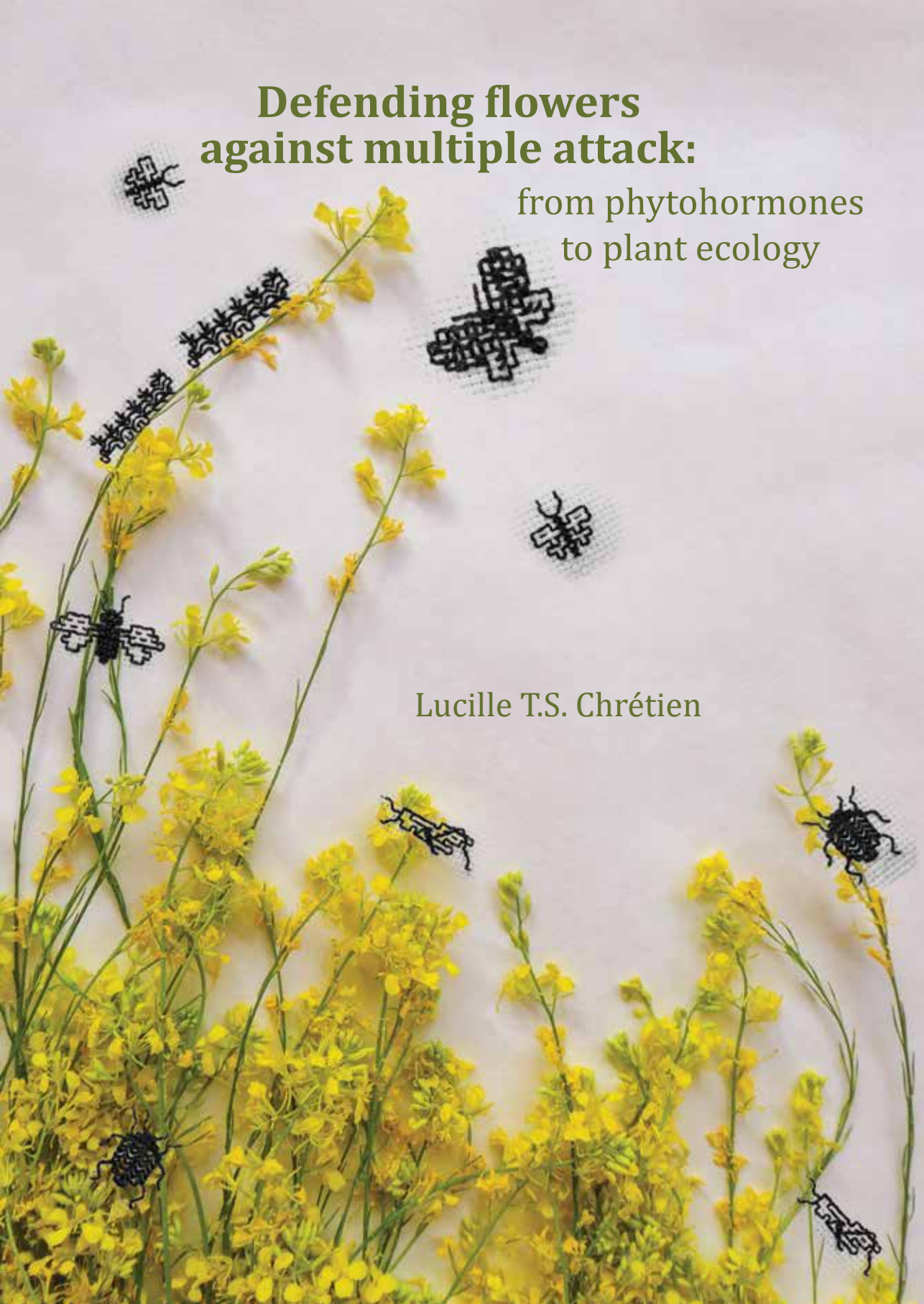
Defending flowers against multiple attack:

from phytohormones to plant ecology



Lucille T.S. Chrétien

Propositions

1. Flowers should be included in ecological studies of plant-insect interactions.
   (this thesis)

2. *Brassica nigra* is more resistant to florivory by a specialist caterpillar than by a specialist aphid.
   (this thesis)

3. Statistical tests are biased methods for unbiased detection of patterns in data.

4. Complexity of developmental sex determination is the basis for going beyond binarism in both biological sex and social gender.

5. The under-representation of insects in textbooks reflects the neglected importance of their ecosystem services.

6. The capital-driven publication system threatens academia's integrity.

7. Changes in languages are necessary for social mutations.

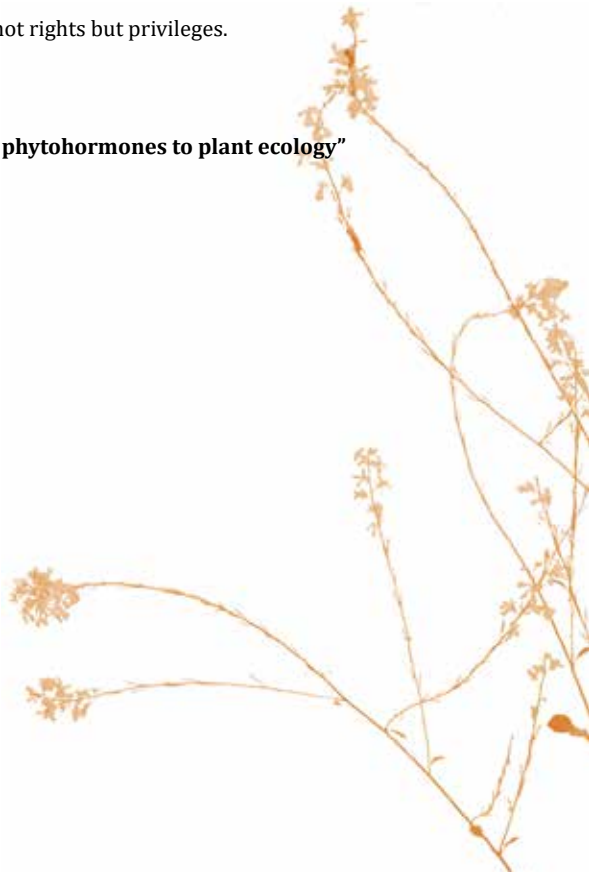
8. Social "rights" that marriage provides are not rights but privileges.

Propositions belonging to the thesis entitled:

"Defending flowers against multiple attack: from phytohormones to plant ecology"

Lucille T. S. Chrétien

Wageningen, 24 May 2019



Defending flowers against multiple attack: from phytohormones to plant ecology

Lucille T.S. Chrétien

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Defending flowers against multiple attack: from phytohormones to plant ecology

Lucille T.S. Chrétien

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“Je viens de voir une chose émouvante : la dernière mue d’un criquet, l’extraction de l’adulte de sa gaine larvaire. C’est magnifique.”

“I have just beheld a stirring sight: the last moult of a Locust, the extraction of the adult from his larval wrapper. It is magnificent.”

Jean-Henri Fabre,
in Souvenirs Entomologiques, Série VI,
Chapitre XVII, Les Acridiens – la dernière mue,
1899

To my family

Abstract

My thesis aimed at investigating the defensive and reproductive strategies of plants when flowers are under multiple attack by two florivorous insect species (aphid, caterpillar) and a phytopathogenic bacterium. The study of *Brassica nigra*, an annual outcrossing Brassicaceae, indicated that inflorescences under attack were more resistant to caterpillars than to aphids. This project identified jasmonates as the main phytohormones mediating plant responses to attack, and jasmonates were particularly upregulated in inflorescences exposed to single or dual attack by caterpillars. Glucosinolates were not induced in inflorescences and these defensive compounds likely did not mediate induced direct resistance against florivores. Attack induced changes in the volatile emission of plants in the flowering stage, especially when caterpillars were among the attackers. However, plants maintained their interaction with parasitoid wasps that mediated indirect defences against florivores. Moreover, changes in primary metabolism may have contributed to plant compensation for damage inflicted by the attackers and overall, to plant tolerance to attacks. Finally, plants maintained interactions with pollinators despite the phytochemical changes induced upon attack.

Court résumé

Ma thèse visait à étudier les stratégies de défense et de reproduction des plantes lorsque leurs fleurs sont attaquées conjointement par plusieurs insectes (puceron, chenille) et pathogènes, et à en comprendre les mécanismes sous-jacents. L'étude de la brassicacée annuelle *Brassica nigra* a indiqué qu'en cas d'attaque, les inflorescences étaient plus résistantes aux chenilles qu'aux pucerons. Ce projet a identifié les jasmonates comme les principales phytohormones induites dans les inflorescences, en particulier à la suite d'attaque simple ou double impliquant les chenilles. L'induction de la résistance directe contre les florivores ne semble cependant pas médiée par les glucosinolates, ces composés de défense n'étant en effet pas induits dans les inflorescences. Les attaques, en particulier par les chenilles, ont modifié l'émission de composés volatiles par les plantes en fleur. Cependant, les plantes ont maintenu leur interaction avec des guêpes parasitoïdes qui contribuent aux défenses indirectes contre les florivores. De plus, les changements du métabolisme primaire ont probablement contribué à la capacité des plantes à compenser, et tolérer, les dommages dus aux attaques. Enfin, malgré ces changements phytochimiques, les plantes ont maintenu leur interaction avec les pollinisateurs.

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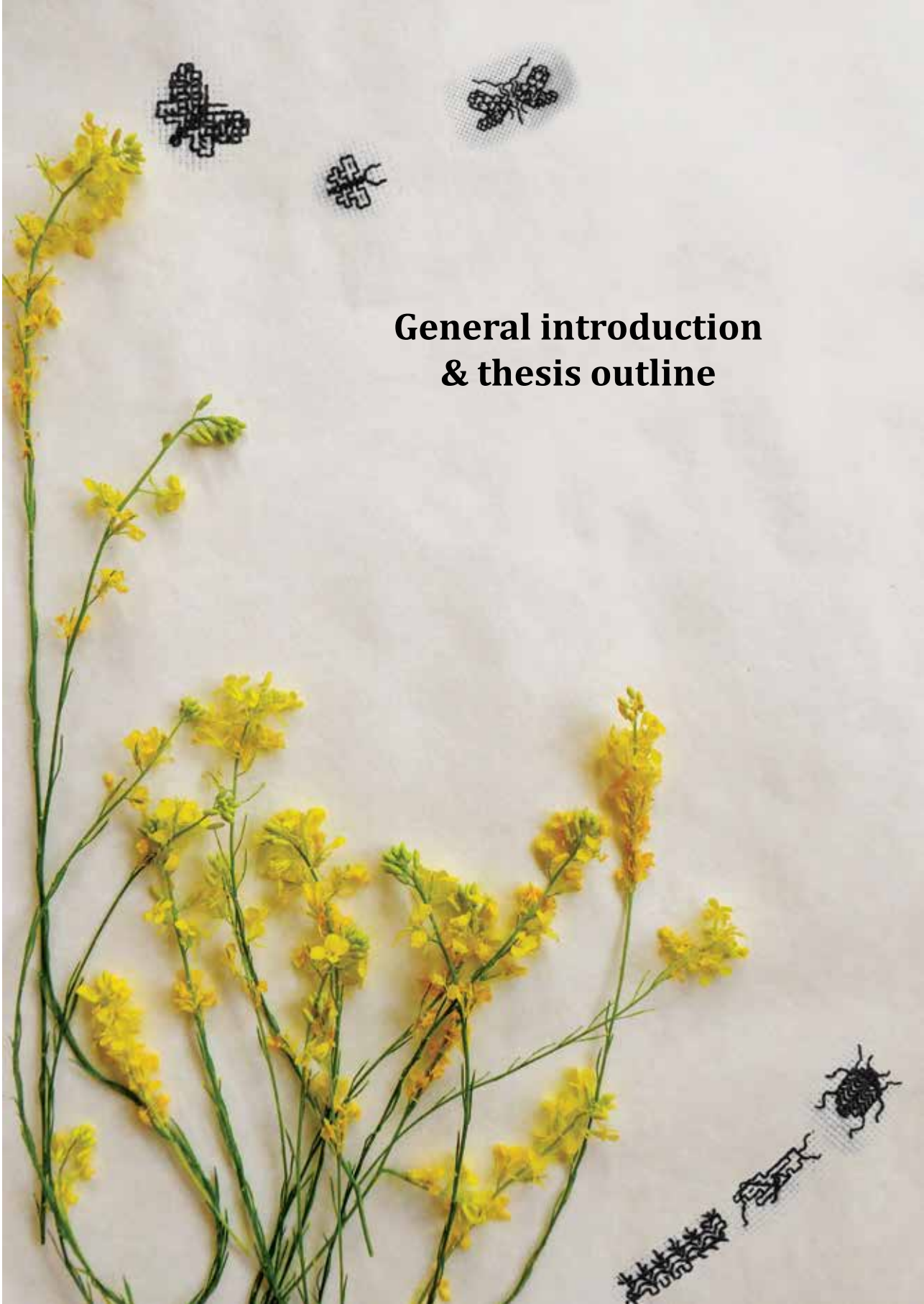

General introduction & thesis outline

General Introduction & thesis outline

Plant biochemistry can influence interactions with plant-associated community members (Ohgushi, 2005; Dicke & Baldwin, 2010; Abdala-Roberts *et al.*, 2016). With their commonly colorful and odorant display, flowers have a key role in this web of interactions. **Flowers** are reproductive structures typical of angiosperms, which is currently the most diverse group of land plants (Frame, 2003; Theissen & Melzer, 2007). **Pollinators** mediate the **reproduction** of as much as 87% of all angiosperm species, and likely contributed to the evolution of the incredible diversity of chemical and morphological structures that attract pollinators to flowers (Crane *et al.*, 1995; Ollerton *et al.*, 2011). Flowers of angiosperms were likely eaten by herbivores before pollinators evolved, and **florivores** likely contributed too to the evolution of flowers traits (Frame, 2003). Yet, defense responses of plants to attack on their flowers is still understudied, although **defense** strategies that protect flowers likely differ from those that protect leaves (McCall & Irwin, 2006). While leaves of plants ensure growth and accumulation of resources as plants defend themselves against attackers, flowers of outcrossing plants need to maintain interactions with mutualist pollinators as plant defend against attackers.

Defense of plants in the flowering stage

Decades of investigation on plants in the vegetative stage have established that plants evolved intricate strategies to defend themselves and limit the fitness impact of attack (Karban & Myers, 1989; Núñez-Farfán *et al.*, 2007; Agrawal, 2011; Karban, 2011; Erb *et al.*, 2012). On the one hand, plants can resist attack by deterring biotic invaders, or by limiting the performance of these attackers. Resistance mechanisms can be present constitutively or can be transiently induced after attack (Agrawal *et al.*, 1999; Dicke & Hilker, 2003; Agrawal, 2011; Karban, 2011). Direct resistance may directly counteract attackers through morphological structures such as trichomes, or *via* chemicals (secondary metabolites) that can be released in plant tissues as toxins (Berenbaum, 1995; Agrawal, 2011). Indirect resistance may indirectly decrease the herbivore load through the attraction of natural enemies of the attackers, often *via* the release on volatile secondary metabolites that act as cues (Dicke & Baldwin, 2010; Kessler, 2015; Dicke, 2016). On the other hand, tolerance limits the effects of attack on plant fitness without interfering with the attacker, for example by regrowth of damaged organs (Strauss & Agrawal, 1999; Núñez-Farfán *et al.*, 2007). It generally involves reallocation of resources among plant tissues (Schwachtje & Baldwin, 2008; Bolton, 2009; Schultz *et al.*, 2013). Resistance and tolerance responses to attack can both be systemic and can modify plant

chemistry and traits on a large scale. For flowering plants that rely on pollinators for reproduction, such systemic induction of defense can conflict with reproduction because plants need to continue attracting pollinators while defending against attackers (Kessler & Halitschke, 2009; Lucas-Barbosa *et al.*, 2011). Most studies have so far addressed plant defense for plants in the vegetative stage and against herbivores that feed on leaves (folivores), although attack of flowers can directly reduce seed production by plants and thus Darwinian fitness.

Herbivores that feed on inflorescences (bracts, flowers, pollen, and ovules, till seed coat development) are called florivores (McCall & Irwin, 2006). Together with pathogens that develop on inflorescences, florivores can directly reduce seed production by eating, damaging or killing parts of the plant's reproductive structures. Since flowers carry the gametes of plants, florivory or infection of floral tissues likely have a stronger impact on plant seed production than folivores, which may consequently affect plant fitness. Damage to flowers can indeed cause a stronger reduction in plant seed set compared to damages to leaves or stems (Wise & Rausher, 2013; Schlinkert *et al.*, 2015). Florivores can be additionally challenging to plants by their numbers and occurrence. Plants in the flowering stage generally attract a greater diversity and abundance of arthropods (Johnson & Agrawal, 2005; Johnson & Agrawal, 2007; Abdala-Roberts *et al.*, 2017). Inflorescences are not only apparent, and, thus, more likely to be found and attacked by herbivorous insects (Schlinkert *et al.*, 2015), but they also contain high levels of primary metabolites that fuel floral development and are nutritious to herbivores (Mooney, 1972). Inflorescences of plants thus provide a diversity of feeding niches to florivores or floral pathogens (Johnson & Agrawal, 2005; Smallegange *et al.*, 2007; Abdala-Roberts *et al.*, 2017), which can reduce plant reproductive success by the removal of flowers or the repellence of pollinators.

Florivore-induced responses may indirectly conflict with plant reproduction. Defense and reproduction can indeed trade off for plants upon to attack, and this trade-off may be particularly strong when inflorescences of plants are attacked (McCall & Irwin, 2006). Florivore-induced changes in plant morphological traits and chemistry may interfere with ecological interactions (ecological costs), and, for example, deter pollinators and carnivores, or attract other florivores (Strauss *et al.*, 2002; Lucas-Barbosa, 2016). Resources invested into defense or reallocated to attacked tissues as a tolerance mechanism may also decrease resources allocated to reproduction (energetic costs) (Herms & Mattson, 1992; Strauss *et al.*, 2002; Orians *et al.*, 2011). As a consequence, plants are expected to finely regulate tolerance and

resistance mechanisms, and to deploy such strategies optimizing investments in defense and reproduction. However, most studies have addressed this for plants in the vegetative stage, whereas little is known about responses of flowering plants to attackers, and even less when regarding the mechanisms and regulation of plant responses in flower tissues.

Plant defense is predicted to be the strongest in tissues that are the most valuable, *i.e.* the most important for plant fitness (Herms & Mattson, 1992; Stamp, 2003). In this regard, flowers are predicted to be more defended than leaves (McCall & Irwin, 2006). Flowers are as well expected to be more constitutively defended and less defended *via* inducible resistance than leaves, because of the high value of flowers as well as their conspicuousness that make florivorous attack predictable (Zangerl, 2003; McCall, 2006; McCall & Karban, 2006). Plants may indeed provide their inflorescences with constitutive levels of defenses as a first filter against potential attackers. For example, glucosinolate concentrations were up to three-fold higher in flowers than in leaves of Arabidopsis (Brown *et al.*, 2003) and five-fold for *Brassica nigra* (Smallegange *et al.*, 2007). Similarly, protease inhibitors, which are anti-digestive for insects, were several hundred times more concentrated in flowers of tomato plants than in leaves (Damle *et al.*, 2005). When investigating induction of compounds that mediate plant direct resistance, studies so far showed varying outcomes and it remains difficult to draw a general pattern. Plants can respond to attack on their inflorescences with an induction of non-volatile secondary metabolites at the scale of the inflorescence (Smallegange *et al.*, 2007; Boyer *et al.*, 2016), within the damaged flower (Ohnmeiss & Baldwin, 2000), and plants can as well show no induction of secondary metabolites (Zangerl & Rutledge, 1996; Smallegange *et al.*, 2007; Godschalx *et al.*, 2016). Plants can also change volatile emission when inflorescences are attacked (Dannon *et al.*, 2010; Lucas-Barbosa *et al.*, 2015), and such changes in odours can attract parasitoids (Dannon *et al.*, 2010). As a complement to resistance, tolerance may be used by plants to cope with attack on inflorescences. Mature leaves are responsible for carbon fixation, roots take care of nutrient uptake, and flowers are a strong resource sink (Mooney, 1972; Orians *et al.*, 2011). Upon florivorous attack, source tissues can still provide flowers with resources and support compensatory mechanisms such as regrowth (McCall & Irwin, 2006; Orians *et al.*, 2011). Tolerance response to florivory or artificial removal of reproductive structures have been reported across various plant species and families, and generally involve regrowth of damaged structures or reallocation of resources to the structures that remained undamaged (Rosenheim *et al.*, 1997; Wise *et al.*, 2008; Lucas-Barbosa, 2016; Lucas-Barbosa *et al.*, 2017). To understand

the strategies that plants evolved when facing attack on flowers, it is now needed to further link changes in plant traits upon response to attack with their ecological consequences on the different beneficial or detrimental members of the flower-associated community.

Phytohormones in plants in the flowering stage

Plant defense and reproduction are regulated by phytohormones that control many physiological processes including the production of an enormous diversity of secondary metabolites having defensive and reproductive functions (Koornneef & Pieterse, 2008; Dudareva *et al.*, 2013; Dicke & van Loon, 2014). Both processes rely on the induction of only a few phytohormones involved in canonical pathways that can interact with each other (Kunkel & Brooks, 2002; Thaler *et al.*, 2012; Dicke & van Loon, 2014). These phytohormones may be produced in leaves as well as in flower tissues.

Phytohormones and defense in plant foliage

The phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) have key positions in an intricate network of signal-transduction pathways that are induced in plants attacked on leaves (Fig. 1). The phytohormone JA is often associated with ET and chiefly orchestrates responses to chewing herbivores and necrotrophic pathogens, whereas the SA pathway is induced in response to phloem-feeding herbivores and biotrophic pathogens (Wu & Baldwin, 2010; Erb *et al.*, 2012; Lazebnik *et al.*, 2014; Okada *et al.*, 2015; Lortzing & Steppuhn, 2016; Checker *et al.*, 2018). Other phytohormones seem to be more specifically induced. Cytokinins (CK), for example, may reconfigure plant defense-related metabolism, and abscisic acid (ABA) is occasionally induced upon caterpillar feeding (Ton *et al.*, 2002; Bari & Jones, 2009; Dicke & Baldwin, 2010; Wu & Baldwin, 2010; Karban, 2011; Vos *et al.*, 2013; Giron & Glevarec, 2014; Brütting *et al.*, 2016; Erb, 2012). Plant responses to attack on leaves are, therefore, specific to the inducing attackers, and this may allow plants to fine-tune their defense (Erb *et al.*, 2012; Dicke & van Loon, 2014).

Phytohormonally regulated pathways specifically induced in response to attack can crosstalk when several attackers are simultaneously damaging a plant (Fig. 1). Such crosstalk can positively, neutrally, or even negatively impact phytohormonal levels or phytohormonal effects (Thaler *et al.*, 2002b; Heidel & Baldwin, 2004; Rodriguez-Saona *et al.*, 2010; Duceppe *et al.*, 2012; Soler *et al.*, 2012; Checker *et al.*, 2018). A diverse family of JA co-receptors and transcriptional repressors, the JAZ (JASMONATE-ZIM DOMAIN) proteins, which seem to have distinct functions (despite

some redundancy), likely regulate crosstalk between the JA-signalling pathway and signalling pathways mediated by other hormones (Farmer, 2007; Kazan & Manners, 2012). In leaves of young plants challenged by different types of attackers, SA and JA typically have an antagonistic relationship and ET and JA often act synergistically (Kunkel & Brooks, 2002; Thaler *et al.*, 2002a; Koornneef & Pieterse, 2008; Thaler *et al.*, 2010; Thaler *et al.*, 2012; Zhu & Lee, 2014), although exceptions have been found (Koornneef and Pieterse 2008). The interplay at the phytohormonal level tends to correlate with patterns observed at the transcriptomic (Heidel & Baldwin, 2004) and proteomic levels (Rodriguez-Saona *et al.*, 2010; Duceppe *et al.*, 2012). Therefore, hormonal responses can affect plant susceptibility or resistance in the context of multiple attack, and this effect can operate at different spatial and time scales (Rostás *et al.*, 2003; Thaler *et al.*, 2012; Hauser *et al.*, 2013; Lazebnik *et al.*, 2014).

Phytohormonal regulation of flowering

These same phytohormones (JA, SA, ET, ABA, CK) that mediate resistance in vegetative plants are also involved in the regulation of plant reproduction (Meilan, 1997; Avanci *et al.*, 2010; Lortzing & Steppuhn, 2016, Fig. 1). SA, CK, and auxins, for example, mediate plant phenology and can promote the transition to flowering (Bavrina *et al.*, 1999; D'Aloia *et al.*, 2011; Rivas-San Vicente & Plasencia, 2011). ABA has a developmental role and can regulate inflorescence architecture (Han *et al.*, 2014). At a smaller scale, these phytohormones contribute to flower development and shape flower traits. JA, for example, is involved in the development of flowers and pollen production (Stintzi & Browse, 2000; Avanci *et al.*, 2010). Signalling based on the active form of JA, the jasmonoyl-L-isoleucine, plays a role in limb expansion and opening, and in floral volatile emission (Stitz *et al.*, 2014; Li *et al.*, 2018). ABA may also regulate flower development, and levels of ABA peaks in ovaries and stamen during flower development (Leng *et al.*, 2017). Additionally, ABA may play a role in flower senescence and associated changes in pigmentation (Ferrante *et al.*, 2006), and ET drives post-pollination changes in flowers (O'Neill, 1997). Because of shared signalling pathways, plant phytohormonal responses to attack may affect flowering.

Induction of signal-transduction pathways upon attack can affect plant traits linked to reproduction. For example, an attack-induced increase in SA level can activate the transition to flowering (Martínez *et al.*, 2004; Wada & Takeno, 2010; Carella *et al.*, 2015). Exogenous application of SA on vegetative plants may as well enhance the number of inflorescences developed later by the plant and increases total flavonoid

content of inflorescences (Pacheca *et al.*, 2012). Additionally, exogenous application of JA on flowers of *Brassica napus* induced floral nectar secretion (Radhika *et al.*, 2010). Yet, only few studies have addressed the phytohormonal regulation of defense mechanisms against attackers in inflorescences.

Phytohormones in defense of flowers

JAs are involved in the production of constitutive levels of defensive compounds in flowers. This likely operates *via* a flower-specific JAZ that may mediate flower-specific defenses (Li *et al.*, 2017). Levels of JA-Ile, for example, negatively correlated with the percentage of damaged buds in the field for *Nicotiana attenuata* (Li *et al.*, 2018). This correlation was associated with the concentration of plant secondary metabolites: flowers that were deficient for JA-Ile biosynthesis or perception contained lower concentrations of various secondary metabolites (nicotine, trypsin protease inhibitors, (*E*)-α-bergamotene, among others) than flowers of control plants, and had three to six times more flowers and buds being damaged by florivores than control plants (Li *et al.*, 2018). Yet, little is known about hormonal changes upon attack of flowers. In the native tobacco *N. attenuata*, no induction of JA and ET was detected in leaves of plants in the flowering stage upon caterpillar folivory, whereas higher levels of these phytohormones were quantified in leaves of plants in the vegetative stage exposed to herbivore attack than on control plants (Diezel *et al.*, 2011). Interestingly, when inflorescences were removed, foliar induction of JA and ET upon damage was recovered (Diezel *et al.*, 2011). A hypothesis is that flowering *N. attenuata* did not respond to folivory by caterpillars with changes in leaves, but only with changes in inflorescences. It remains to be investigated whether and how phytohormones are induced in inflorescences upon attack, and whether such response to attack would promote plant defense and/or interfere with reproduction (Fig. 1).

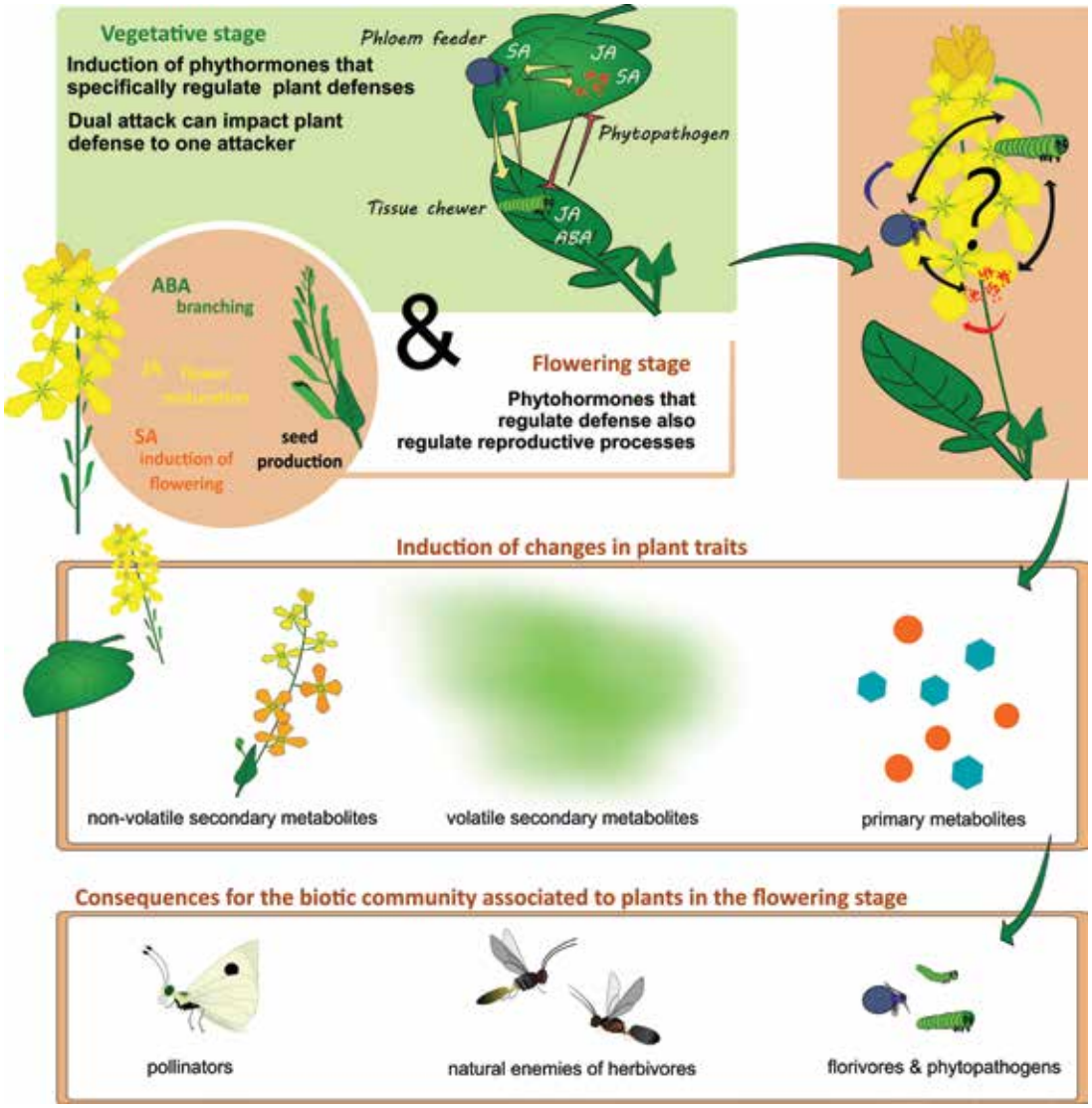

Fig. 1. Schematic representation of functions of phytohormones in plants and the chemical and ecological consequences of the induction of phytohormonally-regulated defense pathways in response to attack on flowers. Phytohormones that are known to regulate defenses of plants in the vegetative stage also regulate flowering processes of plants in the flowering stage. When several attackers are simultaneously attacking a plant, phytohormonally-regulated defense pathways induced upon attack can crosstalk and affect the outcome of plant defenses. When flowers of plants are under attack, phytohormonally-regulated defense responses of plants may alter traits of plants, such as chemical compounds that are commonly involved in direct resistance (non-volatile secondary metabolites), in indirect resistance (volatile secondary metabolites), or supporting tolerance processes. Changes in plant traits can have consequences for the abundance and composition of community members associated with the plant in the flowering stage, ranging from mutualistic members (pollinators, natural enemies of the attackers) to antagonistic members (florivores). Flat-ended red arrows show inhibition, yellow arrows show facilitation.

Flower traits that change upon attack

The impact of folivores on flower traits has received increasing attention in the last decade (Quesada *et al.*, 1995; Kessler & Halitschke, 2009; Diezel *et al.*, 2011), whereas studies focusing on the impact of florivorous insects and pathogens have just begun (Frame, 2003; McCall & Irwin, 2006; Wackers *et al.*, 2007; Lucas-Barbosa, 2016). The concentrations of a broad range of plant primary and secondary metabolites are modified in response to attack, and changes can be local or systemic in the plant (Treutter, 2006; Karban, 2011; Dicke & van Loon, 2014; Dormont *et al.*, 2014). They range from non-volatile chemicals involved in low digestibility and palatability of tissues to volatile blends (Kessler & Baldwin, 2001; Treutter, 2006; Dicke *et al.*, 2009; Textor & Gershenzon, 2009; Schuman *et al.*, 2012). As a consequence, they can interfere with cues and rewards displayed to attract pollinators like flower scent, color and nectar, or pollen quality (O'Neill, 1997; Strauss, 1997; Lucas-Barbosa *et al.*, 2011; Rodriguez-Saona *et al.*, 2011; Pacheca *et al.*, 2012; Bruinsma *et al.*, 2014). Therefore, plants in the flowering stage are expected to fine-tune investments in inducible defenses and reproductive traits (Molyneux & Ralphs, 1992; Robertson *et al.*, 1999; Wright & Schiestl, 2009; Schiestl *et al.*, 2014; Theis *et al.*, 2014). So far, studies addressing induced changes in chemical traits of flowers upon attack mainly focused on plant volatile emission, and little is known about resistance mechanisms of flower tissues. Most data acquired are based on attack with one type of attacker at a time, and experiments with multiple attack would now bring a better understanding of plant responses to attack on flowers in natural situations. Studies also mostly focused on artificial removal of floral tissues and florivory by tissue chewers that directly remove plant tissues, which may trigger very different responses compared to less conspicuous feeders such as phloem- or cell-sucking insects or pathogens.

<u>Induction of non-volatile secondary metabolites</u>

Induction of secondary metabolites upon damage to flowers has been observed at a local scale. Damage to the corolla of a wild tobacco flower (*N. attenuata*, Solanaceae) indeed induced a two-fold increase in nicotine levels in the same flower (Euler & Baldwin, 1996). When considering inflorescence-wide induction of secondary metabolites, results show varying outcomes. Under artificial florivory, flowers of Lima bean (*Phaseolus lunatus*, Fabaceae) showed no induction of cyanogenic compounds, which are toxins that mediate resistance in this plant species (Godschalx *et al.*, 2016). Similarly, upon mechanical damage to one flower of *Impatiens capensis* (Balsaminaceae), no induction of tannins was measured in flowers of the same branch or on parallel branches. Interestingly, when 30% of the

tissue of the induced flower was removed, levels of anthocyanins increased in the same branch for plants with red flower morphs, but decreased in flowers of parallel branches of yellow morphs. No induction was found when a higher percentage (60%) of damage was inflicted to the damaged flower (Boyer *et al.*, 2016). Results are in accordance with measurement of resistance upon attack by *Pieris brassicae* caterpillars. In the wild mustard (*B. nigra*, Brassicaceae), induction of floral secondary metabolites upon caterpillar folivory and florivory was observed in only one out of three plant accessions (Smallegange *et al.*, 2007). The magnitude of the changes in glucosinolate concentrations upon attack was also minor compared to the differences between plant accessions and plant parts (Smallegange *et al.*, 2007). In line with this, florivory by *P. brassicae* caterpillars did not affect the UV-vis profile of flowers of *B. nigra*, an indication that color did not change, compared to control plants and had little effect on non-volatile phenolic compounds (Lucas-Barbosa *et al.*, 2015). Thus, there is so far little evidence for induction of secondary metabolites in inflorescences upon attack, which may contribute to preserving interactions with pollinators upon attack, but may as well limit plant induced resistance to attack on flowers (Fig. 1).

Induction of volatile secondary metabolites

Flower-associated organisms may also affect the composition and quantity of volatile compounds emitted by plants in the flowering stage (Fig. 1). For example, epiphytic bacteria of flowers may directly contribute to the floral volatile blend, or induce or decrease the emission of floral volatiles by the plants (Helletsgruber *et al.*, 2017). In *B. nigra,* both folivory by *P. brassicae* and florivory affected the composition of the whole-plant VOC blend but treatments explained a much larger variation under folivory than florivory (Lucas-Barbosa *et al.*, 2015). Florivory by the Parsnip webworm (*Depressaria pastinacella*), for example, greatly increased VOC emission by the attacked umbel (local), and especially induced an increased emission of octyl esters by umbels of wild parsnip (*Pastinaca sativa*) (Zangerl & Berenbaum, 2009). Similarly, damage by *Helicoverpa zea* caterpillars on flower buds of cotton plants affected floral volatile emissions of the damaged buds (local) and of undamaged leaves (systematic) compared to undamaged control plants (Röse & Tumlinson, 2004). Compounds that were constitutively released by tissue rupture, or whose emission was up- or down-regulated by florivory are known to be involved in the attraction of natural enemies, attraction or repellence of other herbivores, and attraction of pollinators in this system (Röse & Tumlinson, 2004). It remains to be further understood whether such chemical changes in response to florivory are adaptive.

Induction of primary metabolites

Changes in floral primary metabolites have received little attention although they likely contribute to plant defense responses to attack (Fig. 1). Upon artificial removal of buds, aerial parts of cotton plants showed a slight increase in total amounts of both nitrogen and sugars, and qualitative differences were detected compared to non-damaged control plants (Dale, 1959). Flowering *B. nigra* plants exposed to folivory by *P. brassicae* caterpillars had a lower total C/N ratio than control plants, in both inflorescences and leaves (Lucas-Barbosa *et al.*, 2017). However, no effect was found after caterpillars had moved from the leaves to the inflorescences and fed from them (Lucas-Barbosa *et al.*, 2017). Folivory by *P. brassicae* also modified the sugar composition of nectar of flowers of *B. nigra*, and florivory by *Meligethes rufimanus* reduced by three-fold the amount of nectar produced by *Isomeris arborea* flowers (Krupnick *et al.*, 1999). Such phenotypic changes may have extensive consequences for interactions of plants with the surrounding community by directly affecting plant nutritional quality to the attacker, or reward quality to mutualists.

Ecological consequences of attacker-induced changes in flower traits

There is now ample evidence that plant traits can shape plant interactions with mutualistic and antagonistic flower-associated community members (Soler *et al.*, 2005; Poelman *et al.*, 2008; Heil & Karban, 2009; Kessler & Halitschke, 2009; Zangerl & Berenbaum, 2009; Dicke & Baldwin, 2010; Pareja *et al.*, 2012; Stam *et al.*, 2014). Constitutive levels secondary metabolites in flowers can deter herbivores from feeding. In *Raphanus sativus*, for example, generalist herbivores and specialists both preferred to feed from white-color-morph flowers over pink ones, and although color morph did not affect the performance of the specialists, generalists performed better on the white morph (McCall *et al.*, 2013). Such preference may be mediated by the chemical content of floral tissues (Johnson *et al.*, 2008; Tsuji & Sota, 2010; Tsuji & Sota, 2013). As a support to this, the purple areas of petunia flowers that contain phenolics, for example, suffer less damage by caterpillars of *Helicoverpa zea* and *Trichoplusa ni* than white areas do (Johnson *et al.*, 2008). In contrast, VOCs and floral size of a cucurbitaceous species seemed to be the main traits correlating with florivory by specialist beetles, whereas toxic cucurbitacins showed little correlation with florivory (Theis *et al.*, 2014). In terms of primary metabolism, nitrogen and sugar content of plants (*i.e.* the nutritional quality of tissues) has a direct impact on herbivore performance and can sometimes even outweigh the detrimental effect of plant secondary metabolites (Augner, 1995; Behmer, 2009; Hervé *et al.*, 2016; Cao *et al.*, 2017). Caterpillars of *P. brassicae,* for example, preferred to feed from nutrient-rich inflorescences of *B. nigra* than from leaves despite the high levels of glucosinolates of

the inflorescences (Smallegange *et al.*, 2007). Therefore, plant responses to attack of flowers that involve changes in such floral traits can also affect the flower-associated community (Fig. 1). An integrated approach that considers different types of chemical changes induced upon attack will bring a better insight in how plants responses to attack on their inflorescences can affect the community associated to inflorescences.

Consequences for attackers of inflorescences

Upon attack, the induction of changes in plant traits influences arthropod occurrence and abundance in the field (Fig. 1). The response of the community members seems to be especially strong when the initial inducer is a specialist feeder (van Zandt & Agrawal, 2004; Viswanathan *et al.*, 2005; Kessler & Halitschke, 2007; Poelman *et al.*, 2008; Poelman *et al.*, 2010; Poelman & Dicke, 2014; Stam *et al.*, 2018). First studies on plant-mediated interactions and the flower-associated community have only just been initiated (Poelman & Dicke, 2014; Stam *et al.*, 2018). In the perennial White cabbage (*Brassica oleracea*), folivory by early-season attackers in the first year of vegetative growth modified the composition of the herbivore arthropod community associated with flowers in the subsequent year (Stam *et al.*, 2018). Concerning florivores, it was shown that artificial florivory systemically decreased florivory (Boyer *et al.*, 2016). However, high levels of secondary metabolites may as well deter carnivores (Hunter, 2003; Soper Gorden & Adler, 2016).

Consequences of carnivore attraction

There is limited literature addressing indirect defenses of reproductive parts of plants. Flower-based predators have rarely been considered as potential predators of herbivores and thus, as beneficial to the plant (Romero & Vasconcellos-Neto, 2004; Higginson *et al.*, 2010; Knauer *et al.*, 2018). Generalist carnivorous arthropods can greatly contribute to plant indirect defense. For example, florivore-induced emission of the volatile β-ocimene made flowers of the buckler-mustard *Biscutella laevigata* more attractive to crab spiders, and this may benefit the plant because the spiders mainly predated the florivores (Knauer *et al.*, 2018, Fig. 1). Another type of generalist carnivorous insects, social wasps, spent more time searching for caterpillar prey in field plots of flowering *B. nigra* plants that were infested with florivorous *P. brassicae* caterpillars, and wasps exerted a strong predation pressure, effectivey controlling numbers of this damaging florivore (Lucas-Barbosa *et al.*, 2014). However, these generalist predators may conflict with pollination by also predating on pollinators, which is well known for thomisid crab spiders (Heiling *et al.*, 2004; Gonçalves-Souza *et al.*, 2008; Vasconcellos-Neto *et al.*, 2017). Parasitoids, which are generally specialized on a few host species, may offer a

good compromise for plants. Cowpea flowers damaged by caterpillars successfully attracted parasitoids when compared to non-infested flowers (Dannon *et al.*, 2010).

Consequences for pollinator attraction

Attack on the inflorescences of a plant can directly reduce pollinator attraction by the presence of feeding damage, and may indirectly interfere with the attraction of pollinators because of induced changes in floral traits (Fig. 1). For example, *D. pastinacella* caterpillars build a protective silken web that not only prevents the access of carnivores, but also limits the access of umbels of *P. sativa* to pollinators (Krupnick *et al.*, 1999). In *I. arborea* plants, florivory by pollen beetles (*M. rufinamus*) reduced the number of healthy floral branches, of healthy flowers and the volume of nectar produced, and the association of these direct consumptive effects and indirect effects *via* nectar rewards reduced visitation by pollinators (Krupnick *et al.*, 1999). Similarly, artificial removal of petals decreased the attraction of pollinators to flowers of *Eurya japonica* (Tsuji & Ohgushi, 2018). In this same system, pollen export from anthers and pollen deposition on stigmas were reduced in flowers attacked by *M. rufinamus* (Krupnick & Weis, 1999). Florivory can also alter floral traits that are exploited by pollinators for foraging, which can impact the behavior of pollinators. For example, florivory by *P. brassicae* on *B. nigra* altered floral composition of phenolics, which can be linked to tissue color, and the floral volatile emitted (Lucas-Barbosa *et al.*, 2015). Plant responses to *P. brassicae* florivory resulted in a decrease in the attraction of syrphid flies, which visited fewer flowers and spent less time per flower than on non-infested plants (Lucas-Barbosa *et al.*, 2015). Such direct and indirect effects of floral attackers on the attraction of pollinators may be more consequential for plant fitness than attack to leaves that only indirectly affect pollinators.

Both parasitoids and pollinators use VOCs as foraging cues, thus attraction of parasitoids and pollinators may trade off (McCall & Irwin, 2006; Lucas-Barbosa *et al.*, 2011; Lucas-Barbosa, 2016). This conflict has been addressed for flowering plants upon leaf herbivory only and still remains to be investigated for plants under attack by folivores. Flowering *Brassica rapa* prioritized the recruitment of pollinators over the recruitment of parasitoids upon herbivory by *P. brassicae* (Schiestl *et al.*, 2014; Desurmont *et al.*, 2015), whereas *Sinapis alba* attracted both pollinators and parasitoids upon herbivory by *Lipaphis erysimi* and *Myzus persicae* (Pareja *et al.*, 2012). Overall, the outcome for plant reproductive success likely depends on the natural availability of pollinators and carnivores in the plant's environment.

Conclusions

To date, most studies have focused on plants in the vegetative stage when addressing defense responses to biotic stresses, although recent findings suggest that flower feeders may strongly challenge plant defensive mechanisms and interfere with reproduction (McCall & Irwin, 2006; Lucas-Barbosa *et al.*, 2011; Lucas-Barbosa, 2016). In particular, annual plants have no opportunity to postpone investments in reproduction to another year as perennial plants may do, so they need to face all challenges at once. In this context, the current project took a multidisciplinary approach to investigate how plants deal with defensive and reproductive strategies when flowers are exposed to multiple attack. I worked with the Black mustard, *B. nigra*, an annual outcrossing flowering plant, and three inducers that commonly attack brassicaceous plants: the sucking aphid *Brevicoryne brassicae* and the chewing caterpillar *P. brassicae*, which both prefer to feed from flowers, and a bacterial phytopathogen *Xanthomonas campestris* pathovar *raphani* that can infect any developmental stage of the plant**.**

Objectives of this study

The overall objective is to investigate whether and how **plants in the flowering stage** exposed to **multiple biotic stresses** differentially invest in **defense and reproduction** when **inflorescences are attacked**, and to study the role of phytohormones as orchestrators of interactions between plants and their mutualistic and antagonistic flower-associated organisms. To achieve this, this thesis focuses on several layers of biological integration through four sub-objectives:

Objective 1: To measure the concentration of phytohormones that are involved in reproduction and defense of plants when inflorescences are exposed to single or dual attack by a sucking insect, a chewing insect, and a phytopathogen.

This objective aims at quantifying the concentration of phytohormones that have a central role in orchestrating defense and reproduction of plants: SA, ABA, and jasmonates including JA, and at comparing their concentrations in inflorescences of *B. nigra* upon exposure to single and dual attack by a phytopathogen, a sucking insect and a chewing insect.

Objective 2: To identify plant traits involved in resistance, tolerance, and reproduction, which are modified when inflorescences are exposed to single or dual attack by a sucking insect, a chewing insect, and a phytopathogen.

This objective aims at determining how plant respond to attack on inflorescences by

a phytopathogen, a phloem-sucking insect, and a chewing insect, 1) by quantifying the concentration of primary metabolites (amino acids, sugars) and secondary metabolites (glucosinolates and VOCs), and 2) by analysing variation across plant parts and over time in flowering *B. nigra* plants.

Objective 3: To assess whether and how the performance of florivorous insects and their natural enemies changes when inflorescences are exposed to single or dual attack by a sucking insect, a chewing insect, and a phytopathogen.
This objective aims at investigating if and how plant responses to attack on inflorescences by a phytopathogen, a phloem-sucking insect and a chewing insect 1) impact the performance of the sucking and chewing herbivores feeding from flowers in the greenhouse and in the field, and 2) affect the foraging behavior and parasitization success of their respective predators and parasitoids in the greenhouse and in the field.

Objective 4: To determine fitness consequences for plants when inflorescences are exposed to single or dual attack by a sucking insect, a chewing insect, and a phytopathogen.
This objective specifically aims at determining how plant responses to single and dual attack on inflorescences by a phytopathogen, a sucking insect and a chewing insect 1) affect the attraction and foraging of pollinators that mediate reproduction, 2) influence the occurrence and abundance of florivores in the field, and 3) impact plant seed production in the field.

Study system
This project focuses on the **Black mustard *Brassica nigra*** (Brassicales: Brassicaceae), a wild flowering plant species native to Europe, where they naturally grow together in large patches in disturbed open areas (Bell & Muller, 1973; Meyer, 2000). *Brassica nigra* is particularly suitable to study how plants in the flowering stage face florivorous attacks: it is mainly outcrossing (Conner & Neumeier, 1995; Lucas-Barbosa *et al.*, 2013), and thus relies on pollinators for reproduction; it is a fast growing annual plant, meaning that it has only one opportunity to reproduce. Defense mechanisms of Brassicaceae in the vegetative stage are well understood, and there is extensive literature addressing physiological and ecological impacts of multiple attack on leaves of *B. nigra* (Smallegange *et al.*, 2007; Lucas-Barbosa *et al.*, 2013; Pashalidou *et al.*, 2013; Lucas-Barbosa *et al.*, 2014; Ponzio, 2016; Bonnet *et al.*, 2017; Rusman *et al.*, 2018). *Brassica nigra* contains high concentrations of glucosinolates, which are key compounds mediating resistance to herbivores

and pathogens in Brassicales (Textor & Gershenzon, 2009; Bekaert *et al.*, 2012). Glucosinolates breakdown products are detrimental to the consumers (Hopkins et al 2009). Upon tissue damage, and especially when plant cells break, glucosinolates come in contact with myrosinase enzymes that break them down to highly toxic compounds such as isothiocyanates or less toxic nitriles (Hopkins *et al.*, 2009; Brown & Hampton, 2011; Mithöfer & Boland, 2012). Electrophilic isothiocyanates are suggested to react with proteins and nucleic acids, leading to their inactivation (Brown & Hampton, 2011; Mithöfer & Boland, 2012). The pungent taste of glucosinolate breakdown has been used for centuries in cooking worldwide, and glucosinolate-rich seeds of mustards are the base of the typical Dutch and French mustard. In the flowering stage, glucosinolate levels are especially high in flowers where they are up to five times more concentrated than in the leaves (Smallegange *et al.*, 2007). The aliphatic glucosinolate sinigrin, and the indolic glucosinolate 4-hydroxyglucobrassicin, and the phenolic glucosinolate phenylethylglucosinolate are present at significantly higher levels in flowers than in leaves of *B. nigra* (Smallegange *et al.*, 2007).

To the best of my knowledge, there is a limited number of studies extensively describing the community of **florivorous insects and pathogens attacking** B. nigra. Specialist flower-chewing *Meligethes aeneus* (Coleoptera: Nitidulidae) and the specialist phloem-feeder *B. brassicae* aphids have been reported as being the main florivorous insects on *B. nigra* in France, and their abundance varies according to the *B. nigra* populations (Bischoff & Trémulot, 2011). Caterpillars of *Pieris brassicae* have as well been described as florivores on *B. nigra* (Smallegange *et al.*, 2007). Throughout the project, *B. nigra* plants were exposed to two insect species and one bacterial species that are specialists on Brassicales or Brassicaceae. They were selected based on their feeding/infection mode and the distinct responses they induce in the plants, as reported for Brassicaceae in the vegetative stage.

***Brevicoryne brassicae* aphids** (Hemiptera: Aphididae) were selected as phloem feeders, which are expected to mainly induce the SA pathway (Mewis *et al.*, 2005; Koornneef & Pieterse, 2008; Erb *et al.*, 2012). *Brevicoryne brassicae* particularly thrives on inflorescences of *B. nigra* where they develop larger colonies than on leaves (pers. obs.). In leaves of cabbage plants, *B. brassicae* induced mainly SA-mediated signalling pathways, and additionally, JA-mediated signalling-pathways. The induction of the SA-signalling-pathway by aphids is considered to antagonize

JA-mediated induction of resistance (Moran & Thompson, 2001; Moran *et al.*, 2002; Kuśnierczyk *et al.*, 2008; Broekgaarden *et al.*, 2011; Kroes *et al.*, 2015; Kroes *et al.*, 2016). As a specialist feeder, this aphid produces an endogenous myrosinase and can sequester plant glucosinolates, thus rendering the aphid toxic to most non-specialized carnivores (Kos *et al.*, 2011; Kos *et al.*, 2012a). They indeed prefer high concentrations of aliphatic glucosinolates and such levels are positively correlated to the aphid's performance (Kos *et al.*, 2011; Kos *et al.*, 2012a; Züst & Agrawal, 2016). However, *B. brassicae* aphids are susceptible to indole glucosinolates (Kos *et al.*, 2011).

Pieris brassicae (Lepidoptera: Pieridae) was selected as a tissue chewer. As suggested by its Latin name "Pieris", meaning a muse, *P. brassicae* has not only been inspiring the Dutch painter van Gogh (Dicke, 2000), but also researchers for decades and extensive literature is available. Butterflies of *P. brassicae* preferably lay a clutch of eggs on leaves of *B. nigra* in the flowering stage (Lucas-Barbosa *et al.*, 2014). Caterpillars in the first instar (L1) feed on leaves, whereas caterpillars in the second instar (L2) migrate to the inflorescence and become exclusively florivorous (Smallegange *et al.*, 2007; Lucas-Barbosa *et al.*, 2013). Flowers and buds support a higher growth rate of the specialist florivore *P. brassicae* caterpillars than leaves (Smallegange *et al.*, 2007). About 90% of the caterpillar food intake happens in the last larval stage before pupation (L5), and a L5 caterpillar consumes on average 135±21 buds and flowers (Smallegange *et al.*, 2007; Smallegange *et al.*, 2008). As chewing herbivores, caterpillars of *P. brassicae* are expected to induce the JA/ET-mediated signalling pathway as well as the ABA pathway (Mewis *et al.*, 2005; Koornneef & Pieterse, 2008; Erb *et al.*, 2012; Vos *et al.*, 2013). In leaves of *B. nigra*, caterpillars induced higher concentrations of JA compared to control plants, and upregulated genes linked to JA biosynthesis or the JA pathway (Bonnet *et al.*, 2017). Eggs, in contrast, did not induce JA, but locally increased concentrations of SA (Hilker & Fatouros, 2016; Bonnet *et al.*, 2017). *Pieris brassicae* caterpillars are specialized in feeding on Brassicaceae. They limit the toxicity of glucosinolate breakdown products by detoxifying them in their gut. A nitrile specifier protein (NSP) turns these products into less toxic nitriles and catabolites of nitriles (Wittstock *et al.*, 2004), which, however, may still affect the caterpillar immune system and make the host-caterpillar more susceptible to parasitization (Kos *et al.*, 2012b).

***Xanthomonas campestris* pv. *raphani* (Xcr)** (Xanthomonadales: Xanthomonadaceae) was selected as phytopathogen. Xcr is a proteobacterium that can infect leaves of *B. nigra* (Ponzio, 2016). This *Xanthomonas* species generally occurs in cultivated fields, as indicated by its name *campestris* (rural), and the pathovar *raphani* causes the non-vascular leaf spot disease on plants in the Brassicaceae (Machmud, 1982; Vicente *et al.*, 2002; Vicente *et al.*, 2006). When visible, symptoms are 1-3 mm large necrotic spots on the infected leaf (Machmud, 1982). *In vitro*, Xcr forms yellow (*xanthos*-) individual colonies (*monas*-, units). Xcr can be found in seeds of plants initially infected on the leaves (Machmud, 1982), which suggests that the bacteria can migrate to reproductive parts of plants. However, the bacterium rarely kills infected plants and mustards such as *B. nigra* show relatively high resistance to it (McCulloch, 1929; Vicente *et al.*, 2006; Ponzio, 2016; Ponzio *et al.*, 2016b). Pathways involving JA, SA and ET are important in regulating plant resistance against Xcr (Ton *et al.*, 2002), and it was recently confirmed that Xcr can induce the local and systemic production of JA and local production of SA in leaves of *B. nigra* (Bonnet *et al.*, 2017). Insect transmission of a closely related pathovar, *X. campestris* pv. *campestris*, can occur in greenhouses, but seems to be rare in the field (Shelton & Hunter, 1985).

Natural enemies of herbivorous insects on *B. nigra* are mainly carnivorous arthropods and entomopathogens, which are essential components of the defense strategy of *B. nigra* (Lucas-Barbosa *et al.*, 2017). On Brassicaceae, eggs and/or small herbivores are commonly eaten by mostly generalist predators such as larvae and adults of Coccinellidae (Coleoptera), larvae of *Chrysopa* spp. (Neuroptera: Chrysopidae), larvae of Syrphidae (Diptera), larvae and adults of a wide range of Heteroptera species (Hemiptera), and spiders (Araneae) (Lucas-Barbosa *et al.*, 2014; Lucas-Barbosa *et al.*, 2017; Stam *et al.*, 2018). *Vespula* and *Polistes* spp. (Hymenoptera: Vespidae) are particularly efficient predators against larger herbivorous insects such as *P. brassicae* caterpillars on *B. nigra* (Lucas-Barbosa *et al.*, 2014). However, these wasps can also be a threat to pollinators (Higginson *et al.*, 2010). Besides predators, parasitoids are generally more specialized on a type, species, or developmental stage of insects, and can effectively control populations of herbivorous insects. On Brassicaceae, diverse parasitoid species have been recorded, many of them are hymenopteran (Bahana & Karuhize, 1986; Büchi, 2002; Eickermann, 2008; Gols *et al.*, 2008; Poelman *et al.*, 2011). Adult wasps are nectar feeders; thus, the presence of flowers has the potential to increase the population

of parasitoids in agricultural fields (Bianchi & Wäckers, 2008; Jamont *et al.*, 2014), although floral VOCs may interfere with parasitoid attraction in some cases (Desurmont *et al.*, 2015). For greenhouse experiments on indirect resistance of *B. nigra*, two species of endoparasitoid wasps were selected.

Diaeretiella rapae (Hymenoptera: Braconidae) is the main endoparasitoid of *B. brassicae* aphids in The Netherlands and parasitizes mainly late-instar nymphs of aphids associated with Brassicaceae (Hafez, 1961; Bahana & Karuhize, 1986; Vaughn *et al.*, 1996). Although aphids can carry one to fifteen eggs of *D. rapae*, only one *D. rapae* larva eventually develops into an adult per host aphid (Hafez, 1961). When larvae of *D. rapae* are about to pupate, the cuticle of the dead aphid hardens. Larvae of *D. rapae* pupate inside the hardened dead aphids, named mummies (Hafez, 1961). Mummies are easily recognizable and can be counted to estimate parasitization success by *D. rapae* wasps. *D. rapae* mate as soon as they emerge from mummies, and are immediately ready to oviposit (Bahana & Karuhize, 1986). Electroantennogram studies indicate that *D. rapae* can perceive certain plant volatiles (Vaughn *et al.*, 1996), and HIPVs may be used by the wasps to locate plants infested with their host. Specialist parasitoids such as *D. rapae* are generally not affected by the glucosinolates and grow bigger when the host aphid performs better (Kos *et al.*, 2012a).

Cotesia glomerata (Hymenoptera: Braconidae) is a specialist endoparasitoid that oviposits in *Pieris brassicae* caterpillars developing on Brassicaceae. It is the main parasitoid of *P. brassicae* caterpillars (Geervliet & Brodeur, 1992; Brodeur *et al.*, 1998) and preferably parasitizes L1 caterpillars (Mattiacci & Dicke, 1995). Larvae are gregarious and a female parasitoid can lay several dozens of eggs per caterpillar (Karowe & Schoonhoven, 1992). In the field, the parasitoid brood size is about 20 larvae per *P. brassicae* caterpillar on *B. nigra* plants (Smallegange *et al.*, 2008). Parasitoid larvae develop inside *P. brassicae* caterpillars that are maintained alive, and when caterpillars are about to pupate, larvae of *C. glomerata* egress from the caterpillars and metamorphose into adult wasps (Tagawa, 2000). The caterpillar eventually dies. Odour-guided foraging of *C. glomerata* has been extensively investigated for *B. nigra* (Lucas-Barbosa *et al.*, 2014; Ponzio *et al.*, 2014; Ponzio *et al.*, 2016a; Ponzio *et al.*, 2016b).

Pollinators of *B. nigra* belong to a large diversity of orders and cover many families (Conner & Neumeier, 1995; Lucas-Barbosa *et al.*, 2013); thus *B. nigra* flowers can be considered to be "generalist flowers" (Frame, 2003; Gómez *et al.*, 2015). In The Netherlands, the most abundant and diverse pollinators of *B. nigra* are honeybees, bumblebees (Hymenoptera: Apidae), and syrphid flies (Diptera: Syrphidae) (Lucas-Barbosa *et al.*, 2013; Rusman *et al.*, 2018). To a lower extent, flowers of *B. nigra* are pollinated by Lepidoptera (especially Pieridae) (Courtney *et al.*, 1982; Lucas-Barbosa *et al.*, 2013). Pollinators rely on diverse floral traits, such as VOCs and color, to find and select flowers, and different orders of pollinators use different rewards (pollen/nectar) and thus exploit different traits (Knauer & Schiestl, 2015; Parachnowitsch & Manson, 2015; Borghi & Fernie, 2017; Kantsa *et al.*, 2018). The diverse community of pollinators of *B. nigra* exploits different floral rewards and likely uses a wide range of flower traits.

Thesis contents

This project investigates how a flowering annual plant, *B. nigra*, defends itself against florivores while maintaining reproduction. This thesis aims at linking physiological mechanisms of plant responses to plant ecology through four complementary experimental chapters that combine experiments analysing plant physiology, plant biochemistry, and biotic interactions. The studies exploited two complementary approaches: greenhouse experiments in controlled conditions with simplified biotic interactions, which allow the investigation of potential mechanisms underpinning plant responses to attack, and field experiments, which are necessary to investigate the adaptive value of plant responses to attack.

Chapter 2 presents a study that addresses the potential implication of phytohormones induced upon florivory by insects and attack by a phytopathogen on direct and indirect resistance of plants against florivores. *Brassica nigra* plants were exposed to single and dual attack to inflorescences and the study links patterns of phytohormone induction to plant-mediated facilitation or hindrance between florivores attacking the plant simultaneously, as well as their consequences for plant resistance. The study focuses on three types of phytohormones, *i.e.* SA, ABA, and JAs, chosen for their central roles in defense and reproduction.

Chapter 3 presents a study that combines experiments in the greenhouse and in the field to investigate whether changes induced in *B. nigra* when inflorescences are exposed to single and dual attack may interfere with indirect resistance and pollinator attraction, and consequently affect plant seed production. The study analyses the potential contribution of volatiles emitted by *B. nigra* in the flowering stage in mediating interactions between plants and these flower-associated mutualists. This project also addresses the specificity of the plant responses to florivorous insects and a phytopathogen, and focuses on the consequences of plant responses to dual attack compared to the single attack situation.

Chapter 4 presents a study that addresses the metabolic mechanisms associated with flowering, as well as with tolerance and resistance mechanisms of *B. nigra* upon attack by florivores. The study characterizes simultaneously changes in the composition of primary and secondary metabolites of leaves and inflorescences when *B. nigra* are exposed in the flowering stage to single and dual attack by florivorous insects and a phytopathogen. Metabolic changes are further linked to changes in biomass of inflorescences, leaves, and roots of plants upon attack during the flowering period.

Chapter 5 presents a field study that investigates whether *B. nigra* responses to florivory by two florivorous insects and attack by a phytopathogen affect the composition of the florivorous community over the course of plant reproductive period, and what the consequences are for plant seed set.

The **General Discussion** presents the contribution of this project to the understanding of a central paradigm of plant biology, *i.e*. the trade-off between defense and reproduction. It summarizes the relative contribution of resistance and tolerance mechanisms to the defense flowering *B. nigra* when inflorescences are exposed to different types of attackers, and highlights the importance of understanding plant response to attackers in the ecological context. It generalises how annual plants may deal with multiple enemies that attack their reproductive tissues.

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Chapter 2

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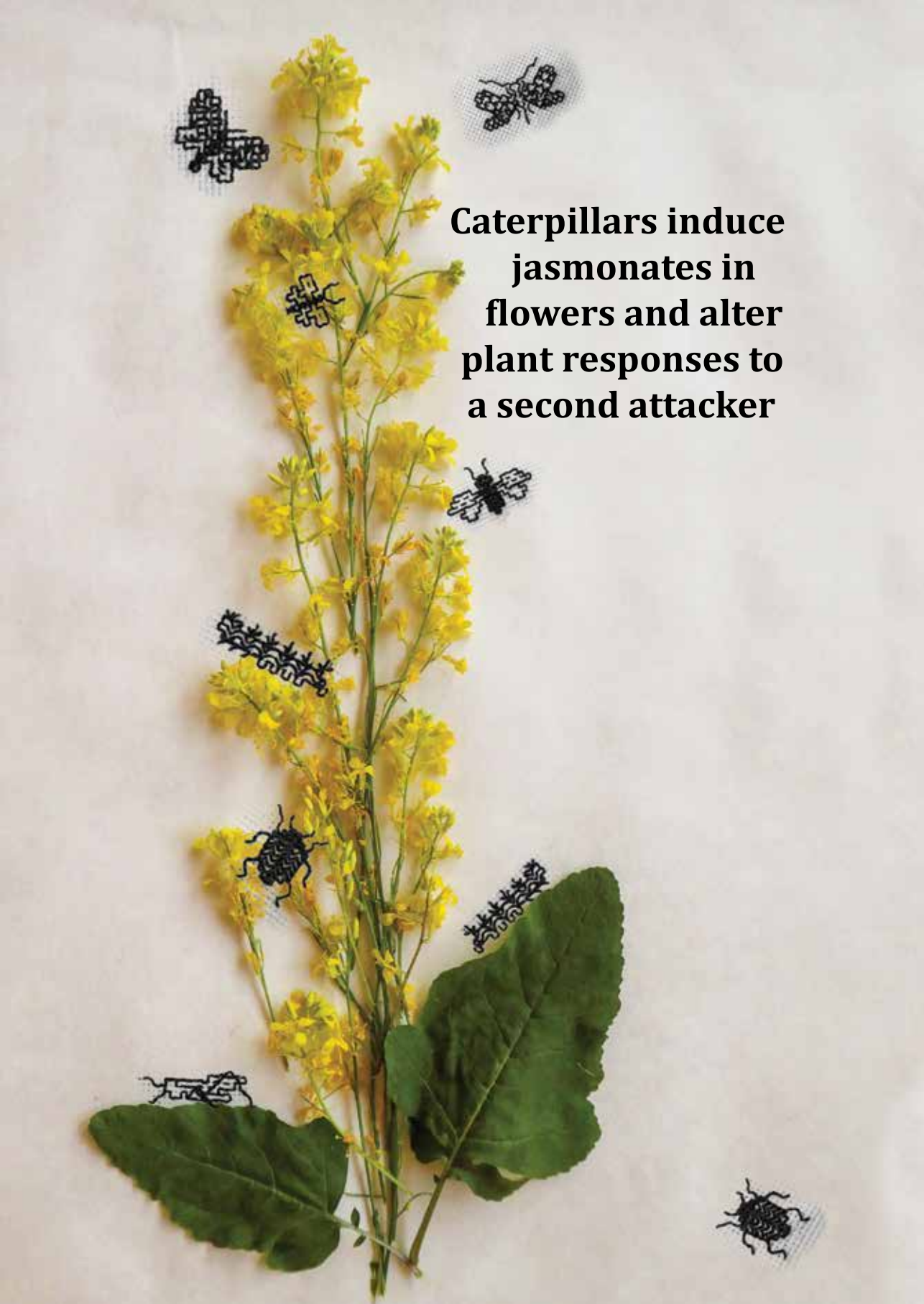

Caterpillars induce jasmonates in flowers and alter plant responses to a second attacker

Abstract

In nature, herbivorous insects and plant pathogens are generally abundant when plants are flowering. Thus, plants face a diversity of attackers during their reproductive phase. Plant responses to one attacker can interfere with responses to a second attacker, and phytohormones that orchestrate plant reproduction are also involved in resistance to insect and pathogen attack. We quantified phytohormonal responses of flowering plants exposed to single or dual attack and studied resistance mechanisms of plants in the flowering stage. Flowering *Brassica nigra* were exposed to either a chewing caterpillar, a phloem-feeding aphid, or a bacterial pathogen, and plant hormonal responses were compared with dual attack situations. We quantified phytohormones in inflorescences and leaves, and determined the consequences of hormonal changes for components of direct and indirect plant resistance. Caterpillars were the main inducers of jasmonates in inflorescences, and the phytohormonal profile of leaves was neither affected by insect nor pathogen attack. Dual attack increased plant resistance to caterpillars, but compromised resistance to aphids. Parasitoid performance was negatively correlated with the performance of their hosts. We conclude that plants prioritize resistance of reproductive tissues over vegetative tissues, and that a chewing herbivore species is the main driver of responses in flowering *B. nigra*.

Key words

Brassica nigra (Brassicaceae), florivorous insects, flowers, multiple attack, parasitoids, phytohormones, phytopathogens, plant resistance

Introduction

During their life time, plants interact with a multitude of organisms, and plant attackers are generally abundant during the flowering period (Lucas-Barbosa *et al.*, 2014; Schlinkert *et al.*, 2015). Plants evolved various defence strategies to defend against a multitude of attackers and to maximize their fitness (Dicke & Hilker, 2003; Howe & Jander, 2008; Agrawal, 2011; Karban, 2011; Dicke & van Loon, 2014). Plant resistance traits can be induced upon attack and directly affect the performance and survival of plant antagonists or enhance the effectiveness of natural enemies of the plant attackers (Dicke & Hilker, 2003; Dicke & Baldwin, 2010; Wu & Baldwin, 2010). Inducible resistance traits of plants can vary depending on the ontogenetic stage of the plant (Barton & Koricheva, 2010; Erbilgin & Colgan, 2012; Quintero *et al.*, 2014), on the identity of the attacker (Erb *et al.*, 2012; Dicke & van Loon, 2014), and on whether the plant is attacked by a single or by multiple species (Soler *et al.*, 2012; Kroes *et al.*, 2015). Such specificity in the induction and regulation of plant responses to attack allows plants to activate resistance traits specifically in targeted tissues and to mount tailor-made resistance to different attackers (Pieterse & Dicke, 2007; Karban, 2011; Mithöfer & Boland, 2012).

A few phytohormones regulate the main biosynthetic pathways in plants, and these can play a role in adjusting plant defence strategies to different attackers (Heidel & Baldwin, 2004; Erb *et al.*, 2012). Jasmonic acid (JA) is the main phytohormone involved in plant responses to chewing herbivores and necrotrophic pathogens, whereas salicylic acid (SA) is the main phytohormone mediating plant responses to phloem-feeding herbivores and biotrophic pathogens (Heidel & Baldwin, 2004; Wu & Baldwin, 2010; Lazebnik *et al.*, 2014). Other phytohormones such as abscisic acid (ABA) and cytokinins (CKs) seem to be more specific, as they accumulate particularly in response to certain species of chewing herbivores and pathogens (Bari & Jones, 2009; Ton *et al.*, 2009). In nature, plants are often simultaneously or successively challenged by multiple attackers, and the synergistic or antagonistic nature of phytohormonal responses can shape a plant's phenotype and determine plant resistance or susceptibility to multiple attackers (Koornneef & Pieterse, 2008; Lazebnik *et al.*, 2014).

When plants are challenged by attackers from different feeding guilds, the induction of distinct phytohormones can have antagonistic effects due to negative crosstalk between signalling pathways. Indeed, although exceptions occur, SA and JA usually have antagonistic effects (Koornneef & Pieterse, 2008; Erb *et al.*, 2012; Thaler *et al.*, 2012), and this can modulate the expression of plant resistance. Plant

indirect resistance can also be influenced by plant responses to multiple attack. Changes in herbivore performance can positively or negatively affect the attraction and performance of their natural enemies (Henry *et al.*, 2005; Rodriguez-Saona *et al.*, 2005; Kos *et al.*, 2012; Soler *et al.*, 2012). Therefore, a plant's response to one attacker can interfere with the response to another attacker, and consequently positively or negatively impact both direct and indirect plant resistance.

To date, chemical and ecological consequences of plant responses to multiple attack have been exclusively studied for plants in the vegetative stage, although resistance of plants in the flowering stage is directly linked to plant fitness. The same phytohormones that mediate resistance to insects and pathogens also influence plant reproduction (Santner & Estelle, 2009; Avanci *et al.*, 2010; Giron *et al.*, 2013; Santino *et al.*, 2013). For instance, SA is involved in the induction of flowering (Martínez *et al.*, 2004; Wada & Takeno, 2010; Rivas-San Vicente & Plasencia, 2011). JA is essential for male fertility (Stintzi & Browse, 2000; Wasternack & Hause, 2013), petal growth (Brioudes *et al.*, 2009), and affects the allocation of resources between different organs (Babst *et al.*, 2005). ABA is involved in pod abscission (Liu *et al.*, 2003) and may induce bud formation and flowering (Samuoliene *et al.*, 2009). The induction of phytohormones by attackers could thus interfere with the regulation of plant reproduction. Consequently, we expect plants that are attacked in the flowering stage and plants attacked in the vegetative stage to have different profiles of phytohormones. Moreover, recent studies have shown that herbivore attack to plants in the flowering stage induces primary and secondary metabolic changes in flowers, rather than in leaves (Pareja *et al.*, 2012; Bruinsma *et al.*, 2014; Lucas-Barbosa, 2016). Such results suggest that plants can differentially allocate resources to leaves or inflorescences, as well as activate resistance traits specifically in flower or leaf tissues. Despite the evidence that herbivore attack to leaves and flowers influences the metabolic profile of flowers (Pareja *et al.*, 2012; Bruinsma *et al.*, 2014; Lucas-Barbosa, 2016), to our knowledge no studies have investigated how plants in the flowering stage deal with multiple attack on flowers, nor what the consequences are for plant hormonal regulation of resistance and reproductive processes.

Here, we investigated phytohormonal responses of flowering plants to single or dual attack, by two insect species and a bacterial pathogen. We expected to detect higher resistance levels in flowers than in leaves, and that the plant phytohormonal profile is characteristic to the type of attacker and combination of attackers. To investigate these questions, we quantified phytohormone concentrations in leaves and inflorescences of plants exposed to single or dual attack, and compared this with

concentrations in plant tissues of non-exposed control plants. We investigated how phytohormonal responses to single or dual attack are reflected in plant resistance to insects, as well as the cascading effects on the natural enemies of the herbivores.

Materials and methods

Study system

The black mustard *Brassica nigra* L. (Brassicales: Brassicaceae) is an annual plant, generally considered to be an outcrossing species (Conner & Neumeier) although some selfing can occur (Lucas-Barbosa *et al.*, 2013; Lucas-Barbosa *et al.*, 2017). In nature, *B. nigra* is attacked by specialist herbivores such as the cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and the large cabbage white butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae), as well as pathogens such as the bacterium *Xanthomonas campestris* pathovar *raphani* (Xcr). This bacterium is the agent of the Leaf Spot Disease that forms small necrotic spots (~1-3 mm) on leaves of many Brassicaceae, but rarely kills the plants (Machmud, 1982; Vicente *et al.*, 2006). The two insect attackers can damage flowers of brassicaceous plants (Lucas-Barbosa *et al.*, 2013 L. Chrétien, pers. obs.), Xcr can spread from infected leaves to mature seeds of broccoli plants (Machmud, 1982). These three attackers are expected to induce distinct responses in *B. nigra*. The phloem-feeding aphid *B. brassicae* is expected to mainly induce the SA-pathway (Mewis *et al.*, 2005; Koornneef & Pieterse, 2008; Erb *et al.*, 2012). Caterpillars of *P. brassicae* are chewing herbivores, which generally induce the JA/ethylene (ET) pathway as well as abscisic acid (ABA) (Mewis *et al.*, 2005; Koornneef & Pieterse, 2008; Erb *et al.*, 2012; Vos *et al.*, 2013). The bacterium Xcr can induce the production of JA and SA (Bonnet *et al.*, 2017), and ET mediates resistance against Xcr (Ton *et al.*, 2002). Both insect herbivores, *B. brassicae* and *P. brassicae,* are frequently attacked by parasitic wasps. The solitary parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae) is the main parasitoid of *B. brassicae* in The Netherlands (Hafez, 1961), and parasitizes aphids associated with Brassicaceae (Bahana & Karuhize, 1986; Vaughn *et al.*, 1996). *Cotesia glomerata* L. (Hymenoptera: Braconidae) is a gregarious specialist parasitoid and the main parasitoid of *P. brassicae* (Geervliet & Brodeur, 1992; Brodeur *et al.*, 1998).

Plant, insect and bacteria cultures

We used a mixture of seeds from at least 20 individual *B. nigra* plants that had been exposed to open pollination in a field of the experimental farm of Wageningen University, The Netherlands (Lucas-Barbosa *et al.*, 2013). Plants grew in pots (Ø17 cm – 2 l) filled with a mixture of potting soil and sand (1:1 v v^{-1}), in a greenhouse compartment (22 ± 2 °C, 50-70 % r. h., L16:D8).

Brevicoryne brassicae aphids were reared on Brussels sprout (*Brassica oleracea* var. *gemmifera*) plants in a greenhouse compartment (21 ± 1 °C, 50-70 % r. h., L16:D8). The parasitic wasp *D. rapae* was reared on *B. brassicae* aphids on Brussels sprout plants in a climate cabinet (25 ± 1 °C, L16:D8). Honey from organic production and water were provided to the adult wasps.

Pieris brassicae caterpillars were also reared on Brussels sprout plants in a climate room (21 ± 1 °C, 50-70 % r. h., L16:D8), and pupae and adult *P. brassicae* were kept in a greenhouse compartment (25 ± 1 °C, 50 - 70 % r. h., L16:D8). Butterflies fed on honey solution (10 % w v^{-1}) from organic production. To rear *C. glomerata*, neonate caterpillars were parasitized by *C. glomerata* and reared on Brussels sprout plants in a climate room (21 ± 1 °C, 50-70 % r. h., L16:D8). Adult wasps were kept in a climate cabinet (25 ± 1 °C, L16:D8) and provided with honey from organic production and water.

Xanthomonas campestris pv. *raphani* was obtained from Utrecht University, the Netherlands (Ponzio *et al.*, 2014). Xcr was cultured in an artificial liquid medium nutrient broth (8 g L^{-1} of Difco™ : beef extract 3.0 g L^{-1} and peptone 5.0 g L^{-1}, BD Diagnostics, New Jersey, USA) for about 22 h at 28 ºC and shaken at 160 rpm. Cells of Xcr were obtained by centrifuging the culture broth twice at 3000 relative centrifugal force for 10 min and re-suspending the pellet containing the bacterial cells in buffer ($MgSO_4$, 10 mM) after each centrifugation. We estimated the concentration of the final inoculum (10^9 cells mL^{-1}) by measuring the light absorbance at 600 nm.

Plant treatment – induction of *B. nigra* plants by single or simultaneous dual attack

Within two days after opening of the first flowers, *B. nigra* plants were exposed to one or two attackers, or kept as control. Plants were exposed to a single attacker, either *B. brassicae*, *P. brassicae*, or Xcr, or simultaneously exposed to two of these three attackers. Control plants were exposed to buffer only, or kept untreated (Fig. 1a). We exposed experimental plants to densities of insect attackers commonly observed in the field to set ecologically relevant conditions (Lucas-Barbosa et al., 2013; Lucas-Barbosa et al., 2014, D. Lucas-Barbosa and L. Chrétien, pers. obs.). To infest *B. nigra* with *B. brassicae* (Fig. 1b), we gently placed 5 young adult females on a bract (flower leaf), at the base of the inflorescence. Shortly after infestation, the aphids moved to the flower stems where they quickly established large colonies by asexual reproduction. It is common to observe an early infestation of *B. nigra* flowers by one to ten *B. brassicae* adults in the field (D. Lucas-Barbosa and L. Chrétien, pers. obs.). *Pieris brassicae* lay eggs in clutches on the leaves of flowering *B. nigra*

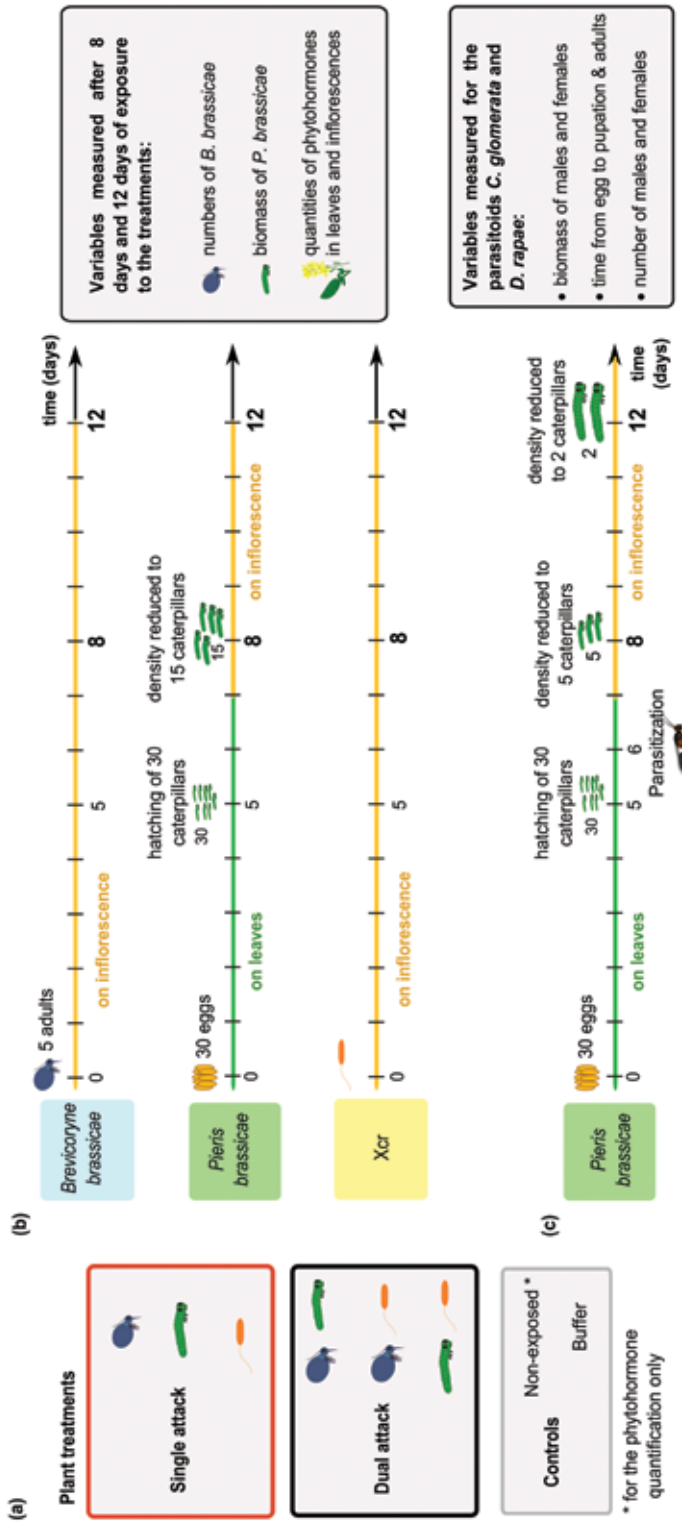

Fig. 1. Schematic representation of the treatments applied to *Brassica nigra* plants and timeline of the experiments (**a**) Description of the treatments. Flowering *B. nigra* were exposed to one attacker, exposed simultaneously to two attackers, to buffer only (control), or were non-exposed. Plant treatments that did not require bacterial infection had a floral leaf exposed to buffer solution instead of the inoculum containing the bacterium. (**b**) Timeline of the experiments with herbivores and/or pathogens. Plants were exposed to one of the treatments for 8 and 12 days, then concentrations of phytohormones or herbivore performance were assessed. Caterpillar density was reduced by 50 % on day 8, to mimic natural dispersal to neighbouring plants. (**c**) Timeline of the experiments with parasitoids. L1 caterpillars were parasitized on day 6 by parasitoid wasps, and caterpillar density was reduced from 30 caterpillars to 5 at 8 days after infestation, and from 5 to 2 at 12 days after infestation.

(Lucas-Barbosa et al., 2014) and after hatching, L1 or L2 caterpillars move to the inflorescence and become florivores (Lucas-Barbosa et al., 2013). To infest *B. nigra* with *P. brassicae* (Fig. 1b), plants were exposed to butterflies until a clutch of at least 30 eggs was laid on a leaf, and any extra eggs were gently removed with forceps. A fine mesh covered the inflorescence to protect open flowers from pollination by the butterflies while plants were exposed to them. *Pieris brassicae* caterpillars hatched from the eggs at five days after oviposition (Fig. 1b). The newly hatched caterpillars fed transiently on leaves (about 2 days), and generally moved to the flowers, at six to seven days after oviposition. When caterpillars had not moved, they were transferred to the flowers at seven days after oviposition (Fig. 1b) to ensure damage to flowers for at least 24 h before the first plant sampling and measurements at day 8. Eight days after infestation, caterpillar density was reduced by 50% to mimic natural predation and dispersal to neighbouring *B. nigra* plants as observed in the field, and to prevent complete consumption of flowers (Fig. 1b). Caterpillar survival was not affected by any of the treatments. For infestation with Xcr (Fig. 1b), 500 µl of the bacterium inoculum (109 cells mL-1 in buffer) was applied on the underside of a bract, at the base of the inflorescence. A soft-clip was used to keep a piece of cotton wool (2 cm x 2 cm) containing the inoculum attached to the bract for 4 h as Xcr enters plant tissues *via* stomata (McCulloch, 1929; Machmud, 1982). The described methodology was adapted from techniques commonly used which consist of either spraying the plant with inoculum (Machmud, 1982; Vicente et al., 2006), applying the inoculum with cotton wool (McCulloch, 1929), or dipping the plant part in inoculum (De Vos et al., 2006). For the experimental plants that were used for phytohormone quantification, we recorded necrotic spots that could either be plant hypersensitive response (HR) or a disease symptom. Mustard plants are relatively resistant to Xcr and the disease rarely spreads throughout the plant (McCulloch, 1929; Vicente et al., 2006; Ponzio, 2016; Ponzio et al., 2016b). For recordings at day 12, necrotic spots were observed on 50 % of the plants per treatment, and for recordings at day 8, necrotic spots were observed on 33 % to 50 % of the plants per treatment. To control for a possible effect of the buffer on plant responses, plants exposed to aphids or caterpillars only, or aphids plus caterpillars simultaneously, were clipped for 4 h with buffer solution containing no bacteria. In addition, two control treatments were added: plants that received no treatment, and plants that were clipped for 4 h with bacteria-free buffer solution. Within a plant, a single bract never received more than one treatment. Exposed and control plants were kept in a greenhouse compartment (21 ± 1 °C, 50 - 70 % r. h., L16:D8) until sampling. Dual attack consisted of simultaneously exposing plants to two attackers (methods same as above).

Sampling and quantification of phytohormones in leaves and inflorescences of *B. nigra* upon single and dual attack

To investigate the induction of phytohormonal responses in flowering *B. nigra* plants exposed to three types of single attackers or simultaneous exposure to two attackers, phytohormone concentrations were quantified in leaves and inflorescences of plants exposed to one of eight different treatments: 1) *B. brassicae*, 2) *P. brassicae*, 3) Xcr, 4) *P. brassicae* plus *B. brassicae*, 5) *P. brassicae* plus Xcr, 6) *B. brassicae* plus Xcr, 7) buffer (control) and 8) non-treated (control) (Fig. 1a). After 8 and 12 days of exposure to the treatments, we sampled leaves and inflorescences for the quantification of phytohormones. Shortly before harvesting, all insects were removed from the plants. All true leaves and inflorescences were harvested, immediately frozen in liquid nitrogen, and kept at -80 ºC. True leaves and inflorescences were then freeze-dried, ground and kept at -20 ºC. The bracts or leaves originally exposed to the insects or to the bacterial inoculum were not harvested. We focused on three key phytohormones, ABA, SA and JA, including precursors, active forms, and degradation forms of the latter. Thus, we quantified the concentration of the phytohormones ABA, SA, JA and the precursor of JA, cis-(+)-12-oxophytodienoic acid (cis-OPDA) (Heitz *et al.*, 2016). In addition, we quantified (+)-7-iso-jasmonoyl-L-isoleucine [(+)-7-iso-JA-Ile] assumed to be the most active form of JA and (-)-jasmonoyl-L-isoleucine [(-)-JA-Ile] a less active form of JA (Fonseca *et al.*, 2009; Avanci *et al.*, 2010), and we quantified the degradation products of JA that are non-active: 12-hydroxy-jasmonate [12-OH-JA], 12-hydroxy-jasmonoyl-isoleucine [12-OH-JA-Ile] and 12-carboxyjasmonoyl-isoleucine [12-COOH-JA-Ile] (Heitz *et al.*, 2016). Phytohormone concentrations (ng g^{-1} of dry mass) were quantified for six plant replicates per treatment, and per time point. Extraction of phytohormones and analyses were performed following the method of Almeida Trapp *et al.* (2014), and as described in Supporting Information (Methods S1).

Effects of dual attack on plant direct resistance to aphids and caterpillars

To investigate whether different induction profiles of phytohormones are reflected in plant direct resistance or susceptibility to herbivorous insects when exposed to single and dual attack, we assessed the performance of *B. brassicae* aphids and of *P. brassicae* caterpillars that fed on *B. nigra* plants exposed to single attack by the herbivores, or to simultaneous attack by another herbivore or the bacteria (Fig. 1b). The performance of *B. brassicae* was assessed on *B. nigra* plants exposed to each of the following three treatments: 1) *B. brassicae*, 2) *B. brassicae* plus *P. brassicae*, and 3) *B. brassicae* plus Xcr. The performance of *P. brassicae* was assessed on *B. nigra* plants exposed to each of the following three treatments: 1) *P. brassicae*, 2)

P. brassicae plus *B. brassicae,* and 3) *P. brassicae* plus Xcr. After 8 and 12 days of exposure to treatments, the number of aphids and the fresh biomass of caterpillars were used as proxies of plant resistance. For this, aphids generated by the five initial young females were counted one by one for colonies smaller than 100 aphids, and for larger colonies, the number of aphids was estimated based on the count of 100 aphids. After 8 days of exposure to treatments, 50 % of the caterpillars (~15 caterpillars per plant) were randomly selected, weighed individually and discarded. After 12 days of exposure to treatment, the remaining caterpillars were weighed (~15 caterpillars per plant), and both caterpillars and plants were discarded. We had seven to eight plant replicates per treatment.

Effects of dual attack on parasitoid performance

The performance of the parasitoid *D. rapae* was assessed in aphid hosts on plants exposed to each of three treatments: 1) *B. brassicae*, 2) *B. brassicae* plus *P. brassicae*, and 3) *B. brassicae* plus Xcr; the performance of the parasitoid *C. glomerata* was assessed in caterpillar hosts on *B. nigra* plants exposed to each of three treatments: 1) *P. brassicae*, 2) *P. brassicae* plus *B. brassicae,* and 3) *P. brassicae* plus Xcr. Host herbivores were parasitized after 6 days of exposure of the plant to the attackers. Female wasps used for parasitization were 3-6 days old, non-experienced (naïve) and mated. For parasitization, fifteen young aphid nymphs (randomly selected) or thirty *P. brassicae* L1 caterpillars were exposed for 90 min to twelve wasps. In the field, *D. rapae* only oviposits in the late-instar nymphs within the aphid colony (Hafez, 1961) and *C. glomerata* parasitizes L1 caterpillars (Mattiacci & Dicke, 1995) and generally oviposits in all caterpillars in a clutch. We assumed that all nymphs and caterpillars were parasitized, and placed them back on the plant to complete their development. Caterpillar density was reduced from thirty caterpillars to five at two days after parasitization (day 8). Six days after parasitization (day 12), only two randomly selected caterpillars were kept on the plant to ensure that there would be enough plant material for the caterpillars to feed (Fig. 1c); the other three caterpillars were discarded. When the first aphid mummies became visible, aphid-infested flower stalks were cut, and we kept the flower stalk with humidified cotton wool around it in a mesh box. Fifth instar (L5) caterpillars were collected before egression of the parasitoid larvae, and individual caterpillars were placed in separate mesh boxes. Boxes with mummies or caterpillars were placed in a climate cabinet (25 ± 1°C, L16:D8) until adult *D. rapae* and *C. glomerata* wasps emerged. Parasitoid performance was assessed by measuring development time (egg to adult), fresh biomass of male and female adult wasps, and number of male and female adult wasps. To determine the developmental time of *D. rapae*, we recorded

the date when the first mummies were observed (pupation of the wasp larvae) and the date of emergence of the first adults. To determine the developmental time of *C. glomerata* we recorded the date when the first pupal cocoons were observed, and the date of emergence of the first adults. Adult parasitoids were sexed and counted on the day they emerged from the mummies or cocoons, and stored at -20 °C until they were individually weighed. For *D. rapae*, we had 15 parasitized aphids per plant and four to six plant replicates per treatment. The biomass of males and females that emerged from parasitized *B. brassicae* feeding on an individual plant was used for statistical tests. For *C. glomerata,* dozens of male and female wasps emerged per caterpillar, and we had two caterpillars per plant, and six to ten plants per treatment. The mean biomass of female wasps and male wasps emerging per caterpillar was calculated and used for statistical tests on a per plant basis.

Statistical analyses

Phytohormone profiles of different plant tissues and of plants subjected to different treatments were analysed by multivariate data analysis, using Projection to Latent Structures Discriminant Analysis (PLS-DA) with Umetrics SIMCA (Umetrics AB, Realeased 2015, Version 14.0, Umeå, Sweden). Data for non-treated plants were not included in the discriminant analyses because phytohormone concentrations were similar to those in plants treated with buffer (Fig. S1 & S2, Table S1 & S2). We used a Generalized Linear Model (GLM) with a Likelihood ratio and Chi-square test to assess whether 1) there was an effect of treatment, plant part, or time point on the concentration of each of the phytohormones (overall), 2) there was an effect of treatment or plant part at each time point separately (day 8, day 12) on the concentrations of each of the phytohormones. We included treatment, time point, and plant part, as main factors plus all interactions in the first case, and treatment and plant part as main factors, and their interaction in the second case. When a significant effect of one of the main factors or of an interaction was detected, a Bonferroni *post-hoc* test was used to test for differences between treatments (overall effect), plant parts (leaves and inflorescences), and between each combination of treatment and plant part. We based the model on a normal distribution and Identity was specified as the link function in the model.

Experimental data on the development time, biomass and numbers of insects were also analysed by a GLM with a Likelihood ratio and Chi-square test. We included in the model as main factors: 1) treatment and time point when analysing number of aphids and biomass of caterpillars; 2) plant treatment and sex, when analysing data related to biomass and numbers of parasitoids, 3) treatment and developmental

stage when analysing data related to the development time of the parasitoids. In all cases, interactions were included. Plant identity was nested within the factor treatment and included in the model. When a significant effect of one of the main factors was detected or when an interaction between factors was significant, a Bonferroni *post-hoc* test was used to test for differences between treatments (overall effect), between the other main factors, and between all combinations of factor levels. Data on insect biomass were analysed by a GLM model that was based on a normal distribution and the function Identity was specified as the link function in the model. The mean biomass of female or male *C. glomerata* wasps that emerged per caterpillar was used for the analysis. Data on insect numbers were assumed to follow a Poisson distribution, a quasi-likelihood function was used to correct for overdispersion, and Log was specified as the link function in the model. Data related to the developmental time of the parasitoids were first log-transformed to meet assumptions of normality.

Results

Phytohormonal profile of leaves and inflorescences

We assessed plant responses to single and multiple attack by quantifying phytohormones in true leaves and inflorescences of plants that were either exposed to different individual attackers or combinations of attackers for 8 or 12 days, or treated with buffer (control). The first principal component of the discriminant analysis (PLS-DA) clearly separated leaf samples from those of inflorescences based on their phytohormonal profile; 58 % and 14 % of the total variance was explained by the first and second principal components respectively (Fig. 2a). The jasmonates (JA, (+)-7-iso-JA-Ile, (-)-JA-Ile) and their catabolites (12-OH-JA, 12-OH-JA-Ile, 12-COOH-JA-Ile), as well as ABA were more abundant in inflorescences than in leaves, whereas SA and OPDA were more abundant in leaves than in inflorescences (Fig. 2b). Irrespective of the time points, the concentrations of jasmonates and their catabolites were 151 % to 2242 % higher in inflorescences than in leaves (Fig. S1 & S2, Table S1 & S2, GLM, Overall, plant part, for (+)-7-iso-JA-Ile, (-)-JA-Ile, 12-OH-JA, 12-OH-JA-Ile, 12-COOH-JA-Ile, JA, $P<0.001$). Concentrations of ABA were 48 % higher in inflorescences than in leaves (Fig. S1, Table S2, GLM, $P < 0.001$). In contrast, concentrations of cis-OPDA and SA were 46 % and 37 % higher in leaves than in inflorescences (Fig. S1, Table S2, GLM, cis-OPDA: $P < 0.001$, SA: $P = 0.020$).

Independent of attacker identity, time influenced the phytohormonal profile of the plants more strongly in leaves than in inflorescences (Fig. 2). SA concentration, for instance, was higher at day 12 than at day 8 in leaves but not in flowers (Fig.

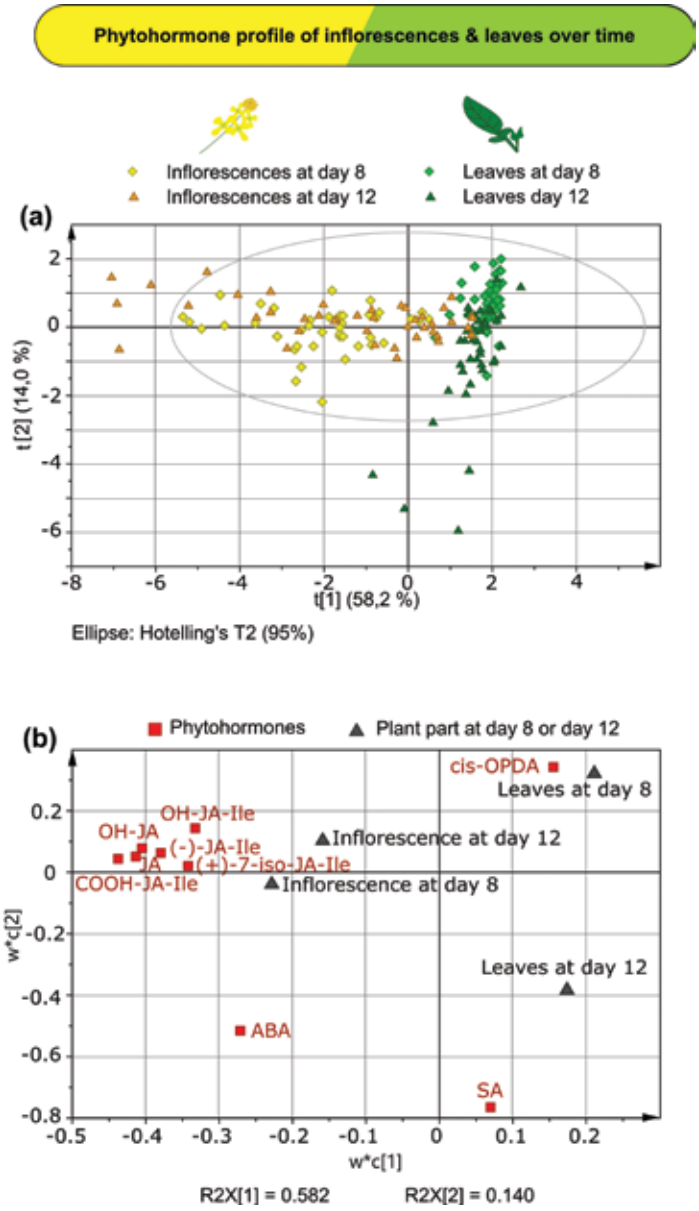

Fig. 2. Phytohormonal profile of leaves and inflorescences of *Brassica nigra* exposed to single or dual attack for 8 or 12 days. Projection to Latent Structures Discriminant Analysis (PLS-DA) of phytohormonal profile in inflorescences and leaves of *B. nigra* after 8 and 12 days of exposure to single or dual attack by *Brevicoryne brassicae*, *Pieris brassicae,* and/or *Xanthomonas campestris* pv. *raphani* (Xcr), or exposure to buffer (control). Six phytohormones were measured: Salicylic acid (SA), Abscisic acid (ABA), Jasmonic acid (JA), cis-(+)-12-oxophytodienoic acid (cis-OPDA), (-)-JA-Ile, (+)-7-iso-JA-Ile, and three catabolites of JA: 12-OH-JA, 12-OH-JA-Ile, 12-COOH-JA-Ile. Phytohomornes concentrations are expressed in ng g^{-1} of dry plant biomass. (**a**) Scatter plots show grouping pattern of samples from inflorescences at day 8, inflorescences at day 12, leaves at day 8, and leaves at day 12 according to the first two principal components. The Hotelling's ellipse confines the confidence region (95 %) of the score plot. (**b**) Loading plots show the contribution of each of the phytohormone quantifications to the first two principal components.

S1, Table S2, GLM, Bonferroni *post-hoc* test, leaves day 8 *vs.* day 12: $P < 0.001$, inflorescence day 8 *vs.* day 12: $P = 1.000$). ABA concentration was also higher at day 12 than at day 8 in leaves but not in flowers (Fig. S2, Table S2, GLM, Bonferroni *post-hoc* test, leaves day 8 *vs.* day 12: $P < 0.001$, inflorescence day 8 *vs.* day 12: $P = 1.000$). For the jasmonates smaller temporal effects were recorded, and here the effects were detected in the inflorescences but not in the leaves. JA and (-)-JA-Ile concentration in inflorescences slightly decreased from 8 to 12 days, whereas the concentration of 12-OH-JA increased. Jasmonic acid concentration was 33 % lower in inflorescences at day 12 than at day 8 (Fig. S2, Table S2, GLM Bonferroni *post-hoc* test, inflorescence day 8 *vs.* day 12: $P < 0.001$, leaves day 8 *vs.* day 12: $P = 1.000$), and (-)-JA-Ile concentration was 15 % lower in inflorescences at day 12 than at day 8 (Fig. S1, Table S1, GLM, Bonferroni *post-hoc* test, inflorescence day 8 *vs.* day 12: $P = 0.029$, leaves day 8 *vs.* day 12: $P = 1.000$), whereas concentration of OH-JA-Ile was 29 % higher in inflorescences at day 12 than at day 8 (Fig. S1, Table S1, GLM, Bonferroni *post-hoc* test, inflorescence day 8 *vs.* day 12: $P = 0.016$, leaves day 8 *vs.* day 12: $P = 1.000$). Time did not influence the concentration of (+)-7-iso-JA-Ile, 12-OH-JA, 12-COOH-JA-Ile, and cis-OPDA (Fig. S1 & S2, Table S1 & S2, GLM, $P > 0.050$).

Phytohormonal profile of inflorescences of plants exposed to single and dual attack by insects and a pathogen

Overall, phytohormone profiles of inflorescences were affected by exposure of plants to single and simultaneous dual attack, and particularly upon 12 days of exposure to the treatments (Fig. 3). The first principal component of the PLS-DA clearly separated inflorescence samples of plants that had been exposed to single attack and dual attack involving caterpillars from inflorescence samples of plants that had not been exposed to caterpillars (Fig. 3c). Induction of biologically active jasmonates and their catabolites was affected by treatments that included *P. brassicae* caterpillars, either as single attackers or in combination with aphids or bacteria (Fig. 3, Fig. S1). The second principal component separated samples of inflorescences that had been exposed to single attack from those exposed to dual attack; 54% and 13 % of the total variance was explained by the first and second principal components, respectively. Especially, single attack by caterpillars and dual attack by caterpillars plus aphids were separated from samples of inflorescences that had been exposed to caterpillars plus bacteria for 12 days (Fig. 3c). Indeed, for the catabolites 12-OH-JA-Ile and 12-COOH-JA-Ile, concentrations were about 50% higher in inflorescences exposed to caterpillars plus bacteria than in inflorescences exposed to caterpillars only (Fig. S1, GLM, Bonferroni *post-hoc* test, caterpillar plus bacteria *vs.* caterpillar, 12-OH-JA-Ile: $P = 0.011$, 12-COOH-JA-Ile: $P < 0.001$).

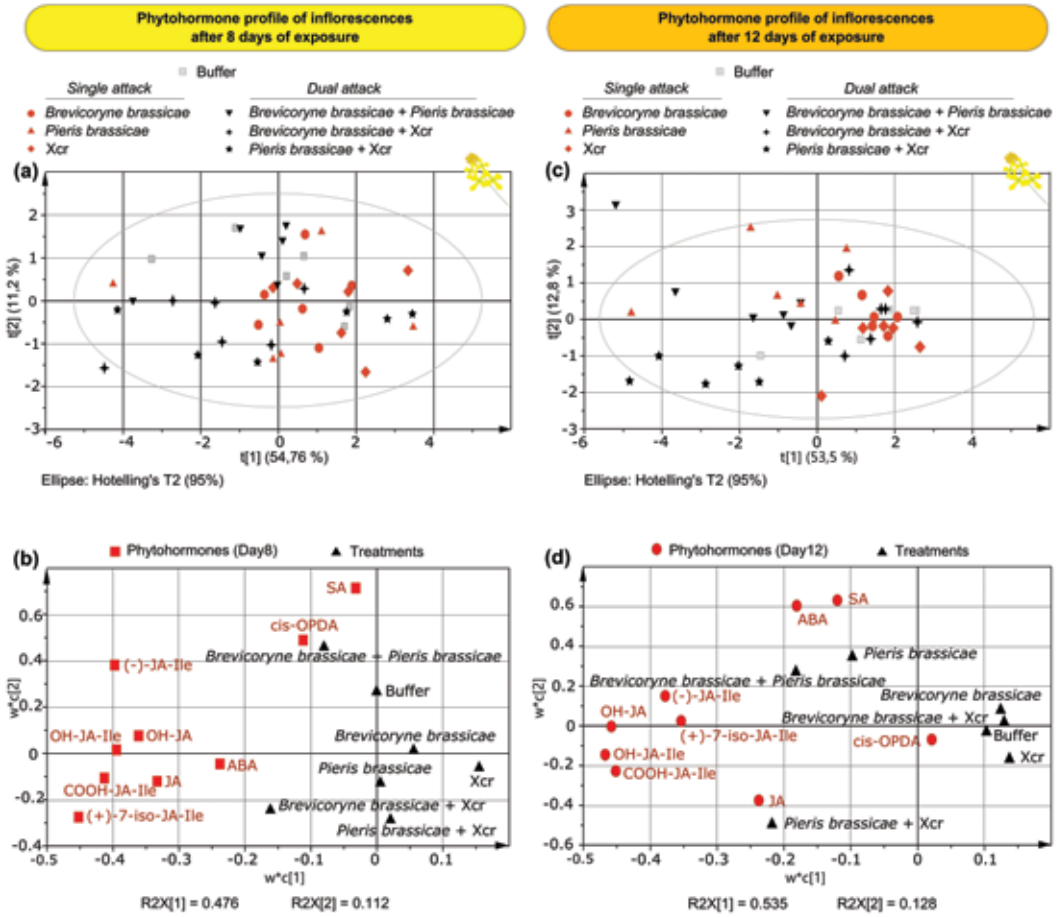

Fig. 3. Phytohormonal profile of inflorescences of *Brassica nigra* exposed to single or dual attack for 8 and for 12 days. Projection to Latent Structures Discriminant Analysis (PLS-DA) separating samples by treatment group for the phytohormonal response of inflorescences after 8 and 12 days of exposure of the plant to treatments. Six phytohormones were measured: Salicylic acid (SA), Abscisic acid (ABA), Jasmonic acid (JA), cis-(+)-12-oxophytodienoic acid (cis-OPDA), (-)-JA-Ile, (+)-7-iso-JA-Ile, and three catabolites of JA: 12-OH-JA, 12-OH-JA-Ile, 12-COOH-JA-Ile. Phytohomornes concentrations are expressed in ng g^{-1} of dry plant biomass. (**a,c**) Scatter plots show grouping pattern of samples from a same treatment according to the first two principal components. The Hotelling's ellipse confines the confidence region (95 %) of the score plot. (**b,d**) Loading plots show the contribution of each of the phytohormone quantifications to the first two principal components.

Overall, exposure of plants to either aphids or Xcr, or to dual attack by aphids plus Xcr, did not influence the phytohormonal profile of inflorescences, neither at day 8 nor at day 12 (Fig. 3). However, differences were present for some phytohormones (Fig. S1, Table S1). For instance, plants exposed to aphids plus bacteria had higher concentrations of (+)-7-iso-JA-Ile than plants exposed to either aphids only (P = 0.002) or bacteria only (P = 0.035).

Changes in the phytohormonal profile upon exposure of plants to attackers were tissue- and time-specific. Flower attackers induced changes in the concentration of phytohormones in the inflorescences but not in the leaves (Fig. S1 & S2, Table S1 & S2). The effect of treatment on the phytohormonal profile was dependent on the time point, and most changes were observed after 12 days of exposure (Fig. 3 & S2, Table S1 & S2). After 8 days of exposure, treatments affected the concentration of one jasmonate, (+)-7-iso-JA-Ile, but after 12 days of exposure, treatments affected the concentration of five jasmonates, *i.e*. (+)-7-iso-JA-Ile, (-)-JA-Ile, 12-OH-JA, 12-OH-JA-Ile, and 12-COOH-JA-Ile (Fig. S1 & S2, Table S1 & S2).

Effects of dual attack on plant direct resistance to aphids and caterpillars

We estimated plant resistance to the insect attackers by counting aphids and weighing caterpillars on plants exposed to single or dual attack. *Brevicoryne brassicae* aphids performed best when feeding on plants that were simultaneously exposed to another attacker than on plants exposed to aphids only (Fig. 4). An overall effect of treatment was detected (Fig. 4; $P < 0.001$): *B. brassicae* numbers were larger on plants exposed to dual attack by aphids plus *P. brassicae* (P = 0.002) or aphids plus Xcr ($P < 0.001$) than on plants infested with aphids only. *Brevicoryne brassicae* were even more abundant on plants that were co-infected with Xcr than on plants co-infested with *P. brassicae* (Fig. 4, $P < 0.001$).

In contrast, *P. brassicae* caterpillars performed worse when feeding on plants that were simultaneously exposed to another attacker than on plants where the caterpillars were the only attacker (Fig. 4). An overall effect of treatment was detected (Fig. 4, P = 0.042). However, this effect was limited to plants that had been exposed to the treatments for 12 days (Fig. 4, interaction Treatment*Day, P = 0.048). After 12 days of exposure of plants to single or dual attack, *P. brassicae* were heavier when caterpillars were the only attackers than on plants exposed to dual attack in the presence of *B. brassicae* (P = 0.026).

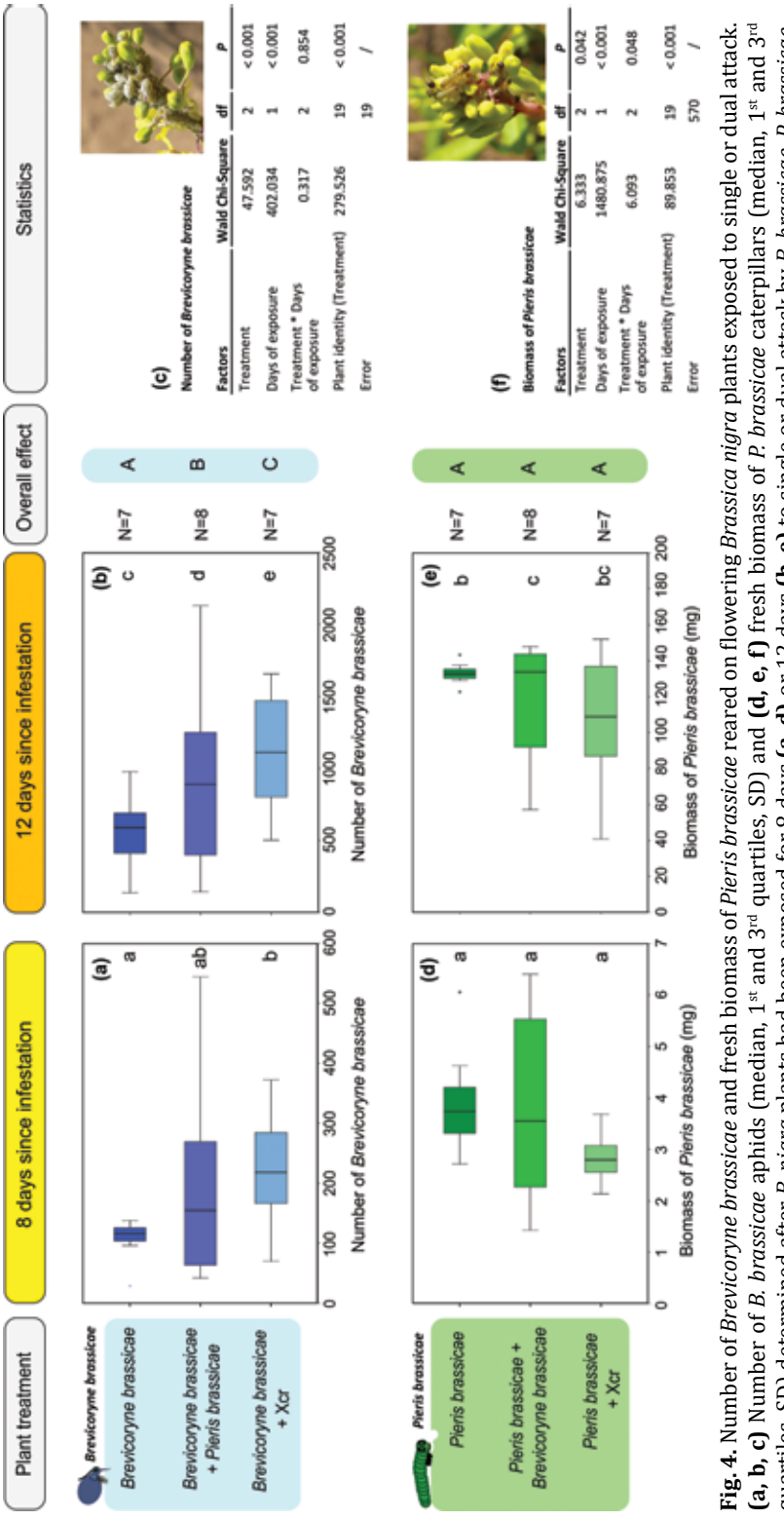

Fig. 4. Number of *Brevicoryne brassicae* and fresh biomass of *Pieris brassicae* reared on flowering *Brassica nigra* plants exposed to single or dual attack. **(a, b, c)** Number of *B. brassicae* aphids (median, 1st and 3rd quartiles, SD) and **(d, e, f)** fresh biomass of *P. brassicae* caterpillars (median, 1st and 3rd quartiles, SD) determined after *B. nigra* plants had been exposed for 8 days **(a, d)** or 12 days **(b, e)** to single or dual attack by *B. brassicae*, *P. brassicae*, and/or *Xanthomonas campestris* pv. *raphani* (Xcr), and statistics **(c, f)**. Overall effects of the treatment and days of exposure to treatments were tested with a General Linear Model with Poisson distribution (*B. brassicae* number) or normal distribution (*P. brassicae* biomass), using likelihood function and Chi-Square test. Interaction between treatment and day was included in the model. Bonferroni *post-hoc* test was used for pairwise comparisons at the 0.05 significance level. Letters (A, B, C) indicate overall significant differences between treatments, lowercase letters (a, b, c, d) indicate significant differences between each treatment of both time points at the 0.05 level. N shows the number of plant replicates. Outliers are represented by "°" (out).

Effects of dual attack on plant indirect resistance

We measured biomass of male and female parasitoids, developmental time, and number of male and female parasitoids and used these parameters to assess the performance of parasitoids on plants exposed to single or simultaneous dual attack. Performance of the aphid parasitoid was affected by exposure of plants to dual attack, and males and females were differentially affected (Fig. 5). Biomass of *D. rapae* males was higher when the host aphids fed on plants that were simultaneously infested by *P. brassicae* caterpillars ($P = 0.046$) than on plants infested by the aphids only or by the aphids plus bacteria. Biomass of female *D. rapae* was similar when the host aphid *B. brassicae* fed from plants exposed to the aphids only, and when the host fed from plants exposed to dual attack by either *P. brassicae* ($P = 0.297$) or Xcr ($P = 1.000$) (Fig. 5). Larvae of *D. rapae* developed slower when their aphid hosts fed from plants exposed to dual attack by aphids plus either *P. brassicae* ($P < 0.001$) or Xcr ($P < 0.001$) than on plants infested with their aphid hosts only (Fig. S3). Furthermore, numbers of male and female *D. rapae* that emerged from aphids were not affected by the treatments (Fig. S4).

In contrast, the caterpillar parasitoid, *C. glomerata,* performed better on plants exposed to dual attack by caterpillars plus bacteria than on plants exposed to caterpillars only or on plants exposed to caterpillars plus aphids (Fig. 5). Moreover, treatments affected males and females in a similar way. Irrespective of the sex, *C. glomerata* were heavier when wasps emerged from host caterpillars that fed on plants exposed to dual attack by caterpillars plus Xcr than on plants infested with *P. brassicae* only (Fig. 5, males, $P < 0.001$; females, $P < 0.001$) or to dual attack by caterpillars plus *B. brassicae* (Fig. 5, males, $P = 0.002$; females, $P < 0.001$). Wasp biomass was similar for wasps that developed in host caterpillars feeding from plants simultaneously infested with *B. brassicae* and in host caterpillars feeding from plants infested with *P. brassicae* only (Fig. 5, males, $P = 0.170$; females, $P = 1.000$). Furthermore, the developmental time of *C. glomerata* was not influenced by dual attack neither by *B. brassicae* nor by Xcr (Fig. S3). Irrespective of the sex, similar numbers of wasps emerged from host caterpillars that fed from plants infested only with the host *P. brassicae* and on plants exposed to dual attack by caterpillars plus either *B. brassicae* or Xcr (Fig. S4).

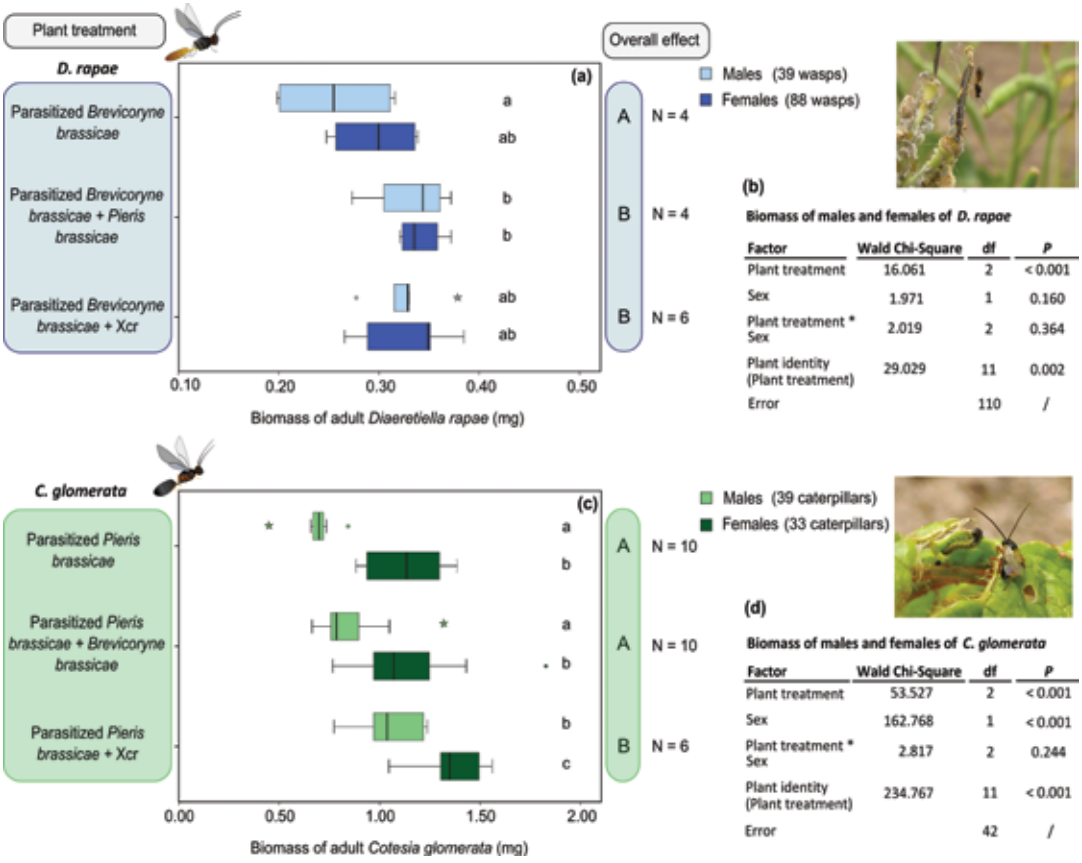

Fig. 5. Fresh biomass of the parasitoid *Diaeretiella rapae* and of the parasitoid *Cotesia glomerata* developing in *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars respectively, reared on flowering *Brassica nigra* exposed to single or dual attack . (a) Fresh biomass of male and female *D. rapae* (median, 1st and 3rd quartiles, SD) and (c) of male and female *C. glomerata* (median, 1st and 3rd quartiles, SD) that emerged from their respective herbivorous hosts. Hosts of the parasitic wasps were reared on plants exposed to single or dual attack by *B. brassicae*, *P. brassicae*, and/or *Xanthomonas campestris* pv. *raphani* (Xcr). (b, d) Overall effects of the treatment were tested with a General Linear Model with normal distribution, using likelihood function and Chi-Square test. Bonferroni *post-hoc* test were used for pairwise comparisons at the 0.05 significance level. Capital letters (A, B, C) indicate overall significant differences between treatments, lowercase letters (a, b, c) indicate significant differences between each treatment for males and females at the 0.05 level. N shows the number of plant replicates, and number of wasps is displayed in brackets. Outliers are represented by " ° " (out) and " * " (far out).

Discussion

This study provides evidence for changes in the phytohormonal profile of the inflorescence upon exposure of flowering plants to single or simultaneous dual attack. Induction was mainly modulated by plant exposure to caterpillars, and was characteristic of flower tissues. Concentrations of jasmonates were especially high in dually-attacked plants compared with plants exposed to single attack. Dual attack rendered plants more resistant to caterpillars but more susceptible to aphids. Furthermore, plant response to dual attack negatively affected the performance of parasitoids of the aphids, whereas it positively affected parasitoids of the caterpillars when compared with the single-attack situation.

The phytohormonal profile of plants exposed to dual attack differed from that of plants exposed to single attack; higher concentrations of jasmonates were recorded in dual-attacked plants than in single-attacked plants. Our results demonstrate that jasmonates were enhanced in flower tissues, whereas no changes in SA and ABA concentrations were recorded following induction. We did not detect phytohormonal responses of plants to single attack by aphids or bacteria, which suggest that plants did not activate responses to these attackers under the conditions of our experiments. However, plants responded strongly to dual attack by caterpillars plus aphids and caterpillars plus bacteria, and to some extent, to aphids plus bacteria. Concentrations of biologically active jasmonates and their catabolites in flowers were higher when plants were exposed to dual attack by caterpillars plus bacteria than when exposed to single attack by caterpillars only, and different from the sum of the effects of both single attacks. This suggests a synergistic or additive effect of caterpillars and Xcr as observed upon interactions with other microorganisms (Rodriguez-Saona *et al.*, 2010; Lazebnik *et al.*, 2014), and this effect may strengthen resistance against both caterpillars and pathogens (Ton *et al.*, 2002; Rostás *et al.*, 2003; Lazebnik *et al.*, 2014). Interestingly, high levels of jasmonates were also induced upon attack by caterpillars plus aphids.

The current knowledge on phytohormonal responses to insects and pathogens shows that aphids generally induce SA in plants at the vegetative stage (Heidel & Baldwin, 2004; Wu & Baldwin, 2010). Moreover, it is commonly accepted that JA and SA pathways crosstalk, meaning that JA induction down-regulates the SA-pathway and SA induction down-regulates the JA-pathway (Kunkel & Brooks, 2002; Koornneef & Pieterse, 2008; Rodriguez-Saona *et al.*, 2010; Thaler *et al.*, 2012), although some synergistic interactions are known as well (Kunkel & Brooks, 2002; Koornneef & Pieterse, 2008). In the present study on flowering plants, no

SA induction was detected upon insect or pathogen attack, neither in leaves nor in flowers, despite the fact that a few hundreds to a thousand aphids were feeding on the plants at the time points recorded. Interestingly, when compared with single attack by caterpillars, dual attack enhanced JA responses irrespective of the identity of the second attacker.

JA induction underlies resistance to chewing herbivores and occasionally to phloem feeders although aphids mainly induce SA (Hansen & Halkier, 2005; Mewis *et al.*, 2005; Mithöfer & Boland, 2012; Guo *et al.*, 2013). Dual attack and the enhanced concentrations of JAs were reflected in stronger resistance of plants to caterpillars when compared with the caterpillar-only attack situation, but compromised plant resistance to aphids. In fact, the development of aphids was not impaired, and these phloem feeders even benefited from dual attack despite the jasmonate induction in the flowers. There was no obvious competition for food between the two insect attackers during the experiment, and we think that direct competition is an unlikely explanation for the results observed. Plant exposure to *P. brassicae* caterpillars results in allocation of resources to flowers in *B. nigra* (Lucas-Barbosa *et al.*, 2017). Thus, we speculate that allocation of resources to flowers could facilitate the development of aphid colonies just below the flowers (Fig. 4), by increasing the nutritional quality of phloem in the inflorescence, and thus, promoting aphid colony growth.

Plant responses to the attackers also affected the performance of parasitoids of the herbivorous insects. Parasitoids performed best when their host performed worse, and we expect that female parasitoids will preferably lay eggs in hosts where their offspring perform best. Our results show that female parasitoids of the aphids (*D. rapae)* developed slower on dual-attacked plants, whereas parasitoids of the caterpillars (*C. glomerata)* were positively affected. Immune responses of the host insect can lead to encapsulation and killing of the parasitoid eggs, or negatively affect the development of the parasitoid larvae (Lackie, 1988). We observed that upon exposure to caterpillars and bacteria, plants exhibit high concentrations of jasmonates which can lead to higher concentrations of resistance compounds. Thus, we speculate that plant immune response possibly benefited the parasitoid by weakening the physiology of the host caterpillar, and herbivore ability to mount an effective immune response against parasitoids (Bukovinszky *et al.*, 2009)2009. We conclude that dual attack compromised important elements of plant direct and indirect resistance to aphids, but increased plant resistance to caterpillars. Based on this, we expect it to be advantageous for parasitoids to also respond to cues that

can be associated with host plants that carry the best quality hosts, and that overall the complex phytohormone-mediated interactions between multiple attackers can attenuate or enhance plant resistance depending on their feeding guild, with synergistic effects between key elements of plant direct and indirect defense.

The constitutive phytohormonal profile of leaves of flowering *B. nigra* plants is very different from that of flowers, and remarkably the phytohormonal profile of leaves remained unaffected when plants were exposed to single or dual attack, although true leaves of plants were directly exposed to eggs and caterpillars. Interestingly, jasmonates, their catabolites, and to some extent ABA, were present in higher concentrations in inflorescences than in leaves (see also (Li *et al.*, 2017), whereas SA and OPDA reached higher concentrations in leaves than in inflorescences. Plants responded to the attackers only with phytohormonal changes in flower tissues. To date, studies of plant responses to multiple attack have been made only for plants in the vegetative stage, and these showed that plant resistance can be negatively or positively affected when plants are exposed to more than one attacker (Soler *et al.*, 2012; Lazebnik *et al.*, 2014). Moreover, inducibility of resistance traits has been assumed to decrease with plant ontogeny (Diezel *et al.*, 2011). Our data supports the idea that inducibility of plant responses in flowering plants is rather canalized to flower tissues, where the phytohormonal profile changes in response to insect and pathogen attack. Indeed, recent studies have demonstrated that herbivore attack to leaves influences the volatile profile of flowers (Pareja *et al.*, 2012; Bruinsma *et al.*, 2014), and that resources can be allocated to flowers upon exposure to insect herbivores (Lucas-Barbosa *et al.*, 2017). For instance, folivory by *P. brassicae* caterpillars induced changes in the volatile blend of *B. nigra* flowers whereas the volatile emission of leaves did not change in response to attack (Bruinsma *et al.*, 2014). It has been speculated that induction of phytohormones in inflorescences in response to attack could indirectly interfere with reproductive processes (Herms & Mattson, 1992; Strauss *et al.*, 2002). Response to attack can modify flower chemistry and affect sugar composition of floral nectar (Euler & Baldwin, 1996; Strauss *et al.*, 2004; Bruinsma *et al.*, 2014), and affect flower-insect interactions, including changes in pollinator behavior (Lucas-Barbosa *et al.*, 2011; Bruinsma *et al.*, 2014).

Our data show that the phytohormonal profile varied with time. To date, most data on phytohormonal responses to attack have been determined for short periods of induction, restricted to from a few hours to 3 days of induction (Stam *et al.*, 2014), despite the fact that in natural conditions, plants are exposed to attackers throughout their development. Duration of exposure to the attackers and the

amount of damage caused to the plants can provide a plausible explanation for the differences quantified over time. Indeed, plant responses can be affected by densities of attackers (Zhang *et al.*, 2009; Kroes *et al.*, 2015; Ponzio *et al.*, 2016a), different larval stages can also induce different responses in plants (Erb *et al.*, 2012), and ontogeny influences the phytohormonal profile of plant tissues (Du *et al.*, 2008; Quintero & Bowers, 2011; Erbilgin & Colgan, 2012; Quintero *et al.*, 2014). Phytohormonal analyses of leaves showed that concentrations were higher at day 12 than at day 8, and this may be the result of senescence of the leaves by day 12 (L.T.S. Chrétien, pers. obs.) supporting the hypothesis that plants redirect resources from leaves to the inflorescences upon attack, and activate resistance traits in flower tissues (Lucas-Barbosa *et al.*, 2013; Pashalidou *et al.*, 2013; Lucas-Barbosa, 2016; Lucas-Barbosa *et al.*, 2016; Lucas-Barbosa *et al.*, 2017), in accordance with the optimal defence theory (Cates & Rhoades, 1977). We speculate that plant response to egg deposition on leaves – which typically induces SA – may have inhibited an early induction of JA in the inflorescence by the caterpillars when recorded at day 8, *i.e*. three days after the caterpillars had hatched from the eggs, providing also a possible explanation of why higher phytohormonal concentrations were quantified at day 12 than at day 8 (Bruessow *et al.*, 2010; Hilker & Fatouros, 2016).

Our study addressed for the first time, to our knowledge, inducible resistance of an annual plant in the flowering stage under multiple attack, and shows that dual attack promotes plant resistance to caterpillars, but compromises plant resistance to aphids. Caterpillars were the main inducers of plant responses, and the biologically active forms of JA were upregulated in flower tissues, overruling ABA and SA responses. We conclude that at the flowering stage of *B. nigra* plants the inducibility of defensive traits is redirected to the protection of reproductive tissues – something we expect to be typical of fast-growing annual plants – and that under multiple attack, chewing herbivores are the main drivers of inducible plant resistance.

Acknowledgments
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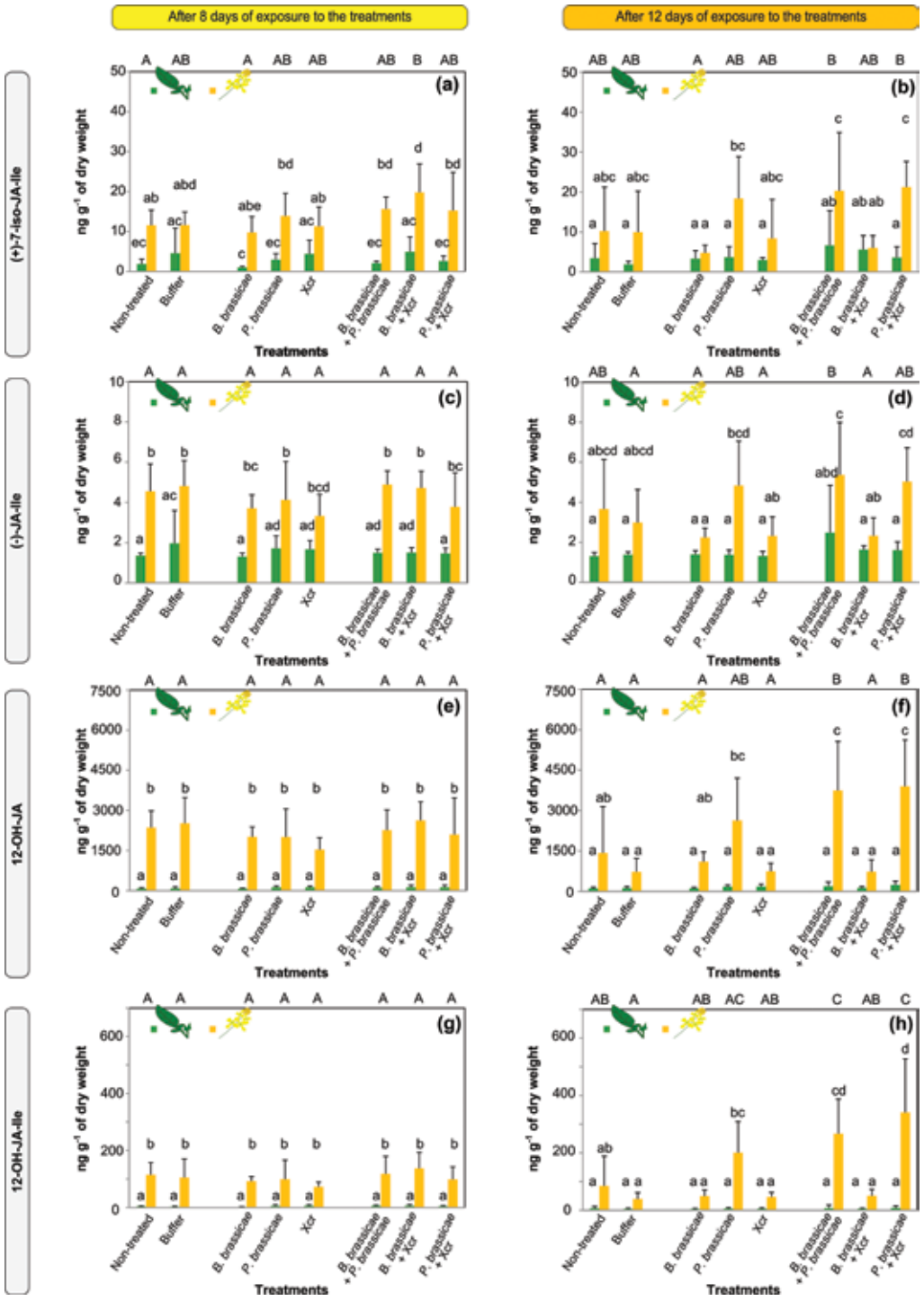
Supplemental information

Methods S1. Protocol for extraction and quantification of the phytohormones and their catabolites

Extraction of phytohormones was done by stirring 20 mg of ground freeze-dried plant material in 1.5 ml of methanol for 30 min, and then centrifuging it twice (at 14,000 rpm, for 10 min at 4 ºC) and combining the supernatants. The final methanolic crude extract was then evaporated (speed-vac at 30 ºC) and re-dissolved in 500 µl methanol. The following internal standards were added to the methanolic extract: 60 ng D_6-abscisic acid (D_6-ABA) (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), 60 ng of D_6-jasmonic acid (D_6-JA) (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4-salicylic acid (D_6-SA) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 12 ng of JA-$^{13}C_6$-isoleucine conjugate [JA-$^{13}C_6$-Ile]. To obtain JA-$^{13}C_6$-Ile, JA was conjugated to $^{13}C_6$-Ile (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) as described by Kramell *et al.* (Kramell *et al.*, 1988).

Resulting extracts were analysed by high performance liquid chromatography (Agilent 1200 HPLC system, Agilent technologies, Santa Clara, USA) coupled with a mass spectrometer (MS) (API 5000, Applied Biosystem, Foster city, USA) and equipped with a Turbospray ion source. Two µl of extracts was separated on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent technologies, Santa Clara, USA). Two solvents formed the mobile phase: formic acid (0.05 %) in ultrapure water as solvent A, and acetonitrile as solvent B. The following gradient was used: 0-0.5 min, 5 % B; 0.5-9.5 min, 5-42 % B; 9.5-9.51 min, 42-100 % B; 9.51-12 min, 100 % B and 12.1-15 min, 5 % B. The flow rate was 1.1 ml min^{-1} and the column was kept at 25 ºC. In the MS, the liquid effluent was ionized by electrospray ionisation in a negative mode (-4500 eV). The turbo gas temperature was set at 700 ºC. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi, and collision gas at 7 psi. The MS was run in multiple reaction monitoring (MRM) mode at *m/z* 263.0 to 153.2 (collision energy (CE) -22 V; declustering potential (DP) -35 V) for ABA; at *m/z* 269.0 to 159.2 (CE -22 V; DP -35 V) for D_6-ABA; at *m/z* 209.1 to 59.0 (CE -24 V; DP -35 V) for JA; at *m/z* 215.1 to 59.0 (CE -24 V; DP -35 V) for D_6-JA; at *m/z* 136.9 to 93.0 (CE -22 V; DP -35 V) for SA; at *m/z* 140.9 to 97.0 (CE -22 V; DP -35 V) for D_4-SA; at *m/z* 290.9 to 165.1 (CE -24 V; DP -45 V) for cis-OPDA, at *m/z* 322.2 to 130.1 (CE -30V; DP -50V) for JA-Ile conjugate; at *m/z* 328.2 to 136.1 (CE -30V; DP -50V) for JA-$^{13}C_6$-Ile conjugate; at *m/z* 338.2 to 130.1 (CE -30V; DP -50V) for 12-hydroxy-jasmonoyl-isoleucine [12-OH-JA-Ile] conjugate; at *m/z* 352.2

to 130.1 (CE -30V; DP -50V) for 12-carboxyjasmonoyl-isoleucine [12-COOH-JA-Ile] conjugate; and at *m/z* 225.1 to 59.0 (CE -24V; DP -35V) for 12-hydroxy-jasmonate [12-OH-JA]. Phytohormones were quantified in ng g^{-1} of dry biomass (Analyst 1.5, Applied Biosystems, Foster city, USA) using their respective internal standards. The D_6-JA was used for the quantification of cis-OPDA with a response factor of 0.5, and for 12-OH-JA with a response factor of 1.0. 12-OH-JA-Ile conjugate and 12-COOH-JA-Ile conjugate were quantified using JA-$^{13}C_6$-Ile conjugate as internal standard applying a response factor of 1.0.


After 8 days of exposure to the treatments
After 12 days of exposure to the treatments
(+)-7-iso-JA-Ile
(-)-JA-Ile
12-OH-JA
12-OH-JA-Ile
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
ng g-1 of dry weight
Treatments
Non-treated
Buffer
B. brassicae
P. brassicae
Xcr
B. brassicae + P. brassicae
B. brassicae + Xcr
P. brassicae + Xcr

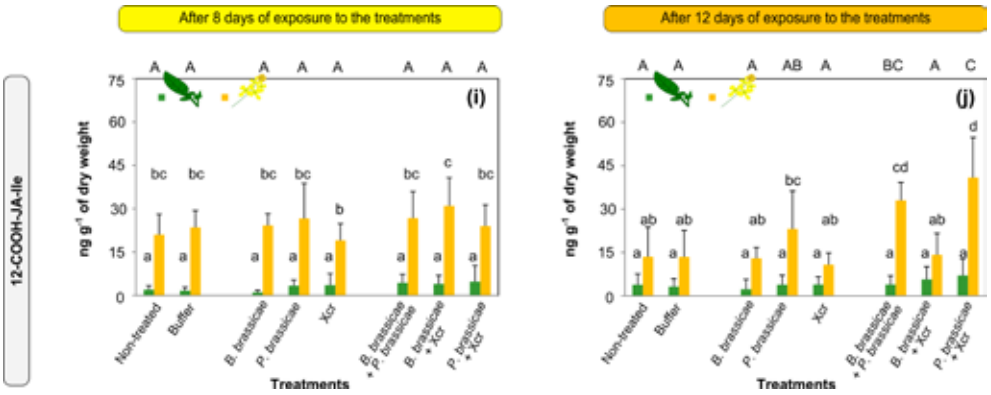


Fig. S1 (left and above). Concentration of active jasmonates and their catabolites (mean + SD) quantified in leaves and inflorescences of *Brassica nigra* plants exposed to single or dual attack for 8 or 12 days. Quantities (ng g^{-1}) in leaves (green) and inflorescences (yellow) of the jasmonate-derived phytohormones: (+)-7-iso-jasmonoyl-L-isoleucine [(+)-7-iso-JA-Ile] in leaves and inflorescences at day 8 (a) and day 12 (b); and (-)-jasmonoyl-L-isoleucine [(-)-JA-Ile] in leaves and flowers at day 8 (c) and day 12 (d), and of the catabolic forms: 12-hydroxy-jasmonate [12-OH-JA] in leaves and inflorescences at day 8 (e) and day 12 (f); 12-hydroxy-jasmonoyl-isoleucine [12-OH-JA-Ile] in leaves and inflorescences at day 8 (g) and day 12 (h); 12-carboxyjasmonoyl-isoleucine [12-COOH-JA-Ile] in leaves and inflorescences at day 8 (i) and day 12 (j), in plants that were non-treated, exposed to buffer, or exposed to single or dual attack by *Brevicoryne brassicae*, *Pieris brassicae*, and/or *Xanthomonas campestris* pv. *raphani* (Xcr). We had 6 replicates per treatment and time point. Capital letters (A, B, C) indicate overall significant differences between treatments, lower case letters (a, b, c) indicate significant differences between each treatment for leaves and inflorescences at the 0.05 level.

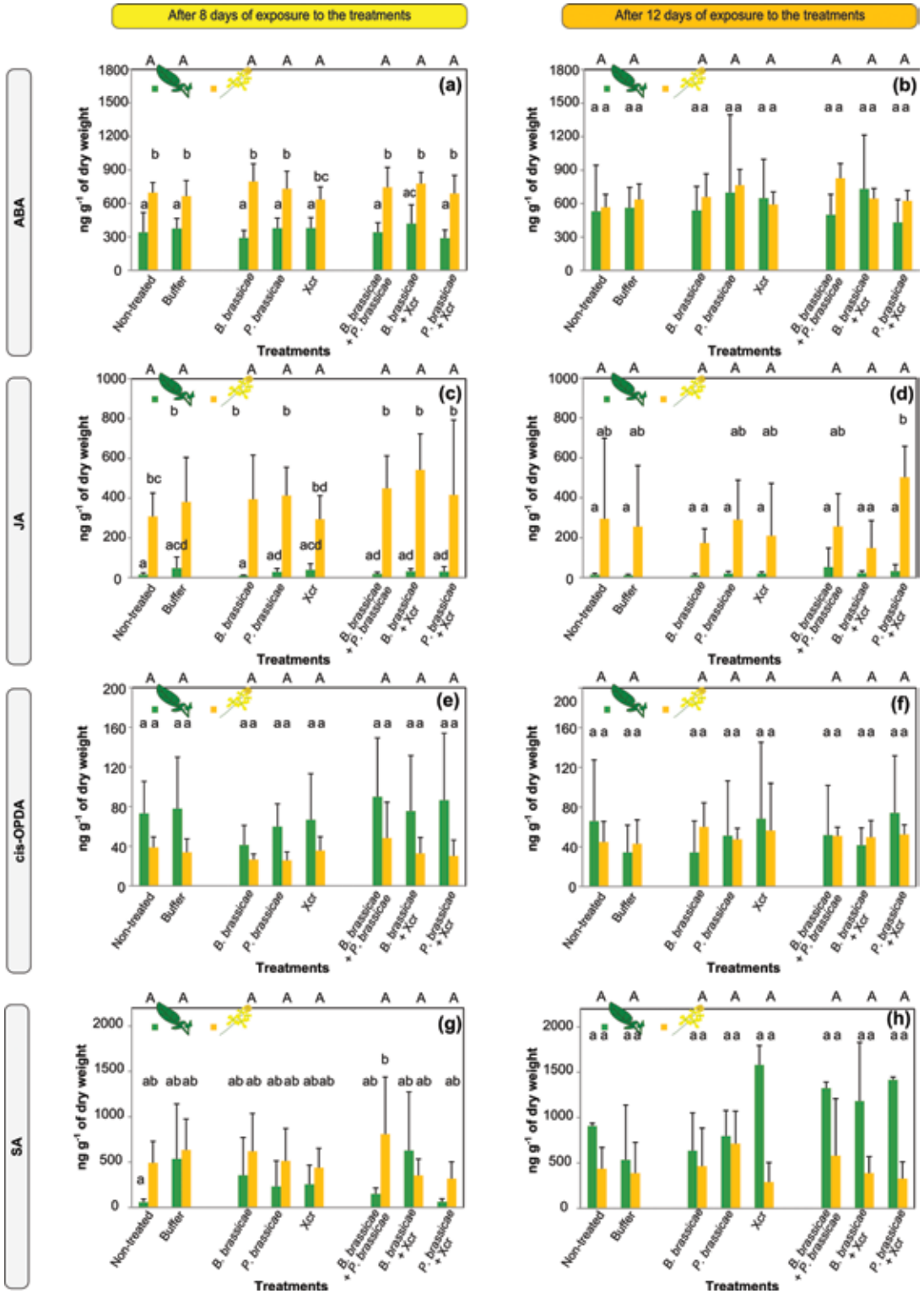

After 8 days of exposure to the treatments
After 12 days of exposure to the treatments
ABA
JA
cis-OPDA
SA
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
ng g-1 of dry weight
Treatments
Non-treated
Buffer
B. brassicae
P. brassicae
Xcr
B. brassicae + P. brassicae
B. brassicae + Xcr
P. brassicae + Xcr

Fig. S2 (left). Concentration of abscisic acid (ABA), jasmonic acid (JA), cis-(+)-12-oxophytodienoic acid (cis-OPDA) and salicylic acid (SA) quantified in leaves and inflorescences (mean + SD) of *Brassica nigra* plants exposed to single and dual attack for 8 and 12 days. Quantities (ng g^{-1}) in leaves (green) and inflorescences (yellow) of the phytohormones: ABA in leaves and inflorescences at day 8 (a) and day 12 (b); JA in leaves and flowers at day 8 (c) and day 12 (d), cis-OPDA in leaves and inflorescences at day 8 (e) and day 12 (f); SA in leaves and inflorescences at day 8 (g) and day 12 (h), in plants that were non-treated, exposed to buffer, or exposed to single or dual attack by *Brevicoryne brassicae*, *Pieris brassicae*, and/or *Xanthomonas campestris* pv. *raphani* (Xcr). We had 6 replicates per treatment and time point. Capital letters (A, B, C) indicate overall significant differences between treatments, lower case letters (a, b, c) indicate significant differences between each treatment for leaves and inflorescences at the 0.05 level.

Table S1. Output of the Generalized Linear Model for the effects of treatment, plant part and day (duration of exposure to the treatments) on the concentration of the jasmonic acid (JA)-related phytohormones: the active forms (+)-7-iso-jasmonoyl-L-isoleucine [(+)-7-iso-JA-Ile] and (-)-jasmonoyl-L-isoleucine [(-)-JA-Ile], and of their catabolic forms 12-hydroxy-jasmonate [12-OH-JA], 12-hydroxy-jasmonoyl-isoleucine [12-OH-JA-Ile], 12-carboxyjasmonoyl-isoleucine [12-COOH-JA-Ile]. We assessed compound concentration in leaves and inflorescences of flowering *Brassica nigra* plants that were exposed to single or dual attack for 8 or 12 days. Output of the analyses including both time points in the statistical model is shown on the left side. On the right side, the output for each of the time points is shown.

(+)-7-iso-JA-Ile

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	29.113	7	< 0.001
Plant part	148.560	1	< 0.001
Day	0.046	1	0.830
Treatment*Plant part	20.323	7	0.005
Treatment*Day	19.421	7	0.007
Plant part*Day	1.699	1	0.192
Treatment*Plant part*Day	14.259	7	0.047

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	20.787	7	0.004
Plant part	163.381	1	< 0.001
Treatment*Plant part	11.333	7	0.125

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	25.611	7	< 0.001
Plant part	41.058	1	< 0.001
Treatment*Plant part	19.592	7	0.007

(-)-JA-Ile

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	29.34	7	< 0.001
Plant part	216.308	1	< 0.001
Day	3.908	1	0.048
Treatment*Plant part	15.373	7	0.032
Treatment*Day	20.146	7	0.005
Plant part*Day	4.046	1	0.044
Treatment*Plant part*Day	13.090	7	0.070

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	11.116	7	0.134
Plant part	206.233	1	< 0.001
Treatment*Plant part	8.615	7	0.281

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	31.726	7	< 0.001
Plant part	60.949	1	< 0.001
Treatment*Plant part	17.110	7	0.017

12-OH-JA

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	44.288	7	< 0.001
Plant part	368.557	1	< 0.001
Day	1.550	1	0.213
Treatment*Plant part	40.043	7	< 0.001
Treatment*Day	47.403	7	< 0.001
Plant part*Day	3.110	1	0.078
Treatment*Plant part*Day	43.510	7	< 0.001

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	8.202	7	0.315
Plant part	349.145	1	<0.001
Treatment*Plant part	8.756	7	0.271

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	63.125	7	<0.001
Plant part	110.69	1	<0.001
Treatment*Plant part	56.934	7	<0.001

12-OH-JA-Ile

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	64.975	7	< 0.001
Plant part	260.717	1	< 0.001
Day	4.996	1	0.025
Treatment*Plant part	61.011	7	< 0.001
Treatment*Day	62.301	7	< 0.001
Plant part*Day	4.071	1	0.044
Treatment*Plant part*Day	60.039	7	< 0.001

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	7.713	7	0.359
Plant part	234.939	1	<0.001
Treatment*Plant part	7.258	7	0.403

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	78.722	7	<0.001
Plant part	104.735	1	<0.001
Treatment*Plant part	74.892	7	<0.001

12-COOH-JA-Ile

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	61.905	7	< 0.001
Plant part	469.932	1	< 0.001
Day	3.259	1	0.071
Treatment*Plant part	34.191	7	< 0.001
Treatment*Day	34.489	7	< 0.001
Plant part*Day	9.861	1	0.002
Treatment*Plant part*Day	34.950	7	< 0.001

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	12.679	7	0.080
Plant part	359.996	1	<0.001
Treatment*Plant part	8.225	7	0.313

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	74.745	7	<0.001
Plant part	150.127	1	<0.001
Treatment*Plant part	54.262	7	<0.001

Table S2. Output of the Generalized Linear Model for the effects of treatment, plant part and day (duration of exposure to the treatments) on the concentration of the phytohormones: salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), and cis-(+)-12-oxophytodienoic acid (cis-OPDA). We assessed compound concentration in leaves and inflorescences of flowering *Brassica nigra* plants that were exposed to single or dual attack for 8 or 12 days. Output of the analyses including both time points in the statistical model is shown on the left side. On the right side, the output for each of the time points is shown.

ABA

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	9.813	7	0.199
Plant part	59.929	1	< 0.001
Day	9.267	1	0.002
Treatment*Plant part	8.787	7	0.268
Treatment*Day	2.392	7	0.935
Plant part*Day	23.332	1	< 0.001
Treatment*Plant part*Day	3.357	7	0.850

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	7.205	7	0.408
Plant part	240.084	1	< 0.001
Treatment*Plant part	9.121	7	0.244

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	5.885	7	0.553
Plant part	2.536	1	0.111
Treatment*Plant part	5.471	7	0.603

JA

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	8.948	7	0.256
Plant part	218.377	1	< 0.001
Day	10.985	1	0.001
Treatment*Plant part	10.470	7	0.163
Treatment*Day	7.075	7	0.421
Plant part*Day	9.512	1	0.002
Treatment*Plant part*Day	10.687	7	0.153

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	7.184	7	0.410
Plant part	181.623	1	< 0.001
Treatment*Plant part	7.311	7	0.397

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	11.687	7	0.111
Plant part	60.951	1	< 0.001
Treatment*Plant part	10.111	7	0.182

cis-OPDA

Overall

Factors	Wald Chi-Square	df	*P*
Treatment	7.594	7	0.370
Plant part	15.660	1	< 0.001
Day	0.033	1	0.856
Treatment*Plant part	5.519	7	0.597
Treatment*Day	5.322	7	0.621
Plant part*Day	12.673	1	< 0.001
Treatment*Plant part*Day	1.749	7	0.972

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	8.761	7	0.270
Plant part	31.468	1	<0.001
Treatment*Plant part	2.903	7	0.894

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	4.583	7	0.711
Plant part	0.072	1	0.789
Treatment*Plant part	4.230	7	0.753

SA

Overall

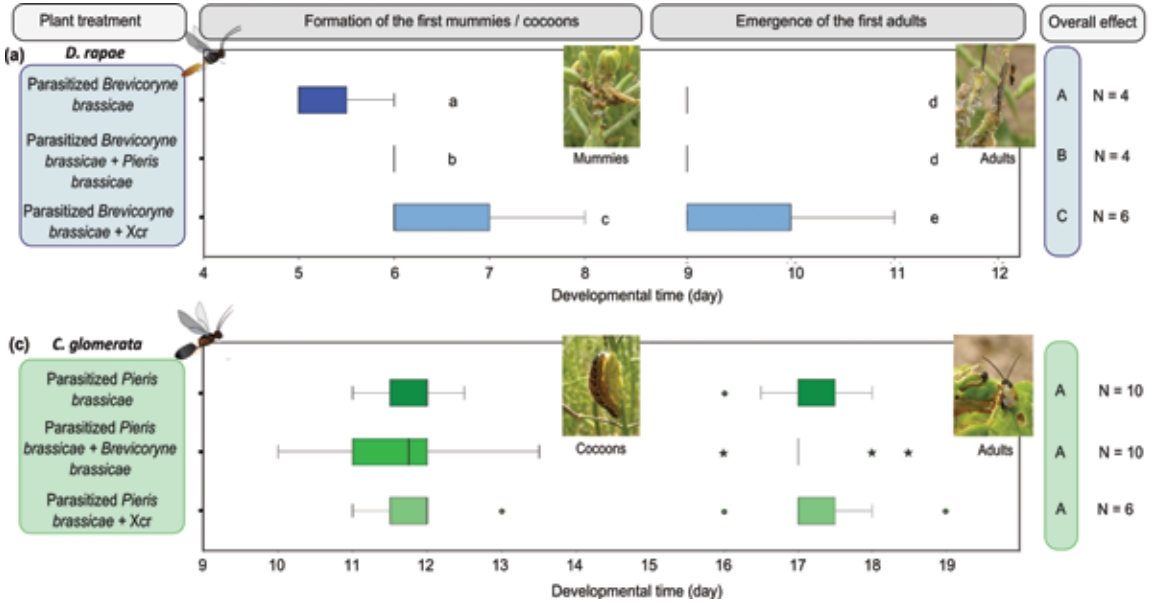
Factors	Wald Chi-Square	df	*P*
Treatment	3.885	7	0.793
Plant part	5.431	1	0.020
Day	19.907	1	< 0.001
Treatment*Plant part	10.932	7	0.142
Treatment*Day	10.344	7	0.170
Plant part*Day	29.203	1	< 0.001
Treatment*Plant part*Day	9.550	7	0.216

Day 8

Factors	Wald Chi-Square	df	*P*
Treatment	13.316	7	0.065
Plant part	12.605	1	<0.001
Treatment*Plant part	14.090	7	0.05

Day 12

Factors	Wald Chi-Square	df	*P*
Treatment	5.685	7	0.577
Plant part	18.404	1	<0.001
Treatment*Plant part	9.354	7	0.228



(b)

Developmental time of *D. rapae*

Factors	Wald Chi-Square	df	P
Plant Treatment	107.634	2	< 0.001
Developmental stage	1394.995	1	< 0.001
Plant treatment * Developmental stage	23.988	2	< 0.001
Plant identity (Plant treatment)	99.645	11	< 0.001
Error		11	/

(d)

Developmental time of *C. glomerata*

Factors	Wald Chi-Square	df	P
Plant treatment	3.215	2	0.200
Developmental stage	3662.661	1	< 0.001
Plant treatment * Developmental stage	0.929	2	0.628
Plant identity (Plant treatment)	213.044	24	< 0.001
Error		50	/

Fig. S3. Developmental time of the parasitoid *Diaeretiella rapae* and of the parasitoid *Cotesia glomerata* developing in *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars respectively, reared on flowering *Brassica nigra* plants exposed to single or dual attack. **(a)** Developmental time of males and females *D. rapae* (median, 1st and 3rd quartiles, SD) and **(c)** of males and females *C. glomerata (*median, 1st and 3rd quartiles, SD) that developed in and emerged from their respective herbivorous hosts. Hosts of the parasitic wasps were reared on plants exposed to single or dual attack by *B. brassicae*, *P. brassicae*, and/or *Xanthomonas campestris* pv. *raphani* (Xcr). **(b, d)** Overall effects of the treatment were tested with a General Linear Mix Model with a normal distribution using likelihood function and Wald Chi-Square test. Bonferroni *post-hoc* test were used for pairwise comparisons at 0.05 significance level. Capital letters (A, B, C) indicate overall significant differences between treatments, lowercase letters (a, b, c) indicate significant differences between each treatment for males and females at the 0.05 level. N represents the number of plant replicates. Outliers are represented by " ° " (out) and " * " (far out)

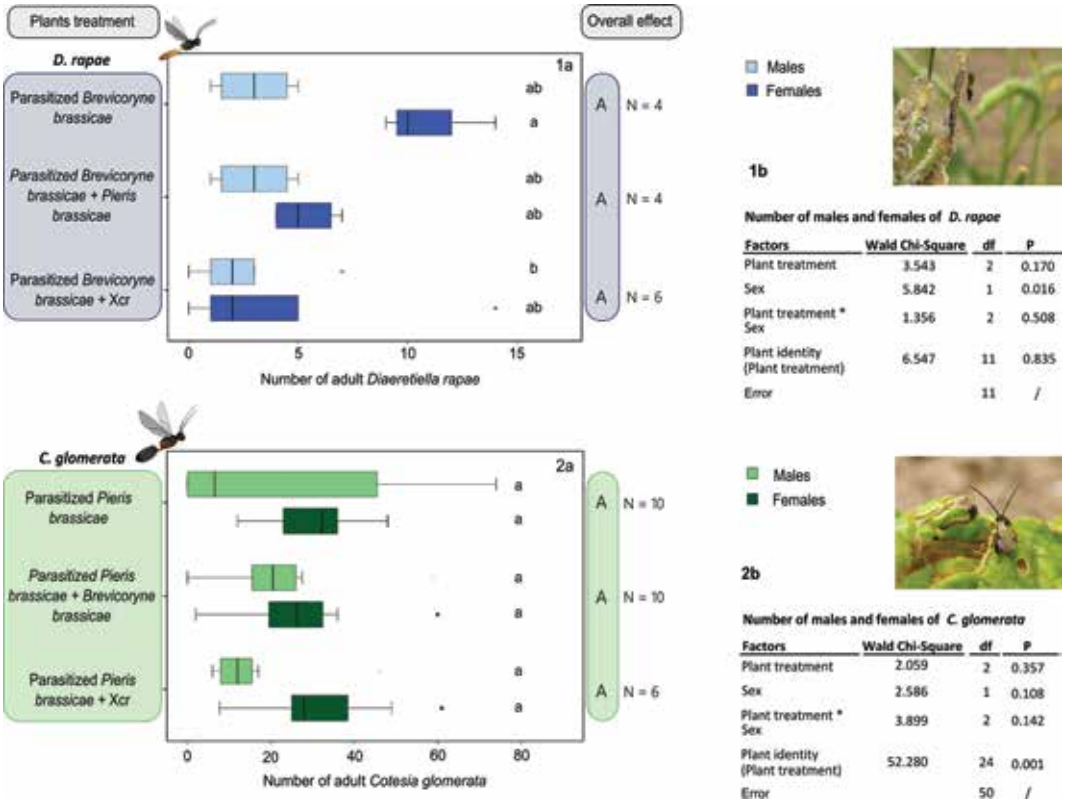
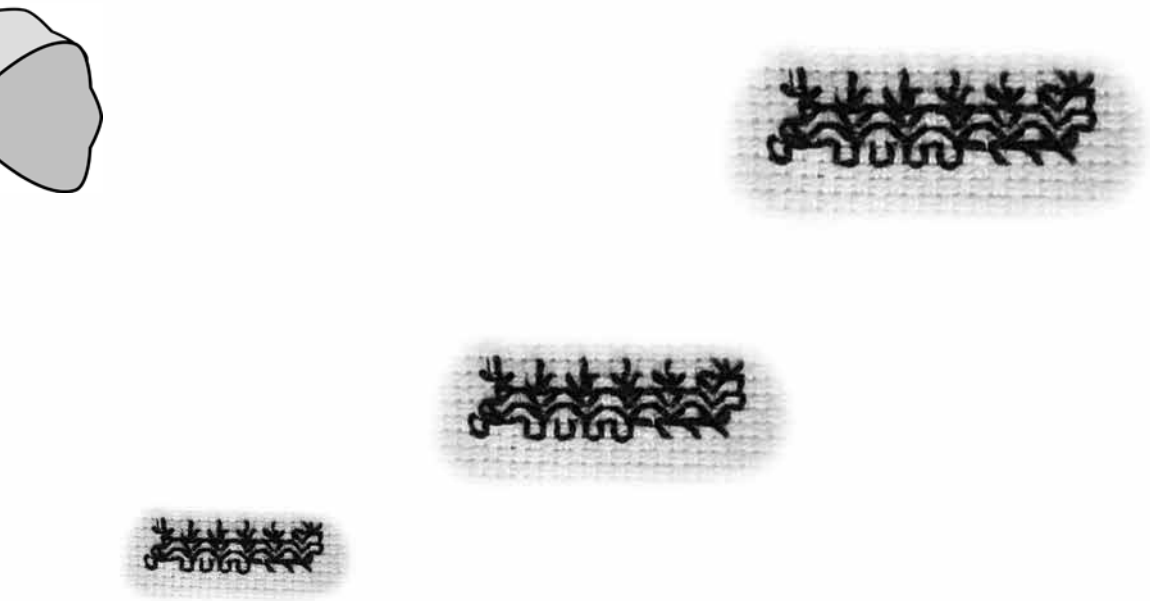


Fig. S4. Number of adults *Diaeretiella rapae* and of adults *Cotesia glomerata* that emerged from *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars respectively, reared on flowering *Brassica nigra* plants exposed to single or dual attack. **(a)** Number of males and females *D. rapae* (median, 1st and 3rd quartiles, SD) and **(a)** of males and females *C. glomerata* (median, 1st and 3rd quartiles, SD) that emerged from their respective herbivorous hosts. Hosts of the parasitic wasps were reared on plants exposed to single or simultaneous dual attack by *B. brassicae*, *P. brassicae*, and/or *Xanthomonas campestris* pv. *raphani* (Xcr). (**b,d).** Overall effects of the treatment were tested with a General Linear Model with a Poisson distribution using likelihood function and Chi-Square test. Bonferroni *post-hoc* test were used for pairwise comparisons at 0.05 significance level. Capital letters (A, B, C) indicate overall significant differences between treatments, lowercase letters (a, b, c) indicate significant differences between each treatment for males and females at the 0.05 level. N represents the number of plant replicates. Outliers are represented by " ° " (out) and " * " (far out).

Chapter 3



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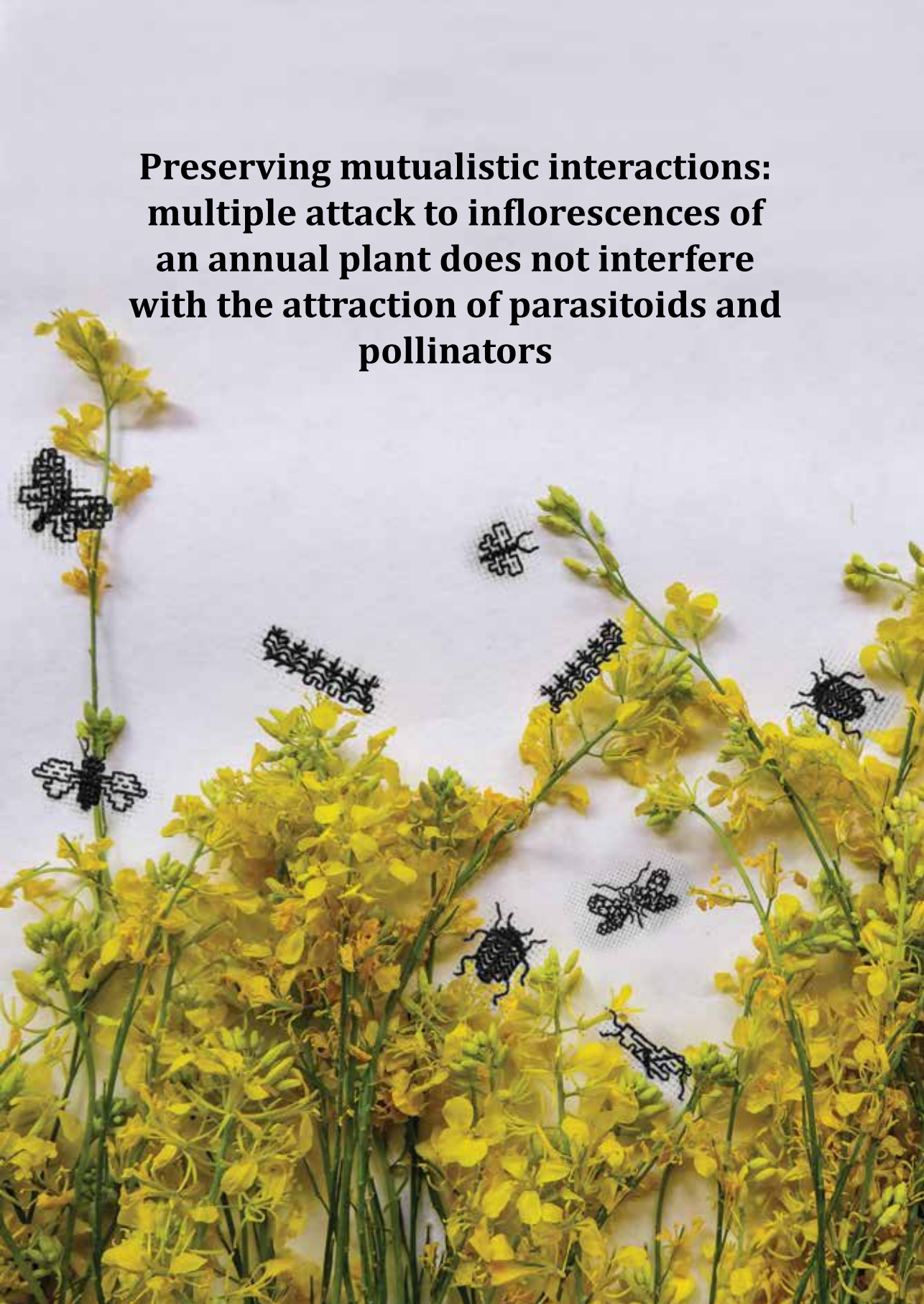
Preserving mutualistic interactions: multiple attack to inflorescences of an annual plant does not interfere with the attraction of parasitoids and pollinators



Abstract

Plants in the flowering stage need to ensure reproduction by protecting themselves from attack and by preserving interactions with mutualist pollinators. Floral traits that change upon attack, such as volatile emission, can provide reliable information to insectivores on the presence of attackers on flowers. Such changes can, however, interfere with the attraction of pollinators. Plants may be particularly challenged upon multiple attack to inflorescences since they need to attract carnivores despite the presence of other organisms that can interfere with plant responses to attack. To address this challenge, we measured volatile emission of flowering *Brassica nigra* plants in response to single or dual attack on their inflorescences, and we recorded the attraction and visitation of flowers by pollinators and the attraction and oviposition by parasitoids. Plants were exposed in the flowering stage to *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars, and *Xanthomonas campestris* pv. *raphani* bacteria, which mostly damaged inflorescences. We found that single attack by caterpillars and dual attack by caterpillar plus aphids induced the strongest changes in plant volatile emission. Despite these changes in plant volatile emission, the preference of parasitoids of the caterpillars or the aphids was not affected by dual attack by a host and a non-host compared to single attack with the host only, when tested in two-choice assays in the greenhouse or in a common garden experiment. The composition of the pollinator community associated to flowers of *B. nigra* was affected by plant exposure to the attackers in a common garden experiment. However, the total number of pollinators attracted to the plants did not change upon attack, and plants exposed to the different combinations of attackers produced similar number of seeds as control plants. We conclude that *B. nigra* exposed to single or dual attack on their inflorescences maintained interactions with natural enemies of the insect attackers and with pollinators. We highlight that the ability of *B. nigra* to cope with multiple attackers is likely supported by the diversity and abundance of mutualistic interactions supported by the plant.

Key words

Brassica nigra (Brassicaceae), flowering plant, indirect resistance, multiple attack, plant volatiles, plant reproductive success, pollination

Introduction

Outcrossing plants in the flowering stage need to protect themselves from attack while maintaining pollination in order to ensure their reproduction. Plant volatile emissions play a key role in these processes by mediating mutualistic interactions with natural enemies of herbivorous insects and with pollinators (Dudareva *et al.*, 2006; Junker *et al.*, 2010; Schiestl, 2010; Kessler *et al.*, 2011; Muhlemann *et al.*, 2014; Schiestl *et al.*, 2014; Lucas-Barbosa, 2016). The composition of a volatile blend is specific to the plant part that emits it, such as leaves and flowers (Pichersky & Gershenzon, 2002; Lucas-Barbosa *et al.*, 2011). Floral volatiles are commonly associated with the attraction of pollinators to flowers, which likely selects for a reliable blend that can be associated with reward quality and quantity (Wright & Schiestl, 2009; Schiestl & Johnson, 2013). Upon herbivore or pathogen attack, odours emitted by plants can change and herbivore-induced volatiles emitted by damaged plants are essential cues for insectivores to find herbivorous insects on plants (Kessler & Baldwin, 2001). While the attraction of pollinators has an evident direct advantage for the reproductive success of most outcrossing plant species, there is increasing evidence that also indirect resistance can benefit plants (van Loon *et al.*, 2000; Fritzsche-Hoballah & Turlings, 2001; Kessler & Baldwin, 2001; Schuman *et al.*, 2012; Gols *et al.*, 2015; Lucas-Barbosa *et al.*, 2017). Thus, when facing attackers in the flowering stage, the attraction of pollinators and natural enemies of herbivores may trade off (Bruinsma *et al.*, 2008; Kessler & Halitschke, 2009; Lucas-Barbosa *et al.*, 2011; Schiestl *et al.*, 2014).

The emission of plant odours can qualitatively and quantitatively vary upon attack (Rostás *et al.*, 2006; Ponzio *et al.*, 2013). Plants can, for instance, differentially respond to herbivore species (Erb *et al.*, 2012; Dicke & van Loon, 2014), herbivore densities (Kroes *et al.*, 2015; Ponzio *et al.*, 2016a), or to different developmental stages of an herbivore (Takabayashi *et al.*, 1995). The induction of specific signal-transduction pathways in response to insect or pathogen attack results in specific changes in plant volatile emission, which can influence the attraction of natural enemies of the plant attackers (Dicke, 1999; Hilker & Meiners, 2002; Dicke & Baldwin, 2010). Plants face multiple attackers in nature, and plant responses to a combination of attackers can differ from the sum of responses induced by the attackers separately (Soler *et al.*, 2012; Kroes *et al.*, 2015; Silva *et al.*, 2016; Ponzio *et al.*, 2017). Dual attack may interfere, for example, with the attraction of parasitoids to their host (Zhang *et al.*, 2009; Ponzio *et al.*, 2013; Kroes *et al.*, 2015), although other studies showed parasitoid host-finding behaviour to be more robust than expected when considering changes in volatile blends (Rostás *et al.*, 2006; Erb

et al., 2010; Ponzio *et al.*, 2014). Multiple attack may, thus, alter a plant's indirect resistance to herbivores. Folivory by *P. rapae* caterpillars on Broccoli, for example, reduced parasitism of *B. brassicae* aphids in the field, which in some sites lead to increased growth of aphid colonies (Blubaugh *et al.*, 2018).

Although indirect resistance of plants in the vegetative stage has been extensively addressed, studies investigating indirect resistance of plants in the flowering stage are scarce, especially when considering florivores. Yet, volatile emission can also mediate indirect resistance of plants to florivores. Florivore-induced emission of β-ocimene, for example, makes flowers of the mustard *Biscutella laevigata* more attractive to crab spiders, which may benefit the plant because the spiders mainly prey on florivores (Knauer *et al.*, 2018). Additionally, cowpea flowers damaged by *Maruca vitrata* caterpillars emit volatiles that attract parasitoids of these herbivores (Dannon *et al.*, 2010). Plant response to single attack by a florivore may interfere with plant response to another attacker on inflorescences. Dual attack to flowers of *Brassica nigra* by combinations of aphids, caterpillars and bacteria induced an increase in levels of jasmonates (JAs) in inflorescences compared to plants exposed to single attack (Chrétien *et al.*, 2018). Moreover, the content of inflorescences in jasmonates changed in a specific manner depending on the combination of attackers (Chrétien *et al.*, 2018). Jasmonates are involved in the production of floral scent (Stitz *et al.*, 2014), and changes in floral levels of jasmonates may translate into different volatile emissions, among other traits. Jasmonates can, for example, regulate the production of the volatile (E)-α-bergamotene, which is known to mediate indirect resistance of *Nicotiana attenuata* (Li, R *et al.*, 2017). Interference with the attraction of carnivores upon multiple attack could have strong negative impacts considering the direct damage they inflict on flowers.

Herbivore-induced plant traits that mediate indirect resistance of plants can also influence pollinator behaviour, and consequently, affect the pollination success of a plant attacked in the flowering stage (Lehtila & Strauss, 1997; Krupnick *et al.*, 1999; Kessler *et al.*, 2011; Lucas-Barbosa *et al.*, 2011). Among those traits, herbivore-induced plant volatiles (HIPVs) seem to be important cues (Lucas-Barbosa *et al.*, 2011; Schiestl *et al.*, 2014). For example, florivory by the parsnip webworm on wild parsnip induced an increased emission of octyl esters that could be linked to an altered pollination success in the field (Zangerl & Berenbaum, 2009). Herbivory on plants in the flowering stage leads to a wide array of consequences for pollinators, ranging from enhanced attraction (Rusman *et al.*, 2018) to deterrence (Kessler & Halitschke, 2009; Kessler *et al.*, 2011; Bruinsma *et al.*, 2014), although herbivory

sometimes does not affect pollinator attraction (Pareja *et al.*, 2012). Herbivory can as well lead to changes in the time a pollinator spends on a flower, or the number of flowers they visit (Lucas-Barbosa *et al.*, 2013; Bruinsma *et al.*, 2014). Such effects of herbivory on pollinator recruitment, and the consequences for seed production by the plants, are highly dependent on the insect species (Rusman *et al.*, 2018), and appear to be specific to the feeding guild of the herbivorous insect (Rusman *et al.*, 2018), and to the feeding site on the plant (Kessler & Halitschke, 2009). When considering florivores, attackers can directly alter flower traits by feeding damage and indirectly induce changes in flower traits *via* inducible responses in plants (Irwin & Adler, 2006; McCall & Irwin, 2006; Zangerl & Berenbaum, 2009).

The conflict between maintaining floral traits attractive to pollinators while inducing changes that attract carnivores may results in a trade-off between indirect resistance and reproduction (McCall & Irwin, 2006; Lucas-Barbosa *et al.*, 2011; Lucas-Barbosa, 2016). *Sinapis alba*, for instance, maintains interactions with both pollinators and parasitoids upon single attack on leaves by aphids (Pareja *et al.*, 2012). *Brassica rapa,* however*,* prioritizes the recruitment of pollinators over the recruitment of parasitoids upon folivory by caterpillars (Schiestl *et al.*, 2014; Desurmont *et al.*, 2015). Little is known about the combined attraction of pollinators and carnivores to plants attacked on their inflorescences. The association of inflorescences with insectivores such as crab spiders can actually threaten pollinators and, thus, may directly interfere with pollination, although they can also benefit plants by predating on florivores (Romero & Vasconcellos-Neto, 2004; Gonçalves-Souza *et al.*, 2008; Higginson *et al.*, 2010; Knauer *et al.*, 2018). Attracting parasitoids that specifically target florivores, may reduce the risk of predation upon pollinators.

So far, few studies have addressed whether the attraction of insectivores to inflorescences upon attack trade off with the attraction of pollinators (Pareja *et al.*, 2012; Schiestl *et al.*, 2014; Lucas-Barbosa, 2016). The aim of this study is to explore whether and how multiple attack, mostly florivorous, by a phytopathogenic bacteria, an aphid and a caterpillar affects the recruitment of pollinators and natural enemies by a plant in the flowering stage. We investigated the role of plant VOCs as a potential mediator of these two mutualistic interactions. To address this question, we collected and analysed volatiles from the headspace of flowering *B. nigra* plants that had been exposed to single or dual attack, and investigated the behavioural responses of parasitoids and pollinators in greenhouse experiments and in the field. To assess the consequences for plant fitness, we quantified seed set of the plants in the field.

Materials and methods

Study system

The black mustard *Brassica nigra* (Brassicales: Brassicaceae) is a common native plant in The Netherlands that grows in dense patches. This annual species relies on pollinating insects for reproduction (Conner & Neumeier, 1995), although some selfing can occur as well (Lucas-Barbosa *et al.*, 2013; Lucas-Barbosa *et al.*, 2017). Pollinators of *B. nigra* belong to different insect orders, especially the Hymenoptera and Diptera, but also the Lepidoptera. *Brassica nigra* is commonly colonized by the cabbage aphid *Brevicoryne brassicae* (Hemiptera: Aphididae), which is a phloem feeder specialized on brassicaceous plants. This aphid species develops large colonies on inflorescences of *B. nigra*, whereas the development on leaves is limited (LTS Chrétien, pers. obs.). The main parasitoid of *B. brassicae* is the solitary wasp *Diaeretiella rapae* (Hymenoptera: Braconidae) (Hafez, 1961; Bahana & Karuhize, 1986; Vaughn *et al.*, 1996) that preferably oviposits in late-instar *B. brassicae* nymphs (Hafez, 1961), and prefers flower-feeding aphids to leaf-feeding aphids (LTS Chrétien, pers. obs.). The gregarious caterpillars of *Pieris brassicae* (Lepidoptera: Pieridae) are specialist herbivores of brassicaceous plants and use *B. nigra* as one of their host plants (Lucas-Barbosa *et al.*, 2014). The butterflies lay eggs on leaves (Lucas-Barbosa *et al.*, 2014) and upon hatching the first-instar (L1) larvae feed on these leaves. The second instar (L2) larvae migrate to the inflorescence and become exclusively florivorous (Lucas-Barbosa *et al.*, 2013). In The Netherlands, caterpillars of *P. brassicae* are often parasitized by the gregarious parasitoid *Cotesia glomerata* (Hymenoptera: Braconidae) which lays a few dozens of eggs in first or second-instar caterpillars of *P. brassicae* (Karowe & Schoonhoven, 1992; Mattiacci & Dicke, 1995). Bacterial diseases, such as *Xanthomonas campestris* pathovar *raphani* (Xcr), can infect *B. nigra*. Xcr causes so-called leaf spot disease that mainly affects plants in the Brassicaceae but rarely kills the plants (Machmud, 1982; Vicente *et al.*, 2006). The bacteria can be found in seeds of plants initially infected on the leaves (Machmud, 1982). Xcr causes 1-3 mm large necrotic spots on the infected leaf (Machmud, 1982) and *B nigra* shows relatively high resistance to Xcr (McCulloch, 1929; Vicente *et al.*, 2006; Ponzio, 2016; Ponzio *et al.*, 2016b).

Plant culture

Plants were cultured in pots (Ø17 cm – 2 L content) filled with a 1:1 (v/v) mix of sand and potting soil (Lentse Potgrond, Lent, The Netherlands). Seeds of *B. nigra* were obtained from 25 plants (CGN06619, Center for Genetic Ressources (CGN), Wageningen, The Netherlands) that were exposed to open pollination in the experimental farm of Wageningen University in spring 2012. Plants for the

greenhouse experiments were grown in greenhouse compartments (22 °C ± 2 °C, 60-70% r.h, 16L:8D) and infested when the first flowers had just opened. For the field experiments, plants were sown in a greenhouse and seedlings (3-4 leaves) were transferred outdoors and grown in an area protected by insect screen. Plants were transplanted to the experimental field within 5 days after the opening of the first flowers. *Brassica nigra* plants in full bloom had several hundreds of open flowers.

Insect and bacterial cultures

Brevicoryne brassicae aphids and *P. brassicae* caterpillars were reared on Brussels sprout plants (*Brassica oleracea* variety *gemmifera*) in a greenhouse compartment (22 ± 2°C, 50-70% r.h., L16:D8). *Pieris brassicae* butterflies were provided with honey solution from organic production (10%, Melvita, Weide & Veldbloemen) as food, and were kept in a greenhouse compartment (25 ± 2 °C, 50-70% r.h., 16L:8D). *Diaeretiella rapae* was reared in a climate cabinet (25 ± 1°C, L16:D8) and *C. glomerata* was reared in a greenhouse compartment (22 ± 2°C, 50-70% r.h., L16:D8). Adult parasitoids were provided with honey from organic production and water.

Xcr was obtained from Utrecht University, the Netherlands (Ponzio *et al.*, 2014). The bacteria were cultured in an artificial liquid medium (8 g L^{-1} of Difco™: beef extract 3.0 g L^{-1} and peptone 5.0 g L^{-1}, BD Diagnostics, New Jersey, USA) kept at 28 °C under gentle shaking at 170 rpm for 21 ± 1 h. The liquid medium with bacterial cells was then centrifuged twice for 10 min at 4080 rotation per min (rpm) and after each centrifugation the pellet containing the bacterial cells was re-suspended in buffer (10 mM M_gSO_4). We estimated the concentration of the inoculum by measuring the light absorbance at 600 nm and adjusted the concentration of the final inoculum to 10^9 cells mL^{-1} by diluting in buffer (10 mM M_gSO_4).

Plant treatments

Within a few days after the opening of the first flowers, plants were exposed to buffer, single attack by either *B. brassicae*, *P. brassicae*, or Xcr, or to dual attack by combinations of these attackers. To infest the plants with *B. brassicae*, five young adult females were gently placed on a bract (inflorescence leaf) at the base of the inflorescence. The aphids dispersed within a few hours, mainly to the flower stalk, where they quickly multiply and form colonies. For the infestation with *P. brassicae*, plants were exposed to mated female butterflies that were allowed to oviposit. We kept a cluster of 30 eggs on the plants and gently removed any surplus of eggs. To infect plants with bacteria, we soaked a 2 x 2-cm piece of cotton wool with 500 µL

of the bacterium inoculum (10^9 cells mL^{-1} in buffer) that we placed on the underside of a bract and maintained for 4 hours by a soft clip; control plants (Buffer) were clipped with cotton wool soaked in buffer solution only (10 mM M_gSO_4) as described in (Chrétien *et al.*, 2018). Plants exposed to single or dual attack with the insects *B. brassicae* and/or *P. brassicae* were also clipped with cotton wool with buffer solution to control for a possible effect of buffer and clipping on plant responses when comparing volatile emission, parasitism in the field, and attraction of pollinators in the field. For this same purpose, whenever a plant was inoculated with Xcr for the two-choice assays testing for parasitoid preference in a greenhouse, the other plant of the pair was clipped with buffer on cotton wool. Plants treated with dual attack were simultaneously exposed to two out of the three attackers, and a bract never received more than one treatment.

In the greenhouse, caterpillars hatched after 5 days, and in the field this took place between 11 to 16 days after oviposition. To ensure that flowers were damaged for at least one day prior to the experiments in the greenhouse, 50 % of the caterpillars were transferred to the inflorescence when they had not yet moved there by themselves; subsequently, cotton wool was placed around the stem as a barrier between the leaves and the inflorescence. In the field experiments, we allowed the caterpillars to freely disperse throughout the plant.

Effect of single and dual attack on volatile emission of *Brassica nigra* at the flowering stage

To investigate whether plant odours are influenced by plant exposure to single *versus* dual attack, we collected volatiles from the headspace of aboveground parts of the plants after eight days of exposure to one of eight treatments: 1) *B. brassicae*, 2) *P. brassicae*, 3) Xcr, 4) *P. brassicae* plus *B. brassicae*, 5) *P. brassicae* plus Xcr, 6) *B. brassicae* plus Xcr, 7) buffer (control) and (8) non-treated (control). At day 8, *B. nigra* carried L2/L3 caterpillars and aphid colonies of about 80-180 individuals. All insects were removed from the plant prior to volatile collection. Apart from the non-treated control plants, all individual plants that were not exposed to the bacteria were exposed to buffer for 4 hours, to control for a possible effect the clip with the buffer-impregnated cotton piece. Volatiles were collected by enclosing the aboveground parts of the plant in an oven bag (Toppits® Brat-Schlauch, polyester; 32 x 32 x 70 cm; Toppits, Minden, Germany). Filtered synthetic air was then flushed into the oven bag at a rate of 300 mL min^{-1} (224-PCMTX*, air-sampling pump Deluxe equipped with an inlet protection filter, Dorset, UK) through a Teflon tube. The air that passed through the bag was then sucked out through a second Teflon tube at a

flow rate of 200 mL min^{-1} and led through a metal tube filled with 90 mg of Tenax TA 25/30 mesh (Grace-Alltech). Both Teflon tubes were inserted in the top of the oven bags through an opening that was then closed tightly. The volatile collection lasted for 1.5 h. Oven bags were discarded after use. We had six replicates per treatment and volatiles were collected in a greenhouse compartment (25 ± 2 °C, 50-70% r.h., 16L:8D).

Volatiles were analysed by a gas chromatograph coupled to a mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Plant volatiles were desorbed from the Tenax using a thermodesorption unit (Ultra 50:50, Markes, Llantrisant, UK) that heated the samples from 25 °C to 250 °C (5 min hold) at a rate of 60 °C min^{-1}. The released compounds were focused in a cold trap (ID 1.80 mm) at 0 °C that was filled with Tenax and charcoal. The volatiles were transferred in splitless mode to the analytical column (30 m x 0.25 mm ID, 1 μm film thickness, DB-5, Phenomenex, Torrence, CA, USA) by flash heating the cold trap at 40 °C sec^{-1} to 280 °C (10 min hold). The temperature program of the oven started at 40 °C and immediately rose to 280 °C (4 min hold) at a rate of 5 °C min^{-1}. Electron impact ionization at 70 eV was used to ionize the column effluent. Mass scanning was carried out from *m/z* 35 to 300 with 4.70 scans sec^{-1}. Compounds were identified (Table S1) by comparing the mass spectra with the mass spectra of Wiley libraries, NIST and the Wageningen Mass Spectral Database of Natural Products. Identified compounds were confirmed based on retention index using the literature (Adams, 1995).

Peak area was calculated based on total ion chromatograms (TIC) or selected-ion chromatograms (SIC). SIC integration technique has a better resolution than TIC technique, therefore, SIC data were used to analyse the effect of treatment on the composition of the volatile blend. The TIC technique allows to cumulate peak area of the eluted peaks of a chromatogram and, thus, was used to calculate total volatile emission of plants. Results of this study are based on plant compounds that were detected in at least 50% of the replicates of one of the treatments, and whose peak area was 3.5-fold higher than in background samples (volatiles collected from empty oven bags in which no plant was present) for peaks that were integrated based on TIC, or five-fold higher for peaks that were integrated based on SIC. Peak area of individual compounds was divided by fresh biomass of the aboveground part of the plant for standardization.

Changes in the composition of the volatile blend was analysed using Projection to Latent Structures - Discriminant Analysis (PLS-DA), and treatment was set as the

grouping factor. We tested whether plant exposure to attackers had an effect on the total emission of volatiles with a Kruskal-Wallis test, and a 0.05 significance level. Since a significant effect of treatment was detected, we analysed which treatments differed from each other by removing treatments with the most extreme median from the analysis until no significant difference was found between treatments. We had four to seven plant replicates per treatment.

Effect of single and dual attack on the parasitisation of *Brassica nigra*'s atttackers by parasitoid wasps - greenhouse experiments

To investigate whether dual attack affects indirect plant resistance, we assessed the preference of the caterpillar parasitoid *C. glomerata* and of the aphid parasitoid *D. rapae* for plants exposed to dual attack by the host and a non-host compared with plants exposed to single attack by the host. We recorded the plant on which the parasitoid landed first and the plant that was preferred for oviposition in the following two-choice situations: 1) *P. brassicae vs*. *P. brassicae* plus *B. brassicae*, 2) *P. brassicae vs. P. brassicae* plus *Xcr* for *C. glomerata*, and 1) *B. brassicae vs*. *B. brassicae* plus *P. brassicae*, 2) *B. brassicae vs. B. brassicae* plus *Xcr* for *D. rapae*. Plants were used in the experiments after 8 days of exposure to the treatments.

Cotesia glomerata

Pairs of plants were placed 70 cm apart on a T-shaped platform inside a flight chamber made of gauze (293 x 200 x 230 cm) that hung in a greenhouse compartment (25 ± 2 °C, 50-70% r.h., 16L:8D). Individual wasps were released at the base of the T, 90 cm away from the plants. Each wasp was given 10 min to locate a host and we recorded on which of the two plants the wasp first landed and parasitized caterpillars. An observation was stopped as soon as the wasp oviposited in a caterpillar because *C. glomerata* generally oviposits in all caterpillars of a chosen clutch of caterpillars (Wiskerke & Vet, 1994). When a wasp did not land on a plant within five minutes, the wasp was removed from the flight chamber and this was recorded as non-response. To compensate for possible positional bias, the position of the plants was swapped after every three wasps tested. Each female wasp was only tested once, and a maximum of 15 wasps were tested per individual pair of plants. Among those 15 wasps, three to ten wasps responded by flying to a pair of plants and landing on one of them, whereas two to seven wasps subsequently responded with oviposition. When only one wasp responded, that plant pair was excluded from the analysis. All behavioural observations were carried out in the afternoons. We had six to eight pairs of plants per combination of treatments.

Diaeretiella rapae

A pair of plants was placed in an igloo tent (70 x 73 x 105 cm) in a greenhouse compartment (22 ± 2 °C, 60-70% r.h., 16L:8D). One female parasitoid (3-to-6-days old) was released per igloo tent and left with the plants for 20 h. Wasps were given 15 min to accommodate to the new environment, and the location of the wasp (on the tent, on a plant) was recorded after 15 min, 1 h, and 2 h following the release. The plant on which the wasp was first recorded was considered to be the plant that was preferred by the wasp; we assumed that this proxy represented the wasp's preference because 95% of the wasps stayed on the same plant during the 2 h of recording. After 20 h, the wasp was removed from the cage and the plants were kept in a greenhouse compartment until aphid mummies developed (25 ± 2 °C, 50-70% r.h., 16L:8D). Number of mummies was then recorded at 7 ± 1 d after the release of the wasp, and we used the highest total number of mummies to determine the plant that was preferred for oviposition. Only in one case both plants had exactly the same number of mummies, and no preference was recorded for this pair. We had 18 to 20 pairs of plants per combination of treatments.

Statistical analyses

For the data on *C. glomerata* preference, the effect of plant pair on wasp first landing and oviposition choice was first tested for each combination of treatment using a Generalized Linear Model (GLM) based on a binomial distribution with logit as a link function, and a 0.05 significance level. No effect of plant pair was detected, and wasp choice was then tested regardless of the plant pair. Thus, landing and oviposition preference of both *C. glomerata* and *D. rapae* was tested with a binomial test, with a probability of 0.50 for a wasp to go to one of the plants. The effect of treatment on the number of mummies per plant was tested with a paired Student's t-test at the 0.05 significance level; assumptions for normal distribution of the data and equal variances were met.

Effect of single and dual attack on parasitisation of the insect attackers and on the visitation of flowers of *Brassica nigra* by pollinators - field experiments

Field layout

To test whether dual attack to flowering *B. nigra* affects visitation by pollinators and parasitism or predation of insect herbivores in the field, we set up common-garden experiments in which plots of *B. nigra* plants were exposed to one out of seven treatments: 1) *B. brassicae*, 2) *P. brassicae*, 3) Xcr, 4) *P. brassicae* plus *B. brassicae*, 5) *P. brassicae* plus Xcr, 6) *B. brassicae* plus Xcr, and 7) buffer (control). Each plot (50

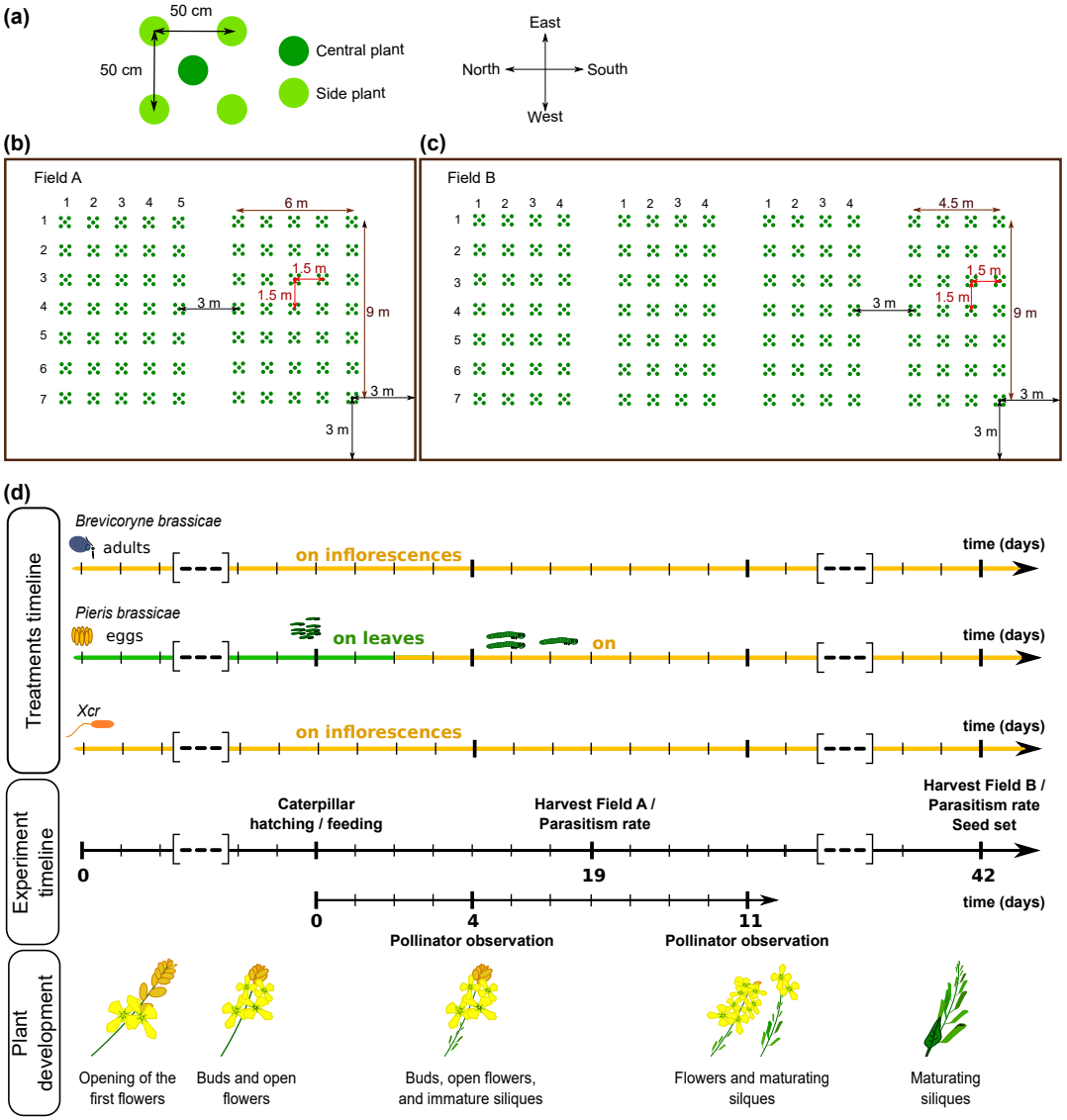


Fig. 1. Schematic representation of the layout of the common garden experiment and timeline of the recordings and of the treatments. The compass indicates the orientation of the plots and of the two fields. **(a)** A plot (50 cm x 50 cm) consists of one central plant (dark green) and four side plants (light green). **(b)** Field A consisted of 2 blocks of 9 m x 6 m, each composed of 35 plots organized in 7 rows and 5 columns. **(c)** Field B consisted of 4 blocks of 9 m x 4.5 m, each composed of 28 plots organized in 7 rows and 4 columns. (**b, c**) Blocks were 3 m apart, and within a block, central plants of each plot were 1.5 m apart. A fence (brown line) was placed around the fields, 3 m apart from the plots. **(d)** *Brassica nigra* plants were infested with either 5 *Brevicoryne brassicae* aphids or 30 eggs of *Pieris brassicae*, or infected with *Xanthomonas campestris* pv. *raphani* (Xcr). Dual attack consisted of simultaneous attack by two of these organisms. Caterpillars hatched from eggs from 9 to 16 days after infestation, and plants were harvested 19 days after infestation (Field A) to measure parasitism rates and 42 days after infestation (Field B) to measure parasitism rates and seed set. Pollinator visitations were recorded at 4 and 11 days after caterpillars had hatched and fed from the plants. Flower status shows the development of the reproductive parts of the plant over the field experiments.

cm x 50 cm) consisted of five plants (Fig 1a). Following the design by Lucas-Barbosa *et al.* (2013), plants were infested/infected in the field directly after transplantation, and only the central plant of a treated plot was originally exposed to one or two of the three attackers. Insect attackers dispersed through the plot and colonized the side plants of a given plot on average within 7 days after infestation for aphids, and about 10 days after hatching for the caterpillars. If fewer than 50 % of the caterpillars hatched from the eggs (*i.e.* < 15 caterpillars), we added neonate caterpillars from the laboratory culture to ensure a minimum of 15 caterpillars per plot. Similarly, when fewer than two aphid colonies were found per plot, we added six adult female aphids from our laboratory rearing. In this way, the central plant of all infested plots carried at least 15 caterpillars and two aphid colonies, to ensure induction of the plants by the insect attackers. Once plants had been transferred to the field, no attempt was made to prevent further infestation by any other herbivores.

The common-garden experiment was divided into fields A and B: 1) in field A, we investigated the effects of dual attack on parasitisation of *B. brassicae* and *P. brassicae* (Fig. 1b); 2) in field B, we investigated the effects of dual attack on flower visitation by pollinators, parasitisation of *B. brassicae* and *P. brassicae*, and on the number of seeds produced by the plants (Fig. 1c). Layout of field A (Fig. 1b) consisted of two blocks of 9 m x 6 m each consisting of 35 plots organized in seven rows and five columns. Fourteen plots were transplanted to the field on each day – two plots for each of the seven treatments – within five consecutive days (between June 3rd 2015 to June 7th 2015). Plants were harvested after 19 days of exposure to the treatments (between June 22nd 2015 and June 26th 2015) to measure parasitism upon *B. brassicae* and *P. brassicae*. We had 10 replicates (plots) per treatment. Layout of field B (Fig. 1c) consisted of four blocks of 9 m x 4.5 m, each composed of 28 plots organized in seven rows and four columns. Fourteen plots were transplanted to the field on each day – two plots for each of the seven treatments – within eight consecutive days (between May 19th 2015 and May 26th 2015). Plants were harvested 42 days after infestation/infection (between June 30th 2015 and July 7th 2015) to measure parasitism of *B. brassicae* and *P. brassicae*, and seed set of plants. We had 16 replicates (plots) per treatment. In both fields A and B, blocks were 3 m apart, and within a block, the central plants of plots were 1.5 m apart. Treatments were assigned to plots according to a Latin square design, so that plants of the same treatment that were infested/infected on the same day were never planted in the same column or row. A fence was placed around each field, 3 m from the nearest plots, to protect the fields from relatively larger herbivores such as rabbits. The ground area around the plots was regularly weeded.

Parasitisation of aphids and caterpillars

The number of aphid mummies on all central plants and on two side plants of a given plot was counted at 19 days after infestation in field B and 42 days after infestation in field A. Plants were first harvested and then living aphids were gently brushed off the plants to uncover all mummies for counting. *Brevicoryne brassicae* are the main aphids developing on *B. nigra* in our field, and mummies are assumed to mainly belong to this species. Numbers of mummies per plant were averaged at the plot level. Numbers of mummies at day 19 were analysed with a Generalized Linear Mixed Model (GLMM) based on a negative binomial distribution and log as link function. Numbers of mummies at day 42 were normally distributed, and variances could be assumed as equal, therefore these data were analysed with a Linear Mixed Model (LMM). In both cases, the main effect of treatment was set as fixed factor and intercept was included, we added the planting day as a random factor. We used a significance level of 0.05.

Parasitisation of *P. brassicae* was estimated by dissecting caterpillars to check for the presence or absence of parasitoid eggs in L1/L2 caterpillars. Caterpillars were collected from the plants of field B after 19 days since infestation of the plants with butterfly eggs.

Pollinator visitation to *Brassica nigra* exposed to multiple attack in the field

We recorded pollinator visitation to plots of treated and control plants of *B. nigra* after 4 d and 11 d of caterpillar feeding. At day 4, caterpillars were at the L2/L3 stage and had been feeding from flowers for 1-2 days; plants mainly had flowers and buds. At day 11 caterpillars were in the L5 stage and had been feeding from flowers for 8-9 days; plants had flowers and unripe siliques. Fewer than 10% of the caterpillars were still on the plants at day 11 as most caterpillars had been predated or had died of disease. The two observation time points were dertermined based on the number of days that plants were exposed to caterpillar feeding because caterpillars hatched from the eggs within a time window of 11 to 16 days after egg deposition, irrespective of the treatments (Fig. S2). Plots with no caterpillars as treatment were observed based on the caterpillar plots of the same planting date.

Each plot was observed for 10 min, using a handheld computer (Psion Workabout Pro TM3, London, UK) and the Observer software (version 10, Noldus Information Technology b.v., Wageningen, The Netherlands; Noldus 1991). Observations were performed between 9 am and 6 pm. To investigate the effect of single and dual attack on the attractivness of flowers, we recorded the number and identity of pollinators

visiting a given plot of plants. Pollinator identity was classified into four groups: 1) bees, 2) flies, 3) bumblebees, 4) butterflies, and we identified the most abundant flower visitors to the species level. Bees included the honeybee *Apis mellifera* and solitary bees. Flies included the syrphid *Eristalis tenax* and other fly species. Bumblebees included *Bombus lapidarius*, *Bombus terrestris*, and other bumblebees visiting the flowers. Butterflies included *Pieris rapae* and other butterflies visiting the flowers. We also recorded the time spent between the moment a pollinator first arrived to the flowers of the plot till the moment it left the plot's plants, and we counted the number of flowers visited over this time period. Thus, we could calculate the time a pollinator spent on average per flower. Once the visitor had left, we would start following a new one, and repeated this over the 10 minutes of observation. We cannot exclude the possibility that the same pollinator returned to the plot after having left, and if so, its visit was recorded as a new visitation.

Effect of treatment of the total number of pollinators was analysed with an LMM, data were normally distributed and met the assumption of equal variances. We used a GLMM based on a normal distribution with identity as a link function to test the effect of treatments on time spent per flower by honeybees and flies and for the number of flowers honeybees visited during their visit to the plot of plants. For the number of flowers that flies visited during their visit to a plot of plants, we used a GLMM based on a negative binomial distribution, with logit as a link function. Data of one individual honeybee (Aphid plus bacteria treatment, day 4) was excluded; this insect spent 10 times more time on a flower than average and was considered as an outlier. For all LMM and GLMM anlyses, the main effect of treatment was set as fixed factor and the intercept was included; in addition, the date when the plot was observed (observation date) was set as a random factor. Total number of pollinators at day 4 and day 11 were compared with a G-test. Effect of treatment of the assemblage of the pollinator community was tested with a Chi-square test at the two time points. When a significant effect of treatment was detected, we analysed which treatment differed from each other by removing treatments with the most extreme distributions from the analysis. The significance level was 0.05 in all cases.

Effect of single and dual attack on the seed set of *Brassica nigra* in the field

We determined the seed set of *B. nigra* plants in field B after 42 days of exposure to the treatments. Siliques were stored to dry at room temperature in the dark in a farm building (Unifarm, Wageningen University) until seeds were processed and counted. Number of seeds was estimated by dividing the weight of the total number of seeds of a given plant by the weight of 100 seeds of this plant. Seeds were counted

for the central plant and the two randomly selected side plants for each plot, and we calculated the average number of seeds produced per plant per plot. In four cases, the central plant had died during the experiment, and in three cases the seed bag of the central plant could not be identified. Data related to these plots were not included in the analyses.

Effect of treatment was analysed with an LMM. Main effect of treatment was set as fixed factor and intercept was included, the planting date was set as a random factor, and we used a significance level of 0.05.

Statistical software and procedures

We used respectively the default GENMIX, GENLIN, and MIXED procedures of SPSS (IBM Corp., IBM SPSS Statistics for Windows, Versions 24, Armonk, NY: IBM Corp.) to run GLMMs, GLMs, and LMMs. Chi-square tests and G-tests were performed in Excel (version 2016, for Windows, Microsoft® office, Redmond, Washington, USA). PLS-DAs were performed in SIMCA (Umetrics AB, Version 15.0, Umeå, Sweden), and we used the default 7-fold cross-validation (CV) procedure to calculate model fit parameters: the number of significant components, the goodness of fit R^2X and R^2Y, and the predictive ability Q^2Y. R^2X and R^2Y represent respectively the percentage of variation explained by the matrix of volatile data (X) and by the matrix of treatments (Y). In poor models, the order of rows in the original data set can affect the value of Q^2Y (Triba *et al.*, 2015). Thus, we ran each PLS-DA for 4 datasets with randomly permuted rows, and we display the averaged Q^2Y value ± standard deviation; models were stable and there was little variation.

Results

Effect of single and dual attack on the volatile emission of flowering *Brassica nigra*

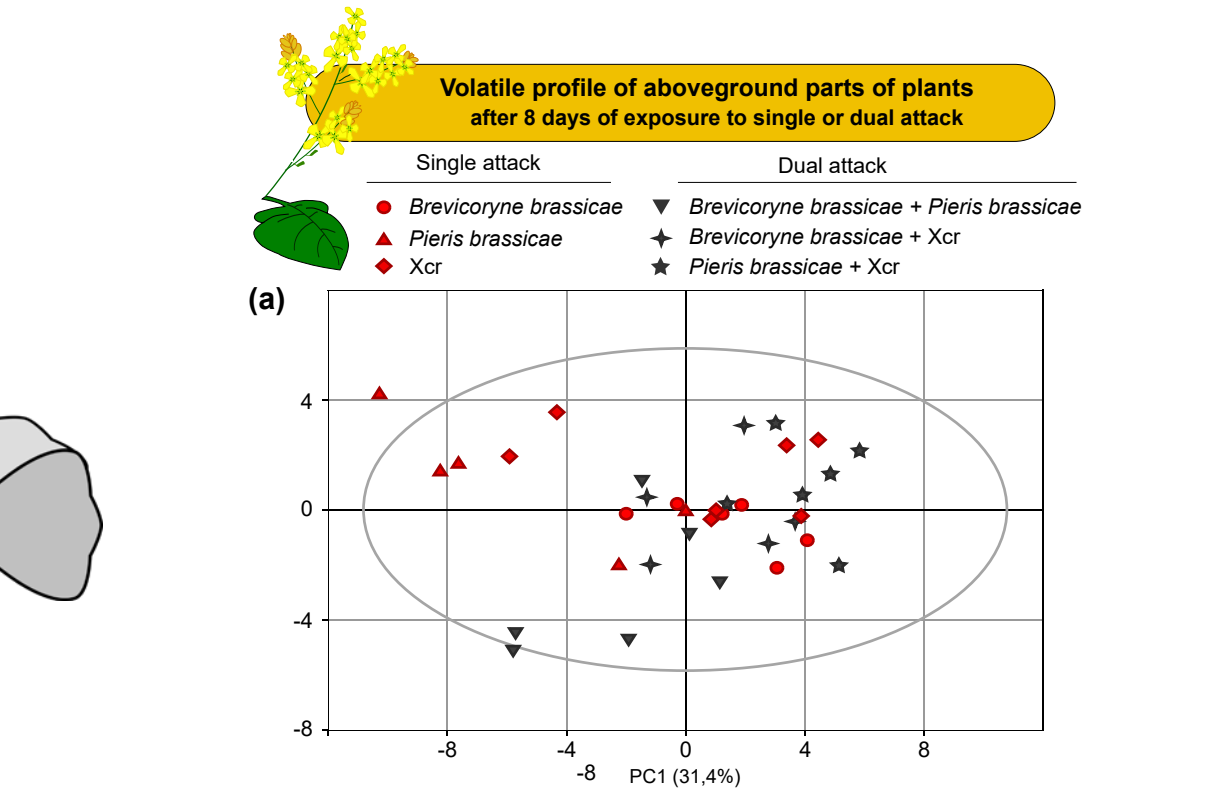
Composition of the volatile blend

We detected, identified and quantified 59 compounds belonging to 6 classes of volatile compounds (Table S1): 36 terpenoids (26 monoterpenoids, 1 homoterpenoid, 8 sesquiterpenoids, 1 homosesquiterpene), 9 aromatic benzenoids and phenylpropanoids, 5 fatty acid derivatives, and 6 nitrogen-containing compounds (including glucosinolate derivatives), and 3 compounds that could not be identified and classified. Only quantitative and no qualitative differences were determined when comparing compounds in the volatile blend emitted by aboveground parts of flowering *B. nigra* exposed to the different treatments.

Volatile profile

Treatments affected the composition of the volatile blend emitted by aboveground parts of *B. nigra* plants exposed to single and simultaneous dual attack by *B. brassicae* aphids, *P. brassicae* caterpillars and Xcr bacteria, which mostly attacked inflorescences (Fig. 2). A Principal Latent Structure Discrimination Analysis (PLS-DA) based on the samples of the six treatment combinations resulted in a model with one significant principal component (R^2X = 0.314, R^2Y = 0.104, Q^2 = 0.045 ± SD 0.007). We display here the projection of the data for plant samples over two principal components for visual representation (Fig. 2a). The first principal component (PC1) explained 31.4% of the variation and separated plant samples according to plant exposure to caterpillars. Blends of plants exposed to caterpillars only, and to a lower extent, plants exposed to aphids plus caterpillars, differed from the blends of plants exposed to the other treatments. Fifty percent (29 VOCs) of the VOCs contributed most to the differentiation of the blends (VIP > 1). Most of them were more associated to the blend of plants exposed to caterpillars and to aphids plus caterpillars, indicating that they were emitted at higher rate by these plants compared to plants exposed to single attack with aphids or with bacteria, or to dual attack with the bacteria plus an insect (Fig. 2b, Table S1). The VOCs that contributed most to the separation described were mainly monoterpenoids (18), representing 70% of all monoterpenoids detected in the blend. Additionally, four out of six nitrogen-containing VOCs detected in the blend contributed to the separation described above, including glucosinolate derivatives.

We analyzed in simpler models whether the blend of plants exposed to single attack differed from the blend of plants exposed to dual attack to further link changes in volatile emission with the attraction of natural enemies of the insect attackers in single attack and dual attack situations. Therefore, the blend of plants exposed to single attack with *P. brassicae* caterpillars was compared to the blends of plants exposed to dual attack with *P. brassicae* and another attacker, similarly the blend of plants exposed to single attack with *B. brassicae* aphids was compared to blends of plants exposed to dual attack with *B. brassicae* and another attacker. A PLS-DA based on the samples of plants exposed to single attack by *P. brassicae*, *P. brassicae* plus *B. brassicae*, or *P. brassicae* plus Xcr, resulted in a model with one significant principal component (R^2X = 0.360, R^2Y = 0.344, Q^2 = 0.228 ± SD 0.008), and confirmed that samples of plants exposed to caterpillars plus Xcr differed from samples of plants exposed to caterpillars and aphids plus caterpillars according to PC1 (36% of the variation, Fig. 3a). However, blends of plants exposed to caterpillars could not be separated from blends of plants exposed to aphids plus caterpillars.


Volatile profile of aboveground parts of plants
after 8 days of exposure to single or dual attack
Single attack
Brevicoryne brassicae
Pieris brassicae
Xcr
Dual attack
Brevicoryne brassicae + Pieris brassicae
Brevicoryne brassicae + Xcr
Pieris brassicae + Xcr
(a)
PC1 (31,4%)

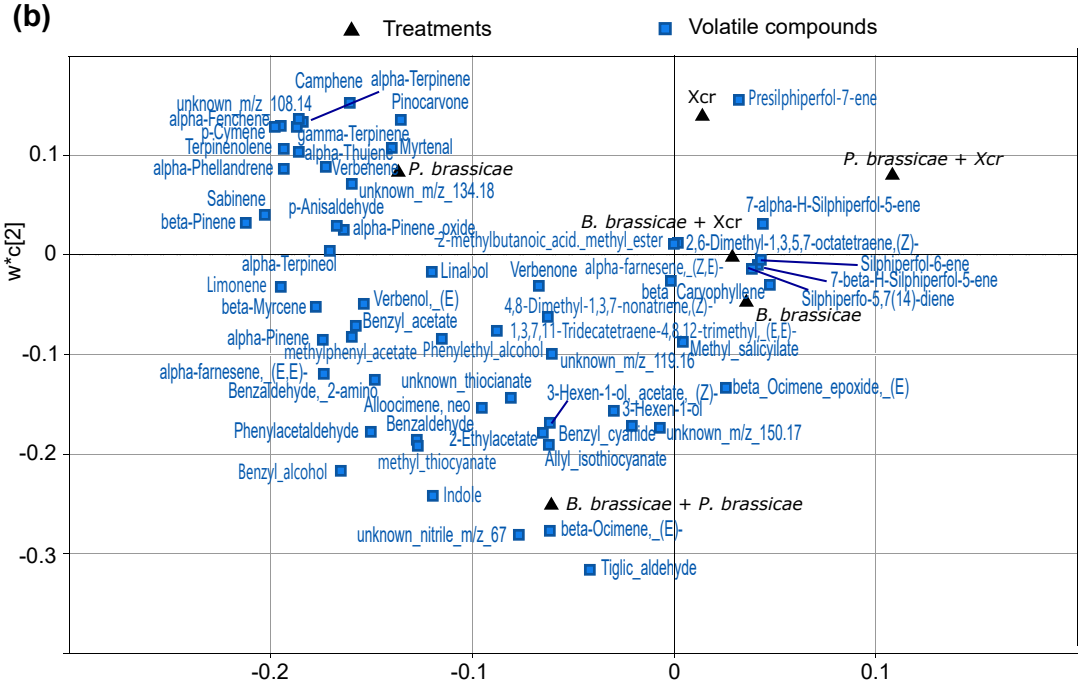

(b)
Treatments
Volatile compounds
w*c[2]
Camphene
alpha-Terpinene
unknown_m/z_108.14
Pinocarvone
alpha-Fenchene
p-Cymene
gamma-Terpinene
Terpinenolene
alpha-Thujene
Myrtenal
alpha-Phellandrene
Verbenene
P. brassicae
unknown_m/z_134.18
Sabinene
p-Anisaldehyde
beta-Pinene
alpha-Pinene_oxide
Xcr
Presilphiperfol-7-ene
P. brassicae + Xcr
7-alpha-H-Silphiperfol-5-ene
B. brassicae + Xcr
2-methylbutanoic_acid,_methyl_ester
2,6-Dimethyl-1,3,5,7-octatetraene,(Z)-
Silphiperfol-6-ene
7-beta-H-Silphiperfol-5-ene
alpha-Terpineol
Linalool
Verbenone
alpha-farnesene,_(Z,E)-
beta-Caryophyllene
Silphiperfo-5,7(14)-diene
Limonene
Verbenol,_(E)
4,8-Dimethyl-1,3,7-nonatriene,(Z)-
B. brassicae
beta-Myrcene
Benzyl_acetate
1,3,7,11-Tridecatetraene-4,8,12-trimethyl,_(E,E)-
alpha-Pinene
methylphenyl_acetate
Phenylethyl_alcohol
Methyl_salicylate
unknown_m/z_119.16
alpha-farnesene,_(E,E)-
unknown_thiocianate
beta_Ocimene_epoxide,_(E)
Benzaldehyde,_2-amino
3-Hexen-1-ol,_acetate,_(Z)-
Alloocimene,_neo
3-Hexen-1-ol
Phenylacetaldehyde
Benzaldehyde
2-Ethylacetate
Benzyl_cyanide
unknown_m/z_150.17
methyl_thiocyanate
Allyl_isothiocyanate
Benzyl_alcohol
Indole
B. brassicae + P. brassicae
unknown_nitrile_m/z_67
beta-Ocimene,_(E)-
Tiglic_aldehyde

Fig. 2 (left). Volatiles profiles of aboveground parts of flowering *Brassica nigra* plants exposed to buffer (light grey), single attack (red), and dual attack (dark grey). Projection to Latent Structures - Discriminant Analysis (PLS-DA) based on the quantity of 59 volatile compounds (expressed as peak area $/10^9$ g^{-1} of plant fresh biomass) that could be detected and quantified using chromatograms based on single ion chromatograms (SIC) in samples of *B. nigra*. Volatile blends were collected for 1.5 h from aboveground parts of *B. nigra* exposed for 8 days to either single or dual attack by *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars, and/or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria. Treatments were set as classes in the PLS-DA. (**a**) Scatter plots show grouping pattern of samples from a same treatment according to the first two principal components (t[1], t[2]). The percentage between brackets indicates the percentage of variation in the data explained by each principal component. The Hotelling's ellipse confines the confidence region (95 %) of the score plot. (**b**) Loading plots show the contribution of each of the volatile compounds' quantifications to the first two principal components.

VOCs contributing to the separation between blends were mostly the same as in the PLS-DA based on all treatments (VIP > 1), and the difference in blend composition was mainly driven by over 70% of the 26 monoterpenoids detected, five out the six nitrogen-containing compounds detected, and three out of the five fatty acid derivatives (Fig. 3b, Table S1). PLS-DA based on VOC emission of plants exposed to single attack by *B. brassicae*, *B. brassicae* plus *P. brassicae*, or *B. brassicae* plus Xcr did not result in a model with a significant PC, indicating that the model could not separate the blends based on their composition.

Differences in the total volatile emission of *B. nigra* upon attack followed similar trends as observed for the composition of the VOC blends. Overall, treatment affected total volatile emission and the effect was of small magnitude (Fig. S1, Kruskal-Wallis, chi-square = 14.159, df = 7, P = 0.048). Although total VOC emission of treated plants did not differ from those of control plants (plants exposed to buffer or non-treated), total emission of plants exposed to aphids plus caterpillars significantly differed from total emission of plants exposed to caterpillars plus bacteria.

Effect of single and dual attack on the parasitisation of *Brassica nigra*'s attackers by parasitoid wasps

In the greenhouse, dual attack did not influence the first landing and oviposition preference of *C. glomerata* in a two-choice assay where *B. nigra* plants were exposed to the host caterpillars alone versus plants exposed to hosts plus a non-host that was either aphids or bacteria (Fig. 4a, b). In the field, across all treatments, 97 % of the 60 caterpillars recollected were parasitized.

Similarly, also landing and oviposition of *D. rapae* in a two-choice assay in a greenhouse with *B. nigra* plants exposed to host aphids alone versus plants exposed

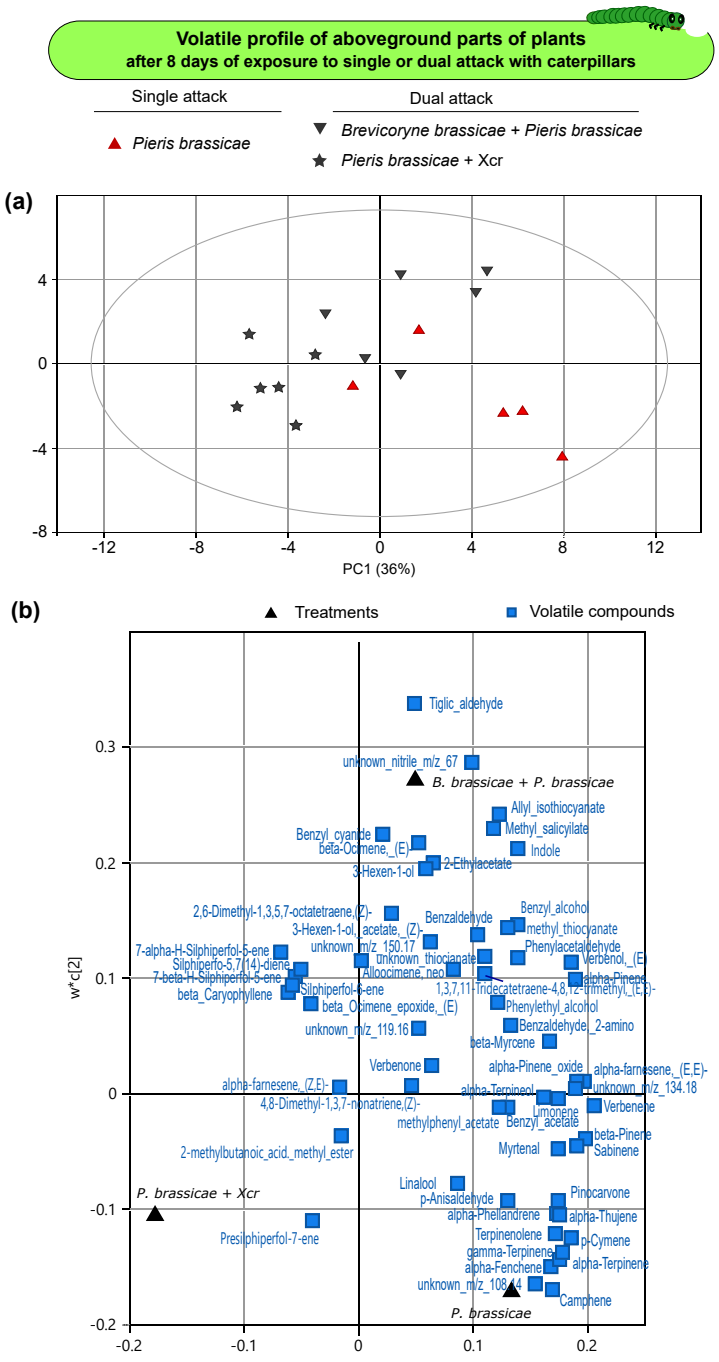


Fig. 3. Volatiles profiles of aboveground parts of flowering *Brassica nigra* plants exposed to single attack with *Pieris brassicae* caterpillars (red), dual attack with *Brevicoryne brassicae* aphids plus *P. brassicae* or with *P. brassicae* plus *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria (dark grey). Projection to Latent Structures - Discriminant Analysis (PLS-DA) based on the quantity of 59 volatile compounds (expressed as peak area $/10^9$ g^{-1} of plant fresh biomass) that could be detected and quantified using chromatograms based on single ion chromatograms (SIC) in samples of *B. nigra*. Volatile blends were collected for 1.5 h from aboveground parts of *B. nigra* exposed for 8 days to either single attack with *Pieris brassicae* caterpillars, dual attack with *Brevicoryne brassicae* aphids plus *P. brassicae* or with *P. brassicae* plus *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria. Treatments were set as classes in the PLS-DA. (**a**) Scatter plots show grouping pattern of samples from a same treatment according to the first two principal components (t[1], t[2]). The percentage between brackets indicates the percentage of variation in the data explained by each principal component. The Hotelling's ellipse confines the confidence region (95 %) of the score plot. (**b**) Loading plots show the contribution of each of the volatile compounds' quantifications to the first two principal components.

to hosts plus a non-host were equally distributed over the two plant treatments (Fig. 4c, d). Moreover, we found similar numbers of mummies on single and dually-treated plants exposed to *D. rapae* (Fig. S2a). The field experiment led to a similar conclusion. In the common-garden experiment, treatments did not influence the number of aphid mummies recorded on plants that were initially exposed to aphids alone or to aphids plus caterpillars or bacteria (Fig. S2b, c).

Effect of single and dual attack on the visitation of flowers of *Brassica nigra* by pollinators

Number of pollinators

Overall, bees were the most abundant pollinators (73.4 % at day 4 and 74.1 % at day 11), followed by syrphid flies (9.5 % on day 4 and 5.4 % on day 11), then bumblebees (6.1 % on day 4 and day 11) and finally butterflies (0.1 % on day 4 and day 11) (Table S2). Similar numbers of pollinators visited the plants on day 4 and on day 11 irrespective of the treatments (Table S2, G-test, $P = 0.168$). We recorded 790 pollinators over 56 plots after 4 days of caterpillar feeding, and 905 pollinators over 60 plots after 11 days of caterpillar feeding. Treatments did not affect the number of pollinators visiting the *B. nigra* flowers neither on day 4 nor on day 11 (Fig. 5 a,c).

Assemblage of the pollinator community

Plant exposure to the attackers had an effect on the assemblage of the community of pollinators at both time points. The pollinator community of plants exposed to dual attack by aphids plus caterpillars particularly differed from the pollinator community of plants exposed to single attack or other combinations of dual attack (Fig. 5b, d, Table S2). At the first time point, this difference seemed to be driven by the number of flies visiting plants exposed to dual attack by aphids plus caterpillars (Table S2). About three times fewer flies visited plants attacked by aphids plus caterpillars when compared with plants attacked by caterpillars only or aphids only, and about four times fewer flies visited plants attacked by aphids plus caterpillars compared with plants exposed to other dually-attacked treatment (Table S2, Fig 5b). At the second time point, almost twice as many flies visited plots attacked by aphids plus caterpillars compared with plots where plants were attacked by caterpillars plus bacteria (Table S2, Fig.5 d).

Time spent per flower

The time that honeybees and flies spent per flower at each of the two observation time points was not influenced by the treatments (Fig. S3), and neither was the number of flowers visited in row by a bee or a fly (Bees, LMM, day 4: $F = 0.678$,

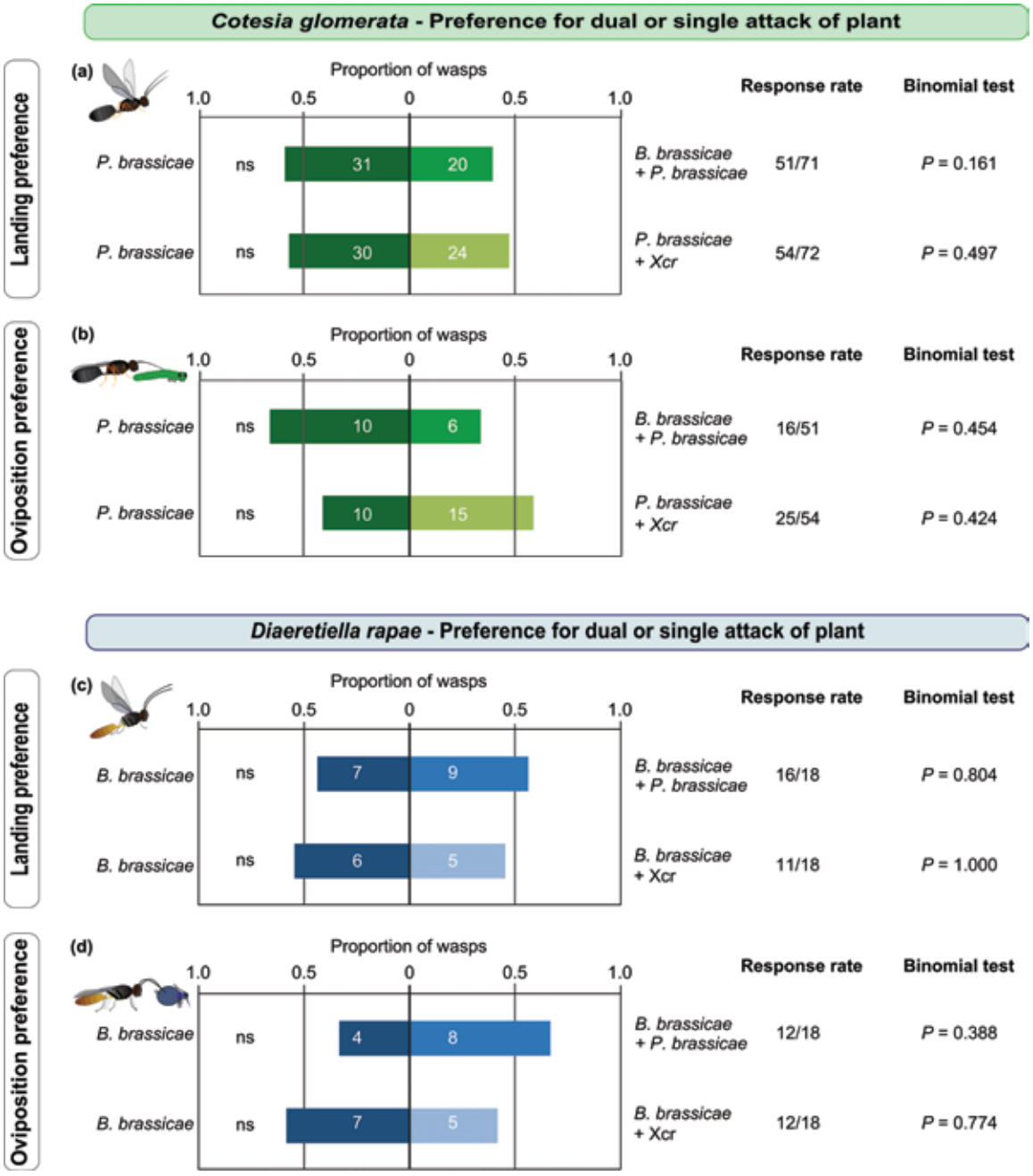

Cotesia glomerata - Preference for dual or single attack of plant
Landing preference
(a)
Proportion of wasps
1.0 0.5 0 0.5 1.0
Response rate
Binomial test
P. brassicae
ns
31
20
B. brassicae + P. brassicae
51/71
P = 0.161
P. brassicae
ns
30
24
P. brassicae + Xcr
54/72
P = 0.497
Oviposition preference
(b)
Proportion of wasps
1.0 0.5 0 0.5 1.0
Response rate
Binomial test
P. brassicae
ns
10
6
B. brassicae + P. brassicae
16/51
P = 0.454
P. brassicae
ns
10
15
P. brassicae + Xcr
25/54
P = 0.424
Diaeretiella rapae - Preference for dual or single attack of plant
Landing preference
(c)
Proportion of wasps
1.0 0.5 0 0.5 1.0
Response rate
Binomial test
B. brassicae
ns
7
9
B. brassicae + P. brassicae
16/18
P = 0.804
B. brassicae
ns
6
5
B. brassicae + Xcr
11/18
P = 1.000
Oviposition preference
(d)
Proportion of wasps
1.0 0.5 0 0.5 1.0
Response rate
Binomial test
B. brassicae
ns
4
8
B. brassicae + P. brassicae
12/18
P = 0.388
B. brassicae
ns
7
5
B. brassicae + Xcr
12/18
P = 0.774

Fig. 4 (left). Proportion of *Cotesia glomerata* and *Diaeretiella rapae* that landed and oviposited on flowering *Brassica nigra* plants exposed to either the parasitoid's host or to the host plus a non-host in two-choice assays in a greenhouse. To test the preference of *C. glomerata* wasps, plants were either exposed to single attack by *Pieris brassicae* caterpillars (host), or exposed to dual attack by *Brevicoryne brassicae* aphids plus *P. brassicae* or by *P. brassicae* plus *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria; to test the preference of *D. rapae* wasps, plants were either exposed to single attack by *B. brassicae* aphids (host), or exposed to dual attack by *B. brassicae* plus *P. brassicae* or by *B. brassicae* plus Xcr. Plants exposed to single and dual attack were combined two by two in a flight chamber where a *C. glomerata* wasp was released for 10 min **(a, b)** or in a tent where a *D. rapae* wasp was released and left for 20 h **(c, d)**. We scored plantson which the wasps landed first (**a, c**) and plants on which *C. glomerata* first oviposited or that had the most aphids parazitized by *D. rapae* (**b, d**). Response rate indicates the number of responding wasps over the number of tested wasps. Proportions were tested using a binomial test), and the significance level was set to $\alpha = 0.05$.

Numerator df = 6, Denominator df = 44.835, $P = 0.668$; day 11: LMM, F = 1.026, Numerator df = 6, Denominator df = 47.680, $P = 0.420$; Flies, GLMM, day 4: F = 1.629, df1 = 6, df2 = 15, $P = 0.207$, day 11: low replication did not allow statistical analyses).

Effect of single and dual attack on seed set of *Brassica nigra* in the field

Plants of plots exposed in the field to an initial attack by *B. brassicae* aphids, *P. brassicae* caterpillars, or Xcr bacteria produced on average similar numbers of seeds as plants of plots exposed to dual combinations of those attackers or to buffer (control plots) (Fig. 6).

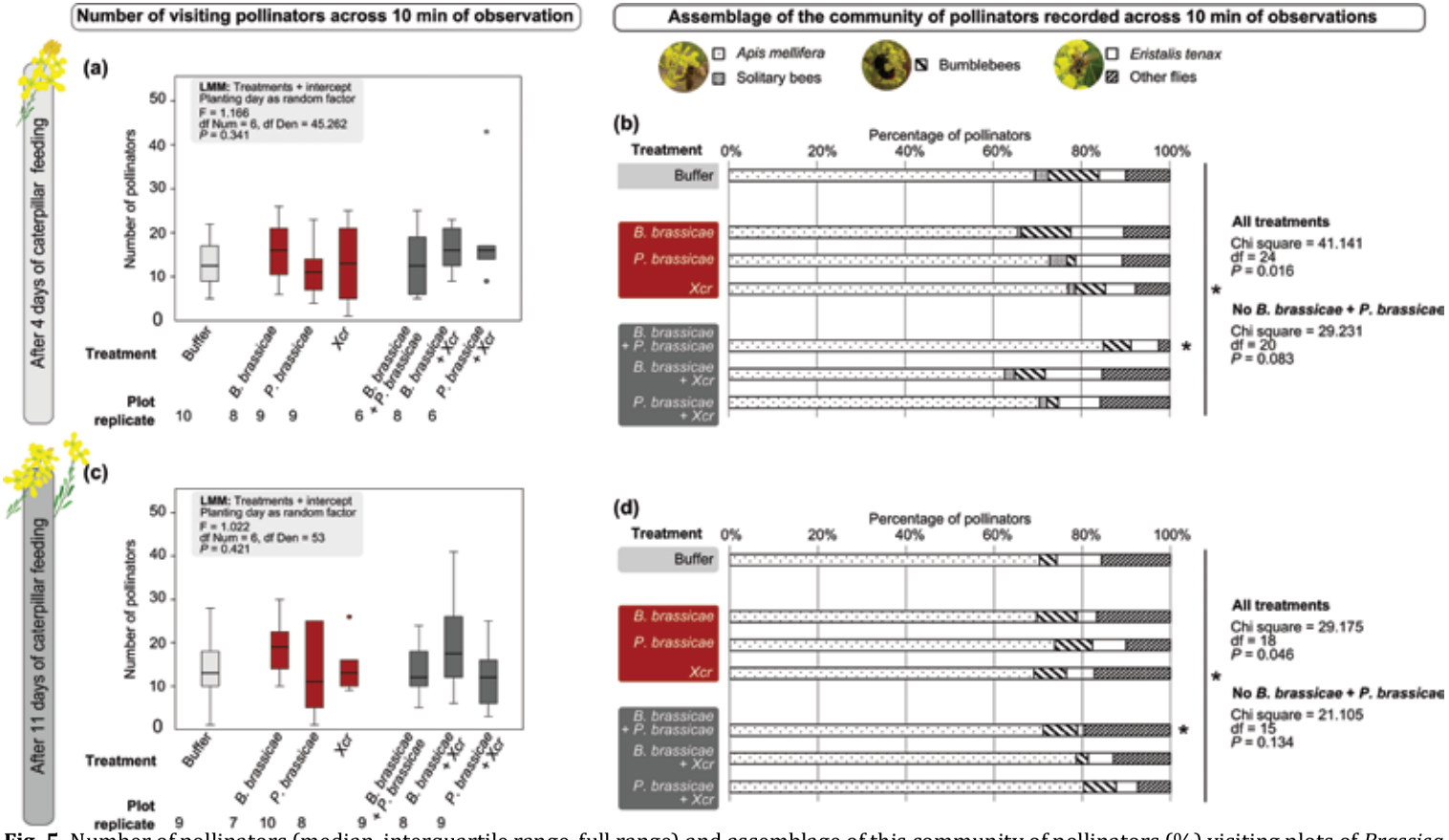


Fig. 5. Number of pollinators (median, interquartile range, full range) and assemblage of this community of pollinators (%) visiting plots of *Brassica nigra* exposed to buffer (light grey), single attack (red), and dual attack (dark grey) in the field. Plots of five *B. nigra* plants were observed for 10 min and all pollinators arriving to the plot were counted and recorded as either *Apis mellifera* (honeybee), solitary bees, bumblebees, *Eristalis tenax,* or other flies. Pollinator observation took place after 4 d of caterpillar feeding (a, b) and after 11 d (c, d). Numbers of pollinators were summed per plot (a, c) and the contribution of each type of pollinator (%) to this total community was calculated (b, d). Butterflies were excluded as they represented less than 1% of the whole community. Plots were exposed in the field to single attack by either *Brevicoryne brassicae, Pieris brassicae,* and/or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by combinations of two of those attackers or exposed to buffer (control). Outliers are represented by " ° " (further than 1.5 x interquartile range). Effect of treatments was analysed with a Linear Mixed Model (LMM), the significance level was set to $\alpha = 0.05$.

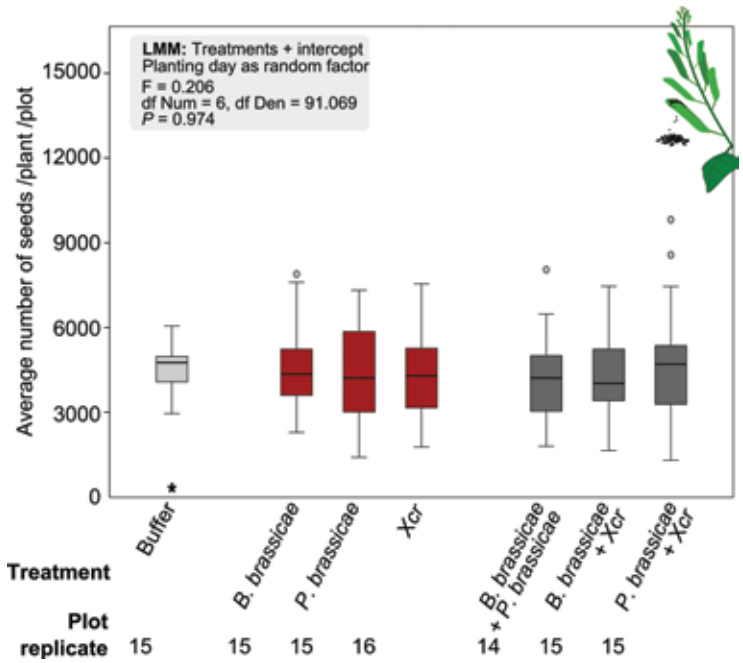


Fig. 6. Average number of seeds (median, interquartile range, full range) produced by plants of each plot of *Brassica nigra* plants exposed to buffer (light grey), single attack (red), and dual attack (dark grey) in the field. Plots of *B. nigra* were exposed in the field to single or dual attack by *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars, and/or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, or exposed to buffer (control). After 42 d, seeds were harvested, weighed and their numbers estimated for the central plant and two side plants of each plot; we show the average number of seeds produced per plant per plot. Outliers are represented by " ° " (further than 1.5 x interquartile range). Effect of treatments was analysed with a Linear Mixed Model (LMM). The significance level was set to $\alpha = 0.05$.

Discussion

Our study shows that exposure of flowering *B. nigra* to single attack with three different attackers, or to dual combinations of these, maintained their ability to attract parasitoids and pollinators despite the fact that treatments affected the volatile blend emitted by the plants. Caterpillars, in single or dual combination with another attacker, were the main inducers of changes in plant volatile emission, and 50% of the 59 VOCs emitted by flowering *B. nigra* strongly contributed to the changes. On the one hand, parasitoid preference was resilient to these induced changes, and plant exposure to non-host attackers neither affected choices of parasitoids in the greenhouse nor parasitism in the field. On the other hand, the pollinator community of plants exposed to dual attack with aphids plus caterpillars differed from the community associated to plants exposed to single attack or other combinations of dual attack. However, plant exposure to attackers did not impact the number of pollinators visiting flowers within the time frame of our observations, and seed set was not affected by plant exposure to attack. *Brassica nigra* interacts with over 10 different pollinator species from at least three insect orders, and negative effects of induced changes in floral traits to a subset of the community may be buffered by the attraction of other pollinator species. Thus, the complex blend of volatiles emitted by *B. nigra* may preserve interactions with a diverse mutualistic community of pollinators and with the main natural enemies of the herbivores. Interacting with diverse mutualists likely supports the maintenance of pollination and indirect resistance upon multiple attack.

The inducible emission of volatiles by plants in the flowering stage has so far mainly been explored for single attack, and most studies focused on plant responses to folivory (Kessler *et al.*, 2011; Lucas-Barbosa *et al.*, 2011; Pareja *et al.*, 2012; Bruinsma *et al.*, 2014; Schiestl *et al.*, 2014). These studies provided considerable information on the specificity of response of plants in the flowering stage to folivory. In *Sinapis alba*, for example, folivory by *Plutella xyllostella* caterpillars had no effect on volatile emission of flowers, whereas attack by aphids did in comparison with non-attacked plants (Pareja *et al.*, 2012). The specialist aphid *Lipaphis erysimi*, especially, had a stronger inhibition on floral volatile emission than the generalist aphid *Myzus persicae* did (Pareja *et al.*, 2012). In our study, most of the attack was on the inflorescences, and we showed that florivory on *B. nigra* resulted in the emission of a volatile blend that is characteristic for the attacker or dual combination of attackers. Indeed, plants exposed to caterpillars only or to caterpillars plus aphids emitted a blend that had a different composition than the blend emitted by plants exposed to caterpillars plus bacteria. Our study indicates

that dual attack induces plant volatile emission that can differ from that of plants exposed to those species of attackers individually, and this was so far mainly known for plants in the vegetative stage (Ponzio *et al.*, 2013). These results are similar to data on flowering cotton plants exposed to multiple attack; cotton plants produced volatiles in different proportions than when plants were exposed to single attack (Magalhães *et al.*, 2018). Such specificity to attackers suggests that *B. nigra* in the flowering stage are able to perceive, recognize and respond to different types of attack to their inflorescences.

Differences in volatile emission are likely to be the result of specific signal-transduction pathways induced upon attack. The mechanism underlying the inducibility of biosynthetic pathways that mediate resistance in plants have received ample attention when regarding plants in the vegetative stage (Stam *et al.*, 2014). Few studies have addressed the molecular pathways that are induced in flowering parts of plants. It appears that plants in the flowering stage can respond to attack on their inflorescences with an induction of the phytohormone jasmonic acid (JA) and its derivatives (Chrétien *et al.*, 2018; Li *et al.*, 2018). Moreover, the JA-mediated pathway is differentially regulated in flower tissues is than in leaf tissues (Li, R *et al.*, 2017). Data from our previous study show that inflorescences of *B. nigra* exposed to *P. brassicae*, *B. brassicae* or *Xcr*, or to dual infestations by combinations of these attackers had distinct phytohormonal profiles, and that caterpillars in particular induced the active forms of JA in inflorescence tissues (Chrétien *et al.*, 2018). Dual attack by caterpillars plus aphids and caterpillars plus bacteria induced higher concentrations of jasmonates in inflorescences than single attack (Chrétien *et al.*, 2018). Effect of treatments on volatile emission seems to be in line with the patterns observed in phytohormonal induction upon attack. Indeed, caterpillars were the main driver of changes in VOC emission by aerial parts of plants, which can result from induced JA levels and direct disruption of plant tissues. Moreover, VOC emission upon caterpillar attack or attack by caterpillars plus aphids differed from VOC emission by *B. nigra* upon attack by caterpillars plus bacteria, although no differences were found between the blend of plants exposed to caterpillars and to caterpillars plus aphids. Besides the role of JA in the production of HIPVs, an increase in JA levels can also lead to reduced nectar production (Bruinsma *et al.*, 2008), and in the present study, herbivore attack has likely affected other floral traits that we did not assess.

Herbivore-induced plant responses are complex, and they can be specifically perceived and exploited by multiple members of the plant community that can all

contribute to plant fitness, in a negative or positive manner (Kessler & Halitschke, 2007). Despite the chemical changes induced by dual attack in flowering *B. nigra,* the co-occurrence of two distinct attackers neither affected the attraction of pollinators to the flowers, nor the attraction and oviposition preference of the parasitoids. For plants in the vegetative stage, some studies highlighted HIPV-driven changes in parasitoid behaviour in the presence of non-hosts (Dicke *et al.*, 2009; Ponzio *et al.*, 2013). However, for *B. nigra* in the vegetative stage, co-infestation of *P. brassicae* caterpillars with *B. brassicae,* eggs of *P. brassicae* or Xcr induced changes in the volatile emission of the blend, but the parasitoid *C. glomerata* could still locate its host in two-choice assays with plants exposed to the host caterpillar *vs.* plants exposed to the host and a non-host (Ponzio *et al.*, 2014; Cusumano *et al.*, 2015). Similarly, *D. rapae* and other aphid parasitoids tend to maintain their ability to locate their host upon multiple attack on *Brassica juncea* (da Silva *et al.*, 2016). Cabbage VOC blends are complex and changes cannot be linked to parasitoid attraction in a straightforward way (Ponzio *et al.*, 2014; Li, Y *et al.*, 2017). The VOC emission that we measured represents the full sampled headspace and is likely broader than the subset of volatiles that is used by the parasitoid (Ponzio *et al.*, 2014). In flowering *B. nigra*, we detected as many as 59 compounds that belong to at least six classes of compounds, thus, we can consider *B. nigra*'s odour as a complex blend (Dudareva *et al.*, 2006). Among the blend components, 50 % contributed most to the separation of the blends upon attack in the multivariate analyses, the others having little or no contribution to the differences between the blends. It is thus possible that the subset of VOCs perceived and used by the parasitoid was little affected upon dual attack. Therefore, complex VOC blends may provide a certain chemical plasticity to attacked plants.

Plants may buffer detrimental effects of attack on some of their pollinators by attracting other ones. Such an ecological plasticity is especially an option for flowers that are not pollinator limited and interact with diverse range of pollinators (Lucas-Barbosa, 2016; Rusman *et al.*, 2018). Inflorescences of *B. nigra* recruited on average one to two pollinators per minute, which visited several flowers in a row. Competition between pollinators was even observed in the field (L.T.S. Chrétien & D. Lucas-Barbosa, pers. obs.). This abundance of pollinators probably explains why the total number of pollinators attracted to the plants was not affected by plant exposure to the attackers. Although the total number of pollinators was not influenced by the plant exposure to the attackers, herbivore attack affected the assemblage of the pollinator community visiting the flowers. Plants exposed to aphids plus caterpillars, in particular, harboured a different community than other

treatments. Different pollinators harvest different types of rewards and can exploit different flower traits. As a consequence, changes in some flower traits in response to herbivory or pathogen attack may affect only a subset of the pollinator community (Junker *et al.*, 2013; Schiestl & Johnson, 2013; Lucas-Barbosa, 2016). *Brassica nigra* interacted with over 10 species of flower visitors belonging to three different orders: Hymenoptera, Diptera and Lepidoptera. In our study, most community changes were actually due to fewer flower visitations by syrphid flies, a pattern that had also been observed in (Rusman *et al.*, 2018). Upon attack, if changes of a flower trait repelled some pollinators, this decrease may have been compensated by an increase in visitation by other types of pollinators. Thus, generalist flowers may benefit from an ecological plasticity upon attack.

Similarly, plants may benefit from the attraction of a diverse community of specialist and generalist insectivores that may be resilient to changes in plant cues used by insectivores when plants are attacked by multiple organisms. Natural enemies of herbivores are diverse and abundant on brassicaceous plants (Lucas-Barbosa *et al.*, 2014; Lucas-Barbosa *et al.*, 2017; Stam *et al.*, 2018). These insectivores are an important component of the resistance strategy of plants in the flowering stage (Lucas-Barbosa *et al.*, 2014; Gols *et al.*, 2015; Lucas-Barbosa *et al.*, 2017; Knauer *et al.*, 2018). On *B. nigra*, mortality of herbivores on the plants was caused by parasitoids and predators, with few of the caterpillars surviving until the adult stage. Like pollinators, insectivores are known to use a wide array of cues to find their prey or host for oviposition, and these cues can be olfactory, visual, or gustatory (Kessler & Halitschke, 2007; Stam *et al.*, 2014). The diversity of insectivores encountered on aboveground parts of *B. nigra* is presumably large leading to nearly 100 % mortality of caterpillars. Insectivores mainly belonged to six orders: Hymenoptera, Diptera, Heteroptera, Coleoptera, Aranea, and Acarina (Lucas-Barbosa *et al.*, 2014), L.T.S. Chrétien and D. Lucas-Barbosa, pers. obs). Thus, abundance and diversity of natural enemies may provide *B. nigra* with a flexible means to indirectly resist to multiple attack by florivores.

When exposed to single or simultaneous dual attack by *B. brassicae* aphids, *P. brassicae* caterpillars and Xcr bacteria, which mostly attacked inflorescences, *B. nigra* produced similar numbers of seeds as control plants, which indicates that the plants compensated for damage and possible interference with mutualistic associations. Plants maintained interactions with both carnivores and pollinators despite changes in plant traits when exposed to single and dual attack to the inflorescences. Our results suggest that the resilience to attack of *B. nigra* may be

supported by the chemical diversity that supports various mutualistic interactions of the plant (Gols *et al.*, 2015; Lucas-Barbosa *et al.*, 2017). Complex VOC blends of flowering plants likely evolved under selection pressure of both pollinators and herbivores, and may limit pleiotropic effects (Schiestl *et al.*, 2014; Schiestl, 2015). We can expect to observe such flexibility for plants that exploit interactions with diverse community members, and compensation in seed production upon folivory and florivory seems to be common in the Brassicaceae (Lucas-Barbosa, 2016). Moreover, exploring the physiological and ecological plasticity of plants will bring complementary insights on the strategies developed by plants to cope with insect and pathogen attack, and on the evolution of plant chemical traits.

Acknowledgments

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Supplemental information

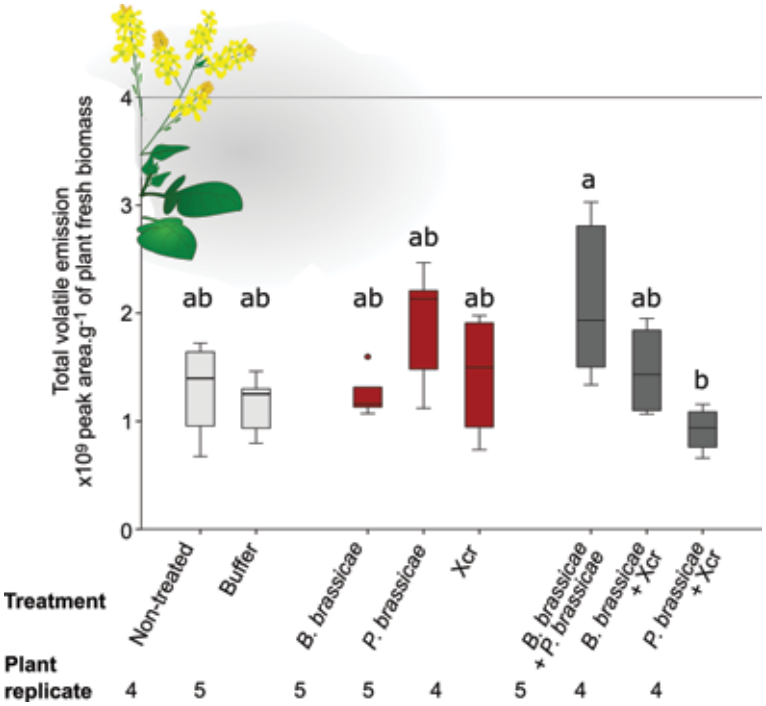

Fig. S1. Total volatile emission (x 10^9 peak area g^{-1} of plant fresh biomass - median, interquartile range, full range) of aboveground parts of flowering *Brassica nigra* left untreated or exposed to buffer (light grey), to single (red) attack, and to dual attack (dark grey). Volatile blends were collected for 1.5 h from aboveground parts of *B. nigra* exposed for 8 d to single or dual attack by *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars, and/or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, exposed to buffer or non-treated (controls). Graph show the sum of the peak area of 59 volatiles that could be detected and quantified using chromatograms based on Total Ion Counts (TIC). Effect of treatments was analysed using a Kruskal-Wallis test: chi-square = 14.159, df = 7, $P = 0.048$). The significance level was set to $\alpha = 0.05$. Outliers are represented by " ° " (further than 1.5 x Interquartile range).

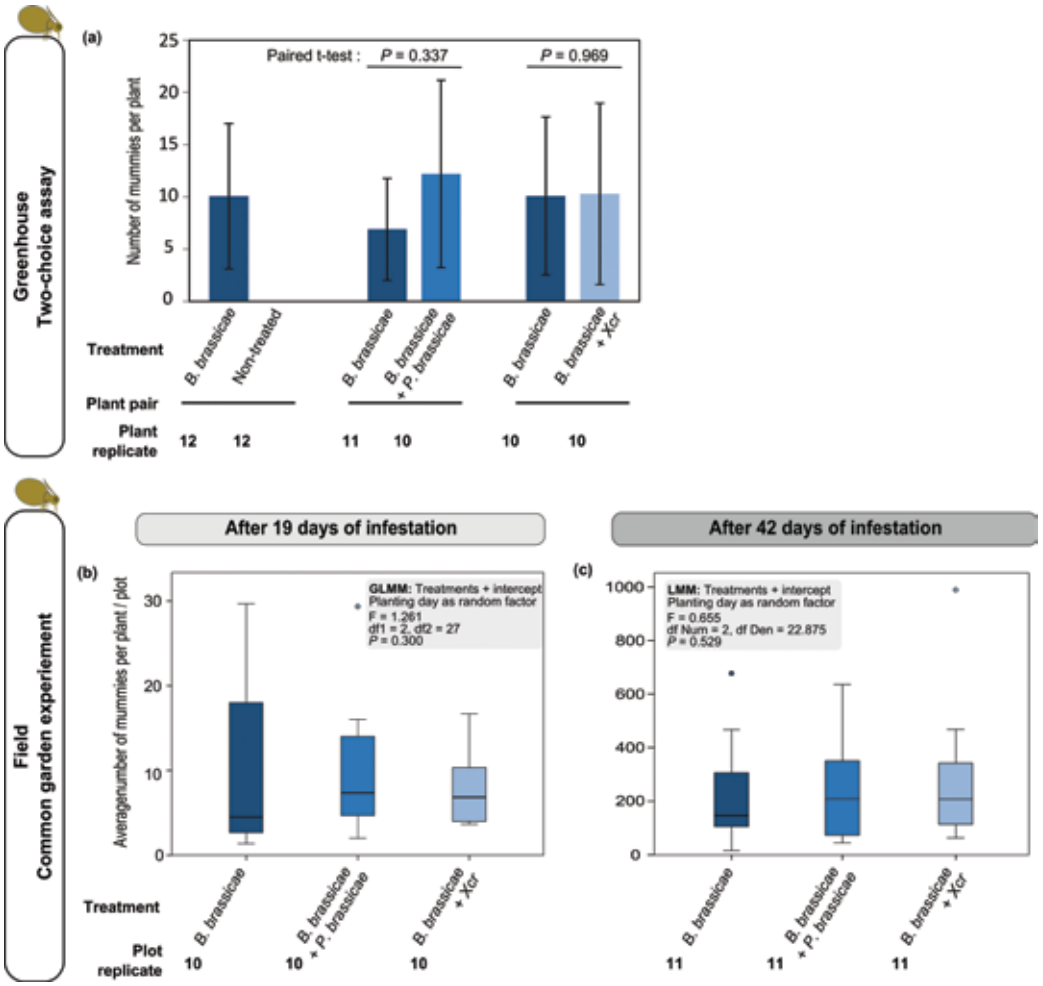

Fig. S2. Number of *Brevicoryne brassicae* aphids that were effectively parasitized (mummies) on *Brassica nigra* plants exposed to single attack by *B. brassicae* (dark blue) or to dual attack by *B. brassicae* plus *Pieris brassicae* (blue) or plus *Xanthomonas campestris* pv. *raphani* (Xcr) (light blue) in a greenhouse and in the field. **(a)** Number of mummies (mean ± SD) in 2-choice assay in a greenhouse. Plants infested with the host aphids only or with another non-host (*P. brassicae* caterpillars or Xcr bacteria) were exposed to *Diaretiella rapae* wasps for 20h. Mummies were counted after 7 ± 1 d. **(b, c)** Average number of mummies of aphids (mostly *B. brassicae*) recorded per plant within a plot (median, interquartile range, full range) in the common garden experiment (Wageningen, The Netherlands, 2015) after 19 d **(c)** and after 42 d of exposure to the attackers **(d)**. Plants were organized in plots of 5 plants, and the central plant of each plot was exposed to either single attack by *Brevicoryne brassicae* aphids, or to dual combinations of these aphids plus *P. brassicae* or plus Xcr. Mummies were counted on the central plants and two side plants per plot, and the number of mummies was averaged at the plot level. *D. rapae* is the main parasitoid of *B. brassicae* in The Netherlands. **(a, b, c)** Effect of exposure to attackers of the number of mummies was analysed with a paired t-test, a Linear Mixed Model (LMM), and a Generalized Linear Mixed Model (GLMM – based on a negative binomial distribution with Logit as link function). The significance level was set to $\alpha = 0.05$. "Num" stands for numerators, "Den" stands for denominator. Outliers are represented by " ° " (further than 1.5 x Interquartile range).

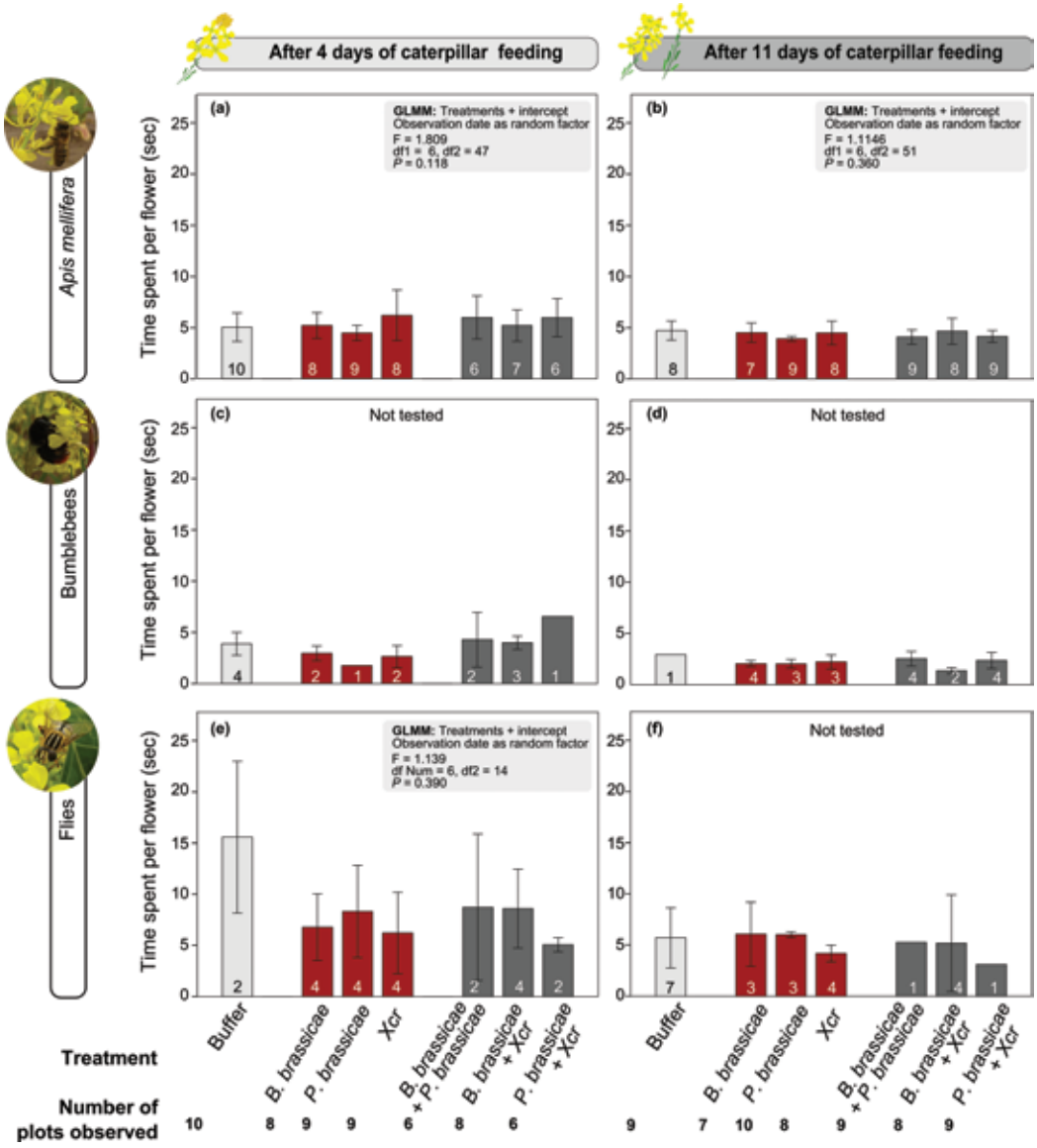

Fig. S3. Time spent per flower (mean ± SD) by three types of pollinators on flowers of *Brassica nigra* plants exposed to buffer (light grey), single attack (red), and dual attack (dark grey). Pollinator visitation to *B. nigra* was recorded in a common garden experiment (Wageningen, The Netherlands, 2015). Plants were organized in plots of five plants, and the central plant of each plot was exposed to either single attack by *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, to dual combinations of those attackers, or to buffer only (control). We recorded the time that *Apis mellifera* (honeybee), bumblebees, and flies would spend per flower of a plot over 10 min of observation. Time spent per flower was recorded at two time points: 4 d and 11 d after *P. brassicae* started feeding from the plant (leaves); at both time points, caterpillars had started feeding from *B. nigra* flowers. Numbers in the bars indicate the number of plot replicate for each pollinator and time point. Effect of the treatments was analysed with a Generalized Linear Mixed Model (GLMM) based on a normal distribution, identity was set as link function. The significance level was set to $\alpha = 0.05$. No statistical test was performed when there were fewer than two plot replicates for a treatment.

Table S1. List of volatile compounds collected from the aboveground part of flowering *Brassica nigra* plants exposed for 8 days to single attack (red) with either *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars or *Xanthomonas campestris* pv. *raphani* bacteria (Xcr), to dual attack with two of these attackers (dark grey), or to buffer (control – light grey) and of non-treated plants (light grey) in the greenhouse, the VIP value of the compounds in the PLS-DA analysis, and peak area.

	PLSDA, VIP>1		Peak area per treatment (mean ± SD) – expressed in 10^3/g of plant aerial fresh biomass							
	all treatments	caterpillar treatments	Non-treated	Buffer	*B. brassicae*	*P. brassicae*	Xcr	*B. brassicae* + *P. brassicae*	*B. brassicae* + Xcr	*P. brassicae* + Xcr
Volatile compounds *										
Benzenoids and phenylpropanoids (9	**4**	**2**								
2-Phenylethanol			247 ± 112	1859 ± 2075	1212 ± 1350	2398 ± 1857	1481 ± 2720	2222 ± 2429	730 ± 512	551 ± 515
Benzylacetate			62 ± 41	61 ± 45	104 ± 88	220 ± 139	78 ± 78	158 ± 139	55 ± 36	76 ± 44
Benzaldehyde			14220 ± 6758	25421 ± 10646	16607 ± 9215	26906 ± 9669	15544 ± 8030	31906 ± 25623	19408 ± 8817	14428 ± 6071
Benzaldehyde 2-amino			2392 ± 731	9513 ± 3199	5526 ± 4595	11326 ± 6640	5280 ± 3854	10273 ± 6562	7180 ± 4102	4886 ± 2301
Benzyl-alcohol			4123 ± 2596	8216 ± 2782	5642 ± 3100	9088 ± 2331	4643 ± 2670	9841 ± 5087	4374 ± 1505	4787 ± 2760
Methyl-phenyl-acetate			22 ± 12	95 ± 57	91 ± 99	306 ± 364	53 ± 62	180 ± 234	60 ± 37	25 ± 12
Methyl-salicylate			85 ± 68	162 ± 264	280 ± 298	83 ± 76	154 ± 146	123 ± 62	102 ± 107	35 ± 26
p-Anisaldehyde			2768 ± 1374	14701 ± 7813	7909 ± 5705	22806 ± 15105	9433 ± 6581	13628 ± 9353	8744 ± 4551	8488 ± 5139
Phenylacetaldehyde			1410 ± 948	12588 ± 8171	9476 ± 8420	19191 ± 15319	8080 ± 12387	19701 ± 17080	8884 ± 7054	3422 ± 3307
Monoterpenoids (26)	**18**	**19**								
2,6-Dimethyl-1,3,5,7-octatetraene (Z)			1232 ± 965	309 ± 288	1205 ± 1488	510 ± 464	1427 ± 3034	1000 ± 1054	452 ± 567	485 ± 693
Alloocimene, neo			6350 ± 3496	4008 ± 2821	4636 ± 2566	5567 ± 2267	3450 ± 2932	6603 ± 5030	3338 ± 1729	3423 ± 3351
alpha-Fenchene			119 ± 89	291 ± 265	121 ± 107	442 ± 287	179 ± 177	189 ± 114	107 ± 67	71 ± 72
alpha-Pinene			9012 ± 4141	12632 ± 5975	9353 ± 4172	17201 ± 3835	11743 ± 3646	16218 ± 7890	14388 ± 10252	5687 ± 4008
alpha-Pinene -oxide			18 ± 18	56 ± 49	19 ± 22	68 ± 40	41 ± 65	49 ± 31	21 ± 20	6 ± 6
alpha-phellandrene			488 ± 310	1195 ± 813	515 ± 283	1427 ± 715	712 ± 504	829 ± 459	678 ± 370	374 ± 313
alpha-Terpinene			247 ± 141	617 ± 503	250 ± 147	838 ± 452	414 ± 465	407 ± 231	265 ± 131	173 ± 151
alpha-Terpineol			251 ± 209	567 ± 409	322 ± 206	791 ± 500	432 ± 388	600 ± 353	406 ± 290	204 ± 186
alpha-Thujene			1815 ± 1117	4387 ± 3039	1954 ± 1103	5038 ± 2500	2766 ± 2120	2921 ± 1530	2453 ± 1241	1305 ± 1083
beta-Myrcene			1449 ± 509	2261 ± 1329	1477 ± 502	2565 ± 1008	1685 ± 861	2257 ± 1199	1681 ± 816	1002 ± 472
beta-Ocimene (E)			15722 ± 7799	6701 ± 7284	11255 ± 6490	9247 ± 8557	7010 ± 3887	17872 ± 12653	7749 ± 6982	7602 ± 7915
beta-Ocimene-epoxide (E)			374 ± 294	231 ± 375	203 ± 125	172 ± 186	139 ± 91	274 ± 178	212 ± 174	270 ± 259
beta-Pinene			836 ± 534	1473 ± 832	847 ± 601	2089 ± 794	1134 ± 581	1404 ± 721	1012 ± 644	464 ± 434
Camphene			769 ± 462	1708 ± 1469	1208 ± 1191	2674 ± 1680	1421 ± 1468	1087 ± 535	720 ± 338	456 ± 378
gamma-Terpinene			226 ± 128	541 ± 402	236 ± 133	735 ± 381	372 ± 393	367 ± 196	246 ± 123	154 ± 136
p-Cymene			568 ± 253	1150 ± 813	665 ± 288	1532 ± 672	887 ± 577	837 ± 430	655 ± 339	382 ± 238
Limonene			4730 ± 1308	6671 ± 3848	4781 ± 1000	8277 ± 3112	4819 ± 2561	6565 ± 3496	4891 ± 1562	3096 ± 1349
Linalool			110 ± 127	206 ± 231	147 ± 143	352 ± 434	64 ± 25	196 ± 237	87 ± 77	119 ± 62
Myrtenal			300 ± 200	526 ± 473	235 ± 135	496 ± 310	395 ± 383	321 ± 179	242 ± 223	102 ± 76
Pinocarvone			171 ± 171	344 ± 406	102 ± 110	314 ± 235	225 ± 307	161 ± 66	122 ± 111	36 ± 30
Sabinene			2513 ± 1410	4993 ± 2783	2543 ± 1333	5889 ± 2239	3377 ± 1845	4073 ± 1899	3211 ± 1750	1670 ± 1382
Terpinolene			549 ± 311	1322 ± 943	542 ± 287	1672 ± 889	815 ± 644	916 ± 470	742 ± 388	427 ± 349
Unknown monoterpene_m/z 134.18			107 ± 71	228 ± 204	113 ± 73	205 ± 89	169 ± 131	155 ± 84	100 ± 47	49 ± 39
Verbenene			829 ± 482	1512 ± 1004	776 ± 535	1526 ± 680	1243 ± 829	1096 ± 402	840 ± 475	315 ± 270
Verbenol (E)			401 ± 223	621 ± 413	404 ± 425	737 ± 288	588 ± 468	711 ± 452	465 ± 389	124 ± 100
Verbenone			2800 ± 3301	6979 ± 7067	3595 ± 2913	6469 ± 5979	4709 ± 2777	6131 ± 8452	6333 ± 5765	3396 ± 2586
Homoterpenoids (1)	**0**	**0**								
4,8-Dimethyl-1,3,7-nonatriene (Z)			2405 ± 2239	6308 ± 6102	2769 ± 1512	3734 ± 1305	2404 ± 1390	3385 ± 1419	2281 ± 2536	2779 ± 3706
Sesquiterpenoids (8)	**1**	**7**								
7-alpha-H-Silphiperfol-5-ene			504 ± 555	1620 ± 1862	490 ± 598	539 ± 354	1540 ± 875	1080 ± 967	1960 ± 2853	1093 ± 684
7-beta-H-Silphiperfol-5-ene			109 ± 117	425 ± 549	129 ± 160	158 ± 102	602 ± 618	560 ± 766	1002 ± 1752	570 ± 841
alpha-Farnesene (E,E)			1029 ± 333	1475 ± 620	1698 ± 962	2351 ± 896	1033 ± 820	1826 ± 737	1241 ± 796	767 ± 303
alpha-Farnesene (Z,E)			976 ± 466	1404 ± 1066	1460 ± 1073	1352 ± 436	1280 ± 720	1432 ± 659	751 ± 482	1501 ± 1459
beta-Caryophyllene (E)			18 ± 12	60 ± 31	63 ± 50	42 ± 16	121 ± 158	144 ± 232	259 ± 455	161 ± 203
Presilphiperfol-7-ene			14 ± 17	175 ± 191	46 ± 61	100 ± 119	120 ± 75	63 ± 91	144 ± 109	121 ± 113
Silphiperfol-5,7(14)-diene			10 ± 14	23 ± 26	8 ± 11	6 ± 5	65 ± 102	60 ± 111	112 ± 232	57 ± 102
Silphiperfol-6-ene			72 ± 79	285 ± 376	84 ± 104	103 ± 66	406 ± 420	369 ± 508	690 ± 1220	393 ± 588
Homosesquiterpene (1)	**0**	**0**								
1,3,7,11-Tridecatetraene-4,8,12-trimethyl (E,E)			1310 ± 1075	734 ± 388	1639 ± 1544	1693 ± 1449	1500 ± 1528	1779 ± 1679	764 ± 526	572 ± 294
Fatty acid derivatives (5)	**1**	**3**								
2- Ethyl-acetate			821 ± 554	417 ± 378	735 ± 501	611 ± 296	621 ± 962	1215 ± 1292	332 ± 141	363 ± 365
2-Methylbutanoic-acid-methyl-ester			219 ± 107	302 ± 220	290 ± 181	279 ± 184	243 ± 95	266 ± 154	180 ± 63	297 ± 211
3-Hexen-1-ol (Z)			501 ± 111	1014 ± 639	1425 ± 1480	896 ± 1111	1107 ± 1094	1519 ± 1147	905 ± 771	657 ± 322
3-Hexen-1-ol-acetate (Z)			2512 ± 1728	3860 ± 3750	3578 ± 3037	3785 ± 3960	2769 ± 1796	4757 ± 3060	3143 ± 2600	2659 ± 1699
Tiglic-aldehyde			1972 ± 697	2391 ± 2520	4146 ± 3885	2450 ± 1514	3354 ± 1478	6969 ± 4011	3413 ± 2210	2536 ± 2013
Nitrogen containing (6)	**4**	**5**								
Allyl-isothiocyanate			7527 ± 2236	13183 ± 7478	18734 ± 13184	18660 ± 14984	15828 ± 11682	27053 ± 12068	30373 ± 28397	8792 ± 2996
Benzyl-cyanide			1760 ± 1536	4547 ± 3372	4007 ± 3278	2864 ± 3565	3816 ± 4443	6400 ± 3744	3243 ± 2178	3334 ± 3443
Indole			2995 ± 1154	5544 ± 2792	4709 ± 3404	5798 ± 2746	3436 ± 2929	7373 ± 3680	5040 ± 3663	2681 ± 1479
Methyl-thiocyanate			36 ± 9	59 ± 31	60 ± 33	104 ± 69	58 ± 19	118 ± 81	106 ± 79	40 ± 11
Unknown nitrile m/z_67			4320 ± 2909	7493 ± 3633	11839 ± 7218	11636 ± 8875	8695 ± 3692	20266 ± 9981	14823 ± 16230	7167 ± 2336
Unknown thiocyanate			565 ± 187	1412 ± 961	2265 ± 2161	3035 ± 4415	1848 ± 2212	3446 ± 2059	3787 ± 3906	752 ± 344
Unclassifed unknown compounds (3	**1**	**1**								
unknown_m/z_108.14			241 ± 144	1245 ± 1362	203 ± 195	1258 ± 1084	375 ± 369	404 ± 225	410 ± 471	101 ± 64
unknown_m/z_119.16			45 ± 21	67 ± 78	49 ± 31	69 ± 65	44 ± 22	78 ± 110	72 ± 38	42 ± 19
unknown_m/z_150.17			704 ± 532	180 ± 158	402 ± 541	256 ± 236	150 ± 113	477 ± 478	235 ± 285	319 ± 507

* Putative identy, based on retention index and mass spectrum

Table S2. Total number of pollinators visiting plots of *Brassica nigra* after 4 d and 11 d of caterpillar feeding in the common garden experiment, Wageningen, The Netherlands, 2015. When comparing numbers across treatments, note that the number of replicate N (number of plots) might be different from one treatment to the other.

		Bees			Bumblebees				Flies			Butterflies				
	Treatments	*Apis mellifera*	Solitary bees	Total bees	*Bombus lapidarius*	*Bombus terrestris*	Other bumblebees	Total bumblebees	*Eristalis tenax*	Other flies	Total flies	*Pieris rapae*	Other butterflies	Total butterflies	Total counts	N (plots)
4 days after caterpillar feeding	Buffer	91	4	95	5	6	0	11	8	13	21	0	0	0	**127**	10
	B. brassicae	82	1	83	9	3	0	12	15	13	28	1	0	1	**124**	8
	P. brassicae	73	4	77	2	0	0	2	11	11	22	0	0	0	**101**	9
	Xcr	87	2	89	6	2	0	8	8	9	17	0	0	0	**114**	9
	B. brassicae + *P. brassicae*	68	0	68	4	0	1	5	5	2	7	0	0	0	**80**	6
	B. brassicae + Xcr	82	3	85	5	2	1	8	17	20	37	0	0	0	**130**	8
	P. brassicae + Xcr	81	2	83	1	0	1	2	11	18	29	0	0	0	**114**	6
	Total counts	**564**	**16**	**580**	**32**	**13**	**3**	**48**	**75**	**86**	**161**	**1**	**0**	**1**	**790**	**56**
	% of the total community	**71.39**	**2.03**	**73.42**	**4.05**	**1.65**	**0.38**	**6.08**	**9.49**	**10.89**	**20.38**	**0.13**	**0.00**	**0.13**	**100**	

		Bees			Bumblebees				Flies			Butterflies				
	Treatments	*Apis mellifera*	Solitary bees	Total bees	*Bombus lapidarius*	*Bombus terrestris*	Other bumblebees	Total bumblebees	*Eristalis tenax*	Other flies	Total flies	*Pieris rapae*	Other butterflies	Total butterflies	Total counts	N (plots)
11 days after caterpillar feeding	Buffer	90	0	90	4	1	0	5	12	20	32	0	0	0	**127**	9
	B. brassicae	92	0	92	9	2	1	12	6	22	28	0	0	0	**132**	7
	P. brassicae	96	1	97	8	1	1	10	8	13	21	0	0	0	**128**	10
	Xcr	76	1	77	5	2	0	7	7	19	26	0	1	1	**111**	8
	B. brassicae + *P. brassicae*	91	1	92	6	1	3	10	2	25	27	0	0	0	**129**	9
	B. brassicae + Xcr	122	3	125	3	1	0	4	8	20	28	0	0	0	**157**	8
	P. brassicae + Xcr	98	0	98	3	4	1	8	6	9	15	0	0	0	**121**	9
	Total counts	**665**	**6**	**671**	**38**	**12**	**6**	**56**	**49**	**128**	**177**	**0**	**1**	**1**	**905**	**60**
	% of the total community	**73.48**	**0.66**	**74.14**	**4.20**	**1.33**	**0.66**	**6.19**	**5.41**	**14.14**	**19.56**	**0.00**	**0.11**	**0.11**	**100**	

Chapter 4

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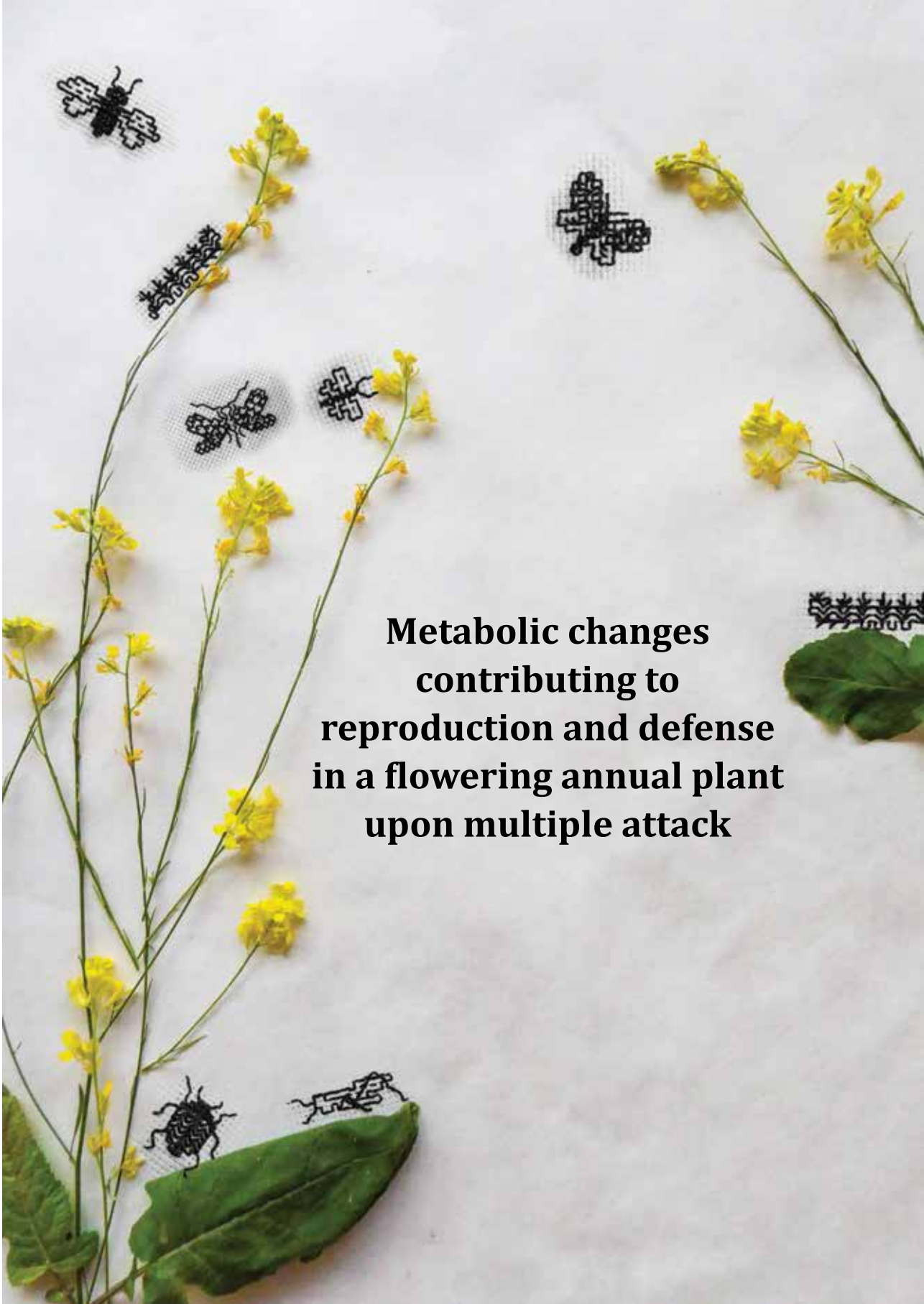


Metabolic changes contributing to reproduction and defense in a flowering annual plant upon multiple attack

Abstract

Resistance and tolerance responses to attack by plants in the vegetative stage are mediated by reprogramming of primary and secondary metabolites. From the vegetative to the flowering stage, plants undergo important physiological changes resulting from inflorescences being a resource sink. Plant ontogeny may therefore affect the defense strategy of plants to attacks, and it was hypothesized that plants favor tolerance and constitutive resistance over induced resistance in inflorescences. This study addresses how single attack by three types of specialist attackers that are mostly attacking or infecting flowers, and dual combinations of them, affect metabolic changes and regrowth in the annual plant *Brassica nigra* during the flowering stage. We measured total amounts and composition of primary metabolites (sugars and amino acids) and secondary metabolites mediating direct resistance (glucosinolates) of leaves and inflorescences, as well as dry biomass of roots, leaves and inflorescences over time after attack. Differences in metabolic profiles and plant dry biomass were mostly explained by time (4, 8, and 12 d since attack) and plant parts (leaves *vs.* inflorescences), and highlighted the intense investment of resources towards inflorescences. Inflorescences contained 1.2 to 4 times higher levels of primary metabolites than leaves, and biomass of inflorescences increased 77 % between 4 and 8 days. Inflorescences were constitutively defended, and had up to 7 times higher levels of glucosinolates than leaves, whereas induction of glucosinolates was only detected in leaves and not in the inflorescence. Attack by insects and a bacterium transiently affected total concentration of soluble sugars in the leaves and inflorescences early after attack, while no changes were observed for free and protein-bound amino acids. Dry biomass of inflorescences was affected by treatment early after attack, but plants eventually compensated for damage. Our results suggest that flowering *B. nigra* invests especially in constitutive resistance in inflorescences to limit colonization by most attackers. Upon attack by specialist attackers, *B. nigra* favoured tolerance to attack over induced direct resistance in inflorescences. This strategy is likely typical of short-lived annual plants.

Key words

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

Introduction

Plants are exposed to attack by a wide variety of organisms including microbial pathogens and herbivorous insects. Plants cannot move away from attackers but have evolved intricate strategies to defend and limit their impact on fitness. These include constitutive and induced resistance that counteract the attackers, as well as tolerance which limits the effects of attack on fitness without interfering with the attacker, for example by regrowth of damaged organs (Berenbaum, 1995; Strauss & Agrawal, 1999; Núñez-Farfán *et al.*, 2007; Agrawal, 2011; Bekaert *et al.*, 2012; Mithöfer & Boland, 2012; Lucas-Barbosa, 2016). Primary metabolites have a primordial role in both plant tolerance and resistance to attack, and secondary metabolites especially mediate plant resistance (Machado *et al.*, 2013; Machado *et al.*, 2015; Machado *et al.*, 2017). When developing from the vegetative to the flowering stage, plants undergo major physiological changes resulting from flowers being a strong resource sink, and ontogeny particularly influences defense trajectories deployed by plants upon attack (Mooney, 1972; Barneix & Causin, 1996; Barton & Koricheva, 2010; Quintero *et al.*, 2014; Barton & Boege, 2017). Attack-induced changes in plants in the flowering stage have, however, received little attention although they are likely involved in defense strategies that protect reproductive parts of plants.

Plant development and interactions with attackers can be mediated by plant metabolic content. Carbohydrates and amino acids are considered as primary metabolites being compounds involved in central metabolism (Berenbaum, 1995). Apart from being structural molecules that represent 30-60% of plant biomass (Mooney, 1972), carbohydrates also provide energy to support physiological activities and are either stored as starch, or transported as soluble sugars, mainly sucrose (Mooney, 1972; Dietze *et al.*, 2014; Yang *et al.*, 2016). Similarly, free amino acids are essential building blocks of proteins, including a wide diversity of enzymes (Mooney, 1972; Keller, 1993; Barneix & Causin, 1996). Primary metabolites may also serve as signalling molecules or can supply carbon and nitrogen to the biosynthesis of secondary metabolites (Bourgaud *et al.*, 2001; Dudareva *et al.*, 2006; Schwachtje & Baldwin, 2008; Bekaert *et al.*, 2012). Glucosinolates, which are secondary metabolites whose breakdown products are toxic to most herbivorous attackers, are a clear illustration of this because they are derived from free amino acids, such as tryptophan, methionine, alanine or phenylalanine, and a glucose molecule (Textor & Gershenzon, 2009). Secondary metabolites represent less than 5% of total C and N and mainly play a role in interspecific interactions that underlie plant resistance to attack (Berenbaum, 1995; Mithöfer & Boland, 2012). Inflorescences,

which are resource sinks, have a high demand of primary metabolites and likely very different profiles from photosynthetic tissues such as leaves (Mooney, 1972; Barneix & Causin, 1996; Borghi & Fernie, 2017).

Optimal defense theory predicts that the most valuable tissues are highly defended, and consequently, it has been proposed that inflorescences are more defended than leaves (Herms & Mattson, 1992; McCall & Irwin, 2006; Barton & Koricheva, 2010). Flowers indeed carry a plant's gametes and, therefore, flower removal can directly alter reproductive output of plants. Damage to flowers can indirectly affect pollinator-mediated interactions (McCall & Irwin, 2006). Flowers are conspicuous and nutrient rich, which increases the likelihood of being attacked. In this context, induced resistance is expected to be rare in reproductive tissues, plants investing preferentially in constitutively produced metabolic compounds (McCall & Karban, 2006). Indeed, there is evidence that inflorescences generally have higher consitutive levels of glucosinolates than leaves of flowering plants (Smallegange *et al.*, 2007; Li *et al.*, 2017); but see (Godschalx *et al.*, 2016). Since florivory damages resource-sink flowers but not the leaves that provide resources to the plant, tolerance is expected to be a common way for plants to cope with attack to their flowers (McCall & Irwin, 2006; Orians *et al.*, 2011).

Attack by pathogens and herbivores induces an extensive reprogramming of secondary and primary metabolic pathways (Schwachtje & Baldwin, 2008; Bolton, 2009; Kerchev *et al.*, 2012; Giron *et al.*, 2013; Pastor *et al.*, 2014; Balmer *et al.*, 2015; Zhou *et al.*, 2015). These metabolic changes supply the increased demand for energy and carbon to sustain physiological responses such as regrowth (Mooney, 1972; Traw, 2002; Cna'ani *et al.*, 2013; Schultz *et al.*, 2013), or induced production of secondary metabolites (Schwachtje & Baldwin, 2008). Changes in C:N ratio have been associated with investments in tolerance mainly, although the C:N ratio can also reflect allocation to resistance (Royer *et al.*, 2013). For example, a low C:N ratio is generally associated with accelerated growth that results in an increase in plant biomass (Royer *et al.*, 2013). Besides quantitative changes, individual primary compounds can be either toxic to attackers or necessary for their development (Augner, 1995; Behmer, 2009). Similarly, tissue composition in secondary metabolites affects attacker preference and performance (Kos *et al.*, 2011; Kos *et al.*, 2012; Züst & Agrawal, 2016). As a consequence, quantity and richness of primary and secondary metabolites contribute to both plant tolerance and resistance to attack (Mooney, 1972). Despite the important changes plants undergo during the transition to the flowering stage, metabolic changes involved in resistance and

tolerance of flowering plants upon attack has been little characterized, and even less is known about changes in reproductive tissues such as inflorescences (McCall & Irwin, 2006; Lucas-Barbosa *et al.*, 2017).

In leaves of plants in the vegetative stage, different attackers may induce different metabolic changes, which may be dependent on attacker identity and potentially on the feeding-guild and specialisation level of the attackers. For instance, caterpillars of *Pieris brassicae* induced more changes than *Brevicoryne brassicae* aphids in the metabolome of Black mustard, *Brassica nigra* (Ponzio *et al.*, 2017). Additionally, the generalist caterpillar *Helicoverpa zea* affected a greater number of metabolites than the specialist caterpillar *Manduca sexta* in tomato plants, and mainly affected concentrations of metabolites linked to resistance whereas *M. sexta* impacted metabolites linked to N and C transport (Steinbrenner *et al.*, 2011). In cotton plants, different types of damage resulted in different growth responses: the removal of buds was associated with an increased axillary branching while prior phloem-sucking by aphids on leaves decreased it, both leading to compensation of damage (Rosenheim *et al.*, 1997). The effects of attack by more than one attacker species on metabolic profiles have been much less studied than the effects of single attack. Yet, attack by single species rarely occurs in nature (Orians *et al.*, 2011; Stam *et al.*, 2014). Previous work by Ponzio *et al.* (2017) showed that in leaves of vegetative *B. nigra* plants, dual attack by aphids and caterpillars induced metabolic changes that were different from the effects of each of the two attackers separately. In flowering *B. nigra*, exposure of leaves to *P. brassicae* induced an acceleration in reproduction and an increase in the allocation of nitrogen to flowers (Lucas-Barbosa *et al.*, 2017). *Brevicoryne brassicae* aphids performed better on inflorescences of *B. nigra* that were also infested with caterpillars (Chrétien *et al.*, 2018), which consequentially may impair plant fitness. Thus, plants in the flowering stage are likely to fine-tune their response to the type and combination of attackers they are facing.

This study investigates how single attack by three types of specialists attackers that are mostly attacking flowers, and dual combinations of them, affect metabolic changes of leaves and inflorescences in the annual plant *B. nigra* over the course of the flowering stage, and link them to tolerance and resistance responses of the plants to attack. We analysed attacker-induced changes in structural (protein-bound amino acids) and non-structural (free amino acids and soluble sugars) primary metabolites, and in a group of secondary metabolites typical of the Brassicaceae, *i.e.* the glucosinolates. We assessed the composition of individual primary and secondary metabolites, total concentrations, and ratios of soluble sugars over free

amino acids as an indication for C:N ratio. To estimate compensation for damage and potential reallocation of resources between plant parts upon attack, we measured dry biomass of inflorescences, leaves, and roots over the course of the flowering period. Based on the literature cited above, we expect inflorescences to display constitutive rather than induced direct resistance, and to show a tolerance response to attack, supported by the reallocation of primary metabolites between plant parts upon exposure to attackers. We hypothesize that herbivore or pathogen attack induces a systemic response in the plant, and that leaves and inflorescences will be differentially affected. Finally, we expect the metabolic response of leaves and flowers to be specific for the identity and combination of attackers, and that changes will be especially driven by caterpillars (Chrétien *et al.*, 2018).

Materials and methods

Study system

We investigated the Black Mustard *B. nigra* (Brassicales: Brassicaceae), which is commonly attacked by specialist insect herbivores and by microbial pathogens. It is a fast growing annual plant that contains high concentrations of nitrogen-containing glucosinolates, which are major defense compounds in the Brassicales (Textor & Gershenzon, 2009). Upon tissue damage, glucosinolates break down into highly toxic compounds such as isothiocyanates (Hopkins *et al.*, 2009; Brown & Hampton, 2011; Mithöfer & Boland, 2012). In the flowering stage, attackers mainly feed from the reproductive parts of the plant that contain high levels of glucosinolates (Smallegange *et al.*, 2007; L.T.S. Chrétien, pers. obs.). From those attackers, we selected two florivorous insects and one phytopathogenic bacterium based on their distinct mode of action on the plant. As a phloem feeder, we chose the cabbage aphid *Brevicoryne brassicae* (Hemiptera: Aphididae), a specialist herbivore that develops large populations of thousands of individuals on inflorescences of brassicaceous plants. As a tissue chewer, we used the specialist caterpillars of the Large Cabbage White butterfly *Pieris brassicae* (Lepidoptera: Pieridae). These butterflies lay eggs in clutches on leaves of flowering *B. nigra* and after hatching, first and second instar (L1 and L2) caterpillars move to the inflorescence and use mainly flowers and buds as a food source (Lucas-Barbosa *et al.*, 2013). Finally, we selected as a pathogen the bacterium *Xanthomonas campestris* pathovar *raphani* (Xcr) which causes Leaf Spot Disease and forms small necrotic spots (~1-3 mm) on leaves of many brassicaceous plants (Machmud, 1982; Vicente *et al.*, 2006). The bacterium can spread through the plant, and in broccoli, Xcr can spread from infected leaves to mature seeds (Machmud, 1982). Mustard plants are relatively resistant to Xcr, and the pathogen rarely kills the plant (Machmud, 1982; Vicente *et al.*, 2006; Ponzio *et al.*, 2016).

Plants, insects and bacterial cultures

Brevicoryne brassicae aphids and *P. brassicae* caterpillars were reared on Brussels sprout plants (*Brassica oleracea* variety *gemmifera*) in a greenhouse compartment (22 ± 2 ° C, 50-70% r.h., L16:D8). 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., 16L:8D). *Xanthomonas campestris* pathovar *raphani* (Xcr) was obtained from Utrecht University, The Netherlands (Ponzio *et al.*, 2014). The bacteria were cultured in an artificial liquid medium (8 g L^{-1} of Difco™ : beef extract 3.0 g L^{-1} and peptone 5.0 g L^{-1}, BD Diagnostics, New Jersey, USA) at 28 ± 1 ° C under gentle shaking at 170 rpm for 21 ± 1 h. The liquid medium with bacterial cells was then centrifuged twice for 10 min at 4080 rotations per min (rpm) and after each centrifugation, the supernantant was discarded and the pellet containing the bacterial cells was re-suspended in buffer (10 mM M_gSO_4). We estimated the concentration of the inoculum by measuring the light absorbance at 600 nm and adjusted the concentration of the final inoculum to 10^9 cells mL^{-1} by diluting in buffer (10 mM M_gSO_4).

Seeds of *B. nigra* were obtained from 25 plants (CGN06619, Center for Genetic Resources (CGN), Wageningen, The Netherlands) that had been exposed to open pollination in the field station of Wageningen University in spring 2012. Plants were cultured in pots (Ø17 cm - 2L content) filled with a 1:1 (v/v) mix of sand and potting soil (Lentse Potgrond, Lent, The Netherlands) and placed in a greenhouse compartment (22 ± 2° C, 60-70% r.h, 16L:8D). We infested the plants 1-2 d after the first flowers had just opened.

Plant treatments

Plants were exposed to single attack by either *B. brassicae*, *P. brassicae*, or Xcr, or to simultaneous dual attack by combinations of two of these three attackers. To infest the plants with *B. brassicae* aphids, five young adult females were gently placed on a bract (flower leaf) at the base of the inflorescence. These aphids dispersed on the plant – mainly onto the inflorescence – over the course of the experiment, and at day 4, day 8 and day 12 respectively 53 ± 18, 240 ± 93, and 1194 ± 312 (mean ± SD) aphids were present per plant. For the infestation with *P. brassicae*, plants were exposed to mated female butterflies that oviposited on a leaf of their choice. We kept a cluster of 30 eggs on the plants and gently removed any surplus of eggs. To infect plants with bacteria, we soaked a 2 x 2-cm piece of cotton wool with 500 µL of the bacterium inoculum (10^9 cells mL^{-1} in buffer) and placed it on the underside of a bract for 4 h with a soft clip. The inoculation method is described in details in

Chrétien *et al.* (2018). To control for an effect of clipping and buffer, 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). Plants exposed to dual attack were simultaneously exposed to two of the three attackers, and a bract never reveived more than one treatment. Caterpillars hatched from the eggs after 5 d and fed from leaves for about 2 d before moving to the flowers. If at 7 d after egg deposition some caterpillars were still on the leaves, they were gently moved to the inflorescence. In this way, we ensured that at 8 d since egg deposition, inflorescences of plants had been damaged by caterpillars for at least 1 d.

Metabolic profiles and total concentrations of metabolites in leaves and inflorescences of *B. nigra* exposed to single attack, to dual attack, or to buffer

To investigate how buffer (control), single attack, or simultaneous dual attack with *B. brassicae*, *P. brassicae*, and/or Xcr, affected the metabolic profile of leaves and flowers of *B. nigra*, we extracted and quantified primary metabolites and secondary metabolites at three time points: after 4, 8 and 12 d of plant exposure to treatments. As primary metabolites, we identified and quantitfied the different protein-bound amino acids, free amino acids, and soluble sugars that could be extracted from leaf and inflorescence samples of *B. nigra*. As secondary metabolites, we identitfied and quantified glucosinolates. Free amino acids, protein-bound amino acids, and sugars, were quantified in three plants per treatment per time point; glucosinolates were quantified in six plants per treatment per time point.

We used multivariate analysis to investigate how treatments, plant parts and time points affected the plant composition in primary and secondary metabolites. We analyzed which metabolic compounds contributed most to the differences explained by these factors. Multivariate analysis was based on the concentration of the different metabolites. Additionally, effect of treatment, plant part, and time point on total concentrations of protein-bound amino acids, free amino acids, soluble sugars, and glucosinolates was calculated by summing concentrations of individual compounds. Based on these total concentrations, we calculated the ratio of free amino acids relative to soluble sugars for leaves and inflorescences of *B. nigra* exposed to the different treatments.

Plants were sampled as described by Chrétien *et al.* (2018). In brief, all true leaves and all inflorescences of *B. nigra* plants were harvested after 4, 8 and 12 d of exposure to treatments. Plant parts were cut at the base of the petiole/stem and immediately frozen in liquid nitrogen and stored at –80º C until they were freeze-

dried and ground. The ground plant material was kept at –20º C until chemical analyses were performed. We did not harvest the bracts and leaves that initially received the treatments. Moreover, insects were removed from the plants shortly before harvest. Six plants were sampled per treatment, per time point.

Extraction, identification and quantification of free and protein-bound amino acids
Amino acids were extracted, derivatized with propyl-chloroformate, and analysed by GC-MS. Extraction and derivatization were performed using the kit EZ:faast (Phenomenex, Aschaffenburg, Germany) that we adapted to our samples; it consisted of a first 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 first extracted from 5 mg of plant material with 1 mL solution of 1:3 acetonitrile 100% and HCl (0.01M) and shaken (twist) 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 sulfonic acid (4M) 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 º 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 (See Protocol S1 for complete description of the procedure). 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 chloroformates do not react with arginine for derivatization; thus, arginine could not be quantified in our samples.

We used an autosampler (Gerstel, Mühleim an der Ruhr, Germany) to inject 2 µL of extract into the column (Zebron ZH-5HT inferno 30 m × 0.25 mm × 0.25 µm, Phenomenex, Aschaffenburg, Germany) in splitless mode (50:1); injector temperature was 250 º C. The column was heated at 15 ºC /min from 110 º C to 320 º C, with a final hold time of 7 min, and 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 ºC. We used Electron Ionisation with electron energy of -70 eV, an ionisation source at 230 º C and the two quadrupole mass analyzers at 180ºC. The scan range was 45 – 450 m/z with 3.7 scans/s.

Amino acids used as external standards and amino acids detected in plant samples were identified based on the mass spectrum of the derivatized compounds, provided by the EZ:faast kit. Area of the identified peak was calculated based on total ion chromatograms (TIC). We used mixtures of corresponding amino acids at 0.2 mM each as external standards to quantify the amino acids identified 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 (LOD) provided in the EZ:faast instructions.

Extraction, identification and quantification of soluble sugars

Soluble sugars were extracted from 5 mg of ground freeze-dried plant material, derivatized, and analysed by GC-MS. To first 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 6,000 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 (BSTFA): Trimethylchlorosilane (TMCS) (Sigma-Aldrich, Merck KGaA, Damstadt, Germany), and heated the solution at 70 ºC 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 homogenised the solution prior to injection into the GC-MS.

We used an autosampler (Gerstel, Mühleim an der Ruhr, Germany) to inject 2 µL of extract into the column (Zebron ZH-5HT inferno, 30 m × 0.25 mm × 0.25 µm, Phenomenex, Aschaffenburg, Germany) in split mode (50:1); the injector was heated at 250 ºC. Column temperature was at 60 ºC for one min, then increased at 30 ºC/min from 60 ºC to 120 ºC, and then at 8 ºC/min to 320 ºC, with a final hold time of 10 min, and total run time of 30 min. Helium was used as a carrier gas, with a constant flow of 1.2 mL/min. The transfer line to the triple quadruple MS was set at 320 ºC. We used Electron Ionisation with electron energy of -70 eV, an ionisation source at 230 ºC and the two quadrupole mass analyzers at 180 ºC. The scan range was 45 – 450 m/z with 3.7 scans/s.

Soluble sugars were identified based on the mass spectra of the derivatized compounds, provided by NIST (National Institute of Standards and Technology). Area of the identified peaks was calculated based on TIC. We used the internal standard to quantify each identified compound, and we multiplied the compound 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. We quantified soluble sugars in three plants per treatment per time point.

Extraction, identification and quantification of glucosinolates

Soluble sugars were extracted from 20 mg of ground freeze-dried plant material, desulfatized, and analysed by GC-MS. Glucosinolates were extracted by adding 1 mL of 80% methanol (v/v) (method described in Doheny-Adams *et al.* (2017), and 50 µM of sinalbin was added as internal standard. Sinalbin was isolated from *Sinapis alba* seeds at the MPI CE, Jena, Germany. The suspension was placed in a shaker for 5 min at 230 rpm for extraction, and centrifuged at 3,200 rpm for 10 min to separate the supernatant containing the glucosinolates from the remaining pellet. Glucosinolates were extracted by solid-phase extraction with a 28 mg column of DEAE-Sephadex® A-25 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). For the extraction, Sephadex was first conditioned with 800 µl MilliQ-Water (Dosieraufsatz), followed by 500 µL 80% methanol, and dried in between using a vacuum manifold. 800 µL 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 Mili-Q water and finally with 500 µL of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (0.02 M, pH 5.2). To desulfatize the glucosinolates, the column was treated with 30 µL of arylsulfatase 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 ºC until analysis. The arylsulfatase was prepared from lyophilized powder of aryl-sulfatase (from *Helix pomatia*, type H1, product number S9626, Sigma, St. Louis MO, USA) as described in Graser *et al.* (2001).

Desulfoglucosinolates were separated using high performance liquid chromatography (HPLC, Agilent 1100 HPLC system, Agilent Technologies, Santa Clara, USA). Solutions were injected onto a reverse-phase C18 column (Nucleodur Sphinx RP, 250 × 4.6 mm, 5 µm, Machrey-Nagel, Düren, Germany). 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 identified by comparing retention times and UV absorption spectra to those of purified standards extracted from *A. thaliana* (Brown *et al.*, 2003). Quantification of identified desulfoglucosinolates was based on peak areas of the 229 nm traces, and was carried out via the internal standard method. Relative response factors used were 2.0 for aliphatic and aromatic glucosinolates, and of 0.5 for indole glucosinolates (Burow *et al.*, 2006).

Assessing dry biomass of roots, vegetative parts and inflorescences of *B. nigra* exposed to single or dual attack

To estimate regrowth of leaves and inflorescence upon attack of the plant and a potential trade-off in plant investment in different organs, we measured dry biomass of roots, leaves and flowers of *B. nigra* exposed to buffer (control), to 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 d 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 grow 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 ºC until the soil could be washed away from the roots. Roots, vegetative parts and inflorescences were dried for 18 h in an oven at 105 ºC and weighed immediately thereafter.

Statistical analyses

Multivariate analyses

Metabolic profiles were analysed by multivariate data analysis using Projection to Latent Structures - Discriminant Analysis (PLS-DA), with SIMCA (Umetrics AB, Version 15.0, Umeå, Sweden). 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 data of samples of leaves at day 4, 8 and 12, and inflorescences at day 4, 8 and 12, irrespective to the treatments. We also analysed how much of

the variation in the chemical/metabolic profiles of leaves and flowers could be explained by plant exposure to the attackers at day 4, 8 and 12. The PLS method is commonly used for multivariate statistical analysis of metabolic data, but can in some cases over-fit data (Triba *et al.*, 2015). 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.

To assure the quality of the models computed, we used the default SIMCA cross-validation (CV) procedure (7-fold cross validation) that calculates the goodness of fit R^2, the predictive ability Q^2Y of the model for the number of significant components, and tests whether the model is significant with a CV-ANOVA ($P_{\text{CV-ANOVA}}$ value, significance level of 0.05). R^2X and R^2Y represent respectively the percentage of the variation explained by the matrix of metabolite data (X) and by the matrix of factors (Y). Robust models show no large discrepancy between R^2Y and Q^2Y (Triba *et al.*, 2015). Moreover, in poor models the value of Q^2Y can be affected by changing the order of the rows in the dataset (Triba *et al.*, 2015). To check the stability of Q^2Y, we ran each PLS-DAs for 6 datasets with randomly permuted rows. There was little variation in the Q^2Y value, and its value displayed in the Results section is averaged over the six permuted datasets. Finally, to confirm that the model does not explain random variation but variation linked to the explanatory variables, we verified that Q^2Y and R^2Y values of the original model differ from the Q^2Y and R^2Y calculated for 999 permuted datasets for the number of significant components determined by the software (Westerhuis *et al.*, 2008a; Westerhuis *et al.*, 2008b).

Contribution of each metabolite to the separation of the explanatory variables (plant part, time-point, treatment) in the validated models was determined graphically in the loading plots and numerically based on VIP values (Variable's Importance in Projection) that are based on the variance explained by the metabolite. 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.

Univariate analyses

Total concentrations of metabolites (protein-bound amino acids, free amino acids, soluble sugars, and glucosinolates) and plant dry biomass were analysed by ANOVA. We tested for an effect of exposure to attack on the total concentrations of metabolites

contained by leaves and inflorescences at day 4, 8 and 12. The effects of plant exposure to attackers and time point on dry plant biomass, and the interactions between these two factors, were also tested for each of the three plant parts. Ratios of concentrations of free amino acids relative to soluble sugars were heteroscedastic across treatments, thus the effect of treatments was tested with a Welch test. When a significant effect of a factor was detected, we used the Bonferroni *post-hoc* test for pairwise comparison after ANOVA, and the Games-Howell *post-hoc* test for pairwise comparisons after the Welch test. Intercept was included in the models. The significance level was set at 0.05. Analyses were performed in SPSS (IBM Corp., IBM SPSS Statistics for Windows, Versions 24 and 25. Armonk, NY: IBM Corp.)

Results

Effect of plant part and time since infestation/infection on the profile of protein-bound amino acids

We detected 19 protein-bound amino acids that were present in all samples of treated and control plants (Table S1). Among these 19 amino acids, three are normally not found in proteins: ornithine, γ-amino-n-butyric acid (GABA), and a leucine derivative. Ornithine can come from deamination of arginin during the derivatization process (Halket *et al.*, 2005), and likely gives a proxy of arginin content. GABA could be produced by decarboxylation of glutamic acid, a reaction nornally catalysed by the glutamate decarboxylase (Morrison *et al.*, 2013), and both GABA and the leucine derivates may be artefacts of protein hydrolysis.

Time since infestation/infection significantly affected the composition of protein-bound amino acids in leaves and flowers of B. nigra plants (Fig. 1). A Principal Latent Structure - Discrimination Analysis (PLS-DA) based on the samples from all treatments and control resulted in a model with 7 significant principal components (R2X = 0.950, R2Y = 0.374, Q2 = 0.254 ± SD 0.014, PCV-ANOVA < 0.001). The first principal component (PC1) explained 57.5% of the variation and separated plant samples by the time-point, i.e. 4, 8 and 12 days since infestation/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 earlier time points (Fig. 1b). This is supported by total concentration of protein-bound amino acids, which were 20% lower at day 8 than at day 4, and was further reduced by 27% from day 8 to day 12 in leaves and flower tissues overall (Table S1). Both variation in samples of different plant parts and time points correlated with the second principal component (PC2), which explained a lower percentage of variation, 12.9%. In terms of total concentrations, inflorescences had

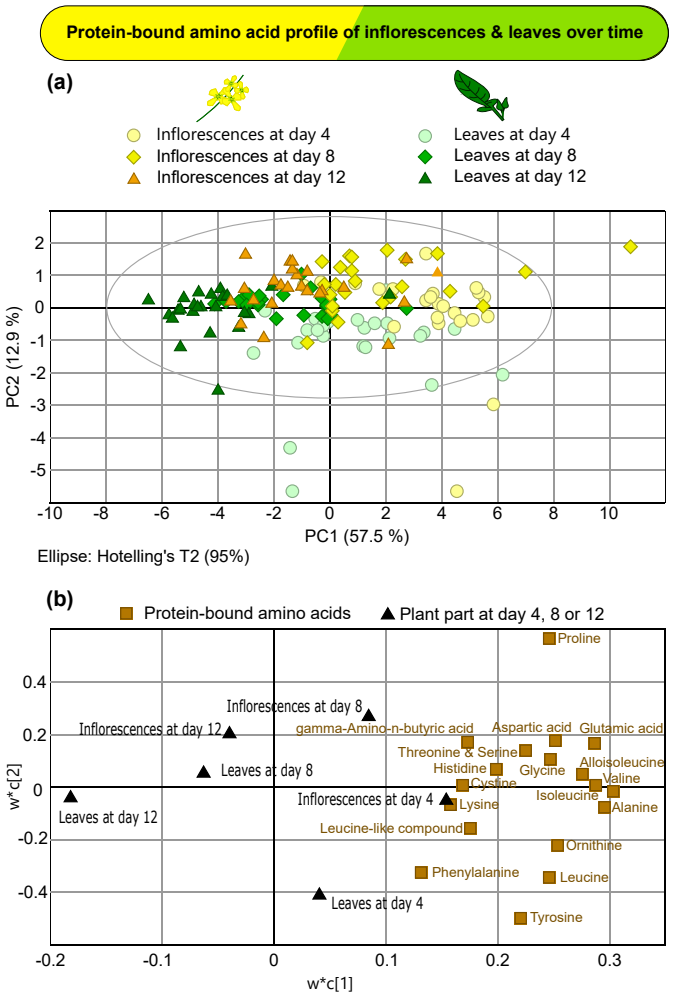


Fig. 1. Profiles of protein-bound amino acids of inflorescences and leaves of *Brassica nigra* plants exposed to single and dual attack for 4, 8 or 12 days. Projection to Latent Structures - Discriminant Analysis (PLS-DA) based on the concentration of 19 protein-bound amino acids (μmol.g^{-1} of plant dry biomass) that were detected and quantified in samples of *B. nigra* exposed to single attack with *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Concentrations of protein-bound amino acids were measured in leaves and infloresecences of three plants per treatment after 4 d, 8 d and 12 d of exposure to the treatments. Plant part and time since infestation/infection were set as classes. The PLS-DA resulted in a model with 7 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 grouping pattern of samples from inflorescences and leaves at day 4, day 8, and day 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.

38.4% higher levels of protein-bound amino acids than leaves (Table S1). Overall, proline, GABA, leucine-like compound, ornithine, leucine and tyrosine contributed most to the combined effect of time and plant part on the composition of protein-bound amino acids (Table S1, VIP values > 1).

Effect of plant part and time since infestation/infection on profile of free amino acids and soluble sugars

We detected 23 free amino acids, two being exclusively found in flowers (Table S2), and 8 soluble sugars that were recorded in all samples of leaves and flower tissues (Table S3). The composition of free and soluble primary metabolites of *B. nigra* plants was more affected by plant part than by time since infestation/infection (Fig. 2). The contribution of plant part to the separation on metabolic profiles was especially striking for free amino acid profiles.

For free amino acids, a PLS-DA based on the samples from all treatments and control resulted in a model with 8 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 (Fig. 2a). The loading plot indicated that all amino acids except 3-hydroxyproline were more positively associated to inflorescence samples than to leaf samples (Fig. 2b). Levels of free amino acids were approximately four times higher in inflorescences than in leaves (Table S2). Histidine and α-aminobutyric acid were exclusively found in inflorescences and glutamine, tyrosine, tryptophane, 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 (Fig. 2a). Time differentially affected the free amino acid profile of leaves and inflorescences, and the difference between leaves and inflorescences decreased with time (Fig. 2a). Profiles of inflorescences at 8 and 12 d since infestation/infection differed less from each other than from profiles of inflorescences at 4 d. Total concentrations of free amino acids indeed indicated a progressive decrease of 42% in leaves from day 4 to day 12, including a strong decrease of 33% in inflorescences from day 4 to day 8. Overall, aspartic acid and glutamic acid were more associated to inflorescences at day 4, and proline, tryptophane, α-aminoadipic acid, and histidine were more associated to inflorescences at day 8 and day 12 (Fig. 2b). These six amino acids contributed most (VIP values > 1) to the discrimination between inflorescences and leaves at days 4, 8 and 12.

For soluble sugars, a PLS-DA based on the samples from 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

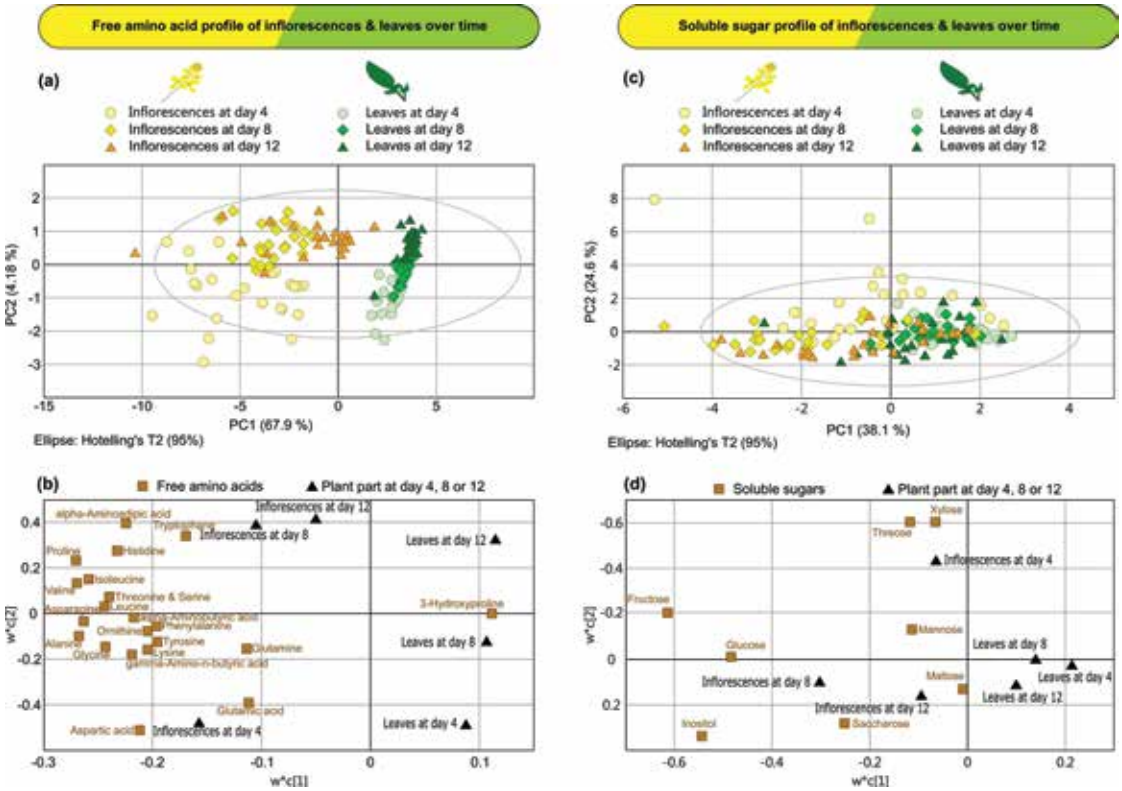


Fig. 2 Profiles of free amino acids and soluble sugars of inflorescences and leaves of *Brassica nigra* plants exposed to single and dual attack for 4, 8, or 12 d. Projection to Latent Structures - Discriminant Analysis (PLS-DA) based on the concentration of 23 free amino acids (**a, b,** µmol.g^{-1} of plant dry biomass) and 8 soluble sugars (**c, d,** µmol.g^{-1} of plant dry biomass) that were detected and quantified in samples of *B. nigra* exposed to single attack with *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Concentrations of primary metabolites were measured in leaves and inflorescences of three plants per treatment after 4, 8, and 12 d of exposure to the treatments. Plant part and time since infestation/infection were set as classes. The PLS-DA resulted in a model with 8 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 5 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 grouping pattern of samples from inflorescences and leaves at 4, 8, and 12 d 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.

and mainly separated samples of leaves from samples of inflorescences (Fig. 2c). Inflorescences were more associated than leaves to high levels of fructose, glucose and inositol, especially inflorescences at d 8 when compared to inflorescences at d 4 and d 12 (Fig. 2d). This was consistent with total concentration of soluble sugars, which was 87.2% higher in inflorescences than in leaves, and overall highest in inflorescences at d 8 than at d 4 and d 12 (Table S3). PC2 explained 24.6% of the variation and separated samples of inflorescences based on time since infestation/ infection, whereas samples of leaves at the three time points clustered together. There was a clear separation between samples of inflorescences at day 4 and samples of inflorescences at days 8 and 12. In the loading plot (Fig. 2d), we can see that inflorescences at day 4 were more associated to high levels of threose and xylose, whereas inflorescences at d 8 and 12 were more associated to saccharose and inositol. Overall, inositol, saccharose, and fructose contributed most (Table S3, VIP values > 1) to the separation between samples of leaves and of inflorescences at the three time points.

Effect of plant part and time since infestation/infection on the glucosinolate profile

We detected six glucosinolates that were present in both plant parts (leaves and inflorescences) at the three time points (Table S4). Plant part and, to a lesser extent, time since infestation/infection, affected the composition of glucosinolate profile of B. nigra plants (Fig. 3a, b). A PLS-DA 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 ± 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 (Fig. 3a). Sinigrin, which accounted for over 98% of total glucosinolates, and 4-hydroxy-indolyl-3-methyl-glucosinolate strongly contributed to this separation (Fig. 3b, Table S4, VIP values > 1), and were present in respectively 4 and 26 times higher levels in inflorescences than in leaves (Table S4). Overall, total glucosinolate levels were 4.8 times higher in inflorescences than in leaves (Table S4). PC2 explained 18.5% of the variation and separated samples of leaves and inflorescences harvested after 12 d from samples of leaves and inflorescences harvested after 4 or 8 d (Fig. 3a). 1-Methoxy-indolyl-3-methyl-glucosinolate drives this separation (Fig. 3b, Table S4 VIP values > 1). Indeed, all leaves and flowers of plants produced 1-methoxy-indolyl-3-methyl-glucosinolate after 12 d, whereas less than 50% of the plants produced it after 4 or 8 d (Table S4).

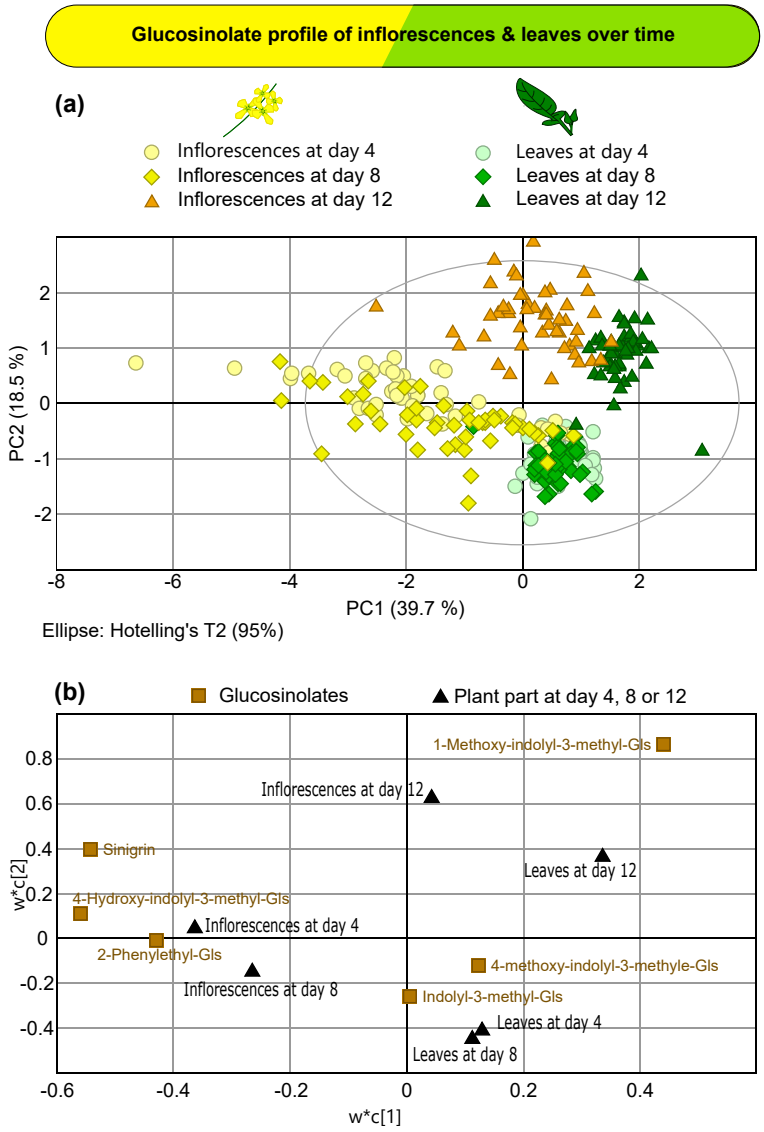


Fig. 3. Profile of glucosinolates of inflorescences and leaves of *Brassica nigra* plants exposed to single and dual attack for 4 d, 8 d, and 12 d. Projection to Latent Structures - Discriminant Analysis (PLS-DA) based on the concentration of six glucosinolates (μmol.g^{-1} of plant dry biomass) that were detected and quantified in samples of *B. nigra* exposed to single attack with *Brevicoryne brassicae* aphids, eggs and caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Concentrations of glucosinolates were measured in leaves and inflorescences of three plants per treatment after 4 d, 8 d, and 12 d of exposure to the treatments. Plant part and time since infestation/infection were set as classes. The PLS-DA resulted in a model with 5 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 indicates the percentage of variation in the data explained by the first two principal components that resulted from the model. (**a**) Scatter plots show grouping pattern of samples from inflorescences and leaves at 4, 8, and 12 d 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.

Fig. 4 (right). Total concentrations of glucosinolates and solubles sugars (average ± SD), and ratio of concentrations of soluble sugars over free amino acids in inflorescences and leaves of *Brassica nigra* that were exposed to single or dual attck for 4 d. *Brassica nigra* plants were exposed to single attack with *Brevicoryne brassicae* aphids, eggs and caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Panels **a - c** show total concentrations of glucosinolates (**a**) and soluble sugars (**b**) in samples of inflorescences (**yellow**) and leaves (**green**) of attacked plants. There were six plant replicates per treatement for the glucosinolates, and 3-4 for the soluble sugars. Effect of treatments was tested with an ANOVA, and when significant, a Bonferroni *post-hoc* test was used for pairwise comparisons. Panel **c** shows the ratios of total concentrations of soluble sugars over total concentrations of free amino acids of inflorescences (**yellow**) and leaves (**green**) of attacked plants. There were three plant replicates per treatment. Effect of treatments was tested with a Welch test, and when significant, a Games-Howell *post-hoc* test was used for pairwise comparisons. All concentrations are expressed in $\mu mol.g^{-1}$ of plant dry biomass and were calculated by summing the concentrations of each detected and quantified compound. For each statistical test, the significance level was set to $\alpha = 0.05$; test parameters are indicated in a gray frame in the panels.

<u>Effect of single and dual attack on metabolic profiles</u>

We investigated the effect of plant exposure to attackers on the composition of primary metabolites, *i.e.* protein-bound amino acids, free amino acids, and soluble sugars, and glucosinolates as secondary metabolites. The effect of treatments on metabolic profiles was tested within inflorescences and within leaves, and separately for day 4, 8 and 12 since infestation/infection. None of the PLS-DAs separating metabolic profiles based on treatments resulted in a significant model. This indicates that the composition in metabolic compounds could not be separated based on treatments.

<u>Effect of single and dual attack on the total concentration of metabolites</u>

Treatments affected the total concentrations of glucosinolates and soluble sugars only at an early stage of exposure to attackers, *i.e.* at 4 d since infestation when plants carried eggs of *P. brassicae* and about 50 aphids (Fig. 4). This effect was not found at day 8 and day 12 (Fig. S2). Attack only affected the total concentration of glucosinolates in leaves, whereas no effect was detected in inflorescences (Fig. 4a). Bacteria seemed to be the main driver of changes in 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 ($P = 0.036$), and 3.4 times higher than leaves of plants exposed to aphids plus *P. brassicae* ($P = 0.017$). Similarly, leaves of plants exposed to dual attack by *P. brassicae* plus bacteria had 3.2 times higher levels of glucosinolates than leaves of plants exposed to *P. brassicae* alone ($P = 0.033$), and 3.6 times higher than leaves of plants exposed to aphids plus *P. brassicae* ($P = 0.004$). The glucosinolate level in leaves of plants attacked by bacteria plus aphids or bacteria plus *P. brassicae*

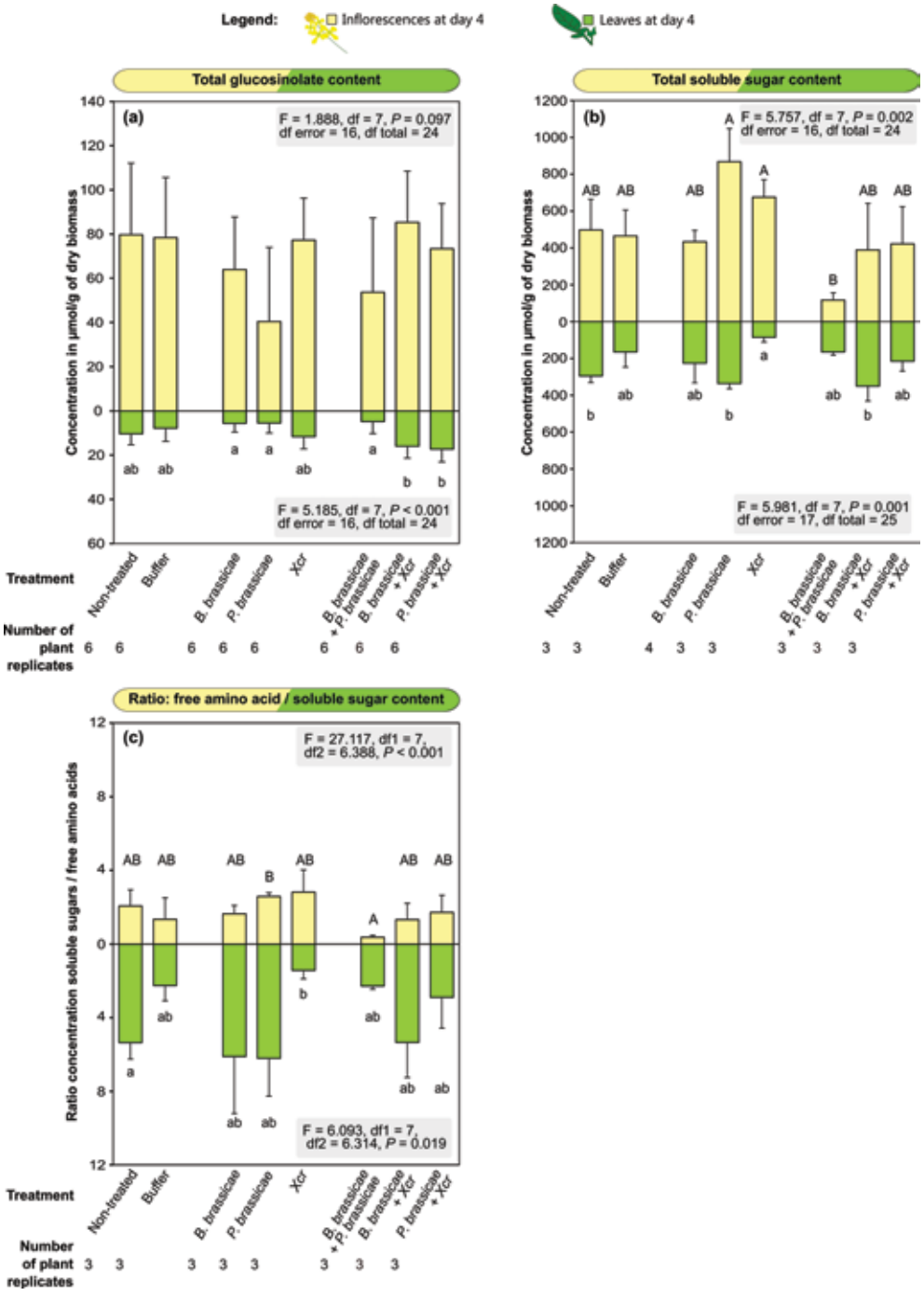
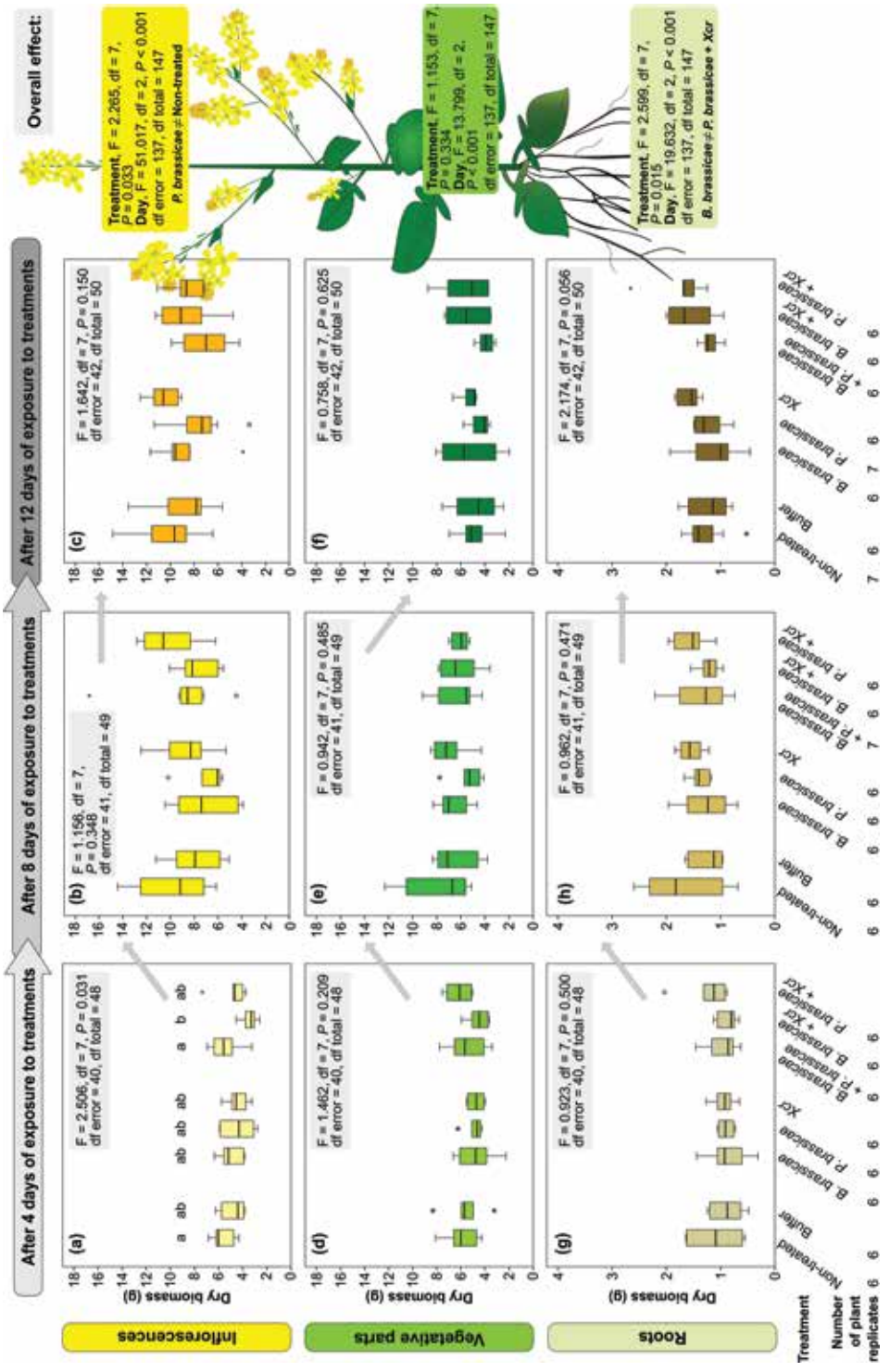

Legend:
Inflorescences at day 4
Leaves at day 4
Total glucosinolate content
(a)
F = 1.888, df = 7, P = 0.097
df error = 16, df total = 24
F = 5.185, df = 7, P < 0.001
df error = 16, df total = 24
Concentration in µmol/g of dry biomass
Total soluble sugar content
(b)
F = 5.757, df = 7, P = 0.002
df error = 16, df total = 24
F = 5.981, df = 7, P = 0.001
df error = 17, df total = 25
Ratio: free amino acid / soluble sugar content
(c)
F = 27.117, df1 = 7, df2 = 6.388, P < 0.001
F = 6.093, df1 = 7, df2 = 6.314, P = 0.019
Ratio concentration soluble sugars / free amino acids
Treatment
Non-treated
Buffer
B. brassicae
P. brassicae
Xcr
B. brassicae + P. brassicae
B. brassicae + Xcr
P. brassicae + Xcr
Number of plant replicates

Fig. 5 (right). Dry biomass (median, interquartile range, full range) of inflorescences, vegetative parts, and roots of *Brassica nigra* that were exposed to single or dual attack for 4 d, 8 d and 12 d. Plants were exposed to single attack with *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Inflorescences (**a, b, c**), vegetative parts (**d, e, f**), and roots (**g, h, i**) were harvested after 4 d (**a, d, g**), 8 d (**b, e, h**), and 12 d (**c, f, i**) of exposure to the treatments; plant parts were then dried and weighed. Grey arrows between graphs represent 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 treatement and time point. Statistics for each plant part and at 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 overall effect of treatment and day were tested with an ANOVA, and when significant, a Bonferroni *post-hoc* test was used for pairwise comparisons. The significance level was set to $\alpha = 0.05$; test parameters are indicated in a gray frame in the panels.

did not significantly differ from that of plants attacked by bacteria only. Foliar concentrations of glucosinolates of plants exposed to attackers did not significantly differ from concentration of control plants exposed to buffer or of non-treated plants.

Attack affected the total level of soluble sugars of both inflorescences and leaves (Fig. 4b). In inflorescences, changes were driven by 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 ($P = 0.001$) and 5.8 times lower levels of soluble sugars than inflorescences of plants attacked by bacteria only ($P = 0.014$). Floral sugar levels of plants exposed to attackers did not significantly differ from those of control plants exposed to buffer or of non-treated plants. In the leaves, changes were mainly driven by exposure to bacteria. Leaves of plants attacked by bacteria had about 4 times lower levels of soluble sugars than plants attacked by *P. brassicae* ($P = 0.006$) and by aphids plus bacteria ($P = 0.003$), and 3.5 times lower levels of soluble sugars than non-treated control plants ($P = 0.030$). Other treatments did not affect foliar concentrations of soluble sugars when compared to control plants exposed to buffer and to non-treated 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 (Fig. S1).

The ratio of concentrations of soluble sugars over free amino acids in inflorescences was affected by plant expsoure to the attackers, and as for sugars, dual attack with the two herbivorous insects were driving the changes (Fig. 4c). The ratio of soluble C:N was 6.9 times lower in inflorescences of plants exposed to *B. brassicae* plus *P.*


Overall effect:
Treatment, F = 2.265, df = 7, P = 0.033
Day, F = 51.017, df = 2, P < 0.001
df error = 137, df total = 147
P. brassicae ≠ Non-treated
Treatment, F = 1.153, df = 7, P = 0.334
Day, F = 13.799, df = 2, P < 0.001
df error = 137, df total = 147
Treatment, F = 2.599, df = 7, P = 0.015
Day, F = 19.632, df = 2, P < 0.001
df error = 137, df total = 147
B. brassicae ≠ P. brassicae + Xcr
After 4 days of exposure to treatments
After 8 days of exposure to treatments
After 12 days of exposure to treatments
Inflorescences
Vegetative parts
Roots
Dry biomass (g)
(a) F = 2.506, df = 7, P = 0.031 df error = 40, df total = 48
(b) F = 1.156, df = 7, P = 0.349 df error = 41, df total = 49
(c) F = 1.642, df = 7, P = 0.150 df error = 42, df total = 50
(d) F = 1.462, df = 7, P = 0.209 df error = 40, df total = 48
(e) F = 0.942, df = 7, P = 0.485 df error = 41, df total = 49
(f) F = 0.758, df = 7, P = 0.625 df error = 42, df total = 50
(g) F = 0.923, df = 7, P = 0.500 df error = 40, df total = 48
(h) F = 0.962, df = 7, P = 0.471 df error = 41, df total = 49
F = 2.174, df = 7, P = 0.056 df error = 42, df total = 50
Treatment
Number of plant replicates
Non-treated
Buffer
B. brassicae
P. brassicae
Xcr
B. brassicae + Xcr
P. brassicae + Xcr

brassicae than in inflorescences of plants attacked by *P. brassicae* only ($P = 0.003$). The ratio was 3.8 times lower in leaves of plants attacked by Xcr than in leaves of non-treated control plants ($P = 0.039$). Plant exposure to attackers did not affect soluble sugars/free amino acids ratio of inflorescences compared to the one of control plants exposed to buffer or of non-treated plants.

Plant dry biomass

Dry biomass of inflorescences, vegetative parts, and roots of *B. nigra* plants exposed to single attack, dual attack, to buffer (control), or non-treated for at 4, 8 or 12 d after treatment was affected by the time point (Fig. 5). Overall, dry biomass of inflorescences and roots increased by 77% and 46% respectively from day 4 to day 8 ($P < 0.001$ for both comparisons), dry biomass of vegetative parts increased by 23% from day 4 to 8 ($P = 0.002$). While dry biomass of inflorescences and roots at day 12 was similar to that at day 8, dry biomass of vegetative parts decreased by 32% from day 8 to day 12 ($P < 0.001$).

Treatments affected inflorescence biomass at day 4 (Fig. 5a): inflorescences of plants exposed to aphids plus bacteria had lower dry biomass than those exposed to aphids plus caterpillars ($P = 0.048$) or non-treated ($P = 0.016$). Dry biomass of inflorescences of other treated plants did not differ from that of inflorescences of control plants exposed to buffer or of non-treated plants. Although we did not find an effect of *P. brassicae* attack on the dry biomass of inflorescence when considering the time points separately, overall *P. brassicae*-treated plants had a lower biomass than non-treated plants ($P = 0.017$). Similarly, we found that, overall, roots of plants exposed to caterpillars plus bacteria had lower dry biomass than roots of plants exposed to buffer ($P = 0.037$) and to single attack with aphids ($P = 0.017$). This effect was not detected for root dry biomass at individual time points.

Discussion

Our data suggest that *B. nigra* invests in consitutive resistance in inflorescences, and in the case of attack at the flowering stage, plants can compensate for damage already soon after exposure to attackers has started. Inflorescences indeed contained higher levels of glucosinolates, which mediate direct resistance, than leaves. Additionally, attack induced changes in the glucosinolate concentration of leaves but not of inflorescences. Plant exposure to pathogen and insect attack had little effect on the content of leaves and inflorescences in primary metabolites. Treatments affected the soluble sugars/ free amino acids ratio, and this was driven by changes in the total concentration of soluble sugar. Attacker-induced metabolic changes were only detected at the first time point (4

d) after exposure to attack. Similarly, attack affected biomas of inflorescences at this first time point, and plants seemed to later compensate for damage upon attack by insects and bacteria. Overall, changes in the metabolic content of leaves and inflorescences over time reflected the high investment of the plants into reproduction. Inflorescences had higher levels of primary metabolites than leaves, and dry biomass of inflorescences almost doubled between the first two time points.

Optimal defense theory predicts that valuable tissues are highly defended (Herms & Mattson, 1992; Stamp, 2003; McCall & Irwin, 2006; Barton & Koricheva, 2010). 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 since nitrogen content is important for insect performance (Schoonhoven et al., 2005; Nation, 2008; Behmer, 2009). Because flowers are a strong sink and directly important for plant fitness, it is predicted that flowers evolve less inducible resistance mechanisms than leaves, and rather use constitutive resistance mechanisms (McCall & Irwin, 2006; Orians et al., 2011). In this line, *B. nigra* had six times higher constitutive concentrations of glucosinolates in inflorescences compared to leaves. Moreover, no induction of glucosinolates was detected in inflorescences upon attack and profiles were similar across treatments. When considering foliar glucosinolates, data indicate that after 4 days of exposure to dual attack by insects plus bacteria glucosinolate levels were induced compared to situations with the insects alone, or with dual attack by insects. Glucosinolates can indeed negatively affect pathogens, especially necrotrophic bacteria (Textor & Gershenzon, 2009), and *B. nigra* is known for its resistance to Xcr (Machmud, 1982; Vicente *et al.*, 2006; Ponzio *et al.*, 2016). For inflorescences, limiting the induction of toxic compounds may as well reduce the risk to alter rewards and floral cues used by mutualist pollinators (Strauss *et al.*, 2002; Lucas-Barbosa *et al.*, 2017). For example, sinigrin and 4-hydroxy-indolyl-3-methyl-glucosinolate, that were both constitutively produced in inflorescences of *B. nigra*, are also constitutively present in nectar of *B. nigra* (Bruinsma *et al.*, 2014).

Constitutive resistance may hamper colonization of inflorescences by the most damaging herbivores. Six glucosinolates were found in both leaves and inflorescences of flowering *B. nigra*: one aliphatic (sinigrin) that acounts for 98% of glucosinolate concentration of inflorescences, four indolic and one aromatic glucosinolates. Aliphatic glucosinolate such as sinigrin are indeed particularly toxic to chewing herbivores, especially when they are not specialized on Brassicales plants (Textor & Gershenzon, 2009; Bekaert *et al.*, 2012). Such levels of sinigrin may be a barrier

to chewing herbivores, which physically remove buds and flowers. Chewing insects such as *P. brassicae* caterpillars in their final instar can, for example, remove about 135 buds and flowers on *B. nigra*, and significantly decrease seed production compared to non-infested plants in greenhouse tests (Smallegange *et al.*, 2007; Smallegange *et al.*, 2008). Aphids may as well be an important threat to *B. nigra*, and infestation by *B. brassicae* aphids can hamper the development of siliques in the field (L.T.S. Chrétien, pers. obs.). The diversity of indolic glucosinolates found in inflorescences of *B. nigra* may be a defense against phloem- or cell-feeders. It has indeed been proposed that indolic glucosinolates evolved under the pressure of aphids escaping from the glucosinolate-myrosinase system (Züst & Agrawal, 2016) while aliphatic glucosinolates have little negative impact on aphid performance (Barth & Jander, 2006; Kos *et al.*, 2012; Züst & Agrawal, 2016). To our knowledge, up to 15 glucosinolates have been reported in leaves of vegetative *B. nigra* (7 aliphatic, 4 indolic, 4 aromatics) (van Dam *et al.*, 2004; Bonnet *et al.*, 2017; Ponzio *et al.*, 2017). Thus, it seems that from the vegetative stage to the flowering stage, *B. nigra* maintains a large diversity of indole glucosinolates while the diversity in aliphatic and aromatic decreases. Additionally, one indolic glucosinolate, 1-methoxy-indolyl-3-methyl-glucosinolate, was produced late in the flowering phenology. It is known to reduce reproduction of *M. persicae* (Kim & Jander, 2007), and in our system, could protect maturing siliques or be translocated to the seeds that commonly contain high concentrations of toxic compounds (Bellostas *et al.*, 2007).

For plants in the vegetative stage, studies generally show that attack by pathogens or herbivores induces phytohormonal responses and lead to changes in primary metabolism of leaves (Schwachtje & Baldwin, 2008; Bolton, 2009; Giron *et al.*, 2013; Zhou *et al.*, 2015). Such changes depend on the identity and combination of attackers (Schwachtje & Baldwin, 2008; Steinbrenner *et al.*, 2011; Ponzio *et al.*, 2017). Leaves and inflorescences of blooming plants can also specifically respond to different attackers in terms of phytohoromonal induction, and in *B. nigra* mainly jasmonates were induced (Chrétien *et al.*, 2018). Besides the role of jasmonates as mediator of induced plant resistance to attack, jasmonates may also mediate allocation of carbohydrates in vegetative plants (Schwachtje *et al.*, 2006; Machado *et al.*, 2013; Machado *et al.*, 2017). Attack to flowering *B. nigra* indeed affected the total concentration of soluble sugars of leaves and/or inflorescences, which could be interpreted as a requirement to support an increased demand of resources to sustain tolerance and resistance activities to face attack (Heil & Bostock, 2002; Kerchev *et al.*, 2012; Mithöfer & Boland, 2012; Schultz *et al.*, 2013; Machado *et al.*, 2017). Plants attacked by Xcr had lower foliar levels of sugars than plants dually

exposed to aphid plus Xcr and non-treated plants, whereas floral amounts did not differ. Leaves may respond to attack by the bacteria to protect themselves in order to fuel the response of the inflorescence. Additionally, inflorescences of plants dually infested with *B. brassicae* plus *P. brassicae* (as eggs at 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 compensation of damage to tissues (Kerchev *et al.*, 2012). In line with this, inflorescences of plants attacked by *B. brassicae* plus *P. brassicae* had a lower C:N ratio than inflorescences of plants attacked by *P. brassicae* only, which generally indicates an investment in growth (Royer *et al.*, 2013). Upon dual attack with herbivores that both remove tissues and fluids from the inflorescences, plants may, therefore, invest into re-growing eaten or damaged parts. This hypothesis is supported by the observation that inflorescences of plants attacked by *B. brassicae* plus *P. brassicae*, *P. brassicae* only, or by Xcr, had similar dry biomass despite that caterpillars were eating floral buds, flowers and stems. Our data suggest that attacker-specific changes in primary metabolism may translate into distinct metabolic changes that support tolerance of inflorescences to attack.

We are just starting to explore inducible responses of plants in the flowering stage to folivores and florivores (McCall & Irwin, 2006; Lucas-Barbosa, 2016). The present study shows that attack induces metabolic changes early after plant exposure to insects and a pathogen, at 4 d since attack, when plants started flowering, and no changes were measured at later time points when plants were in full bloom. Young flowering plants need to ensure reproduction and may be more inducible than plants that are further advanced in the flowering (Barton & Koricheva, 2010).

Exposure to insects and pathogen attack induced changes in metabolic profiles of *B. nigra* leaves and inflorescences that were of small magnitude compared with the differences quantified between leaves and inflorecences and between 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 d since 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, 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 (Mooney, 1972; Barneix & Causin, 1996). 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 all primary metabolic profiles that inflorescences underwent important changes between day 4 and day 8. Notably, free-circulating aspartic acid and glutamic acid were relatively more concentrated in inflorescences that started flowering compared to older ones. Aspartic acid and glutamic acid are among the most concentrated amino acids in phloem sap of leaves of boulting brassicaceous species such as *Brassica juncea*, *Brassica napus*, and *Brassica campestris* (Weibull & Melin, 1990). Our data could reflect phloem recruitment to inflorescences that had initiated flower opening, which can provide resources necessary to 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 relatively more important 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 favors tolerance to attack by specialists over induced resistance. Tolerance may be more effective against specialists attackers than resistance as specialist attackers are little affected by plant direct resistance traits (Orians *et al.*, 2011). Plants responded to attackers early after attack infection and 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 germination and survival of young seedlings. Additionally, reprogramming of secondary and primary metabolic

pathways upon attack is also likely to influence arthropod communities associated to plants in the flowering stage, with cascading effects on plant fitness that still needs to be unravelled in the natural ecological context.

Acknowledgements

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Supplemental information

Protocol S1. Procedure for amino acid extraction and derivatization adapted from EZ:faast kit (Phenomenex, Aschaffenburg, Germany

Amino acids were extracted and derivatized using the kit EZ:faast (Phenomenex, Aschaffenburg, Germany) that we adapted to our samples. We used two extraction techniques, one for the free amino acids and one for the protein-bound amino acids. Free amino acids were first extracted from 5 mg of plant material with 1 mL solution of 1:3 acetonitrile (100%) and HCl (0.01M), and shaken (twist) for 1 h. We took 200 µL of the solution and added 100 µL of internal standard (Norvalin 0.2 mM in N-propanol 10%). The solution was then slowly passed through an SPE column (2 ± 1 min per sample), followed by 200 µL of N-propanol to elute compounds that were not fixed by the column. Amino acids were then eluted from the SPE column with 200 µL of 3:2 of sodium hydroxide:N-propanol. For the protein-bound amino acids, the peptide bonds first had to be hydrolysed. For this, we introduced 500 µL of methane sulfonic acid (4M) for 5 mg of plant sample, purged the air of the vial with N_2, and incubated the closed vials in an oven at 150 ºC for 2 h. At the end of the incubation, vials were quickly cooled in ice; we then took 100 µL of the liquid hydrolysate and added 240 µL of sodium carbonate solution to stop the hydrolysis reaction. We then took 25 µL of this mix and added 100 µL of internal standard (Norvalin 0.2 mM in N-propanol 10%). The extract solution was slowly passed through an SPE column (2 ± 1 min per sample), after which 200 µL of HPLC-grade water was passed through the column to elute compounds that were not fixed by the column. Amino acids were then eluted from the SPE column with 200 µL of 3:2 of sodium hydroxide:N-propanol.

To derivatize both free and protein-bound amino acids by addition of an ester function on the carboxyl and amine groups, 50 µL of propyl-chloroformate was added to the eluted solution and the mix was vortexed twice for 5 – 8 s with an interval time of 1 min. The derivatization resulted into two phases: an organic phase with the derivatized amino acid, and an aqueous phase. 100 µL of iso-octane was then added to help the derivatized amino acid migrate to the organic layer. The organic layer was then extracted and dried under a light flow of N_2 for a maximum time of 10 min. The dry precipitate was then re-disolved in 50 µL (free amino acids) or 80 µL (protein amino acids) of a solution of iso-octane (80%) and chloroform (20%), and the final solution was homogenised for injection in the GC-MS. A drawback of this method is that chloroformates do not react with arginine for derivatization, thus, arginine could not be detected in our samples.

Table S1. List of protein-bound amino acids that were identified and quantified in leaves (green) and inflorescences (yellow) of *B.nigra* exposed to attack, and their contribution (VIP value) to separation in the PLS-DA of Fig. 1.

Identified amino acids	Common name	VIP value	Average concentration ± SD (µmol / g of plant DW) day 4		day 8		day 12	
Proteogenic α-amino acids								
Aliphatic side chain								
alanine		0.91	479.2 ± 133.3	582.2 ± 85.7	355.7 ± 94.0	490.5 ± 124.3	214.3 ± 94.5	387.1 ± 108.5
alloisoleucine		0.93	26.0 ± 8.8	32.1 ± 10.5	20.8 ± 6.7	34.9 ± 18.5	12.8 ± 6.3	19.4 ± 6.5
isoleucine		0.98	504.1 ± 80.0	571.4 ± 75.3	388.8 ± 82.8	532.5 ± 104.0	280.2 ± 78.7	431.8 ± 82.3
leucine		**1.03**	754.2 ± 139.1	751.1 ± 104.8	561.7 ± 108.1	665.2 ± 122.9	456.7 ± 97.4	608.1 ± 142.5
valine		0.82	582.2 ± 130.9	665.1 ± 108.7	464.1 ± 98.8	620.6 ± 136.5	305.9 ± 100.4	497.8 ± 108.6
Aromatic side chain								
phenylalanine		0.94	663.4 ± 189.9	612.7 ± 335.7	490.1 ± 97.2	536.3 ± 132.6	398.6 ± 188.4	496.5 ± 187.4
tyrosine		**1.08**	547.1 ± 182.9	529.0 ± 221.9	321.9 ± 95.4	409.7 ± 144.0	222.7 ± 91.0	299.8 ± 106.1
Charged side chain - positive								
histidine		0.91	490.3 ± 255.4	690.8 ± 772.1	349.3 ± 107	584.2 ± 179.5	197.3 ± 144.0	360.2 ± 173.9
lysine		0.85	1083.7 ± 354.6	1555.1 ± 1585.3	719.6 ± 160	1132.7 ± 443.2	549.3 ± 329.8	836.7 ± 597
Charged side chain - negative								
glutamic acid		0.87	524.8 ± 173.9	731.3 ± 149.3	447.4 ± 98.3	627.8 ± 122.1	276.7 ± 125.2	507.4 ± 137.7
aspartic acid		0.82	193.7 ± 63.1	253.8 ± 48.1	178.2 ± 39.5	235.1 ± 70.6	105.9 ± 51.7	180.7 ± 66.3
Dimers								
cystine		0.96	41.5 ± 51.9	66.3 ± 55.1	25.9 ± 32.9	68.7 ± 47.9	3.1 ± 10.6	14.4 ± 25.9
Polar uncharged side chain								
threonine + serine *		0.88	109.1 ± 50.1	156.4 ± 43.6	94.8 ± 28.4	136.2 ± 63.7	45.2 ± 31.2	90.2 ± 44.5
Irregular								
glycine		0.82	370.4 ± 121.1	454.1 ± 96.2	304.5 ± 72.3	422.1 ± 117.8	175.1 ± 79.2	300.3 ± 88.5
proline		**1.31**	300.4 ± 94.1	446.0 ± 78.2	292.9 ± 62.0	465.7 ± 134.6	180.8 ± 78.7	391.8 ± 120.4
Non-proteogenic amino acids								
γ-amino-n-butyric acid	GABA	**1.08**	39.5 ± 70.2	222.3 ± 321.2	0 ± 0	129.5 ± 66.9	0 ± 0	67.9 ± 68.1
Leucine-like-compound		**1.12**	25 ± 35.6	41 ± 31.4	8 ± 18.8	24.5 ± 35.1	1.8 ± 8.7	6.5 ± 17.6
ornithine		**1.44**	35.4 ± 16.9	73.7 ± 46.3	14.3 ± 6	35 ± 17	9.3 ± 10.1	13.7 ± 9.3
Total concentration of protein-bound amino acids			6769.9 ± 1332.8	8434.4 ± 2921.9	5037.9 ± 1011.6	7151.1 ± 1493.4	3435.3 ± 1122.5	5510.1 ± 1557.4
			24 plants		24 plants		24 plants	

* threonine and serine coeluted

Table S2. List of free-circulating amino acids that were identified and quantified in leaves (green) and inflorescences (yellow) of *B.nigra* exposed to attack, and their contribution (VIP value) to separation in the PLS-DA of Fig. 2.

Identified amino acids	Common name	VIP value	Average concentration ± SD (µmol / g of plant DW)					
			day 4		day 8		day 12	
Proteogenic α-amino acids								
Aliphatic side chain								
alanine		0.83	5.94 ± 2.07	23.29 ± 3.86	2.97 ± 1.11	17.62 ± 2.67	3.08 ± 2.06	14.39 ± 5.88
isoleucine		0.84	1.09 ± 0.37	9.17 ± 2.81	0.87 ± 0.24	8.67 ± 1.79	1.01 ± 0.39	6.43 ± 3.13
leucine		0.76	2.88 ± 0.93	11.52 ± 4.76	2.10 ± 0.46	10.27 ± 2.15	1.76 ± 0.54	7.80 ± 3.65
valine		0.84	1.52 ± 0.55	10.13 ± 2.15	1.16 ± 0.32	9.10 ± 1.23	1.23 ± 0.57	7.37 ± 2.82
Aromatic side chain								
phenylalanine		0.89	1.41 ± 0.73	6.76 ± 5.03	1.03 ± 0.37	5.79 ± 2.36	1.11 ± 0.55	3.29 ± 2.32
tryptophan		**1.19**	0.13 ± 0.24**	1.76 ± 1.71	0.04 ± 0.08	2.20 ± 1.37	0.18 ± 0.30	2.24 ± 1.91
tyrosine		0.89	0.35 ± 0.45**	3.69 ± 3.15	0 ± 0	2.38 ± 1.03	0.04 ± 0.15	1.71 ± 1.53
Charged side chain - positive								
histidine		**1.02**	0 ± 0	8.05 ± 4.73	0 ± 0	8.61 ± 2.50	0 ± 0	7.26 ± 4.39
lysine		0.88	1.62 ± 1.09	9.91 ± 7.04	0.77 ± 0.27	6.66 ± 2.73	0.54 ± 0.49	4.65 ± 3.48
Charged side chain - negative								
glutamic acid		**1.27**	21.28 ± 10.32	28.59 ± 13.8	19.82 ± 3.74	22.6 ± 8.15	12.28 ± 5.12	20.25 ± 7.07
aspartic acid		**1.56**	10.20 ± 2.27	14.45 ± 1.35	7.60 ± 1.51	11.44 ± 1.34	4.82 ± 2.15	10.31 ± 2.97
Polar uncharged side chain								
asparagine		0.86	0.88 ± 0.28	13.59 ± 4.13	0.55 ± 0.15	10.44 ± 3.02	0.58 ± 0.26	6.81 ± 3.16
glutamine		0.97	4.04 ± 5.80**	99.42 ± 172.49	0.40 ± 0.86	32.49 ± 22.14	3.52 ± 10.00	44.00 ± 53.02
threonine + serine *		0.77	3.68 ± 1.90	20.99 ± 7.52	2.09 ± 0.69	17.20 ± 7.04	2.27 ± 2.23	15.69 ± 7.93
Irregular								
glycine		0.82	1.50 ± 0.82	9.08 ± 4.07	0.69 ± 0.20	6.27 ± 1.30	0.74 ± 0.29	4.51 ± 2.32
proline		**1.17**	3.10 ± 1.24	33.23 ± 6.24	2.21 ± 0.91	30.33 ± 3.39	1.59 ± 0.54	29.52 ± 9.56
Non-proteogenic amino acids								
α-aminoadipic acid		**1.31**	0.08 ± 0.10	0.95 ± 0.48	0.20 ± 0.15	1.21 ± 0.30	0.29 ± 0.18	0.75 ± 0.32
α-aminobutyric acid		0.8	0 ± 0	0.14 ± 0.09	0 ± 0	0.09 ± 0.04	0 ± 0	0.08 ± 0.07
γ-amino-n-butyric acid	GABA	0.84	3.95 ± 4.09	15.63 ± 7.90	1.14 ± 0.42	10.94 ± 3.57	0.70 ± 0.47	8.39 ± 6.34
3-hydroxyproline		**1.1**	1.02 ± 0.59	0.50 ± 0.35	0.89 ± 0.68	0.51 ± 0.32	1.49 ± 1.47	0.46 ± 0.35
ornithine		0.92	0.01 ± 0.04**	0.35 ± 0.22	0 ± 0	0.28 ± 0.13	0.02 ± 0.05	0.11 ± 0.19
Total concentration of free amino acids			64.65 ± 18.1	321.17 ± 185.47	44.52 ± 8.57	215.08 ± 44.55	37.25 ± 24.84	196.01 ± 99.76
			24 plants		24 plants		24 plants	

* threonine and serine coeluted

**compound present in less than 50% of the samples for the day and plant part considered

Table S3. List of soluble sugars that were identified and quantified in leaves (green) and inflorescences (yellow) of *B. nigra* exposed to attack, and their contribution (VIP value) to the separation in the PLS-DA of Fig. 2.

Identified soluble sugars	Common name	VIP value	Average concentration ± SD (µmol / g of plant DW)					
			day 4		day 8		day 12	
Monosaccharides (5)								
fructose		**1.09**	42.22 ± 25.76	167.37 ± 108.27	55.88 ± 33.49	232.06 ± 85.38	63.41 ± 36.06	123.07 ± 75.13
glucose		0.84	155.56 ± 67.12	250.60 ± 126.38	194.44 ± 61.58	358.08 ± 106.36	189.93 ± 107.01	241.45 ± 106.45
mannose		0.77	5.93 ± 4.10	11.89 ± 11.10	11.40 ± 4.65	11.16 ± 15.40	8.20 ± 6.45	9.25 ± 10.88
threose		0.86	0.25 ± 0.21	0.75 ± 0.56	0.36 ± 0.18	0.36 ± 0.21	0.30 ± 0.30	0.28 ± 0.20
xylose	wood sugar	0.92	4.84 ± 4.85	21.18 ± 24.05	5.08 ± 3.30	5.12 ± 4.34	6.31 ± 7.64	4.86 ± 5.14
Cyclitols (1)								
inositol		**1.29**	10.06 ± 4.81	12.69 ± 6.39	9.16 ± 3.34	24.76 ± 7.27	10.12 ± 4.55	25.81 ± 11.39
Disaccharides (2)								
maltose		0.98	1.41 ± 1.13	2.00 ± 1.27	2.57 ± 1.00	2.45 ± 0.91	3.67 ± 4.03	2.06 ± 0.79
saccharose	sucrose	**1.13**	8.82 ± 8.25	17.18 ± 16.40	20.13 ± 10.75	36.05 ± 17.52	37.12 ± 25.47	22.46 ± 14.79
Total concentration of sugars			229.09 ± 102.39	483.65 ± 246.90	298.96 ± 99.11	670.04 ± 209.76	319.05 ± 159.94	429.22 ± 194.17
			24 plants		24 plants		24 plants	

Table S4. List of glucosinolates that were identified and quantified in leaves (green) and inflorescences (yellow) of *B. nigra* exposed to attack, and their contribution (VIP value) to separation in the PLS-DA of Fig. 3.

Identified glucosinolates	Common name	VIP value	Average concentration ± SD (µmol / g of plant DW)					
			day 4		day 8		day 12	
Olefin (allyl) (1)								
2-propenyl-glucosinolate	sinigrin	**1.2**	9.67 ± 6.53	68.40 ± 28.70	14.28 ± 9.58	50.09 ± 22.40	11.85 ± 5.56	56.44 ± 22.01
Indole (4)								
4-hydroxy-indolyl-3-methyl-glucosinolate	4-hydroxy-glucobrassicin	**1.0**	0.01 ±0.01	0.27 ± 0.18	0.00 ± 0.01	0.22 ± 0.15	0.01 ± 0.01	0.08 ± 0.08
indolyl-3-methyl-glucosinolate	glucobrassicin	0.7	0.08 ± 0.05	0.06 ± 0.03	0.09 ± 0.04	0.08 ± 0.05	0.06 ± 0.04	0.05 ± 0.04
4-methoxy-indolyl-3-methyl-glucosinolate	4-methoxy-glucobrassicin	0.6	0.03 ± 0.03	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.04 ± 0.03	0.02 ± 0.02
1-methoxy-indolyl-3-methyl-glucosinolate	neoglucobrassicin	**1.5**	0.00 ± 0.01**	0.00 ± 0.01**	0.00 ± 0.00**	0.00 ± 0.01**	0.13 ± 0.03	0.12 ± 0.04
Aromatic (1)								
2-phenylethyl-glucosinolate	gluconasturtiin	0.7	0.09 ± 0.10	0.26 ± 0.23	0.11 ± 0.08	0.29 ± 0.21	0.03 ± 0.04	0.14 ± 0.10
Total concentration of glucosinolates			9.89 ± 6.64	69.01 ± 28.96	14.51 ± 9.61	50.71 ± 22.69	12.13 ± 5.56	56.86 ± 22.10
			48 plants		48 plants		48 plants	

**compound present in less than 50% of the samples for the day and plant part considered

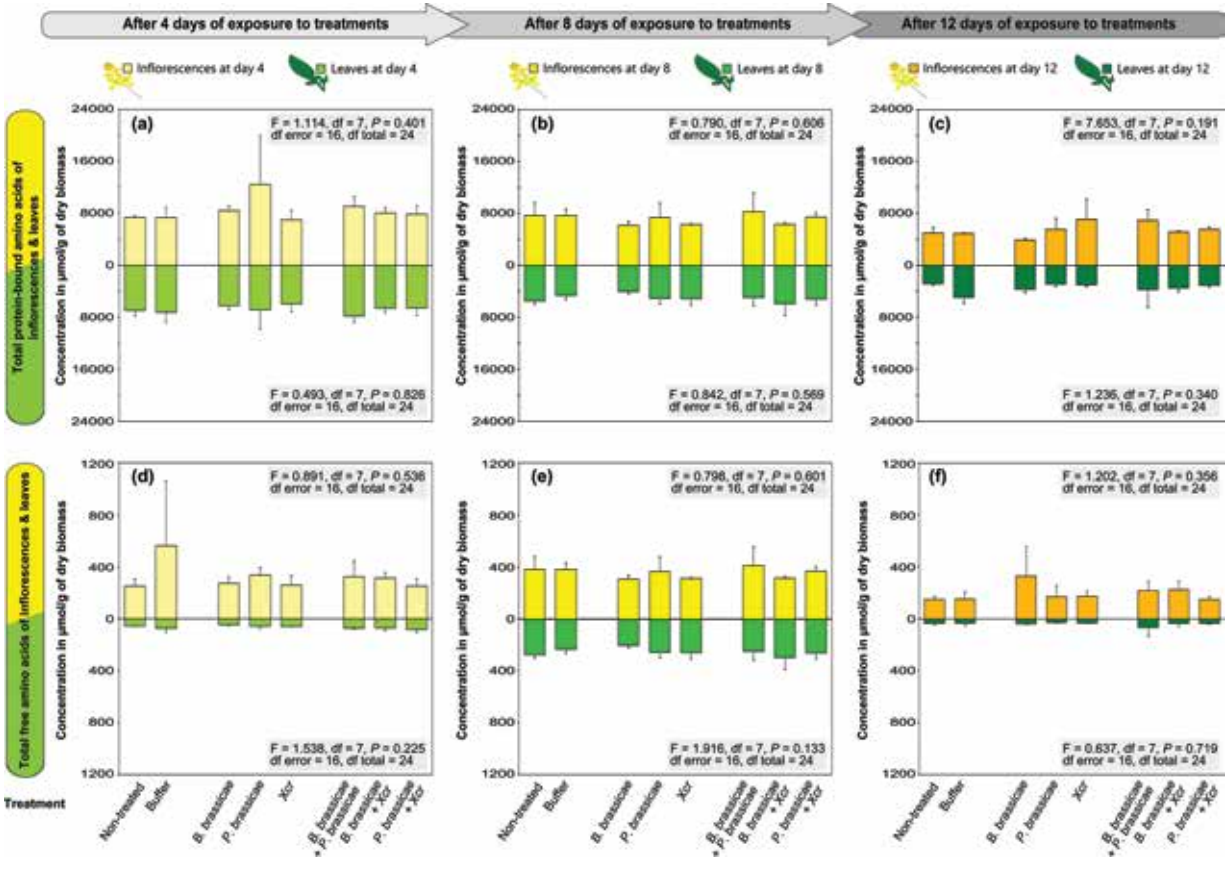


Fig. S1 Total concentrations (average ± SD) of protein-bound amino acids and free amino acids of inflorescences and leaves of *Brassica nigra* plants that had been exposed to single or dual attck for 4 d, 8d, and 12 d. Plants were exposed to single attack with *Brevicoryne brassicae* aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Panels a - f show total concentrations of protein-bound amino acids (a -c) and free amino acids (d - f) at d 4, 8 and 12 in samples of inflorescences and leaves of attacked plants. All concentrations are expressed in µmol.g-1 of plant dry biomass and were calculated by summing the concentrations of each detected and quantified compound. There were three plant replicates per treatements. Effect of treatment was tested with an ANOVA, and when significant, a Bonferroni post-hoc test was used for pairwise comparisons. The significance level was set to $\alpha = 0.05$; test parameters are indicated in a gray frame in the panels.

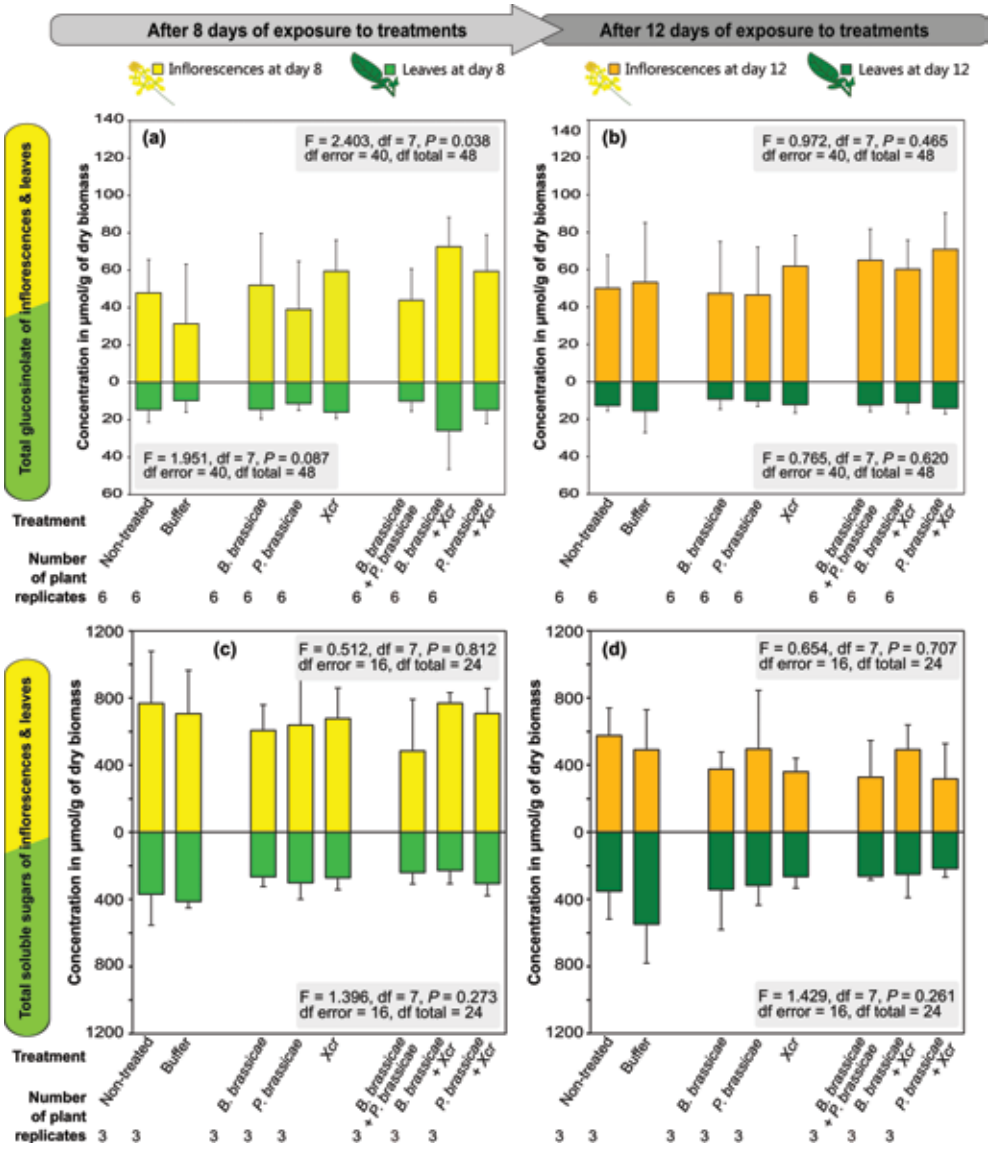
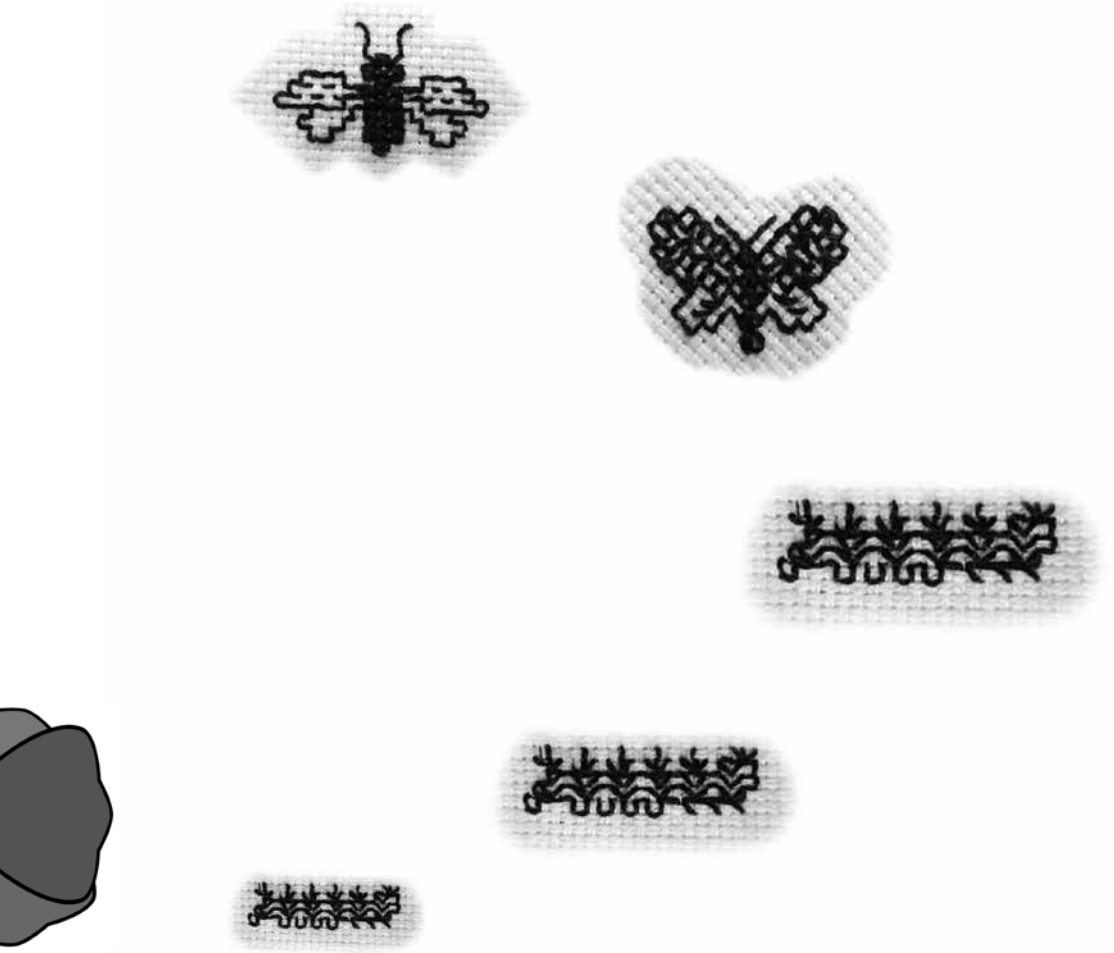


Fig. S2. Total concentrations (average ± SD) of glucosinolates and soluble sugars of inflorescences and leaves of *Brassica nigra* plants that had been exposed to single or dual attack for 8 and 12 d. Plants were exposed to single attack with aphids Brevicoryne brassicae aphids, eggs followed by caterpillars of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr), to dual attack by simultaneous infestion/infection by two of these three attackers, or exposed to buffer (control), or non-treated. Panels a - d show totals concentrations of glucosinolates (a - b and soluble sugars (c - d) at d 4, 8 and 12 d in samples of inflorescences and leaves of attacked plants. All concentrations are expressed in µmol.g-1 of plant dry biomass and were calculated by summing the concentrations of each detected and quantified compound. There were 6 plant replicates per treatement for the glucosinolates, and 3-4 for the soluble sugars. Effect of treatments was tested with an ANOVA, and when significant, a Bonferroni post-hoc test was used for pairwise comparisons.The significance level was set to $\alpha = 0.05$; test parameters are indicated in a gray frame in the panels.

Chapter 5



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Impact of multiple attack to inflorescences of *Brassica nigra* on its florivorous insect community

Abstract

Plant responses to attack influence plant phenotype and consequently can shape plant-associated biotic communities. Therefore, it is important to investigate changes in plant-associated communities in the natural context to understand the ecological consequences of plant responses to attack. Because damage on floral tissues (florivory) can reduce seed production by plants, changes in the florivorous community associated to inflorescences of plants upon attack likely affect the reproductive success of a plant. Here, we studied how responses of flowering *Brassica nigra* plants to single and dual attack influence the composition of the community of florivorous insects during the plant's reproductive period, and ultimately plant fitness. Plants that had just started flowering were either kept as control plants or exposed to single or dual combinations of three types of attackers that mostly infested/infected inflorescences (initial attackers): *Brevicoryne brassicae* aphids, *Pieris brassicae* caterpillars, and *Xanthomonas campestris* bacteria. As a proxy for plant fitness, we measured seed set and seed biomass of the plants. Plants were strongly defended against the initial attacker *P. brassicae* and most caterpillars died after a week of florivory. In contrast, *B. brassicae* were more abundant on plants exposed to dual attack than on plants exposed to single attack in the first week following the initial attack. Additionally, *B. brassicae* remained abundant on plants on which they had been introduced as the initial attacker. Plant responses to attack only transiently affected the composition of the colonizing florivorous community, and the composition of the colonizing florivorous community homogenized across treated plants in the second half of the flowering period. These changes were mediated by the abundance of only one species out of at least 27 species recorded: the specialist aphid *Lipaphis erysimi*, which occurred in the first half on the plant flowering period and had low abundance. We did not find a treatment-related fitness impact. We conclude that responses of *B. nigra* to attack on their inflorescences mainly impact the florivorous insects introduced as initial attackers, and had little effect on the subsequently colonizing florivorous community. Understanding the fitness consequences of plant responses to attack for plant fitness needs a dynamic approach with the plants' ecological context.

Key words

Inflorescences, florivore community, multiple attack, plant-mediated interactions, reproductive success

Introduction

From seedling to seed production, plants need to cope with attack by pathogens and herbivorous organisms. There is ample evidence that plant traits such as chemistry and morphology shape the community of organisms associated to plants (Newton *et al.*, 2009; Poelman *et al.*, 2009). As a consequence, changes in plant traits induced by herbivores and pathogens have the potential to restructure interactions between community members (Ohgushi, 2005; Kessler & Halitschke, 2007; Poelman *et al.*, 2010; Poelman & Dicke, 2014; Stam *et al.*, 2014; Ohgushi, 2016). Due to the specificity of plant responses to attack, early attack by an herbivorous insect can specifically shape patterns of herbivore occurrence and abundance through a plant's growing season (van Zandt & Agrawal, 2004; Viswanathan *et al.*, 2005; Poelman *et al.*, 2008a; Poelman & Dicke, 2014). Taking plant-mediated interactions between members of the plant-associated community into account can, therefore, be essential to understand the fitness consequences of plant responses to attack (Fordyce, 2006; Whitham *et al.*, 2006; Wise & Rausher, 2013; Ohgushi, 2016; Poelman & Kessler, 2016). The impact of changes in flower traits in response to attack on the florivore community has received little attention despite the strong negative impact that florivores can have on plant fitness (McCall & Irwin, 2006).

The first colonizers of a plant are expected to play a major role in structuring the herbivore community (Kessler & Halitschke, 2007; Poelman & Dicke, 2014; Stam *et al.*, 2014). Early-season herbivores, which can induce responses in plants at the beginning of the growing season, may indeed have a strong impact on plant fitness by initiating plant responses that will further cascade on the community members arriving later on the plant (Poelman & Dicke, 2014; Poelman & Kessler, 2016; Stam, 2016; Wise & Rausher, 2016). Different early-season insect attackers can, for example, lead to the development of distinct arthropod communities on plants in the vegetative stage (van Zandt & Agrawal, 2004; Viswanathan *et al.*, 2005; Poelman *et al.*, 2008a; Li *et al.*, 2016). Plant responses to phytopathogenic microbes can similarly play a role in structuring the plant-associated community (Tack *et al.*, 2012; Tack & Dicke, 2013; Sugio *et al.*, 2014). When manipulating the order of arrival of early-season insect herbivores, the first insect of the sequel can have the strongest structuring effect on the subsequent arthropod community (Stam *et al.*, 2018). It also appears that specialization of the initial attacker particularly influences the outcome of plant-mediated interactions between community members. In the Brassicaceae, for example, specialist feeders seem to be the main drivers shaping arthropod communities (Poelman *et al.*, 2008b; Poelman & Dicke, 2014).

Herbivore-induced changes in community composition do not only depend on the identity of the early-season attacker, but also on the identity of the colonizers that potentially settle on the attacked plants. Herbivore-induced phytochemical and morphological changes can reduce or enhance the attraction of subsequent colonizers, as well as the plant suitability for the colonizers according to their preference and performance (Kessler & Halitschke, 2007; Poelman *et al.*, 2008a; Poelman *et al.*, 2008b; Stam *et al.*, 2014; Meiners, 2015). Herbivore-induced plant volatiles, for example, mediate plant selection for oviposition, feeding, or predation (Kessler & Halitschke, 2007; Dicke & Baldwin, 2010), and can affect herbivore occurrence and abundance under field conditions (Kessler & Baldwin, 2001; Schuman *et al.*, 2012; Xiao *et al.*, 2012). After colonization of the plant, interspecific competition between community members can occur *via* plant quality, which has consequences for the performance of herbivores and carnivores (Denno *et al.*, 2000; Rodriguez-Saona *et al.*, 2005; Soler *et al.*, 2005; Poelman *et al.*, 2011). A large meta-analysis including arthropods, molluscs and mammals indicated that generalists and specialists respond differently (Leimu & Koricheva, 2006). Specialist insects tend to be attracted to induced plants and to proliferate on them compared to generalist herbivores (van Zandt & Agrawal, 2004; Poelman *et al.*, 2008a; Poelman *et al.*, 2010).

Plant phenology strongly shapes arthropod communities, and flowering phenology can be more important than other plant traits such as resistance in leaves (Johnson & Agrawal, 2005). Transition to flowering often correlates with higher arthropod abundance, diversity and richness, which is likely mediated by the new feeding niches that inflorescences bring to herbivores (Johnson & Agrawal, 2005; Abdala-Roberts *et al.*, 2017). It is estimated that florivores and frugivores are more detrimental to plant fitness than folivores (Schlinkert *et al.*, 2015; Wise & Rausher, 2016), although this has not always received experimental support (Godschalx *et al.*, 2016). Flowers generally contain high nutritional value but are also highly defended, which probably selects for a community of specialist feeders (Smallegange *et al.*, 2007; Hopkins *et al.*, 2009). Studies on plants in the vegetative stage highlight specialists as strong drivers of plant-mediated interactions, and they are likely to inflict extensive damage on inflorescences. However, few studies have so far linked the florivore community to plant fitness parameters (McCall & Irwin, 2006; Wise & Rausher, 2013; Stam *et al.*, 2018). For perennial wild cabbage, manipulating the type of attacker and order of arrival in the first year did not affect the leaf-associated insect community in the second year, but did affect flower-associated herbivores (Stam *et al.*, 2018). The subsequent effect on seed production

was subtle (Stam *et al.*, 2018). Plant responses to attack to their inflorescences may lead to stronger fitness consequences.

There is growing evidence that plants in the flowering stage can respond to attack to their inflorescences with phenotypic changes that affect flower tissues. *Brassica nigra* plants, for example, can respond to florivory by *Pieris brassicae* caterpillars with an induction of jasmonates in their inflorescences, whereas attack by *Brevicoryne brassicae* aphids and *Xanthomonas* bacteria has little effect on phytohormonal profile (Chrétien *et al.*, 2018). Jasmonates can mediate the biosynthesis of volatile compounds in inflorescences (Li *et al.*, 2017; Li *et al.*, 2018), and are also known to regulate primary and secondary metabolism in tissues of vegetative plants (Bari & Jones, 2009; Wasternack & Hause, 2013). Therefore, plants responses to attack on their inflorescences may affect subsequent plant selection by other florivores as well as their performance on the plants (Tsuji & Sota, 2010; McCall & Barr, 2012; McCall *et al.*, 2013). In line with this, attack by *P. brassicae* can induce changes in the floral volatile blend emitted by *B. nigra* and in the carbon and nitrogen content of flowering *B. nigra* (Lucas-Barbosa *et al.*, 2015; Lucas-Barbosa *et al.*, 2017). Floral volatiles and flower morphology of gourds correlated with attraction of specialist florivorous beetles, and this correlation was stronger than with resistance traits such as cucurbitacin concentration (Theis *et al.*, 2014). Florivory can also affect the performance of other florivores (McCall, 2006; Boyer *et al.*, 2016; Chrétien *et al.*, 2018). *Brevicoryne brassicae* aphids, for example, were more abundant on *B. nigra* exposed to dual attack with this aphid species plus *P. brassicae* or plus *Xanthomonas* bacteria than on plants exposed to *B. brassicae* only. Therefore, responses of plants in the flowering stage to attack with changes in floral traits may influence flower-associated species and, thus, the composition of the florivorous community. Because florivores can impact plant fitness, we predict that plants have evolved defense strategies that minimize the indirect consequences of plant response to attack on the subsequent florivorous community.

We investigated whether and how responses of plants, which had just started to flower, to attack on their inflorescences influenced the community of florivorous insects colonizing inflorescences over the course of the plant's reproductive period. To understand the potential fitness consequences of plant responses to attack, we assessed the number of seeds produced and the biomass of individual seeds, when plants carried mature siliques at the end of the flowering period (female fitness). We focused on the black mustard *B. nigra* (Brassicales: Brassicaceae), which is an annual plant species native from The Netherlands, where it grows in dense patches.

We assessed and compared the composition of the florivorous insect community on *B. nigra* exposed in the early flowering stage to single or dual attack by three types of specialist attackers: *B. brassicae* aphids*, P. brassicae* eggs plus caterpillars, and *Xanthomonas campestris* pathovar *raphani* bacteria (Xcr). The cabbage aphid *B. brassicae* (Hemiptera: Aphididae) develops large populations of thousands of individuals on inflorescences, and is specialized on brassicaceous plants (Hughes, 1963). Caterpillars of the Large Cabbage White butterfly *P. brassicae* (Lepidoptera: Pieridae) are specialist herbivores of Brassicaceae and use *B. nigra* as one of their host plants (Lucas-Barbosa *et al.*, 2014). Female *P. brassicae* butterflies lay eggs in clutches on the leaves of flowering *B. nigra* and after hatching, first and second instar (L1 and L2) caterpillars gregariously move to the inflorescence and use mainly flowers and buds as a food source (Lucas-Barbosa *et al.*, 2013; Lucas-Barbosa *et al.*, 2014). Xcr (Xanthomonadales: Xanthomonadaceae) is a phytopathogen causing leaf spot disease that forms small necrotic spots on leaves of many brassicaceous plants and can spread from infected leaves to mature seeds (Machmud, 1982; Vicente *et al.*, 2006). Mustard plants are particularly resistant to Xcr (McCulloch, 1929; Vicente *et al.*, 2006; Ponzio *et al.*, 2016b).

Materials and methods

Plant and insect cultures

Seeds of *B. nigra* were obtained from 25 plants that were exposed to open pollination in the field station of Wageningen University in the spring of 2015. Maternal plants descended from the CGN06619 line (Center for Genetic Resources (CGN), Wageningen, The Netherlands) that had been exposed to open pollination at the experimental farm of Wageningen University (The Netherlands) for several generations. Plants were sown in pots (Ø17 cm - 2L, 1:1 (v/v) mix of sand and potting soil, Lentse Potgrond, Lent, The Netherlands) in a greenhouse and seedlings (3-4 leaves) were transferred to an outdoor area protected by insect screen. Plants were transplanted to the field within five days after the opening of the first flowers.

Brevicoryne brassicae aphids and *Pieris brassicae* caterpillars were reared on Brussels sprout plants (*Brassica oleracea* variety *gemmifera*) in a greenhouse compartment (22 ± 2°C, 50-70% r.h., L16:D8). *Pieris brassicae* butterflies were provided with honey solution from organic production (10%, Melvita, Weide & Veldbloemen) as food, and were kept in a greenhouse compartment (25 ± 2 °C, 50-70% r.h., 16L:8D). *Xanthomonas campestris* pathovar *raphani* was obtained from Utrecht University, The Netherlands (Ponzio *et al.*, 2014). The bacteria were cultured in an artificial liquid medium (8 g L^{-1} of Difco™ : beef extract 3.0 g L^{-1} and

peptone 5.0 g L^{-1}, BD Diagnostics, New Jersey, USA) and kept at 28 °C under gentle shaking at 170 rpm for 21 ± 1 h. The liquid medium with bacterial cells was then centrifuged twice for 10 min at 4080 rotations per min and after each centrifugation the pellet containing the bacterial cells was re-suspended in buffer (10 mM M_gSO_4). We estimated the concentration of the inoculum by measuring the light absorbance at 600 nm and adjusted the concentration of the final inoculum to 10^9 cells mL^{-1} by diluting in buffer (10 mM M_gSO_4).

Field layout and plant treatments

To test whether plant responses to single and dual attack of flowering *B. nigra* plants affected the community of herbivorous insects feeding from the inflorescences, we exposed plots of *B. nigra* to one of the seven following treatments: 1) *B. brassicae*, 2) *P. brassicae*, 3) Xcr, 4) *P. brassicae* plus *B. brassicae*, 5) *P. brassicae* plus Xcr, 6) *B. brassicae* plus Xcr, and 7) buffer (control). Each plot (50 cm x 50 cm) consisted of five plants (Fig S1a). Only the central plant of a treated plot was originally exposed to attackers, and insect attackers could later disperse from the central plant to the side plants of the same plot. Central plants of each plot were infested in an outdoor area protected by insect-proof screen, about 3 ± 1 h prior to transplantation to the field. To infest plants with *B. brassicae*, five young adult females were gently placed on a bract (flower leaf) at the base of the inflorescence (Fig S1b). For the infestation with *P. brassicae*, plants were exposed to a mated female butterfly that was allowed to oviposit until about 30 eggs were laid, and we gently removed extra eggs to keep a clutch of 30 eggs (Fig S1b). To infect plants with bacteria, we soaked a 2 x 2 cm piece of cotton wool with 500 µL of the bacterium inoculum (10^9 cells mL^{-1} in buffer) that we placed on the underside of a bract and maintained for 4 hours by a soft clip. Control plants (buffer) were clipped with cotton wool soaked in buffer solution only (10 mM M_gSO_4) (Fig S1b). To additionally control for a possible effect of clipping and buffer, plants that did not receive the bacterial inoculation were clipped with buffer only, as described above. Plants assigned to dual treatment were infested or infected with two of these attackers simultaneously. An individual bract never received more than one treatment.

Each plot was covered with an insect-proof mesh for 2 d in order to prevent external colonisation of the plant during the early phase of the treatment and ensure that initial attackers settled on the plants. To have a standardized minimal amount of damage, we ensured that the central plant was exposed to at least five aphid colonies and 15 caterpillars. Therefore, we counted the number of adult *B. brassicae* on plants of plots that had received a *B. brassicae* treatment just after removing the mesh. When

fewer than the five initial aphids were recovered, we added adult aphids from the laboratory culture to reach five aphids per plot. Caterpillars hatched and started feeding after 8 to 9 days since oviposition, regardless of treatment. When fewer than 50 % of the caterpillars hatched from the eggs (*i.e.* < 15 caterpillars), we added neonate caterpillars from the laboratory culture to have a final count of 15 caterpillars.

The field consisted of two blocks of 9 m x 10.5 m, each consisting of 56 plots organized in seven rows and eight columns (Fig S1c). Blocks were 3 m apart, and within a block, the central plants of each plot were 1.5 m apart. Fourteen plots were transplanted to the field on each day – two plots for each of the seven treatments – within eight consecutive days (between May 18th 2017 to May 25th 2017). We had 16 replicates (plots) per treatment. Treatments were assigned to plots according to a Latin square design, and plants of the same treatment that were infested/infected on the same day were never planted in the same column or row. A fence was placed around the field, 3 m from the nearest plots, to protect the field from mammalian herbivores such as rabbits and hares.

Recording of herbivorous insects on inflorescences of *B. nigra* plants in the field
We identified and counted all herbivorous insects found on the inflorescences of *B. nigra* at 8 d, 16 d, 24 d and 32 d after plants had been exposed to the treatments. When possible, insects were identified to the species level; otherwise, we restricted the identification to the family level (Büchi & Roos-Humbel, 1991; Alford & Nilsson, 2003; Marczali *et al.*, 2007; Ahuja *et al.*, 2010; Opitz *et al.*, 2012; Schlinkert *et al.*, 2015). Two types of *Ceutorhynchus* spp. beetles were recorded but could not be identified to the species level; thus, we named them based on their phenotype: large gray *Ceutorhynchus*, small shiny-blue *Ceutorhynchus.* Similarly, we differentiated small gray *Lygus* from other *Lygus.* Species richness was calculated based on the lowest level of identification we could reach, species or family. Because not all insects could be identified to the species level, our data represent an underestimation of the actual species richness and should be considered as a proxy.

Recorded insects were either exclusive plant feeders or omnivores, and we observed their feeding behavior to verify that they were feeding from *B. nigra* and not only using the plant as a shelter. Florivores are plant feeders that inflict damage to bracts, floral display, pollen, and/or ovules, until seed coat development (McCall 2006), and we also recorded insects feeding on seeds and siliques. Additionally, we specified from which plant parts the insects were feeding (floral parts: stalk, buds, flowers, siliques, or bracts). Considering eggs, we recorded single eggs, egg clutches,

and counted the number of eggs per clutch. Single eggs of Pieridae or Noctuidae were identified as respectively "pierid egg" and "noctuid egg"; they could have been laid by a female from either a solitary species or from a gregarious species that was disturbed during the oviposition process. For mines, we distinguished mines containing a larva, mines containing a pupa, and empty mines (insects leave after pupation). We eventually summed these three categories in the final data set and analyzed the cumulative number of mines for each recording dates. The herbivore community was assessed for the central plants of each plot, and for two randomly selected side plants. The same plants were assessed at the four time points. For each assessed plant, we determined the developmental stage of the inflorescence (estimated proportion of buds/flowers/siliques).

Characterization of the insect community feeding on reproductive parts of *B. nigra* in the field

Identified insect species and families were further classified based on functional traits: specialization level and feeding guild (Hatfield *et al.*, 1983; Varis, 1995; Vierbergen, 2002; Dietrich, 2005; Opitz *et al.*, 2012; Marullo & De Grazia, 2013; Traugott *et al.*, 2015). The occurrence of each identified species or family was calculated based on presence/absence data across all plants throughout the field season (Overall occurrence), at each of the four time points (Occurrence over time), and for floral parts and bracts within a plant (Feeding location). Occurrence shows the percentage of plants or plant parts that harbor the given insect species relative to the total number of plants within the category considered. Based on the overall occurrence, species were classified from very rare to very common according to the following criteria: very rare ($0 \leq X \leq 5\%$); rare ($5\% < X \leq 10\%$); occasional ($10\% < X \leq 25\%$); common ($25\% < X \leq 50\%$); very common ($X > 50\%$). Feeding location was classified into five categories based on the relative occurrence of the insect species on bracts and floral parts: bracts (more than 10 times more on bracts than on floral parts), more on bracts (more than twice more on bracts than on floral parts), bracts and floral parts (less than a two-fold difference), more on floral parts (more than twice more on floral parts than bracts), and floral parts (more than 10 times more on floral parts than bracts).

Relative and total abundance of insect species occurring on bracts and floral parts of control *B. nigra* plants

To analyze community development, we calculated relative abundance of the different species on bracts and floral parts compared to the total abundance on the plant. For this, we summed the abundance for each insect species across plant

replicates, and divided it by the total number of insects recorded across the plant replicates. We focused on plants that were not exposed to an initial attack, *i.e.* control plants exposed to buffer only, and separated central and side plants at day 8, 16, 24 and 32. Total abundance per plant was calculated by taking the average of the total insect abundance of each assessed plant, and species richness was calculated by taking the average of the number of different species recorded on each assessed plant. Abundance of *Plutella xylostella* and *Athalia* sp. included eggs counted individually, and abundance of *Mamestra brassicae* included numbers of eggs clutches.

Abundance of the insects that were experimentally introduced on inflorescences of *B. nigra* plants initially exposed to single or dual attack and on control plants

Effect of initial plant exposure to *B. brassicae* aphids, eggs of *P. brassicae,* or Xcr bacteria, to two of these attackers simultaneously, or to buffer (control), on the abundance of *B. brassicae* and *P. brassicae*, was analyzed separately from the other insect species recorded on *B. nigra*. *Brevicoryne brassicae* and *P. brassicae* had been artificially introduced onto the plant as an initial treatment and could later not be distinguished from conspecifics colonizing the plant from the field. Because only central plants of plots initially received the attackers, we separated the attacker abundance of central and side plants of plots. Counts for the two side plants were averaged per plot, and we used this averaged value for statistical analyses and graphs.

In several cases, no *B. brassicae* or *P. brassicae* were found on plots. This absence of *B. brassicae* or *P. brassicae* did not allow statistical models to compute estimates. Thus, we ran separate models testing: 1) when possible, the effect of all seven treatments on the abundance of *B. brassicae* and *P. brassicae*, 2) in all cases, the effect of treatment on the number *B. brassicae* and *P. brassicae* that developed on plants initially exposed to those attackers: *B. brassicae* only and simultaneous dual attack with *B. brassicae* plus *P. brassicae* or plus Xcr, or *P. brassicae* only and simultaneous dual attack with *B. brassicae* plus *P. brassicae* or plus Xcr.

Community composition of florivorous insects on inflorescences of *B. nigra* exposed to single or dual attack and on control plants

To investigate whether the response of *B. nigra* to single and dual attack at the beginning of the flowering stage affects the subsequent colonization of inflorescences by florivores, we recorded the composition of the florivorous community of *B. nigra* plants initially exposed to *B. brassicae* aphids, eggs of *P. brassicae,* or the bacterium

Xcr, to two of those attackers simultaneously, or exposed to buffer (control). Vegetative parts of plants were not assessed. Assessing community composition was based on the abundance of the different insect species recorded, and analysed using multivariate models. We restricted the analysis to species that occurred in at least 5% of the plants for at least one time point. Thus, we excluded very rare species that may result in a high risk of false positive outcome, but took into account the dynamics of the community by including species that had a transient high occurrence. We excluded abundance of *B. brassicae* and *P. brassicae* from the analysis, because they had been initially introduced onto the plants as treatment. Abundance of *P. xylostella* and *Athalia* sp. included eggs counted individually.

Seed set, seed biomass and fresh biomass of control *B. nigra* plants and *B. nigra* initially exposed to single or dual attack

We tested the effect of plant exposure to single and dual attack by *B. brassicae*, *P. brassicae,* or Xcr, on the reproductive success of *B. nigra* plants by comparing with control plants exposed to buffer only. We used the number of seeds produced and seed biomass as proxies for plant reproductive success. Fresh weight of inflorescences was measured at the end of the experiment. Plants were harvested at 41 ± 1 d after they had been infested/infected and transplanted into the field. Reproductive plant parts were separated from the leaves by cutting at the base of the main inflorescence, and all side inflorescences were cut at the base of their stalk. Fresh weight of the reproductive parts was then assessed and siliques were stored at room temperature in the dark, to dry at the facilities of the experimental farm of Wageningen University (Unifarm). Dry siliques were then crushed open to harvest the seeds. We weighed a sample of 100 randomly selected seeds as well as the total number of seeds harvested per plant. The total number of seeds was estimated by dividing the weight of the total number of seeds by the weight of 100 seeds of this plant and multiplying by 100. Fresh biomass of plant reproductive parts, number of seeds, and biomass of seeds were assessed for the central plants and the two randomly selected side plants of plots that were used for insect community recordings. Data for the two side plants of a same plot were averaged, and we used this average for statistical analyses and graphs. For one *B. brassicae* treatment, seed data of one of the two side plants was missing. We analyzed data separately for the central plants and side plants of plots.

Univariate statistical analyses

The time-dependent treatment effects on abundance, species richness, number of seeds and seed biomass were analyzed with Generalized Linear Mixed Models (GLMM) and Generalized Linear Models (GLM) for count data (O'Hara & Kotze, 2010; Ives, 2015; Warton *et al.*, 2016), and Linear Mixed Models (LMM) and Linear Models (LM) for continuous data and number of seeds (Warton *et al.*, 2016) in SPSS (Versions 24 and 25, for Windows, IBM Corporation, Armonk, NY, USA). Model parameters are detailed in the Supporting Information. Continuous data and seed number data met the assumptions of normality and equal variances across treatments. We specified a Poisson distribution for count data, and when data were overdispersed, we either fitted a quasi-Poisson or a negative binomial distribution (Ver Hoef & Boveng, 2007; Huang *et al.*, 2016); Log was used as link function. We specified a normal distribution for continuous data and seed number, and identity was specified as link function. We used a backward method for model selection (Kincaid; Bolker *et al.*, 2009). We first computed models with fixed intercept, all fixed factors to be tested, and related random factors, *i.e.* Plot identity, Block and/or Planting Day. Random factors were modelled as random intercepts, with scaled identity or variance component as covariance structure. Non-converging models were then simplified by removing irrelevant random factors until we obtained a model able to converge (Bolker *et al.*, 2009), as described in the Supporting Information. Non-significant random factors were excluded from the models. We used GLMMs to test the effect of time (day 8, 16, 24 and/or 32) on insect abundance and richness, to account for the covariation between observations on the same plant or plot. We accounted for repeated measurements through modeling the multiple residuals for each subject, in the covariance matrix of the residuals (R matrix) (Kincaid). Either Plot identity or Plant identity were defined as subject. We defined the covariance structure by testing correlation between time points for the tested parameters, calculation and graphical observation of variances, and by model comparisons (Littell *et al.*, 2000; Kincaid). We used the Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) for model selection when working with linear models (LM and LMM). For generalized models (GLM and GLMM), the -2 pseudo-log-likelihood and related model fit estimators cannot be reliably compared. Thus, we compared covariance structures for covariance matrix parameters that matched the data and were significant in a Wald Z test. For all model selection procedures, we verified that the estimates of the fixed factors, their standard error, and the 95% confidence intervals did not appear as over- or underestimated and we checked the stability of the estimates (Littell *et al.*, 2000). Additionally, we compared plots of predicted values plotted against observed data

for the best fit. The final model, the distribution fitted, and covariance structures, are indicated in the figures or tables in the Supporting Information (Casals *et al.*, 2014). When an effect of a fixed factor was detected, we performed *post-hoc* tests for pairwise comparisons of factor levels using a significance threshold of 0.05. We applied the Least Significant Difference (LSD) *P*-value adjustment when there were up to three pairwise comparisons, and a Bonferroni (L(M)Ms), or sequential Bonferroni (GL(M)M), *P*-value adjustment when there were more. *Post-hoc* tests following GL(M)Ms were performed on the linear predictors.

We tested whether the abundance of *B. brassicae* aphids and *P. brassicae* caterpillars were associated to the number and biomass of seeds produced by *B. nigra*. For this, we plotted regression lines for the best-fit slope and intercept linking seed data (response) to abundance (predictor), and computed the related R-squared value (R^2, ranging from 0 to 1). Number and biomass of seeds were normally distributed. A linear regression was calculated for both attackers using the abundance recorded at the time points taken separately, and data of all individual plants were used, regardless of treatments. Regressions were performed in Excel (version 2016, for Windows, Microsoft® office, Redmond, Washington, USA).

Multivariate statistical analyses

The time-dependent treatment effects on the composition of the insect community that colonized inflorescences of *B. nigra* were analyzed by Principal Response Curve analysis (PRC) (van den Brink & ter Braak, 1999), and related ordination methods using CANOCO version 5.11 (ter Braak & Šmilauer, 2018). The data were log(y+1) transformed. The PRC was based on a partial Canonical Correspondence Analysis (pCCA) (ter Braak *et al.*, 1988), instead of the usual partial redundancy analysis because they have a gradient length of 3.9 SD units of species turnover (ter Braak & Šmilauer, 2018). The usual species scores in correspondence analysis-like methods tend to exaggerate the importance of rare species. A solution is to represent species by their contribution score to an ordination or PRC axis (Greenacre), the contribution score being the usual score multiplied by the square-root of the total abundance after transformation. Testing of statistical significance was performed by Monte Carlo permutation testing using 999 random permutations of plots consisting of one central plant and the two side plants. This test is presumably liberal in this context as it treated measurements on the same plot at different times as independent. For this reason, partial Canonical Correspondence Analysis (pCCA) was also applied per time point, in particular to analyze the variation explained by treatments, and how the insect community differed across treatments.

Both PRC-CCA and pCCA analysis were adjusted for the effects of block, planting day and plant location within the plot and plant location in the field (row and column) by specifying them as covariates in CANOCO. Additionally, we executed CCAs where treatments and these covariates were considered as explanatory variables to test their relative importance. For this, we computed the conditional effects of the different explanatory variables, and tested their significance in a permutation test using the false discovery rate to adjust the *P*-values

Results

Characterization of the insect community on reproductive parts of *B. nigra* plants in the field

Throughout the season and regardless of treatments, we recorded 30 insect categories (later considered as species for simplification) from at least 27 species and 14 families, which were feeding from the reproductive parts of *B. nigra* plants (Table 1). At least 10 of these species were sucking insects, all Hemiptera and Thysanoptera. About 25% of the sucking insects were specialist feeders, the others being generalists. Around 60% of the sucking insects were found at least twice more often on floral parts than on bracts, 30% were found equally on bracts and floral parts, and 10% were found at least twice more often on bracts than on floral parts. At least 17 species recorded on inflorescences of *B. nigra* were tissue-chewing insects from the orders Coleoptera, Lepidoptera, Hymenoptera and likely Diptera (mining insects). About 80% of the tissue-chewing insects were specialist feeders, the others being generalists. Around 40% of the chewing insects were found at least twice more often on bracts than on floral parts or twice more on floral parts than on bracts, while about 20% were found equally on bracts and floral parts.

If we exclude *B. brassicae* and *P. brassicae*, which were experimentally placed on the plant as inducers, the overall occurrence of the different species was low. Over 50% of the insect species occurred on less than 5% of the plants and, consequently, were considered to be very rare (Table 1). Only *Meligethes* sp. beetles and thrips occurred on more than 50% of the plants. Overall, 12 species had an occurrence higher than 5%, and were considered as the core community. Five of them were sucking insects: small gray *Lygus*, *Myzus persicae*, *Myzus persicae* sub. *nicotianae*, unidentified winged aphids, and thrips; seven of them were chewing insects: large gray *Ceutorhynchus*, small shiny-blue *Ceutorhynchus*, *Meligethes* sp., striped *Phyllotreta*, *Plutella xylostella*, *Athalia* sp., and mining insects. Occurrence of species was highly dynamic over time. The striped *Phyllotreta* beetle, and the *Ceutorhynchus* beetles from the core community had a transient pattern of occurrence and were

Table 1. List of herbivorous insects recorded in the field on reproductive parts of treated and control *Brassica nigra* plants at four different time points.

Order or suborder	Family	Common name	Developmental stage	Feeding-guild	Specialization level	Feeding location	Bracts	Floral parts	Overall occurrence	Occurence over time* day 4 (42 plots, 126 plants)	day 16 (43 plots, 129 plants)	day 24 (28 plots, 84 plants)	day 32 (39 plots, 117 plants)
Coleoptera (≥9 species)													
Large gray *Ceutorhynchus*[a]	Curculionoidae	Weevil	adults	tissue chewing	specialist on Brassicaceae	floral parts	9.1%	90.9%	**rare (7.3%)**	**5.6%**	**17.1%**	3.5%	0.9%
Small shiny-blue *Ceutorhynchus*[aa]	Curculionoidae	Weevil	adults	tissue chewing	likely specialist on Brassicaceae	bracts & floral parts	47.8%	52.2%	**rare (5.1%)**	**9.5%**	**6.2%**	3.6%	
Small matte-dark *Ceutorhynchus*[aaa]	Curculionoidae	Weevil	adults	tissue chewing	likely specialist on Brassicaceae	floral parts	7.7%	92.3%	very rare (2.7%)	**7.9%**	1.5%		0.9%
Meligethes sp.[b]	Nitidulidae	Pollen beetle	larvae & adults	tissue chewing	specialist on Brassicaceae	floral parts	1.6%	100%	**very common (81.0%)**	**83.3%**	**93.8%**	**89.3%**	**61.4%**
Phaedon cochleriae	Chrysomelidae	Mustard beetle	larvae	tissue chewing	specialist on Brassicaceae	bracts	100%	0%	very rare (1.3%)		1.6%	1.2%	2.6%
Striped *Phyllotreta*[c]	Chrysomelidae	Striped fleabeetle	adults	tissue chewing	specialist on Brassicaceae	more on bracts	71.0%	35.5%	**occasional (13.7%)**	**24.6%**	**20.9%**	2.4%	1.6%
Black *Phyllotreta*[cc]	Chrysomelidae	Fleabeetle	adults	tissue chewing	specialist on Brassicaceae	more on floral parts	28.6%	71.4%	very rare (1.5%)	4.0%	0.8%	1.2%	
Unidentified Chrysomelidae	Chrysomelidae	/	adults	tissue chewing	/	bracts	100%	0%	very rare (0.7%)				2.6%
Unidentified Elateridae	Elateridae	Click beetle	adults	tissue chewing	generalist[1]	floral parts	0%	100%	very rare (0.4%)		0.8%		0.9%
Unidentified eggs & larvae	/	/	eggs and larvae	tissue chewing	/	bracts	100%	0%	very rare (1.1%)		0.8%	1.2%	2.6%
Hemiptera (≥10 species)													
Auchenorrhyncha (≥1)													
Unidentified Cicadellidae	Cicadellidae	Leafhopper	adults	sap sucking	/[2]	bracts	91.7%	8.3%	very rare (2.6%)	1.6%	1.6%	2.4%	**5.3%**
Heteroptera (≥2)													
Small gray *Lygus*	Miridae	Lygus bug	nymphs & adults	sap & tissue sucking	generalist[3]	bracts & floral parts	61.5%	38.5%	**rare (5.7%)**			**6.0%**	**18.4%**
Other *Lygus*	Miridae	Lygus bug	nymphs & adults	sap & tissue sucking	generalist[3]	more on floral parts	23.5%	76.5%	very rare (3.8%)	0.8%	1.6%		**12.3%**
Sternorrhyncha (≥5)													
Aphis fabae	Aphididae	Black bean aphid	nymphs & adults	phloem sucking	generalist	more on floral parts	33.3%	66.7%	very rare (2.0%)	0.8%		2.4%	**5.3%**
Brevicoryne brassicae[d]	Aphididae	Cabbage aphid	nymphs & adults	phloem sucking	specialist on Brassicaceae	more on floral parts	37.1%	91.9%	/ (46.1%)	20.6%	38.0%	59.5%	74.6%
Lipaphis erysimi	Aphididae	Mustard aphid	nymphs & adults	phloem sucking	specialist on Brassicaceae	more on floral parts	22.9%	88.3%	very rare (2.0%)	0.8%	**5.4%**	1.2%	
Myzus persicae	Aphididae	Green peach aphid	nymphs & adults	phloem sucking	generalist	bracts & floral parts	68.1%	45.2%	**common (30.7%)**	**27.0%**	**33.3%**	**39.3%**	**25.4%**
Myzus persicae sub. nicotianae	Aphididae	Tobacco aphid	nymphs & adults	phloem sucking	generalist	bracts & floral parts	58.9%	46.4%	**occasional (12.1%)**	**15.1%**	**17.1%**	4.8%	**9.6%**
Winged forms - unidentified	Aphididae	/	nymphs & adults	phloem sucking	/	more on floral parts	30.5%	76.8%	**occasional (17.9%)**	**19.8%**	**17.1%**	**15.5%**	**19.3%**
Thysanoptera (≥2)													
Unidentified Thrips	Thripidae (?)	Thrips	nymphs & adults	cell sucking	likely generalist[4]	floral parts	9.2%	98.0%	**very common (76.2%)**	**78.60%**	**72.10%**	**95.20%**	**64.90%**
Lepidoptera (≥6 species)													
Evergestis extimalis	Crambidae	/	caterpillars	tissue chewing	specialist on Brassicaceae	more on floral parts	33.3%	66.7%	very rare (1.3%)				**5.3%**
Evergestis forficalis	Crambidae	Garden pebble moth	caterpillars	tissue chewing	specialist on Brassicaceae	bracts	100%	0%	very rare (1.1%)		0.8%		3.5%
Mamestra brassicae	Noctuidae	Cabbage moth	eggs, caterpillars	tissue chewing	generalist	bracts	100%	0%	very rare (1.1%)		3.1%		0.9%
Pieris brassicae[d]	Pieridae	Large cabbage white butterfly	caterpillars	tissue chewing	specialist on Brassicaceae	bracts & floral parts	66.7%	41.7%	/ (7.9%)		20.9%	10.7%	
Pieridae eggs	Pieridae	/	eggs	tissue chewing	specialist on Brassicaceae	bracts	100%	0%	very rare (0.2%)		0.8%		
Plutella xylostella	Plutellidae	Diamondback moth	eggs, caterpillars, pupae	tissue chewing	specialist on Brassicaceae	bracts & floral parts	54.1%	63.5%	**occasional (19.0%)**		**7.0%**	**19.0%**	**53.5%**
Unidentifed loopers	Noctuidae/Geometridae	Looper moths	caterpillars	tissue chewing	/	more on floral parts	28.6%	71.4%	very rare (1.5%)			4.8%	2.6%
Noctuidae eggs	Noctuidae	/	eggs	tissue chewing	generalist	bracts	100%	0%	very rare (0.2%)			1.2%	
Hymenoptera (≥1 species)													
Athalia sp.[e]	Tenthredinidae	Sawfly	eggs, larvae	tissue chewing	specialist on Brassicaceae[5]	more on bracts	94.6%	12.5%	**occasional (12.1%)**			**19.0%**	**35.1%**
Diptera/Coleoptera/Lepidoptera (≥1 species)													
Mining insects	/	Miners	larvae, pupae, empty mines	internal tissue chewing	/	bracts	100%	0%	**common (40.2%)**		**10.9%**	**73.8%**	**93.9%**

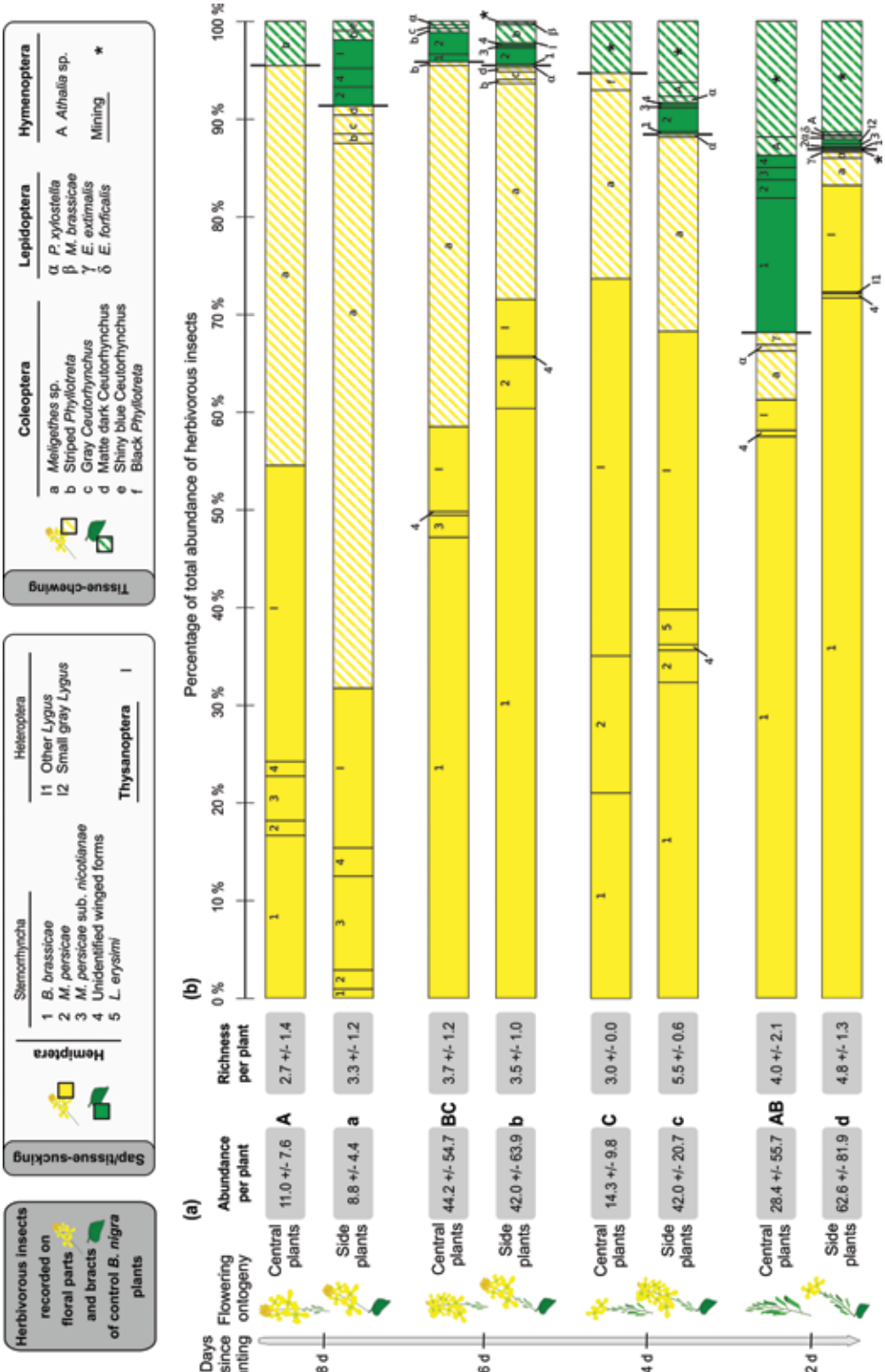
[a]Likely a mixture of *Ceutorhynchus obstrictus and C. pallidactylus* (Marczali *et al.*, 2007; Ahuja *et al.*, 2010)
[aa]Possibly *Ceutorhynchus erysimi* or *C. sulcicollis*
[aaa]Possibly *Ceutorhynchus assimilis* (Marczali *et al.*, 2007; Ahuja *et al.*, 2010)
[b]Likely a mixture of *Meligethes aeneus* (mainly) and *M. viridescens* (Büchi & Roos-Humbel, 1991 ; Ahuja *et al.*, 2010)
[c]Likely a mixture of *Phyllotreta striolata*, *P. nemorum* and *P. undulata* (Ahuja *et al.*, 2010)
[cc]Likely a mixture of *Phyllotreta atra* and *P. cruciferae* (Ahuja *et al.*, 2010)
[d]**Herbivore species used for the early season induction.**
[e]Likely a mixture of *Athalia rosae* (mainly), *A. lugens*, *A. liberta* and A. *cornubiae* (Ahuja *et al.*, 2010; Opitz *et al.*, 2012)
[1]Traugott *et al.*, 2015
[2]Dietrich, 2005
[3]Hatfield *et al.*, 1983; Varis, 1995
[4]Vierbergen, 2001 (p359); Marullo & De Grazia, 2013
[5]Opitz *et al.*, 2012

Fig. 1 (right). Relative abundance of herbivorous insects recorded on floral parts and bracts of central and side *Brassica nigra* plants of control plots at four different time points. The abundance of each insect species or insect group is shown in percentage of total abundance for central and side plants of control plots at 8, 16, 24 and 32 days after buffer exposure. Insects recorded feeding on reproductive parts of *B. nigra* were categorized into two groups: 1. sap/tissue-sucking, and 2. tissue-chewing, on floral parts (yellow) and bracts (green). Within each group, we distinguished the orders (bold) and suborders (normal). Total abundance and richness in insect species or groups (as indicated in the legend) are indicated in grey boxes as average ± standard deviation. Flowering ontogeny indicates the flowering phenology of the plants. For each plot of five plants, florivorous insect community was recorded on the reproductive parts of the central plant and of two randomly selected side plants. Insect community of the two side plants was averaged. Effect of time point and plant location was analysed with a Generalized Linear Mixed Model for total abundance and with a Generalized Linear Model for species richness. Interaction between time point and plant location was significant for total abundance, thus we performed a sequential-Bonferroni *post-hoc* test based on pairwise comparisons between times points for central plants (capital letters) and for side plants (lower case letters), and between side plants and central plants at each time points (non-significant). Different letters indicate significant differences ($P < 0.05$).

mostly present at the first two time points. Additionally, some species that were overall very rare had transient occurrence higher than 5% at certain time points. This was the case for the following species: Other *Lygus* and *Aphis fabae* at day 32, and *Lipaphis erysimi* at day 16.

Relative and total abundance of insect species occurring on bracts and floral parts of control *B. nigra* plants

Control *B. nigra* were only exposed to buffer and did not receive any initial infestation or infection. On inflorescences of central plants of control plots, total abundance of insects increased over time until day 16, and then decreased, while the total abundance gradually increased over time on inflorescences of the side plants (Fig. 1a, Table S1a). Abundance on central and side plants was similar at days 8, 16, and 24, while side plants had about twice as many insects as central plants at day 32 (Fig. 1a, $P = 0.063$). Species richness was lower on central plants (3.3 ± 1.4 insect species per inflorescence on average) than on side plants (4.4 ± 1.4 insect species per inflorescence on average, $P = 0.028$, Fig. 1a & Table S1b), and slightly, but not significantly, increased over time from 3.0 ± 1.3 insect species per inflorescence at day 8 to 4.4 ± 1.7 insect species per inflorescence at day 32 (Fig. 1a & Table S1b, Time point: $P = 0.069$). Over 90% of insect abundance was due to insects recorded on floral parts, and the remaining insects were on bracts (Fig. 1b). Among these 90% on floral parts, abundance of aphids, thrips and *Meligethes* sp. accounted for over 85%. Counts of aphids on floral parts mainly represented *B. brassicae*, which reached up to 80% of the total abundance on plants.


Herbivorous insects recorded on floral parts and bracts of control B. nigra plants
Sap/tissue-sucking
Hemiptera
Sternorrhyncha
1 B. brassicae
2 M. persicae
3 M. persicae sub. nicotianae
4 Unidentified winged forms
5 L. erysimi
Heteroptera
I1 Other Lygus
I2 Small gray Lygus
Thysanoptera I
Tissue-chewing
Coleoptera
a Meligethes sp.
b Striped Phyllotreta
c Gray Ceutorhynchus
d Matte dark Ceutorhynchus
e Shiny blue Ceutorhynchus
f Black Phyllotreta
Lepidoptera
α P. xylostella
β M. brassicae
γ E. extimalis
δ E. forficalis
Hymenoptera
A Athalia sp.
Mining *
(a)
(b)
Percentage of total abundance of herbivorous insects
0 %
10 %
20 %
30 %
40 %
50 %
60 %
70 %
80 %
90 %
100 %
Days since planting
Flowering ontogeny
Abundance per plant
Richness per plant
8 d
16 d
24 d
32 d
Central plants
Side plants
11.0 +/- 7.6 A
2.7 +/- 1.4
8.8 +/- 4.4 a
3.3 +/- 1.2
44.2 +/- 54.7 BC
3.7 +/- 1.2
42.0 +/- 63.9 b
3.5 +/- 1.0
14.3 +/- 9.8 C
3.0 +/- 0.0
42.0 +/- 20.7 c
5.5 +/- 0.6
28.4 +/- 55.7 AB
4.0 +/- 2.1
62.6 +/- 81.9 d
4.8 +/- 1.3

Abundance of the insects that were experimentally introduced on inflorescences of *B. nigra* plants initially exposed to single or dual attack and on control plants

Brevicoryne brassicae aphids were more abundant on plots where plants had been initially exposed to single infestation with *B. brassicae* or dual attack with *B. brassicae* plus *P. brassicae* or Xcr, than on plants of plots that did not receive the initial aphid attack (Fig. 2 & Fig. S2). When comparing *B. brassicae*-infested plants after 8 d of exposure to treatments, central plants initially exposed to dual attack by *B. brassicae* plus *P. brassicae* or Xcr had 1.8 times more *B. brassicae* than plants exposed to *B. brassicae* only (Fig. 2a; resp. P = 0.001 and P = 0.050). Only few *B. brassicae* had migrated to side plants at day 8 (Fig. 2b). Similarly at day 24, central plants initially exposed to dual attack by *B. brassicae* plus Xcr had 2.5 times more *B. brassicae* than plants exposed to *B. brassicae* only (Fig. S2c, P = 0.006), and abundance of *B. brassicae* on plants exposed to *B. brassicae* plus *P. brassicae* did not differ from the abundance of the other two treatments (Fig. S2c, P = 0.474 with *B. brassicae*-treated plants, P = 0.683 with *B. brassicae* plus Xcr-treated plants). The positive effect of dual attack on *B. brassicae* abundance, when compared to single attack, was not detected anymore at day 32 (Fig. 2c, d). Side plants had a lower abundance of *B. brassicae* than central plants at each of the four time points, with respectively 24.4 times less, 7.6, 4.5, and 4.0 times less at 8 d, 16 d, 24 d, and 32 d since infestation (Table S2). The abundance of *B. brassicae* increased over time and this was especially true for central plants that harbored on average 42 ± 20 *B. brassicae* at 8 d, 127 ± 118 at 16 d, 389 ± 423 at 24 d, and 1,664 ± 1,729 at 32 d (Table S2). At day 8, plants were starting the full bloom period, and at day 32, plants mainly carried maturing siliques.

After hatching from eggs that had been deposited on leaves, *P. brassicae* caterpillars reached the inflorescences between day 8 and day 16. At day 16 and day 24, almost exclusively plants that were initially exposed to single attack with *P. brassicae*, and dual attack with *P. brassicae* plus *B. brassicae* or Xcr, carried *P. brassicae* caterpillars (Fig. 3). No *P. brassicae* were found anymore at day 32. Among caterpillar-infested plants, the abundance of *P. brassicae* was similar on plants across treatments, both among central and among side plants at day 16 and day 24 (Fig. 3). The abundance of *P. brassicae* decreased by 14 times on central plants between day 16 and day 24 (Table S3, $P < 0.001$), and abundance remained low and on average below one caterpillar per side plants at both days. Abundance was 11 times lower on side plants than on central plants at day 16, while abundance on central and side plants of plots did not differ anymore at day 24 (Table S3, $P < 0.001$). At day 16, caterpillars

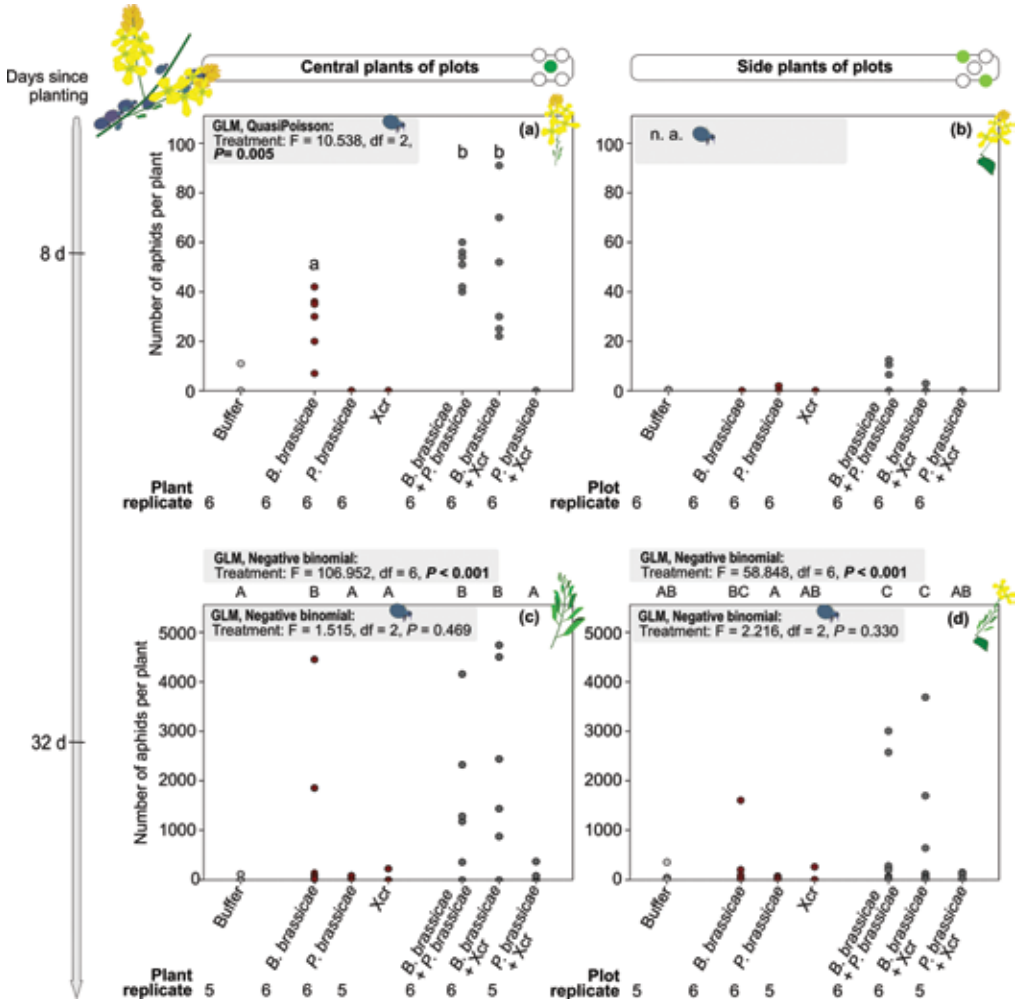


Fig. 2. Abundance of *Brevicoryne brassicae* aphids on inflorescences of central and side *Brassica nigra* plants of plots of which the central plant had been experimentally exposed to single attack, dual attack, or buffer for 8 or 32 days. Panels show the number of *B. brassicae* aphids recorded on inflorescences of *B. nigra* plants after 8 days (a, b) or 32 days (c, d) of exposure to an initial introduction of *B. brassicae* aphids, eggs of *Pieris brassicae*, or *Xanthomonas campestris* pv. raphani (Xcr) bacteria, by two of those attackers, or exposed to buffer (control). Only central plants of plots initially received the treatments. The number of *B. brassicae* were recorded for central (a, c) and side (b, d) plants. Two side plants per plot were randomly assessed and we averaged *B. brassicae* abundance over the two side plants. We had five to six plots per treatment. Effect of treatment on *B. brassicae* abundance was tested with a Generalized Linear Model (GLM) for the three treatments involving this attacker: single attack with *B. brassicae,* dual attack with B. brassicae plus *P. brassicae* and with *B. brassicae* plus Xcr; statistical results are indicated in the grey box within the graph frame. When possible, we also tested the effect of all seven treatments with a GLM, and test results are indicated in the grey frame above the graphs. Letters indicate differences between treatments when testing pairwise combinations with a sequential Bonferroni P-value adjustment. At day 8, plants were starting the full bloom period, and at day 32, plants were mainly carrying maturing siliques.

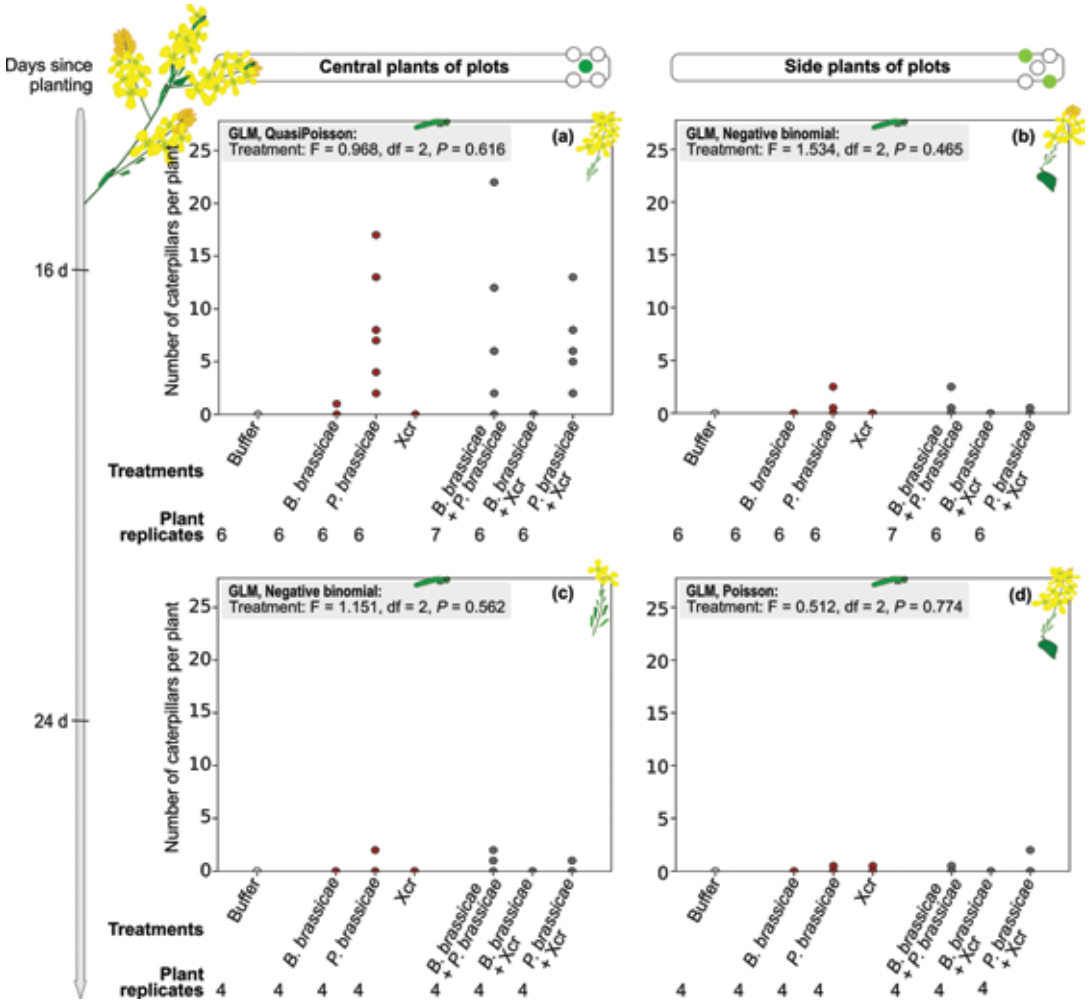


Fig. 3. Abundance of *Pieris brassicae* caterpillars on inflorescences of central and side *Brassica nigra* plants of plots of which the central plant had been experimentally exposed to single attack, dual attack, or buffer for 16 or 24 days. Panels show the number of *P. brassicae* caterpillars recorded on inflorescences of *B. nigra* plants after 16 days (a, b) or 24 days (c, d) of exposure to an initial introduction of *B. brassicae* aphids, eggs of *Pieris brassicae*, or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, by two of those attackers, or exposed to buffer (control). Only central plants of plots initially received the treatments. The number of *P. brassicae* was recorded on central (a, c) and side (b, d) plants. Two side plants per plot were randomly assessed for *P. brassicae* and we averaged *P. brassicae* abundance over the two side plants. We had four to seven plots per treatment. Effect of treatment on *P. brassicae* abundance was tested with a Generalized Linear Model for the three treatments involving this attacker: single attack with *P. brassicae*, dual attack with *P. brassicae* plus *B. brassicae* and *P. brassicae* plus Xcr; test results are indicated in the grey box within the graph frame. At day 16, plants were in full bloom period, and at day 24, plants were stopping bud production and had mainly open flowers and maturing siliques.

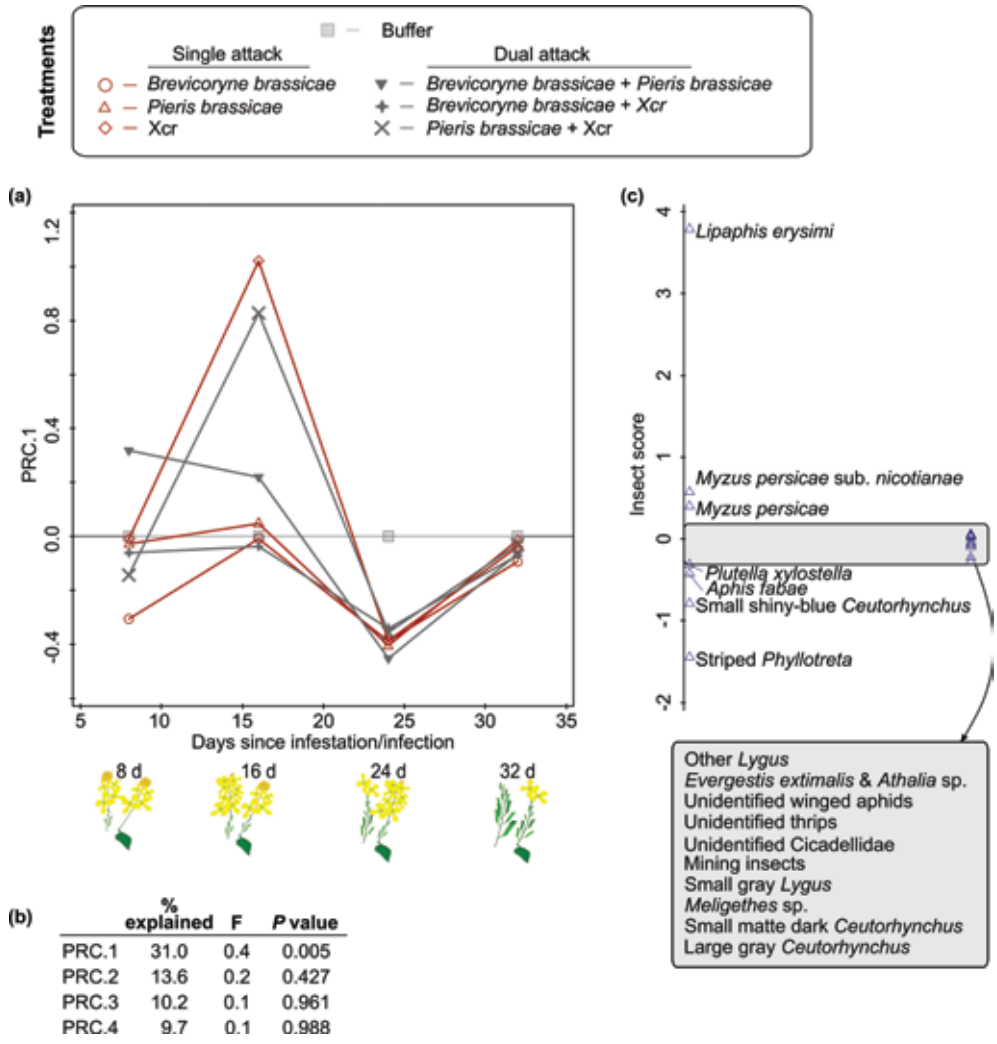
were moulting from the second to the third larval stage, and at day 24, from the fourth to the fifth stage, which is the last larval stage before pupation.

Community composition of florivorous insects on inflorescences of *B. nigra* plants exposed to single or dual attack and on control plants

We visualized the time-dependent effects of plant response to attack on the composition of the florivorous community colonizing the inflorescences of *B nigra* with a PRC-CCA (Fig. 4, 6% of the total variation). The first axis (PRC-1) shows the most important effect, which represents 30% of the total variation explained by the time-dependent effects of treatments (Fig. 4a, b). PRC-1 graphically expresses the time-dependent effect of treatment as differences to the control treatment (Buffer), and the effects were largest on the specialist aphid *L. erysimi* (Fig. 4a, c). *Lipaphis erysimi* had relatively higher abundance on plants exposed to single attack by Xcr and to dual attack by *P. brassicae* plus Xcr treatments at 16 d compared to other treatments. To a lower extent, *Lipaphis erysimi* had relatively higher abundance on plants exposed to dual attack by *B*. *brassicae* plus *P. brassicae* treatments at 8 days compared to other treatments. However, *L. erysimi* had overal low occurrence (Table 1) and were present on more than 5 % of the plants at 16 d only. Its contribution to the separation of the treatments should thus be considered with care. The relative abundance of six other insect species contributed to the differentiation between community composition of experimentally treated plants (Fig. 4c, insect scores ≥ |0.3|): the generalist aphids *M. persicae*, *M. persicae* sub. *nicotianae*, and *A. fabae*, the specialist striped *Phyllotreta* beetles and small shiny-blue *Ceutorhynchus* beetles, and the specialist lepidopteran *P. xylostella*. At the early time points (8 d and 16 d), insect communities were more heterogeneous across treatments than at later time points (24 d and 32 d).

Analysis of the effect of plant exposure to attack on the composition of the florivorous community per time point showed differences at 8 d and 16 d, but not at later days (Fig. S3a, b). Note that the differences are significant without correction for multiple testing (P = 0.037 and 0.014, respectively), but not with correction, because the Bonferroni-threshold is 0.05/4 = 0.0125.

The percentage of variation explained by treatments was small when compared to the variation explained by other field-design variables such as block, plant location within the plot, plot location in the field (row and column), and planting day (Fig S3c, d).



(b)

	% explained	F	*P* value
PRC.1	31.0	0.4	0.005
PRC.2	13.6	0.2	0.427
PRC.3	10.2	0.1	0.961
PRC.4	9.7	0.1	0.988

Fig. 4. Principal response curve (PRC) analysis showing variation over time and between treatments in the composition of the herbivore community colonizing inflorescences of *Brassica nigra* plants initially exposed to single attack, dual attack, or buffer. We recorded in a field experiment all herbivorous insects colonizing reproductive parts of *B. nigra* plants exposed to an initial introduction of *Brevicoryne brassicae* aphids, eggs of *Pieris brassicae,* or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, by two of those attackers, or exposed to buffer (control). The PRC analysis is based on insect species that occurred on at least 5% of the plants for at least one time point; thus we selected 18 insect species/groups over 456 plants across the four time points (8, 16, 24 and 32 d since treatment). Recordings of *B. brassicae* and *P. brassicae* were excluded. Planting day, block, column and row number in the field, and plant position within the plot were included as covariates. The initial treatment was set as the explanatory variable, and it accounted for 7.17% of the partial variation in a PRC-CCA. Panel (a) shows the projection over time according to the first PRC axis. Panel (b) summarizes the contribution of the first four PRC axes in explaining the variation, the fitted variation in the PRC analysis, and their significance. The insect scores (c) show the contribution of each insect species to the separation between treatments. Only scores of insects that contributed most to the separation are displayed, and the grey box presents insect species with scores near zero sorted by descending order.

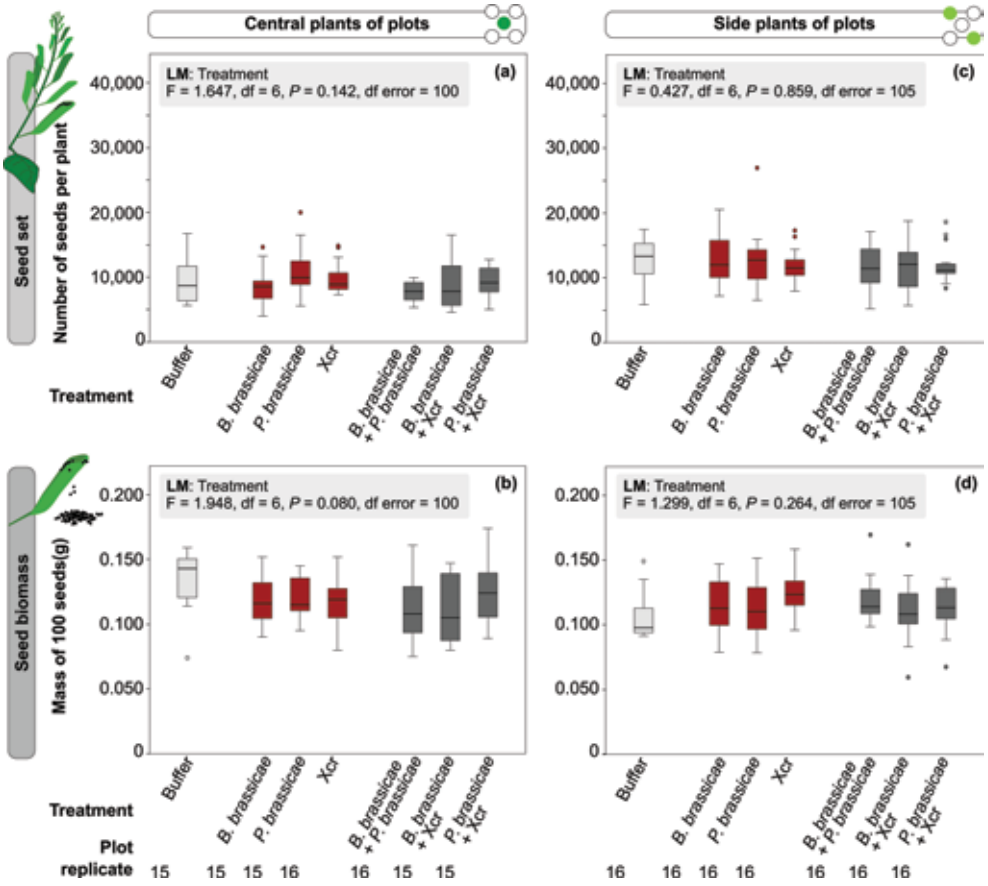


Fig. 5. Number and biomass of seeds (median, interquartile range, full range) produced by *Brassica nigra* plants exposed to single attack, dual attack or buffer. Seed set (a, b) and biomass of 100 seeds (c, d) of *B. nigra* were measured 42 days after plants had been exposed to an initial introduction of *B. brassicae* aphids, eggs of *Pieris brassicae,* or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, by two of those attackers, or exposed to buffer (control). Only central plants of each plot initially received the treatments. Assessments were made for central plants (a, c) and for side plants (b, d). Two side plants per plot were harvested and we averaged number of seeds and biomass of 100 seeds over the two side plants. There were 15 to 16 plots per treatment. Effect of treatment was tested with a Linear Model (LM - ANOVA), and test results are indicated in the grey frame. Outliers are represented by " ° " (further than 1.5 x interquartile).

Seed set, seed biomass and fresh biomass of control *B. nigra* plants and *B. nigra* initially exposed to single or dual attack

Brassica nigra plants exposed to an initial attack by *B. brassicae* aphids, *P. brassicae* caterpillars, or Xcr bacteria produced similar numbers of seeds, and seeds of similar biomass, as plants of plots exposed to combinations of these attackers or to

buffer (control) (Fig. 5). Plant position within the plot affected seed production, and central plants produced 25% fewer seeds than side plants (Table S4, $P < 0.001$). In terms of seed biomass, central plants exposed to buffer (control) produced seeds that were 1.26 times heavier than side plants of the same plot (Table S4, $P = 0.010$), whereas there was no difference in the biomass of seeds produced by central plants and side plants of plots exposed to attackers (Table S4). No correlation was found between the abundance of the attackers *B. brassicae* and *P. brassicae* at days 8, 16, 24, and 32, and the number of seeds and mass of seeds produced per plant at the end of the experiment (Table S5). Reproductive parts of plants exposed to an initial attack by *B. brassicae* aphids, *P. brassicae* caterpillars, or Xcr bacteria had similar fresh biomass as plants exposed to dual combinations of those attackers or to buffer (control) (Fig. S4).

Discussion

Our study shows that responses of plants in the flowering stage to single or dual combinations of specialist attackers that mostly attacked inflorescences of *B. nigra* only transiently influenced the florivore community developing on the inflorescence. Most differences in community composition between treatments were explained by the abundance of the specialist aphid *B. brassicae*, which was introduced as initial attacker. Yet, *Brevicoryne brassicae* were more abundant on plants that were exposed to dual attack by *B. brassicae* plus *P. brassicae* caterpillars or plus Xcr bacteria than on plants exposed to single attack with *B. brassicae*. Differences in the composition of the florivorous community subsequently colonizing inflorescences of *B. nigra* were explained by changes in the abundance of the specialist aphid *L. erysimi*, colonizing plants in the first two weeks following the initial treatment. Plants exposed to Xcr or to *P. brassicae* plus Xcr harbored more *L. erysimi* than plants exposed to single attack by *P. brassicae* or *B. brassicae*, to dual attack by *B. brassicae* plus *P. brassicae* or by *B. brassicae* plus Xcr, or exposed to buffer (control). However, the magnitude of this effect was statistically minor compared to the effect of external factors, such as field-design variable, on the variation measured in the composition of the florivorous community. At the end of the flowering period, the community of florivores that colonized *B. nigra* was similar across treatments, and only plants that were initially exposed to single or dual attack with *B. brassicae* aphids differed from plants that were not exposed to the aphid by carrying higher abundance of *B. brassicae*. Nevertheless, exposure to initial attack did not affect the number of seed and the biomass per seed produced by *B. nigra* compared to control plants. The present study highlights that understanding fitness effects of plant responses to attack in the natural context requires a dynamic approach.

The present study shows that the insect community associated to inflorescences can be differently affected by plant responses to single and dual attack on their inflorescences by *P. brassicae* caterpillars, *B. brassicae* aphids, or Xcr bacteria. Such changes in the inflorescence-associated community composition upon attack may be mediated by changes in plant metabolic content. Among a diverse florivorous community consisting of at least 27 insects from at least six insect orders, plant response to attack affected the abundance of two specialist aphid species. Both *B. brassicae* and *L. erysimi* are phloem-sucking species specialized on plants in the Brassicaceae family, and mainly occurred on stems near flowers and buds where they consume nutrients flowing to the flowers. In our study, the abundance of *B. brassicae* aphids was higher on plants exposed to dual attack by *B. brassicae* plus *P. brassicae* or plus Xcr than on plants exposed to single attack by *B. brassicae. Brevicoryne brassicae* aphids seem to benefit from the dual treatment, and this matches with previous data from greenhouse experiments (Chrétien *et al.*, 2018). Additionally, *L. erysimi* were more abundant on plants exposed to Xcr or to dual attack by *P. brassicae* plus Xcr than on plants exposed to single attack by *P. brassicae* or *B. brassicae*, to dual attack by *B. brassicae* plus *P. brassicae* or by *B. brassicae* plus Xcr, or exposed to buffer (control). Induced direct resistance is generally associated with glucosinolates in Brassicaceae (Fahey *et al.*, 2001; Textor & Gershenzon, 2009). However, the performance of specialist aphids is rarely impaired by an increase in glucosinolates, and *B. brassicae* can even benefit from an increase in aliphatic glucosinolates (Kos *et al.*, 2012; Woodard *et al.*, 2012). In contrast, aphids are more sensitive to plant nutritional quality based on primary metabolites (Cole, 1997). Data from our previous study showed that exposure of *B. nigra* plants to different attackers differentially affected the ratio of soluble sugar/amino-acid in inflorescences, but we did not detect induction of the production of glucosinolates (Chapter 4). Thus, our results suggest that changes in primary metabolisms, which likely support plant tolerance to attack, could as well shape the flower-associated insect community by affecting abundances of florivorous aphids (Denno *et al.*, 2000; Utsumi & Ohgushi, 2008; Ohgushi, 2016).

Infestation of inflorescences by *B. brassicae* aphids at the beginning of the flowering period seems to be important in determining the future abundance of *B. brassicae* on the reproductive parts, whereas *P. brassicae* caterpillar performed poorly on the plants. Almost all *P. brassicae* were eliminated from the inflorescences before the caterpillars pupated, likely *via* indirect resistance. Survival rate of *P. brassicae* caterpillars was close to zero in a previous study (Chapter 3), and predation and parasitisation can largely contribute to decreasing abundance of *P. brassicae*

caterpillars on *B. nigra* (Lucas-Barbosa *et al.*, 2013; Lucas-Barbosa *et al.*, 2014; Lucas-Barbosa *et al.*, 2017). In contrast, *B. brassicae* aphids built up large colonies reaching over a few thousands of individuals on plants that were initially treated with single or dual attack with *B. brassicae*, whereas colonies did not go over 500 individuals on plants that did not receive the initial treatment with *B. brassicae*. Parasitoids of *B. brassicae* aphids, mainly *Diaeretiella rapae* in the Netherlands (Hafez, 1961), prefer smaller densities of aphids over large colonies (Ponzio *et al.*, 2016a; Cascone *et al.*, 2019). This preference may contribute to the increased abundance of *B. brassicae* on dually-attacked plants compared to plants exposed to *B. brassicae* only. Despite the high abundance of *B. brassicae* on inflorescences that had received the aphid as an initial attacker, *B. nigra* reproductive success was not affected by aphid treatments in terms of seed set and seed biomass. This suggests that *B. brassicae* may not be a critical threat for *B. nigra*, as it does not impact the plant's seed production. In contrast, *B. nigra* effectively resists *P. brassicae* caterpillars, which are more damaging than aphids because they directly remove large amounts of flowers and buds (Smallegange *et al.*, 2008).

The response of the florivore community to the initial treatment was time dependent and was strongest in the first two weeks following treatment. Towards the end of the experiments, florivore communities converged across treatments and all plants initially infested with *B. brassicae* aphids or *B. brassicae* aphids plus another attacker carried similar numbers of *B. brassicae.* This short-term effect was similar to results on *Solanum dulcamare* (Viswanathan *et al.*, 2005), although long-lasting effects have been observed on *B. oleracea* (Stam *et al.*, 2018). Temporal dynamics of the community response to initial attack on inflorescences can be mediated by plant traits or can be linked to natural dynamics in florivore occurrence in nature. There is a confounding effect of plant ontogeny and the course of induction processes explaining temporal patterns of plant responses to attack. Induced plant responses to herbivory can take place within a few hours or days following attack (Stam *et al.*, 2018). Additionally, plant ontogeny may influence plant response to attack. As the inflorescence ages, floral parts of plants may become less responsive (Quintero *et al.*, 2014; Quintero & Bowers, 2018). Thus, the effect of treatment on plant traits may be strongest in the days following attack on plants that are still fully blooming. Temporal dynamics of herbivore abundance may also play a role in the time-dependant effect of treatments. The majority of insects found in temperate zones have seasonal peaks with transient maximum abundance and occurrence (Wolda, 1988). Overall, a larger proportion of transient species were contributing to the differences in community between treatments than core species did. Insects

forming the core community in our experiment were present on more than 5 % of the plants throughout the season and may be more adapted to variation in host-plant traits than transient species. Transient species may be more sensitive to attacker-induced changes in plants and their cascading effect than more common species.

The effect of early attack on florivorous colonizers had relatively minor magnitude compared to external factors such as recording day or plant location within the plot. Previous studies showed that most variation in the composition of the insect communities in response to initial attack was correlated with the attraction of specialists and repellence of generalists (Poelman *et al.*, 2008a). The florivorous community on inflorescences of *B. nigra* consisted of 80 % of specialists and 20 % of generalist feeders in terms of occurrence; specialists also accounted for about 90 % of the total abundance when looking at control plants. This dominant abundance of specialists may minimize contrasting effects that induced responses usually have on insects colonizing the plants. Furthermore, the observation that especially transient species contributed to the variation in insect communities after treatment may limit the negative fitness impact of differences in community composition. Indeed, even if transient species reached high abundances on some treatments at an early time point, plants apparently compensated for it by the time seeds were harvested, 41 days after the initial attack. Contrary to plant exposure to attack, plant-plant competion had a strong effect on the seed set produced. Central plants of plots experienced competition from 4 neighbors and produced fewer seeds than the surrounding side plants. Interestingly, seeds produced by unattacked central plants had higher biomass than seeds produced by unattacked side plants (control plots), which indicates that *B. nigra* can compensate the production of a smaller seed set with an increase in seed biomass. However, this compensation was lost upon attack and central plants of attacked plots produced as heavy seeds as side plants surrounding the central plant. Therefore, competition may alter plant ability to tolerate attack to their inflorescences (Gãmez & Fuentes, 2001).

Our study shows that, although occurrence and abundance of florivores colonizing inflorescences were affected by the specificity of plant response to a given attacker, and to combination of attackers, the magnitude of variation in florivore communities that could be statistically attributed to treatment was minor. Annual plants such as *B. nigra* probably have accumulated enough resources in the vegetative stage to support high constitutive defenses in inflorescences. For instance, glucosinolate levels in *B. nigra* flowers are up to five times higher than in its leaves (Smallegange *et*

al., 2007; Chapter 4). As a consequence, flowers of *B. nigra* mainly face florivory by specialist feeders that are adapted to such high levels of defense and that may only respond to changes in plant primary metabolites. In a life-long community context, the history of damage in the vegetative stage may lead to stronger consequences for plant fitness than damage to inflorescences only. Recent work has indeed shown a legacy effect from the vegetative stage to the flowering stage in perennial wild cabbage plants (Stam *et al.*, 2018). The present results highlight the importance of taking plant ontogeny and community context into account when understanding fitness consequences of plant responses to attack.

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Supplemental information

Models parameters for univariate analyses in SPSS

We used respectively the GENMIX, GENLIN, and MIXED procedures of SPSS for GLMMs, GLMs, and L(M)Ms. In GLMMs, the fixed-effect and random-effect parameters estimation method was the pseudo-quasilikelihood (PQL) and GLMs were based on the fisher method with a scale of 1. For both models, the estimation of the standard errors of the estimates and of the coefficients was based on the Huber-White method (robust method), which can handle violation of model assumption (SPSS user guide). Only for the analysis of species richness, we used the model-based estimation method because the robust method did not result in reliable estimates. Degrees of freedom (df) were estimated with the residual method in GLMMs. We used an ascending order for both categorical targets and predictors. In the L(M) Ms, we used the Restricted Maximum Likelihood (REML) as an estimation method. The confidence interval of the estimates was calculated with the Wald method, and the estimates for the fixed factors were tested with a Wald F test in GLMMs, LMMs and LMs and Wald chi-square in GLMs. The estimates for the random factors were tested with a Wald Z test.

Simplification of non-converging GLMM and LMMs in SPSS

GLMMs and LMMs with all random factors would generally not converge and/or the Hessian matrix was not positive definite or was null, which is commonly means that there is no variation in the data for the considered effect (Bolker *et al.*, 2009). Thus, we tested correlation between central plants and side plants using the R-squared (Plot identity), and we graphically verified that Block and Planting Day were not affecting the parameters measured.

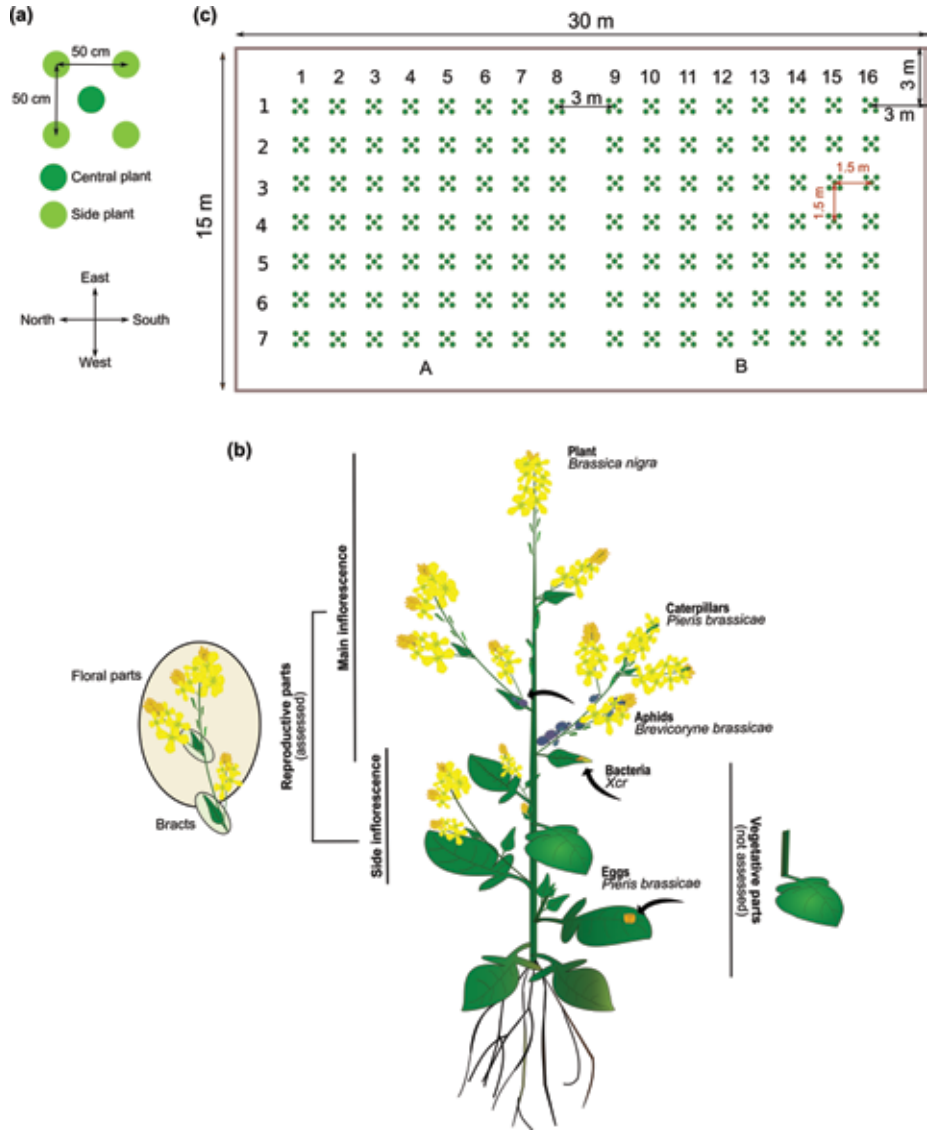


Figure S1. Schematic representation of the common garden experimental layout and description of the parts of *Brassica nigra* plants that were assessed for herbivorous insects. The compass indicates the orientation of the plots and the field. **(a)** A plot (50 cm x 50 cm) consisted of one central plant (dark green) and four side plants (light green). **(b)** *B. nigra* plants were infested with either 5 *Brevicoryne brassicae* aphids or 30 eggs of *Pieris brassicae* or infected with *Xanthomonas campestris* pv. *raphani* bacteria (Xcr). Dual attack consisted of combinations of two of these treatments simultaneously. Caterpillars hatched from eggs about 8 to 9 days after infestation and migrated to the inflorescence within a few days. We distinguished vegetative parts and reproductive parts of plants, and only reproductive parts were assessed for herbivorous insects. In the reproductive parts we included the main inflorescence and side inflorescences; side inflorescences were growing from axillary buds at the base of foliar petioles. We recorded whether insects fed from bracts, or from floral parts (flowers, buds, stalk, and siliques). **(c)** The field consisted of two blocks, A and B, each composed of 56 plots organized in 7 rows and 8 columns. Blocks were 3 m apart, and within a block, central plants of each plot were 1.5 m apart. A fence (brown line) was placed around the field, 3 m away from the plots.

Table S1. (a) Output of the Generalized Linear Mixed Model testing the effect of Time point (days 8, 16, 24 and 32), Plant location within plot (Central and Side), and their interaction, on the total abundance of herbivorous insects on inflorescences of *Brassica nigra* plants exposed to Buffer (control); **(b)** Output of the Generalized Linear Model testing the effect of Time point (days 8, 16, 24 and 32), Plant location within plot (Central and Side), and their interaction, on the species richness in herbivore insects on inflorescences of *B. nigra* plants exposed to Buffer.

(a) Total abundance

Fixed effects	Factor levels	F	df1	df2	*P*
Time point	4	17.233	3	34	< 0.001
Plant location	2	0.670	1	34	0.419
Time point * Plant location	4*2	7.255	3	34	0.001
Corrected model		33.611	7	34	< 0.001

Negative binomial distribution

Negative binomial coefficient: 0.877

N = 42 plants

Residual effect	Variance estimate	Std. error	Z	*P*	95% confidence interval
Diagonal	2.871	0.997	2.880	0.004	1.454 < > 5.670
Rho	0.958	0.020	47.154	< 0.001	0.893 < > 0.984

Autoregressive 1 structure

Subject specification: Plant identity

(b) Species richness

Fixed effects	Factor levels	Wald chi-square	df	*P*
Time point	4	7.105	3	0.069
Plant location	2	4.836	1	0.028
Time point * Plant location	4*2	4.280	3	0.233
Intercept		571.931	1	< 0.001

quasi-Poisson distribution

Pearson chi-square / df = 0.452

N = 42 plants

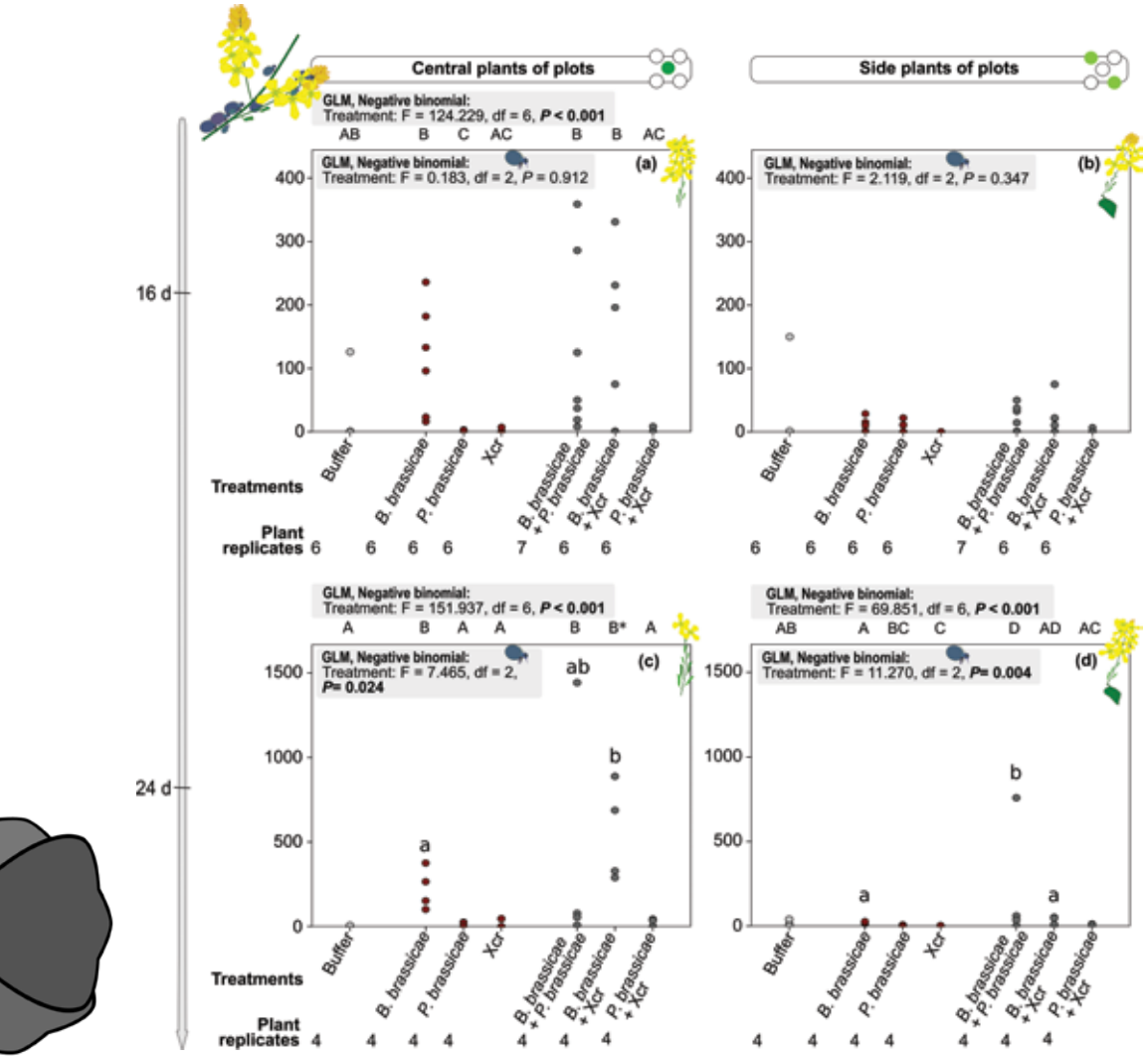


Figure S2. Abundance of *Brevicoryne brassicae* aphids on inflorescences of central and side *Brassica nigra* plants of plots of which the central plant had been experimentally exposed to single attack, dual attack, or buffer for 16 and 24 days. Panels show the number of *B. brassicae* aphids recorded on inflorescences of *B. nigra* plants after 16 days (a, b) or 24 days (c, d) of exposure to an initial introduction of *B. brassicae* aphids, eggs of *Pieris brassicae,* and/or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, by two of those attackers, or exposed to buffer (control). Only central plants of plots initially received the treatments. The number of *B. brassicae* was recorded on central (a, c) and side (b, d) plants. Two side plants per plot were randomly assessed and we averaged *B. brassicae* abundance over the two side plants. We had four to seven plots per treatment. Effect of treatment on *B. brassicae* abundance was tested with a Generalized linear model (GLM) for the three treatments involving this attacker: single attack with *B. brassicae*, dual attack with *B. brassicae* plus *P. brassicae* and *B. brassicae* plus Xcr; tests results are indicated in the grey box within the graph frame. When possible, we also tested the effect of all seven treatments with a GLM, and test results are indicated in the grey frame above the graphs. Letters indicate differences between treatments when testing pairwise combinations with a sequential Bonferroni *P*-value adjustment. At day 16, plants were in full bloom period, and at day 24, plants were stopping bud production and had mainly open flowers and maturing siliques.

Table S2. Output of the Generalized Linear Mixed Model testing the effect of Treatment, Time point (days 8, 16, 24 and 32), Plant location (Centre and Side), and their interaction, on the abundance of the attacker *Brevicoryne brassicae* on reproductive parts of *Brassica nigra* plants exposed to *B. brassicae* only or to *B. brassicae* plus another attacker (*Pieris brassicae* or *Xanthomonas campestris* pv. *raphani*).

Fixed effects	Factor levels	F	df1	df2	*P*
Treatment	3	5.391	2	116	0.006
Time point	4	131.197	3	116	< 0.001
Plant location	2	105.921	1	116	< 0.001
Time point * Plant location	4*2	12.322	3	116	< 0.001
Time point * Treatment	4*3	2.946	6	116	0.010
Treatment * Plant location	3*2	2.929	2	116	0.057
Corrected model		68.085	17	116	< 0.001

Negative binomial distribution

Negative binomial coefficient: 1.754

N=134 plants

Random effect	Variance estimate	Std. error	Z	*P*	95% confidence interval
Random intercept for Plot identity	0.378	0.193	1.961	0.050	0.139 < > 1.027

Scaled identity covariance structure

Subject specification: Plot identity

Residual effect	Variance estimate	Std. error	Z	*P*	95% confidence interval
Day 8	0.453	0.129	3.515	< 0.001	0.259 < > 0.791
Day 16	0.707	0.194	3.647	< 0.001	0.413 < > 1.210
Day 24	0.461	0.161	2.869	0.004	0.233 < > 0.913
Day 32	0.858	0.254	3.382	0.001	0.481 < > 1.532

Diagonal covariance structure

Subject specification: Plot identity*Plant identity

Pairwise comparaisons - based on transformed data

P-values based on the least significant difference adjusted significance to the level of 0.05

Treatments	Overall effect	At day 8	At day 24
B. brassicae vs. B. brassicae plus *P. brassicae*	0.002	≤ 0.001	0.037
B. brassicae vs. B. brassicae plus *Xcr*	0.022	0.012	0.160
B. brassicae plus *P. brassicae vs. B. brassicae* plus *Xcr*	0.399	0.074	0.174

Time points	Overall effect	Central plants	Side plants
Pairwise contrasts between Day 8, 16, 24, and 32	≤ 0.001	≤ 0.001	≤ 0.001

Plant location	Overall effect	At each time point
Central plants *vs.* Side plants	≤ 0.001	≤ 0.001

Table S3. Output of the Generalized Linear Model testing the effect of Treatment, Time point (day 16 and 24), Plant location (Centre and Side), and their interaction, on the abundance of the attacker *Pieris brassicae* on reproductive parts of *Brassica nigra* plants exposed to *P. brassicae* only or to *P. brassicae* plus another attacker (*Brevicoryne brassicae* or *Xanthomonas campestris* pv. *raphani*).

Fixed factors	**Factor levels**	**Wald chi-square**	**df**	***P***
Treatment	3	0.749	2	0.688
Time point	2	17.647	1	< 0.001
Plant location	2	12.986	1	< 0.001
Time point * Plant location	2*2	10.112	1	0.001
Time point * Treatment	2*3	0.095	2	0.954
Treatment * Plant location	3*2	0.099	2	0.952
Intercept		0.032	1	0.858

Negative binomial distribution

Pearson chi-square/df = 0,832

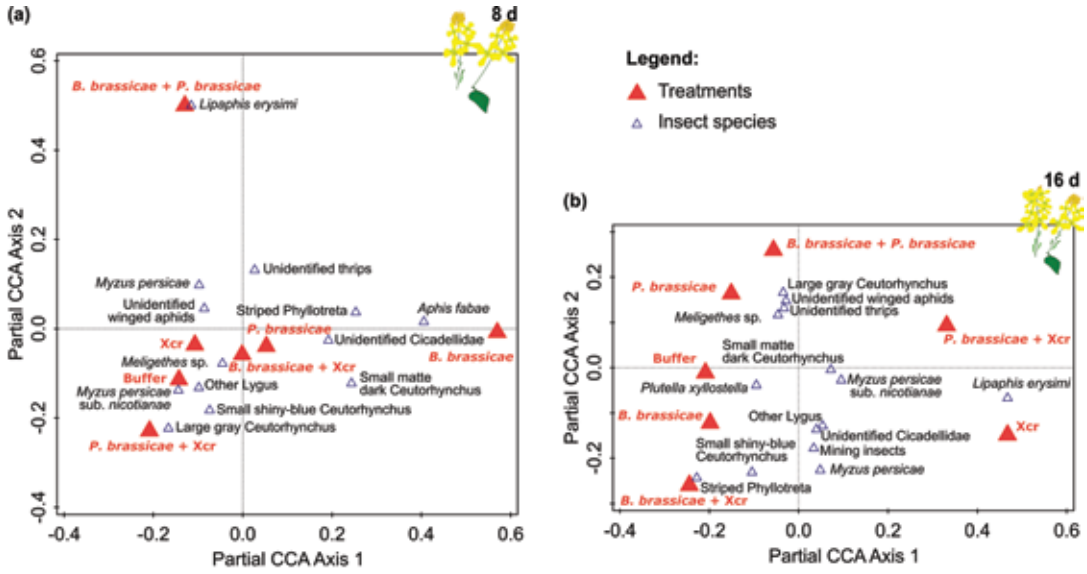
N=62 plants

Pairwise comparaisons - based on transformed data

P-values based on the least significant difference adjusted significance to the level of 0.05

Time points	**Overall effect**	**Central plants**	**Side plants**
Day 16 *vs.* Day 24	≤ 0.001	≤ 0.001	0.487

Plant location	**Overall effect**	**At Day 16**	**At Day 24**
Central plants *vs.* Side plants	≤ 0.001	≤ 0.001	0.787



(c) 8 d

	% explained	pseudo -F	*P* value	adjusted *P* value
Planting day	3.13	4.0	0.001	0.011
B. brassicae	1.94	2.5	0.033	0.182
B. brassicae + P. brassicae	1.70	2.2	0.050	0.183
P. brassicae + Xcr	1.24	1.6	0.121	0.266
Block A	0.97	1.3	0.227	0.401
Block B	0.97	1.3	/	/
Field column	1.18	1.6	0.090	0.248
B. brassicae + Xcr	0.89	1.2	0.255	0.401
Plant location Side	0.78	1.0	0.869	0.915
Plant location Centre	0.78	1.0	/	/
Field row	0.68	0.9	0.484	0.609
Buffer	0.64	0.8	0.498	0.609
P. brassicae	0.32	0.4	0.915	0.915
Xcr	0.32	0.4	/	/

(d) 16 d

	% explained	pseudo -F	*P* value	adjusted *P* value
Xcr	2.14	2.8	0.021	0.072
P. brassicae + Xcr	1.76	2.3	0.026	0.072
Block A	1.78	2.4	0.007	0.044
Block B	1.78	2.4	/	/
Planting day	1.71	2.3	0.008	0.044
Field row	1.16	1.6	0.095	0.209
B. brassicae + Xcr	0.81	1.1	0.328	0.601
B. brassicae	0.69	0.9	0.491	0.751
Buffer	0.64	0.9	0.546	0.751
Plant position Side	0.40	0.5	0.999	0.999
Plant position Centre	0.40	0.5	/	/
P. brassicae	0.38	0.5	0.889	0.999
B. brassicae + P. brassicae	0.38	0.5	/	/
Field column	0.34	0.5	0.917	0.999

Figure S3. Composition of florivorous insect communities on inflorescences of *Brassica nigra* at 8 and 16 days after they had been exposed to single attack, dual attack, or buffer. Analyses were based on the abundance of insect species/groups that occurred on at least 5% of the plants for at least one time point (as for the PRC); thus we had 13 insect species for 126 plants at day 8 and 14 insect species for 129 plants at day 16. Recordings of *Brevicoryne brassicae* and *Pieris brassicae* were excluded. Ordination plots (a, b) show the contribution in a Partial Canonical Correspondence Analysis (pCCA) of each insect species or group (blue-lined triangles) to the differences between treatments at day 8 (a) and day 16 (b) in terms of the insect community colonizing *Brassica nigra* plants exposed to an initial attack by aphids *B. brassicae*, eggs of *Pieris brassicae,* and/or bacteria *Xanthomonas campestris* pv. *raphani* (Xcr), by two of those attackers, or exposed to buffer (control). Plain red triangles indicate the centroid of ordination scores of all plants of a same treatment. Scores are shown for the first and second axis, which respectively explained 2.20% and 1.84% of the total variation at day 8, and 3.41% and 1.35% of the total variation at day 16. Planting day, Block, column and row number in the field, and plant position within the plot were included as covariates. Tables (c, d) summarize the conditional term effects for treatments and covariates after performing a CCA using these factors as explanatory variables for day 8 (c) and day 16 (d). Tables display the percentage of variation explained by the different terms and their significance. *P*-value was adjusted for multiple comparisons using false discovery rate, and adjusted *P*-values below 0.100 indicate significant differences.

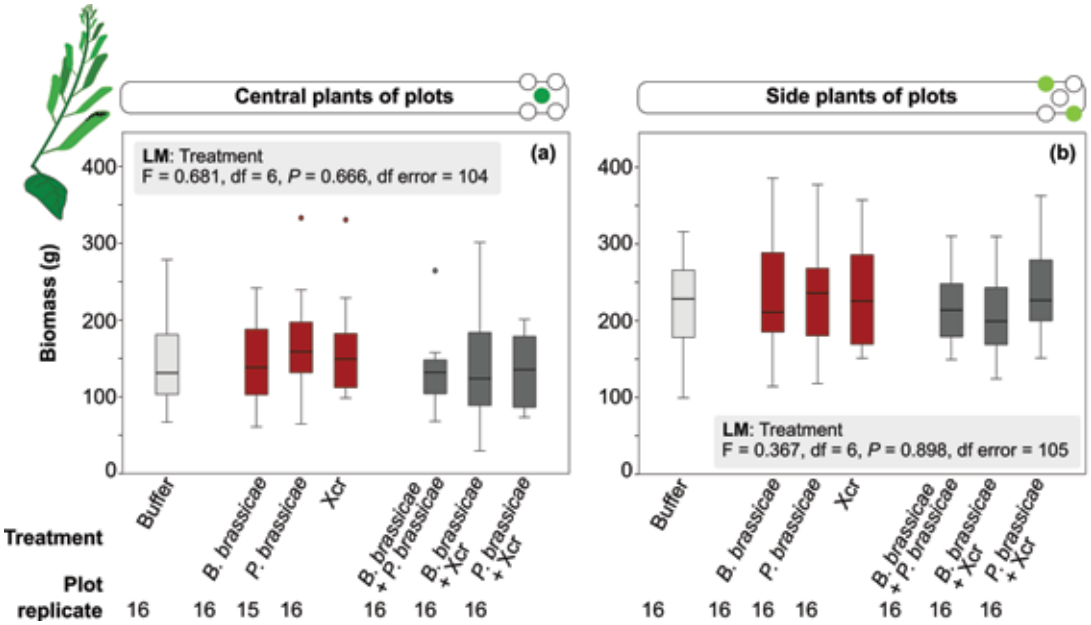


Figure S4. Fresh biomass (median, interquartile range, full range) of reproductive parts of *Brassica nigra* plants exposed to single attack, dual attack or buffer. *Brassica nigra* plants were harvested from the field after 41 days and fresh biomass of reproductive parts was measured for plants of plots initially exposed to *Brevicoryne brassicae* aphids, eggs of *Pieris brassicae,* and/or *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria, by two of those attackers, or exposed to buffer (control). Only central plants of plot initially received the treatments, and we separated biomass of central (a) and side (b) plants. Two side plants per plot were harvested and we averaged fresh biomass over the two side plants. We had 15 to 16 plots per treatment. Effect of treatment on the fresh biomass was tested with a Linear Model (LM - ANOVA), and test results are indicated in the grey frame. Outliers are represented by " ◦ " (further than 1.5 x Interquartile).

Table S4. Output of the Linear Model (LM - ANOVA) testing the effect of Treatment, Plant location (centre and side), and their interaction, on the number of seeds and the biomass of 100 seeds produced by *Brassica nigra* at 41 days after exposure to an initial attack by *Brevicoryne brassicae* aphids, eggs of *Pieris brassicae,* and/or *Xanthomonas campestris* pv. *raphani* bacteria, by two of those attackers, or exposed to buffer (control).

(a) Number of seed per plant

Fixed factors	Factor levels	F	df1	df2	*P*
Treatment	7	1.280	6	205	0.268
Plant location	2	46.181	1	205	< 0.001
Treatment * Plant location	7*2	0.622	6	205	0.713
Intercept		2.52x10^10	1	205	< 0.001

N = 219 plants

(b) Biomass of 100 seeds

Fixed factors	Factor levels	F	df1	df2	*P*
Treatment	7	0.802	6	205	0.570
Plant location	2	3.078	1	205	0.081
Treatment * Plant location	7*2	2.592	6	205	0.019
Intercept		9,843.84	1	205	< 0.001

N = 219 plants

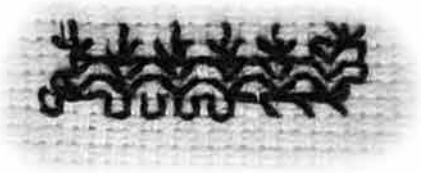
Pairwise comparaisons - based original data	
P-values based on the Bonferroni adjusted significance to the level of 0.05	
Buffer-treated central plants *vs.* Buffer-treated side plants	*P* = 0.010
Other comparisons	non-signififcant

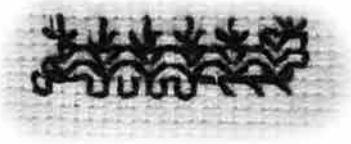
Table S5. Summary of the best fit lines and R-squared (R^2) correlating the number of seeds and biomass of seeds of *Brassica nigra* to the abundance of *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars on inflorescences assessed at days 8, 16, 24 and 32 after the initial infestation/ infection of the plants.

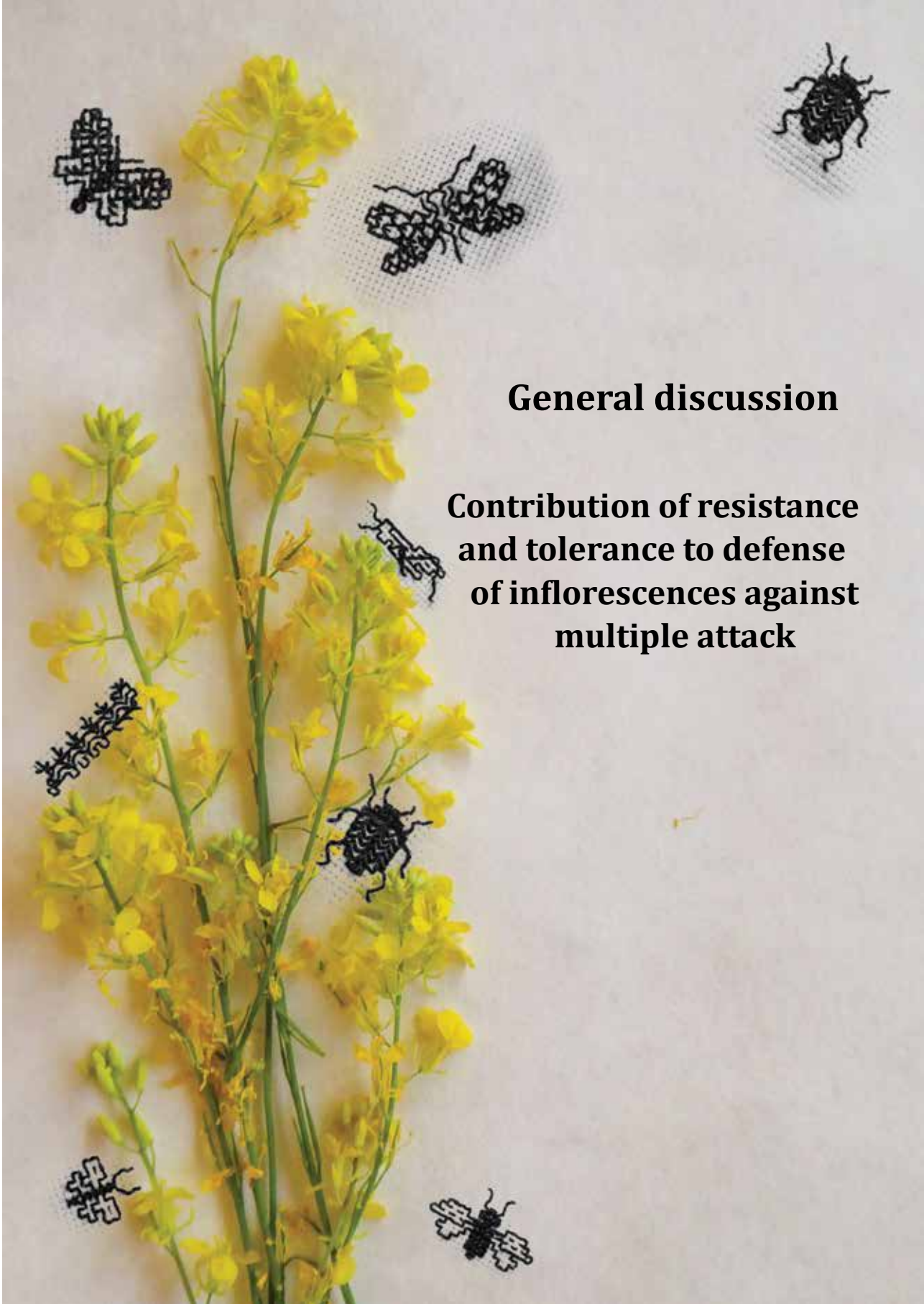
Abundance of *Brevicoryne brassicae* aphids (x)				
	at day 8	**at day 16**	**at day 24**	**at day 32**
Correlation with seed set (y)	y=12,379-63.164x	y=11,452-10.722x	y=12,253-0.881x	y=11,421-0.399x
R^2	0,0486	0,0228	0,0022	0,0073
Correlation with seed biomass (y)	$y=0.1130-6.10^{-5}x$	$y=0.1187-2.10^{-6}x$	$y=0.1114-1.10^{-6}x$	$y=0.1182-3.10^{-6}x$
R^2	0,0014	0,0036	9.10^{-5}	0,0093
Number of plants	124	127	83	115

Abundance of *Pieris brassicae* caterpillars (x)		
	at day 16	**at day 24**
Correlation with seed set (y)	y=11,198-38.5x	y=12,158-236.7x
R^2	0,0006	0,0009
Correlation with seed biomass (y)	y=0.1179-0.0002x	y=0.1113+0.0015x
R^2	0,0005	0,0010
Number of plants	127	80

Chapter 6







General discussion

Contribution of resistance and tolerance to defense of inflorescences against multiple attack

Introduction: flowers and florivores

Flowers are shoots that bear reproductive structures made of fertile and sterile appendages. They are typical of gymnosperms (reproductive cones or strobili) and angiosperms, and lead to the formation of seeds that represent the next generation (spermatophytes) (Frame, 2003; Theissen & Melzer, 2007). **Flowers of angiosperms** are a combination of key innovations that likely contributed to the evolutionary and ecological success of this group of plants: angiosperms are currently acknowledged as the most diverse group of land plants, with around 300,000 species representing about 80 % of green plants (Theissen & Melzer, 2007; Specht & Bartlett, 2009). A major characteristic, which gave angiosperms their name (*angio-* enclosure), is the protection of ovules in an ovary (**carpel**) that will form the **fruit** (Theissen & Melzer, 2007), which conferred plants with a variety of seed dispersal opportunities. The embryo (zygote) is additionally provided with resources by storage tissue, the endosperm, that results from a double fertilization (Theissen & Melzer, 2007). Flowers of angiosperms are generally hermaphrodites and display a perianth that often includes organs of petaloid appearance (Theissen & Melzer, 2007). This display particularly attracts and sustains a great diversity of biotic interactions, including interactions with pollinators that mediate reproduction of about 87 % of all angiosperms (Frame, 2003; Ollerton *et al.*, 2011).

Contrary to gymnosperms, angiosperm plants and flowers are particularly edible (Frame, 2003). For over 135 million years, the evolution of angiosperms has been intertwined with that of insects, most of them being plant feeders (Frame, 2003). Angiosperms are generally associated with an incredible diversity of interactions between species as well as within species (Ehrlich & Raven, 1964; Janz, 2011). A plant in the flowering stage is generally associated with more abundant and more speciose communities of arthropods, including pollinators as well as herbivores, when compared to the vegetative stage (Johnson & Agrawal, 2005; Johnson & Agrawal, 2007; Abdala-Roberts *et al.*, 2017). Herbivores that consume bracts, buds, and flowers, including pollen and ovules until the seed coat is formed, are called florivores (McCall & Irwin, 2006). Florivory as well as pathogen infection of flowers can directly affect reproductive success of plants by removal of flowers, or indirectly by altering the reproductive function of flowers (Louda & Potvin, 1995; Lohman *et al.*, 1996; Krupnick *et al.*, 1999; McCall & Irwin, 2006; Smallegange *et al.*, 2008; Zangerl & Berenbaum, 2009). Therefore, florivores and phytopathogens that infect inflorescences are predicted to have the most drastic impact on plant fitness compared with other types of herbivores (Wise & Rausher, 2013; Schlinkert *et al.*, 2015).

Flowers protect plant gametes and due to this high fitness value, flowers are predicted to be the most defended organs within a plant in the flowering stage (Herms & Mattson, 1992; Stamp, 2003; McCall & Irwin, 2006). To defend themselves, plants can either target the attackers and counteract them, and/or tolerate them (Agrawal, 2011). Several non-exclusive strategies have evolved. A first line of protection consists of morphological structures or chemicals that are constitutively present in plants, and refrain attackers from eating or settling on the plant (Mithöfer & Boland, 2012; Kessler, 2015). Against attackers that pass this first barrier, plants can specifically respond by directly affecting the performance and survival of the attackers, or indirectly by recruiting natural enemies of the herbivores (Textor & Gershenzon, 2009; Dicke & Baldwin, 2010; Karban, 2011; Mithöfer & Boland, 2012; Dicke & van Loon, 2014). Plants can also limit the fitness impact of attack by tolerance strategies, and rely on compensatory mechanisms such as regrowth of damaged tissues (Strauss & Agrawal, 1999; Núñez-Farfán *et al.*, 2007). Tolerance and resistance can provide effective defense to the plant, but they also come at a cost. Defense can for example trade off with reproduction when defense and reproduction directly compete for resources, or when defense interferes with the biotic community associated to the plant (Herms & Mattson, 1992; Agrawal *et al.*, 1999; Strauss *et al.*, 1999; Strauss *et al.*, 2002; Agrawal, 2011; Orians *et al.*, 2011; Dicke & van Loon, 2014). Therefore, plants in the flowering stage face this challenge of fine-tuning their investments in defense and reproduction, especially when attacked by florivores (McCall & Irwin, 2006; Lucas-Barbosa *et al.*, 2011; Schiestl *et al.*, 2014; Lucas-Barbosa, 2016). Scientists are starting to consider the importance of integrating responses of plants in the flowering stage when studying plant defenses, since flower feeders may strongly challenge plant defensive mechanisms and interfere with reproduction.

In this project, I explored how plants in the flowering stage fine-tune defense and reproduction mechanisms when inflorescences are exposed to multiple biotic stresses. I aimed at identifying physiological and chemical changes of plants in the flowering stage that contribute to plant resistance and tolerance strategies upon multiple attack to inflorescences. I additionally aimed at identifying the consequences of plant defenses against floral attackers for the attraction of pollinators, as well as for plant fitness parameters. To address those objectives, I used the annual outcrossing plant *Brassica nigra* (Brassicales: Brassicaceae). When the plants started flowering, I exposed inflorescences to combinations of attackers that are specialized on plants in the Brassicales: florivorous aphids (*Brevicoryne brassicae*) and caterpillars (*Pieris brassicae*), and a phytopathogenic bacterium (*Xanthomonas campestris* pv.

raphani - Xcr). The three attackers were selected for their different mode of attack, and for the distinct phytohormonal pathways they can induce in plants. I measured changes in phytohormones, primary metabolites, and secondary metabolites (glucosinolates, volatiles) in leaves and inflorescences, when inflorescences of *B. nigra* were exposed to the attackers. Through a combination of greenhouse and field experiments, I linked these chemical changes measured to the plant's interactions with mutualists (pollinators, natural enemies of herbivorous insects), antagonists (florivores), and to plant fitness parameters. *Brassica nigra* effectively defended against floral attackers. Results suggest that flowering *B. nigra* protect its inflorescences with constitutive resistance, which likely limits floral colonization by most attackers (Chapter 4). When inflorescences were attacked by single or dual combinations of the specialist feeders, plants responded with an induction of phytohormones in their inflorescence whereas no induction was measured in leaves (Chapter 2). The results presented in this thesis suggest that *B. nigra* plants effectively defend their inflorescences against single and dual attack by three types of specialist attackers, and dealt with attackers *via* induced resistance and tolerance mechanisms (Chapter 2 and 4). When regarding inducible direct resistance, induction of glucosinolates was observed in leaves, but not in flowers (Chapter 4). Despite that no induction of glucosinolates was measured in inflorescences, caterpillars performed worse upon dual attack than upon single attack, and this could be mediate by other defensive compounds. Compared to phloem-sucking aphids and a phytopathogen, the chewing caterpillars were the main driver of responses in flowering *B. nigra* and inflorescences effectively defended themselves against them. In contrast, aphid infestation appeared to be facilitated by the plant's tolerance response to dual attack (Chapter 2, 3, and 5). *Brassica nigra* maintained interactions with pollinators upon attack of inflorescences. Responses of *B. nigra* to attack did not interfere with the number of pollinators visiting the plant, although the composition of the pollinator community may change upon attack (Chapter 3). Moreover, attacked plants produced similar numbers of seeds as unattacked plants (Chapter 3 and 5). In this final chapter of my thesis, I summarize how this project advances our understanding of the relative contribution of resistance and tolerance mechanisms to the protection of flowers, and discuss possible future perspectives that may bring further understanding on plant defense strategies against attack on flowers.

Contribution of constitutive resistance to the defense of flowers

Besides morphological structures such as trichomes, the constitutive production of defensive compounds that directly affect attacker performance and survival

can protect flowers from attackers (McCall & Irwin, 2006). Constitutive resistance is predicted to be stronger in flowers than in leaves (McCall & Irwin, 2006). This prediction derives from the fitness value of flowers and the high probability of attack on flowers due to their conspicuousness and high resource richness (Zangerl & Bazzaz, 1992; Zangerl, 2003; McCall & Irwin, 2006). In accordance with this prediction, enriched levels of floral non-volatile defense compounds in comparison to leaves seem to be relatively common across plant species and orders (Zangerl & Rutledge, 1996; Brown *et al.*, 2003; Strauss *et al.*, 2004; Damle *et al.*, 2005; Smallegange *et al.*, 2007; McCall & Fordyce, 2010). Data gathered during my project confirmed that *B. nigra* follows this prediction too. The overall concentration of total glucosinolates in inflorescences of *B. nigra* was indeed on average five times higher than in leaves over the course of the flowering period (Chapter 4), as previously reported (Smallegange *et al.*, 2007). Glucosinolates are non-volatile secondary metabolites stored in plant cells. Their breakdown products have a detrimental impact on herbivore performance, especially for attackers that are not specialized feeders on glucosinolate-rich species in the Brassicales (Hopkins *et al.*, 2009; Textor & Gershenzon, 2009). In line with this, 60 % of the 30 florivore species recorded on *B. nigra* were specialists and only these specialist species reached such high abundance on plants when compared with generalists (Chapter 5). Such high levels of compounds mediating direct resistance in inflorescences most likely contribute to the selection of a florivore community that mainly consist of specialists.

Plants may have evolved towards the constitutive production of defensive compounds that prevent the most threatening attackers from settling on inflorescences. It can be expected that chewing herbivores that directly remove flowers, buds or even eat the meristems of the floral stem are more likely to reduce seed production than sucking insects (Stamp, 2003; McCall & Irwin, 2006). A last instar *Pieris brassicae* caterpillar can, for example, remove on average 135 buds and flowers, and can damage a large proportion of floral branches on *B. nigra*, which can reduce seed production (Smallegange *et al.*, 2007; Smallegange *et al.*, 2008). The constitutive glucosinolate composition of *B. nigra* indicates that the plant's inflorescences have a stronger constitutive resistance than leaves. The glucosinolate concentration of inflorescences of *B. nigra* consisted of about 99 % of sinigrin (Chapter 4), which is an aliphatic glucosinolate that mostly affects the performance of chewing insects, whereas aliphatic glucosinolates have little effect on aphid performance (Kos *et al.*, 2012a; Kos *et al.*, 2012b). Results suggest that the glucosinolate composition of floral tissue may provide a targeted protection to flowers by preventing plant colonisation by the most detrimental attackers.

Compounds that confer resistance are energetically costly for a plant and, thus, hypotetically only beneficial when florivory would otherwise reduce seed production (Strauss *et al.*, 2002; Bekaert *et al.*, 2012; Mithöfer & Boland, 2012). In lima bean plants (*Phaseolus lunatus*), for example, flower removal did not affect seed production, and flowers of lima bean expectedly had a constitutive cyanogenesis potential that was inferior to that of of young and intermediate leaves (Godschalx *et al.*, 2016). Therefore, it is important in future studies to estimate the fitness consequences of removal of flowers in order to understand patterns in the levels of constitutive direct resistance across plant species: the content of constitutive defense compounds in flowers may increase when the fitness value of a flower increases. The composition of defense-compound mixtures constitutively present in flowers may specifically protect flowers from the colonization by the most detrimental attackers. To further understand the evolution of complex mixtures of defense compounds as a constitutive barrier to attack on flowers, studies could use natural variation in the composition of floral constitutive defense and associate it to the composition of the flower-associated community. Such approach may allow to specifically pinpoint which compounds are detrimental to certain attackers.

Contribution of inducible resistance to the defense of flowers

Inducible resistance is thought to limit the energetic costs of resistance by inducing specific resistance responses only when an attack occur (Agrawal, 1998; Agrawal *et al.*, 1999; Dicke & Hilker, 2003; Zangerl, 2003; Karban, 2011). There are only few studies that investigated whether and how plant responses to attack on flowers provide inflorescences with induced resistance (McCall & Irwin, 2006; Lucas-Barbosa *et al.*, 2011; Lucas-Barbosa, 2016). So far, studies mostly focused on artificial florivory or chewing insects that directly remove floral tissues. In the present project, I measured volatile emission by aerial parts of *B. nigra* as well as glucosinolate concentrations in leaves and inflorescences (flowers, buds & stem pooled together) of *B. nigra* exposed to single and dual attack by *P. brassicae* caterpillars*, B. brassicae* aphids and Xcr bacteria, which are specialists and mostly damage the inflorescences. By combining these attackers, I explored the contrasting effect of single and multiple attack on plant inducible resistance and highlight constraints that plant may face when dealing with multiple attackers.

Inducible direct resistance

Plants can respond to artificial or chewing damage on their floral tissues with either no induction of secondary metabolites in floral tissues (Zangerl & Rutledge, 1996; Godschalx *et al.*, 2016), or with an induction (Ohnmeiss & Baldwin, 2000;

Boyer *et al.*, 2016). However, induction of secondary metabolites in flowers has so far rarely been linked to consequences on florivore performance (McCall & Irwin, 2006; Boyer *et al.*, 2016). In *Impatiens capensis*, damage to one flower corolla marginally reduced the number of flowers subsequently damaged in the field and had no effect on the amount of damage per flower and did not match patterns of induction or reduction of anthocyanins (Boyer *et al.*, 2016). This suggests reduction in the occurrence of florivory measured upon attack may have been mediated by traits such as volatiles, rather that toxins that typically affect performance (Boyer *et al.*, 2016). The present study attempted to link induction of glucosinolates to the performance of the insect florivores, and explored the possible role of phytohormones in the resistance response of *B. nigra* to multiple attackers. Exposure of plants to combinations of attackers that included caterpillars induced the highest upregulation of phytohormones in flowers, and plants responded with an induction of jasmonates (JAs) in inflorescences, but not in leaves (Chapter 2). Levels of JAs were especially high upon dual attack compared to single attack. JAs are known to regulate signalling pathways involved in induced plant resistance (Erb *et al.*, 2012; Lazebnik *et al.*, 2014). In accordance to the levels of JAs, results suggested that dual attack increased plant resistance to the caterpillars, whereas dual attack facilitated aphid performance, when compared to single attack. However, no induction of glucosinolates was recorded in inflorescences upon attack. Plants only responded with changes in levels of foliar total glucosinolates when Xcr was one of the attackers, which may protect leaves from Xcr infection (Chapter 4). This result suggests the involvement of other mechanims affecting caterpillar performance, such as other compounds, direct competition, or other plant defense strategies. Induction of defense compounds may be a poor strategy against specialist feeders that survives the high consitutive defenses of inflorescences, in particular when one compound mediates both constitutive and induced direct resistance.

In order to further understand the adaptive function of induction of non-volatile secondary meatbolites in response to attack on flowers, future studies may explore whether and how induced induction of these metabolites can be linked to the performance of the attackers. A possible approach could be to compare the performance of a flower attacker on lines of plants that have different induction levels of a certain non-volatile secondary metabolite. Higher levels of a compound that is detrimental to the attacker would then be associated with a lower performance of the attacker. Additionally, particular focus should be given to whether specialist and generalist attackers of flowers induce different responses in terms of non-volatile compounds, and how this further affects their performance. Since induction

of non-volatile secondary metabolites may impact floral traits (nectar, color) that are exploited by pollinators (McCall & Irwin, 2006), exploring the possible consequences of induced direct resistance to the attraction of pollinators will bring further insights into constraints that may limit plant direct resistance strategies (Rusman *et al.*, 2019).

Inducible indirect resistance

Upon attack, plants can attract natural enemies of the attackers, and thus indirectly resist the attackers (Dicke & Hilker, 2003; Zangerl, 2003; Agrawal, 2011). Predation or parasitization of florivorous attackers can have clear benefit for plants in terms of seed production (Smallegange *et al.*, 2008; Gols *et al.*, 2015; Lucas-Barbosa *et al.*, 2017). *Brassica nigra* plants attacked by parasitized caterpillars of *P. brassicae* produced more seeds than plants exposed to non-parasitized caterpillars, and as many seeds as undamaged control plants (Smallegange *et al.*, 2008). Plant volatiles that are induced by different attackers can be major cues used by natural enemies to locate their prey or host (Dicke & Baldwin, 2010). Florivory by *Maruca vitrata* caterpillars induced changes in the volatile blend of flowers of *Vigna unguiculata* (cowpea) that effectively attracted a parasitoid of the florivorous caterpillars (*Apanteles taragamae*) compared with control plants (Dannon *et al.*, 2010). Previous work indeed reported that *Cotesia glomerata* preferably landed on flowering *B. nigra* attacked by the caterpillar *P. brassicae*, either on inflorescences or on leaves, compared to non-infested control plants (Lucas-Barbosa *et al.*, 2014). When several organisms simultaneously attack flowers, the recruitment of natural enemies of attackers may be altered.

A plant response to one attacker may interfere with the response to another organism attacking the plant simultaneously, and this has been largely explored for plants in the vegetative stage (Das *et al.*, 2013; Ponzio *et al.*, 2013; Lazebnik *et al.*, 2014; Stam *et al.*, 2014). Herbivory or infection by host and non-host herbivores can indeed reduce the attraction of natural enemies to the attacked plant as well as the parasitism rate by natural enemies compared to plants attacked by the host only (Mauck *et al.*, 2015; Blubaugh *et al.*, 2018). Such interference can be mediated by changes in volatile emission, or by alteration of the suitability of the insect as host or food (Rodriguez-Saona *et al.*, 2005; Soler *et al.*, 2012; Ponzio *et al.*, 2013; Cusumano *et al.*, 2015; de Rijk *et al.*, 2016; Kaplan *et al.*, 2016). The present study showed that the volatile blend of flowering *B. nigra* exposed to caterpillars differed from the blend of flowering *B. nigra* plants exposed to caterpillars plus aphids or caterpillars plus bacteria. When comparing volatile blends of flowering plants

exposed to *B. brassicae* only or *B. brassicae* plus caterpillars or plus bacteria, no differences in composition was detected (Chapter 3, Fig. 1). Changes in volatile emission matched with differences in levels of jasmonates that single or dual attack by *P. brassicae* induced in inflorescences (Chapter 2). JAs can mediate the induction of volatiles in flowers (Li *et al.*, 2017; Li *et al.*, 2018) as well as in leaves (Dicke & Baldwin, 2010), and may have driven the changes in volatile emission measured in my project. About 60 % out of the 57 volatile compounds detected in the blend of *B. nigra* accounted for the differences in volatile emission upon attack. Additionally, parasitoid performance was negatively correlated with the performance of their host: *C. glomerata* larvae performed best in caterpillars feeding on plants exposed to the dual attack treatments, whereas *D. rapae* performed worse on plants exposed to the dual attack treatments (Chapter 2). Although dual attack interfered with plant volatile emission and host suitability for parasitoid development, parasitoids showed no preference for plants exposed to only their host over plants exposed to their host plus a non-host, neither in a greenhouse assay nor in the field (Chapter 3, Fig. 1). Thus, there was no disruption of the attraction of natural enemies despite changes in plant volatile emission and host quality, as well as potentially other floral traits. Parasitoid host-searching behavior was similarly not affected by changes in the volatile blend on *B. nigra* exposed to one or two different attackers in the vegetative stage (Ponzio *et al.*, 2014; Cusumano *et al.*, 2015; Ponzio *et al.*, 2016). The emission of a complex VOC blend may minimize the disruption of the attraction of natural enemies when plants are exposed to multiple attackers.

Despite its clear benefit for plants, induced indirect resistance may interfere with the attraction of pollinators that advertise the plant to other florivores or mediate reproduction (Irwin *et al.*, 2004; McCall & Irwin, 2006; Schiestl, 2010; Schiestl *et al.*, 2014; Jacobsen & Raguso, 2018). Induction of volatiles attracting natural enemies may indeed alter plant traits that are also exploited by pollinators to locate flowers and assess reward quality (Schiestl *et al.*, 2014), although both mutualists may be resilient to changes (Pareja *et al.*, 2012). Only a subset of the volatile blend is used by different members of the community associated to the plant (de Boer *et al.*, 2004; Bruce & Pickett, 2011). Therefore, the emission of a complex blend, such as the one of *B. nigra*, may allow plants to differentially advertise their presence to pollinators and parasitoids. In the field, the composition of the pollinator community was quite resilient to changes in plant traits mediated by attack, and overall, plants exposed to *P. brassicae*, *B. brassicae*, or Xcr, in single or dual combination, attracted as many pollinators as non-treated plants (Chapter 3, Fig. 1). For flowers that interact with a diverse range of pollinators (generalist flowers), changes in floral traits may be

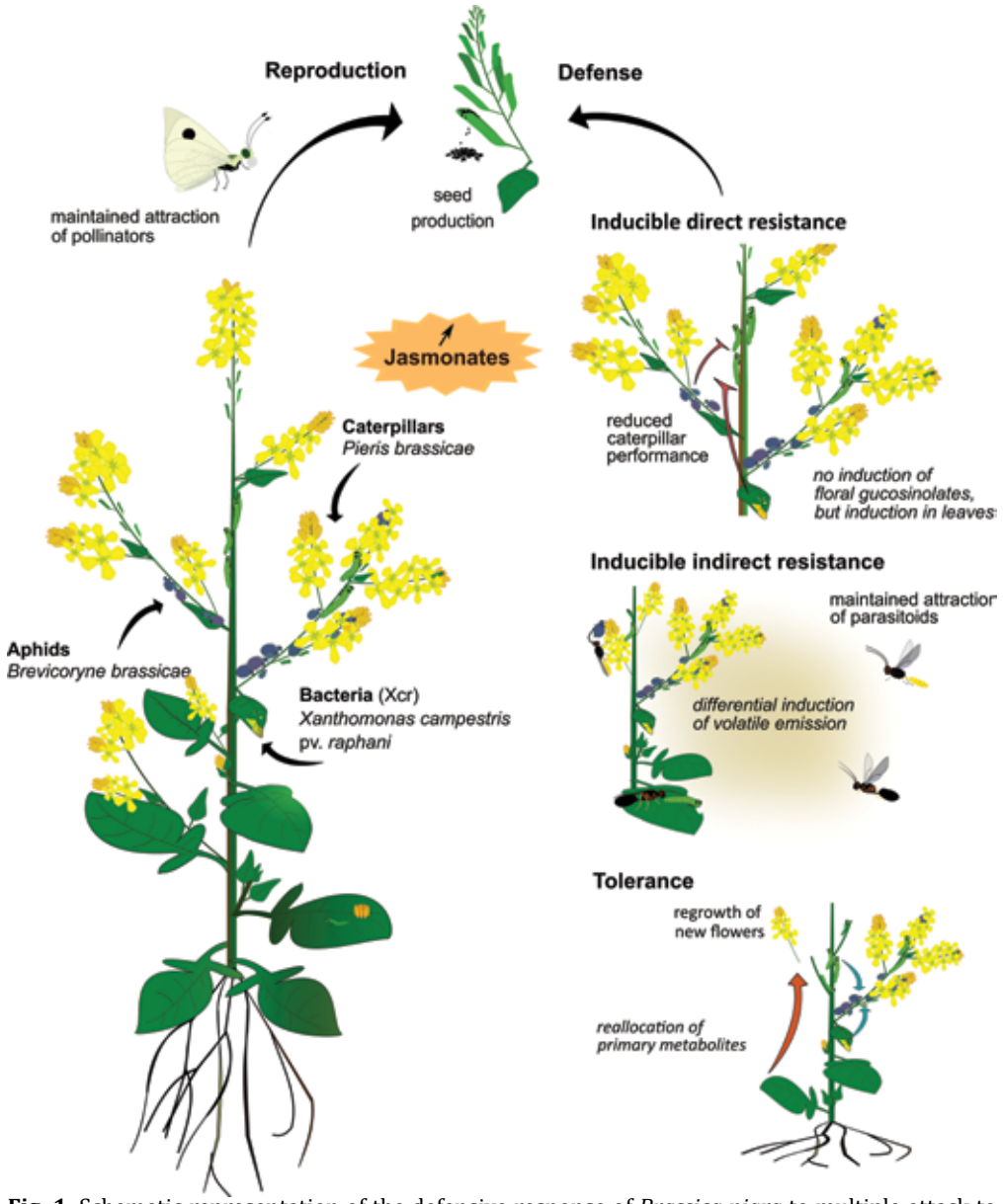


Fig. 1. Schematic representation of the defensive response of *Brassica nigra* to multiple attack to their inflorescences. Plants responded to single or dual attack by distinct types of attackers (aphids, caterpillars, bacteria) with an induction of jasmonates in inflorescences, but not in leaves. Upon attack, plants defended their inflorescences *via* three strategies: induced direct resistance that reduced caterpillar performance, and that was not mediated by an induction of glucosinolates; induced indirect resistance mediated by the induction of plant volatile emission that attracted parasitoids to their host; and tolerance, which was likely mediated by the regrowth of damaged flowers and supported by the reallocation of primary metabolites from vegetative parts to inflorescences. Red flat-ended arrow indicates that dual attack decreased caterpillar performance. Blue arrow indicates that dual attack increased aphid performance. Orange arrow represents allocation of primary metabolites. Attack to inflorescences neither affected the total attraction of pollinators to flowers nor reduced the production of seeds by *B. nigra*.

buffered by the diversity and abundance of mutualistic interactions associated with flowers (Frame, 2003; McCall & Irwin, 2006; Lucas-Barbosa, 2016). Additionally, it is important to consider that induced changes may affect the community of florivores subsequently settling on the plants for their possible effect on plant reproductive output. In *B. nigra*, initial attack to flowers only had a transient effect on the community composition and affected only one species out of the 18 most occurring species (Chapter 5). As a consequence, induction of indirect resistance may not necessarily be constrained by subsequent ecological consequences.

To understand the contribution of indirect resistance to the protection of flowers, future studies may compare abundance of florivores in the presence and in the absence of natural enemies of the florivores. The present project suggests that *B. nigra* may indirectly resist more effectively against the specialist chewer than against the specialist phloem sucker. However, I did not consider predators such as syrphid larvae or ladybeetles, which may be important in the control of aphids. In the absence of natural enemies, populations of *B. brassicae* can reach more than one thousand individuals within 12 days, whereas populations reached such levels in the field within 32 d (Chapters 2 and 5). Therefore, in a more general view, an extensive assessment on the natural communities of natural enemies contributing to the indirect protection, their occurrence, and their effectiveness in controlling florivore populations on flowers would provide a better understanding of the mediation of indirect resistance to florivorous insects. Furthermore, exploring the consequences of induction of volatile compounds or of the attraction of carnivorous animals on the attraction of pollinators will indicate possible constraints that may restrict the evolution of indirect resistance against attackers of flowers. In this regard, outcrossing plants may be particularly constrained in the expression of defense strategies since they rely on pollinators for reproduction. Outcrossing plants with flowers that are associated with a generalist community of pollinators may be more resilient to changes in flower traits upon attack than specialized flowers.

Contribution of tolerance to the defense of flowers

Plants can use tolerance strategies to overcome the effect of attack on their fitness. Leaves and bracts that synthesize carbohydrates, as well as roots that take up nitrogen provide plants with energy to tolerate attack to flowers, which are resource sink organs (McCall & Irwin, 2006; Orians *et al.*, 2011). Plant responses to chewing florivores or artificial florivory can partially compensate, fully compensate or overcompensate for the attack in terms of seed set (Hendrix, 1984; Lohman *et al.*, 1996; McCall & Irwin, 2006; Wise *et al.*, 2008; Zangerl & Berenbaum, 2009; Lucas-

Barbosa *et al.*, 2013). So far, the exploration of plant tolerance strategies upon attack of flowers mainly focused on plant responses to artificial florivory or tissue chewing by caterpillars. In this project, I tested whether and how *B. nigra* tolerate single and dual attack by three specialist attackers that mostly damage flowers. I measured seed production by the plants in a field experiment, as well as biomass of inflorescences, leaves and roots, and the metabolic composition of inflorescences and leaves (soluble sugars, free amino acids, protein-bounds amino acids) in a greenhouse experiment. *Brassica nigra* tolerated different types of damage on flowers by specialist attackers: attack by *P. brassicae* caterpillars, *B. brassicae* aphids and attack by the phyotopathogen Xcr, and dual combinations of them, did not affect the number of seeds by *B. nigra* in the field (Chapters 3 and 5, Fig. 1). By comparing plant tolerance in response to single attack, and dual attack, my project brings further understanding on the differential response of plants.

Mechanisms contributing to tolerance of attack on flowers

Regrowth of damaged plant parts is a common tolerance mechanism (Strauss & Agrawal, 1999; Agrawal, 2011). Upon florivory, plants can initiate new floral branches or new buds and flowers (Dale, 1959; McCall & Irwin, 2006; Smallegange *et al.*, 2008; Wise *et al.*, 2008). In the horsenettle *Solanum carolinensis*, the ability of generating new flower buds after florivory was the trait that was most correlated to plant tolerance (Wise *et al.*, 2008). Biomass data suggest that in the greenhouse and in the field, *B. nigra* compensated for physical damage on inflorescences (Chapters 4 and 5, Fig. 1). Dry biomass of inflorescences of *B. nigra* was not affected by attack compared to unattacked control plants despite the observation that the caterpillars consume a large number of flowers. It is difficult to quantify how many resources were consumed by *B. brassicae* aphids because they feed on phloem of flower branches. However, aphid feeding can lead to bud or flower death (pers.obs.). Xcr incoculation did not lead to visible death of floral parts, but symptoms of diseases (necrotic spots) could be observed on the infected bract of about 50 % of the plants. It remains to be investigated whether this compensation was mediated by new branching or by the production of new flowers on the same branch, for example.

Instead of regrowing tissues, plants may invest more resources in the remaining tissues or allocate resources to healthy flowers instead of to the damaged flowers (Krupnick & Weis, 1999; McCall & Irwin, 2006). In this way, plants may increase the reproductive output of the remaining flowers (Hurd *et al.*, 1979; McCall & Irwin, 2006). Plants may, for example, abort a floral branch on which aphids have settled, and reallocate those resources to branches or flowers left undamaged that would

otherwise have been invested into the aborted branch. As an illustration of this strategy, the annual fabaceous plant *Cassia fasciculata* upon artificial removal of a floral branch allocated resources to other branches (Garrish & Lee, 1989). Such responses may allow plants to cope with attackers that do not remove tissues such as aphids. On *B. nigra*, colonization of a floral branch by *B. brassicae* aphids sometimes led to an absence of siliques in the segment covered by aphids and only the floral petioles or dry buds remained visible (personal observation). Plants may abort the formation of siliques as an effect of aphids sucking phloem, deterrence of pollinators, or as a strategy to allocate resources to non-infested floral branches. Interestingly, attack by *B. brassicae* aphids, Xcr bacteria, or *P. brassicae* caterpillars did not affect the biomass per seed compared to control plant (Chapter 5), which suggests that *B. nigra* did not respond to attack with an increased allocation of resources into seeds as a way to increase viability of the remaining seeds. It would be interesting to determine the number of siliques, seeds per siliques, to further understand the contribution of allocation of available resources to flowers left undamaged.

Plants can as well mitigate the fitness effect of damage by enhancing mutualistic interactions with pollinators (Strauss & Murch, 2004; McCall & Irwin, 2006; Lucas-Barbosa, 2016). Attack may induce changes in floral size, or nectar and pollen, and in this way increase the interaction with pollinators. Such a strategy may benefit both female and male fitness by increasing ovule fertilization and pollen exportation. *Brassica nigra* attacked on the inflorescences by either caterpillars, aphids or bacteria, or by dual combinations of them, were visited by similar numbers of pollinators as did non-attacked plants. Moreover, attack did not affect the time that pollinators spent per flower. Attacker-induced changes in the pollinator community composition were subtle, and no plants had a pollinator community that differed from the one associated with control plants (Chapter 3). It remains to be investigated whether the changes in the community composition translate into increased or decreased pollinator activity on the flowers. Nevertheless, my results suggest that tolerance of *B. nigra* to attack is unlikely to be mediated by increased attraction of pollinators.

In general, tolerance may be an effective strategy against specialist florivores or pathogens that are little affected by induction of secondary metabolites (Stamp, 2003; Orians *et al.*, 2011). It is interesting in future studies to explore how plants tolerate attackers that differ in terms of specialization level. Since different attackers from different feeding guilds, or even from different species within a feeding guild,

can inflict different types of damage, further studies could assess whether and how plants respond with regrowth of damaged or killed tissues (new branching or by the production of new flowers); whether and how plants increase resource allocation to flowers that remained undamaged (more seeds per fruit, heavier seeds); whether and how pollination is enhanced (pollen grain deposited on pistil or removed from anthers by pollinators for example). Such studies may estimate the type and amount of damage on flowers inflicted by different types of attackers, and link this to different tolerance strategies upon attack by distinct organisms on flowers.

Resource allocation supporting tolerance mechanisms

Regrowing lost parts of plants requires resources, and for sink inflorescences regrowth may therefore be supported by an allocation of primary metabolites (Schwachtje & Baldwin, 2008; Bolton, 2009; Schultz *et al.*, 2013). Artificial debudding of cotton slightly increased total sugar and nitrogen in aerial parts which may support the development of new buds (Dale, 1959). In *B. nigra*, attack by *P. brassicae* can result in a reduction in total C/N ratio in inflorescences, which could favor growth and the associated compensation for damage (Lucas-Barbosa *et al.*, 2017). In the present project, plants responded to exposure to caterpillars plus aphids with a decrease in the C/N ratio on inflorescences compared with plants exposed to caterpillars only, and this was correlated with a reduction of soluble sugars that may fuel regrowth of damaged flowers (Chapter 4, Fig. 1). Regrowth of damaged parts may be especially important to cope with insects that remove or kill a large proportion of plant tissues. Rapid response to attack as observed for *B. nigra* requires directly available resources. In cotton plants, compensatory potential via enhanced vegetative growth increased with the avalaibility of resources in the soil, and under poor resource conditions this perennial plant favored resource allocation for storage in roots rather than immediate regrowth (Sadras, 1996). Similarly, competition in high plant densities can affect the ability to recover from loss of flowers (Gãmez & Fuentes, 2001). *Brassica nigra* is a species that typically grows in patches (Bell & Muller, 1973; Meyer, 2000). My results suggest that seed production by *B. nigra* is sensitive to plant-plant competition when plants are attacked in the flowering stage, and competition for resources may reduce the plant ability to limit fitness impact of attacks (Chapter 5). Additionally, competition experienced by plants from the beginning of the growing season can impact the plant reproductive output (de Vries *et al.*, 2018; de Vries *et al.*, 2019). Therefore, resource acquisition at the vegetative stage, as well as at the time when inflorescences are attacked, may constribute to a plant's abilility to tolerate damage to flowers.

Plant tolerance to attack is interconnected with indirect and direct resistance to the attackers. Indirect resistance may facilitate tolerance. For example, *B. nigra* exposed to parasitized *P. brassicae* caterpillars can compensate for seed production compared to non-infested plants, whereas plants exposed to non-parasitized *P. brassicae* did not (Smallegange *et al.*, 2008). However, tolerance of plants to attackers may as well reduce plant ability to directly resist to attackers. Increased resource allocation to flowers upon attack may interfere with plant direct resistance by increasing nutrient content of the floral tissues or phloem being consumed, which can benefit the attackers. Higher nutritional quality of plant tissues may indeed compensate for the detrimental effect of floral defensive compounds on the performance of the attackers (Smallegange *et al.*, 2007; Cao *et al.*, 2017). When *B. nigra* were exposed to dual attack with *B. brassicae* aphids plus caterpillars or plus Xcr, *B. brassicae* colonies reached higher numbers than on plants exposed to *B. brassicae* only. This effect was observed in controlled greenhouse assays as well as in the field within the first four days following the initial attack to flowering *B. nigra* (Chapters 2 and 5, Fig. 1). This facilitiation may be linked to changes in plant primary metabolism upon attack. Additionally, plant responses to attack had little effect on the subsequent community of florivores colonizing inflorescences of *B. nigra,* and only temporary changes in the abundance of another specialist aphid, *Lipaphis erysimi,* were measured (Chapter 5). In a field experiment, neither the facilitation of *B. brassicae* upon dual attack nor the increase in the abundance of *L. erysimi* altered seed production by *B. nigra* (Chapter 5). Tolerance to attack on flowers can remain an effective strategy to cope with attack in a more complex community context when it does interfere with direct resistance, or when interference with direct resistance do not have impact on plant fitness.

Future studies should explore physiological processes that mediate plant tolerance to attack. Possible approaches could be, for example, to measure the photosynthetic activity or to track sugar or amino acid allocation in response to attack. The present project highlights the importance of testing the effect of plant responses to attack in a natural context to further understand their impact on plant fitness: resource allocation to flowers may benefit plant antagonists, which can be a constraint if such facilitation reduces plant fitness. Leaf herbivory changes resource acquisition in the vegetative stage, which may influence plant ability to later defend against floral attackers. Leaf herbivory can influence plant competition with neighbouring plants and if a plant loses this competition it is overshadowed, and receives less sunlight and thus cannot produce high levels of resources for the flowers (de Vries *et al.*, 2018; de Vries *et al.*, 2019). Further studies could investigate whether and how shadowing, or resource limitation affect a plant's ability to tolerate attack onto flowers.

High resource investment in plant reproduction when flowering

When developing from the vegetative to the flowering stage, plants undergo major physiological changes resulting from flowers being a strong resource sink (Mooney, 1972; Barneix & Causin, 1996). Inflorescences and flowers are generally richer and more concentrated in primary metabolites than leaves (Mooney, 1972). In lima bean, the concentration of soluble proteins is higher in buds and flowers than in leaves (Godschalx *et al.*, 2016). In line with this, phytohormonal content and metabolic composition of *B. nigra* strongly differed between leaves and inflorescences. Compared to leaves, inflorescences were enriched in soluble sugars, free amino acids, and jasmonates, which are major phytohormonal regulators in plants. Resources invested in inflorescences likely support the high levels of energetically costly constitutive resistance, which seems to be common for inflorescences, as well as the production of flowers. This investment of the plant into inflorescences was particularly clear at the beginning of the flowering period. Biomass of inflorescences of *B. nigra* indeed increases by nearly 80% between 4 d and 8 d after the first flower opened, when plants were infested (Chapter 4). Similarly, metabolic content was overall higher in young inflorescences than in old inflorescences (Chapters 2, 3, and 4). High investment in reproduction may be typical of annual plants that accumulate resources in the vegetative stage to invest in their single reproductive period before the plant dies (Mooney, 1972). Since annual plants only have one opportunity to reproduce and invest resources accumulated during the vegetative stage into reproduction before dying, annual plants may need to rapidly face attackers. Whereas perennial plants can spread their defense strategies over years and may sequester resources in roots upon attack to inflorescences to re-invest them into reproduction later, under more favorable conditions. Adressing how annual plants and perennial plants differently defend against attackers that damage flowers will provide further understanding of how time limitation in lifespan can influence plant defense stratetegies against attack on inflorescences.

General conclusions of the project and perspectives

To date, most studies have focused on plants in the vegetative stage when addressing defense responses to biotic stresses, although recent findings suggest that flower feeders may strongly challenge plant defensive mechanisms and interfere with reproduction. To survive and reproduce, plants face the challenge to balance investments in defense and flowering processes. Defending against florivores may be particularly challenging for plants. Florivores can not only directly damage gametes that flowers potect, but plant response to attack can also indirectly alter floral traits and the pollination. So far, little is known about defenses of plants in

the flowering stage, and plant responses to attack on flowers have received little attention. In this study, I analyzed plant defenses against distinct types of attack on flowers. I focused on plant constitutive direct resistance, plant inducible direct and indirect resistance, and how plants can tolerate attack to their flowers. I analyzed the consequences of plant responses to attack of flowers on other plant-associated community members (florivorous antagonists, and mutualistic pollinators) in the context of plant defense against attackers on inflorescences.

Bioactive jasmonates were identified as the main phytohormones mediating responses of *B. nigra* to attack to their inflorescences, and jasmonates were particularly upregulated when plants were exposed to single or dual attack that involved caterpillars (Fig. 1). Bioactive forms of JA can be involved in a diverse range of physiological and defensive processes in plants (Avanci *et al.*, 2010; Erb *et al.*, 2012), induction of jasmonates may affect a wide array of traits in plants in the flowering stage. Therefore, the differential regulation of jasmonate-mediated signalling pathways involved in reproduction and defense should be further investigated to understand how plants regulate their responses to attack by florivores (Li *et al.*, 2017; Li *et al.*, 2018). Despite the induction of phytohormones in flowers in response to attack, results suggest that *B. nigra* maintained interactions with pollinators. *Brassica nigra* was visited by a diverse community of pollinators that consisted of more than 10 species that exploit different plant cues and rewards. Such a community may provide the plant with certain plasticity: when a certain pollinator species is deterred by a change in plant traits, another may be attracted, and overall attraction to attacked flowers may remain the same as for flowers of non-attacked plants leading to unaffected seed production (Garibaldi *et al.*, 2013).

Results suggest that *B. nigra* plants effectively defend their inflorescences against three types of specialist attackers (Fig. 1). The project found evidence for induced resistance of *B. nigra* to attack by *P. brassicae*; caterpillars had a lower performance on dual attacked plants than on plants exposed to dual attack. However, no induction of glucosinolates was detected in inflorescences, and the mechanisms mediating the reduction in caterpillar performance remain to be identified. Since the insects and the bacteria used as plant attacker are specialized on dealing with glucosinolates, and also survive the high constitutive levels of glucosinolates of flowers, an induction of glucosinolates may not be an effective strategy against such specialist attackers. In complement to direct resistance, results suggest that *B. nigra* protected their flowers *via* inducible indirect resistance as well. Plants responded to attack with changes in the volatile blend emitted by aerial parts of the plants, and

caterpillars were the main driver of changes in the volatile blend. Plants exposed to attack recruited natural enemies of the attackers, and the attraction and parasitism rate was not affected by the simultaneous attack with another non-host organism. The complex blend of volatiles emitted by *B. nigra* may favor the maintenance of interactions with natural enemies to plants upon multiple attack. Induced resistance mechanisms likely reduced the damages inflicted by the attackers, and plants used tolerance strategies to mitigate the fitness impact of damages. Results suggest that plants regrew lost flowers upon attack. Changes in plant primary metabolisms that occurred within a few days after attack may have contributed to plant compensation for damage. Such rapid compensation for damage may be typical of annual plants that have only one opportunity to reproduce and likely try to rapidly reach full compensation for the effects of attack.

Plant chemical diversity mediates an intricate web of interactions and can influence community composition and biodiversity (Swain, 1977; Futuyma & Agrawal, 2009; Kursar *et al.*, 2009; Abdala-Roberts *et al.*). In particular, some plant secondary metabolites have key roles in important functions for plant survival and reproduction: in plant-attacker interactions, plant-carnivore interactions, plant-pollinators interactions, for example (Schoonhoven *et al.*, 2005; Raguso, 2008; Dicke & Baldwin, 2010). The emission of complex blends of volatiles may allow plants to maintain interactions with pollinators and natural enemies of attackers upon attack. Complex chemical diversity generally supports a diverse network of interactions. Maintaining sufficient interactions with mutualist organisms is essential for plant survival and reproduction. Results of this study suggest that preferences may be buffered by the large number and diverse community of pollinators still visiting flowers of a plant, which may be especially true for plants that are not limited in terms of pollinators. At the scale of a field, flowering plants provide an incredible amount of colors, odors, and feeding niches, which support a wide array of interactions. The presence of plants in the flowering stage in the proximity of agricultural fields can benefit the protection of neighboring plants against attackers and promote plant interactions with diverse pollinators (Holzschuh *et al.*, 2007; Bianchi & Wäckers, 2008; Kohler *et al.*, 2008). For example, the presence of plants in the flowering stage in agricultural fields can increase the population of parasitoids, which are typically nectar feeders as adults (Bianchi & Wäckers, 2008). Further understanding of how this diversity, at the plant level as well as at the landscape level, can provide protection to plants in response to attack, could help to implement agricultural system with environmentally friendly ways to protect crop plants (Jenke-Kodama *et al.*, 2008). Maintaining diversity in

agricultural fields and in natural environments, and therefore supporting diverse biotic interactions, may be even more crucial than the drastic decline in insects that may also reduce their ecological services (Hallmann *et al.*, 2017).

This project contributed to the general understanding of plant defenses against floral attack. Plant defense against attack to flowers, or to inflorescences, have so far been little explored despite that flowers mediate plant reproduction, and therefore, have a strong contribution to plant fitness. Florivory can be a major threat because florivores can not only directly consume or kill flowers, but also indirectly alter pollinator attraction and natural enemies of the herbivores. Plants probably evolved under this constraint of effectively protecting their flowers while maintaining mutualistic interactions. These selection pressures likely drove the incredible diversity of shapes, colors, chemistry and smell that flowers display (Crane *et al.*, 1995; Schiestl, 2010; Buchanan & Underwood, 2013).

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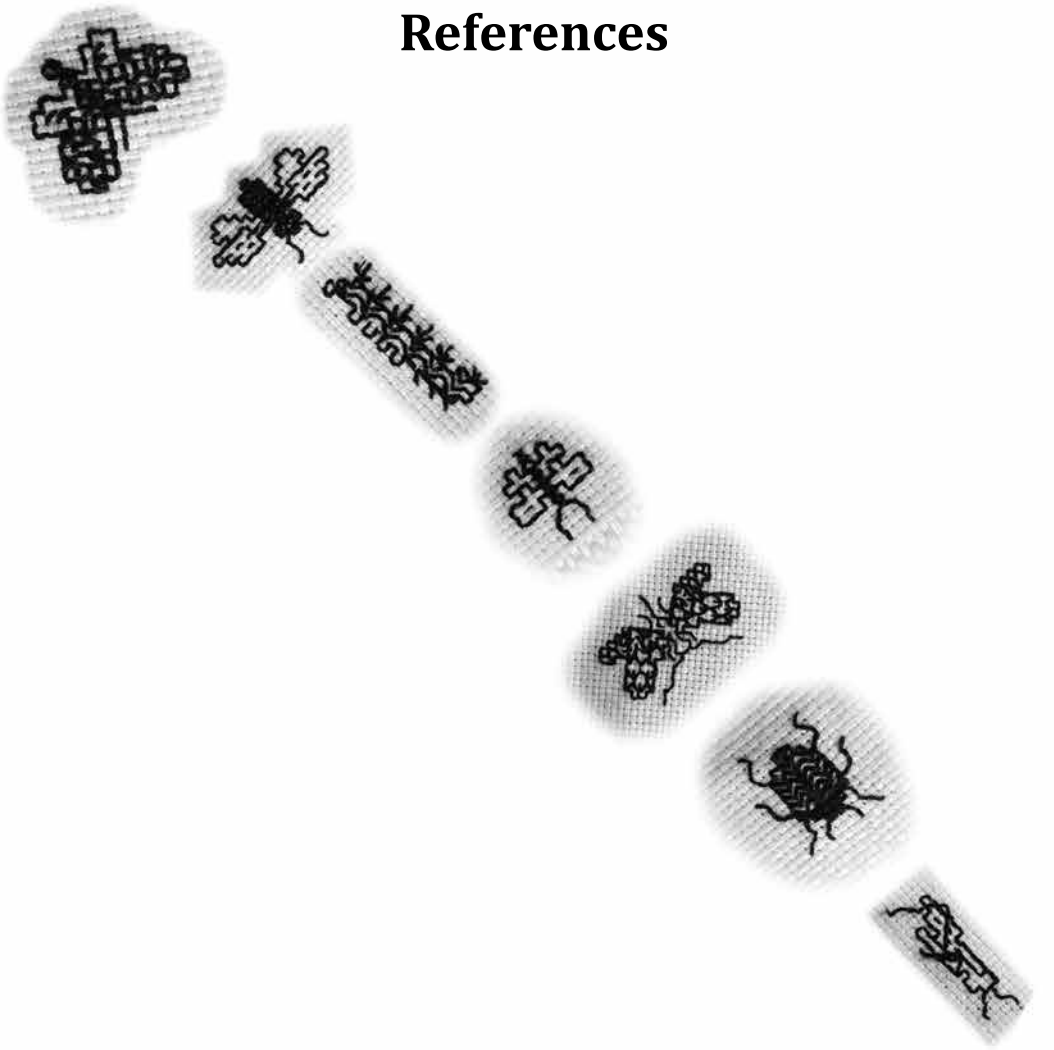

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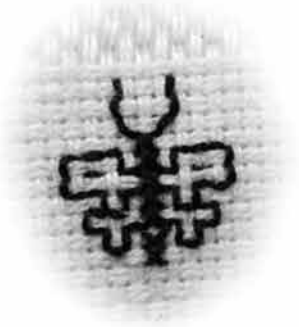
Summary



During the transition from vegetative to flowering stage, plants allocate a large amount of resources to flowers that make them nutritious for herbivores. Within a plant's lifespan, the flowering stage is generally associated with a diverse herbivore community. Flowers directly mediate plant reproduction, and feeding damage on flowers (florivory) can more strongly reduce plant seed production than damage on leaves. Due to this high fitness value, flowers are predicted to be highly defended. However, few studies have addressed plant defenses against attack on flowers so far, despite the challenge faced by plants to protect their flowers while simultaneously attracting pollinators. Plant defenses and reproduction are regulated by phytohormones that control the production of an enormous diversity of secondary metabolites having defensive and reproductive functions. It is well established that plants in the vegetative stage can specifically respond to different species of attackers: the phytohormone jasmonic-acid (JA) orchestrates responses to chewing herbivores and necrotrophic pathogens, sometimes in association with ethylene and/or abscisic acid (ABA), whereas the salicylic-acid (SA)-pathway is induced in response to phloem-feeding herbivores and biotrophic pathogens. These same phytohormones are also involved in the regulation of plant reproduction, and, for example, regulate various floral traits. In response to attack on flowers, phytohormonally regulated defense-pathways may protect flowers of plants, but changes in flower traits may as well interfere with the reproductive processes. Beyond the impact of defensive traits on plant resistance against herbivores and pathogens, such responses can cascade and affect flower-associated members of the community, including mutualistic carnivores and pollinators. Therefore, plants likely evolved mechanisms that optimize defenses while limiting negative consequences for reproduction.

In this project, I explored how plants **in the flowering stage** fine-tune **defense and reproduction** mechanisms when inflorescences are exposed to **multiple biotic stresses**. I investigated plant responses to single or dual attack on inflorescences, when plants had just started to flower, and linked these to plant fitness. I approached this question through four sub-objectives: 1) to measure the concentration of phytohormones that are involved in reproduction and defense; 2) to identify plant traits involved in resistance, tolerance, and reproduction that are modified in response to attack; 3) to assess whether and how the performance of florivorous attackers and of their natural enemies changes in response to attack; 4) to determine consequences of attack on the attraction of pollinators and plant fitness. I focussed on the Black mustard *Brassica nigra*, an annual outcrossing flowering plant. **Inflorescences** of *B. nigra* were exposed to three types of inducers

that commonly attack brassicaceous plants, and that are known to induce distinct phytohormonal pathways in plants in the vegetative stage: the phloem-sucking aphid *Brevicoryne brassicae* and the chewing caterpillar *Pieris brassicae*, which are florivores, and a bacterial phytopathogen *Xanthomonas campestris* pathovar *raphani* (Xcr) that can infect any developmental stage of the plant and can spread to seeds.

The hormonal mediation of plant responses to attack and their consequences for plant resistance remain largely unexplored for plants in the flowering stage, especially when attackers are on flowers. In **Chapter 2**, I present a study that addressed the potential implication of phytohormones, induced upon attack of inflorescences, on direct and indirect resistance of plants against florivores. To address this question, I exposed *B. nigra* plants to single and dual attack to inflorescences by *B. brassicae* aphids, *P. brassicae* caterpillars, and Xcr bacteria. I quantified phytohormonal responses of leaves and inflorescences of *B. nigra* upon attack, and linked concentrations of phytohormones to the performance of two florivorous insects and their parasitoids. I focused on three types of phytohormones, SA, ABA, and jasmonates (active forms and inactive catabolites of JA), selected for their central role in defense and reproduction. Caterpillars were the main drivers of the induction of phytohormones upon attack to inflorescences. Plants responded to single attack by caterpillars and to dual attack by caterpillars plus aphids or bacteria with an induction of jasmonates in the inflorescences, but not in the leaves. Levels of jasmonates were particularly high upon dual attack, and dual attack decreased the performance of the florivorous caterpillar, but increased the performance of the florivorous aphid. Additionally, parasitoids performed better when their hosts performed worse. Thus, dual attack may increase resistance of plants to caterpillars, but may as well decrease resistance to the aphids. For plant upon dual attack, the increased resistance to caterpillars may be mediated by an induction of defense compounds, whereas facilitation of aphids may be mediated by changes in plant primary metabolism.

Induced emission of volatile compounds upon attack can attract natural enemies of the herbivores, which mediate plant indirect resistance. However, volatiles also mediate the attraction of pollinators to flowers. Thus, changes in plant volatile emission upon attack may disrupt the attraction of pollinators, which can consequently affect plant reproductive output. In **Chapter 3**, I investigated whether and how *B. nigra* preserves interactions with pollinators and parasitoids when inflorescences are exposed to single and dual attack. I particularly focused

on changes in plant volatile emissions as a common cue exploited by both plant mutualists. Flowering *B. nigra* were exposed to single or dual attack by *B. brassicae* aphids, *P. brassicae* caterpillars, and Xcr bacteria, or left unattacked as control. I recorded parasitoids preference in two-choice assays comparing plants exposed to single attack with their host (either caterpillar or aphid) *vs.* plants exposed to dual attack with their host and a non-host in the greenhouse, as well as parasitism in a field experiment. Effect of single and dual attack on pollinator visitation was recorded in a common-garden experiment. Aboveground parts of *B. nigra* emitted a complex blend; about 60 compounds were detected. Caterpillars were the main inducers of changes in volatile emission, and the blend composition differed according to the combination of attackers. Changes in the emission of about 30 compounds contributed the most to these differences. Neither the preference of the parasitoid of the caterpillars (*Cotesia glomerata*) nor of the parasitoid of the aphids (*Diaeretiella rapae*) was affected by the presence of a non-host. This robust host-searching behavior, despite changes in plant volatile emission, may rely on the fact that parasitoids only exploit a subset of the volatile blend. Plant response to attack did not affect pollinator attraction, although the composition of the community may change. Plants interacted with at least 10 species of pollinators that exploit different cues and rewards, and their foraging may be differentially influenced by changes in plant traits. Thus, there was no evidence of a trade-off between the attraction of parasitoids and pollinators on *B. nigra* upon attack. The resilience of the parasitoids and the pollinator community to changes in volatile emission, among other plant traits, may be mediated by the complex volatile blend and the diversity of pollinators associated to flowers of *B. nigra*.

In **Chapter 4,** I explore metabolic mechanisms associated with tolerance and resistance of flowering *B. nigra* against attack to their inflorescences. This study characterized how single and dual attack by two florivorous insects and a phytopathogen affect concentrations of metabolites that mediate plant resistance and that support mechanisms of tolerance to attack. I measured the composition and total concentrations of 1) primary metabolites (protein-bound amino acids, free amino acids, and soluble sugars) that provide building blocks and energy to plants physiological processes; 2) secondary metabolites that typically mediate direct resistance in *B. nigra* (glucosinolates), in leaves and inflorescences of attacked plants. Changes in biomass of inflorescences, leaves, and roots of plants upon attack were measured to determine whether and how plants compensate for damaged tissues over the flowering period. Primary metabolites were indeed 1.2 to 4 times more concentrated in inflorescences than in leaves, which reflected the strong resource

investment of *B. nigra* to reproduction. Inflorescences were constitutively defended with 7 times higher concentration of glucosinolates than leaves. In particular, 99 % of the quantity of floral glucosinolate consisted in sinigrin, an allyl glucosinolate generally detrimental to chewing insects. Plants did not respond to attack with an induction of glucosinolates in inflorescences, and only responded with changes in the total concentration of foliar glucosinolates. Among the different single and dual attack situations, only attacks that involved Xcr induced changes in the foliar concentration of glucosinolates, which possibly prevents bacteria from spreading to leaves. Florivorous caterpillars can remove large amounts of buds and flowers, and aphid feeding can sometimes kill flowers. Biomass data suggest that *B. nigra* readily regrew parts of inflorescences that were damaged upon attack. Regrowth may have been supported by the changes in total concentrations of soluble sugars in leaves and flowers measured after attack; no changes were measured for concentrations of free amino acids and protein-bound amino acids. Results suggest that *B. nigra* resist attack through constitutive and inducible direct resistance, but the study did not find evidence of glucosinolate induction in flowers. Specialist attackers that survive constitutive levels of glucosinolates may not be affected by an increase in glucosinolate concentrations upon attack. Other defensive compounds may mediate direct resistance of *B. nigra.* Against attackers that survive the constitutive barrier, plants may tolerate attack through processes such as regrowth, which may be fuelled by changes in plant soluble sugars. Inflorescences of plants are strong resource sink, and upon attack on flowers, undamaged leaves and roots may provide resources necessary to compensate for damages.

Early-season attack of plants in the vegetative stage can influence the composition of the plant-associated community settling on a plant over the growth season of a plant. Since florivores can strongly affect the reproductive output of plants, the effect of florivory on the composition of the florivore community subsequently colonizing plants may affect plant fitness. In **Chapter 5**, I present a field study that investigated whether responses of *B. nigra* to attack on flowers affect the composition of the florivorous community over the course of plant reproductive period, and what the consequences are for plant seed set. I identified and counted florivorous insects that occurred on inflorescences of *B. nigra* exposed to single or dual attack by *B. brassicae* aphids, *P. brassicae* caterpillars, or Xcr bacteria, or of plants left untreated. As a proxy of plant fitness, I quantified the numbers of seeds and the biomass of the seeds produced by the plants. About 30 different species of florivores were recorded. Over 60 % of these were specialists, and only specialists reached high abundances on inflorescences. Plants were strongly defended against

the initial attacker *P. brassicae* and most caterpillars died after a week of florivory. In contrast, *B. brassicae* remained abundant on plants that received it as an initial attacker compared to plants that were not initially exposed to *B. brassicae* aphids. *Brevicoryne brassicae* were also more abundant on plants exposed to dual attack than on plants exposed to single attack in the first week following the initial attack, as previously measured in Chapter 2. Plant responses to attack affected only transiently the composition of florivorous community that subsequently colonized inflorescences. These changes were mediated by the abundance of only one species: the specialist aphid *Lipaphis erysimi*, which occurred in the first half on the plant's flowering period and had overall low abundance. Therefore, its statistical significance needs to be considered with care. In the second half of the flowering period, no difference was observed anymore in the composition of the florivorous community, and exposure to single and dual attack did not affect the reproductive output of *B. nigra*. Thus, results suggest that responses of *B. nigra* to attack on their inflorescences mainly impacted the florivorous insects introduced as initial attackers and had little effect on the colonizing florivorous community. Aphids may have benefited from changes in primary metabolisms upon some treatments.

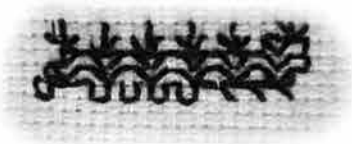
The **General Discussion** presents the contribution of this project to the understanding of a central paradigm of plant biology, *i.e.* the trade-off between defense and reproduction. *Brassica nigra* effectively defended its inflorescences against multiple attack by a chewing insect, a phloem-sucking insect, and a phytopathogenic bacterium. This project identified bioactive jasmonates as the main phytohormones mediating the response of *B. nigra* to attack on their inflorescences. Jasmonates are involved in a wide array of physiological processes that not only contribute to plant defense, but also plant reproduction. Despite the hormonal response to attack, results suggest that plants maintained interactions with pollinators upon attack. *Brassica nigra* interacts with diverse and abundant pollinators, and deterrence of certain pollinators upon attack may be compensated by the attraction of other pollinators.

Previous work on ontogenetic defense trajectory tend to indicate a decrease in inducible direct resistance and an increase in constitutive resistance as plants transit from the vegetative stage to the flowering stage. The present study suggests that *B. nigra* constitutively protected their inflorescences from colonization through high levels of defensive compounds (glucosinolates). Constitutive resistance is predicted to be a common strategy developed by plants to protect flowers, which have strong fitness value and have high risk of being attacked (nutritious and conspicuous).

High constitutive defense likely selected for the community of specialist florivores recorded on *B. nigra*. In response to attack by specialist florivorous insects or a bacterium that infected inflorescences, plant did not protect their inflorescences *via* an induction of glucosinolates. However, other defensive compounds could mediate plant resistance. Additionally, plants effectively recruited natural enemies of the attackers, and dual attack by an insect host and a non-host did not disrupt the overall parasitism on plants. This may be mediated by volatile emission by aerial parts of plant that changed upon attack, in particular when *P. brassicae* caterpillars were one of the attackers. Survival of *P. brassicae* caterpillars in the field was about 1 %, and the contribution of predators to the indirect resistance of *B. nigra* against flower attackers now need further investigation. *Brassica nigra* also mitigated the fitness impact of attack through plant tolerance that was likely mediated by a regrowth of damaged parts of the inflorescence and changes in plant metabolism. Rapid compensation for damages may be typical of annual plants. Annual plants indeed have only one opportunity to reproduce and likely try to rapidly reach full compensation for the effects of attack, whereas perennial plants could sequester resources in roots upon florivory, and reinvest sequestered resources into reproduction once attackers are gone or in the following years.

This project contributed to the general understanding of plant defense against floral attack. Attack of flowers has so far been little explored despite that flowers mediate plant reproduction, and therefore, have a strong contribution to plant fitness. Pollinators mediate the reproduction of about 87 % of angiosperms and for these outcrossing plants, florivory can be a major threat since florivores can not only directly consume or kill flowers, but also indirectly alter pollinator attraction through induced responses of plants upon attack. Plants probably evolved under these constraints of effectively protecting their flowers while maintaining interactions with pollinators, which likely drove the tremendous diversity of shapes, colors, and odors that one can now observe in flowers.

Resumé



Pendant la transition du stade végétatif au stade floral, les plantes allouent une grande quantité de ressources vers leurs fleurs, ce qui rend les fleurs particulièrement nutritives pour les herbivores. Au cours du développement de la plante, le stade floral est en effet généralement associé à une communauté d'herbivores très diverse. Les fleurs étant directement impliquées dans la reproduction des plantes, les dommages faits aux fleurs par des animaux florivores ou des pathogènes peuvent réduire la production de graines plus fortement que les dommages faits aux feuilles. Du fait de cette contribution essentielle des fleurs à la fitness de la plante, une hypothèse dérivée de la Théorie de Défense Optimale propose que les plantes aient évolué d'importants moyens pour défendre leurs fleurs. Cependant, peu d'études ont abordé le sujet de la défense des plantes contre l'attaque des fleurs jusqu'à présent, alors que les plantes en fleur font face au défi particulier de protéger leurs fleurs tout en restant attractives pour les pollinisateurs. Les défenses et la reproduction des plantes sont orchestrées par des phytohormones qui contrôlent la production d'une énorme diversité de métabolites secondaires, ces derniers ayant des fonctions à la fois défensives et reproductives. Il est maintenant bien établi que les plantes au stade végétatif peuvent répondre spécifiquement à différentes espèces de phytophages et phytopathogènes: la phytohormone jasmonic-acid (JA) régule les réponses aux insectes masticateurs et aux pathogènes nécrotrophes, parfois en association avec l'éthylène et/ou l'acide abscissique (ABA), alors que l'acide salicylique (SA) est induit en réponse aux herbivores qui aspirent la sève du phloème et aux agents pathogènes biotrophes. Ces mêmes phytohormones sont également impliquées dans la régulation de la reproduction des plantes et, par exemple, régulent divers traits floraux. En réponse à l'attaque de leurs fleurs, les voies de défense induites dans la plante et médiées par ces phytohormones peuvent donc protéger les fleurs, mais également interférer avec les processus de reproduction. Au-delà de l'impact des traits défensifs sur la résistance des plantes contre les herbivores et les agents pathogènes, ces réponses peuvent avoir des conséquences plus larges et affecter les membres de la communauté associés aux fleurs, y compris leurs mutualistes carnivores et leurs pollinisateurs. Par conséquent, les plantes ont probablement développé des mécanismes qui optimisent les défenses des fleurs tout en limitant les conséquences négatives pour leur reproduction.

Dans ce projet, j'ai exploré comment les plantes **au stade floral** régulent leur **mécanismes de défense et de reproduction** lorsque les inflorescences sont **attaquées conjointement par plusieurs insectes florivores et par un pathogène**. J'ai étudié les réponses des plantes à un ou deux types d'attaquants, qui ont été introduits sur les plantes lorsque celles-ci commençaient à fleurir, et quel

était l'impact de ces réponses sur la fitness de la plante. J'ai abordé cette question à travers quatre sous-objectifs: 1) mesurer la concentration des phytohormones qui sont impliquées dans la reproduction et la défense; 2) identifier les traits des plantes qui sont impliqués dans la résistance, la tolérance et la reproduction et qui sont modifiés en réponse aux attaques; 3) évaluer si, et comment, la performance des insectes florivores et de leurs ennemis naturels change en réponse aux attaques; 4) déterminer les conséquences de l'attaque des fleurs sur l'attraction des pollinisateurs ainsi que sur la fitness de la plante. Je me suis concentrée sur la moutarde noire *Brassica nigra*, une angiosperme annuelle généralement allogame. **Les inflorescences** de *B. nigra* ont été exposées à trois types d'attaquants qui infestent ou infectent couramment les plantes de la famille des Brassicacée, et qui sont connus pour induire des voies phytohormonales distinctes dans les plantes au stade végétatif: deux insectes florivores, *Brevicoryne brassicae* en tant que puceron qui aspire la sève du phloème et *Pieris brassicae* en tant que chenille qui mastique les tissues végétaux, et, en tant que phytopathogène, la bactérie *Xanthomonas campestris* pathovar *raphani* (Xcr) qui peut infecter n'importe quel stade de développement de la plante et peut se propager jusqu'aux graines.

La régulation hormonale des réponses des plantes aux attaques et leurs conséquences pour la résistance des plantes restent largement inexplorées pour les plantes au stade floral, en particulier lorsque les fleurs sont attaquées. Dans le **Chapitre 2**, je présente une étude qui aborde l'implication potentielle des phytohormones induites lors de l'attaque des inflorescences sur la résistance directe et indirecte des plantes contre les florivores. Pour répondre à cette question, j'ai exposé les inflorescences de *B. nigra* à un ou deux types d'attaquants parmi les insectes et le pathogène suivants : les pucerons *B. brassicae*, les chenilles *P. brassicae* et les bactéries Xcr. J'ai quantifié des phytohormones des feuilles et des inflorescences de *B. nigra* lors de l'attaque, et j'ai lié les concentrations de ces phytohormones à la performance de deux insectes florivores et de leurs parasitoïdes. Je me suis concentrée sur trois types de phytohormones : SA, ABA et les jasmonates (formes actives et catabolites inactifs de JA), sélectionnées pour leur rôle central dans la défense et la reproduction. Les chenilles étaient les principales responsables de l'induction des phytohormones lorsque les inflorescences étaient attaquées par les différentes combinaisons de phytophages et phytopathogènes. Les plantes ont répondu aux chenilles, seules ou conjointement avec un autre attaquant, par une induction de jasmonates dans les inflorescences mais pas dans les feuilles. Les niveaux de jasmonates étaient particulièrement élevés lors des attaques conjointes, et les attaques conjointes ont réduit la performance des chenilles mais

ont augmenté la performance des pucerons sur les inflorescences. En outre, une réduction de la performance de ces insectes était généralement associée à une augmentation de la performance de leur guêpe parasitoïde respective. Ainsi, les attaques conjointes peuvent augmenter la résistance des plantes aux chenilles, mais peuvent également diminuer leur résistance aux pucerons. Pour les plantes exposées à des attaques conjointes, cette résistance accrue aux chenilles pourrait être due à une augmentation de la teneur en composés défensifs dans les tissus de la plante, tandis que cette réduction de la résistance contre les pucerons pourrait être médiée par des changements dans le métabolisme primaire de la plante.

Les ennemis naturels des herbivores peuvent être attirés par l'émission de composés volatils par les plantes lorsque celles-ci sont attaquées. Cette attraction de prédateurs contribue aux mécanismes de résistance indirecte des plantes. Cependant, les composés volatils sont également impliqués dans l'attraction des pollinisateurs vers les fleurs. Ainsi, les changements dans les émissions volatiles des plantes induits lors d'une attaque pourraient aussi perturber l'attraction des pollinisateurs, et ainsi altérer la reproduction des plantes. Dans le **Chapitre 3**, j'ai étudié si et comment *B. nigra* préserve ses interactions avec les pollinisateurs et les parasitoïdes lorsque les inflorescences sont exposées à un ou deux types d'attaquants. Du fait que les composés volatiles émis par les plantes soient exploités par deux mutualistes des plantes, je me suis particulièrement concentrée sur les changements au niveau de l'émission de composés volatiles lorsque les inflorescences de la plante sont attaquées. Les inflorescences de *B. nigra* ont soit été exposées à un ou deux types d'attaquant parmi les insectes et le pathogène suivants : les pucerons *B. brassicae*, les chenilles *P. brassicae* et les bactéries Xcr, ou n'ont soit pas été attaquées et ont été utilisées comme groupe témoin. J'ai étudié la préférence des parasitoïdes lors d'expérience en serre en leur proposant de choisir entre une plante exposée à une attaque par l'insecte hôte seul ou à une attaque conjointe par l'insecte hôte et un non-hôte. J'ai également mesuré les taux de parasitisme sur ces plantes et l'effet d'une attaque simple et double sur la visite des pollinisateurs lors d'une expérience sur le terrain. Les parties aériennes de *B. nigra* ont émis un mélange complexe de composés volatils constitué d'environ 60 composés. Ces changements dans les émissions de composés volatils ont été principalement induits par les attaques simples ou conjointes impliquant des chenilles, et la composition du mélange différait selon la combinaison des attaquants. Une modification de l'émission d'environ 30 composés a contribué le plus à cette différence d'odeurs produites par les plantes attaquées par les chenilles. Ni la préférence du parasitoïde des chenilles (*Cotesia glomerata*), ni celle du parasitoïde des pucerons (*Diaeretiella rapae*),

n'ont été affectées par la présence d'un non-hôte. Les parasitoïdes n'exploitant qu'un sous-ensemble du mélange de composés volatiles, des changements dans l'émission de ces composés par les plantes pourraient n'avoir que peu d'influence sur l'efficacité de la recherche d'hôtes par les parasitoïdes. La réponse des plantes aux attaques n'a pas affecté le nombre total de pollinisateurs attirés vers les plantes, bien que la composition de la communauté de pollinisateurs ait changé après certains types d'attaques. *Brassica nigra* interagit avec au moins 10 espèces de pollinisateurs qui exploitent des indices et des récompenses différentes, et leur alimentation peut être influencée de façon différenciée par des changements dans les traits des plantes. Pour conclure, lorsque leurs inflorescences sont attaquées, il ne semble pas que *B. nigra* ait à faire face à un compromis entre l'attraction des parasitoïdes et l'attraction des pollinisateurs. La résilience des parasitoïdes et de la communauté de pollinisateurs face aux changements dans les émissions volatiles, parmi d'autres traits végétaux, pourrait être facilitée par le mélange complexe de composés volatiles ainsi que par la diversité de pollinisateurs associés aux fleurs de *B. nigra*.

Dans le **Chapitre 4**, j'explore les mécanismes métaboliques associés à la tolérance et à la résistance de *B. nigra* contre l'attaque de leurs inflorescences. Cette étude a caractérisé la façon dont deux insectes florivores et un phytopathogène, attaquant les inflorescences seuls ou conjointement, affectent les concentrations de métabolites qui interviennent dans la résistance des plantes et qui contribuent aux mécanismes de tolérance aux attaques. J'ai mesuré la composition et les concentrations totales 1) des métabolites primaires (acides aminés contenus dans les protéines, acides aminés libres, et sucres solubles) qui fournissent des blocs de construction et de l'énergie aux processus physiologiques des plantes; 2) de métabolites secondaires qui sont généralement impliqués dans la résistance directe chez *B. nigra* (glucosinolates) ; les concentrations de ces métabolites ont été mesurées dans les feuilles et les inflorescences des plantes attaquées. Les changements dans la biomasse des inflorescences, des feuilles et des racines des plantes attaquées ont été mesurés pour déterminer si et comment les plantes en fleur compensent les dommages infligés à leurs tissues par les insectes phytophages et la bactérie. Les métabolites primaires étaient en effet 1,2 à 4 fois plus concentrés dans les inflorescences que dans les feuilles, ce qui reflétait le fort investissement de ressources vers la reproduction chez *B. nigra*. Les inflorescences étaient défendues constitutivement grâce à une concentration 7 fois plus élevée de glucosinolates que dans les feuilles. En particulier, 99% de la quantité de glucosinolates contenus dans les inflorescences était de la sinigrin, un allyle glucosinolate généralement néfaste aux insectes

masticateurs. Les attaques n'ont pas induit d'augmentation de la concentration en glucosinolates dans les inflorescences, et seulement les concentrations de glucosinolates contenus dans les feuilles ont changé sous l'effet de certains types d'attaques. Parmi les différentes situations d'attaque simple par une seule espèce ou conjointe par deux espèces, seules les attaques qui impliquaient la bactérie Xcr ont induit des changements dans la concentration des glucosinolates foliaires, ce qui pourrait éventuellement limiter la propagation des bactéries des inflorescences vers les feuilles. Les chenilles florivores peuvent manger et endommager une grande quantité de boutons et de fleurs, et le prélèvement de sève du phloème par les pucerons peut parfois tuer les fleurs. Les données sur la biomasse de *B. nigra* suggèrent que les parties des inflorescences qui ont été endommagées lors de l'attaque repoussent rapidement, ce qui permettrait à la plante de compenser l'effet des dégâts dus aux attaques. Cette repousse pourrait être favorisée par des changements dans la concentration en sucres solubles mesurés dans les feuilles et les inflorescences après l'attaque de la plante; aucun changement n'a été perçu pour les concentrations d'acides aminés libres et d'acides aminés contenus dans les protéines. Les résultats suggèrent que *B. nigra* résiste aux attaques grâce à des mécanismes de résistance directe qui sont constitutifs et inductibles, même si l'étude n'a montré une induction de glucosinolates que dans les feuilles et non dans les inflorescences de la plante. Les attaquants étant des spécialistes adaptés aux niveaux constitutifs élevés en glucosinolates, il est possible qu'ils ne soient pas affectés par une augmentation des concentrations de glucosinolate lors de l'attaque et la plante ne bénéficierait donc pas d'une induction des glucosinolates. D'autres composés défensifs, qui n'ont pas été étudiés dans ce projet, pourraient intervenir dans la résistance directe de *B. nigra*. Contre les attaquants qui survivent à cette barrière constitutive, les plantes peuvent tolérer leurs dégâts en ayant recourt à des processus tels que la repousse, qui peuvent être facilités par des changements dans le contenu de la plante en sucres solubles. Les inflorescences des plantes sont d'importants puits de ressources, et lorsque les fleurs sont attaquées, les feuilles et les racines restées intactes peuvent fournir les ressources nécessaires pour compenser les dommages faits aux fleurs.

Lorsque les plantes sont attaquées au début de leur période de développement végétatif, leur réponse aux attaques peut influencer la composition de la communauté d'arthropodes qui s'installe sur la plante, et cet effet peut persister au cours de la saison de développement de la plante. Du fait que les florivores puissent fortement affecter le succès reproductif des plantes, l'effet de l'attaque des fleurs sur la composition de la communauté de florivores qui colonise les plantes au stade

floral pourrait réduire la fitness de la plante. Dans le **Chapitre 5**, je présente une étude de terrain qui avait pour objectif de tester si les réponses de *B. nigra* à l'attaque de leurs fleurs modifient la composition de la communauté de florivores au cours de la période de reproduction des plantes, et quelles en sont les conséquences pour la production de graines par les plantes attaquées. J'ai identifié et compté les insectes florivores qui ont colonisé les inflorescences de *B. nigra* exposées à une attaque simple ou double par les pucerons *B. brassicae*, les chenilles de *P. brassicae*, ou les bactéries Xcr, ou qui ont colonisé les inflorescences de plantes qui n'étaient pas soumises à une attaque. Pour approximer la fitness des plantes, j'ai quantifié le nombre et la biomasse des graines produites. Environ 30 espèces différentes de florivores ont été recensées. Plus de 60% d'entre elles étaient des spécialistes, et seuls ces spécialistes atteignaient des abondances élevées sur les inflorescences. Les plantes se sont efficacement défendues contre l'attaquant initial *P. brassicae*. En effet, au bout d'une semaine la densité de chenilles était presque nulle. En revanche, les pucerons *B. brassicae* sont restés abondants sur les plantes qui l'ont reçu comme attaquant initial par rapport aux plantes qui n'étaient pas exposées initialement à ces pucerons. Au cours de la première semaine suivant l'attaque initiale, *B. brassicae* étaient également plus abondants sur les plantes exposées aux attaques doubles que sur les plantes exposées à un seul type d'attaquant, ce qui confirme les résultats obtenus en serre dans le Chapitre 2. Les réponses des plantes aux attaques initiales n'ont eu qu'un effet transitoire sur la composition de la communauté de florivores qui a ensuite colonisé les inflorescences. Ces changements ont été dus à l'abondance d'une seule espèce: le puceron *Lipaphis erysimi*, un spécialiste des brassicacées, qui n'était présent sur les plantes qu'au cours de la première moitié de la période de floraison et dont l'abondance était globalement faible. Par conséquent, la valeur statistique de cet effet doit être interprétée avec précaution. Dans la seconde moitié de la période de floraison, aucune différence n'a été observée dans la composition de la communauté florivores, et l'attaque des inflorescences par un ou deux florivores ou pathogène n'a pas affecté la production de graines par *B. nigra*. Ainsi, les résultats suggèrent que les réponses de *B. nigra* aux attaques de leurs inflorescences ont principalement influencé les insectes florivores introduits comme attaquants initiaux et ont eu peu d'effet sur la communauté florivores colonisant ensuite les plantes. Les pucerons ont peut-être bénéficié de changements dans les métabolismes primaires sur certains traitements, favorisant le développement de colonies.

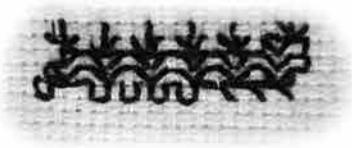
La **Discussion G**énérale présente la contribution de ce projet à la compréhension

d'un paradigme central de la biologie végétale : le compromis entre la défense et la reproduction chez la plante. *Brassica nigra* a efficacement défendu ses inflorescences contre les attaques par un insecte masticateur, un insecte aspirant la sève du phloème, et une bactérie phytopathogénique, attaquant seul ou conjointement les inflorescences. Ce projet a identifié les jasmonates bioactives comme les principales phytohormones régulant la réponse de *B. nigra* contre les attaques de leurs inflorescences. Les jasmonates sont impliquées dans un large éventail de processus physiologiques qui non seulement contribuent à la défense des plantes, mais aussi à leur reproduction. Malgré les changement phytohormonaux induits par l'attaque des inflorescences, les résultats suggèrent que les plantes maintiennent leurs interactions avec les pollinisateurs. *Brassica nigra* interagit avec des pollinisateurs divers et abondants, et le fait que certains pollinisateurs évitent les fleurs de plantes dont les inflorescences sont attaquées pourrait être compensé par l'attraction accrue d'autres types de pollinisateurs.

L'étude de la défense des plantes au cours de leur ontogénie tend à indiquer que lorsque les plantes passent du stade végétatif au stade floral, l'induction de mécanismes de résistance directe diminue tandis que les mécanismes de résistance constitutive augmentent. Les résultats de ma thèse suggèrent que *B. nigra* protègent leurs inflorescences de la colonisation en utilisant des mécanismes de résistance constitutives, notamment grâce à des niveaux élevés de composés défensifs (glucosinolates). La Théorie de la Défense Optimale des plantes propose en effet que la résistance constitutive est une stratégie communément développée par les plantes pour protéger leurs fleurs, puisque celles-ci ont un rôle important pour la fitness de la plante et que la probabilité que les fleurs soient attaquées est particulièrement élevée du fait qu'elles soient nutritives et visibles. La concentration élevée en glucosinolates contenus par les inflorescences a probablement sélectionné l'établissement une communauté de florivores spécialistes, adaptés aux glucosinolates des inflorescences de *B. nigra*. En réponse aux attaques par deux insectes florivores et d'une bactérie spécialiste des Brassicacées, les plantes n'ont pas protégé leurs inflorescences par induction de glucosinolates. Cependant, d'autres composés défensifs pourraient intervenir dans la résistance directe des plantes contre l'attaque de leurs inflorescences. De plus, le mélange de composés volatiles émis par des parties aériennes de la plante a changé après certaines attaques, en particulier lorsque les chenilles *P. brassicae* étaient l'un des attaquants. Malgré ces changements, l'attaque double par un insecte hôte (chenille ou puceron) et un non-hôte n'a pas perturbé le taux de parasitisme des chenilles et des pucerons sur les plantes. La survie des chenilles *P. brassicae* dans le champ était d'environ

1%, et la contribution des prédateurs à la résistance indirecte de *B. nigra* contre les attaquants des fleurs pourrait être l'objet d'études complémentaires. En plus de mécanismes de résistance, les stratégies de tolérance face aux attaques ont probablement permis aux plantes d'atténuer les effets des attaques sur leur fitness. La tolérance des plantes a probablement été médiée par une repousse des parties endommagées des inflorescences, et soutenue par des changements dans le métabolisme des plantes. Cette compensation rapide des dommages infligés aux inflorescences pourrait être typique des plantes annuelles. Les plantes annuelles n'ont en effet qu'une seule occasion de se reproduire et tentent probablement d'atteindre rapidement une compensation complète des conséquences des attaques, alors que les plantes vivaces pourraient séquestrer leurs ressources dans les racines en cas d'attaque, afin de les réinvestir plus tard dans la reproduction, une fois que les attaquants ont disparu ou dans les années suivantes.

Ce projet a contribué à la compréhension générale de la défense des plantes contre les attaques florales. L'attaque des fleurs a jusqu'ici été peu explorée malgré le rôle central des fleurs dans la reproduction des plantes et, par conséquent, leur contribution essentielle à la fitness de la plante. Les pollinisateurs interviennent dans la reproduction d'environ 87% des angiospermes et, pour ces plantes, l'attaque des fleurs par des florivores ou des pathogènes peut être une menace majeure puisque ces derniers peuvent non seulement directement endommager et tuer des fleurs, mais aussi indirectement altérer l'attraction de pollinisateurs par l'induction de changements dans la plante. Les plantes ont probablement évolué sous cette contrainte de protéger efficacement leurs fleurs tout en conservant leurs interactions avec les pollinisateurs, ce qui a probablement conduit à l'énorme diversité de formes, de couleurs et d'odeurs que l'on peut maintenant observer chez les fleurs.



Acknowledgements

When I heard that one could study insects as a job, it turned on a switch in me. Entomologist, my teacher said, *entomo* – for insect. Until then, I had decided to be a palaeontologist, because everyone said that insects were a transient hobby. Now insects became part of my job, and they still contribute to my daily happiness. This teacher, like many other people, guided my way and supported my choices. Maybe I would have been a palaeontologist, and probably I would have been happy too. I realized that it is not so much the outcome of your choices but the reasons why you made them that make a choice a good choice. People I encountered were one of these reasons.

In your own way, from close or afar, you all contributed to keeping me going.

My passion for snails, plants, insects could fully develop under the patient support and everlasting enthusiasm of my family. You always accepted that I bring kilos of rocks into the house, bones or any type of living being. Despite the snails that I once placed underneath your bed to hide them from my cousin and special snails or dead birds that I would keep in the freezer to preserve them and study them, you kept on supporting me. You bought whole fishes for me to dissect their heads, and most of all, you showed me that with passion and care, one could find a path that makes one happy. My parents, Colette and Christian – you have accomplished so much for me to arrive to this point! Zoé – thank you for tolerating all my little ones, even when my lizards would escape into your sleeves! I am glad you found a path you enjoy so much, just keep going toward what makes you passionate. Bernard and Mariette – I am sure that part of my passion for plants and little beasts and drawing comes from you! Anne-Marie, Jacques, Alain, Carole, Martine, Patrick, Catherine, Julien, Linda, Céline, Cali, Alice, Anaïs, Thomas, Léon, Evelyne, Christine, Philippe, Paulette, Gilbert, Martine, Corentin, Julien, Samuel, Jerôme, Cédric, Romain, Sophie, François, Noémie, Alexis, Roland, Véronique, Marius, Geneviève, Bébert, Claudette – I realize the luck I have to have you all around. I always look forward to our enormous family reunions in the middle of the fields, the fishing sessions, the sculptures and wooden art you make, our annual bird watching outings for Christmas with the majestic grey cranes in the Brenne Natural parks, and simply, spending some time with you all. There are also all the little ones, my birds - Isidore, Leica, Azad, Phylllis, Peluche, Perséphone, Boule de neige, Phénol, Aniline, Ambre, Rose, Feu-follet, Chance, Jade, our family cat – Bambou, my fishes, and of course my snails, centipedes, insects, and plants, who always travelled along with me and are part of what I consider my "mobile" home.

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for others, this deep kindness and understanding beyond borders, make you very special to my heart. You have this fascinating ability of making your way towards your aims that keeps on building the admiration I have for you. Marta – sorry for all the more-than-two-legs-pet invasions through the apartment, my enjoyment for weird dishes, and my disgust for your most favoured staple food - cooked tomato and olives! Home always felt lonely without you. I could not have hoped for anyone else but you, mountain-lover, to hike with me across the ravines and tops of the PhD adventure, carried by the rhythm of Queen melodies beating in the air. Even if this hike is now over, I hope to share more rock and rocky adventures with you. The show must go on!

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I am deeply grateful to my teachers, who have accompanied me for many many years. You all opened me to many things that I did not even think were possible. Maryvonne and Jean Couillandre, Annick Diaz – I am always so happy to meet you whenever I come back to France. Thank you Gérard Coupechoux, François Vidril, Ms. Maudet, Anne l'Haridon, Dominique Trumeau, Francis Denise, Guy Monniax, Florent Loué, Philippe Delmas, and many more, at the schools of Ferrière-sur-Beaulieu, Beaulieu-les-Loches, and Loches. Martine Monin – one day you suggested me to read the science popularization book "Le Kama sutra des demoiselles" by Marc Giraud, which had a section on "Le cri du chou",

i.e. the cabbage's cry for help upon attack by herbivorous insects. I was 14, and got fascinated by these plant-insect interactions! I did not imagine at that time that I would later actually work on this topic.

// À tous mes enseignant.e.s et ami.e.s qui m'ont accompagnée pendant de nombreuses années – je vous suis profondément reconnaissante, et c'est une chance d'être passée par votre classe. Vous m'avez tous ouverte à de nombreuses choses que je ne pensais pas possibles. Maryvonne et Jean Couillandre, Annick Diaz – c'est avec joie que je vous revois quand je reviens en France. Merci Gérard Coupechoux, François Vidril, Mme Maudet, Anne l'Haridon, Dominique Trumeau, Francis Denise, Guy Monniaux, Florent Loué, Philippe Delmas et bien d'autres, des écoles de Ferrière-sur-Beaulieu, Beaulieu-les-Beaulieu Loches, et Loches. Martine Monin - un jour, vous m'avez suggéré de lire le livre de vulgarisation scientifique «Le Kama sutra des demoiselles» de Marc Giraud, *et qui comportait une section sur «Le cri du chou», l'appel à l'aide du chou lorsque celui-ci est attaqué par des insectes phytophages. J'avais 14 ans et j'ai été fascinée par ces interactions plante-insecte! Je n'imaginais pas à cette époque que je travaillerais plus tard sur ce sujet.*

Catherine, Klaus, Noah, Mathis, Marie, and Colette – you opened me to unique travel opportunities and developed my taste for languages and travelling. I am not sure I would be hopping from country to country with so much ease and pleasure if I had not met you, some 14 years ago.

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pour ces merveilles quotidiennes, toi montant tes chevaux et moi sur mon vélo.

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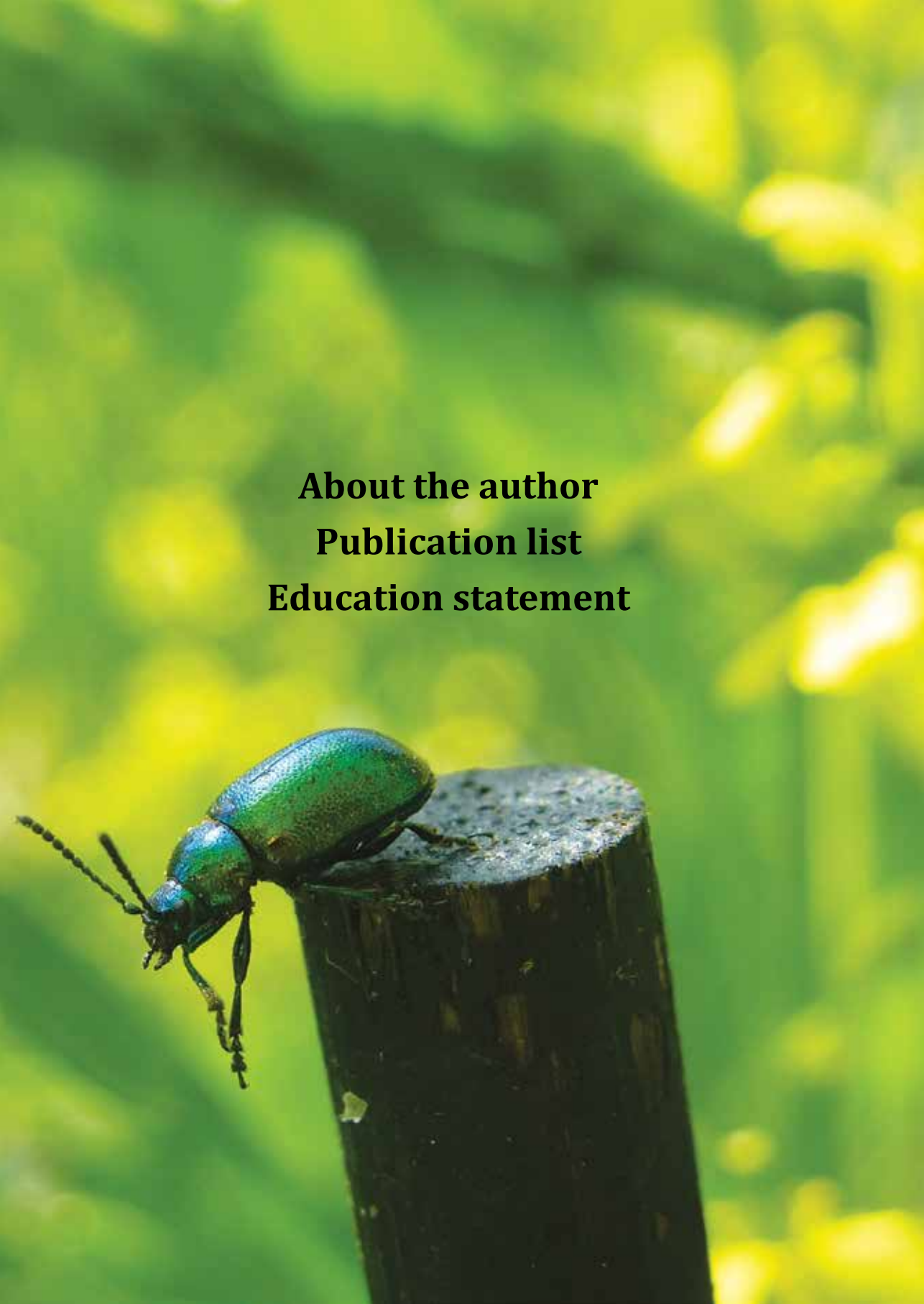
« In every job that must be done, there is an element of fun, you find the fun and snap! The job's a game. » Mary Poppins

About the author

Publication list

Education statement



Lucille Tiphaine Séphora Chrétien was born on September 15th 1990 in Tours, France. She grew up in the countryside of Loches, where she spent weekends biking or hiking through the forest, fields, and along the nearby rivers. This offered her great opportunities to observe nature and to discover the local landscapes, which bear a fascinating historical legacy from Gallo-Roman times to nowadays.

Lucille started collecting rocks and rearing arthropods, molluscs, plants and birds from a very young age. In primary school, she learnt that one could study nature as a job, and she pursued this purpose. Lucille attended secondary school Georges Besse and Alfred de Vigny in Loches. Driven by the wish to develop a scientific carrier, she enrolled at the preparatory school (CPGE) of the Lycée Descartes in Tours, in the "Biology, Chemistry, Physics, and Geology" section (BCPST). This led her to the École Normale Supérieure of Lyon, and a four-year scholarship. After hesitating between geology and biology specialisation, she decided to follow the path of life sciences. She specialized in entomology through Bachelor projects and an internship at the Institute of Research in Insect Biology (IRBI, Tours, France), where she studied mating behaviour of Tettigonidae. During this internship, she heard about the Laboratory of Entomology (Wageningen, The Netherlands), where she decided to go for a Master thesis. In this first Master thesis, she studied plant resistance to a succession of attacks by insects. As Lucille wished to further learn about techniques of chemical ecology, she did a second Master thesis at the Max Plank Institute for Chemical Ecology (MPI CE, Jena, Germany). At the MPI CE, Lucille investigated the influence of plant circadian rhythm on volatile-mediated indirect resistance. She eventually returned to the Laboratory of Entomology to study plant-pollinator interactions in a third Master thesis. In this project, she explored the combined effects of herbivory and pollination on flower preferences by two types of pollinators.

After she graduated from her Master, in September 2014, Lucille worked for a year at the Laboratory of Entomology on developing her PhD project about plant defenses against insects and pathogens attacking their flowers. This PhD project got granted in 2015 and Lucille joined the IRBI and the Laboratory of Entomology. It yielded the results presented in this thesis. Lucille hopes to continue investigating plant-animal interactions. She currently works at the University of Plymouth (England), in collaboration with the German Centre for Integrative Biodiversity Research (iDiv, Leipzig, Germany), on a post-doctoral project studying geographical patterns in defence strategies of plants in the seedling stage along latitudinal and altitudinal gradients, using snails as major threat to seedlings.

List of publications

Joo, Y., Goldberg, J.-K., **Chretien, L.T.S.**, Kim, S.-G., Baldwin, I.T., Schuman, M.C. (2019), The circadian clock contributes to diurnal patterns of plant indirect defense in nature. Accepted for publication in Journal of Integrative Plant Biology.

Chrétien, L. T. S., David, A., Daikou, E., Boland, W., Gershenzon, J., Giron, D., Dicke, M. and Lucas-Barbosa, D. (2018), Caterpillars induce jasmonates in flowers and alter plant responses to a second attacker. New Phytol, 217(3): 1279-1291. doi : 10.1111/nph.14904

Stam, J. M., **Chrétien, L.**, Dicke, M. and Poelman, E. H. (2017), Response of *Brassica oleracea* to temporal variation in attack by two herbivores affects preference and performance of a third herbivore. Ecol Entomol, 42(6): 803–815. doi : 10.1111/een.12455

Chapters 3, 4, 5 to be submitted

Education Statement of the Graduate School

Experimental Plant Sciences

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Date: 24 May 2019
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
▶ **First presentation of your project**		
Challenging the balance between defence and reproduction: hormonal interplay and ecological	06 Mar 2015	1.5
▶ **Writing or rewriting a project proposal**		
Challenging the balance between defence and reproduction: hormonal interplay and ecological	06 Mar 2015	6.0
▶ **Writing a review or book chapter**		
▶ **MSc courses**		
Subtotal Start-Up Phase		7.5

2) Scientific Exposure	date	cp
▶ **EPS PhD student days**		
Get2Gether EPS PhD days, Soest, The Netherlands	28-29 Jan 2016	0.6
Get2Gether EPS PhD days, Soest, The Netherlands	15-16 Feb 2018	0.6
▶ **EPS theme symposia**		
Symposium: EPS theme 2, Utrecht, The Netherlands	20 Feb 2015	0.3
Symposium: EPS theme 2, Leiden, The Netherlands	22 Jan 2016	0.3
Symposium: EPS theme 2, Wageningen, The Netherlands	23 Jan 2017	0.3
Symposium: EPS theme 2, Amsterdam, The Netherlands	24 Jan 2018	0.3
▶ **National meetings (e.g. Lunteren days) and other National Platforms**		
Entomologendag, Ede, The Netherlands	19 Dec 2014	0.3
Netherlands Annual Ecology Meeting, Lunteren, The Netherlands	10-11 Feb 2015	0.6
Entomologendag, Ede, The Netherlands	18 Dec 2015	0.3
Entomologendag, Ede, The Netherlands	15 Dec 2017	0.3
▶ **Seminars (series), workshops and symposia**		
Symposium: 3rd Wageningen PhD Symposium, Wageningen, The Netherlands	26 Mar 2016	0.3
Workshop: Insect-Plant Interaction workshops, Utrecht, The Netherlands	03 Nov 2014	0.3
Workshop: Volatiles workshop, NIOO, Wageningen, The Netherlands	02 Mar 2016	0.3
Workshop: Journée de l'Institut de Recherche sur la Biologie de l'Insecte (IRBI), Tours, France	30 Jun 2016	0.2
Workshop: Insect-Plant Interaction workshops, Leiden, The Netherlands	22 Nov 2016	0.3
Workshop: Insect-Plant Interaction workshops, Wageningen, The Netherlands	07 Nov 2017	0.3
Workshop: Yearly Entomology Laboratory Research Exchange Meeting (YELREM), Renkum, The Netherlands	10 Jun 2015	0.3
Workshop: Yearly Entomology Laboratory Research Exchange Meeting (YELREM), Renkum, The Netherlands	01 Jun 2016	0.3
Workshop: Yearly Entomology Laboratory Research Exchange Meeting (YELREM), Renkum, The Netherlands	01 Jun 2017	0.3
Workshop: Yearly Entomology Laboratory Research Exchange Meeting (YELREM), Renkum, The Netherlands	24 May 2018	0.3
▶ **Seminar plus**		
▶ **International symposia and congresses**		
Symposium: European PhD Network in Insect Sciences, Nice, France	27-29 Oct 2016	0.9
Symposium: Gordon Research Seminar on Plant-Herbivore Interactions, Ventura, USA	11-12 Feb 2017	0.6
Conference: Gordon Research Conference on Plant-Herbivore Intereactions, Ventura, USA	12-17 Feb 2017	1.5
Symposium: 16th Symposium on Insect-Plant Interactions, Tours, France	02-06 Jul 2017	1.5
▶ **Presentations**		
Talk: How to reach out with your science? 3rd Wageningen PhD Symposium	26 Apr 2016	1.0
Talk: Flowers under multiple attack by two insect species and a pathogen: facilitation or competition? European PhD Network in Insect Sciences (Nice, france)	28 Oct 2016	1.0
Talk: Can flowering plants resist insect and pathogen attack? EPS Theme 2 Symposium	23 Jan 2017	1.0
Talk: Can flowering plants resist insect and pathogen attack? Induction of phytohormones and resistance of plants under multiple attack. 16th Symposium on Insect-Plant Interactions (Tours, France)	04 Jul 2017	1.0
Talk: Can flowering plants resist insect and pathogen attack? Induction of phytohormones and resistance of plants under multiple attack. EPS PhD days - Get2Gether	15-16 Feb 2018	1.0
Poster: Challenging the balance between defence and reproduction, Netherlands Annual Ecology	11 Feb 2015	1.0
Poster: Challenging the balance between defence and reproduction, Entomologendag	18 Dec 2015	0.0
Poster: Can a flowering plant resist insect and pathogen attack, Gordon Research Seminar on Plant-Herbivore Interactions	11 Feb 2017	1.0
Poster: Can a flowering plant resist insect and pathogen attack, Gordon Research Conference on Plant-Herbivore Interactions	15 Feb 2017	0.0
▶ **IAB interview**		
▶ **Excursions**		
Subtotal Scientific Exposure		18.3

3) In-Depth Studies	date	cp
► **EPS courses or other PhD courses**		
Advanced Statistics Course Design of Experiments (WIAS & PE&RC)	25-27 May 2016	0.8
Generalized Linear Models (PE&RC & SENSE)	20-21 Jun 2016	0.6
Mixed Linear Models (PE&RC & SENSE)	27-28 Jun 2016	0.6
► **Journal club**		
Insect-Plant Interaction discussion group at Entomology	2014-2018	1.5
Laboratory of Entomology PhD discussion group	2014-2018	1.5
► **Individual research training**		
Subtotal In-Depth Studies		5.0

4) Personal Development	date	cp
► **Skill training courses**		
PhD Competence Assessment (WGS)	02 Feb 2016	0.3
Course: EPS introduction	11 Feb 2016	0.3
Course: Techniques for Writing and Presenting a Scientific Paper	08-11 Mar 2016	1.2
Mini-symposium: Working outside academia, NIOO, Wageningen, The Netherlands	29 Sep 2016	0.2
Course: Reviewing a scientific paper	23 Mar 2017	0.1
Course: Adobe Indesign Essential training	04-05 Jun 2018	0.6
Workshop: Last stretch of the PhD & writing propositions	19 Jun 2018	0.0
► **Organisation of PhD students day, course or conference**		
Organization of the Insect-Plant-Interaction meeting every other week at the Laboratory of Entomology	2014-2016	1.5
► **Membership of Board, Committee or PhD council**		
Subtotal Personal Development		4.2

TOTAL NUMBER OF CREDIT POINTS*	**35.0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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