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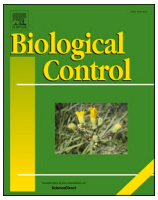

Constitutive and inducible tomato defenses contribute to *Bacillus thuringiensis* lethality against *Spodoptera exigua*

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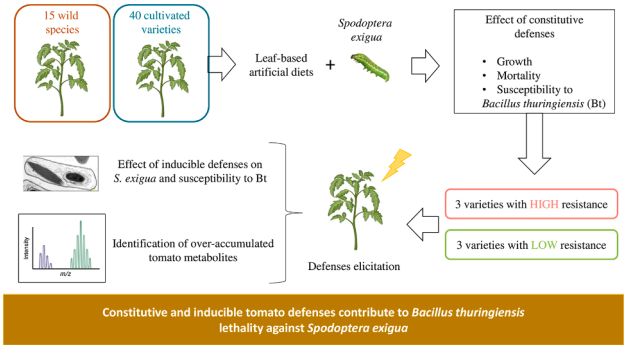
HIGHLIGHTS

- Leaf-based artificial diets allow to study the effect of chemical defenses on insects.
- Tomato defenses enhanced the lethality of a bacterial entomopathogen.
- Over-accumulated plant metabolites were identified after elicitation of defenses.
- Plant defense elicitors contribute to the action of *Bacillus thuringiensis*.

GRAPHICAL ABSTRACT



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ABSTRACT

In nature, insect herbivory exerts continuous selective pressure on plants that, in turn, have developed a wide array of constitutive and inducible defenses to fight against attackers. Since plant defenses may affect higher trophic levels, including entomopathogens, further research is required to understand how plant compounds influence insect-pathogens interactions and their implications for integrated pest management programs. Here, we evaluated the impact of tomato defenses on the lethality produced by the bacterial entomopathogen *Bacillus thuringiensis* (Bt) against second instar larvae of *Spodoptera exigua*. We first examined the effect of constitutive defenses from fifteen wild tomato species and forty cultivated varieties (*Solanum lycopersicum*) on *S. exigua* larval growth and susceptibility to Bt. The results showed larvae fed on wild tomato species had a reduced larval growth compared to larvae fed on cultivated varieties, whereas susceptibility to Bt was similar between both groups. We then selected six cultivated varieties, including those with high and low impacts on larval growth and Bt-induced mortality, to further explore the effect of inducible defenses. Elicitation of defenses by methyl jasmonate (MeJA) reduced larval growth and increased basal mortality. Additionally, when larvae were infected with Bt, MeJA treatment further increased their susceptibility to the entomopathogen. Metabolomic analysis confirmed a reprogramming of tomato leaf metabolism following MeJA elicitation, with an induced accumulation of bioactive compounds, such as saponins or flavonoids, known for their anti-herbivory properties in most

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tomato varieties. Overall, these data reveal that both constitutive and inducible tomato defenses not only protect the plant by directly affecting the insect pest but also enhance the efficacy of bacterial entomopathogens.

1. Introduction

The interaction between plants and herbivorous insects started around 400 million years ago, giving rise to a coevolutionary process wherein plants have been compelled to acquire different defensive strategies (Labandeira, 2013). These strategies encompass the production of a wide array of direct defenses, such as physical barriers (*i.e.* trichomes, cuticle, etc…) or toxic and antinutritional compounds (*i.e.* secondary metabolites, proteinase inhibitors, etc…) (War et al., 2018; Fernández de Bobadilla et al., 2022). Plant defenses may be constitutively produced, or induced following herbivory damage or detection (Hilker and Meiners, 2006; Felton et al., 2014; Watts et al., 2023). In response, insects have developed mechanisms to counteract plant defenses, including sequestration, detoxifying or avoiding behaviours (Heidel-Fischer and Vogel, 2015; Dussourd, 2017). Furthermore, plants encompass additional layers of defense, such as indirect defenses that involve the emission of volatiles acting as prey cues to attract natural enemies of the herbivores, including arthropod predators or parasitoids (Aljbory and Chen, 2018). Plant inducible defenses against herbivores are quickly triggered upon herbivory and finely regulated by signaling pathways coordinated by jasmonic acid and related compounds, collectively known as jasmonates (JAs) (Turner et al., 2002; Howe and Jander, 2008). Indeed, exogenous treatment with JAs induced proteinase inhibitors, alkaloids and other bioactive secondary metabolites (Thaler et al., 1996; War et al., 2015; Flores-Sanchez et al., 2016). For example, exogenous application of methyljasmonate (MeJA) is a common strategy used to trigger inducible plant responses against insect herbivores, such as chewing herbivores (Rodriguez-Saona et al., 2001; Tian et al., 2014; Senthil-Nathan, 2019).

In nature, insect herbivores are exposed to a wide range of microbial entomopathogens. This term includes all the microorganisms that have the ability to infect and kill different species of arthropods (Ruiu, 2018). Due to their host specificity and lack of toxicity to non-target organisms or the environment, microbial entomopathogens are widely used as control agents in integrated pest management programs (Lacey et al., 2015; Irsad et al., 2023). Among entomopathogens, bacteria, baculoviruses, fungi, and nematodes are the most applied as biopesticides. Currently, their commercial use accounts for about 5 % of the entire crop protection market with expectations that this percentage will rise from 10 % to 20 % in the coming years (Sabbahi et al., 2022). The bacterium *Bacillus thuringiensis* (Bt) is the most commonly used entomopathogen in pest control, with formulations based on it representing around 75–95 % of the microbial biopesticide market (Jurat-Fuentes et al., 2021). Bt is a gram-positive bacterium which is present in different ecological niches including soil, water, plants and dead insects, and it is effective against several insect orders such as Lepidoptera, Coleoptera and Diptera (Palma et al., 2014). The infection process begins with the oral ingestion of the bacterial sporulated cells, and/or their formulated spores and the associated insecticidal crystals, which are present in the soil or on the phylloplane. Once inside the insect's gut, the crystals are solubilized, leading to the activation of the insecticidal proteins that form the crystal (Cry toxin). These proteins bind to receptors in the midgut cell membrane, inducing pore formation and ultimately causing the death of the insect (Bravo et al., 2011).

Entomopathogens may be targeted by the plant defensive strategies, which may promote the action of these herbivores' enemies for their own benefit (Rasmann et al., 2005; Mohan et al., 2008a; Lin et al., 2016; Hay et al., 2020). For example, maize defensive responses target the caterpillars' protective gut barrier. This accelerates the invasion of gut bacteria into the larval body cavity where they exacerbate the negative impact of plant defenses on the insect (Mason et al., 2019).

It is known that the effectiveness of Bt formulations against insect pests can be influenced by various plant traits. For instance, nutrition quality of plants can impact Bt efficacy (Shikano and Cory, 2014), as well as plant-produced compounds modulate the insect interaction with the bacterium (Macintosh et al., 1990; Mohan et al., 2008b; Gasmi et al., 2019). Additionally, certain plant defenses are known to interfere with the digestive process of the insect (Chen et al., 2005; Bhonwong et al., 2009), impact on its immune system (Shi et al., 2020; Chen et al., 2022) or disturb the integrity of the peritrophic membrane (Pechan et al., 2002; Kariyat et al., 2017). These alterations can increase the susceptibility of the target insect to *B. thuringiensis*, being the basis of the contribution of the host plant genotype in shaping insect-pathogen interaction (Janmaat and Myers, 2005; Carrière et al., 2005; Bird and Akhurst, 2007). In this context, Shikano et al. (2018) found that tomato induced defenses enhanced the efficacy of Bt against the polyphagous caterpillar *Helicoverpa zea*. They suggested that orthoquinones, resulting from the oxidation of chlorogenic acid by plant PPO enzymes, were responsible for alkylating Bt prototoxins, which increased their solubilization and/or proteolysis, thereby enhancing the effectiveness of the toxins (Ludlum et al., 1991).

Given this, we hypothesized that plant defenses could modulate insect-Bt interactions by increasing the susceptibility of the insect pest to the bacterial entomopathogen. In the present work, we investigated the interaction between constitutive and inducible foliar defenses in tomato (*Solanum lycopersicum*) and Bt infection in the polyphagous caterpillar *Spodoptera exigua*. To explore the diversity of constitutive chemical defenses, we compared 55 tomato genotypes, including fifteen wild tomato species and forty cultivated tomato varieties. We established a bioassay system using leaf-based artificial diets to assess the impact of constitutive chemical defenses from tomato on *S. exigua* performance, in the presence or absence of Bt. We then selected six varieties showing contrasting levels of resistance to *S. exigua* herbivory and performed further experiments to assess the combined effects of constitutive and inducible defenses on Bt-infected larvae. To induce defenses, plants were treated with MeJA to activate JA-dependent responses, and the effects on larval growth and susceptibility to Bt were recorded. Finally, we characterized the chemical changes in elicited leaves that may have contributed to the observed effects. For that, we performed an untargeted metabolomic analysis to identify metabolites that accumulated after elicitation of plant defenses and that may play a role in defense against *S. exigua* and increase susceptibility to Bt. Overall, our findings show that both constitutive and herbivore-inducible tomato chemical defenses increase the action of Bt against *S. exigua*, and we identified key metabolites that may regulate this interaction.

2. Materials and methods

2.1. Insects

The *Spodoptera exigua* caterpillars used in this study were sourced from a laboratory colony established over 10 years ago from eggs supplied by Andermatt Biocontrol AG (Grossdietwil, Switzerland). The colony was maintained in a growth chamber under controlled conditions, with a temperature of 25 ± 3°C, relative humidity of 70 ± 5 % and a photoperiod of 16 h light: 8 h dark. Larvae were reared on artificial diet (Elvira et al., 2010), while adults were provided *ad libitum* access to a 10 % sugar solution.

2.2. Tomato varieties and leaf-based diet preparation

The wild tomato species and cultivated varieties were provided by

the Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV) of the Universitat Politècnica de València (Spain). The selection of the varieties for this study (Table 1, Supplementary) was based on their diversity in fruit metabolic composition (Antonio Granell, Personal communication; Pons et al., 2022; 2023). Tomato seeds were sown in jiffys (0.4 cm), composed by pressed coconut fiber, inside seedbeds (6.5 x 6.5 cm). They were grown in a controlled greenhouse with a temperature ranging 20–26 °C during the day and 19–22 °C at night, and with a diurnal photoperiod of 16 h light and 8 h dark (natural light supplemented with fluorescent tube lighting at dawn and dusk). Plant growth was monitored, no phytosanitary treatments were applied in the greenhouse, and no auxiliary insects were released to avoid untargeted effects on plant defenses. Tomato plants with 6–8 expanded true leaves were harvested after four weeks of growth. Only plants with lack of disease symptoms were employed. A minimum of 3 plants per variety were processed together, with an average of 8 plants per sample. Leaves were immediately frozen in liquid nitrogen and stored at −80 °C. Leaf samples were grounded in a mortar with liquid nitrogen to obtain a fine leaf powder, which was then lyophilized in 10 mL tubes using an Alpha1-2 lyophilizer (Christ, Germany) for 48 h (−52 °C, vacuum pressure 0.2 mm Hg), and stored at room temperature in the dark until their use for plant-based larval diet preparation.

Plant-based diets were formulated by mixing 0.23 g of lyophilized material with 4 mL of agar solution supplemented with 5 % of the compounds used for the standard artificial diet (without antibiotics) for rearing *S. exigua* larvae (Frattini et al., 2022). To prevent heat inactivation of enzymes and metabolites, the leaf powder was mixed with the agar once it had cooled to approximately 35–40 °C.

2.3. *S. exigua* larval performance

Larval performance was assessed by measuring the relative growth of newly molted second instar *S. exigua* larvae (Herrero et al., 2002). The bioassay spanned 144 h, during which larvae were fed for the first 48 h on the plant-based diet, followed by a switch to artificial diet for the remainder of the experiment. Since the *S. exigua* larvae used in these bioassays were sourced from a laboratory colony reared exclusively on artificial diet, the feeding period on plant diets was restricted to 48 h to avoid larval mortality due to a lack of dietary adaptation and to minimize the interference from other factors, such as the nutritional properties of the different plants. At the start of the experiment, larvae were weighed, individually placed in separate wells of a bioassay tray (product no. 9074; Frontier Agricultural Sciences), which was sealed with microperforated adhesive tape (product no. 9074-L; Frontier Agricultural Sciences). The trays were maintained in the growth chamber under the same conditions of the laboratory colony mentioned above. After 144 h, larvae were weighted again, and the relative growth was calculated as milligrams of biomass acquired per milligram of initial body weight. Larval weight was determined using a precision balance (Sartorius MC-1 Analytic AC 120S; Göttingen, Germany) with an accuracy of 0.1 mg. A total of 16 larvae were used per tomato species/variety, and two biological replicates of the experiment were performed at different periods.

Table 1
Effect of the selected cultivated varieties on *S. exigua* growth and susceptibility to Bt.

Constitutive resistance [1]	Internal code	Selected variety	Relative growth ± SEM	Bt mortality± SEM (%)
High	20 T	1	27.9 ± 3.2	46.9 ± 15.6
High	35 T	2	13.1 ± 3.9	62.5 ± 6.3
High	39 T	3	28.0 ± 13.7	60.7 ± 1.9
Low	3 T	4	55.1 ± 15.5	12.1 ± 0.4
Low	25 T	5	66.7 ± 28.6	32.1 ± 10.7
Low	16 T	6	109.9 ± 35.0	6.3 ± 0.0

[1] High group correspond to cultivated tomato varieties (1, 2, 3) with a high constitutive resistance against *S. exigua* (low larval growth and high mortality with Bt). Low group correspond to cultivated tomato varieties (4, 5, 6) with a low constitutive resistance against *S. exigua* (high larval growth and low mortality with Bt).

2.4. *B. thuringiensis* susceptibility bioassays

Bt susceptibility assays were conducted as described by Frattini et al. (2022). Newly molted second instar *S. exigua* larvae were infected with the bacterial entomopathogen using the droplet feeding method. Four µL droplets containing 10 % sucrose, phosphate-buffered saline (PBS; pH 7.4), 10 % (v/v) tracking dye phenol red, and a sublethal concentration of *B. thuringiensis* subsp. *aizawai* (XenTari® GD, 15 million U.I./g; Kenogard S.A, Barcelona, Spain) were prepared. The sublethal concentration of 0.6 mg/mL was determined on the basis of previous bioassays conducted under the same experimental conditions (second instar *S. exigua* larvae, feeding on plant diet for 48 h). Control larvae received a mock treatment with the same procedure but without Bt. Larvae were allowed to feed on the droplets for 15 min, and after visually confirming ingestion, they were immediately placed individually in bioassay trays and provided with plant-based diets. The trays were kept for 48 h in a growth chamber under controlled conditions. After this period, the plant-based diet was replaced with artificial diet. Mortality was registered every 24 h for a period of 6 days. Larval relative growth of larvae at 144 h was calculated and analyzed as described above. A total of 16 larvae were used per species/variety and treatment, and two biological replicates were performed at different time periods. For statistical analysis, differences in larval relative growth between wild tomato species and cultivated varieties were calculated using Student's *t*-test. Mortality in control and Bt-infected larvae were compared using one-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Software Inc., San Diego, CA). The Spearman and Shapiro-Wilk tests were applied to determine homoscedasticity and normality of data, respectively. Additionally, the Pearson correlation coefficient was used to measure the linear correlation of growth and basal mortality values of non-infected larvae with the mortality of Bt-infected larvae.

2.5. *Elicitation of MeJA-inducible plant defenses and evaluation of their effects on S. exigua*

The results of the growth inhibition and Bt susceptibility bioassays with plant-based diet derived from the fifteen wild tomato species and forty cultivated tomato varieties, allowed us to selected six cultivated varieties with contrasting levels of constitutive resistance (i.e., leading to low or high relative larval growth and Bt-associated mortality in plant-based diets, Suppl. Table 1). These were chosen to further investigate the effect of JA-inducible defenses on larval growth and susceptibility to Bt (Table 1). To this purpose, tomato seeds were surface-sterilized by immersion in 4 % NaHClO (10 min), then thoroughly rinsed with sterile distilled water, and germinated in sterile vermiculite at 25 °C in a phytotron for 10 days. Seedlings at two-cotyledon stage were then transplanted to 250 mL pots containing a sterile sand: vermiculite (1:1) mixture (Rivero et al., 2021). Plants were randomly distributed on greenhouse benches and grown in a greenhouse at 24 °C/16 °C with a 16 h/8h diurnal photoperiod and 70 % humidity. After 6 weeks of growth, half of the plants were treated by spraying plant shoots with a solution of 100 µM MeJA (Sigma-Aldrich) in water, which was prepared from a 100 mM MeJA stock solution in 96 % (v/v) ethanol. To ensure uniform application, Silwett 77 (0.02 % v/v) was added as a surfactant, and leaves were sprayed until run off using an aerograph. Control plants were similarly sprayed with a mock solution containing only the solvent and surfactant. This exogenous application of 100 µM MeJA has been shown to efficiently elicit plant defenses in various species, including tomato (Martínez-Medina et al., 2017). Five plants were treated per variety. Leaves were harvested 72 h after MeJA or mock

application to ensure the accumulation of the JA-inducible compounds. The harvested leaves were immediately frozen with liquid nitrogen and stored at −80 °C.

For the *S. exigua* bioassays, frozen leaves were pooled by treatment and variety (5 plants), grounded, and lyophilized as described previously to prepare the plant-based diets. Bioassays to evaluate larval growth and susceptibility to Bt were conducted as described above, with the only modification being the use of a sublethal concentration of XenTari® at 1 mg/mL. Three independent biological replicates were performed, with 16 larvae used per treatment, variety and replicate. Relative growth was calculated after 48 h of feeding on plant-based diet and mortality was recorded every 24 h for a period of 7 days. Statistical differences in relative growth and mortality after Bt infection between treatments were analyzed using two-way ANOVA, with MeJA application and constitutive resistance level as factors (GraphPad Software Inc., San Diego, CA). The Spearman and Shapiro-Wilk tests were applied to determine homoscedasticity and normality of data, respectively. The Pearson correlation coefficient was used to measure the linear correlation of growth and basal mortality values of non-infected larvae with mortality values of Bt-infected larvae.

2.6. Untargeted metabolomic analysis

A comparative metabolomic analysis was conducted to identify metabolites that were over-accumulated in the selected varieties after MeJA treatment. For this purpose, the apical leaflet of the fourth true leaf (young, fully expanded leaf) from each plant sprayed with either MeJA or the solvent was harvested and kept at −80 °C for molecular analysis. In total, there were five biological replicates per treatment (control or MeJA application) for each of the six selected varieties, resulting in 60 samples in total. Each leaflet was individually ground with liquid nitrogen, lyophilized, and 10 mg of powder were resuspended in 1 ml of the extraction buffer (MeOH:H_2O 30:70 containing 0.01 % of HCOOH). After incubating on ice for 40 min, the homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant was filtered with 0.2 μm cellulose filters (Regenerated Cellulose Filter, 0.20 μm, 13mmØ. Pk/100; Teknokroma, St Cugat, Spain). The filtered supernatants (50 μL) were diluted 1:3 using the extraction buffer, and an aliquot of 20 μL of each sample was resolved by an Acquity UPLC system (Waters, Mildford, MA, USA) in positive (ESI+) and negative (ESI-) ion modes for electrospray ionization, coupled to a hybrid quadrupole time-of-flight equipment (QTOF MS Premier, Waters, Mildford, MA, USA) for detection of metabolites. Identification of the signals was performed by introducing a second fragmentation function into the TOF analyzer in a t-wave ranging from 5 to 45 eV.

Raw data were obtained from Masslynx v.4.2 software (Waters, USA) and transformed into.cdf files using the DataBridge tool. Chromatographic signals from positive and negative ESI were processed separately with R software v.4.3.2 (https://cran.r-project.org), and the XCMS algorithm was used for filtering and peak identification, grouping, and signal corrections. Metabolite amounts were quantified by normalizing peak area units to the dry weight of each sample. MarVis Suite 2.0 software allowed signal comparations between treatments using Kruskal-Wallis test ($P < 0.05$) followed by adduct and isotope correction, and clustering. Only peaks with signal-to-noise ratio >10 were included in the analysis. Compounds whose signal intensity was significantly increased a minimum of 2-fold after MeJA treatment and were shared by at least four tomato varieties were selected. Identification of metabolites was based on their exact mass, retention time, and spectrum fragmentation (Gamir et al., 2014; Schymanski et al., 2014). Signals were identified with the use of different online databases such as Massbank, MassBank of North America, PubChem and Human Metabolome databases (www.massbank.jp; https://mona.fiehnlab.ucdavis.edu; https://pubchem.ncbi.nlm.nih.gov; https://hmdb.ca).

In addition, data from positive and negative ESI were combined and normalized by median, transformed by cube root and scaled by Pareto method using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca) to obtain sparse partial least squares discriminant analysis (sPLSDA) plots and heatmaps for each variety. Heatmaps were generated with those metabolites which differed significantly ($P < 0.05$, Kruskal-Wallis test) in signal intensity after MeJA induction compared to the control.

3. Results

3.1. Screening of constitutive defenses of tomato genotypes on S. exigua performance and susceptibility to B. thuringiensis

To explore the effects of natural diversity in constitutive chemical defenses in tomato on *S. exigua* performance and mortality by Bt, we performed a plant-based screening. Specifically, we assessed both direct effects by measuring the *S. exigua* relative growth and mortality, and indirect effects by measuring the susceptibility of *S. exigua* larvae to Bt when feeding on different tomato genotypes, including wild species and cultivated varieties.

We measured the relative growth of *S. exigua* larvae after 48 h of continuous feeding on plant-based diet, followed by feeding on artificial diet until 144 h. When larvae were fed exclusively on the artificial diet, their relative growth at 144 h averaged 130. In contrast, feeding on plant-based diets resulted in noticeably reduced larval relative growth, ranging from 10 to 110 with a median of 32.1 (Suppl. Table 1). A significant influence of plant genotype on *S. exigua* growth was detected (ANOVA, $F_{54,55} = 1.7$, $P = 0.026$). The mean relative growth recorded from larvae fed on wild species was lower compared to that of larvae fed on cultivated varieties (Student's *t*-test, $P = 0.0383$) (Suppl. Table 1, Fig. 1A).

We next evaluated the effect of plant genotypes on Bt performance. Feeding mock-infected larvae on the different plant diets resulted in around 20 % mortality. This increased up to 40 % after Bt infection in both wild (Tukey HSD test, $P = 0.0003$) and cultivated (Tukey HSD test, $P < 0.0001$) varieties (Suppl. Table 1, Fig. 1B), confirming the efficacy of Bt. However, no significant differences were observed between wild species and cultivated varieties in terms of larval mortality, either with (Tukey HSD test, $P = 0.7282$) or without Bt (Tukey HSD test, $P = 0.9982$) (Suppl. Table 1, Fig. 1B).

In addition, we found a clear increase in the mortality of larvae fed on plant-based artificial diets compared with those exclusively reared on artificial diet, either with or without Bt infection (mock-infected: 0–5 %, Bt-infected: 5–10 %). The latter mortality values were obtained from previous assays conducted multiple times, being these data consistent among the bioassays.

Analyzing the combined data, we found that both larval relative growth and basal mortality triggered by each genotype correlated with susceptibility to Bt (Fig. 2). Specifically, relative growth and susceptibility to Bt showed an inverse correlation ($r = -0.6137$, $P < 0.0001$, Fig. 2A), whereas mortality induced by the plant diet alone and mortality triggered by Bt exhibited a positive correlation ($r = 0.5207$, $P < 0.0001$, Fig. 2B). These results suggest that constitutive chemical defenses in tomato, which negatively affect larval development and survival, also contribute to exacerbate the effect of Bt.

3.2. Effect of inducible defenses on larval performance and susceptibility to B. thuringiensis

We further investigated how the inducible defenses in six different cultivated tomato varieties affected larval growth and influenced susceptibility to Bt. The six tomato varieties selected for this set of experiments were grouped based on their constitutive resistance levels observed in the previous screening results. Varieties exhibiting low larval growth and high mortality in response to Bt were classified as having high constitutive resistance (varieties 1, 2, 3), while those showing high larval growth and low mortality to Bt were classified as

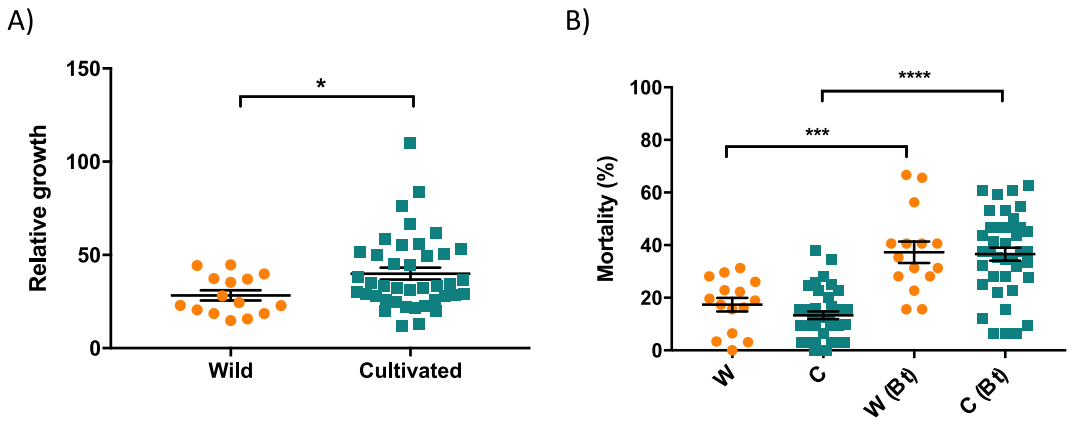

Fig. 1. Effect of constitutive chemical defenses from wild species and tomato cultivated varieties on *S. exigua* growth and mortality. (A) Relative growth of larvae fed on plant-based diets for 48 h and replaced by artificial diet until 144 h. Mean values were analyzed with Studenís *t*-test. Whiskers plot represent the average and standard error of the mean (SEM). (B) Mortality of larvae reared on plant-based diets and infected or not with *Bacillus thuringiensis* (144 h post infection). One-way ANOVA followed by Tukey's multiple comparisons test was used to analyze differences. Whiskers plot represent the average and SEM. Asterisks indicate significant differences between groups (*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$). Color coding and abbreviations: W, wild species, are depicted in orange; C, cultivated varieties are depicted in aquamarine.

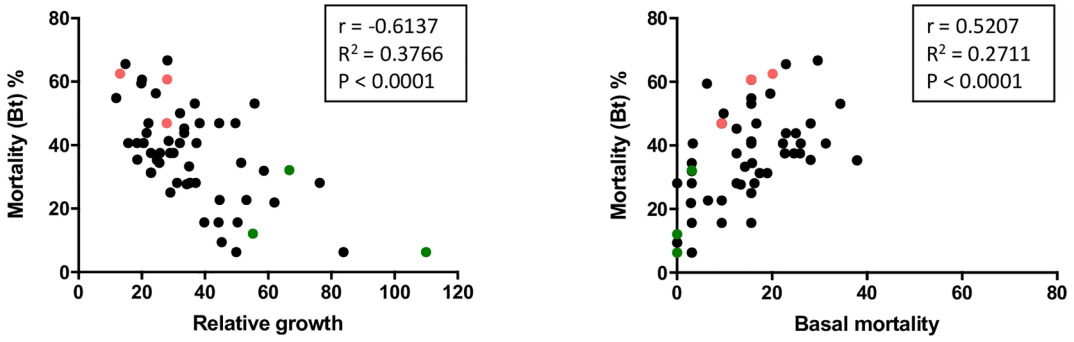

Fig. 2. Correlation between impact of plant compounds on *S. exigua* and its susceptibility to Bt. Scattered plot and Pearson correlation analysis between relative growth of non-infected larvae (A) or basal mortality (B) and mortality values of Bt-infected larvae. Highlighted points correspond to values corresponding to the selected cultivated varieties (red: high constitutive resistance, varieties 1–3; green: low constitutive resistance, varieties 4–6).

having low constitutive resistance (varieties 4, 5, 6) (Table 1).

Inducible defenses were triggered by the application of MeJA, the active form of JA, which is well known for inducing plant defenses (Turner et al., 2002). As expected, MeJA application significantly reduced larval relative growth in the absence of Bt (Fig. 3A), with no observed differences between the high and low constitutive resistance groups (MeJA: $F_{1,24} = 17.64$, $P = 0.0003$; resistance level: $F_{5,24} = 1.663$, $P = 0.1819$; interaction: $F_{5,24} = 0.4834$, $P = 0.7851$; Fig. 3A). Regarding the mortality of Bt-infected larvae, we observed that the MeJA treatment led to an overall increase of approximately 15 % compared to larvae raised on non-induced plant-based diets (Fig. 3B), revealing the contribution of induced defenses to higher susceptibility to Bt. The combined effect of the plant constitutive resistance group (high or low) and MeJA application on Bt susceptibility was not significant (MeJA: $F_{1,24} = 12.13$, $P = 0.0019$; resistance level: $F_{5,24} = 4.193$, $P = 0.007$; interaction: $F_{5,24} = 0.1260$, $P = 0.9851$; Fig. 3B), indicating that inducible defenses had an effect regardless the constitutive defenses. Hence, combining both factors (constitutive resistance and induced defense) had an additive, but not synergistic effect on larval susceptibility to Bt. Once again, we found that lower relative growth and higher basal mortality, triggered by the effects of MeJA-elicited chemical defenses, correlated with higher susceptibility of larvae to Bt ($r = -0.8808$, P=0.0002, Fig. 3C; and $r = 0.5876$, $P = 0.045$, Fig. 3D), highlighting that inducible tomato defenses that directly impair larval growth and survival also increase Bt toxicity against *S. exigua*.

3.3. Impact of MeJA elicitation on foliar metabolic profile

As our data show a clear negative effect of JA-inducible defenses on larval performance, we aimed to explore the changes in the leaf chemical composition triggered by the MeJA treatment potentially responsible for the observed phenotypes. For that, we analyzed the metabolomic profiles by LC-MS/MS. MeJA application led to a rearrangement of the leaf metabolome in all the 6 varieties, as indicated by supervised PCA analysis (Fig. 4A). Heatmap plots were generated to visualize differentially abundant signals between the control and MeJA treatment groups. Depending on the variety, the number of signals significantly differentially accumulated ranged from 100 to 300, and the clustering confirmed the impact of MeJA treatment on the tomato metabolic profile (Fig. 4B).

A more detailed analysis of the metabolomic profiles led to the identification of six compounds which were significantly over-accumulated (over 2-fold) after MeJA treatment in at least four out of the six selected tomato varieties (Fig. 5 and Table 2). Among them, four compounds were identified in the positive ionization mode: 5-hydroxyindole-3-acetic acid, (+)-catechin, an unidentified compound (*m/z* 826.10) and soyasapogenol B base + O-HexA, Hex, dHex, 1malonyl (SB). In the negative ionization mode, the metabolites syringaldehyde, and phosphatidylglycerol 42:10 were found. Remarkably, SB was the only one significantly over-accumulated in the six cultivated varieties.

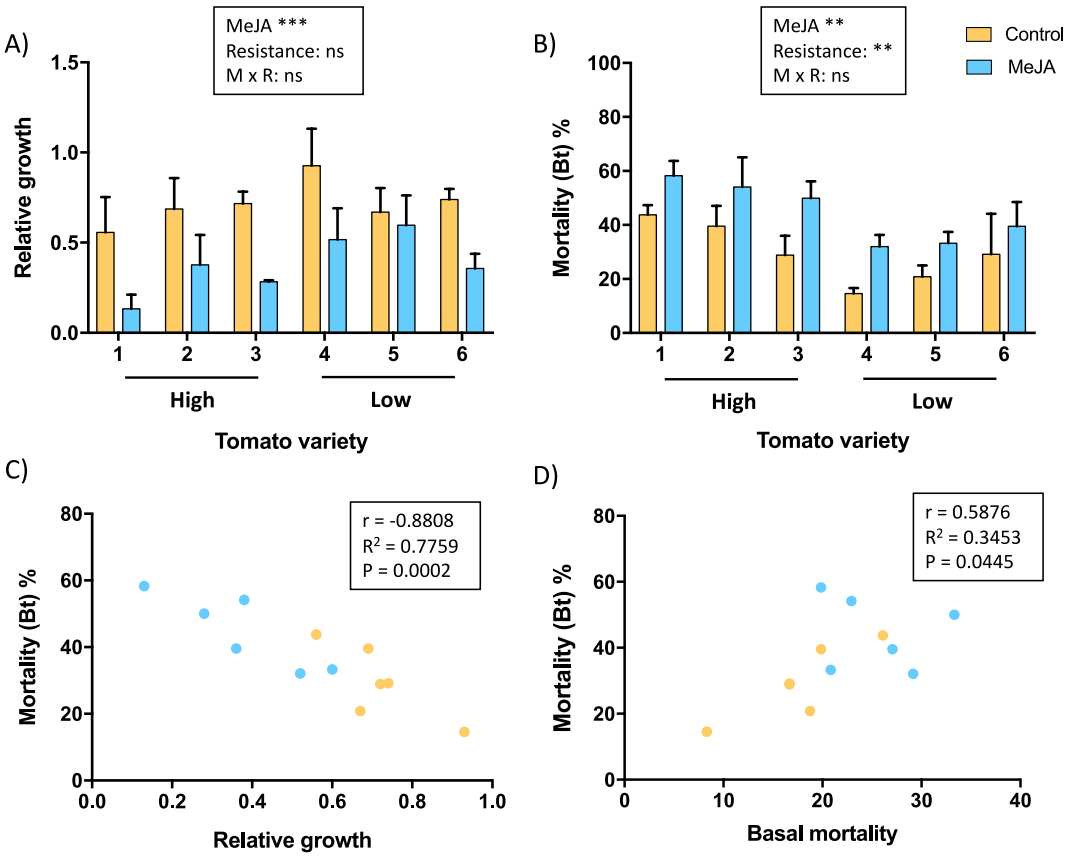

Fig. 3. Effect of inducible chemical defenses from the selected tomato varieties on *S. exigua* growth and susceptibility to Bt. (A) Relative growth of larvae fed on plant-based diets for 48 h. (B) Percentage of mortality of larvae reared on plant-based diets and infected with *Bacillus thuringiensis* (168 h post infection). In both growth and mortality plots, mean values were analyzed with two-way ANOVA using MeJA and constitutive resistance as factors. Error bars depict standard error of the mean (SEM). Scattered plot and Pearson correlation analysis between relative growth (C) and basal mortality (D) of non-infected larvae and percentage of mortality of Bt-infected larvae. Asterisks show significant differences (**$P < 0.01$; ***$P < 0.001$).

4. Discussion

Plants rely on constitutive and inducible defenses to protect themselves from herbivorous insects, such as caterpillars. These defenses serve various functions, including deterrence, antifeedant properties, or toxicity against the herbivore itself, as well as attraction of natural enemies, like predators and parasitoids (Mithöfer and Boland 2012). However, their interaction with microbial entomopathogens, another major group of insect natural enemies, has been poorly addressed. In this study, we demonstrate that both constitutive and inducible defenses in tomato increase the lethality of the bacterial entomopathogen *Bacillus thuringiensis* against the caterpillar *Spodoptera exigua*.

Unlike previous studies that focused solely on the effects of individual plant defensive compounds on Bt activity (Felton and Dahlman 1984; Krischik et al., 1988; Ludlum et al., 1991; Sivamani et al., 1992; Appel and Schultz 1994), our approach involves the use of chemical elicitation of plant defenses and lyophilized leaf material to assess the influence of overall chemical defenses, including the contribution of both constitutive and inducible components. Physical defenses such as leaf thickness, shape, or hardness were excluded, as the leaf material was grounded into powder. Moreover, by comparing different plant genotypes, we demonstrate that the effect on larval growth is genotype-dependent. Our results reveal the positive contribution of tomato defenses to the effectiveness of the entomopathogen *B. thuringiensis* against a generalist caterpillar.

Tomato plants synthesize a plethora of chemical compounds to defend against insect herbivores, such as alkaloids, phenolic compounds, or triterpenoids, which possess antifeedant properties and direct toxicity against herbivores (Howe and Jander 2008; War et al., 2012; Paudel et al., 2019). However, many of these compounds are not produced constitutively due to their high cost to the plant (Karban and Baldwin 1997). Instead, their accumulation is induced upon detection of herbivory. The balance between constitutive and induced defenses within a plant is often influenced by the domestication process, which can either enhance or reduce the rates of inducibility in response to herbivore attack (Whitehead et al., 2017; Ferrero et al., 2020; Szymański et al., 2020). For instance, many ornamental plant species exhibit lower constitutive and higher induced resistance in domesticated species (Kempel et al., 2011). The findings of our study, which assessed the effect of constitutive chemical defenses on the relative growth of *S. exigua* using plant-based diet bioassays, corroborated this trend. Indeed, non-induced cultivated tomato varieties allowed for a greater relative growth of *S. exigua* compared to wild species. This may be a consequence of selective breeding over the years selecting for yield and fruit characteristics, but not for resistance (Bergougnoux 2014). In contrast, the mortality of Bt-challenged larvae fed on different plant diets was similar, regardless of whether the plants were wild or cultivated. This suggests that while domestication altered the levels of constitutive chemical defenses against the caterpillars, it did not affect those defenses that could increase the efficacy of Bt.

The clear correlation between the mortality produced by Bt and the detrimental effects on larval performance (growth decrease and mortality) from non-induced plant-based diets reveals that constitutive tomato defenses enhance larval susceptibility to Bt. These finding was further corroborated by observations from MeJA-elicited plants, which exhibited an analogous pattern. A similar trend was observed in another

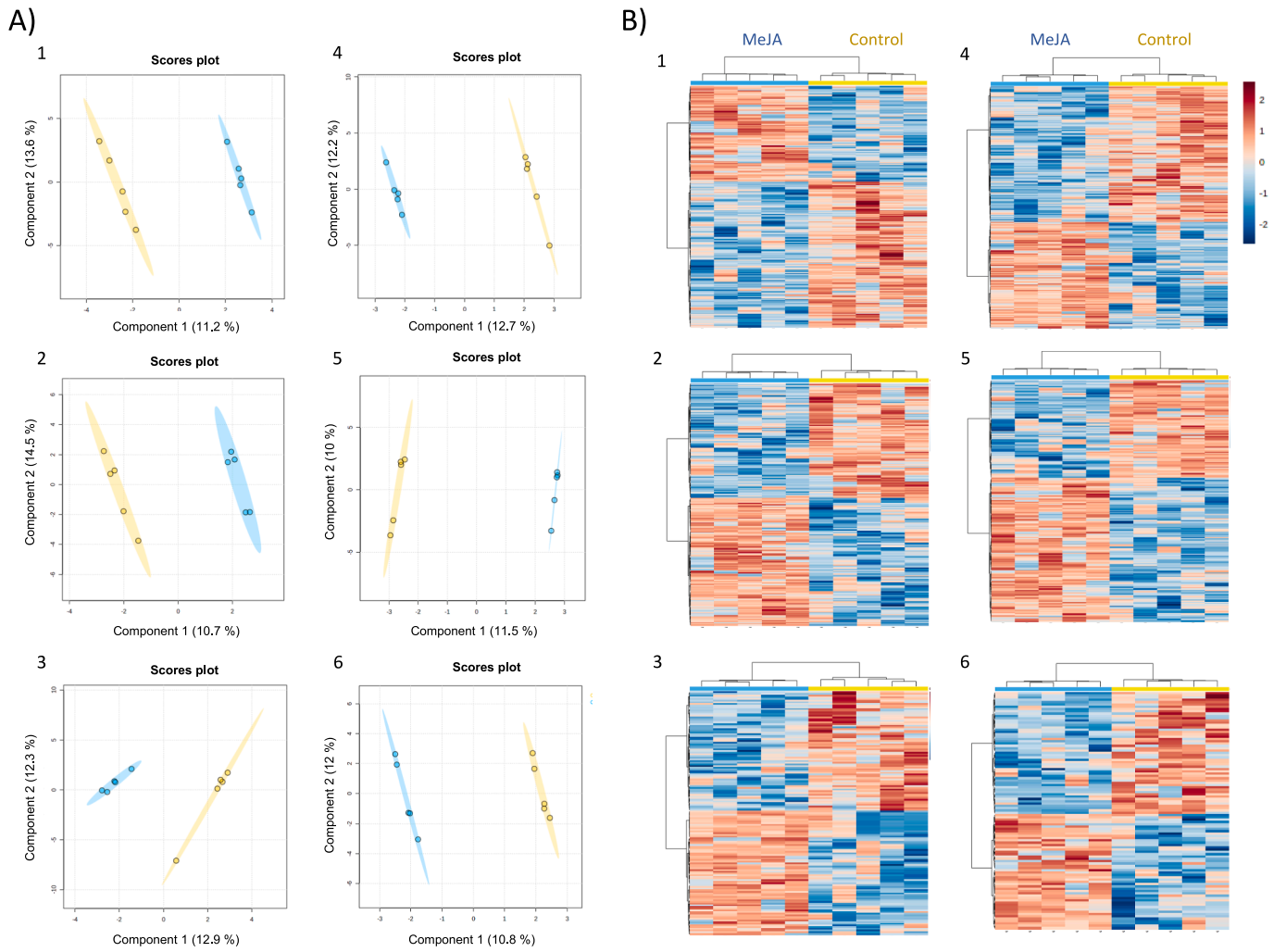

Fig. 4. Metabolomic profile of MeJA-elicited tomato plants from the selected varieties. A non-targeted metabolic analysis of the leaves was performed to assess metabolic changes after treatment of plant shoots with MeJA. Signals from positive and negative electrospray ionization were combined. A) Sparse partial least squares discriminant analysis (sPLSDA) representation of the major sources of variability from the obtained signals. B) Heatmap representing metabolites that significantly differed in signal intensity after MeJA induction compared to the control ($P < 0.05$, Kurskal-Wallis test). Yellow: control plants; blue: MeJA-elicited plants. Varieties 1–3 (high constitutive resistance), varieties 4–6 (low constitutive resistance).

pathosystem: a tomato-based diet exacerbated the effect of the opportunistic pathogen *Serratia marcescens* in *Helicoverpa zea*, another generalist lepidopteran from the Noctuidae family (Mason et al., 2023). Based on these data, we hypothesized that the enhanced action of Bt provides a protective advantage to the plant, allowing for more effective control of insect herbivores.

Plants may use different strategies to weaken insects and enhance Bt activity. *B. thuringiensis* kills its hosts through the production of pore-forming toxins, namely Vip and Cry proteins (Bravo et al., 2011). These toxins bind to the gut epithelium of susceptible insects, where they insert into the brush border membrane of columnar cells, creating an osmotic imbalance that leads to gut destruction. The death of the insect may be further facilitated by the action of opportunistic gut bacteria, which benefit from the gut destruction produced by Bt to invade the host body and cause septicaemia (Mason et al., 2011; Caccia et al., 2016). We hypothesize that plant defenses may contribute to Bt action in several ways. First, several plant phytochemicals directly target the digestion process, jeopardizing the insect's ability to obtain nutritional resources. For example, phenolics are oxidized by polyphenol oxidases (PPOs) resulting in the formation of orthoquinones that alkylate aminoacids and proteins (Felton et al., 1992; Constabel and Barbehenn 2008). Lectins bind to glycosyl groups and damage the luminal epithelial membrane (Vandenborre et al., 2011), and proteinase inhibitors obstruct the activity of digestive enzymes (Zhu-Salzman and Zeng 2015). The interaction between these molecules, which limit the bioavailability of nutrients, and the destruction of the gut epithelial cells prompted by Bt, may further diminish the insect's ability to obtain nutritional resources, thus accelerating their death. In line with this, a previous observation related the Bt-mortality of *H. zea* in JA-induced tomato with the plant PPO activity (Shikano et al., 2018b). Secondly, other plant compounds like triterpenoids, alkaloids, flavonoids or lectins may directly weaken the immune system of the insect, interfering with its ability to remove the pathogen and eventually enhancing the larval susceptibility to Bt due to their inherent toxicity (War et al., 2012). In this regard, we previously showed that *S. exigua* raised on plant-based diet from plants already exposed to herbivory impaired caterpillar growth, increased susceptibility to Bt, and decreased larval PPO activity in the hemolymph, a key marker of the insect immune status (Frattini et al., 2022). Thirdly, some phytochemicals and defensive proteins like tannins, chitinases or proteases disrupt the peritrophic membrane (Mason et al., 2019), a physical barrier against pathogens and mechanical damage that covers the entire larval gut (Barbehenn and Peter Constabel 2011; Konno and Mitsuhashi 2019), whose absence increases the susceptibility of caterpillars to Bt (Guo et al., 2019; Güney et al., 2024).

Although we observed an increase in the susceptibility of *S. exigua*

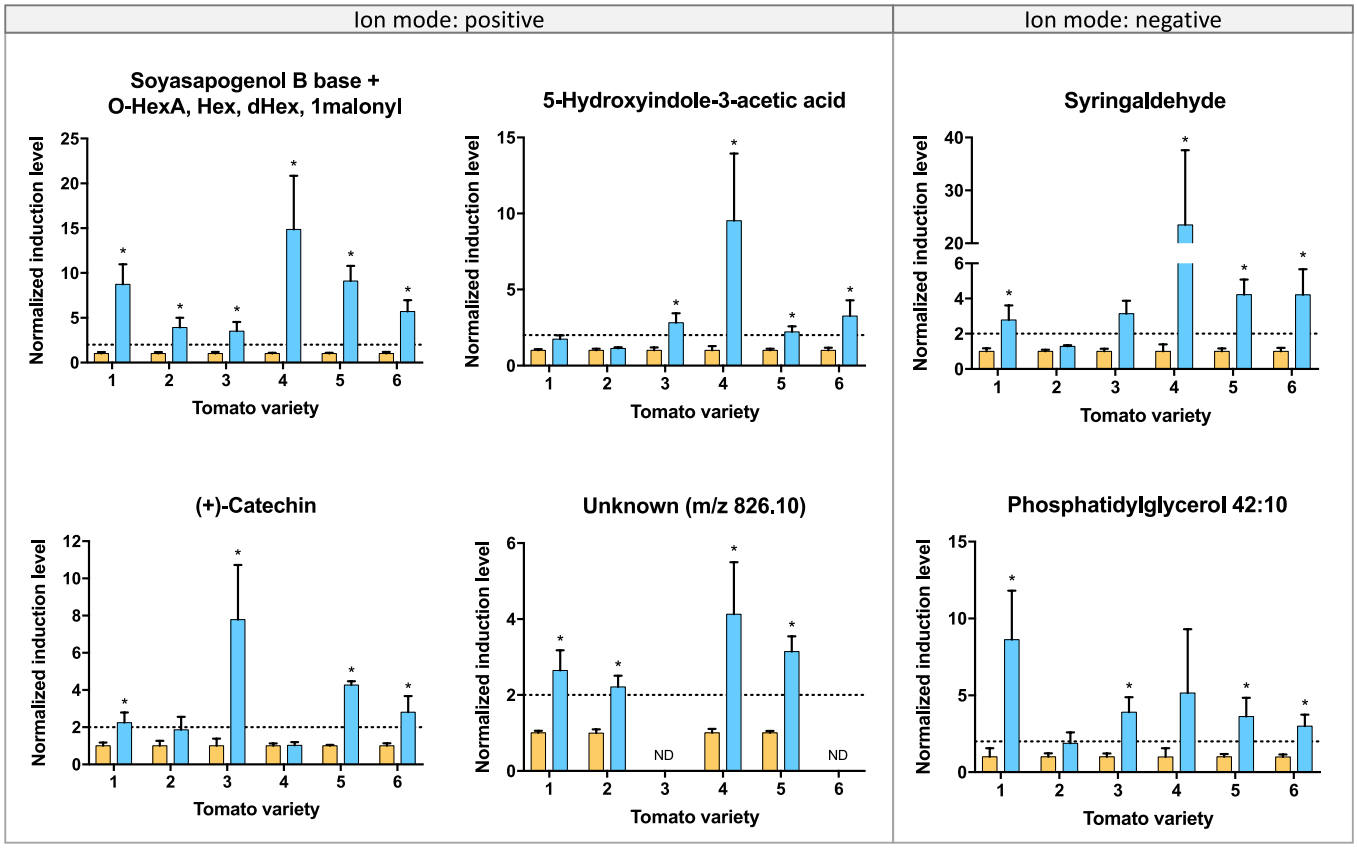

Fig. 5. Relative abundance of tomato metabolites elicited across selected tomato cultivated varieties. Relative abundances are shown as normalized signal intensities of MeJA-elicited metabolite compared to the control within each variety. Four compounds were identified in positive mode ionization (left), while two others were identified in negative mode ionization (right). Error bars depict standard error of the mean (SEM). The color code indicates control plants in yellow and MeJA-elicited plants in blue. Asterisks indicate significant differences between MeJA-elicited and the respective control plants (Kruskal-Wallis test, $P < 0.05$, FC ≥ 2, n = 5).

Table 2
Metabolites with significant induction following MeJA application across the six selected tomato varieties.

Ion mode	Rt (s)[1]	Exact mass	Parent ion	Major fragments	Compound identity	Varieties[2]	Induction level[3]
Positive	4.81	1029.14	1030.02	528.95 > 145.07	Soyasapogenol B base + O-HexA, Hex, dHex, 1malonyl	1–6	3.5–14.9
Positive	2.66	191.03	192.10	147.04 > 117.03	5-Hydroxyindole-3-acetic acid	3–6	2.2–9.5
Positive	3.73	290.09	291.08	207.08 > 123.09	(+)–Catechin	1, 3, 5, 6	2.2–7.8
Positive	4.81	826.10	827.35		Unknown	1, 2, 4, 5	2.2–4.1
Negative	2.64	182.05	181.04	123.03 > 151.01	Syringaldehyde	1, 3–6	2.8–23.5
Negative	2.41	843.31	842.24	153.01 > 327.01	Phosphatidylglycerol 42:10	1, 3–6	3–8.6

[1] Rt: retention time.
[2] *S. lycopersicum* cultivated varieties grouped as high (1–3) or low (4–6) constitutive resistance based on their impact on *S. exigua* performance and mortality.
[3] Relative abundances of MeJA-elicited metabolites were compared with those from control treatment to obtain the induction level values (Kruskal-Wallis test, $P < 0.05$).

larvae to Bt after feeding on the tomato plants, other factors, such as larval instar, body size, or timing of exposure to Bt (before or after plant diet feeding), should be considered for a more complete understanding of the effects of plant defense compounds on the activity of Bt. Previous studies have shown that smaller larval body size or slower larval growth is associated to a higher susceptibility to pathogens (Vogelweith et al., 2013; Shikano et al., 2018a), likely due to a lower allocation of resources to combat infection and/or a prolonged period of vulnerability to the natural enemies. Based on this, it is plausible that infecting younger *S. exigua* larvae (neonates or first instars) with Bt would result in a higher efficacy of the bacterial entomopathogen against the caterpillar.

Timing of pathogen challenge is a critical factor when evaluating the role of plants in the interaction between insects and pathogens (Cory and Hoover 2006; Shikano 2017). Depending on the timing of pathogen ingestion and plant material ingestion, we may encounter different scenarios; (1) If plant material is ingested well in advance of pathogen-challenge, this may result in plant-mediated variation in host physiology, for example the weakening of peritrophic membrane may facilitate the further action of the entomopathogen; (2) If plant material and the pathogen are co-ingested, direct interactions between plant chemicals and the pathogen may occur in the insect's gut, for example in the case of the potential alkylation of Bt protoxins triggered by quinones produced by tomato defensive enzyme PPO; and (3) If plant material is ingested after pathogen-challenge, the plant quality may affect the insect's immune function. In our study, we infected larvae with Bt prior to feeding on plant diets, with a 15-minute time window between the treatments. After 48 h of feeding on the plant diets, larvae were shifted to an artificial diet. We hypothesize that the enhanced Bt mortality

related to the ingestion of plant material, such as that observed in MeJA-induced plant, is likely related to changes in the host physiology. For example, it could be associated with the opening of pores in the insect gut by Bt pore-forming toxins, which may allow plant chemicals to enter the hemocoel and have greater toxic effect. However, future research should assess the effect of leaf compounds on Bt lethality when plant diets are ingested simultaneously with, or prior to, pathogen challenge.

To date, only few individual defensive plant compounds have demonstrated to enhance Bt action (Felton and Dahlman 1984; Krischik et al., 1988; Sivamani et al., 1992; Appel and Schultz 1994). To identify bioactive molecules potentially responsible for both detrimental effects on larval growth and mortality and Bt susceptibility, we compared the profiles of the six MeJA-elicited tomato varieties. Our results revealed a substantial rearrangement of the leaf metabolic profile in all varieties after MeJA treatment, regardless of their constitutive resistance classification. This metabolic shift in leaves after exogenous application of MeJA is a well-documented phenomenon in various plants (Flores-Sanchez et al., 2016; Papazian et al., 2019; Ramabulana et al., 2020), attributed to JA-mediated induction of secondary metabolite biosynthesis (Wasternack and Hause 2013; Wasternack 2014; Yu et al., 2019). We looked for metabolites consistently over-accumulated in elicited plants across the tested varieties. Among them, we identified six metabolites over-accumulated across varieties, five of them with significant increases in abundance in at least four out of the six tested tomato varieties: 5-hydroxyindole-3-acetic acid, (+)-catechin, syringaldehyde, phosphatidylglycerol 42:10 and an unidentified compound with a *m/z* of 826.10. Among these, the flavonoid catechin showed an acute toxicity against *Spodoptera litura* larvae (Ruttanaphan et al., 2023), and reduced the larval growth of the lepidopteran pest *Ectropis grisescens* (Li et al., 2022) when supplemented in artificial diet. Interestingly, the compound flavone, belonging to the flavonoid family, has been reported to interact in a synergistic way with the Bt toxin Cry1Ac when inducing mortality against *Helicoverpa armigera* (Wang et al., 2021). Similarly, the polyphenol syringaldehyde negatively affected the mobility of the bean weevil *Acanthoscelides obtectus* and increased its mortality (Regnault-Roger et al., 2004).

A single compound was significantly over-accumulated in all six tomato varieties: soyasapogenol B base + O-HexA, Hex, dHex, 1 malonyl. This compound corresponds to a triterpenoid saponin, a secondary metabolite widely distributed in plants and whose production is influenced by biotic environmental factors (Szakiel et al., 2011; de Costa et al., 2013). Saponins play a role in plant defenses against herbivores, particularly due to their amphipathic properties, which lead to the formation of strong complexes with cholesterol, triggering cellular toxicity and altering insect molting (Weng et al., 2011). Moreover, saponins disturb insect digestion by inactivating digestive enzymes and altering insect gut microbiota (Singh and Kaur 2018). Previous studies where saponins were added to the artificial diet have shown a negative effect on growth and/or mortality of other lepidopteran insects such as *Spodoptera littoralis* (De Geyter et al., 2007), *Ostrinia nubilalis* (Nozzolillo et al., 1997) and *Helicoverpa zea* (Dowd et al., 2011). Moreover, a synergistic interaction between a tea saponin, a mycotoxin from *Metarhizium anisopliae* and Bt var. *kurstaki* was detected against *S. exigua* in terms of mortality (Rizwan-Ul-Haq et al., 2009). The reported anti-herbivory effects of these compounds support their role in the growth reduction observed in larvae fed on MeJA-induced plants and may be responsible of the increased mortality upon Bt infection.

In conclusion, our study demonstrated that both constitutive and inducible tomato defenses increase the susceptibility of the generalist caterpillar *S. exigua* to *B. thuringiensis*. We identified some compounds which may contribute to these toxic effects. These results strongly support the notion that tomato defenses, which directly affect the insect herbivore, may also indirectly enhance the action of a third trophic level, the entomopathogen group. This finding has positive implications for sustainable crop management programs, which may combine the selection of plant defense traits with the use of entomopathogens.

CRediT authorship contribution statement

Ada Frattini: Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Rosa M. González-Martínez:** Resources, Methodology. **Juan M. García:** Methodology, Resources. **Zhivko Minchev:** Writing – review & editing, Software, Formal analysis. **María J. Pozo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Víctor Flors:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Cristina M. Crava:** Writing – review & editing, Validation, Supervision, Software, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Salvador Herrero:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105624.

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