Effects of soil salinity on the expression of direct and indirect defences in wild cotton Gossypium hirsutum

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Abstract
1. Previous studies have frequently reported effects of abiotic factors on herbivore-induced plant defences based on effects on single plant traits. However, plants commonly express multiple defences simultaneously and these traits are often correlated. Thus, a fuller understanding of abiotic-context dependency in plant defence requires measuring multiple traits and addressing their patterns of correlated expression.

2. We evaluated the effects of soil salinity on the expression of direct (phenolic compounds, gossypol gland density) and indirect (volatile organic compounds, extrafloral nectar) defensive traits in wild cotton Gossypium hirsutum. Specifically, we asked whether soil salinity affects the induction of these traits, and whether it shapes trait correlations potentially underlying altered patterns of trait induction. We conducted a factorial experiment with 16 cotton genotypes where we manipulated soil salinity and defence induction by applying artificial leaf damage (25% mechanical damage and caterpillar oral secretions) and measured defence levels at different time points post damage.

3. Leaf damage induced most traits except gossypol gland density, whereas salinity did not have a mean effect (across constitutive and induced levels) on any of the measured traits. Nonetheless, salinity prevented the induction of phenolic compounds (condensed and hydrolysable tannins), and also affected trait correlations. Specifically, phenolic compounds were negatively associated with nectar production only under salinized conditions, an apparent trade-off that could affect the induction of phenolic compounds. In addition, positive correlations between phenolic compounds and gland density and root biomass observed under control conditions were lost under salinized conditions.

4. Synthesis. By investigating the effects of soil salinity on the expression of multiple direct and indirect defensive traits and their underlying correlations, these findings build towards a better understanding of how abiotic context dependency shapes plant allocation to and expression of multiple defensive traits.
1 | INTRODUCTION

Ceaseless attack by herbivores has led over time to the evolution of a diverse array of anti-herbivore defences in plants (Agrawal, 2007, 2011; Marquis, 1992). Direct defences include chemical (e.g. phenolic compounds, alkaloids, terpenoids) or structural (e.g. trichomes, spines) traits that deter herbivores, reduce their consumption or decrease their survival (Agrawal, 2007; Carmona, Lajeunesse, & Johnson, 2011; Mithöfer & Boland, 2012 and references therein). On the other hand, indirect defences involve chemical [e.g. volatile organic compounds (VOCs)] or structural [e.g. extrafloral nectar (EFN), domatia] traits that provide shelter, food or information on herbivore presence to natural enemies (i.e. predators and parasitoids; Dicke, Poecke, & de Boer, 2003; Turlings & Erb, 2018). Both types of defences are expressed constitutively (i.e. baseline levels) but can also be actively induced in response to herbivore damage (Agrawal, 2007; Karban, 2011; Karban & Baldwin, 1997). A central theme of research on plant defences has been to understand the ecological mechanisms and plant endogenous processes behind the multiple expression of plant direct and indirect defensive traits under different biotic and abiotic contexts.

Abiotic factors such as light availability, soil nutrients and water availability can exert substantial influences on plant defence expression (Moore, Andrew, Carsten, & Foley, 2014), particularly on changes in patterns of defence induction in response to herbivore damage (Donaldson, Kruger, & Lindroth, 2006; Gouinguené & Turlings, 2002; Sampedro, Moreira, & Zas, 2011). The effects of soil nutrient or light availability have received much attention, where reduced defensive levels (and increased growth) are usually found under nutrient-rich or high light availability (e.g. Abdala-Roberts, Moreira, Cervera, & Parra-Tabla, 2014; Moreira, Zas, Solla, & Sampedro, 2015; Orians, Lower, Fritz, & Roche, 2003; Osier & Lindroth, 2006). However, other abiotic factors have been largely ignored and thus much of what we know about abiotic forcing on plant defences derives from a fairly narrow assessment restricted to nutrient and light effects. One factor that has received less attention is soil salinity, which is a pervasive influence in both natural (e.g. coastal or marine) and human-altered habitats (FAO, 2001; Setia et al., 2012), and has been commonly studied with respect to plant physiological responses including effects on cell water relations, hormonal balance, and carbon supply (reviewed by Hernández, 2019; Munns, 2002). Injuries in leaves due to salinity stress result from an excess of Na⁺ and/or Cl⁻ in tissues which causes the loss of photosynthetic surface (Munns, 2002; Munns et al., 2002), which ultimately reduces resource uptake and use. In turn, these physiological effects can influence plant allocation to and synthesis of secondary metabolites and physical defences (Baldwin & Preston, 1999; Huberty & Denno, 2004). To date, however, the effects of salinity on direct and indirect plant defences, and in particular their inducibility (i.e. difference between induced and constitutive levels of defences) by insect herbivore attack, have received little attention (for studies on plant salt stress responses and pathogens see: Bai, Kissoudis, Yan, Visser, & van der Linden, 2018; Besri, 1993; Snapp, Shennan, & van Bruggen, 1991). The few studies that have looked at salinity effects on the expression of plant defences associated with herbivory have involved aquatic or wetland species (e.g. Bueno-Sudatti, Toyota Fujii, Vianna Rodrigues, Turra, & Crespo Pereira, 2011; Loreto & Delfine, 2000; Moon & Stiling, 2000), as well as a few cultivated terrestrial plants (e.g. Forieri, Hildebrandt, & Rostás, 2016; Wang, Eneji, Kong, Wang, & Dong, 2015). However, most of these studies did not assess plant defence induction in response to herbivory and research involving terrestrial wild plant species remains scarce.

One crucial challenge for advancing our understanding of the context-dependency of plant defence expression, is determining how abiotic factors simultaneously affect the induction of multiple traits in response to herbivory. Plants are expected to modify allocation to different defensive traits as a function of the relative costs of each trait under varying environmental conditions (Stamp, 2003; Strauss, Rudgers, Lau, & Irwin, 2002; Zangerl & Rutledge, 1995). For example, relative allocation to different traits is predicted to be driven by trade-offs between direct and indirect defences (Koricheva & Romero, 2012), or between constitutive and herbivore-induced defences (Brody & Karban, 1992; Kempel, Schadler, Chrobock, Fischer, & van Kleunen, 2011; Rasmann, Erwin, Halitschke, & Agrawal, 2011). Recent studies have looked at correlations between inducibility of direct versus indirect defences along ecological gradients (e.g. Defossez, Pellissier, & Rasmann, 2018; Moreira et al., 2014), and a few studies have experimentally manipulated abiotic factors and tested the effects on inducibility of multiple defences and their underlying trade-offs (Descombes, Kergunteuil, Glauser, Rasmann, & Pellissier, 2020; Sampedro et al., 2011). Following from studies that have addressed abiotic controls over plant growth-defence trade-offs (Abdala-Roberts et al., 2014; Moreira et al., 2014), an untested prediction is whether allocation costs among defensive traits are more severe or only arise under conditions of abiotic stress or low resources.

In the present study, we evaluated the effects of soil salinity on the expression of direct and indirect defensive traits in wild cotton Gossypium hirsutum. Specifically, we asked: (a) Does soil salinity affect constitutive levels of direct and indirect defensive traits and their inducibility in response to insect herbivory? (b) Are there negative correlations between defensive traits suggestive of allocation constraints leading to trade-offs, or, alternatively, patterns of trait co-expression (i.e. positive correlations)? and (c) Does soil salinity shape these trait correlations? We predicted that any such trade-offs between defensive traits would arise or become stronger under...
Throughout its coastal distribution, wild cotton is exposed to moderate
abundant in the coastal scrubland along the northern coast of the
mesticated in southeast Mexico where wild populations are especially
dominated by numerous species of (native) insect herbivores, among which
leaf chewers represent the most common guild (e.g. Orthoptera,
Lepidoptera and Coleoptera; Abdala-Roberts, Pérez-Niño, Moreira,
et al., 2019). Insect herbivory results, on average, in $23 \pm 2.08\%$ of leaf
area consumed (range: 9%–52%) per plant ($n = 26$ populations; Abdala-
Roberts, Pérez-Niño, Moreira, et al., 2019; Abdala-Roberts, Quijano-
Medina, Moreira, Vázquez-González, et al., 2019). In turn, wild cotton
produces leaf traits that are putatively associated with either direct
(e.g. gossypol, phenolic compounds and glandular trichomes) or indirect
(VOCs and EFN) defence (Agrawal, Karban, & Colfer, 2000; Loughrin,
Manukian, Heath, Turlings, & Tumlinson, 1994; McAuslane, Alborn, &
Toth, 1997; McCall, Turlings, Loughrin, Proveaux, & Tumlinson, 1994;
Opitz, Kunert, & Gershenzon, 2008). For example, previous studies
have found that wild and cultivated $G. \text{ hirsutum}$ synthesize phenolic
compounds and terpenoids (stored in pigment glands) that confer direct
resistance against insect herbivores (e.g. piercing sucking insects and
caterpillars; Agrawal & Karban, 2000; Mansour, Zohdy, El-Gengaïhi,
& Amr, 1997; Nix, Paull, & Colgrave, 2017; Opitz et al., 2008). In addition,
as an indirect defence, EFN attracts natural enemies of herbivores
such as predatory ants and parasitic wasps (Abdala-Roberts, Pérez-
have also been shown to release VOCs in response to leaf damage,
which are known to attract natural enemies (Chappuis & Egger, 2016;
Loughrin et al., 1994; McCall et al., 1994).

### 2 | MATERIALS AND METHODS

**2.2 | Plant material**

In January 2018, seeds were collected from 16 plants (hereafter ma-
ternal lines or genotypes) separated by least 1 m at a site located
nearby the coastal town of Celestun, Yucatan (Mexico; 21°00'50.2''N
90°19'26.9''W). Seeds were germinated in Petri dishes at 35°C and
individually sown in 25 x 30 cm low-density polyethylene nursery
bags containing sandy soil collected from the plant genotype source
site (see above) mixed with perlite (1:1). All plants were maintained in
a greenhouse at the Campus de Ciencias Biológicas y Agropecuarias
of the University of Yucatan (Yucatan, Mexico) for a 3-month period
before starting the experiment. Plants were treated with a fungicide
(Bravo, Syngenta, Mexico) and an insecticide (BIODe® Promotora
Técnica Industrial, Mexico) 1 month prior to the experiment. In addition,
also 1 month before treatment, all plants were fertilized (amino
acids 9%, N 1.73% Amino Terra-F, Farmacia Agroquimica de México,
Mexico).

**2.3 | Experimental design and treatments**

Once plants were 3.5 months old, we conducted a fully crossed
factorial experiment with soil salinity (two levels: tap water with

![FIGURE 1 Predicted scenarios of defensive trait correlations and effects of abiotic stress caused by soil salinization on such associations. (a) There is no correlation between traits under control conditions, but a negative correlation (suggestive of a trade-off) arises under salinized conditions. (b) There is a negative correlation between traits under control conditions (low or ambient salinity), which strengthens under salinized (high salinity) conditions. A positive correlation between traits is also possible, whereby co-expression patterns could (c) weaken or (d) disappear under salinization. Null scenarios (not shown) include no effects of salinization on trait correlations or no trait associations whatsoever regardless of soil salinity.](image-url)
added salt vs. tap water without added salt), leaf defence induction (two levels: control vs. damage-induced) and plant genotype (16 levels) as main factors. In total, the experiment included 320 plants (i.e. two salinity treatments × two induction treatments × 16 genotypes × five replicates). First, we randomly assigned each plant to two levels of salinity, namely: irrigation with tap water (i.e. control) or irrigated with salinized tap water. The desired salinity level (i.e. salt concentration) for the latter treatment was achieved by adding NaCl to the water. Plants belonging to soil salinization were first watered once with 1.25‰ saline water (salt concentration) for the latter treatment were spiked with 10⁻¹⁵ M internal standards solution [n-octane and n-heptane, both chemicals] each to previous to GC-MS analysis. Samples were subsequently taken to the laboratory to measure sugar content (expressed in °Brix) with a refractometer (Atago Master T 0 to 33 °Brix, Germany).

We collected above-ground VOC emissions 24 hr after leaf damage using a subset of 65 plants (4–5 plants per genotype) which may not be qualitatively or quantitatively equivalent to induction by native herbivory. (Heil et al., 2012), various studies have demonstrated that combining mechanical leaf removal and caterpillar secretions provides an effective proxy of natural damage in several cultivated species (e.g. maize: Turlings et al., 1993; McAuslane et al., 1997; tobacco: Halitschke, Schittko, Pohnert, Boland, & Baldwin, 2001), including cultivated (Röse & Tumlinson, 2005) and wild cotton (Abdala-Roberts, Pérez-Niño, Moreira, et al., 2019; Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019; Chappuis & Egger, 2016).

2.4 Estimation of direct and indirect defences

2.4.1 Indirect defences

We quantified EFN production and concentration 24 hr after applying leaf damage (N = 256 plants, N = 4 plants per genotype for each leaf damage by salinization combination: Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). To this end, we used 5-μl capillary tubes (Micropipettes Blaubrand ® intraMARK, colour code white, Germany) to collect and measure the amount of nectar production by nectaries found on the apical leaves. Nectar was collected between 06.00 and 08.00 hr, and samples were subsequently taken to the laboratory to measure sugar content (expressed in °Brix). We quantified EFN production and concentration 24 hr after applying leaf damage (N = 256 plants, N = 4 plants per genotype for each leaf damage by salinization combination: Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). To this end, we used 5-μl capillary tubes (Micropipettes Blaubrand ® intraMARK, colour code white, Germany) to collect and measure the amount of nectar production by nectaries found on the apical leaves. Nectar was collected between 06.00 and 08.00 hr, and samples were subsequently taken to the laboratory to measure sugar content (expressed in °Brix). We quantified EFN production and concentration 24 hr after applying leaf damage (N = 256 plants, N = 4 plants per genotype for each leaf damage by salinization combination: Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). To this end, we used 5-μl capillary tubes (Micropipettes Blaubrand ® intraMARK, colour code white, Germany) to collect and measure the amount of nectar production by nectaries found on the apical leaves. Nectar was collected between 06.00 and 08.00 hr, and samples were subsequently taken to the laboratory to measure sugar content (expressed in °Brix).

We collected above-ground VOC emissions 24 hr after leaf damage using a subset of 65 plants (4–5 plants per genotype) which were different from those used for EFN sampling. Following Turlings et al. (1998), plants were bagged within a Polyacetate bag (Reynolds, Inc; see also Raguso & Pellmyr, 1998), and VOCs were adsorbed on filters containing 25 mg of 80/100 mesh Hayesep-Q adsorbent (Sigma, Switzerland). One of the filter ends was inserted into the bag and the other end was connected with tubing to a micro air sampler (Supelco PAS-500) at a flow rate of 500 ml/min. For each sampling period, we also collected an air sample from empty bags which served as an ambient control. After collecting volatiles for 2 hr, traps were eluted with 150 μl dichloromethane. Samples were sealed with caps and Teflon, stored at −30°C and carried to the FARCE lab (Neuchâtel Switzerland) for GC-MS analysis. The elutions were spiked with 10-μl internal standards solution [n-octane and nonyl-acetate, (20 μg/μl each) previous to GC-MS analysis. Samples were analysed with a gas chromatograph (Agilent7890B) coupled with a mass spectrometer detector (Agilent 5977B). A 1.5-μl aliquot of each sample was injected in pulsed splitless mode onto an Agilent HP-5MS column (30-m length × 250-μm diameter and 0.25-μm film thickness). After injection, temperature was maintained at 40°C for 3.5 min, increased to 100°C at a rate of 8°C per min, and subsequently to 230°C at a rate of 5°C per min followed by a post run of 3 min at 250°C. Helium was used as carrier gas and kept at constant flow of 0.9 ml/min. Compounds were subsequently identified by comparing their mass spectra with those from the NIST mass spectral library and comparisons with authentic standards. Compound quantities were estimated based on peak areas comparison with internal standards.

| Table S1 | Percent salinity values were estimated from direct measurements of water potential following standard procedures (Campbell, 1988; Katerji, van Hoorn, Hamdy, & Mastrorilli, 2004). One week after the first application of saline water, we randomly assigned plants of each salinity level to one of two leaf induction treatments: undamaged control or 25% of leaf area removed for all fully expanded leaves. We achieved the desired level of damage by removing the sides of each leaf with a scissor (equivalent to c. 25% of leaf area), as well as puncturing the remaining leaf tissue with an awl (4–5 holes per leaf). Immediately after applying mechanical damage, we exposed the punctured tissue to oral secretions of third instar larvae of the hypergeneralist Spodoptera frugiperda (Abdala-Roberts, Pérez-Niño, Moreira, et al., 2019; Chappuis & Egger, 2016; Quijano-Medina, Covelio, Moreira, & Abdala-Roberts, 2019; Turlings, McCall, Alborn, & Tumlinson, 1993). We obtained caterpillar secretions by gently poking the abdomen of each larva until it regurgitated (Turlings et al., 1993). Larvae were sourced from a colony reared on artificial diet for several generations at the Chemical Ecology Lab in ECOSUR (Chiapas, Mexico), and originally collected from maize fields. Spodoptera frugiperda (like other Spodoptera species) is known to attack cultivated cotton, and has been shown to induce both direct (e.g. terpenoids, phenolic compounds) and indirect defences (VOCs, EFN) in both wild and cultivated G. hirsutum (Chappuis & Egger, 2016). Although induced responses to S. frugiperda may not be qualitatively or quantitatively equivalent to induction by native caterpillars, our test was aimed at mimicking a response by wild cotton plants to feeding by a generalist insect.

Applying mechanical damage and insect oral secretions has the important advantage (over natural herbivory) of allowing to precisely control the amount of leaf area that is damaged. Although it is admittedly less realistic than actual insect feeding. (Heil et al., 2012),...
2.4.2 | Direct defences

We collected two or three fully expanded new (undamaged) leaves per plant to quantify phenolic compounds. We sampled the same plants used to quantify EFN, and in this case, leaf sampling was conducted at two time points: 1 week after herbivore induction (‘short-term’ measurement; \( N = 128 \) plants), and 6 weeks after induction (‘long-term’ measurement; \( N = 128 \) plants). The latter time point involved sampling leaves that were starting to expand (e.g. leaf buds) at the moment of damage application (totally or almost totally expanded at the time of collection) and was aimed at quantifying induction in new leaf tissues grown after induction. We followed this approach as previous work has shown that phenolic compounds are not only induced a few days after leaf damage in pre-existing tissue, but also exhibit induced levels in new tissues grown several weeks after damage (Abdala-Roberts, Pérez-Niño, Moreira, et al., 2019; Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). We used a different subset of plants for each time point because mechanical damage from short-term leaf collection would induce defences and therefore affect long-term measurements. Phenolic compounds were extracted from 20 mg of dry plant tissue with 1 ml of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al., 2014). For phenolic compound identification, 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 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 until 14 min, from 100% to 10% B (14–15 min), hold 10% B until 17 min. The injection volume was 3 µl, 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 1,200 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 4,500 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 to 50 eV. Individual compounds were identified based on the data obtained from the standard substances or published literature including RT, \( \lambda_{\text{max}} ([M–H]−), \) and major fragment ions. For phenolic compound quantification, 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) 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. Concentrations were expressed in mg/g tissue on a dry weight basis.

We also quantified leaf pigment gland density and trichome density for the same samples collected for the long-term measurement of phenolic compounds. These traits are not induced immediately after damage, but are rather expressed in new tissues grown after treatment application (Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). Preliminary assessments indicated that trichomes were absent for most of the plants and genotypes sampled, so this trait was dropped from the analyses. We counted the number of pigment glands on the adaxial surface of each of two 28 mm² discs obtained from one leaf using a stereoscopic microscope. These two values were converted to density (glands/cm²) and then averaged per plant for statistical analysis.

At the end of the experiment, we harvested all plants, separated leaf, stem and root tissues, and dried them to test for effects of salinization on plant growth, as well as to assess relative changes in allocation to growth versus defences.

2.5 | Statistical analyses

2.5.1 | Effect of soil salinity on defence induction

We ran GLMMs using data at the plant level to test for the effects of salinity, leaf damage, and plant genotype, as well as the two-way interaction between damage and salinity on direct (gland density, trichome density, and phenolic compounds) and indirect (EFN amount and concentration, VOCs) putative defensive traits. Phenolic compounds and VOCs were analysed both as totals as well as by group of compounds (flavonoids, condensed and hydrolysable tannins and lignins) in the former case and by individual compound in the latter case. All variables were normally distributed except data for VOC concentration which were log-transformed to achieve normality of residuals. Whenever the interaction was significant, we conducted pre-planned contrasts to statistically compare induction treatment level means within each salinity level. All models were run with PROC MIXED in SAS ver. 9.4 (SAS Institute, 2015).

2.5.2 | Soil salinity effects on trait correlations

We ran ANCOVAs using plant genotype means computed separately for control and treated (salinized soil) plants. Each of these models included the effect of salinity, a predictor trait, and their interaction on a second trait acting as a response (see details ahead). The effect of the predictor trait tested for a correlation between traits and the interaction tested whether salinity altered any such correlation. We did not run models including EFN concentration, since this trait was not quantifiable for several of the cotton genotypes which precluded reliable
tests at the genotypic level. Rather than running ANCOVAs for all pairwise combinations of traits (which would inflate Type I error), we only ran models for traits showing a significant (p < 0.05) salinity by induction interaction from the plant-level GLMMs as this was indicative that salinization altered the induction of that trait. In this sense, inspection of results from the plant-level models indicated a significant salinity by leaf damage interaction only for phenolic compounds (see Section 3). We therefore ran genotypic ANCOVAs to investigate whether effects of salinity on correlations between these chemical defences (for both short- and long-term data) and the other defensive traits (EFN amount, gland density and VOCs) could possibly explain the salinity effect on the induction of the phenolic compounds. In each of these genotypic ANCOVAs, phenolic compounds were treated as the response (rather than predictor). In the case of EFN amount and VOCs concentration, the rationale being that these traits are induced more quickly than phenolic compounds (Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019; Chappuis & Egger, 2016; McCall et al., 1994), which might set the stage for reduced allocation of subsequently induced phenolic compounds (Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). However, treating phenolic compounds as a response of pigment gland density is debatable since both traits may have similar induction time frames. Despite this, we decided to use phenolic compounds as a response in this case also in order to be consistent, and because the goal was to evaluate plant endogenous processes responsible for salinity effect on the induction of phenolic compounds (which implies treating this trait as a response). Each of the above ANCOVAs was run separately for constitutive and induced trait values, where induced values were measured as: genotype trait mean under the leaf damage treatment – genotype trait mean for controls. Aside from defensive traits, we also calculated inducibility for root biomass (dry weight) as a measure of below-ground compensatory ability, and ran models testing whether constitutive or induced root biomass traded off with phenolic compounds and whether salinity affected these correlations. All ANCOVA models were run with PROC MIXED in SAS ver. 9.4.

All ANCOVAs included the effect of cotton genotype to account for using two measures per genotype, that is, one for each salinity level (control vs. salinized). To visualize results from these models, 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 effect for each observation using the broom package in R ver. 3.6.1 (R Core Team, 2013). By doing so, we obtained model predicted values under each salinization level after accounting for the genotype effect.

3 | RESULTS

3.1 | Effects of leaf damage and soil salinity on cotton defensive traits

Artificial leaf damage resulted in significant 19.48% and 13.83% increases in short-term (control = 10.58 ± 0.75; damaged = 12.64 ± 0.75 mg/g) and long-term (control = 26.99 ± 2.45; damaged = 30.73 ± 2.45 mg/g) concentration of total phenolic compounds, respectively (Table 1). Analyses by group of phenolic compounds indicated similar patterns of short-term induction of flavonoids and condensed tannins (15.26% and 27.76% increments relative to controls respectively; marginally significant in the latter case; Table 1), as well as long-term induction of lignins and condensed tannins (16.48% and 20.26% increases relative to controls respectively; Table 1). In contrast, there was no significant effect of leaf damage on pigment gland density (control = 76.45 ± 3.16; damaged = 80.78 ± 3.16 glands/cm²; Table 1). For indirect defences, leaf damage significantly increased (240%) EFN production (control = 0.10 ± 0.033; damaged = 0.34 ± 0.033 μl), but did not significantly affect nectar concentration (control = 1.65 ± 0.64; damaged = 1.94 ± 0.37 °Brix; Table 1). In addition, leaf damage tended to increase (33%), albeit non-significantly (p = 0.08), total VOCs emissions (control = 911.5 ± 162.1 ng/2 hr; damaged = 1,215.9 ± 158.83 ng/2 hr; Table 1). Analyses of individual VOCs indicated a significant (threefold) increase in linalool emission for damaged plants (control = 10.86 ± 2.82; damaged = 30.26 ± 2.71 ng/2 hr), whereas all other compounds showed no significant change (Table S2).

The main effect of soil salinization was not significant for any of the direct defensive traits measured (Table 1), but the leaf damage by salinity interaction was significant for condensed and hydrolysable tannins (Table 1). Specifically, leaf damage significantly increased (relative to controls) the concentration of these tannins at the long-term measurement but did not affect their expression under salinized soil (Figure 2a,b). Although the interaction was not significant, a similar trend was observed for short-term condensed tannins (Figure 2a), long-term lignins (Figure 2c), and short-term total phenolic compounds (Figure 2e). In addition, we found no interaction for short-term lignins (Figure 2c), short- or long-term flavonoids (Figure 2d) or gland density (Figure 2f; Table 1). In the case of indirect defences, we found no main effect of salinity or salinity by induction interaction for either nectar volume, nectar concentration or total VOCs (Table 1; Figure 3a-c), though in the last case a trend for reduced induction of volatiles under salinization was observed (Figure 3c). We found no interaction for most of the volatile compounds when analysed separately except for β-myrcene, which increased due to leaf damage for control plants but decreased due to damage for plants grown with high salinity (Table S2).

We found no effects of salinization, leaf damage or their interaction on cotton above- or below-ground biomass (Table S3).

3.2 | Effects of soil salinity on defensive trait correlations

Models based on constitutive trait levels indicated a marginally significant negative effect of EFN production, and, more importantly, a significant EFN production by salinity interaction on total phenolic compounds at the short-term time point (Table 2A). This
**TABLE 1** Results from general linear mixed models testing for the effect of artificial leaf damage (or undamaged), salinity (control or salinized soil), and its interaction (leaf damage × salinity) on wild cotton *Gossypium hirsutum* direct and indirect defence traits, and compensation traits. Phenolic compounds were analysed 1 (short-term measurement, ST) and 5 (long-term, LT) weeks after leaf damage. Values given are $F$ tests, degrees of freedom, and $p$-values. All models also included the effect of genotype (random). Significant effects ($p < 0.05$) are shown in bold.

<table>
<thead>
<tr>
<th>Defence type</th>
<th>Trait</th>
<th>Leaf damage (LD)</th>
<th>Salinity (S)</th>
<th>LD × S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$F$</td>
<td>$df$</td>
<td>$p$</td>
</tr>
<tr>
<td>Indirect</td>
<td>Extrafloral nectar volume</td>
<td>26.66</td>
<td>1, 256</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>Extrafloral nectar concentration</td>
<td>0.16</td>
<td>1, 96</td>
<td>0.69</td>
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<tr>
<td></td>
<td>Total volatiles</td>
<td>3.28</td>
<td>1, 52</td>
<td>0.08</td>
</tr>
<tr>
<td>Direct</td>
<td>Pigment glands</td>
<td>2.02</td>
<td>1, 127</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Flavonoids (ST)</td>
<td>5.12</td>
<td>1, 109</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Lignins (ST)</td>
<td>2.47</td>
<td>1, 109</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Condensed tannins (ST)</td>
<td>3.36</td>
<td>1, 109</td>
<td>0.07</td>
</tr>
<tr>
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<td>Hydrolysable tannins (ST)</td>
<td>1.88</td>
<td>1, 109</td>
<td>0.17</td>
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<td></td>
<td>Total phenolic compounds (ST)</td>
<td>7.87</td>
<td>1, 109</td>
<td>&lt;0.01</td>
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<td></td>
<td>Flavonoids (LT)</td>
<td>0.96</td>
<td>1, 127</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Lignins (LT)</td>
<td>5.38</td>
<td>1, 127</td>
<td>0.02</td>
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<tr>
<td></td>
<td>Condensed tannins (LT)</td>
<td>4.32</td>
<td>1, 127</td>
<td>0.04</td>
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<tr>
<td></td>
<td>Hydrolysable tannins (LT)</td>
<td>0.47</td>
<td>1, 127</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Total phenolic compounds (LT)</td>
<td>4.29</td>
<td>1, 127</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**FIGURE 2** Effects of soil salinity on the induction of wild cotton *Gossypium hirsutum* direct defensive traits due to artificial leaf damage. (a–e) Short- and long-term total phenolic compounds (1 and 5 weeks after leaf damage, respectively; see Section 2), (f) pigment gland density. Values are model least-square means and standard errors accounting for the effect of plant genotype. In those cases where the salinity by leaf damage interaction was significant (i.e. condensed and hydrolysable tannins), we compared the means for control (undamaged) and leaf damage treatment within each level of soil salinity (*$p < 0.05$, **$p < 0.01$). CT, condensed tannins; HT, hydrolysable tannins.
depicted a pattern whereby EFN and short-term total phenolic compounds were negatively correlated for plants subjected to salinized soil ($R^2 = 0.79, p < 0.001$), whereas for control plants there was no detectable association ($R^2 = 0.15, p = 0.58$), that is, this apparent trade-off arose only under salinized soil (Figure 4a). In addition, we also observed a significant positive correlation between gland...
salinity and other traits (gland density, VOCs, root biomass) predict pounds (Table 2A), and this also held true when testing constitutive levels of either short- or long-term phenolic compounds, as well as a significant correlation between these traits for control plants ($R^2 = 0.44, p = 0.08$), whereas for plants subjected to salinized water there was no significant correlation ($R^2 = 0.39, p = 0.13$; Figure 4c). All other models using inducibility values for traits predicting inducibility of total phenolic compounds yielded non-significant interactions (Table 2B), and the same held true when testing individual groups of phenolic compounds (Tables S4B and S5B), except for a significant interaction between salinity and inducibility of root biomass on inducibility of long-term hydrolysable tannins (Table S5B). Finally, we also ran models with inducibility values for phenolic compounds and constitutive values for EFN, VOCs and gland density which in all cases yielded non-significant salinity by predictor trait interactions (Tables S6–S8).

### 4 | DISCUSSION

#### 4.1 | Overview

There were no overall effects of soil salinity on either constitutive or induced levels of any of the direct or indirect defensive traits measured in wild cotton. Leaf damage, however, triggered an increase in the expression of several traits, namely EFN amount, total VOC emissions (marginally significant), total phenolic compounds and some groups of phenolic compounds (e.g. condensed and hydrolysable tannins). Furthermore, leaf damage significantly interacted with soil salinity in the case of phenolic compounds, whereby hydrolysable tannins and condensed tannins were significantly induced in controls, but induction no longer occurred in plants subjected to salinization. These interactive effects were not observed for other direct (e.g. pigment gland density) defensive traits or any of the indirect defensive traits measured (though a trend was observed for VOCs). Importantly, we also found that soil salinization altered wild cotton defensive trait correlations. Namely, a negative association (suggesting a trade-off) between total phenolic compounds and EFN production was found for plants with salinized soil but not for controls. Similarly, we found a positive association between phenolic compounds and gland density for control plants but no association for plants subjected to salinization. These results indicate that soil salinity alters defensive trait correlations in wild cotton and that such effects potentially influence patterns of allocation to multiple traits and their inducibility in response to herbivory.

#### 4.2 | Effects of soil salinity on wild cotton defences

We found no main effect of soil salinization on any of the measured direct or indirect defensive traits. In contrast, two previous studies with transgenic varieties G. hirsutum reported that soil salinization caused a significant increase in direct defences including gossypol, flavonoids and levels of individual groups of phenolic compounds (Tables S4A and S5A).

Models using inducibility trait values indicated a significant interaction between inducibility of root biomass and inducibility of long-term total phenolic compounds (Table 2B). This trait association was marginally significantly positive for control plants ($R^2 = 0.44, p = 0.08$), whereas for plants subjected to salinized water there was no significant correlation ($R^2 = 0.39, p = 0.13$; Figure 4c). All other models using inducibility values for traits predicting inducibility of total phenolic compounds yielded non-significant interactions (Table 2B), and the same held true when testing constitutive values for EFN, VOCs and gland density which in all cases yielded non-significant salinity by predictor trait interactions (Tables S6–S8).
tannins (Luo, Dong, Li, Ming, & Zhu, 2008; Wang et al., 2015). However, these studies tested multiple salinity levels and reported effects were only detectable at 200 mM, c. 11.7‰ salinity, which was substantially higher than in our study. Work with other agricultural species has reported mixed effects of soil salinization on plant defences. For example, in the case of maize, soil salinization (1–100 mM, c. 0.06‰–5.8‰ salinity) increased the production of jasmonic acid and several secondary metabolites, but reduced the induction of VOC emissions (Forieri et al., 2016). Likewise, in water-stressed tomato plants, salinization (100 Mm NaCl, c. 5.85‰) caused a significant increase in tomatidine but did not affect other secondary metabolites (α-tomatine 1, α-tomatine 2, dehydrotomatine; Han et al., 2016). Finally, a boost in the expression of secondary metabolites was found in a species of alga in response to moderate levels of water salinity (25‰–30‰; Bueno-Sudatti et al., 2011). Combined, work conducted to date indicates that salinity effects on plant defences are contingent on the amount of salinity and the type of secondary metabolites studied, but the underlying causes for such variation remain elusive without further mechanistic assessments of physiological and metabolic changes underlying changes in defence expression.

The amount of soil salinity achieved in our experiment at the time of leaf damage application (i.e. 7.1‰, a week after the second application of salinized water) was 4.7-fold higher than soil salinity of control plants (1.5‰, i.e. ambient salinity at source site) and close to double the upper limit of variation in soil salinity observed in situ at wild cotton coastal sites (3.5‰). Our results therefore suggest that the studied cotton genotypes were able to overcome substantial short-term increases in soil salinity with no apparent effects on plant growth or constitutive plant defence levels (though for induced defences this was not the case, see ahead). A few previous studies have reported variation in salt tolerance of G. hirsutum commercial varieties (Gossett, Millhollon, & Lucas, 1994; see also Luo et al., 2008), but to our knowledge, the present work is the first to test for soil salinization effects on wild cotton defences. A higher level of soil salinity than used in our study or multiple, more prolonged exposures would presumably be required to cause more substantial physiological effects which alter the expression of constitutive direct and indirect defences. It is also possible that the studied wild cotton populations, which grow near the coast, exhibits higher salt tolerance (and thus weaker effects on defence expression) than agronomic varieties, but this remains to be tested. It is important to note that levels of salinity can vary across these wild cotton populations, which could select for contrasting patterns of tolerance to soil salinity. Similarly, local-scale variation within sites could be also potentially important but has not been yet evaluated. Follow-up work involving multiple populations and levels of salinity combined with several exposure events (over a longer time period) is needed to build on the present findings.

### 4.3 Effect of simulated herbivory on wild cotton defences

Leaf damage induced both direct (e.g. phenolic compounds) and indirect (e.g. EFN, VOCs) defensive traits in wild cotton, which is consistent with previous work of ours with wild (Abdala-Roberts, Pérez-Niño, Moreira, et al., 2019; Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019) as well as studies addressing leaf herbivory with cultivated cotton (e.g. EFN: Wäckers & Wunderlin, 1999; VOCs: Paré & Tumlinson, 1997; Röse & Tumlinson, 2005). Some of these studies used mechanical damage, whereas others used real caterpillar damage (EFN: Wäckers & Bonifay, 2004; VOCs: McCall et al., 1994; Loughrin, Manukian, Heath, & Tumlinson, 1995; Rodriguez-Saona, Crafts-Brandner, & Canas, 2003).

The lack of an effect of leaf damage on EFN concentration is worth noting and agrees with a previous study of ours where it did not find an effect of leaf damage on this trait either (Abdala-Roberts, Pérez-Niño, Moreira, et al., 2019). Similarly, work by Wäckers, Zuber, Wunderlin, and Keller (2001) also found no effect of mechanical leaf damage on nectar concentration for cultivated cotton. Together with our findings, suggests that the induction of nectar production in response to herbivory is associated with a concomitant increase in carbohydrate secretion (i.e. preventing nectar dilution). However, this result should be taken with caution since nectar concentration may have been influenced by dilution due to changes in surrounding air humidity or evaporation (von Arx, Goyret, Davidowitz, & Raguso, 2012). Measurements of soluble solids in the nectar would be desirable to overcome this limitation (e.g. Wäckers et al., 2001).

Additionally, leaf tissue removal necessarily reduces the amount of photosynthetically active leaf area involved in nectar synthesis (Radhika, Kost, Mithöfer, & Boland, 2010). Although this did not prevent the detection of an effect on nectar production (which was strong), it may have influenced the detection of an effect on nectar concentration differences.

Our findings also indicated no effect of leaf damage on terpenoid gland density which also agrees with a previous study of ours with wild cotton suggesting this trait has low plasticity (Abdala-Roberts, Pérez-Niño, Moreira, et al., 2019). However, terpenoid concentrations are thought play a key role in cotton induced resistance to insect herbivory (Chen, Schmelz, Wäckers, & Ruberson, 2008; McAuslane & Alborn, 1998; Wu, Chen, Ge, & Sun, 2007; Zebelo, Disi, Balusu, Reeves, & Fadamiyo, 2017), and previous studies have reported increased gland density in response to herbivory in cultivated cotton (McAuslane et al., 1997; Opitz et al., 2008). Interestingly, a previous study with cultivated cotton found that increased terpenoid concentrations can be achieved not only by increasing the number of glands but also increasing the filling of individual glands (Opitz et al., 2008). Accordingly, future work with wild cotton should involve the quantification of terpenoid concentrations by gas chromatography methods.

### 4.4 Interactive effects of soil salinity and leaf damage

A key finding was that increased soil salinization hampered wild cotton induction of phenolic compounds, specifically hydrolysable and condensed tannins (and a similar tendency for lignins). Although this
result was significant only for long-term measurements, a similar trend was observed for short-term condensed tannins and total phenolic compounds suggesting that salinity affected the induction of phenolics not only in new leaves grown several weeks after damage but also in fully grown leaves shortly after damage. Likewise, though the interaction was not significant either for VOCs (likely due to low statistical power), there was also a trend for salinization to constrain the induction of these compounds. This suggests that indirect defense could also be compromised when plants grow in high salinity soils. At least one previous study has tested for effects of soil salinity on defense induction in cultivated cotton, in which Luo et al. (2008) reported that it boosted gossypol induction in cotton several varieties, and that this effect was stronger under water stress (for examples of cultivated cotton studies with other abiotic stressors see: Chen et al., 2008; Coviella, Stipanovic, & Trumble, 2002; Wu et al., 2007). Likewise, previous work with maize reported that salinity decreases the induction of secondary metabolites after herbivory (Forieri et al., 2016). Overall, studies conducted to date thus indicate that soil salinization decreases the inducibility of secondary metabolites (involved in both direct and indirect defence in the case of wild cotton), and that this effect could interact with other abiotic stressors (e.g. water availability) to shape plant defense expression. The underlying physiological or biochemical mechanisms for such patterns of defense inducibility remain unstudied in wild cotton.

4.5 | Effects of soil salinity on defensive trait correlations

There is a long tradition of theoretical and empirical work on the influence of allocation costs on multiple plant functions (Hahn & Maron, 2016; Simms, 1992; Stamp, 2003). Within this body of literature, studies have addressed whether patterns of defensive trait correlation (co-expression or trade-offs) influence the evolution of plant allocation to multiple defences (Herms & Watson, 1992). However, some studies have argued that defence allocation constraints leading to trade-offs are uncommon (Agrawal, 2011; Koricheva, Nykänen, & Gianoli, 2004), and others have found that in those cases where they do occur these are usually strongly context-dependent (Agrawal & Hastings, 2019; Kergunteuil, Descombes, Glauser, Pellissier, & Rasmann, 2018). Our findings agree with this latter view, showing abiotic context dependency in wild cotton trait correlations due to soil salinization. Specifically, we found a negative correlation between short-term constitutive levels of phenolic compounds and EFN production only when plants were subjected to high soil salinity (Figure 4a). This result agrees with studies showing that resource limitation exacerbates trade-offs between plant growth-defence trade-offs (Abdala-Roberts et al., 2014; Donaldson et al., 2006; Sampedro et al., 2011), presumably because nutrient limitation or abiotic stress exacerbate allocation constraints. Our results uniquely suggest that soil salinity sharpens these defensive constraints, presumably via physiological changes affecting resource uptake and acquisition (Munns et al., 2002).

Our previous work showed seemingly disparate patterns of induction of EFN and phenolic compounds in wild cotton which suggests that these traits antagonize each other (Abdala-Roberts, Quijano-Medina, Reyes-Hernández, et al., 2019). Accordingly, we speculate that rapidly induced EFN in response to leaf damage, combined with EFN being induced to the same extent under salinization, adversely affected the induction of phenolic compounds through an underlying trade-off. It should be noted that this trade-off involved short-term constitutive phenolic compounds whereas salinity significantly hampered their long-term induction (though salinity also tended to affect their short-term induction). However, an examination of correlations between constitutive and induced phenolic compounds points at how salinity could have influenced the induction of these compounds. For example, short-term constitutive phenolic compounds such as hydrolysable tannins were positively correlated with long-term constitutive hydrolysable tannins ($r = 0.53$, $p = 0.03$), and the latter were positively correlated with long-term induced tannins ($r = 0.64$, $p = 0.008$); these genetic correlations were only observed for plants under salinized soil (data from this study). Correspondingly, a structural equation model relating these variables showed a significant indirect (positive) effect of short-term constitutive tannins on long-term induced tannins (coefficient = 0.34, $p = 0.02$). Hence, it is possible to argue that a salinity-driven reduction in short-term constitutive phenolic compounds resulting from a trade-off with EFN production indirectly weakened (via lowered constitutive long-term levels) the long-term induction of these compounds. Although this interpretation remains speculative, evidence from other studies similarly suggests that abiotic factors affect defensive trait correlations (e.g. Kempel et al., 2011; Moreira et al., 2018), particularly those underlain by allocation constraints. This possibility should therefore be addressed in more detail in future work addressing abiotic context dependencies in the expression of multiple defences in wild cotton.

Interestingly, we found two additional lines of evidence for soil salinity effects on wild cotton trait correlations. In one case, we found a significant positive correlation between constitutive total phenolic compounds and pigment gland density for control plants, but this association was not significant for plants grown with salinized soil. Thus, rather than a sharpening of trade-offs, this suggests that the co-expression of these traits is weakened or lost under high soil salinity. In the other case, we found a positive correlation between the inducibility of total phenolic compounds and the inducibility of increased root biomass (proxy of root compensatory growth) for control plants (marginally significant). For plants subjected to salinized soil this association turned non-significant. These findings suggest the possibility that soil salinity shapes concomitant allocation to above-ground induced defences and below-ground compensatory ability.

5 | CONCLUSIONS

This study underscores the importance of endogenous constraints imposed by soil salinity on the induction of plant direct and indirect
defences, as well as its effects on trait correlations potentially affecting their simultaneous expression. Overall, these findings stress the need for providing integral assessments of abiotic context dependency on plant defensive phenotypes by considering multiple defensive traits simultaneously and their correlated expression patterns.

To the extent that trait correlations are commonplace, a likely case for many plant taxa, our work shows that explicitly considering these associations and further investigating their causes (e.g., metabolic or physiological constraints, genetic linkages) can illuminate our understanding of how realistically complex (multivariate) plant defensive phenotypes interact respond to and evolve under different environmental contexts. Further work should involve multiple and more prolonged exposures to soil salinization to identify thresholds in tolerance to salinity, as well as a more detailed assessment of temporal changes in induction (e.g., terpenoids and phenolic compounds) to better describe the time frames of induction for each trait and how these could set the stage for allocation constraints. Finally, manipulations of other biotic (e.g., mycorrhizae) or abiotic (e.g. water availability) factors that covary in situ with soil salinity and their effects on herbivore performance are currently being addressed to achieve a more complete understanding of context dependency in defence expression and its implications for herbivore resistance in wild cotton.

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AUTHORS’ CONTRIBUTIONS
T.Q.-M., L.A.-R. and X.M. conceived the ideas and designed the methodology; T.Q.-M., P.S. and J.C.C. collected the data; T.Q.-M. and L.A.-R. analysed the data; T.Q.-M., L.G. and T.C.J.T. performed the chemical analyses; T.Q.-M., L.A.-R. and X.M. wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA AVAILABILITY STATEMENT
Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.0k6dh9xh (Abdala-Roberts et al., 2020).

REFERENCES


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