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ORIGINAL ARTICLE

Solar UV-B radiation and ethylene play a key role in modulating effective defenses against *Anticarsia gemmatalis* larvae in field-grown soybean

Francisco M. Dillon[1,3] | M. Daniela Tejedor[1] | Natalia Ilina[1] | Hugo D. Chludil[2] | Axel Mithöfer[4] | Eduardo A. Pagano[1] | Jorge A. Zavala[1,3]

[1] Cátedra de Bioquímica, Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453, C1417DSE Buenos Aires, Argentina

[2] Cátedra de Química de Biomoléculas, Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453, C1417DSE Buenos Aires, Argentina

[3] INBA/CONICET, Avenida San Martín 4453, C1417DSE Buenos Aires, Argentina

[4] Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena 07745, Germany

Correspondence
J. A. Zavala, INBA/CONICET, Avenida San Martín 4453, C1417DSE Buenos Aires, Argentina.
Email: zavala@agro.uba.ar

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Abstract

Solar UV-B radiation has been reported to enhance plant defenses against herbivore insects in many species. However, the mechanism and traits involved in the UV-B mediated increment of plant resistance are unknown in crops species, such as soybean. Here, we studied defense-related responses in undamaged and *Anticarsia gemmatalis* larvae-damaged leaves of two soybean cultivars grown under attenuated or full solar UV-B radiation. We determined changes in jasmonates, ethylene (ET), salicylic acid, trypsin protease inhibitor activity, flavonoids, and mRNA expression of genes related with defenses. ET emission induced by *Anticarsia gemmatalis* damage was synergistically increased in plants grown under solar UV-B radiation and was positively correlated with malonyl genistin concentration, trypsin proteinase inhibitor activity and expression of *IFS2*, and the pathogenesis protein *PR2*, while was negatively correlated with leaf consumption. The precursor of ET, aminocyclopropane-carboxylic acid, applied exogenously to soybean was sufficient to strongly induce leaf isoflavonoids. Our results showed that in field-grown soybean isoflavonoids were regulated by both herbivory and solar UV-B inducible ET, whereas flavonols were regulated by solar UV-B radiation only and not by herbivory or ET. Our study suggests that, although ET can modulate UV-B-mediated priming of inducible plant defenses, some plant defenses, such as isoflavonoids, are regulated by ET alone.

KEYWORDS

jasmonic acid, plant-insect interactions, salicylic acid

1 | INTRODUCTION

Defoliation of soybean crops is caused by different insect species that can reduce yield and quality of production, especially when it occurs at reproductive stages (Begum & Eden, 1965). The velvet bean caterpillar (*Anticarsia gemmatalis*) is one of the main defoliator pests of soybean (*Glycine max*) in Argentina and Brazil. In fast-growing herbaceous plants, such as soybean, consumption of plant material by herbivores can be inhibited by toxins or deterrents present in foliage before (constitutive) and after (inducible) insects feed on leaves.

Plant constitutive and inducible defenses are modulated by variations in light quality generated by the canopy and associated to plant density (Ballaré, Mazza, Austin, & Pierik, 2012). Solar ultraviolet-B (UV-B; λ 280–315 nm) radiation is not only used by plants to sense competition (Boccalandro, Mazza, Mazzella, Casal, & Ballaré, 2001) but also increases plant resistance to herbivorous insects under field conditions (Ballaré, Cadlwell, Flint, Robinson, & Bornman, 2011), including soybean crops (Dillon, Chludil, & Zavala, 2017; Mazza, Giménez, Kantolic, & Ballaré, 2013; Zavala, Mazza, Dillon, Chludil, & Ballaré, 2015; Zavala, Scopel, & Ballaré, 2001). One of the main defenses induced typically by solar UV-B radiation in leaves are the phenolic compounds (Ballaré et al., 2011). Solar UV-B radiation induced transcription of *chalcone synthase*, *flavanone 3 β-hydroxylase*, and *flavonol synthase* genes in soybean, increasing flavonol content in leaves (Jenkins, 2009; Kim, Kim, Kim, Lee, & Ahn, 2008). Moreover, other type of flavonoids, such as isoflavonoids can be induced in damaged soybean leaves by lepidopteran larvae, which negatively affected larval growth (Dillon et al., 2017; Murakami et al.,

2014; O'Neill et al., 2010; Piubelli, Hoffmann-Campo, Moscardi, Miyakubo, & Neves De Oliveira, 2005). Isoflavonoid production in soybean is mainly regulated by isoflavone synthase (IFS) enzymes, and although expression of the *IFS2* gene is induced by biotic and abiotic stresses, *IFS1* expression is not affected by these environmental stresses (Dhaubhadel, McGarvey, Williams, & Gijzen, 2003; Gutierrez-Gonzalez, Sleper, & Nguyen, 2009).

Another important plant defense against lepidopteran larvae is the trypsin proteinase inhibitors (TPI; Farmer & Ryan, 1990; Farmer & Ryan, 1992; Jongsma, Bakker, Peters, Bosch, & Stiekema, 1995; Zavala, Patankar, Gase, Hui, & Baldwin, 2004). Two types of TPI have been characterized in soybean (Kunitz, 1946; Birk, Gertler, & Khalef, 1963). TPI are induced in soybean leaves after *A. gemmatalis* attack and other chewing insects, such as *Spodoptera litura* and *Popillia japonica*, which induced the expression of Kunitz type and Bowman-Birk type inhibitors (Casteel et al., 2008; da Silva Fortunato et al., 2007; Dillon et al., 2017; Wang, Wang, Fan, Yang, & Yu, 2014). TPI induction in response to damage or to methyl jasmonate (MeJA) applications is enhanced by solar UV-B radiation in *Nicotiana* sp. and tomato plants (Demkura, Abdala, Baldwin, & Ballaré, 2010; Izaguirre, Scopel, Baldwin, & Ballare, 2003; Stratmann, Stelmach, Weiler, & Ryan, 2000), suggesting that UV-B radiation enhances defensive hormone pathways.

Plants response to herbivore damaged inducing defenses mainly regulated by jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA), which are fine-tuned by the crosstalk among these and other phytohormones (Pieterse, Van Der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012). Although ABA signalling antagonizes down-regulating SA-related defenses (Cao, Yoshioka, & Desveaux, 2011; De Torres-Zabala, Bennett, Truman, & Grant, 2009), certain JA-related defenses depend on ABA and vice versa (Lackman et al., 2011). In addition, JA signalling can be reciprocally suppressed by the SA pathway. Although the antagonistic effect of JA on SA-related responses has been mainly studied in the interactions between pathogens and plants (Brooks, Bender, & Kunkel, 2005; Nomura, Melotto, & He, 2005), recently, this antagonistic interaction was found in developing seeds of soybean after stink bug attack (Giacometti et al., 2016). Moreover, up regulation of the SA signalling pathway suppressed the JA-related gene *VSP2* in *Arabidopsis* regardless the induction of JA-regulated genes by herbivores, or by JA, JA-isoleucine (Ile), or its precursors 12-oxo-phytodienoic acid (OPDA; Koornneef, Leon-Reyes, Ritsema, Verhage, & Den Otter, 2008; Leon-Reyes et al., 2010).

Although plant modulation of defense compounds against herbivores, such as phenolics and TPI, is mainly orchestrated by JA, a concomitant induction of ET with JA can modify plant responses to herbivory (Howe & Jander, 2008). Whereas ET and JA have synergistic effects on up regulating TPI in tomato (O'Donnell et al., 1996), leaf damage produced by larvae of *Manduca sexta* induced ET emission and decreased nicotine production in *Nicotiana attenuata* (Winz & Baldwin, 2001). Also, ET applied exogenously or produced during pathogen infection or stink bug attack eliminates the JA-SA antagonistic relationship, allowing the induction of both JA- and SA-regulated defenses (Giacometti et al., 2016; Leon-Reyes et al., 2009). Although few studies have focused on the effects of ET alone on plant defenses, recently, it has been suggested that ET can increase the production of isoflavonoids in soybean leaves (Yuk et al., 2016). Because it is not clear how solar UV-B radiation synergistically increases chemical defenses in damaged leaves, we proposed that UV-B radiation induces ET emission that increases the production of phenolic compounds and TPI in soybean leaves.

Field experiments of plant responses against herbivores performed with either ambient or attenuated solar UV-B radiation clearly demonstrated that the effects of solar UV-B on reducing herbivory is mediated by production of plant defenses (Ballaré, 2014). To study the role of the phytohormones, SA, JA, ABA, and ET in mediating the regulation by solar UV-B radiation and herbivory in soybean leaves, we cultivated soybean at field conditions under either ambient or attenuated solar UV-B radiation levels generated by plastic filters. At beginning of flowering stage of the soybean crop (R1; Fehr, Caviness, Burmood, & Pennington, 1971), we performed herbivory manipulative treatments and exogenously applied the phytohormones on leaves. We determined TPI activity, content of individual phenolic compounds and endogenous phytohormones, and expression of genes related with defenses in damaged and undamaged leaves. Our results showed associations between ET emission and isoflavonoid inductions, and this association was amplified in soybean leaves by the combination of solar UV-B radiation and herbivory of *A. gemmatalis* larvae.

2 | MATERIAL AND METHODS

2.1 | EXPERIMENTAL

2.1.1 | Plant and insect material

Experiments were carried out in the experimental fields of the University of Buenos Aires (34°35′S, 58°29′W), Buenos Aires, Argentina, during the summer of 2012–2013 and 2013–2014. Two soybean (*Glycine max* L. Merril, Leguminosae) cultivars: Williams (maturity group: III) and Charata (maturity group: VII) were grown in four plots with an inter-row spacing of 20 cm, and a spacing between plants within each row of 15 cm. The plots were watered as needed, and weeds were controlled manually. Eggs of *A. gemmatalis* Hübner (Lepidoptera) were obtained from a laboratory colony maintained at the IMIZA, INTA Castelar, Buenos Aires Province, Argentina.

2.1.2 | UV-B treatment

Soybean plants were allowed to emerge and to grow in the field in four independent split-plots under two adjacent 1.8 × 1.4 m aluminium frames covered with either clear polyester films (Mylar-D, Du-Pont, Wilmington, DE; 0.1 mm thick), which virtually cut-off all solar UV radiation below 310 nm (UVB- treatment), or "Stretch" films (Bemis Co. Minneapolis; 0.025 mm thick), which had very high transmittance (more than 80%) over the whole UV waveband (UVB + treatment; Izaguirre, Mazza, Svatos, Baldwin, & Ballaré, 2007). Both filters have high and similar transmittance to PAR and UV-A (more than 80%) and generate similar conditions of temperature and humidity in the canopy and soil (Ballaré, Scopel, Stapleton, & Yanovsky, 1996; Izaguirre et al., 2007; Zavala et al., 2015). In each plot, two light treatments were performed: attenuated solar UV-B (daily BE-UV-B approximately 0 kJ m^{-2}) and near-ambient UV-B (daily BE-UV-B approximately 8 kJ m^{-2}). Furthermore, four treatments were

generated: (a) cv. Williams grown under transparent filters to solar UV-B radiation, (b) cv. Charata grown under transparent filters to solar UV-B radiation, (c) cv. Williams grown under filters that attenuate solar UV-B radiation, and (d) cv. Charata grown under filters that attenuate solar UV-B radiation. For all measurements, only plants from the centre of the plot were used (where solar UV-B radiation is less than 5% of ambient levels in the UV-B- treatment; Zavala et al., 2001). The filters were raised periodically to maintain them approximately 10 cm above the upper leaf layer. On each individual plot, the filters were changed at least twice during the course of the growing season because the plastics tended to deteriorate and accumulate dust.

2.1.3 | Herbivory and exogenous phytohormone treatments and leaf consumption

To determine plant responses to herbivory and to measure larval consumption, three 3rd instars larvae were placed in transparent tull bags on the youngest full expanded leaves from four plants from each UV-B treatment and genotype within each plot at the R1 stage of soybean crop (Fehr et al., 1971). Larvae were allowed to feed on leaves for 72 hr, and leaves damaged by larvae were photographed with a scale to later calculate consumption using Image J software (NIH, USA). Also, at midday, half of damaged leaves were collected 24 hr and the other half 72 hr after the treatments were initiated. Similarly, control leaves from the same position covered with empty tull bags (four per plot) were collected at 24 and 72 hr after treatments were initiated. Because leaves of two plants per plot equally treated were combined to conform a replicate at each time of collection, plots were considered as true replicate (n = 4). Collected leaves were fast frozen in liquid nitrogen for further analysis. Although phenolic content and TPI activity in leaves were determined both 24 and 72 hr after treatments, gene expression was determined from leaf material collected 24 hr after treatments. ET emission quantification was performed 2 hr after treatments, and the other phytohormones (JA, JA-Ile; OPDA, SA, and ABA) were quantified 24 hr after larvae started to feed on soybean.

To determine the role of JA, SA, and ET on TPI activity and phenolic compounds content in plants grown under either UV-B− or UV-B+ treatments, phytohormones were applied exogenously to two plants from each soybean cultivar and UV-B treatment of each plot. Either precursors or phytohormones were applied to youngest fully expanded leaves by spraying 1 ml of (a) water, (b) 1 mM MeJA, (c) 1,5 mM SA, or (d) 1 mM aminocyclopropane-carboxylic acid (ACC), and then covered with an empty tull bag. Within each plot, leaves from untreated or treated plants were combined to conform a replicate, and the samples collected from the four plots were analysed (n = 4). TPI activity and phenolic content in treated and untreated leaves were determined 24 and 72 hr after treatments. Collection of plant material was done in every case at midday.

2.1.4 | Pupal weight

To determine pupal weight, neonates of *A. gemmatalis* were placed in transparent tull bags on the youngest fully expanded leaves of plants from the four treatments (cv. Williams UVB+ or UVB− and cv. Charata UVB+ or UVB−) at the V6 stage of soybean crop (Fehr et al., 1971). A population of 240 neonates was divided into 24 groups (six repetitions) of 10 larvae (60 larvae × 2 treatments = 120 per cultivar). To ensure food availability for larvae, 6 days after the treatment was started, larvae were changed to an intact leaf from the same treatment. The same procedure was performed whenever larvae removed around 70% of foliar area, until the larvae turn to pupae. Collected pupae were dry at 60 °C and weighted.

2.1.5 | TPI determination

To determine TPI activity in leaves, 150 mg of fresh leaf material were ground in a mortar and transferred to an Eppendorf with 0.525 ml of extraction buffer. Samples were vortexed for 5 min and centrifuged at 12,000 *g* for 15 min and the supernatant was used for TPI activity determination. The extraction buffer was made by adding to 1 L of buffer 0.1 M Tris–HCl (pH: 7.6): 50 g polyvinylpyrrolidone (PVPP), 2 g phenylthiourea, 5 g diethyldithiocarbamate, 18.6 g ET diamine tetraacetic acid. TPI activity was measured using bovine trypsin (Sigma) and as substrate *N*α-benzoyl-*D*, *L*-arginine-*p*-nitroanilide (*D*-*L*-BA*p*NA). The reaction was performed at 37 °C in a microplate reader (Biotec ELx808; Vermont, USA) and measured at λ 410 nm. Activity of trypsin inhibitors was relativized to soluble protein content measured by Bradford (Bradford, 1976) and using Bovine Serum Albumin as standard.

2.1.6 | Phenolic compounds quantification and identification

To determine individual phenolic compounds, leaves were grounded and 0.1 g was combined with 900 µl of 80% methanol aqueous solution in Eppendorf tubes of 2 ml. Samples were vortexed for 1 min and left at room temperature for 1 hr. Afterwards, samples were sonicated for 1 min and left at room temperature 2 hr and then centrifuged at 15,000 rpm for 5 min. After taking a clear supernatant, 400 µl of chloroform were added and after hand-shaking to homogenize, 200 µl of H_2O were added to reach an emulsion. Afterwards, the extracts were centrifuged for 5 min at 11,000 *g*, and the supernatant (free of pigments) was taken for high-pressure liquid chromatography (HPLC) analysis. Chromatography analysis was performed in an HPLC Agilent 1100 A series equipped with a UV detector (Agilent Technologies, Inc., Wilmington, DE, USA), using an Eclipse XDBC18 reversed phase HPLC column (5 µm, 4.6 × 150 mm). The mobile phase consisted of 0.1% aqueous acetic acid (Solvent A) and 0.1% acetic acid in acetonitrile (Solvent B). Solvent B was increased from 15% (at 0 min following injection) to 36% over 30 min. The solvent flow rate was 1 ml min^{-1}. The wavelength of the UV detector was set at λ 254, 270, and 360 nm. Quantification of flavonoids was done as rutin or genistin equivalents, as described in Dillon et al. (2017). Analytical and HPLC degree solvents used for determination of leaf phenolic compounds were purchased from Sintorgan (Argentina). True standards of rutin and genistin used in HPLC analysis were purchased from Sigma-Aldrich.

2.1.7 | Ethylene measurements

To determine ET emission, three leaflets from three plants from the same treatment: either undamaged or damaged by two 3rd instar larvae during 2 hr were collected at midday and incubated in hermetic glasses (600 ml) for 5 hr at room temperature (25 °C) and with white light (approx. PPFD 700 μmol m^{-2} s^{-1}). The petioles were kept in a matrix of water-saturated cotton to prevent leaflets from desiccation. This procedure was done in each of the 4 plots and UV-B treatment. Replicates were composed by the combination of 3 samples collected from each plot (n = 4). ET was measured with a gas chromatograph (Hewlett Packard 4890, Agilent) equipped with a Porapak N 80/100 column of 2-m long and a flame ionizing detector. Temperatures of the injector, column, and detector were 110, 90, and 250 °C, respectively. Flow rate of the carrier N_2 gas was 3.5 kg cm^{-2}. The flow rates of oxygen and hydrogen were 0.5 and 0.6 kg cm^{-2}, respectively. The detector response was standardized by injecting known amounts of standard ET gas (Sigma–Aldrich, St. Louis, MO, USA) by serial dilutions. Retention time was 1.8 min. Amount of ET produced was quantified by the peak area of the standard and expressed as nmoles g^{-1} hr^{-1} fresh weight for leaves.

2.1.8 | Quantification of jasmonic acid, JA-Ile, cis-OPDA, salicylic acid, and abscisic acid measurements

JA, JA-Ile, *cis*-OPDA, SA, and ABA content in soybean leaves were analysed as described by Vadassery et al. (2012). Briefly, dry finely grounded plant material was weighted (25 mg) and extracted with 1.5 ml of methanol containing D_6-JA (60 ng), D_4-SA (60 ng), D_6-ABA (60 ng), and JA-[$^{13}C_6$]Ile conjugate (15 ng) as internal standards. The homogenate was mixed for 30 min and centrifuged at 14,000 rpm for 20 min at 4 °C. After the supernatant was collected, the homogenate was re-extracted with 500 ml of methanol, mixed, and centrifuged, and supernatants were pooled. The combined extracts were evaporated in a SpeedVac at 30 °C and re-dissolved in 500 μl of methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6 mm, 1.8 mm; Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was as follows: 0 to 0.5 min, 5% B; 0.5 to 9.5 min, 5% to 42% B; 9.5 to 9.51 min, 42% to 100% B; 9.51 to 12 min, 100% B; and 12.1 to 15 min, 5% B. The mobile phase flow rate was 1.1 ml min^{-1}. An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in the negative ionization mode, and ions were analysed by MRM in a triple quadrupole. Phytohormones were quantified relative to the signal of their corresponding internal standard.

2.1.9 | RNA extraction and cDNA quantification

Polyadenylated RNA was extracted from soybean leaves by using the mRNA Isolation Kit (Roche Molecular Biochemicals, Gmbh, Mannheim, Germany) and immediately quantified using Qubit Reagent (Thermo Fisher Scientific, USA) to make cDNA with 100 ng of mRNA sample by means of Reverse Transcriptase system and using oligo (dT) 18 primer (Thermo Fisher Scientific, USA). The obtained cDNA samples were diluted 1:10 before use. Quantitative real-time PCR (qPCR) was performed in a 7500 Fast Real Time PCR System (Applied Biosystems) following the manufacturer's standard method for absolute quantification using Fast-Start Universal SYBR Green Master Mix (Roche Applied Science) and primers at a final concentration of 400 nM. Primer pairs are listed in Table S1. Target and housekeeping (®-tubulin) gene expression was individually calculated based on the respective efficiencies. Target expression relative to the housekeeping and normalized was used for two-way analysis of variance (ANOVA).

2.1.10 | Statistical analyses

Statistical analyses were carried out using INFOSTAT software (Student Version 1.1). Phenolic compound and phytohormone concentration, TPI activity and gene expression were analysed using a three-way ANOVA with UV-B, herbivory, and genotype as factors. When the interaction of three principal effects was significant, Duncan Test was performed. Contrast test between undamaged versus damaged leaves was performed for each cultivar/UVB treatment. Consumption and pupal weight were analysed by a two-way ANOVA with genotype and UV-B radiation as factors. Square root transformations of the primary data were used when needed to meet the assumptions of the ANOVA. Principal component analysis (PCA) analysis was done with the normalized data.

3 | RESULTS

3.1 | Leaf consumption and pupal mass

Larvae of *A. gemmatalis* that fed on cv. Charata consumed more leaf area (p = .02) and had more pupal mass than those that fed on cv. Williams (p = .01; Figure 1). Although solar UV-B exclusion increased the leaf area consumed by larvae (p = .007), pupal mass of larvae that fed on plants grown under either UV-B treatment was not different (Figure 1).

3.2 | TPI activity and phenolic compound induction by herbivory and UV-B

Whereas TPI activity in leaves of cv. Charata was neither affected by UV-B treatments nor herbivory, damage produced by *A. gemmatalis* larvae during 72 hr increased TPI activity in leaves of cv. Williams, and this induction was amplified by solar UV-B (p = .05; Figure 2). There were no differences in TPI activity between treatments and cultivars 24 hr after the experiments were initiated (Figure S1).

Herbivory induced an unknown phenolic derivative independently of the cultivar or UV-B treatment (p < .0001; Figure 3a). Genistin and malonyl genistin were only induced in leaves of cv. Williams by the combination of herbivory and solar UV-B treatment (p = .02 and p = .01, respectively; Figure 3b,c). Independently of UV-B radiation, cv. Williams had higher genistin and malonyl genistin concentrations compared to cv. Charata (p < .0001 and p = .05, respectively; Figure 3).

Although solar UV-B radiation induced all flavonols studied in leaves of both soybean cultivars (p < .005), 24 or 72 hr of herbivory

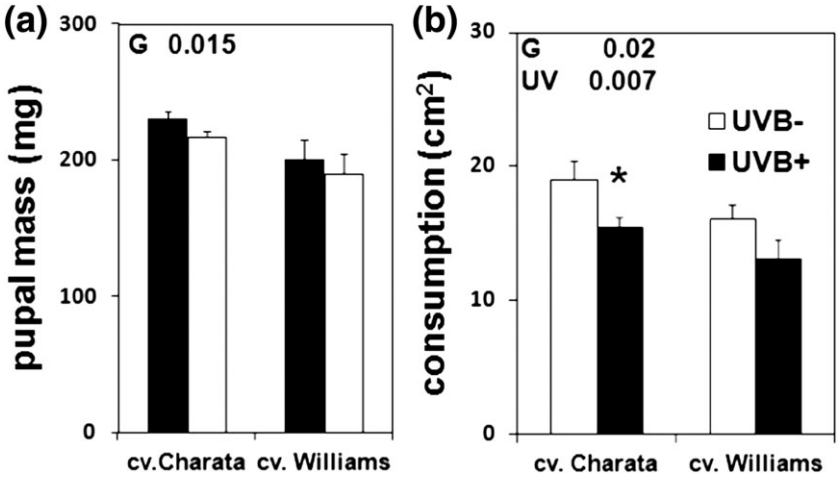

FIGURE 1 Pupal mass (a) and leaf consumption during 72 hr (b) of *Anticarsia gemmatalis* larvae that fed on two field-grown soybean commercial genotypes (cv. Charata; cv. Williams) under two levels of solar UV-B radiation: Attenuated UV-B (white bars) and ambient UV-B (black bars). Error bars represent 1 SEM (n = 6) for pupal mass and (n = 8) for leaf consumption; p values of two-way ANOVA (genotype [G]; UV) are shown. Asterisks indicate significant contrast (p < .05) between UV-B treatments in the same cultivar

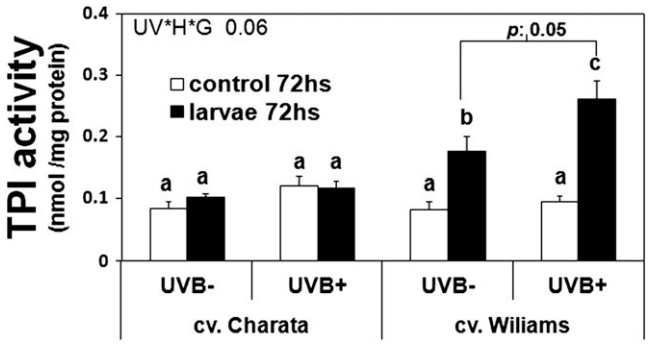

FIGURE 2 TPI activity from undamaged (white bars) and damaged (black bars) leaves by *Anticarsia gemmatalis* larvae during 72 hr of two soybean cultivars (cv. Charata; cv. Williams) grown under two levels of UV-B radiation (UVB−; UVB+). Error bars represent 1 SEM (n = 4); p values of interaction term of three-way ANOVA are shown (G: Genotype; H: Herbivory; UVB radiation). Different means are represented with different letters (Duncan test p < .05). TPI = trypsin protease inhibitor

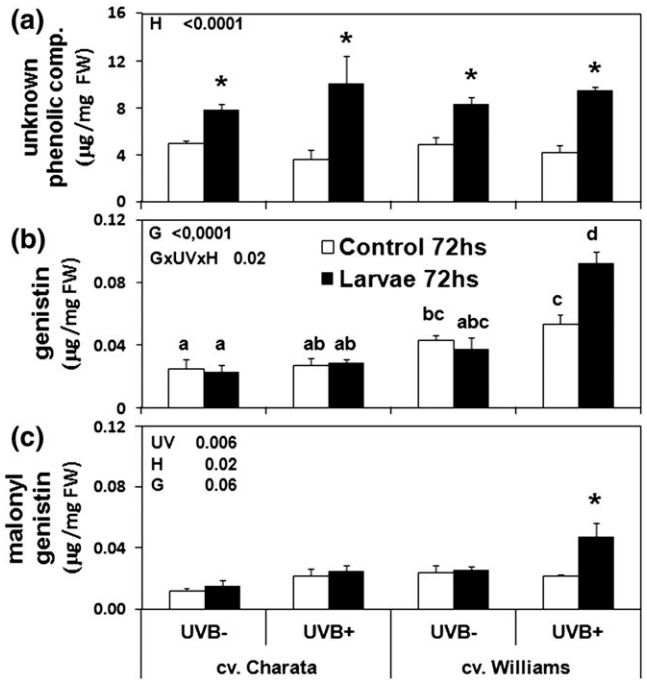

FIGURE 3 Unknown phenolic compound (a), genistin (b), and malonyl genistin (c) concentrations in undamaged (white bars) and damaged (black bars) leaves during 72 hr by *Anticarsia gemmatalis* larvae of two soybean cultivars (cv. Charata; cv. Williams) grown under two levels of UV-B radiation (UVB−; UVB+). Error bars represent 1 SEM (n = 3); p values of significant effects of three-way analysis of variance are shown (G: Genotype; H: Herbivory; UV-B radiation). Different means are represented with different letters (Duncan test p < .05). Asterisks indicate significant differences (p < .05) between control and larvae treated leaf

did not affect flavonols concentration (Figure 4 and Figure S3). Although quercetin triglycoside 1 & 2 (QT1 & 2), kaempferol triglycoside 1 (KT1), and kaempferol triglycoside 2 and isorhamnetin triglycoside 1 (KT2 + IT1) were only present in cv. Charata, quercetin triglycoside 3 (QT3), quercetin diglycoside 1 & 2 were present in cv. Williams (Figure 4). In addition, kaempferol triglycoside 3 was present in both soybean cultivars (Figure 4). UV-B mediated increase of flavonols was higher for quercetin derivatives than for kaempferol derivatives (Figure 4). There were no differences in leaf content of isoflavonoids or the unknown phenolic compound between UV-B treatments and cultivars 24 hr after the experiments were initiated (Figure S2).

3.3 | Gene expression quantification

To explain the effects of herbivory in combination with solar UV-B radiation on phenolic compounds content and TPI activity on leaves, we determined the expression of genes related with the regulation of the isoflavonoid biosynthesis pathway and TPI in soybean. The expression levels of *CHS7/8*, *CHS1*, and *IFS1* genes did not show clear pattern in response to either UV-B treatments or herbivory (Figure 5). Although herbivory induced expression of Bowman-Birk in both soybean cultivars (p = .01), caterpillar attack induced expression of Kunitz only in cv. Williams (p = .04; Figure 5). Expression of *IFS2* and *PR2* was strongly induced by herbivory in combination with solar UV-B radiation only in damaged leaves of cv. Williams (p = .02; Figure 5). Although *PR10* expression levels were higher in cv. Williams

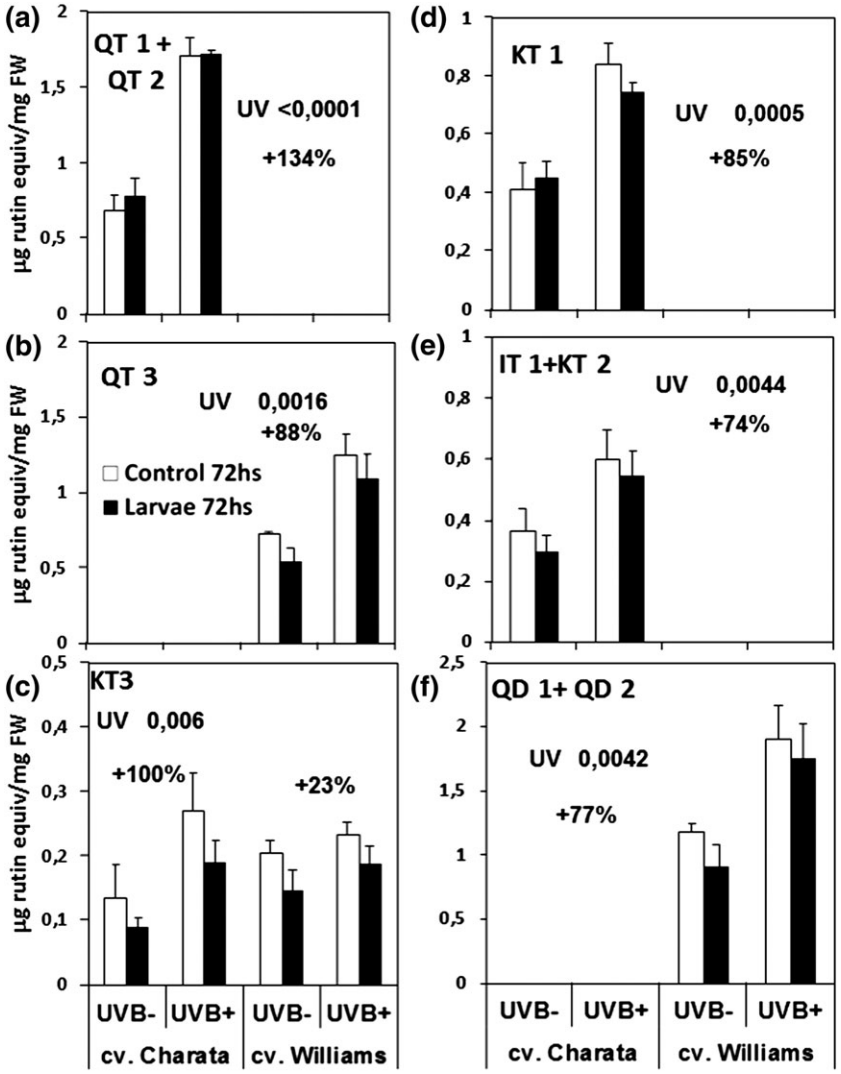

FIGURE 4 Quercetin triglycoside 1 & 2 (a), quercetin triglycoside 3 (b), rutin & rutin isomer (c), kaempferol triglycoside 1 (d), unresolved isorhamnetin triglycoside plus kaempferol triglycoside 2 (e), and kaempferol triglycoside 3 (f) concentrations from undamaged (white bars) and damaged (black bars) leaves for 72 hr days by *Anticarsia gemmatalis* larvae of two soybean cultivars (cv. Charata; cv. Williams) grown under two levels of solar UV-B radiation (UVB-; UVB+). Error bars represent 1 SEM (n = 3); p values of significant effects of a two-way analysis of variance are shown (UVB: UV-B treatment; H: Herbivory). Percentage numbers represent the increment in concentration of flavonols mediated by solar UV-B radiation (calculated from the average of control and damage leaves)

than in cv. Charata (p = .04), *VSPA* was induced in response to herbivory in leaves of both cultivars (p = .05; Figure 5).

3.4 | Jasmonic acid, JA-Ile, cis-OPDA, salicylic acid, and abscisic acid concentrations and ET emission

Herbivory induced the emission of ET from leaves of both cultivars, and the combination of solar UV-B radiation with herbivory increased the emission levels of this hormone (p = .05; Figure 6). Solar UV-B radiation did not modify the concentration of SA, ABA, OPDA, JA, and JA-Ile (Figure 6). Although SA concentration was higher in leaves of cv. Charata than in cv. Williams (p = .02), no differences were found in the concentration of ABA among treatments and cultivars (Figure 6). Although herbivory induced JA and JA-Ile in the leaves of both soybean cultivars, leaf contents of JA-Ile were higher in cv. Williams than in cv. Charata (p < .0001 and p = .003, respectively; Figure 6). Herbivory induced OPDA levels in leaves of both cultivars (p = .006; Figure 6).

3.5 | PCA analysis

To analyse soybean cultivar responses to the combination of herbivory with solar UV-B radiation, we analysed hormonal contents, gene expression, and phenolic compounds and TPI activity with a PCA. PCA analysis aggregated gene expression of *IFS2*, Kunitz, *PR2*, and *PR10* in a group, together with the phytohormones ET and JA-Ile and the genistein derivatives and TPI activity (Figure 7). A second group was formed by *VSPA* and Bowman-Birk gene expression, which was associated with OPDA, JA, and the unknown phenolic compound (Figure 7). Gene expression of *IFS 1*, *CHS 7/8*, *CHS 1*, and *F3H* were aggregated in another group that was associated with ABA contents (Figure 7). Finally, levels of SA and KT3 were distributed each one alone (Figure 7). Our results suggest that malonyl genistin and TPI activity are regulated by ET and JA-Ile.

3.6 | TPI activity and phenolic compound induction by exogenous application of phytohormones

To test the hypothesis that solar UV-B radiation induces ET emission and increases the production of phenolic compounds and TPI in soybean leaves, different phytohormones were applied to leaves of both cultivars, grown either with or without solar UV-B radiation. Although phytohormone treatment did neither affect flavonol concentration (Figure S3) nor the unknown phenolic derivative, application of the ET precursor, ACC (1 mM) strongly induced the isoflavonoids, genistin, and malonyl genistin (p < .0001; Figure 9). In addition, application of ACC increased the concentration of other isoflavonoids, such as

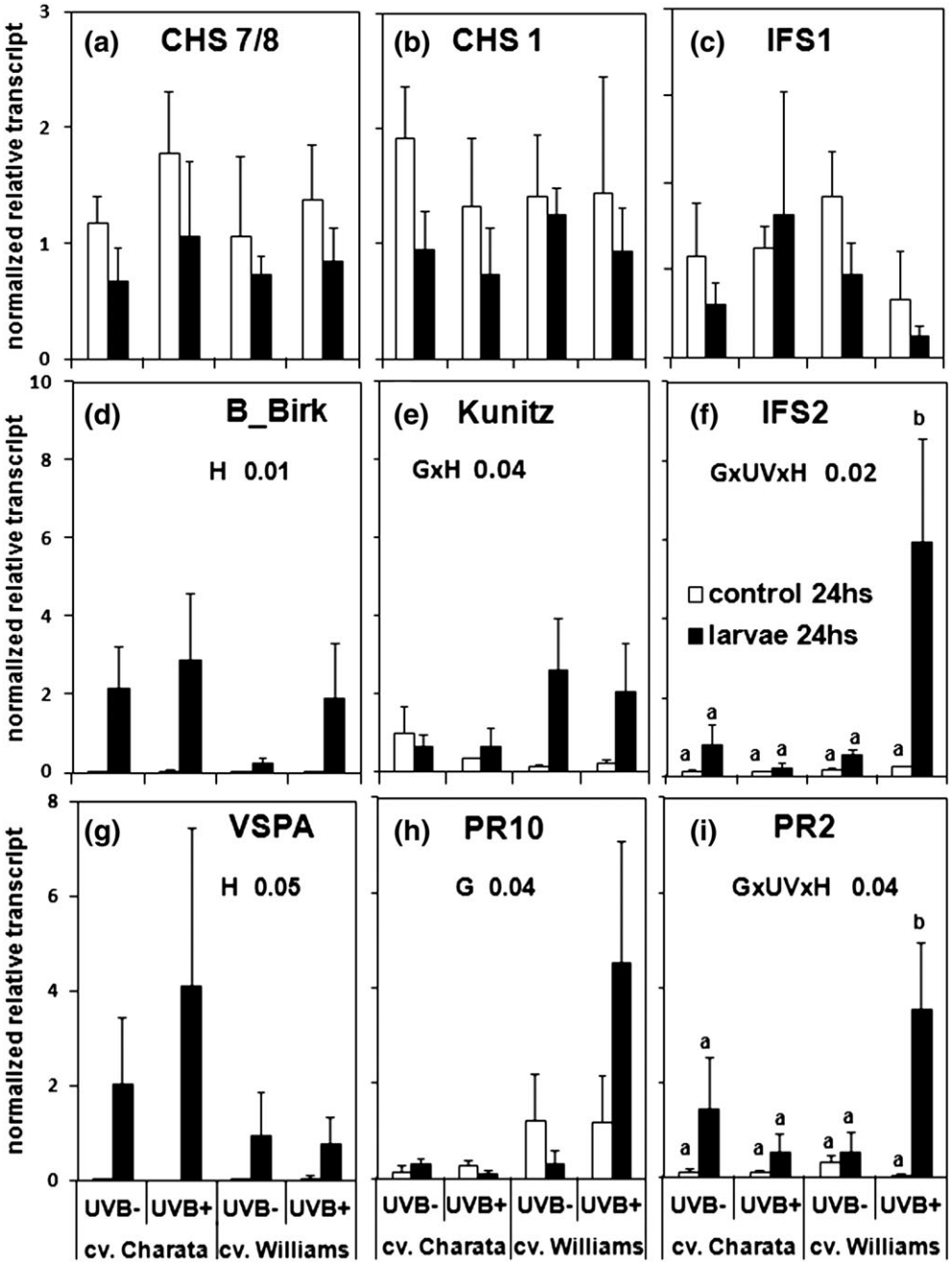

FIGURE 5 Gene expression of *CHS 7/8*, *CHS 1*, *IFS1*, Bowman-Birk, Kunitz, *IFS 2*, *VSPA*, *PR10*, and *PR2* of undamaged (white bars) and damaged (black bars) leaves during 24 hr by *Anticarsia gemmatalis* larvae of two soybean cultivars (cv. Charata; cv. Williams) grown under two levels of UV-B radiation (UVB−; UVB+). Gene expression was normalized and results shown are relative to ®-tubulin. Error bars represent 1 SEM (n = 3); p values of significant effects of three-way analysis of variance are shown (G: Genotype; H: Herbivory; UV-B radiation). Different means are represented with different letters (Duncan test $p < .05$). Asterisks indicate significant differences ($p < .05$) between control and larvae treated leaf

daidzin and malonyl daidzin (Table S2), suggesting that ET alone is a modulator of certain phenolic compounds. MeJA exogenous application induced TPI activity in both soybean cultivars, but only in plants exposed to solar UV-B radiation (Figure 8).

4 | DISCUSION

Many studies have demonstrated the benefits of UV-B-protective secondary metabolites against herbivore insects induced by solar radiation (Ballaré et al., 2011). However, some mechanisms involved in plant resistance against herbivores of important field crops and regulated by solar UV-B radiation are still unknown. To address this question, we used two soybean cultivars that differentially responded to herbivory and solar UV-B radiation (Dillon et al., 2017; Zavala et al., 2015). Whereas in leaves of cv. Williams, solar UV-B radiation induced the accumulation of certain UV-B-protective flavonols (QD1 + QD2, QT3, and KT3) and in combination with herbivory synergistically increased the production of genistin, malonyl genistin, and TPI activity levels, in leaves of cv. Charata solar UV-B radiation increased the accumulation of KT3 and other flavonols (QT1 + QT2, KT1, IT1 + KT2) and with no effect on plant responses to herbivory (Figures 2–4). In

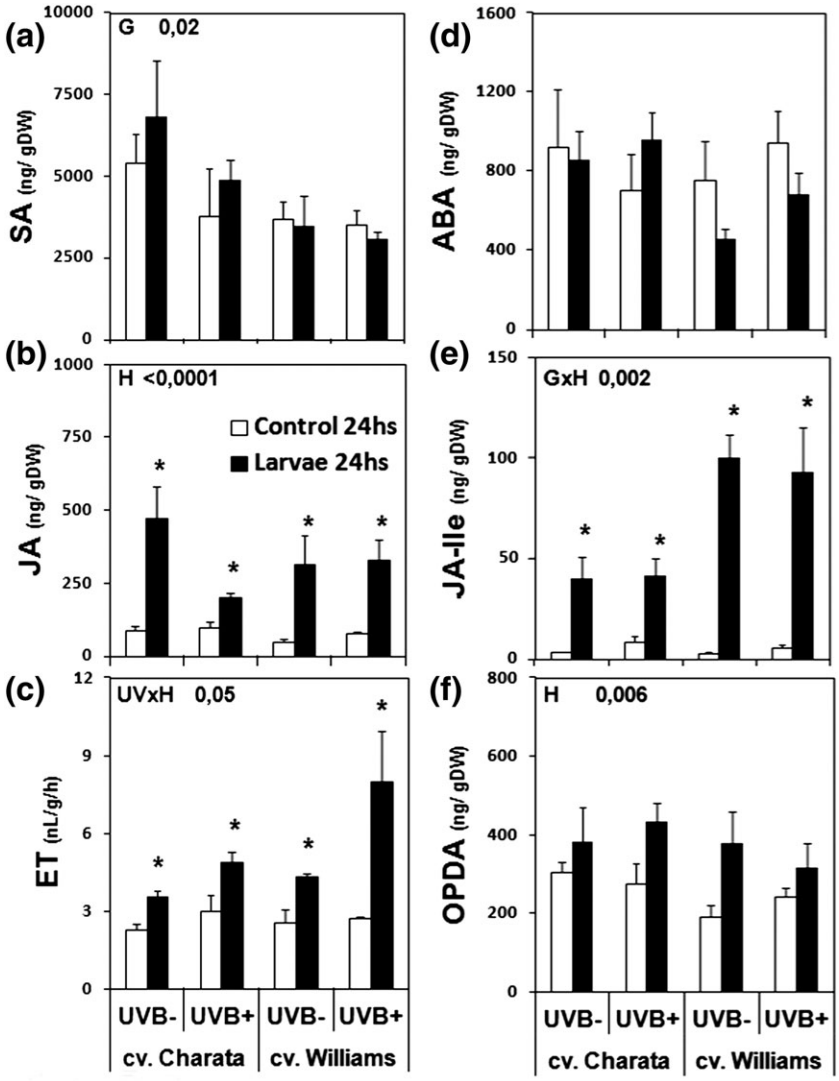

FIGURE 6 Salicylic acid (SA; a), abscisic acid (ABA; b), jasmonic acid (JA; c), jasmonic isoleucine (JA-Ile; d), ethylene (ET; e), and oxo-phytodienoic acid (OPDA; F) concentrations in undamaged (white bars) and damaged (black bars) leaves by larvae of *Anticarsia gemmatalis* during 24 hr of two soybean cultivars (cv. Charata; cv. Williams) grown under two levels of UV-B radiation (UVB−; UVB+). Error bars represent 1 SEM (n = 4); p values of significant effects of three-way analysis of variance are shown (G: Genotype; H: Herbivory; UV-B radiation). Different means are represented with different letters (Duncan test p < .05). Asterisks indicate significant differences (p < .05) between control and larvae treated leaf

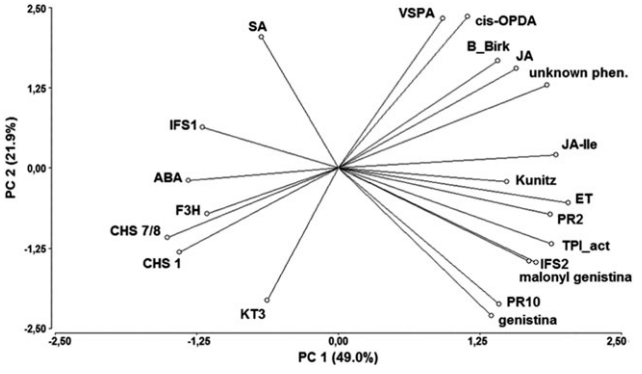

FIGURE 7 Bi-plot of a principal component analysis of defenses (trypsin protease inhibitor activity, genistin, malonyl genistin, kaempferol triglycoside, and the unknown phenolic derivative), phytohormones (ABA, SA, JA, JA Ile, OPDA, ET) and related gene expression (*CHS 7/8*, *CHS 1*, *IFS1*, Bowman-Birk type inhibitor, Kunitz type inhibitor, *IFS 2*, *VSPA*, *PR10*, and *PR2*)

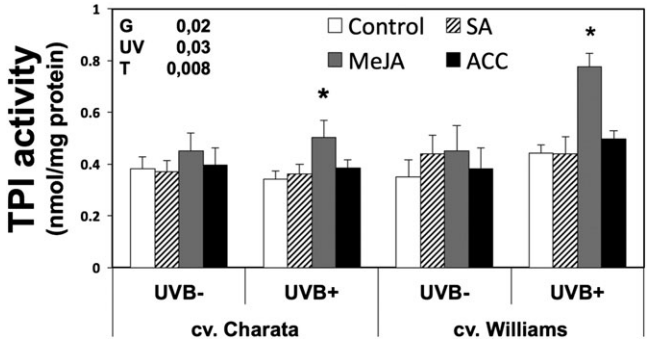

FIGURE 8 Trypsin protease inhibitor (TPI) activity from treated leaves with water (white bars), 1.5 mM SA (black bars), 1 mM MeJA (green bars), and 1 mM ACC (blue bars) of two field grown soybean crops (cv. Charata; cv. Williams) under two levels of solar UV-B radiation (UVB+; UVB−). Error bars represent 1 SEM (n = 4). Asterisks indicate significant differences (p < .05) between control and herbivory leaves; p values of significant effects of three-way analysis of variance are shown (G: Genotype; H: Herbivory; UV-B radiation). SA = salicylic acid; MeJA = methyl jasmonate; ACC = aminocyclopropane-carboxylic acid

addition, we detected in both soybean cultivars an unknown phenolic compound induced by herbivory, but not regulated by SA, JA, ET, or solar UV-B radiation (Figures 3 and 9), suggesting that this compound is not regulated by any pathway studied here.

Although solar UV-B radiation can induce phenolic compounds simultaneously with JA, *N. attenuata* impaired in the JA pathway grown

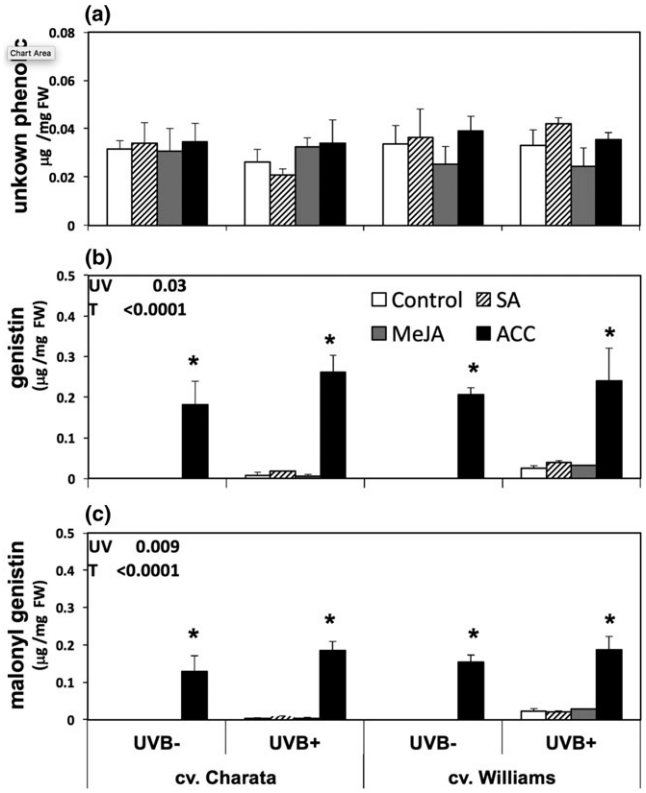

FIGURE 9 Unknown phenolic compound (a), genistin (b), and malonyl genistin (c) concentrations in soybean treated leaves with water (white bars), 1.5 mM SA (black bars), 1 mM MeJA (green bars), and 1 mM ACC (blue bars) of two field grown soybean crops (cv. Charata; cv. Williams) under two levels of UV-B radiation (UVB+; UVB−). Error bars represent 1 SEM (n = 3). Asterisks indicate significant differences (p < .05) between water and phytohormone treatment leaves; p values of significant effects of three-way analysis of variance are shown (G: Genotype; H: Herbivory; UV-B radiation). SA = salicylic acid; MeJA = methyl jasmonate; ACC = aminocyclopropane-carboxylic acid

under solar UV-B radiation increased rutin and chlorogenic acid production, demonstrating that certain phenolic compounds induced by solar UV-B are not regulated by JA pathway (Demkura et al., 2010). Our field experiments demonstrated that exogenous application of the ET precursor ACC alone, but not MeJA, SA, or ABA induced the phenolic compounds genistin and malonyl genistin in field-grown soybean (Figure 9). These results explained the consequences of increased ET emission in cv. Williams produced by the combination of solar UV-B and herbivory, which in turn increased the expression of ET-regulated genes and the production of the isoflavonoids genistin and malonyl genistin (Figures , 5–7). To our knowledge, no study have shown before that in field-grown soybean ET emission induced by solar UV-B radiation is associated to increases on the production of isoflavonoids.

Although ET is one of the main components of the blend of defense signals that increases in plant tissues after herbivore damage, this hormone is considered as an important modulator of plant immunity with secondary participation in induction of plant defenses (Broekaert, Delaure, De Bolle, & Cammuel, 2006; Van Loon, Geraats, & Linthorst, 2006; Von Dahl & Baldwin, 2007). However, our field study suggested that ET alone plays a primary role in mediating the increment of inducible defenses in soybean. The combination of herbivory and solar UV-B radiation not only increased isoflavonoid production in cv. Williams but also induced the highest levels of ET emission and gene expression of *IFS2* and *PR2* (Figures 3, 5, and 6). Although *IFS2* enzyme is the one involved in isoflavonoid production in soybean in response to biotic stresses (Dhaubhadel et al., 2003; Gutierrez-Gonzalez et al., 2009), it has been reported that ET and no other hormones, such as SA or JA, induced *PR2* expression (Mazarei, Elling, Maier, Puthoff, & Baum, 2007). Because flavonols were not synergistically induced by the combination of herbivory and solar UV-B radiation or after ACC application, our results suggest that only isoflavonoids are induced by ET alone (Figures 3, 4, and 6; Figures S3 and S4). Recently, a study have shown that both ethephon and ET induced isoflavonoids in potted soybean grown in a greenhouse (Yuk et al., 2016), suggesting the primary role of ET in the induction of certain defenses.

A more studied role of ET in plant defenses is the synergistically effects on the outcome of the JA response to herbivory (Pieterse et al., 2012). Previous studies have demonstrated that solar UV-B radiation enhances the induction of TPI activity in leaves of *Nicotiana* and tomato after insect damage, by increasing the JA signalling pathway (Demkura et al., 2010; Izaguirre et al., 2003; Stratmann et al., 2000). Similarly, solar UV-B radiation was crucial to induce TPI activity in our field-grown soybean after MeJA application (Figure 8). Moreover, our field experiments suggested that the higher TPI activity levels found in damaged leaves of cv. Williams were up regulated mainly by the induction JA-Ile, and that solar UV-B radiation induced ET and modulated the synergistically increased of TPI activity levels (Figures 2 and 6). This response was explained by expression of the JA-regulated genes *VSPA*, *PR10*, Kunitz, and Bowman-Birk inhibitor, and the ET-regulated gene *PR2* (Figures 5 and 7). Two branches of the JA signalling pathway have been described, the MYC branch and the ethylene response factor (ERF) branch. The MYC branch is controlled by MYC-type transcription factors and includes the JA-responsive marker gene vegetative storage protein2 (*VSP2*) and is associated with plant responses to herbivores (Kazan & Manners, 2012; Lorenzo, Chico, Sanchez-Serrano, & Solano, 2004). Moreover, after induction of JA by herbivore damage ABA can synergistically induce the expression of the MYC branch, while it antagonizes the ERF branch (Abe et al., 2003; Anderson et al., 2004). In our study, although ABA was not induced in soybean grown either with or without solar UV-B radiation, ET and JA were up regulated by herbivory (Figure 6). Activation of the ERF branch of the JA pathway requires both JA and ET signalling, which can act synergistically to defend plants via a regulatory model in which JA induces degradation of JAZ proteins and depresses ET-stabilized EIN3 and EIL1 and interact with JAZs (Zhu et al., 2011).

Conversely, our field experiments also showed that expression of JA-regulated genes and up regulation of ET and JA were not revealed in the production of defense compounds in cv. Charata (Figures 2, 3, 5, and 7), which was reflected in higher pupal mass and leaf area consumed by *Anticarsia* larvae in cv. Charata than in cv. Williams (Figure 1). Pupal mass is an insect growth parameter that depends on consumption and digestibility of plant material and has good correlation with the fecundity of moths (Lindroth, Hofmann, Campbell, McNabb, & Hunt, 2000). Although solar UV-B radiation did not have effects on pupal mass, UV-B radiation reduced leaf consumption of larvae in cv. Charata (Figure 1). Flavonoids genistin and quercetin derivatives of soybean foliage decreased survivorship and larval mass

of *Anticarsia* larvae (Dillon et al., 2017; Piubelli et al., 2005). The low content of genistein derivatives and lack of induction of TPI activity, genistin, and malonyl genistin by herbivory in leaves of cv. Charata may explain the higher pupal mass and leaf consumption by larvae in cv. Charata compared to cv. Williams (Figures 1–3).

Solar UV-B radiation can have direct positive effects on yield of field crops or affects them indirectly by reducing insect herbivory (Ballaré et al., 2011; Mazza et al., 2013; Zavala & Botto, 2002). Many field studies have shown that plants suffer less damage by herbivory when they grow under solar UV-B conditions than when the levels of UV-B radiation are attenuated (Ballaré et al., 2011; Escobar-Bravo, Klinkhamer, & Leiss, 2017). Insect herbivory can be reduced by solar UV-B radiation in field-grown soybean and consequently increased crop yield (Mazza et al., 2013). Solar UV-B radiation can change the chemistry of soybean foliage, which acts indirectly on insect herbivores that avoid plant organs previously exposed to solar UV-B, and also decreased growth and survivorship of insects that feed on them (Dillon et al., 2017; Zavala et al., 2001; Zavala et al., 2015). Some UV-B-protective compounds are also a good defense against herbivores and can be modulated within minutes to hours, such as flavonoids that have the double function and are induced by both herbivory and solar UV-B radiation (Barnes et al., 2015; Izaguirre et al., 2003). In our soybean field experiments, we found phenolic compounds that responded differentially to solar UV-B radiation and herbivory. Whereas flavonols were regulated by solar UV-B radiation and not by herbivory or ET, isoflavonoids were regulated by herbivory and solar UV-B inducible ET. In addition, we found an unknown phenolic compound only regulated by herbivory and not by solar UV-B radiation. Our study suggests that although ET can modulate UV-B-mediated priming of inducible plant defenses, some plant defenses, such as isoflavonoids, are regulated by ET alone.

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ORCID

Jorge A. Zavala http://orcid.org/0000-0001-6265-0920

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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