount of damage as plants exposed to a single moderate (50% damage) event of herbivory (i.e. total amount of damage is held constant in comparing these two levels; Karban & Niiho 1995) and these two treatment levels differed significantly in nectar production). As such, this pattern of induction could be viewed as an 'immunological memory' response in wild cotton, where only repeated attack triggers a significant increase in nectar production. This induction can therefore be assumed to come from a change in the plant's ability to respond to herbivory after repeated attack, and takes place after accounting for the total (final) amount of damage inflicted (Underwood 2012). Although it is difficult to mimic natural patterns of repeated herbivory, it should be noted that during the peak of the rainy season we have observed intense bouts of herbivory (>50% leaf area loss), distributed more or less discretely over one or two periods of attack occurring throughout a 2-week period (e.g. Katyidae and Acrididae). Thus, treatments involving one or two applications of repeated herbivory may come close to simulating temporal patterns of herbivore attack, at least for some guilds of insect herbivores on wild cotton. Nonetheless, including multiple repeated events of herbivory (rather than one) should be considered in order to more fully characterise cotton induced responses to recurrent herbivory.

Different patterns of induction were observed for leaf phenolics. In this case, single events of low and moderate leaf damage significantly increased the concentration of total leaf phenolics relative to control plants, whereas recurrent damage

did not. The fact that a single event of moderate damage did not differ from a single event of low damage suggests that increasing herbivory does not lead to a concomitant increase in the magnitude of induction of phenolics. Some type of limit to induction of these secondary compounds (e.g. physiological constraints or energetic costs) may therefore be in place in wild cotton, beyond which increasing amounts of leaf damage do not lead to a concomitant increase in the magnitude of induction (Karban 2011). On the other hand, the lack of difference in total phenolics between controls and plants subjected to repeated leaf damage indicates no overall induction of these compounds after repeated herbivory (Underwood 2012). Likewise, no difference was found between plants subjected to moderate (50%) leaf damage and those exposed to repeated leaf damage, which (i.e. keeping total leaf damage constant) is consistent with a previous study with cultivated *G. hirsutum* reporting that repeated attack did not boost induced resistance to spider mites relative to single attack (Karban & Niiho 1995). Another key aspect to consider is also whether initial damage affects the plant's ability to respond to subsequent damage, where the level of induction after multiple attacks is determined by the response to new damage and any induced levels remaining from the prior attack (Underwood 2012). The baseline against which to compare induced levels due to repeated damage would be the mean level in plants from the single 25% damage, and we found that the former exhibited a lower level of phenolics than the latter. This suggests that resource depletion or some other mechanism (e.g. diminishing benefits from repeated induction; Underwood 2012) affected the plant's capacity to subsequently induce these compounds. Such a scenario is expected when induced responses to initial and subsequent attacks overlap, which is less likely for extrafloral nectar (at least based on episodes of attack separated by a week or more), as this latter trait exhibits faster and shorter-term induced responses. In addition, extrafloral nectar production is expected to be relatively less costly to produce (Koricheva & Romero 2012; Heil 2015), particularly compared to some types of secondary metabolites which are more costly, including phenolic compounds (Simms 1992; Cipollini *et al.* 2014). This latter aspect would contribute to weaken constraints on induction of extrafloral nectar in response to repeated attacks, but this should be taken with caution since a detailed quantification of defence costs is necessary and because extrafloral nectar may incur significant costs under some conditions (see Blue *et al.* 2015).

Our results also indicated contrasting patterns of local *versus* systemic induction of extrafloral nectar and phenolics in wild cotton. Extrafloral nectar was significantly induced in damaged leaves (i.e. locally) relative to control leaves, whereas undamaged leaves from treated plants did not differ from control leaves, suggesting that this trait was not induced systemically. This pattern is not surprising given previous work with *G. hirsutum* (e.g. Wäckers & Bonifay 2004) and with other species (reviewed by Heil 2015), showing that the induction of extrafloral nectar is usually restricted to the site of damage. Interestingly, other studies have found that induction of *G. hirsutum* leaf volatiles appears to occur both locally and systemically (Olson *et al.* 2008), suggesting differences in the nature of induced responses for these two indirect defensive traits. On the other hand, we found that leaf phenolics were significantly induced in undamaged leaves of treated plants (i.e.

systemically) relative to control leaves, whereas evidence of local induction was lacking as damaged leaves did not significantly differ from controls. This result is somewhat surprising since local induction of chemical defences is arguably more common and stronger (Moreira *et al.* 2018), but is nonetheless consistent with some studies reporting on strong systemic induced responses to herbivory involving direct chemical defences such as terpenoids in *G. hirsutum* (e.g. McAuslane & Alborn 1997) and phenolic compounds in other species (reviewed by Kant *et al.* 2015).

In interpreting the above patterns, inference drawn from these results based on mechanical leaf damage and exposure to *S. frugiperda* oral secretions should be made with some caution. This insect exhibits extreme polyphagy and has been shown to induce chemical defences in both wild and cultivated cotton (Chappuis & Egger 2016; Quijano-Medina *et al.* 2019; Abdala-Roberts *et al.* 2019b). It was therefore considered a good proxy for assessing cotton induced responses to herbivory by a generalist insect. Nonetheless, it is not common in natural populations of wild cotton (T. Quijano-Medina, personal observation), and induced responses to this caterpillar may not necessarily mimic responses to native herbivores, particularly specialist insects. In this sense, future work comparing induced responses by generalist herbivores such as *Spodoptera* spp. (pests on cultivated cotton) relative to, for example, native caterpillars specialised on cotton, would inform on the specificity of these responses and contingency on herbivore traits such as diet breadth or feeding guild.

Our results provide a useful basis for future tests of the effects of herbivory intensity and periodicity on the induction of direct and indirect defences in wild cotton. Experiments testing different combinations of intensity of initial and subsequent damage are needed, as well as manipulations of varying levels of herbivory magnitude and recurrence to test for non-linearity and thresholds in cotton induced defences. In addition, measurements of other defensive traits would provide complementary information to more fully characterise multivariate induction patterns in wild *G. hirsutum*. In this sense, ongoing work with wild cotton in our laboratory involves the induction of additional traits potentially associated with plant resistance to herbivory, including leaf volatiles, terpenoids (e.g. gossypol) and trichomes. A more detailed assessment of potential constraints (e.g. *via* allocation trade-offs) or co-expression of defence traits would be highly valuable to understand the mechanisms (e.g. coupling or antagonism) of simultaneous induction of multiple defence traits. Finally, measuring the effects of these plant responses on herbivore

performance is a necessary next step to determine whether induction consistently translates into effects on herbivores (*i.e.* induced resistance). Experiments using real insect herbivory under natural or semi-natural conditions are also desirable to assess the concomitant influences of induction of direct and indirect defences on herbivores.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Results from general linear mixed models testing for the effect of leaf damage (control, single-event damage [25% or 50% leaf area removal], or multi-event damage [25% applied twice over a week]) and plant genotype on wild cotton (*Gossypium hirsutum*) leaf flavonoids, lignins, and condensed tannins ( $\text{mg}\cdot\text{g}^{-1}$ ). Values shown are either *F*- (for treatment effect) or chi-square (for genotype effect) tests, degrees of freedom, and *P*-values. All models also included the effect of plant (random) to account for repeated samples per plant (statistics not shown). Significant effects ( $P < 0.05$ ) are in bold, marginally significant effects ( $0.05 < P < 0.10$ ) are in italics.

**Table S2.** Results from general linear mixed models testing for the effect of induction (local *versus* systemic) on wild cotton (*Gossypium hirsutum*) leaf flavonoids, lignins, and condensed tannins ( $\text{mg}\cdot\text{g}^{-1}$ ). These models tested for differences between leaves from control (undamaged) plants, undamaged leaves from damaged plants, and damaged leaves (across all leaf damage treatment levels) from damaged plants. Undamaged leaves from treated plants represent trait values due to systemic induction whereas damaged leaves represent values for local induction. Values shown are *F*-values, degrees of freedom, and *P*-values. All models also included the effect of plant genotype and plant (both random, statistics not shown). Significant effects are in bold.

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