Received: 20 January 2022 | Revised: 25 April 2022 | Accepted: 23 May 2022

DOI: 10.1111/pce.14363

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

Plant metabolism and defence strategies in the flowering stage: Time-dependent responses of leaves and flowers under attack

Lucille T. S. Chrétien[1,2] | Alix Khalil[2] | Jonathan Gershenzon[3] | Dani Lucas-Barbosa[1] | Marcel Dicke[1] | David Giron[2]

[1]Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands

[2]Institut de Recherche sur la Biologie de l'Insecte (IRBI), UMR 7261, CNRS/Université de Tours, Tours, France

[3]Max Planck Institute for Chemical Ecology (MPI CE), Department of Biochemistry, Jena, Germany

Correspondence
Lucille T. S. Chrétien, Laboratory of Entomology, Wageningen University, Droevendaalsesteeg 1, Radix Bldg, 6708PB Wageningen, The Netherlands.
Email: lucille.nat@orange.fr

Present addresses
Lucille T. S. Chrétien, Ecology and Evolution Group, University of Plymouth, Drake CircusPlymouthPL4 8AA, UK.

Alix Khalil, Laboratory of Process Engineering, Environment, Agri-food (GEPEA), UMR 6144, ONIRIS, Nantes, France.

Dani Lucas-Barbosa, Institute of Parasitology, National Centre for Vector Entomology, Vetsuisse and Medical Faculty, University of Zürich, Winterthurerstraße 266a, 8057Zürich, Switzerland.

Funding information
Région Centre-Val de Loire; Nederlandse Organisatie voor Wetenschappelijk Onderzoek, Grant/Award Number: SPI87-313; COST FA1405 programme; Centre National de la Recherche Scientifique; Ecole normale supérieure de Lyon

Abstract

Plants developing into the flowering stage undergo major physiological changes. Because flowers are reproductive tissues and resource sinks, strategies to defend them may differ from those for leaves. Thus, this study investigates the defences of flowering plants by assessing processes that sustain resistance (constitutive and induced) and tolerance to attack. We exposed the annual plant *Brassica nigra* to three distinct floral attackers (caterpillar, aphid and bacterial pathogen) and measured whole-plant responses at 4, 8 and 12 days after the attack. We simultaneously analysed profiles of primary and secondary metabolites in leaves and inflorescences and measured dry biomass of roots, leaves and inflorescences as proxies of resource allocation and regrowth. Regardless of treatments, inflorescences contained 1.2 to 4 times higher concentrations of primary metabolites than leaves, and up to 7 times higher concentrations of glucosinolates, which highlights the plant's high investment of resources into inflorescences. No induction of glucosinolates was detected in inflorescences, but the attack transiently affected the total concentration of soluble sugars in both leaves and inflorescences. We conclude that *B. nigra* evolved high constitutive rather than inducible resistance to protect their flowers; plants additionally compensated for damage by attackers via the regrowth of reproductive parts. This strategy may be typical of annual plants.

KEYWORDS

Brassica nigra (Brassicaceae), direct resistance, florivorous insects, flowering stage, multiple attack, phytopathogens, plant primary metabolism, plant secondary metabolism, tolerance

1 | INTRODUCTION

Ontogeny influences defense trajectories deployed by plants upon attack (Barton & Boege, 2017; Barton & Koricheva, 2010; Quintero et al., 2014), and in this regard, the defence of reproductive tissues such as flowers is key to plant reproduction (McCall & Irwin, 2006). During the transition from the vegetative to the flowering stage, plants undergo major physiological changes that fuel flower development and ensure seed production (Barneix & Causin, 1996; Mooney, 1972). Flower tissues, therefore, are more likely to be found and attacked by herbivores due to their visibility and high levels of primary metabolites that make them nutritious targets to attackers (Mooney, 1972; Schlinkert et al., 2015). Herbivores that feed on inflorescences—florivores—can not only strongly reduce the reproductive output of plants by damage to floral structures but they can also interfere with plant–pollinator interactions, with consequences on seed production and fitness (McCall & Irwin, 2006; Rusman et al., 2019a).

Optimal defence theory predicts that the most valuable tissues are highly defended, and therefore, flowers are expected to be more defended than leaves (Barton & Koricheva, 2010; Herms & Mattson, 1992; McCall & Irwin, 2006; Rhoades & Cates, 1976). When considering resistance, for example, which can deter attackers or reduce their performance via constitutive or induced mechanisms, theory predicts that plants invest more in constitutive resistance rather than inducible resistance in flowers because of their high value and predictability of attack (McCall & Irwin, 2006; Zangerl, 2003). Recent literature supports that inflorescences generally have higher constitutive levels of defensive metabolites than leaves of flowering plants (Brown et al., 2003; Damle et al., 2005; Smallegange et al., 2007), with some exceptions (Godschalx et al., 2016). Our understanding of plant responses to attack in the flowering stage, however, remains limited and rarely integrated into the context of plant defence strategies despite the increasing number of studies covering such topics (Lucas-Barbosa, 2016; McCall & Irwin, 2006; Rusman et al., 2019a). Yet, the trade-off between defence and reproduction may be particularly strong when inflorescences are attacked because florivore-induced resistance, or tolerance responses (which reduces the fitness effect of attack without targeting the attackers), can both lead to changes in floral traits and resource allocation to reproduction (Lucas-Barbosa, 2016; McCall & Irwin, 2006; Orians et al., 2011; Rusman et al., 2019a).

A substantial body of literature based on plants in the vegetative stage has demonstrated that attack by pathogens and herbivores can induce an extensive reprogramming of primary and secondary metabolic pathways among plant tissues (Giron et al., 2013; Pastor et al., 2014; Zhou et al., 2015). These metabolic changes supply increased demand for energy and carbon to sustain physiological responses, such as regrowth, or the synthesis and emission of secondary metabolites, which respectively support plant tolerance and resistance to attack (Bekaert et al., 2012; Schultz et al., 2013; Schwachtje & Baldwin, 2008). There is evidence that plants can respond to floral damage with an induction of secondary metabolites either locally within the damaged flower (Euler & Baldwin, 1996; Ohnmeiss & Baldwin, 2000) or systematically at the scale of the inflorescence (Boyer et al., 2016). These results suggest an investment in inducible resistance, although not all studies detected such responses in flowers (Smallegange et al., 2007; Zangerl & Rutledge, 1996). Additionally, florivorous attackers feed on plant parts that are resource sinks, and they do not damage leaves and roots that provide resources to the plant. Plants are thus left with the capacity to absorb and produce resources and can reallocate primary metabolites to support compensatory mechanisms such as regrowth of damaged floral structures or reallocation to reproductive organs that remained undamaged (Lucas-Barbosa et al., 2017; Rosenheim et al., 1997; Wise et al., 2008). As a consequence, tolerance to damage may be a common strategy deployed by plants upon florivory (McCall & Irwin, 2006; Orians et al., 2011).

The present study aims at characterizing the defence strategies of plants in the flowering stage by simultaneously studying mechanisms of plant constitutive and inducible resistance, as well as tolerance to floral attackers. We took a dynamical approach that compares plant responses to a range of threats they would face in the wild to increase ecological realism and identify patterns of defence. To capture attacker-specific defence strategies, we exposed the annual plant *Brassica nigra* to combinations of two distinct florivorous insects and a pathogen inoculated on inflorescences: *Brevicoryne brassicae*, *Pieris brassicae* and *Xanthomonas campestris* pv. *raphani* (Xcr), and measured whole-plant responses after 4, 8 and 12 days of exposure to attackers, plants being attacked when the first flowers opened. We simultaneously analysed changes in the composition and concentration of structural (protein-bound amino acids) and nonstructural (free amino acids and soluble sugars) primary metabolites, and in a group of secondary metabolites: the glucosinolates. Glucosinolates are inducible and support the resistance in Brassicaceae (Hopkins et al., 2009). To estimate compensation for damage and potential reallocation of resources among plant parts, we also measured and compared the dry biomass of inflorescences, leaves and roots.

We previously showed that *B. nigra* can specifically respond to florivory with an induction of phytohormones in their inflorescences: dual attack with *P. brassicae* plus Xcr and *P. brassicae* plus *B. brassicae* induced higher levels of jasmonates than did *P. brassicae*, *B. brassicae* or Xcr alone (Chrétien et al., 2018). *Brevicoryne brassicae* performed better on inflorescences that were also infested with *P. brassicae* or with Xcr when compared to a single attack with *B. brassicae*, whereas *P. brassicae* performed worse on inflorescences coinfested with *B. brassicae* when compared to single attack (Chrétien et al., 2018). This suggested either change in plant defence compounds (linked to resistance) and/or nutritional quality (possibly linked to tolerance). Moreover, exposure of *B. nigra* leaves to *P. brassicae* induced changes in C/N due to an increase in the allocation of nitrogen to flowers, which may be linked to plant tolerance to attack (Lucas-Barbosa et al., 2017). Indeed, Rusman et al. (2019b) counted the number of flowers on *B. nigra* after 7 days of exposure to various florivores, including chewing florivores that physically remove flowers, and found that the number of flowers and inflorescences did not change upon attack.

Consequently, we hypothesize that flowering *B. nigra* responds to attackers with an induction of secondary and/or primary metabolites in their inflorescences. Here, we aim to understand the relative importance of constitutive and inducible resistance and tolerance in the defence of flowering *B. nigra* against floral attackers. We discuss attacker-specific responses and time-dependent changes at the plant scale in relation to resistance and tolerance and propose general patterns of plant defence in the flowering stage.

2 | MATERIALS AND METHODS

2.1 | Study system

We studied the Black Mustard, *B. nigra* (Brassicales: Brassicaceae), which is a fast-growing annual plant that contains high concentrations of nitrogen-containing glucosinolates, a class of compounds that have a major function in the resistance of Brassicales (Textor & Gershenzon, 2009). Upon tissue damage, glucosinolates break down into compounds such as isothiocyanates that are highly toxic to generalist herbivores (Brown & Hampton, 2011; Hopkins et al., 2009; Mithöfer & Boland, 2012). Smallegange et al. (2007) reported that glucosinolate levels in *B. nigra* inflorescences can be five times higher than in leaves. In the flowering stage, attackers mainly feed on the reproductive parts of the plant and specialist herbivores are the most frequent and abundant (L. T. S. Chrétien, personal observation). From those specialist attackers, we selected two florivorous insects and one phytopathogenic bacterium based on their distinct modes of action and reported the effect on flowering *B. nigra* (Chrétien et al., 2018, 2021): (1) the aphid *B. brassicae* (Hemiptera: Aphididae) as a phloem feeder, (2) the Lepidoptera *P. brassicae* (Lepidoptera: Pieridae) eggs that develop into chewing caterpillars, (3) the bacterium *X. campestris* pathovar *raphani* (Xcr) as a necrotrophic pathogen. *Brevicoryne brassicae* can sequestrate plant glucosinolates as defences against nonspecialized carnivores (Kos et al., 2012a). The performance of *B. brassicae* is generally positively correlated to the concentration of aliphatic glucosinolates, but *B. brassicae* are susceptible to indole glucosinolates (Kos et al., 2011, 2012a; Züst & Agrawal, 2016). Caterpillars of *P. brassicae* can reduce the toxicity of glucosinolates breakdown products by turning them into nitriles and their catabolites (Winde & Wittstock, 2011; Wittstock et al., 2004). Detoxification products may, however, affect the caterpillar immune system and increase their susceptibility to carnivores such as parasitoids (Kos et al., 2012b). Xcr causes Leaf Spot Disease and forms small necrotic spots on the leaves of many brassicaceous plants (Machmud, 1982; Vicente et al., 2006). It can spread through the plant from infected leaves to mature seeds (Machmud, 1982). Glucosinolates can negatively affect pathogens and in particular necrotrophic bacteria (Textor & Gershenzon, 2009).

2.2 | Plants, insects and bacterial cultures

We reared *B. brassicae* aphids and *P. brassicae* caterpillars on Brussels sprout plants (*Brassica oleracea* variety *gemmifera*) in a greenhouse compartment (22 ± 2°C, 50%–70% relative humidity [r.h.], L16:D8). A honey solution from organic production (10%, Melvita, Weide & Veldbloemen) was provided to *P. brassicae* butterflies as food, and the butterflies were kept in a greenhouse compartment (25 ± 2°C, 50%–70% r.h., 16 L:8D). Xcr bacteria were obtained from Utrecht University, The Netherlands (Bonnet et al., 2017; Ponzio, 2016; Ponzio et al., 2017b) and cultured as described in Chrétien et al. (2018). The concentration of the inoculum was calculated by measuring the light absorbance at 600 nm of the bacterial solution (in buffer 10 mM M_gSO_4) and adjusted to 10^9 cells/ml.

Brassica nigra were cultured in a greenhouse compartment (22 ± 2°C, 60%–70% r.h, 16L:8D). We germinated seeds from a mixture of seeds from 20 to 25 plants (accession no. CGN06619; Center for Genetic Resources) that had been exposed to open pollination for several generations in the field station of Wageningen University. Seedlings were grown in pots (Ø17 cm–2 L content) with a soil mixture of 1:1 (v/v) sand and potting soil (Lentse Potgrond). Five- to six-week-old plants initiated flowering, and several flowers opened daily. *Brassica nigra* produced several hundred flowers over the course of the flowering period.

2.3 | Plant treatment and selection of time points

Plants were infested in the conditions set for their culture (22 ± 2°C, 60%–70% r.h., 16L:8D) 1–2 days after the first flowers had opened. We exposed *B. nigra* to a single attack by either *B. brassicae*, *P. brassicae* or Xcr, to simultaneous dual attack by combining two of these three species of attackers, or we kept the plants as unattacked controls. The infestations and inoculations were performed as illustrated in Figure 1 of Chrétien et al. (2018). In short, we placed five young-adult apterous females *B. brassicae* and/or an inoculum of Xcr on the lowest bracts of the inflorescence, and/or 30 eggs of *P. brassicae* on a leaf. Plants exposed to dual attack were simultaneously exposed to two of the three attackers, and a bract never received more than one treatment. We placed a 2 × 2-cm piece of cotton wool with 500 µl of the bacterium inoculum (10^9 cells/ml in a buffer) on the underside of a bract for 4 h with a soft clip (Chrétien et al., 2018). To control for the effect of clipping and buffer, a set of control plants (Buffer) and plants that only received insect attackers were clipped with cotton wool soaked in buffer solution only (10 mM M_gSO_4). The other set of control plants remained untreated. In an earlier study, we recorded necrotic spots (~1–3 mm) on the inoculated bract for plants sampled on Days 8 and 12 for chemical analyses, which can indicate plant hypersensitivity response or disease symptoms (Chrétien et al., 2018).

We selected three time points based on the behaviour of *P. brassicae* and the development of the plant: 4, 8 and 12 days since the attack. We considered plants as early flowering at 4 days, in full bloom at 8 days and late flowering at 12 days, with leaves starting senescence. At 4 days, plants carried eggs of *P. brassicae* on a leaf. Eggs can induce responses in *B. nigra* with consequences for plant fitness (Lucas-Barbosa et al., 2013; Pashalidou et al., 2013). Caterpillars hatched from the eggs after 5 days and fed from leaves for about 2 days before moving to the

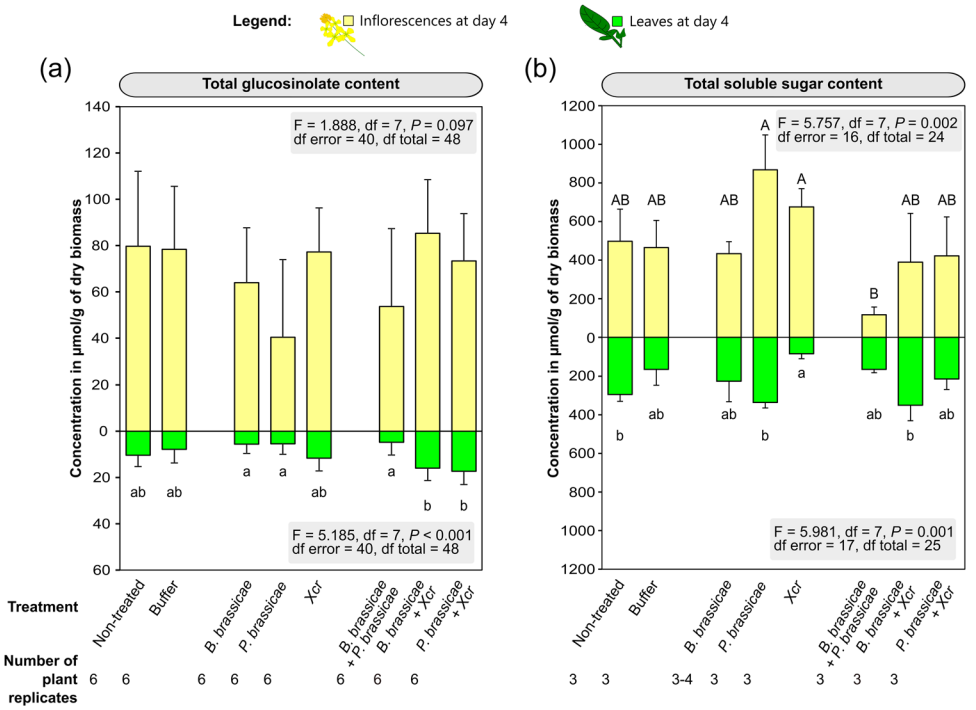

FIGURE 1 Cumulative concentrations of glucosinolates and soluble sugars in inflorescences and leaves of *Brassica nigra* that were exposed to single or dual attack for 4 days. *Brassica nigra* plants were exposed to a single attack by *Brevicoryne brassicae* aphids, eggs and caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or nontreated. Panels (a, b) show total concentrations (average ± SD) of glucosinolates (a) and soluble sugars (b) in samples of inflorescences (yellow) and leaves (green) of attacked plants. There were six plant replicates per treatment for the glucosinolates, and three to four for the soluble sugars. The effect of treatments was tested with an analysis of variance, and when significant, a Bonferroni post hoc test was used for pairwise comparisons. Lower-case letters above the bars indicate statistical outcomes of post hoc test. All concentrations are expressed in µmol/g of plant dry biomass. For each statistical test, the significance level was set to $\alpha = 0.05$; test parameters are indicated in a grey frame in the panels.

flowers (at the L1–L2 stage). If at 7 days after egg deposition some caterpillars were still on the leaves, they were gently moved to the inflorescence to ensure florivory for at least 1 day before the sampling of 8 days. The density of caterpillars was reduced to 15 at 8 days to prevent complete consumption of the flowers and simulate the natural decline in caterpillar density in the field. Caterpillars were in the L3–L4 larval stage at 12 days; a caterpillar can eat 135 flowers and buds during its L5 (final) stage (Smallegange et al., 2007). Aphids freely dispersed on the plants and mainly colonized inflorescences. At 4, 8 and 12 days, we counted, respectively, 53 ± 18, 240 ± 93 and 1194 ± 312 (mean ± SD, $N = 6–8$) aphids per plant. Direct interactions were rarely observed among attackers, and florivores spread across the numerous inflorescences of *B. nigra*. Plants grown in similar conditions by Rusman et al. (2019b) had on average 34 inflorescence branches and 364 flowers 7 days after the start of flowering.

2.4 | Sampling of leaves and inflorescences for metabolic analyses

We excluded the bracts and leaves that initially received the treatments to focus on the systemic response of the plant. All other leaves and all inflorescences (without bracts) of *B. nigra* plants were harvested after either 4, 8 or 12 days of exposure to treatments, including control plants of matching age. As described in Chrétien et al. (2018), plant parts were cut at the base of the petiole/stem, immediately frozen in liquid nitrogen and stored at −80°C, freeze-dried and kept at −20°C until chemical analyses were performed. We used six different individual plants (biological replicates) for each treatment and time point. Glucosinolates were quantified in all six plants per treatment and time point; free amino acids, protein-bound amino acids and sugars were quantified in three of these plants per treatment and time point. Insects, faeces and honeydew were carefully removed with a brush and a wet paper towel shortly prior to sampling; the same procedure was applied to control plants (nontreated and buffer).

2.5 | Extraction, annotation and quantification of glucosinolates

Glucosinolates were extracted from 20 mg of ground, freeze-dried plant material, desulfated and analysed by high-performance liquid chromatography (HPLC), as described by Doheny-Adams et al. (2017).

Glucosinolates were extracted by adding 1 ml of 80% methanol (v/v) and 50 µM of sinalbin was added as an internal standard. Sinalbin was isolated from *Sinapis alba* seeds at the MPI CE. The suspension was placed in a shaker for 5 min at 230 rpm for extraction and centrifuged at 3200 rpm for 10 min to separate the supernatant containing the glucosinolates from the remaining pellet. Glucosinolates were extracted by anion exchange chromatography with a 28 mg column of DEAE-Sephadex® A-25 (Sigma-Aldrich, Merck KGaA). For the extraction, the column was first conditioned with 800 µl MilliQ-Water (Dosieraufsatz), followed by 500 µl 80% methanol and dried in between using a vacuum manifold. Eight hundred microliters of supernatant was then loaded onto the column and the column was rinsed with 500 µl of 80% methanol, followed by two times 1 ml of Milli-Q water and finally with 500 µl of 2-(*N*-morpholino) ethanesulphonic acid (MES) buffer (0.02 M, pH 5.2). To desulfatize the glucosinolates, the column was treated with 30 µl of aryl-sulfatase and incubated overnight at room temperature. Columns were finally eluted with 0.5 ml of Milli-Q water; eluted solutions of desulfoglucosinolates were stored at −20°C until analysis. The aryl-sulfatase was prepared from lyophilized powder of aryl-sulfatase (from *Helix pomatia*, type H1, product number S9626; Sigma) as described in Graser et al. (2001).

Desulfoglucosinolates were separated using HPLC (Agilent 1100 HPLC system; Agilent Technologies). Samples were injected onto a reverse-phase C18 column (Nucleodur Sphinx RP, 250 × 4.6 mm, 5 µm; Machrey-Nagel). The eluent consisted of water (solvent A) and acetonitrile (solvent B), with the following gradient: 0–1 min, 1.5% B; 1–6 min, 1.5%–5% B; 6–8 min, 5%–7% B; 8–18 min, 7%–21% B; 18–23 min, 21%–29% B; 23–23.1 min, 29%–100% B; 23.1–24 min, 100% B; and 24.1–28 min, 1.5% B. The flow rate was 1.0 ml/min and the column was kept at room temperature. Eluted compounds were detected with a photodiode array detector.

Desulfated glucosinolates were annotated by comparing retention times and ultraviolet absorption spectra to those of purified standards extracted from *Arabidopsis thaliana* (Brown et al., 2003). Quantification of the desulfoglucosinolates was based on peak areas of the 229 nm traces and was carried out via the internal standard method. Response factors used were 2.0 for aliphatic glucosinolates and 0.5 for indole glucosinolates (Burow et al., 2006), and 2.0 for 2-phenylethyl-glucosinolate (an aromatic glucosinolate) relative to the internal standard sinalbin. We chose the same response factor for 2-phen ylethyl-glucosinolate as for aliphatic glucosinolates because Brown et al. (2003) reported a response factor of 1.0 for both aliphatic glucosinolates and 2-phenylethyl-glucosinolate relative to sinigrin.

2.6 | Extraction, annotation and quantification of free and protein-bound amino acids

Amino acids were extracted, derived with propyl-chloroformate and analysed by gas chromatography-mass spectrometry (GC-MS). Extraction and derivatization were performed using the kit EZ:faast (Phenomenex) that we adapted to our samples; it consisted of a solid-phase extraction (SPE), followed by derivatization and then by a liquid/liquid extraction. We used two extraction techniques, one for the free amino acids and one for the protein-bound amino acids. Free amino acids were extracted from 5 mg of plant material with 1 ml solution of 1:3 acetonitrile 100% and HCl (0.01 M) and shaken for 1 h. Then, 200 µl of the solution was subjected to the EZ:faast procedure according to the manufacturer's instructions for liquid-phase hydrolysates, using 50 µl of iso-octane 80% and chloroform 20% to dissolve the dry precipitate during the last step. For the protein-bound amino acids, the peptide bonds first had to be hydrolysed. For this, we introduced 500 µl of methane sulphonic acid (4 M) per 5 mg of plant sample, purged the air of the vial with N_2 and incubated the closed vials in an oven at 150°C for 2 h. At the end of the incubation, vials were quickly cooled in ice; subsequently, 100 µl of the liquid hydrolysate was subjected to the EZ:faast procedure according to the manufacturer's instructions using 240 µl of sodium carbonate solution and 80 µl of iso-octane 80% and chloroform 20% to dissolve the dry precipitate during the last step. A complete description of the procedure is provided in Supporting Information: Protocol S1. The kit is designed for more than 60 aliphatic and aromatic amino acids. A drawback of using propyl-chloroformate as a derivatizing agent is that arginine could not be quantified in our samples (Mink et al., 2013; Waldhier et al., 2010).

We used an autosampler (Gerstel) to inject 2 µl of extract into the column (Zebron ZH-5HT inferno 30 m × 0.25 mm × 0.25 µm; Phenomenex) in splitless mode (50:1); injector temperature was 250°C. The column was heated at 15°C/min from 110°C to 320°C, with a final hold time of 7 min, and a total run time of 21 min. Helium was used as a carrier gas, with a constant flow at 1.2 ml/min. The transfer line to the MS was set at 320°C. We used electron ionisation with electron energy of −70 eV, an ionisation source at 230°C and the two quadrupole mass analysers at 180°C. The scan range was 45–450*m/z* with 3.7 scans/s.

Peaks of amino acids used as external standards and amino acids detected in plant samples were annotated based on the mass spectra of the derived compounds, provided by the EZ:faast kit. The area of the peaks of interest was calculated based on total ion chromatograms (TICs). We used mixtures of corresponding amino acids at 0.2 mM each as external standards to quantify amino acids in our samples (in µmol/g of plant dry biomass). After quantification, we verified that the quantity of amino acids in the extract did not reach the saturation level of the SPE column (1.2 µmol) and that the extracted quantity of each amino acid was above the limit of detection provided in the EZ:faast instructions.

2.7 | Extraction, annotation and quantification of soluble sugars

Soluble sugars were extracted from 5 mg of ground freeze-dried plant material, derived and analysed by gas chromatography-mass spectrometry (GC-MS). To remove the chlorophyll, 1 ml of acetone was added to the sample. The solution was shaken for 1 h and the acetone supernatant was discarded. Sugars were then extracted with 1 ml of methanol 80%, the solution was shaken for 1 h, spun for 10 min at

6000 rpm and 80 µl of the supernatant containing the soluble sugars was collected and dried under a gentle nitrogen flow. For the analyses by GC-MS, sugars were derivatized. We added to the precipitate of sugars 50 µl of pyridine and 100 µl of a 99:1 solution of N,O-bis (trimethylsilyl)trifluoroacetamide:trimethylchlorosilane (Sigma-Aldrich, Merck KGaA), and then heated the solution at 70°C in a bain-marie for 1 h under agitation. The solution was then dried under a gentle nitrogen flow, and we finally added 50 µl of the internal standard methyl undecanoate (0.5 mM) and 50 µl of acetonitrile, and then homogenized the solution before injection into the GC-MS.

We used the same injection and MS parameters for the analysis of amino acids, as well as the same column. Only the GC method differed: column temperature was at 60°C for 1 min, then increased at 30°C/min from 60°C to 120°C, and then at 8°C/min to 320°C, with a final hold time of 10 min and total run time of 30 min.

We annotated compounds based on the mass spectra of the derivatized compounds provided by the National Institute of Standards and Technology. The area of the identified peaks was calculated based on TIC. We used the internal standard to quantify each soluble sugar, and we multiplied the soluble sugar peak area by the concentration of the internal standard divided by its peak area. Concentrations of soluble sugars are expressed in µmol/g of plant dry biomass. When several stereoisomers of the same sugar were detected and identified, we added the concentrations of the different stereoisomers to express the total concentration of the compound.

2.8 | Dry biomass of roots, vegetative parts and inflorescences of *B. nigra* exposed to single or dual attack

We measured dry biomass of roots, leaves and flowers of *B. nigra* that were kept nontreated or exposed to buffer (controls), or exposed to a single attack with *B. brassicae*, *P. brassicae* or Xcr, or to simultaneous dual attack by two of these attackers. Plants were harvested after 4, 8 and 12 days of exposure to treatments. Roots were cut from the main vegetative stem, and the aboveground vegetative part was separated from the inflorescence just below the first phytomere of the main inflorescence. Side inflorescences that grew from the base of the leaves' petioles were cut at the base of the flower stalk and pooled with the main inflorescence. Roots and surrounding ground were stored in a plastic bag at 4°C until the soil could be washed away from the roots. Roots, vegetative parts and inflorescences were dried for 16 h in an oven at 105°C and weighed immediately thereafter. There were six to seven plant replicates per treatment and time point.

2.9 | Data processing and statistical analyses

2.9.1 | Calculation of total concentrations

We looked at both qualitative and quantitative changes in the four classes of metabolites analysed in this study. Regarding quantitative changes, we calculated the total concentrations of each metabolic class, that is, glucosinolates, soluble sugars, free amino acids and protein-bound amino acids, by summing concentrations of individual compounds within classes for each factor considered (treatment, plant part and time point). Supporting Information: Tables S1–S4 show compound-specific total concentrations in leaves and inflorescences at each time point, and concentrations were averaged across treatments.

2.9.2 | Univariate analyses

Total concentrations of metabolites (protein-bound amino acids, free amino acids, soluble sugars and glucosinolates) and plant dry biomass were analysed by analysis of variance (ANOVA). Data met the assumptions of normality and equality of variance, respectively, tested with a Shapiro–Wilk test and a Levenes'test. We tested for an effect of exposure to attack on the total concentrations of metabolites contained by leaves and inflorescences on Days 4, 8 and 12 separately. The effects of plant exposure to attackers and time points on dry plant biomass, and the interactions between these two factors, were also tested for each of the three plant parts. When a significant effect of a factor was detected, we used the Bonferroni post hoc test for pairwise comparison. The intercept was included in the models, and the significance level was set at 0.05. Analyses were performed in SPSS (IBM SPSS Statistics for Windows, Versions 24 and 25; IBM Corp.).

2.9.3 | Multivariate analyses

Metabolic profiles were analysed by multivariate data analysis using projection to latent structures-discriminant analysis (PLS-DA), with SIMCA (Version 15.0; Umetrics AB). We analysed how much of the variation in the metabolic profiles of plants could be explained by time and plant part (score plots) and which metabolites had the highest discriminatory power in separating profiles of leaves and inflorescences at the three time points (loading plots). Analysis was based on the concentration of the different metabolites of samples of leaves on Days 4, 8 and 12, and inflorescences on Days 4, 8 and 12, irrespective of treatment. We also analysed how much of the variation in the chemical/metabolic profiles of leaves and flowers was explained by plant exposure to the attackers on Days 4, 8 and 12. The PLS method is commonly used for multivariate statistical analysis of metabolic data, but can in some cases overfit the data (Triba et al., 2015). We describe in the Supporting Information how we ensured the quality of our models (see validation of the PLS-DA in SIMCA). No significant correlation was found between total concentrations of leaves and inflorescences of the same plant samples in terms of protein-bound amino acids, free amino acids, soluble sugars and glucosinolates (data not shown); thus, we considered leaf and inflorescence samples as independent. The contribution of each metabolite to the separation of the explanatory variables (plant part,

time-point and treatment) in the validated models was determined graphically in the loading plots and numerically based on VIP values (variable's importance in projection). A tolerance ellipse was computed around the data points on the score plots. This ellipse is based on Hotelling's T2 calculation, and data points outside this ellipse are considered as outliers.

3 | RESULTS

3.1 | Effect of single and dual attacks on the total concentration and profiles of metabolites

Treatment affected the total concentration of glucosinolates and soluble sugars at an early stage of exposure to attackers, that is, at 4 days since infestation, when plants carried eggs of *P. brassicae* and about 50 aphids (Figure 1). This effect was neither detected on Days 8 and 12 (Supporting Information: Figure S1) nor for amino acids at any of the three time points (Supporting Information: Figure S2). Treatment affected the total concentration of glucosinolates in leaves, whereas no effect was detected in inflorescences (Figure 1a). Bacteria seemed to be the main driver of changes in the total concentration of glucosinolates in leaves. Leaves of plants exposed to dual attack by aphids plus bacteria had 2.9 times higher levels of glucosinolates than leaves of plants exposed to aphids alone (ANOVA, Bonferroni post hoc test, $p = 0.036$), and 3.3 times higher than leaves of plants exposed to aphids plus *P. brassicae* (ANOVA, Bonferroni post hoc test, $p = 0.017$). Similarly, leaves of plants exposed to a dual attack by *P. brassicae* plus bacteria had 3.2 times higher levels of glucosinolates than leaves of plants exposed to *P. brassicae* alone (ANOVA, Bonferroni post hoc test, $p = 0.009$), and 3.6 times higher than leaves of plants exposed to aphids plus *P. brassicae* (ANOVA, Bonferroni post hoc test, $p = 0.004$). The glucosinolate levels in leaves of plants attacked by bacteria plus aphids or bacteria plus *P. brassicae* did not significantly differ from that of plants attacked by bacteria only. Foliar concentrations of glucosinolates of plants exposed to one or two types of attackers did not significantly differ from the concentration of control plants exposed to buffer or of nontreated plants.

Treatment affected the total level of soluble sugars of both inflorescences and leaves (Figure 1b). In inflorescences, changes were driven by a dual attack by the two herbivorous insects. Indeed, inflorescences of plants attacked by aphids plus *P. brassicae* had 7.4 times lower levels of soluble sugars than inflorescences of plants exposed to *P. brassicae* only (ANOVA, Bonferroni post hoc test, $p = 0.001$) and 5.8 times lower levels of soluble sugars than inflorescences of plants attacked by bacteria only (ANOVA, Bonferroni post hoc test, $p = 0.014$). Floral sugar levels of plants exposed to single or dual attackers did not significantly differ from those of control plants exposed to buffer or of nontreated plants. In the leaves, changes were mainly driven by exposure to bacteria. Leaves of plants attacked by bacteria had, respectively, 4.0 and 4.1 times lower levels of soluble sugars than plants attacked by *P. brassicae* (ANOVA, Bonferroni post hoc test, $p = 0.006$) and by aphids plus bacteria (ANOVA, Bonferroni post hoc test, $p = 0.003$), and 3.5 times lower levels of soluble sugars than nontreated control plants (ANOVA, Bonferroni post hoc test, $p = 0.030$). Other treatments did not affect foliar concentrations of soluble sugars when compared to control plants exposed to buffer and to nontreated plants. Total concentrations of protein-bound amino acids and free amino acids were not influenced by treatment, neither for inflorescences nor for leaves after either 4, 8 or 12 days of attack (Supporting Information: Figure S2).

3.2 | Effect of plant part and time on the glucosinolate profile

Plant part and, to a lesser extent, time since infestation/infection affected the composition of glucosinolate profile of *B. nigra* plants (Figure 2a,b). A principal latent structure-discrimination analysis based on the samples from all treatments and controls resulted in a model with five significant principal components ($R^2X = 0.952$, $R^2Y = 0.374$, $Q^2 = 0.346 \pm$ SD 0.004, $P_{CV\text{-}ANOVA} < 0.001$). PC1 explained 39.7% of the variation and clearly separated samples of inflorescences from samples of leaves (Figure 2a). Sinigrin, which accounted for over 98% of total glucosinolates, and 4-hydroxy-indol-3-yl-methyl-glucosinolate strongly contributed to this separation (Figure 2b and Supporting Information: Table S4; VIP values > 1) and were present in, respectively, 4 and 26 times higher levels in inflorescences than in leaves (Supporting Information: Table S1). Overall, the total concentration of all six glucosinolates was 4.8 times higher in inflorescences than in leaves (Supporting Information: Table S1). PC2 explained 18.5% of the variation and separated samples of leaves and inflorescences harvested at 12 days from those harvested at 4 or 8 days (Figure 2a). 1-Methoxy-indol-3-yl-methyl-glucosinolate drove this separation (Figure 2b and Supporting Information: Table S1; VIP values > 1): all leaves and flowers of plants produced 1-methoxy-indol-3-yl-methyl-glucosinolate after 12 days, whereas less than 50% of the plants produced it after 4 or 8 days (Supporting Information: Table S1).

3.3 | Effect of plant part and time on the profile of free amino acids and soluble sugars

The composition of the primary metabolites free amino acids and soluble sugars of *B. nigra* plants was more affected by plant part than by time (Figure 3). The contribution of plant part to the separation of metabolic profiles was especially striking for free amino acid profiles, for which differences were both qualitative and quantitative.

PLS-DA based on the amino acids detected in all treatments and control resulted in a model with eight significant principal components ($R^2X = 0.951$, $R^2Y = 0.457$, $Q^2 = 0.324 \pm$ SD 0.012, $P_{CV\text{-}ANOVA} < 0.001$). PC1 explained 67.9% of the variation and clearly separated leaf samples from inflorescence samples (Figure 3a). The loading plot indicated that all 23 amino acids, except 3-hydroxyproline, were more positively associated with inflorescence samples than with leaf

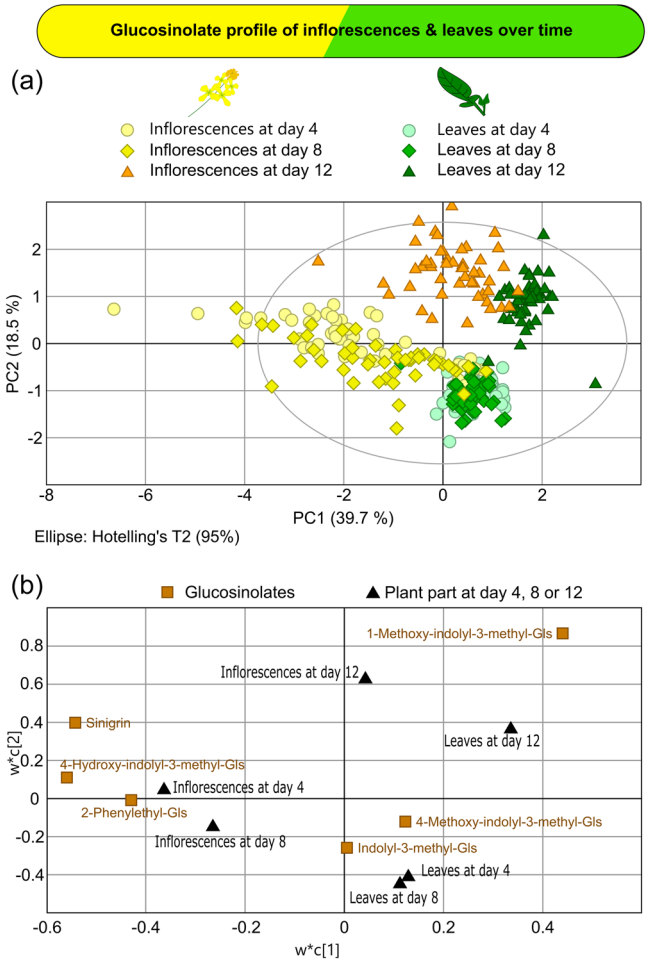

FIGURE 2 Profile of glucosinolates of inflorescences and leaves of *Brassica nigra* plants exposed to single and dual attacks for 4, 8 and 12 days. Projection to latent structures-discriminant analysis (PLS-DA) based on the concentration of six glucosinolates (µmol/g of plant dry biomass) that were detected and quantified in samples of *B. nigra* exposed to a single attack by *Brevicoryne brassicae* aphids, eggs and caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or nontreated. Concentrations of glucosinolates were measured in leaves and inflorescences of six plants per treatment after 4, 8 and 12 days of exposure to the treatments. Plant part and time since infestation/infection were set as classes. The PLS-DA resulted in a model with five significant principal components, and model parameters were: $R^2X = 0.952$, $R^2Y = 0.374$, $Q^2 = 0.346 \pm$ SD 0.004, $P_{CV\text{-}ANOVA} < 0.001$. Percentages between brackets indicate the percentage of variation in the data explained by the first two principal components that resulted from the model. (a) Scatter plots show the grouping pattern of samples from inflorescences and leaves on Days 4, 8 and 12 according to the first two principal components; the Hotelling's ellipse confines the confidence region (95%). (b) Loading plots show the contribution of each of the glucosinolates to the first two principal components. *Gls* stands for glucosinolate. [Color figure can be viewed at wileyonlinelibrary.com]

samples (Figure 3b). Levels of free amino acids were approximately four times higher in inflorescences than in leaves (Supporting Information: Table S2). Histidine and α-aminobutyric acid were exclusively found in inflorescences and glutamine, tyrosine, tryptophan and ornithine were found in less than 50% of the leaf samples (Table S2). PC2 explained 4.2% of the variation and separated samples based on time since infestation/infection (Figure 3a). Time differentially affected the free amino acid profile of leaves and inflorescences, and the difference between leaves and inflorescences decreased with time (Figure 3a). Profiles of inflorescences at 8 and 12 days since infestation/infection differed less from each other than from profiles of inflorescences at 4 days. Total concentrations of free amino acids indeed indicated a progressive decrease of 42% in leaves from Days 4 to 12, including a strong decrease of 33% in inflorescences from Days 4 to 8. Overall, aspartic acid and glutamic acid were more associated with inflorescences on Day 4, and proline, tryptophan, α-aminoadipic acid and histidine were more associated with inflorescences on Days 8 and 12 (Figure 3b). These six amino acids contributed most (VIP values > 1) to the discrimination between inflorescences and leaves on Days 4, 8 and 12.

The PLS-DA of the soluble sugars detected in all treatments and control resulted in a model with five significant principal components ($R^2X = 0.886$, $R^2Y = 0.316$, $Q^2 = 0.240 \pm$ SD 0.008, $P_{CV\text{-}ANOVA} < 0.001$). PC1 explained 38.1% of the variation and mainly separated samples of leaves from samples of inflorescences (Figure 3c). Inflorescences were more associated than leaves with high concentrations of three out of eight soluble sugars: fructose, glucose and inositol, especially inflorescences at 8 days when compared to inflorescences at 4 and 12 days (Figure 3d). This was consistent with the total concentration of soluble sugars, which was 87.2% higher in inflorescences than in leaves, and overall higher in inflorescences at 8 days than at 4 and 12 days (Supporting Information: Table S3). PC2 explained 24.6% of the variation and separated samples based on time since infestation/infection. There was a clear separation between samples of inflorescences on Day 4 versus samples of inflorescences on Days 8 and 12. In the loading plot (Figure 3d), we can see that inflorescences on Day 4 were more associated with high levels of threose and xylose, whereas inflorescences on 8 and 12 days were more associated with saccharose and inositol.

3.4 | Effect of plant part and time on the profile of protein-bound amino acids

Time affected the composition of protein-bound amino acids in the leaves and flowers of *B. nigra* plants (Figure 4). A PLS-DA based on the samples from all treatments and control resulted in a model with seven significant principal components ($R^2X = 0.950$, $R^2Y = 0.374$, $Q^2 = 0.254 \pm$ SD 0.014, $P_{CV\text{-}ANOVA} < 0.001$). The first principal component (PC1) explained 57.5% of the variation and separated plant samples by the time-point, that is, 4, 8 and 12 days since infestation/

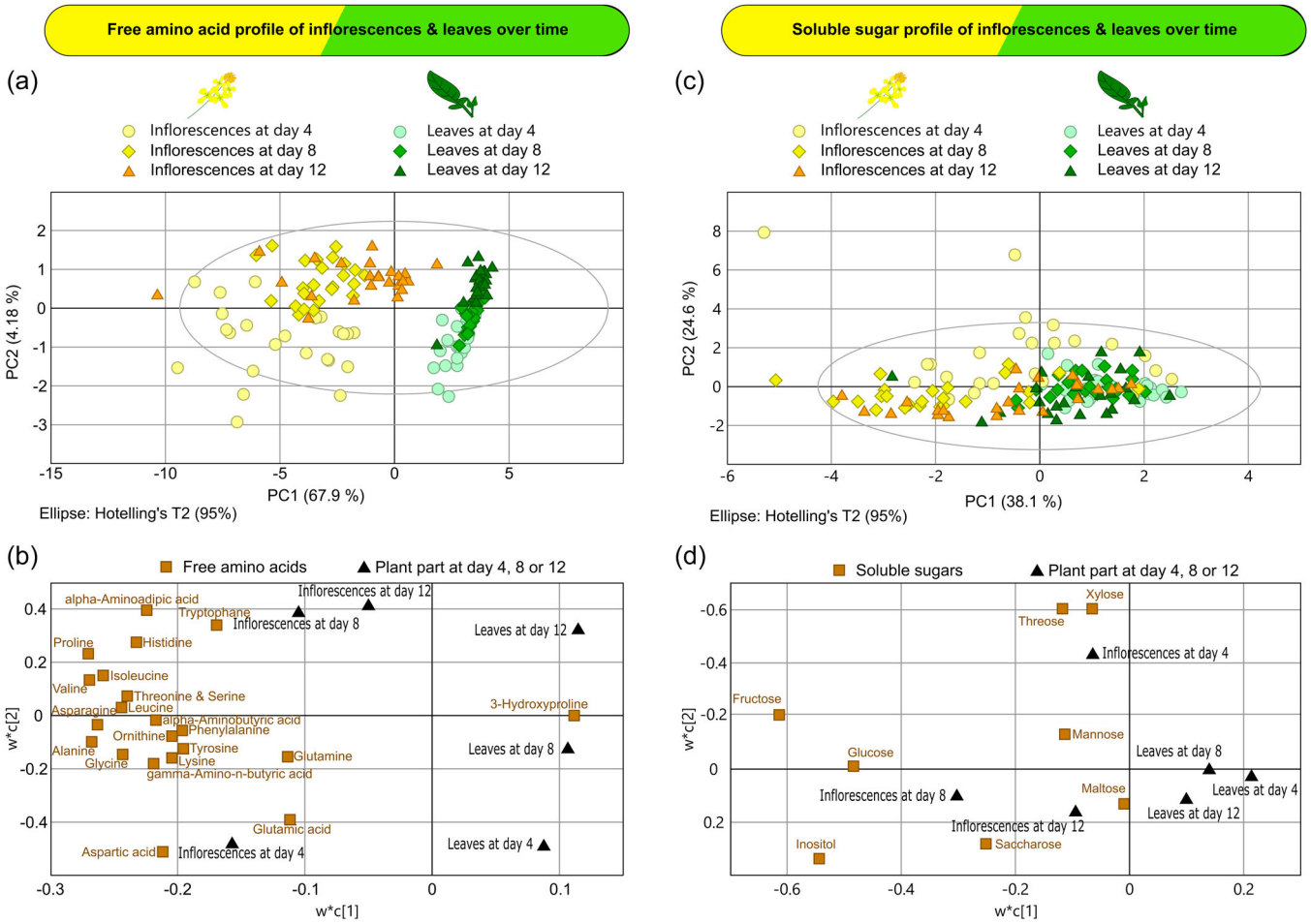

FIGURE 3 Profiles of free amino acids and soluble sugars of inflorescences and leaves of *Brassica nigra* plants were exposed to single and dual attacks for 4, 8 or 12 days. Projection to latent structures-discriminant analysis (PLS-DA) based on the concentration of 23 free amino acids (a, b, µmol/g of plant dry biomass) and 8 soluble sugars (c, d, µmol/g of plant dry biomass) that were detected and quantified in samples of *B. nigra* exposed to single attack by *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or nontreated. Concentrations of primary metabolites were measured in leaves and inflorescences of three plants per treatment after 4, 8 and 12 days of exposure to the treatments. Plant part and time since infestation/infection were set as classes. The PLS-DA resulted in a model with eight significant principal components, and model parameters were: $R^2X = 0.951$, $R^2Y = 0.457$, $Q^2 = 0.324 \pm$ SD 0.012, $P_{CV\text{-}ANOVA} < 0.001$ for free amino acids (a, b), and five significant principal components, and model parameters were: $R^2X = 0.886$, $R^2Y = 0.316$, $Q^2 = 0.240 \pm$ SD 0.008, $P_{CV\text{-}ANOVA} < 0.001$ for soluble sugars (c, d). Percentages between brackets indicate the percentage of variation in the data explained by the first two principal components that resulted from each model. (a, c) Scatter plots show the grouping pattern of samples from inflorescences and leaves on Days 4, 8 and 12 according to the first two principal components; the Hotelling's ellipse confines the confidence region (95%). (b, d) Loading plots show the contribution of each of the metabolites to the first two principal components. [Color figure can be viewed at wileyonlinelibrary.com]

infection. The loading plot, displaying the contribution of each amino acid to this separation, indicates that most of the protein-bound amino acids were positively correlated with tissues at the early time points (Figure 4b). This result is supported by the total concentration of protein-bound amino acids, which was 20% lower on Day 8 than on Day 4, and was further reduced by 27% from Days 8 to 12 in leaves and flower tissues overall (Supporting Information: Table S4). In terms of total concentrations, inflorescences had 38.4% higher levels of protein-bound amino acids than leaves (Supporting Information: Table S4). Proline, leucine and tyrosine were among the amino acids that contributed most to the combined effect of time and plant part on the composition of protein-bound amino acids (Supporting Information: Table S4; VIP values > 1).

3.5 | Plant dry biomass

On Day 4, inflorescences of plants exposed to aphids plus bacteria had lower dry biomass than those exposed to aphids plus *P. brassicae* (still eggs at Day 4) (ANOVA, Bonferroni post hoc test, $p = 0.048$) or nontreated (ANOVA, Bonferroni post hoc test, $p = 0.016$) (Figure 5a). The dry biomass of inflorescences of the other treated plants did not

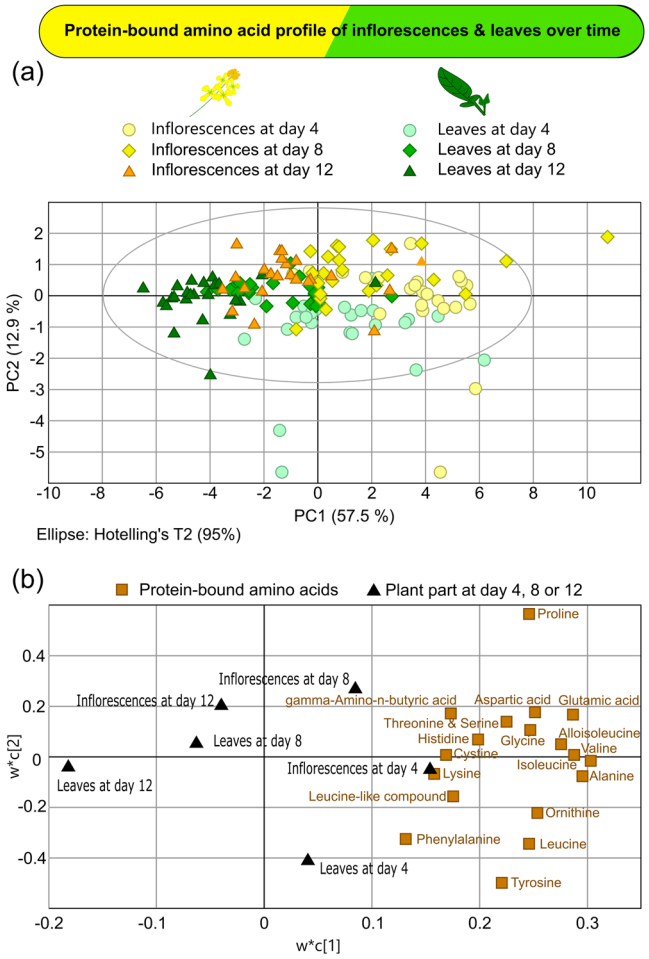

FIGURE 4 Profiles of protein-bound amino acids of inflorescences and leaves of *Brassica nigra* plants exposed to single and dual attack for 4, 8 or 12 days. Projection to latent structures- discriminant analysis (PLS-DA) based on the concentration of 19 protein-bound amino acids (µmol/g of plant dry biomass) that were detected and quantified in samples of *B. nigra* exposed to a single attack by *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or nontreated. Concentrations of protein-bound amino acids were measured in leaves and inflorescences of three plants per treatment after 4, 8 and 12 days of exposure to the treatments. Plant part and time since infestation/infection were set as classes. The PLS-DA resulted in a model with seven significant principal components, and model parameters were: $R^2X = 0.950$, $R^2Y = 0.374$, $Q^2 = 0.254 \pm SD\ 0.014$, $P_{CV\text{-}ANOVA} < 0.001$. Percentages between brackets indicate the percentage of variation in the data explained by the first two principal components that resulted from the model. (a) Scatter plots show the grouping pattern of samples from inflorescences and leaves on Days 4, 8 and 12 according to the first two principal components; the Hotelling's ellipse confines the confidence region (95%). (b) Loading plots show the contribution of each of the protein-bound amino acids to the first two principal components. [Color figure can be viewed at wileyonlinelibrary.com]

differ from that of inflorescences of control plants (buffer and nontreated) at this time point. At the later time points, on Days 8 and 12, inflorescences of attacked plants had similar biomass to inflorescences of control plants despite the physical damages inflicted by caterpillars and aphids (Figure 5b,c). Dry biomass of roots and vegetative parts was not affected by treatments at individual time points (Figure 5d,i), although when looking at the overall effect, roots of plants exposed to caterpillars plus bacteria had higher dry biomass than roots of control plants exposed to buffer (ANOVA, Bonferroni post hoc test, $p = 0.037$) and to single attack with aphids (ANOVA, Bonferroni post hoc test, $p = 0.017$).

Inflorescences had the highest increase in dry biomass over time, with an increase of 77% from Days 4 to 8 (ANOVA, Bonferroni post hoc test, $p < 0.001$), while dry biomass of roots and vegetative parts, respectively, increased by 46% and 23% from Days 4 to 8 (ANOVA, Bonferroni post hoc test, respectively, $p < 0.001$ and $p = 0.002$). While dry biomass of inflorescences and roots on Day 12 was similar to that on Day 8, dry biomass of vegetative parts decreased by 32% from Days 8 to 12 (ANOVA, Bonferroni post hoc test, $p < 0.001$).

4 | DISCUSSION

Our data suggest that *B. nigra* protect their reproductive parts with constitutive resistance rather than inducible resistance. Inflorescences contained up to seven times higher concentrations of glucosinolates than leaves, and attack induced changes in the glucosinolate concentration of leaves but not of inflorescences. Treatments affected both floral and foliar primary metabolites within 4 days following attacks, and this was driven by changes in the total concentration of soluble sugar. These changes in primary metabolism might have supported the early compensation for the damage that we observed, likely via the regrowth of inflorescence. Overall, the changes in the levels of metabolites we measured in leaves and inflorescences over time reflected the high investment of the plants into reproduction.

Optimal defense theory predicts that valuable tissues are highly defended (Barton & Koricheva, 2010; Herms & Mattson, 1992; McCall & Irwin, 2006; Stamp, 2003). A consequence of a plant's high investment in reproductive parts is that tissue richness in primary metabolites makes flowers a high-quality target for herbivorous insects, especially because nitrogen content is important for insect performance (Behmer, 2009; Nation, 2008; Schoonhoven et al., 2005). Because flowers are a strong sink and directly important for plant fitness, it is predicted that flowers evolve constitutive resistance mechanisms rather than inducible ones, as opposed to leaves (McCall & Irwin, 2006; Orians et al., 2011). In agreement with this, *B. nigra* had six times higher constitutive concentrations of glucosinolates in inflorescences compared to leaves, and no induction of glucosinolates was detected in inflorescences upon attack, only in leaves. Although concentrations based on dry plant biomass do not necessarily reflect the quantity of secondary metabolites effectively ingested by the attackers due to tissue-specific differences in structural carbons, such levels of glucosinolates in the inflorescences likely reflect plant investment in preventing floral damage. High constitutive resistance in inflorescences likely selects for a florivorous community mostly composed of specialists attackers that developed mechanisms to

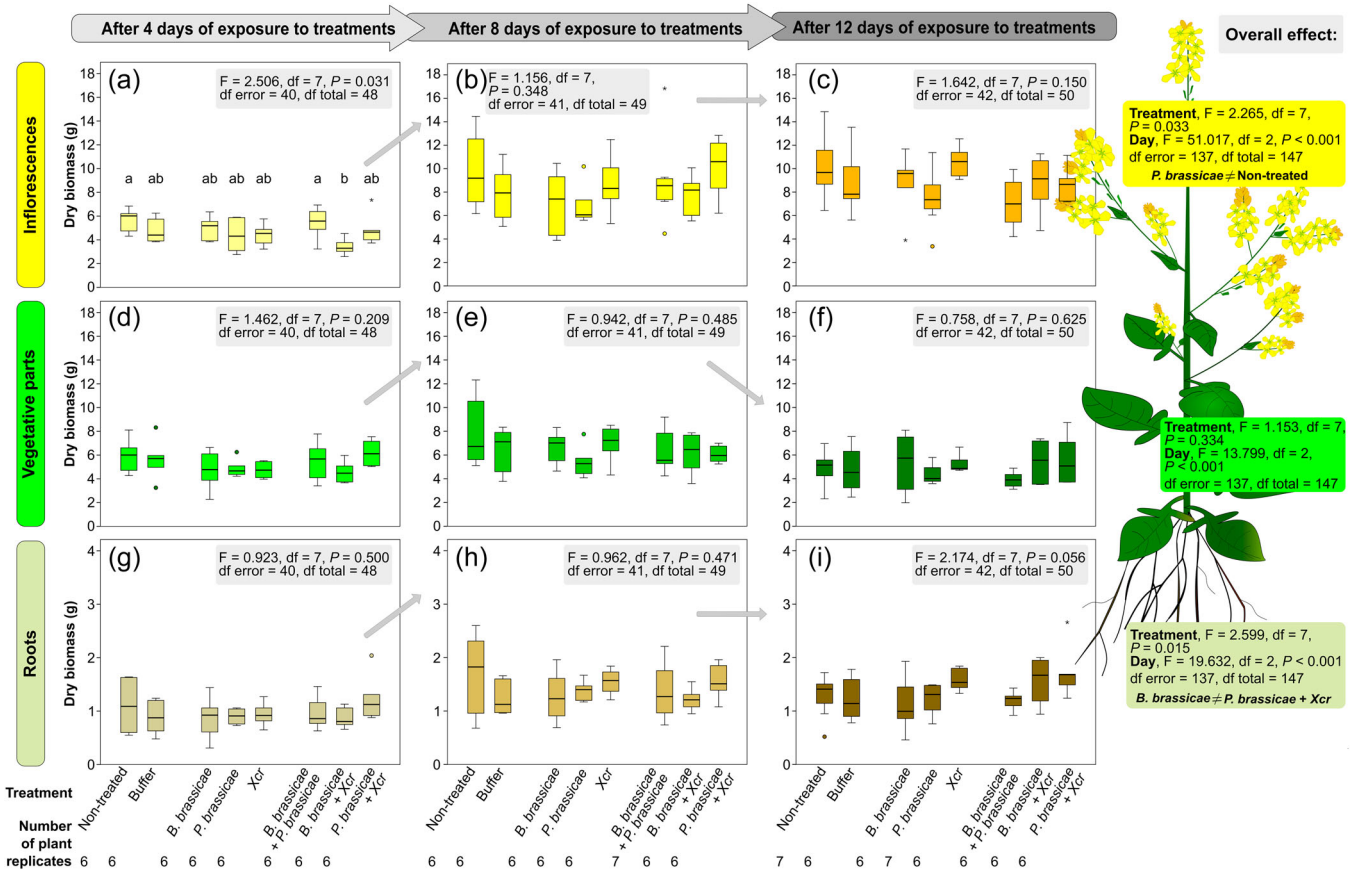

FIGURE 5 Dry biomass of inflorescences, vegetative parts and roots of *Brassica nigra* that were exposed to single or dual attack for 4, 8 and 12 days. Boxes indicate the interquartile range, the horizontal line shows the median, and the whiskers indicate the full range. Plants were exposed to a single attack by *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or nontreated. Inflorescences (a–c), vegetative parts (d–f) and roots (g–i) were harvested after 4 days (a–g), 8 days (b–h) and 12 days (c, f, i) of exposure to the treatments; plant parts were then dried and weighed. Grey arrows between graphs represent a significant increase (up) or decrease (down) in plant biomass from one time-point to the other, or no significant changes (horizontal). There were six plant replicates per treatment and time point. Statistics for each plant part and the three harvesting days are indicated in the panels. Overall statistics for the main effect of treatments and day are indicated on the right of the panels for each plant part. Effects of treatment in each panel and the overall effect of treatment and day were tested with an analysis of variance, and when significant, a Bonferroni post hoc test was used for pairwise comparisons. Lower-case letters above the bars indicate statistical outcomes of post hoc test. The significance level was set to $\alpha = 0.05$; test parameters are indicated in a grey frame in the panels. [Color figure can be viewed at wileyonlinelibrary.com]

reduce the toxicity of glucosinolates or sequestrate them, and against which induction of secondary metabolites might be an ineffective strategy. Alternatively, since glucosinolates such as sinigrin and 4-hydroxy-indolyl-3-methyl-glucosinolate can also be constitutively present in the nectar of *B. nigra* (Bruinsma et al., 2014), limiting the induction of toxic compounds in inflorescences may as well reduce the risk to alter rewards and floral cues used by mutualist pollinators (Rusman et al., 2019a; Strauss et al., 2002). When considering foliar glucosinolates, leaves of plants exposed to dual attack by insects plus bacteria for 4 days had higher levels when compared to situations with the insects alone, or with a dual attack by the two insect species. Plants may be preventing the spread of the pathogen from the inoculation site (inflorescences) to the leaves.

The glucosinolate composition of inflorescence could be primarily targetting chewing herbivores, which can physically remove floral structures. In our study, the composition was dominated by the aliphatic glucosinolate sinigrin, which accounted for about 99% of the glucosinolate concentration of inflorescences. Aliphatic glucosinolates are particularly toxic to chewing herbivores, especially when they are not specialized in Brassicales plants (Bekaert et al., 2012; Textor & Gershenzon, 2009), but have a little negative impact on aphid performance (Barth & Jander, 2006; Kos et al., 2012a; Züst & Agrawal, 2016). The other 1% represented four indolic and one aromatic glucosinolates. Up to 15 glucosinolates have been previously reported throughout the literature in leaves of vegetative *B. nigra*: seven aliphatic, four indolic and four aromatic glucosinolates (Bonnet et al., 2017; Ponzio et al., 2017a; van Dam et al., 2004). Thus, it seems that from the vegetative stage to the flowering stage, *B. nigra* maintains a large diversity of indolic glucosinolates, which may be a barrier against phloem feeders (Züst & Agrawal, 2016). For

example, the indolic glucosinolate 1-methoxy-indol-3-ylmethyl-glucosinolate, which was produced late in the flowering phenology, is known to reduce the reproduction of the aphid *Myzus persicae* (Kim & Jander, 2007), and could protect maturing siliques or be translocated to the seeds (Bellostas et al., 2007).

Despite the high constitutive concentrations of secondary metabolites, specialist attackers can still inflict strong damage on inflorescences. The final instar of *P. brassicae* caterpillars can, for example, remove 135 buds and flowers on *B. nigra* and significantly decrease seed production compared to noninfested plants in greenhouse tests (Smallegange et al., 2007, 2008). When facing such threats, plants may alternatively deploy tolerance strategies to mitigate the fitness effect of floral damage without targeting the attacker (Agrawal, 2011; Strauss & Agrawal, 1999). Our data suggest that attacker-specific changes in primary metabolism may translate into distinct metabolic changes that support tolerance of inflorescences to attack. Plants attacked by Xcr had lower foliar levels of sugars than nontreated plants and plants exposed to aphids plus Xcr, whereas floral levels did not differ. This might result from increased use of foliar resources to sustain tolerance and resistance activities in inflorescences in the face of attack (Schultz et al., 2013). Additionally, inflorescences of plants dually infested with *B. brassicae* plus *P. brassicae* (as eggs on day 4) had lower levels of soluble sugars than plants treated with *P. brassicae* only, or with Xcr, whereas foliar sugar concentrations did not differ. Foliar levels may be kept constant by adapting photosynthesis activity to fuel the floral demand of primary metabolites that contribute to the regrowth of tissue (Kerchev et al., 2012). Plant investment in growth is further supported by the biomass data. Indeed plants attacked by *B. brassicae* plus *P. brassicae*, *P. brassicae* only, or by Xcr, had similar dry biomass despite the fact that caterpillars were eating floral buds, flowers and stems. Previous work showed that leaves and inflorescences of blooming plants can specifically respond to different attackers in terms of phytohormonal induction, mainly via an induction of jasmonates (Chrétien et al., 2018). Besides the role of jasmonates as mediators of induced plant resistance to attack, they can also mediate the allocation of carbohydrates in vegetative plants (Machado et al., 2013; Schwachtje et al., 2006). Thus, changes observed in the primary metabolism of *B. nigra* may connect with the induction of jasmonates measured in a previous study (Chrétien et al., 2018).

Attack induced early metabolic changes, that is, within 4 days of exposure to lepidopteran eggs, aphids and a pathogen, coinciding with blooming, and no changes were measured at later time points when plants were in full bloom. This suggests that plants responded early to attack on their inflorescence, likely to activate coping strategies and maintain their reproductive success. This has been previously observed in *B.* nigra; they can flower earlier and produce seeds earlier than control plants after oviposition by *P. brassicae* butterflies (Lucas-Barbosa et al., 2013; Pashalidou et al., 2013). Inflorescence can respond to *P. brassicae* by an early reduction in total C/N ratio, which may reflect this acceleration of flowering upon attack (Lucas-Barbosa et al., 2017). *Brassica nigra* infested with *P. brassicae* can eventually compensate for herbivory in terms of biomass, and produce as many seeds as noninfested plants (Lucas-Barbosa et al., 2013). Young flowering plants need to ensure reproduction and may, besides, be more inducible than plants that are further advanced in their flowering (Barton & Koricheva, 2010).

Exposure to insects and pathogen attacks induced changes in metabolic profiles of *B. nigra* leaves and inflorescences that were of small magnitude compared with the differences quantified in leaves versus inflorescences and at different time points. Plants seemed to continue with physiological processes despite attack, using resources accumulated in the vegetative stage and investing them into reproduction. For example, leaves and flowers of *B. nigra* plants that had started flowering (4 days since the attack) had higher levels of protein-bound amino acids than older plants. Those high concentrations probably provided building blocks to sustain the development of flower stalks, flowers and buds, and some last leaf expansion (Borghi & Fernie, 2017). Additionally, our data show that inflorescences and leaves clearly had different primary metabolic profiles, especially in terms of free-circulating metabolites, which likely reflects plant investment in the development of these reproductive organs (Barneix & Causin, 1996; Mooney, 1972). We indeed measured four times higher levels of free amino acids in inflorescences than in leaves, and 50% higher levels of soluble sugars. High investment into inflorescences is commonly observed in annual plants that have only one opportunity to reproduce (Mooney, 1972).

It was clear from the primary metabolic profiles characterized in our study that inflorescences underwent important changes between Day 4 and Day 8 for both control and attacked plants. Notably, free-circulating aspartic acid and glutamic acid were more concentrated in inflorescences that started flowering than in older ones. Aspartic acid and glutamic acid are among the most concentrated amino acids in the phloem sap of leaves of boulting brassicaceous species such as *Brassica juncea*, *Brassica napus* and *Brassica campestris* (Weibull & Melin, 1990). Our data may reflect phloem flow to inflorescences that had initiated flower opening, which can provide resources necessary for the fast development to full bloom (Savage et al., 2016). Previous studies indeed showed that Arabidopsis plants with a mutation in a gene coding for a phloem protein suffered from delayed flowering, which was probably mediated by a lack of phloem allocation to the inflorescence (Kloth et al., 2017). In terms of soluble sugars, fructose, glucose and inositol were more abundant in inflorescences than in leaves. Besides their function as building blocks and energy supply (Mooney, 1972), fructose and glucose are the most abundant sugars in *B. nigra* nectar (Bruinsma et al., 2014) and proline is a reward to pollinators (Borghi & Fernie, 2017).

Defense mechanisms in inflorescences are still little understood, and our data suggest that *B. nigra* invests in strong constitutive resistance of flowers to limit colonization by attackers, and favours tolerance to attack by specialists over inducible resistance. Tolerance may be more effective against specialist attackers than resistance because specialist attackers are little affected by direct resistance traits of plants (Orians et al., 2011). Plants responded to attackers early after infection or infestation with changes in the profile of soluble sugars and free amino acids that likely supported

compensatory growth. Compensatory mechanisms could be typical of annual plants, which invest more resources accumulated during vegetative growth into reproduction before dying compared to perennials (Mooney, 1972). This pattern may be especially true for fast-growing plants, such as *B. nigra*. (Agrawal, 2011). Changes in primary metabolites may have trans-generational effects by influencing the composition of nutrients allocated to the seeds produced by the maternal plant upon attack, which can impact the germination and survival of young seedlings. Additionally, reprogramming of secondary and primary metabolic pathways upon attack is also likely to influence arthropod communities associated with plants in the flowering stage, with cascading effects on plant fitness that still need to be unravelled in the natural ecological context.

ACKNOWLEDGEMENTS

We acknowledge financial support provided from the École Normale Supérieure de Lyon (Quatrième année de normalien & Contrat Doctoral Spécifique aux Normaliens; to Lucille T. S. Chrétien), the Netherlands Organisation for Scientific Research, SPI87-313 (NWO, Spinoza award to Marcel Dicke), the Région Centre-Val de Loire and CNRS (to David Giron) and the COST FA1405 programme. We thank Michael Reichelt for his contribution to the analysis of glucosinolates, and Alexandre Villela and Karen Kloth for fruitful discussion, and advice on the chemical analyses.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available fromThe data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Lucille T. S. Chrétien http://orcid.org/0000-0002-4400-1086
Dani Lucas-Barbosa https://orcid.org/0000-0003-3440-1040
Marcel Dicke http://orcid.org/0000-0001-8565-8896
David Giron https://orcid.org/0000-0001-8356-0983

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SUPPORTING INFORMATION

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How to cite this article: Chrétien, L.T.S., Khalil, A., Gershenzon, J., Lucas-Barbosa, D., Dicke, M. & Giron, D. (2022) Plant metabolism and defence strategies in the flowering stage: Time-dependent responses of leaves and flowers under attack. *Plant, Cell & Environment*, 45, 2841–2855. https://doi.org/10.1111/pce.14363