Ecologie chimique de l'interaction colza - méligèthe : vers de nouvelles stratégies de contrôle des insectes ravageurs ?

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PhD thesis

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Chemical ecology of the oilseed rape – pollen beetle interaction: towards new control strategies for insect pests?

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– Table of contents –

– INTRODUCTION –

– Scientific and agronomical context –

THE PLANT – PHYTOPHAGOUS INSECTS ARMS RACE

In all ecosystems taken together, phytophagous insects are considered to consume around 20 % of the biomass produced annually by plants (Agrawal 2011). During the 415 million years of coevolutionary history shared by these two taxa (Ehrlich & Raven 1964; Labandeira 2007), plants have evolved complex and multiple defense strategies against these enemies while insects evolved sophisticated counteradaptations (Gatehouse 2002; Mello & Silva-Filho 2002).

Plant defenses against insects

Apart from a few gene-for-gene resistances (Flor 1942, 1945) known especially in plant – aphid systems (Smith & Boyko 2007), plant resistance to insects is often complex, involving many different traits (Walling 2000; Mello & Silva-Filho 2002; Agrawal & Fishbein 2006; Agrawal 2011). It is often more appropriate to speak about 'defense syndromes' than singleton strategies (Agrawal & Fishbein 2006). Defense traits can be either physical or chemical, but can also be related to plant phenology or growth rate (Mello & Silva-Filho 2002; Carmona *et al.* 2011).

Since the founding paper of Fraenkel (1959), plant resistance to insects is mainly seen through the prism of so-called secondary metabolites, those compounds not involved in primary physiological functions such as growth and reproduction (Berenbaum & Zangerl 2008; Hartmann 2008). Secondary substances can act as repellents, antifeedants, digestibility reducers or toxins (Mello & Silva-Filho 2002; Hartmann 2007). Many of them are typical of one or a few plant families, *e.g.* cardenolides (Agrawal *et al.* 2012), furanocoumarins (Murray *et al.* 1982), glucosinolates (Fahey *et al.* 2001) or pyrrolizidine alkaloids (Hartmann 1991). This irregular distribution of defensive compounds in the plant kingdom is one of the main factors explaining the ecological specialization of the majority of herbivorous insects, for either feeding or oviposition resources (Fraenkel 1959; Ehrlich & Raven 1964; Jaenike 1990; Hartmann 2007). Indeed, species feeding on more than three plant families (*i.e.* generalists) are thought to represent less than 10 % of all phytophagous insects (Bernays & Graham 1988). According to the well-accepted theory specialists should be more efficient than generalists in tolerating specific defense compounds (those of the plant family on which they are specialized), but on the other hand should tolerate a smaller diversity of toxic metabolites (Krieger *et al.* 1971; Whittaker & Feeny 1971). These specific substances should even attract specialists toward their host plant and possibly act as feeding and/or oviposition stimulants, whereas the effect on generalists should be at the opposite (Bruce *et al.* 2005; Hartmann 2007; Ali & Agrawal 2012).

However, the 'generalist – specialist' paradigm, although practical as a first approximation, has to be put into perspective for at least two reasons. Firstly because 'generalist species' often hide complexes of specialized but still opportunistic populations, or even complexes of cryptic species (Loxdale *et al.* 2011). Secondly because examples are known of specialist species being negatively affected by secondary metabolites produced by their host plant (Ali & Agrawal 2012). Furthermore, the same species can be considered either as generalist or as specialist depending of its life stage. Many phytophagous insects (especially lepidopteran species) are generalist pollen or nectar feeders as adults, whereas larvae are specialists of a certain plant family.

If secondary metabolites proved to be an important part of the plant – insect interaction puzzle, primary compounds were probably too neglected (Berenbaum 1995). Low nutritional quality is a selected direct defense strategy in some plant species (Agrawal & Fishbein

2006). Clancy & Price (1987) suggested that it could also be an indirect defense, by lengthening insect development time and so extending the vulnerability window to natural enemies (the 'slow growth – high mortality' hypothesis). Primary metabolites play a major role in determining plant nutritional quality (Awmack & Leather 2002). Hence, concentration in some of these compounds might be under selection due to herbivore pressure (Berenbaum 1995). Certain primary substances are also important phagostimulant compounds, especially sugars (Chapman 2003). Even if insects are to some extent able to adjust their dietary intake depending on the nutritional value of their food (Behmer 2009), appetability (*i.e.* ability to chemically stimulate feeding) of plant tissues may partly explain patterns of food consumption.

However, plant chemistry may not be the sole key to understand the ecology and evolution of plant – insect interactions. In a recent meta-analysis, Carmona *et al.* (2011) found that life-history traits (*e.g.* flowering time or growth rate) were by far the most efficient to predict plant susceptibility to insect herbivores. Architecture and plant size were also shown to play an important role, supporting the 'plant vigor hypothesis' proposed by Price (1991) that states that insect herbivores should prefer more vigorous (*i.e.* faster-growing and larger-size) plants (Cornelissen *et al.* 2008; Carmona *et al.* 2011). Finally, plant physical traits such as tissue toughness or trichome shape/size/density are recognized resistance factors, although they are much less studied than plant chemistry (Clissold *et al.* 2009; Carmona *et al.* 2011).

Defense traits are generally not expressed at their highest level when the plant is healthy. Since constitutive defenses arise with a cost for plants (Strauss *et al.* 2002; Wittstock & Gershenzon 2002; Kempel *et al.* 2011), an important part of plant defense strategies relies on inducible defenses, which are expressed only after herbivore recognition or attack (Howe & Jander 2008; Wu & Baldwin 2010). This recognition is based either on microbial-, pathogen- or damage-associated molecular patterns (MAMPs, PAMPs and DAMPs, respectively), elicitors that are often present in insect oral secretions or oviposition fluids (Howe & Jander 2008; Wu & Baldwin 2010; Hilker & Meiners 2011; Bonaventure 2012; Erb *et al.* 2012). Through a cascade of transcriptomic, metabolomic and proteomic rearrangements regulated essentially by the phytohormones jasmonic acid, salicylic acid and ethylene, novel or higher-intensity defenses are then expressed, locally or systemically (Howe & Jander 2008; Wu & Baldwin 2010; Erb *et al.* 2012). Part of these defenses are indirect; they attract herbivore natural enemies (especially parasitoids but also invertebrate predators and birds) by means of modified and specific odor bouquets (Dicke & van Loon 2000; Holopainen 2004; Howe & Jander 2008; Unsicker *et al.* 2009; Karban 2011). Even without being attacked, plant defenses can be primed by volatile methylated phytohormones (methyl-jasmonate and methyl-salicylate) emitted by conspecifics (Holopainen 2004; Howe & Jander 2008; Karban 2011).

Finally, plant defense cannot be reduced to resistance. Indeed, a second but less studied part of defense strategies is tolerance, *i.e.* capacity to regrow and/or reproduce after being damaged by phytophagous insects (Marquis 1996; Strauss & Agrawal 1999; Fornoni 2011). Although a trade-off between tolerance and resistance is classically supposed, it seems to be more context-dependent than previously thought (Fornoni 2011). Tolerance is a complex mechanism that depends both on the plant species and on the feeding guild of the herbivorous insect (Marquis 1996). Many different processes leading to tolerance have been shown, including increased photosynthetic or growth rate, increased branching or increased resource allocation from root to shoot (Strauss & Agrawal 1999).

Insect counteradaptations to plant defenses

Insects are not passive victims of plant defenses. The coevolutionary process between them and plants led to adaptations allowing avoidance, tolerance or even reuse

of plant chemical defenses (Gatehouse 2002; Karban & Agrawal 2002; Mello & Silva-Filho 2002; Després *et al.* 2007). This arms race is thought to be the cause of both the enormous phylogenetical diversification in the two taxa and the co-cladogenesis that they share (Fraenkel 1959; Ehrlich & Raven 1964; Farell *et al.* 1992; Després *et al.* 2007).

Study of signal transduction and genetic bases of resistance is much more recent on the 'insect side' compared to the 'plant side'. However, mechanisms leading to resistance to plant chemicals are now well described and numerous examples are known. Insects can avoid hosts, or organs within an individual plant, that are the most concentrated in defensive metabolites (Karban & Agrawal 2002; Després *et al.* 2007). Chemically-mediated host selection behavior can concern either feeding or oviposition, be genetically determined or learnt after having experienced these compounds (Karban & Agrawal 2002). Herbivores can also rapidly excrete plant toxins or sequester them for further reuse against their own natural enemies (Karban & Agrawal 2002; Després *et al.* 2007). Sequestration has been well-studied in the Lepidoptera family (Nishida 2002). An important and widely expressed mechanism preventing poisoning by plant chemicals is metabolic resistance, *i.e.* detoxification. It is realized essentially by three enzyme super-families: cytochrome P450 monooxygenases, glutathione S-transferases and carboxylesterases (Després *et al.* 2007). Overproduction of these enzymes has been shown in several insect species to be induced after contact with plant allelochemicals (Karban & Agrawal 2002; Després *et al.* 2007). Production of protease inhibitors is another well-known plant defense against phytophagous insects. These proteins tend to block insect digestive process (Casaretto & Corcuera 1995). In reaction, detection of such proteins often induces massive production of inhibitor-insensitive proteases in the insect gut (Gatehouse 2002; Mello & Silva-Filho 2002).

Rather than just being in a defensive position, insects also evolved 'aggressive' adaptations. These traits have been grouped under the term 'herbivore offense' (Karban & Agrawal 2002). They all involve manipulation of the host plant, often to avoid defense induction. For example, cutting canals transporting defensive compounds prior to feeding, or inhibiting the signal transduction at the basis of plant response to herbivory by means of salivary elicitors (Karban & Agrawal 2002; Després *et al.* 2007).

Resistance traits are not exclusive, and several are often used in the same species. The caterpillar of the monarch butterfly (*Danaus plexippus*) for example, which feeds on milkweeds (*Asclepias* spp., plants that produce toxic cardenolides), uses at the same time vein cutting behavior, sequestration of certain compounds and detoxification of others (Marty & Krieger 1984; Nishida 2002; Helmus & Dussourd 2005). As for plant defense traits, insect resistance traits are not always expressed at their highest level. Metabolic costs of resistance are at the origin of an equilibrium between constitutive and induced defenses, which partly depends on the degree of specialization of the herbivore (Gatehouse 2002; Després *et al.* 2007).

Understanding the fundamental bases of the plant – insect arms race has been a challenge for decades. However, considerable progress has been made and studies range now from the single gene to the whole community, also including higher trophic levels (predators and parasitoids), insect symbionts and soil microbial communities. The development of 'ecogenomics' since the 2000's is a striking example of how integration of several levels that were previously studied separately is now possible (Zheng & Dicke 2008; Anderson & Mitchell-Olds 2011). From an applied point of view, the increasing comprehension of plant – insect interactions allows the consideration of new methods of crop protection against insect pests.

INSECT PESTS AND CROP PROTECTION

Insects are one of the major threats to agricultural production, being responsible for about 10-15 % of yield losses (Oerke 2006). Since the middle of the 20th century, strategies to control their damage to crops have been aimed essentially at eradicating their populations. Different methods such as insecticides, Bt-transformed plants or qualitatively resistant plant (through introgression of R genes, *e.g.* Gallun & Hatchett 1969; Hatchett & Gallun 1970 or McKenzie *et al.* 2002)) have been used but their objective was always the same: free the field of pests.

These 'qualitative' strategies proved to be very efficient in the short term (*e.g.* Carpenter 2010), but of low durability. The selection pressure they impose on pest populations is so strong that any mutant resistant to the mechanism employed has an enormous fitness gain. Consequently, resistance to qualitative strategies is likely to occur. It is not surprising that insecticide resistance is so common in pest populations. It is also probable that the methods that still work today will be circumvented by targeted insects in the near future.

The alternative to qualitative strategies is 'quantitative' control, *i.e.* limiting damage caused by insect pests rather than trying to completely suppress it. The immediate benefit for the crop is lower compared to qualitative control, but the strategy can still be efficient, durable and more considerate of ecosystem functioning. Several control tactics can be used simultaneously, increasing both efficiency and durability. This is the basis of Integrated Pest Management (IPM). Besides improved agronomical or cultural practices, IPM can for example rely on behavioral manipulation of the pest and/or its natural enemies. Some examples of such strategies are pheromone lures, trap cropping (the 'push and pull' strategy) or flower strips to enhance biological control by natural enemies (Foster & Harris 1997; Cook *et al.* 2007).

Another interesting and complementary strategy that could be used in IPM is increasing, by means of classical selection, natural plant resistance to insect pests (Gatehouse 2002). Major limitations to this strategy are presented in detail in *Article 1* of the thesis. To summarize, most of these hurdles come from the phenotyping process. Indeed, logistical and conceptual constraints imposed by insects make even medium-scale phenotyping extremely difficult to achieve. In this thesis we propose an alternative method to avoid using insects for phenotyping (detailed in *Article 1*). Briefly, it consists of identifying key plant traits that determine how much/well the targeted pest (i) is attracted to this plant, (ii) feeds upon it, (iii) produces and lays eggs on it, and (iv) develops on/in it. The identification of these key traits can be performed through studies, in strictly controlled conditions, using a small panel of genotypes of the plant species to be protected. Candidate key traits are first identified by correlating data obtained on the insect (attraction, feeding intensity etc) and data obtained on the plant (primary and secondary metabolites, trichomes, size etc). These traits can then be validated by specific experiments (*e.g.* supplementation, under- or over-expression). If key traits are identified, *i.e.* if they are good predictors of plant resistance, it should then be possible to conduct selection on the sole basis of these traits, without needing any insect. Testing this approach is the objective of this thesis.

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– Study system –

This work focuses on the bipartite interaction between a cultivated plant, oilseed rape (*Brassica napus* L.; Brassicaceae: Brassiceae), and a major insect pest of this crop, the pollen beetle (*Meligethes aeneus* F.; Coleoptera: Nitidulidae).

Note: a recent re-examination of the taxonomy of the subfamily Meligethinae separated the paraphyletic genus *Meligethes* into several new genera. Among numerous taxonomic rearrangements, species feeding on brassicaceous plants were attributed to the new genus *Brassicogethes* (Audisio *et al.* 2009). Although the pollen beetle that we studied should be called *Brassicogethes aeneus*, we chose to stay consistent with the existing bibliography (even most recent articles) by using the name *Meligethes aeneus*.

OILSEED RAPE

At the evolutionary scale, oilseed rape is a very recent species. It seems to result from the hybridization of two *Brassica* species that were cultivated in the same gardens in the Middle Ages: turnip rape (*Brassica rapa*) and cabbage (*B. oleracea*) (U 1935; Doré & Varoquaux 2006; Allender & King 2010). *Brassica napus* is probably only cultivated, since no wild form is known (Gómez-Campo & Prakash 1999). This, combined with a strong selection aiming essentially at improving seed quality and increasing oil yield, led to a relatively low genetic diversity in actual varieties (Hasan *et al.* 2006).

Oilseed rape is one of the four major oil crops in the world (the other three being oil palm, soybean and sunflower), and is the first to be grown in Europe (FAOSTAT). Two types of cultivars exist: (i) winter oilseed rape, sown in late summer and flowering in early spring (winter vernalization is obligatory to induce bolting), and (ii) spring oilseed rape, sown in early spring and flowering in summer (no vernalization is needed). French production is comprised almost exclusively of winter oilseed rape (99.8 % in 2014) (CETIOM). In this thesis we focus on this type.

The long cycle of winter oilseed rape cultivation (about 10-11 months from sowing to harvest) exposes

Fig. 1 Some insect pests of oilseed rape in France: the cabbage stem flea beetle *Psylliodes chrysocephala*, the turnip sawfly *Athalia rosae*, the rape stem weevil *Ceutorhynchus napi*, the rape winter stem weevil *Ceutorhynchus picitarsis*, the Brassica pod midge *Dasineura brassicae* and the pollen beetle *Meligethes aeneus*

plants to attacks from many different pests, among which are several insects (Fig. 1). Consequently, pesticide application level is high in this crop compared to common wheat (1.5 times more pesticide application on oilseed rape) or maize (2.9) (French Ministry of Agriculture 2013). Introgression of resistance to diseases has been conducted for many years, protecting fields against several devastating pathogens (*e.g.* the Phoma stem canker *Leptosphaeria maculans*, the clubroot agent *Plasmodiophora brassicae* or the light leaf spot agent *Pyrenopeziza brassicae*). However, no factor of resistance to insects is known to date, explaining why insecticides represent the largest part (44 %) of applied pesticides to the crop (French Ministry of Agriculture 2013).

THE POLLEN BEETLE

Life cycle

Adult pollen beetles overwinter in the leaf litter, essentially in semi-natural habitats, from late summer to early the next spring (Williams 2010; Rusch *et al.* 2012). Diapause termination is induced when temperatures reach 10 °C (Nilsson 1988a). Adults are generalist pollen feeders (Free & Williams 1978; Ekbom & Borg 1996; Carrié *et al.* 2012; Marques & Draper 2012). At emergence they feed on pollen from plants of many different families, which is obligatory for female sexual maturation (Williams 2010). Oviposition, however, is restricted to brassicaceous plants (Free & Williams 1978; Ekbom & Borg 1996). Beetles quicly seek host plants where to lay eggs. Flights are induced when temperatures reach 10 °C but major migrations occur above 15 °C (Free & Williams 1978; Ferguson *et al.* 2014). At this time period (usually from late February to mid-March in France), winter oilseed rape fields are at the bud stage, *i.e.* no flowers are open yet. Adults mate on the plant (Fig. 2A) and females oviposit in flower buds, after having made a small hole at the bud base (Fig. 2B) (Free & Williams 1978; Nilsson 1998b; Williams 2010). Adults feed on the same plant where eggs are laid, by piercing flower buds when flowers are still closed (Fig. 2C) and on open flowers as soon as blossoming starts (Fig. 2D) (Williams 2010). Eggs hatch inside the bud where they were laid (Fig. 2E) and first-instar larvae stay inside this bud, feeding essentially from the pollen contained in anthers (Cook *et al.* 2004a). The second (and last) instar is reached approximately at bud opening (Fig. 2F). Larvae move from flower to flower, still feeding on pollen (Williams & Free 1978). After about two weeks, larvae drop down to the soil where they pupate, usually in the first centimeters (Williams 2010). New generation adults emerge at the beginning of summer, feed from the pollen of many different plant families and seek overwintering sites in late summer (Williams & Free 1978).

Fig. 2 A: *Meligethes aeneus* adults mating on an oilseed rape bud. B: oviposition holes. C: adult feeding on a closed flower bud. D: adults feeding in an open flower. E: eggs laid inside a flower bud. F: first (right) and second (left) larval instars

Agronomical damage

Pollen beetles are florivores that most of the times act as pollinators. Agronomical damage is usually only caused when adults feed from flower buds, before floewering starts (Williams 2010). Indeed, to reach the pollen they seriously damage the bud (Fig. 3A), leading to its abscission (Fig. 3B). On the other hand, oviposition, adult feeding on open flowers and larval feeding provoke little damage unless populations are unusually large (Williams 2010). The vulnerability window of oilseed rape crops to the pollen beetle is hence very limited in comparison to the duration of the growing phase. The plant also shows a high ability to compensate for beetle damage, mainly by producing more racemes, more pods per raceme and more seeds per pod (Williams 2010). However, if pollen beetle attacks are massive, arise at the early bud stage and if plants are stressed (*e.g.* because of a lack of nitrogen in winter), yield losses can reach up to 70 % (*e.g.* Nilsson 1987).

Control strategies

Pollen beetles were historically controlled by spraying insecticides, especially pyrethroids. However, resistance to these compounds appeared in the late 1970's and is now widespread throughout Europe (Lakocy 1977; Hansen 2003; Slater *et al.* 2011; Heimbach & Müller 2012; Wrzesińska *et al.* 2014; Zimmer *et al.* 2014). Research to find novel compounds or compound mixtures is still going on (*e.g.* Palagacheva *et al.* 2014), but new strategies are also developed.

Considerable efforts have been made to set up trap cropping methods. One possibility is to grow plants that flower before oilseed rape, when the crop is at its susceptible stage. Since pollen beetles are more attracted to flowering than non-flowering hosts, they should prefer the trap plants. Turnip rape, *B. rapa*, appeared to be a good candidate (Cook *et al.* 2004b, 2006, 2007a; Nerad *et al.* 2004; Nilsson 2004; Frearson *et al.* 2005). Another possibility is to use closely related companion plants that would attract pollen beetles, avoiding intensive colonization of the crop to be protected (Veromann *et al.* 2012; Kaasik *et al.* 2014a, 2014b).

In parallel, enhancement of biological control by pollen beetle natural enemies is another option that is studied. It consists either of attracting larval parasitoids by means of companion plants or conservation strips (Büchi 2002; Kaasik *et al.* 2014a, 2014b; Kovács *et al.* 2014; Scheid *et al.* 2011), spraying pathogenic fungi (Hokkanen 1993; Husberg & Hokkanen 2001) or using entomopathogenic nematodes (Nielsen & Philipsen 2005).

In line with the principles of IPM, research is being also conducted on several other methods: adapting agronomical practices (Valantin-Morison *et al.* 2007; Valantin-Morison & Meynard 2008; Veromann *et al.* 2009, 2013), diffusing repellent volatile compounds

Fig. 3 A: adult *Meligethes aeneus* destroying a flower bud to get the pollen it contains. B: difference between a bud abscissed due to pollen beetle damage (black arrow) and buds naturally desiccated (white arrows)

(Mauchline *et al.* 2005, 2008, 2013; Pavela 2011), using botanical insecticides (Pavela 2011; Dorn *et al.* 2014), transforming oilseed rape to make it produce toxic lectins (Åhman & Melander 2003; Melander *et al.* 2003; Åhman *et al.* 2006, 2009; Lehrman *et al.* 2008) or even… manipulating oilseed rape petal color (Cook *et al.* 2013)!

All these alternative strategies are currently in development. Among these, trap cropping within the 'push and pull' principle (Cook *et al.* 2007b) appears particularly promising. However, the pollen beetle remains a major threat for oilseed rape cropping. Other, complementary, tactics are still needed. In particular, no cultivar is known to be resistant to this pest.

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– Objectives and organization of the manuscript –

The objective of this thesis is to identify candidate key traits determining oilseed rape resistance to the pollen beetle, using the approach we propose. The four points we mentioned in the 'Scientific and agronomical context' section are studied: attraction, feeding intensity (of adults), egg production and oviposition, and larval development.

These four points have been more or less extensively studied at the interspecific scale, by comparing different plant species of the Brassicaceae family. Results of these studies are summarized in Fig. 1. From an agronomical point of view, two main conclusions emerge from this summary: (i) most of studies that have been conducted concern oviposition; however, neither oviposition *per se* nor larval development cause significant damage to the crop. (ii) On the contrary, almost nothing is known about the crucial behavior of the beetle that cause damage, *i.e.* adult feeding.

At the intraspecific scale and to our knowledge, very few elements are available. Three studies linked the glucosinolate content (secondary metabolites that are typical of a few plant families including Brassicaceae (Fahey *et al.* 2001)) of different lines/cultivars and their colonization by the pollen beetle. All three had a different conclusion: Milford *et al.* (1989) found no relationship, Giamoustaris & Mithen (1996) found a negative relationship (Fig. 2A) and Cook *et al.* (2007) found a positive relationship (Fig. 2B). On other aspects, a recent three-year field experiment comparing four oilseed rape cultivars (Tölle & Ulber 2013) led to two

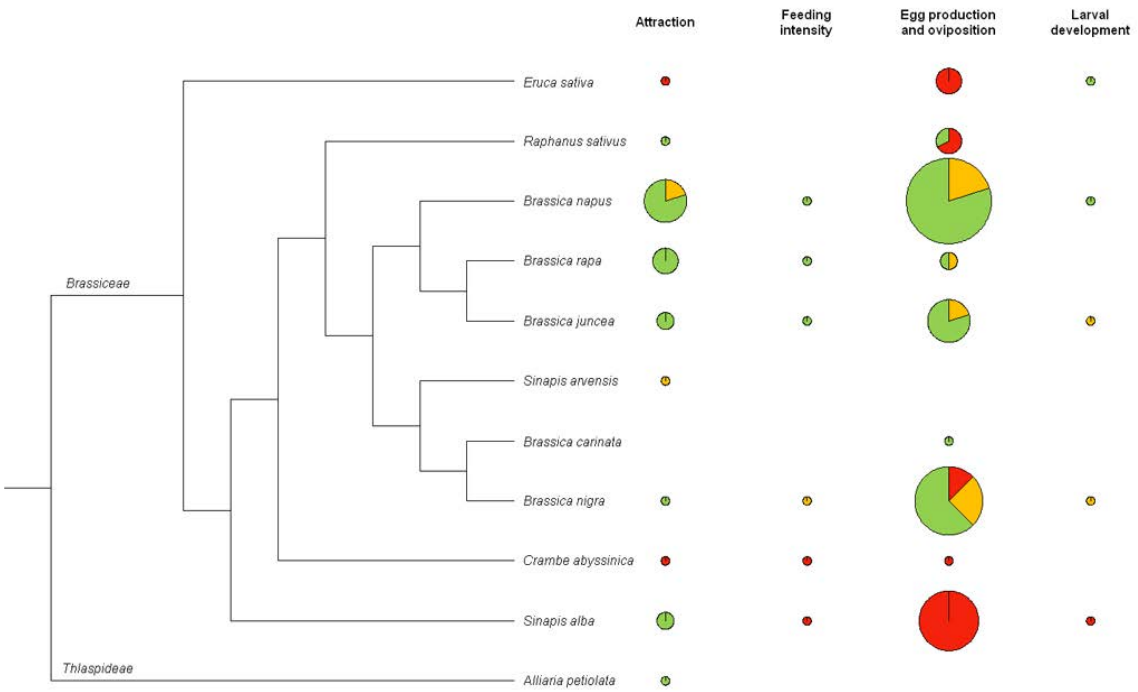

Fig. 1 Summary of results obtained in interspecific comparisons of brassicaceous species for their suitability for the pollen beetle (Free & Williams 1978; Borg & Ekbom 1996; Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Hopkins *et al.* 1998; Cook *et al.* 2007; Veromann *et al.* 2012; Kovács *et al.* 2013; Kaasik *et al.* 2014a, 2014b). For each paper, studied species were broadly classified as 'good host' (green), 'medium host' (orange) or 'bad host' (red). Size of circles is proportional to the number of studies having tested the corresponding species (N = 1 minimum, 10 maximum). Phylogenetic relationships of studied species are shown on the left of the diagram (Brassibase)

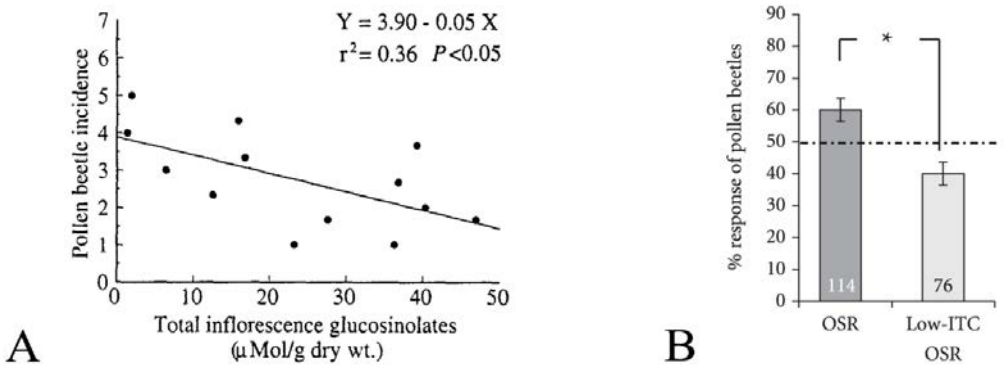

Fig. 2 A: relationship between inflorescence glucosinolate content of different oilseed rape lines grown in the field and pollen beetle colonization (Giamoustaris & Mithen 1996). B: attractiveness of two cultivars having different concentrations of alkenyl glucosinates (the lesser this concentration, the lesser the concentration of volatile isothiocyanates (ITC) emitted when the plant is wounded) in the laboratory (same conclusions from semi-field and field experiments) (Cook *et al.* 2007)

conclusions. First, early-flowering cultivars were more attractive to *Meligethes aeneus* when they were flowering while late-flowering cultivars were at the bud stage. This result extended the current knowledge on interspecific comparisons, *i.e.* flowering plants are generally more attractive than non-flowering plants to the pollen beetle (Free & Williams 1978; Cook *et al* 2006, 2007). Secondly, growth rate of the *M. aeneus* population was reduced on early-flowering compared to late-flowering cultivars. The hypothesis suggested was that an earlier flowering time led to a faster flower fall, thus reducing the period where food is available for beetle larvae and causing higher larval mortality.

The present work was conducted using a selection of six oilseed rape genotypes (Table 1). No data were available on the interaction between the pollen beetle and any of these genotypes; they were chosen because of their genetic diversity and contrasts for other traits: oilseed rape type (winter or spring), erucic acid and glucosinolate content in the seeds and resistance to a pathogenic unicellular eukaryote (*Plasmodiophora brassicae*) (Wagner *et al.* 2012).

In the present thesis, we first detail major hurdles involved in introducing resistance to insects in cultivated plant species, and propose an alternative approach to classical phenotyping (section 'General approach' of the Introduction, *Article 1*). This approach can be conducted on four important biological steps, which we tested in the next chapters: attraction (Chapter 1, *Article 2*), feeding intensity (Chapter 2, *Article 3*), egg production and

Table 1 Previously known characteristics of the six genotypes used in this thesis and vernalization time. WOSR: winter oilseed rape; SOSR: spring oilseed rape. '0' genotypes were selected for a low concentration of erucic acid or glucosinolates in the seeds, whereas '+' genotypes contain high concentrations. Evaluation of resistance to *Plasmodiophora brassicae* come from Wagner *et al*. (2012). Vernalization is necessary only for WOSR but was applied to SOSR to facilitate synchronization of growth stages among genotypes during experiments. The genotype 'Darmor' has to be vernalized for a longer duration to induce development of reproductive organs

Genotype	Type	Country of origin	Seed content		Resistance to *P. brassicae*	Vernalization (weeks)
			Erucic acid	Glucosinolates		
Darmor	WOSR	France	0	0	High	10
Express	WOSR	Germany	0	0	High	8
Liho	SOSR	Germany	+	+	Low	3
Mar	WOSR	Poland	0	0	Medium	8
Markus	WOSR	France	+	+	Low	8
Yudal	SOSR	Korea	+	+	Medium	3

oviposition (Chapter 3, *Articles 4* and *5*), and larval development (Chapter 4, *Article 6*).

The method was consistant: we compared insect behavior/physiology on the six plant genotypes, and tried to link the obtained results with data on plant chemistry (only odor bouquet characterization is not available at the time of writing this thesis). Our objective was to identify, by crossing these two datasets, candidate key plant traits that are the most important in determining the intensity of the interaction.

All experiments were conducted in the laboratory and in no-choice situations, with plants grown in controlled conditions (detailed in *Article 3*) and pollen beetles sampled from the field (due to its long diapause, rearing of this species cannot easily be performed). Except for one experiment in Chapter 4, all experiments were conducted on entire plants to stay as close as possible to the 'natural' interaction.

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– General approach –

Protecting crops against insect pests by selecting for increased plant resistance: barriers to success and an alternative approach

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Insect pests are a major problem in terms of crop protection, and this problem is getting worse with the global increasing resistances to insecticides. Selecting for resistant cultivars to decrease disease damage has been done for decades, but this strategy faces critical issues with insects. We detail these issues, which are both logistical and conceptual. They are essentially in relation with the phenotyping process with insects. We then propose an alternative approach that may break down the actual barriers. Briefly, this entails going back to the laboratory, studying in depth a small panel of plant genotypes and identifying key plant traits that determine the intensity of the interaction with the insect. If such traits are correctly identified, a sort of biochemically-assisted selection process may further be employed to introgress resistance into elite varieties, without needing any insect. We discuss the potential efficiency and durability of this approach. It may be especially valuable against insect pests that cause damage at a temporary susceptible crop stage, by delaying or reducing crop colonization and damage.

INTRODUCTION

Insect pests are considered to be responsible for about 10-15 % of yield losses worldwide (Oerke 2006). Many strategies have been developed in order to decrease damage caused by these organisms, including synthetic and naturally-occuring insecticides, mixed cropping, pheromone traps, biological control by natural enemies or genetically modified crops (Foster & Harris 1997; Kogan 1998; Isman 2006; Carpenter 2010). One tactic, however, has received limited attention: *breeding plants to increase their natural resistance to insect pests*. Although widely used against diseases, this strategy was indeed rarely really implemented against insects even if envisaged in some studies (*e.g.* Dosdall & Kott 2006; Alagar *et al.* 2007; de Sena Fernandes *et al.* 2011; Eickermann *et al.* 2011; Beres *et al.* 2013; Kher *et al.* 2013 or Tefera *et al.* 2013). Few notable examples exist, among which the use of resistant wheat varieties against the wheat midges *Sitodiplosis mosellana* (Ding *et al.* 2000; McKenzie *et al.* 2002) and *Mayetiola destructor* (Gallun & Hatchett 1969; Hatchett & Gallun 1970).

The first reason of this important contrast between insects and diseases comes from the complexity of the interaction with the plant. Plant resistance is classically divided into *qualitative* resistance based on gene-for-gene relationships (Flor 1942, 1955), and *quantitative* resistance based on Quantitative Trait Loci (QTL). Increasing crop resistance to pathogens has been conducted for decades by introgressing R genes, *i.e.* qualitative resistance, into elite varieties. The simplicity of this system allowed rapid progress in disease management and led to production of many resistant cultivars. Very few examples of such simple interactions have been reported in plant – insect systems, *e.g.* the wheat – orange wheat blossom midge (McKenzie *et al.* 2002) and wheat – Hessian fly systems (Gallun & Hatchett 1969; Hatchett & Gallun 1970), some plant – aphid systems (reviewed *in* Smith & Boyko 2007) and a few others (Bonaventure 2012). The interaction between plants and herbivorous insects is, most of the time, complex, involving many traits which integration defines the global resistance level of the plant (Walling 2000; Sarfraz *et al.* 2006; Mitchell-Olds 2010; Kloth *et al.* 2012).

Considering quantitative resistance only, the gap is still huge between plant – disease and plant – insect studies. A simple search on the Web of Science with the keywords "QTL + 'disease resistance'" gives 2,287 results whereas "QTL + 'insect resistance'" gives only 120 (research date: July 23rd 2014). Most of the QTL known for resistance to insects have been discovered in model species, especially *Arabidopsis thaliana* (*e.g.* Pfalz *et al.* 2007, 2009), while a minority of studies concerned cultivated plant species (*e.g.* Blake *et al.* 2011; Tan *et al.* 2013 or Kim *et al.* 2014). Here appears the second reason why crop resistance to insects has not been more developed so far: difficulties in the phenotyping process. Studies aiming at identifying QTL need more than a hundred genotypes (and, of course, replicates within genotypes) to be achieved, either using family-based QTL mapping or genome-wide association (GWA) mapping, an increasingly-used complementary method (Mitchell-Olds 2010). If feasible when working on plant physiology, phenology or resistance to pathogens (*e.g.* Atwell *et al.* 2010), this is *often unrealistic* when considering resistance to insects. Kloth *et al.* (2012) argued that GWA mapping would be of primary interest to understand the genetic bases of how plants resist to insects, and to develop crop resistance to insect pests. However, they underline that the main, yet unresolved, problem with this approach is to set up high-throughput phenotyping methods for insect preference and performance.

In this paper we first summarize the main barriers restricting large-scale phenotyping of plant resistance to insects. These are essentially logistical, but some conceptual issues are also evident. We then propose an alternative method by which resistance to insect pests may be implemented in cultivated plant species. We

finally discuss the potential efficiency and durability of the proposed approach.

MAJOR HURDLES TO LARGE-SCALE PHENOTYPING FOR RESISTANCE TO INSECTS

Logistical difficulties

An obvious but critical barrier to large- (or even medium-) scale phenotyping is that it requires at least hundreds, if not thousands of insects. Adults and larvae – but also different larval instars or adults of different ages, physiological states or differently experienced – generally differ in both their behavior and nutritional requirements (Scriber & Slansky 1981). Therefore, all individuals tested need to be standardized as much as possible. In the laboratory, this involves large numbers of reared insects, which in turn often requires lots of space and manpower. In the field, the entire process depends on natural colonization which fluctuates unpredictably with insect population size in the local environment. This difficulty in getting sufficient numbers of insects probably explains why many studies are conducted on aphids, which produce offspring parthenogenetically, at a high rate and only a few days after their own birth. Insect species reproducing sexually generally have an intrinsic rate of population increase that is lower than asexual species (Lively & Lloyd 1990). Moreover, many pests produce only one generation per year (several wireworms devastating potato crops need four to five years to achieve a complete cycle!) and some have an obligate diapause (*e.g.* the Colorado potato beetle *Leptinotarsa decemlineata*, the Western corn rootworm *Diabrotica virgifera virgifera* or the cabbage stem flea beetle *Psylliodes chrysocephala*). Laboratory rearing is therefore often complicated or even impossible, and periods where field trials can be conducted are often restricted.

Comparing a great number of plant accessions against insects in the laboratory needs extensive space and manpower, especially when the interaction is studied on whole plants. Insects also have to be isolated to avoid colonization of neighboring plants during experimentation. Many systems are available to isolate insects very simply (clip cages, plastic bags, nylon or acrylic glass cages etc), but this constraint makes protocols often onerous. For these two reasons, most phenotyping studies take place in the field.

Field trials have the great advantage that dozens or even hundreds of accessions can be tested simultaneously. However, it comes with four major difficulties that can introduce important bias to the results. All these difficulties arise from the fact that insects colonize the trial by a natural process, which is based mainly on volatile and visual cues provided by the tested plants (Bruce *et al.* 2005; Prokopy & Owens 1983). (i) Volatile compounds are carried by the wind. Consequently, insects move essentially upwind to trace back attractive molecules until finding the odor source (Beyaert & Hilker 2014). The spatial arrangement of accessions in a field trial, in relation to wind direction, may hence play an important role in determining the number of insects found on the different accessions. 'Edge effects', *i.e.* preferential colonization of the first plants encountered if they are suitable for the insect, may also contribute to bias results. (ii) Plot size, *i.e.* number of plants per accession or per replicate of an accession, must reach a minimum critical value to provide signals that are important enough to be detected by insects. A low number of insects on certain accessions may then be caused by too few host-location stimuli due to too small plots (which can also be interpreted by insects as insufficient resources), and has nothing to do with plant resistance. (iii) Many insects behave differently when confronted by flowering and non-flowering plants. Color differences are obvious, but odor bouquets are also likely to differ since flowers are important volatile-emitting organs (Muhlemann *et al.* 2014). If phenotyping is conducted on an insect species that is sensitive to plant flowering phenology, it needs to be homogenized among

accessions. This sole constraint can be very challenging, as differences in accession colonization may be completely biased by differences in flowering phenology. More generally, the same may arise with any difference in phenology or even in growth rate. Indeed, insects are known to select their host plant partly for their 'vigor', for which the simplest measure is size (Cornelissen *et al.* 2008). (iv) Finally, a crucial point lies in the process of choice made by the insect. The basic hypothesis of field trials is that insects would colonize the accessions they prefer. This is true if and only if signals emitted by all accessions are perceived by the insect, which can then make an active choice after a comparison of these signals (Martel & Boivin 2011). Ensuring this condition to be true is hardly possible. However, it is certain that the more accessions are compared, the more signals are simultaneously emitted. Therefore, the less it is likely that insects distinguish all of them (and the more it is likely that signals interact with each other, disturbing insect recognition).

Conceptual issues

In order to reduce space constraints and study more accessions in the laboratory, alternative methods to using entire plants have long been used: leaf disks, stem sections, cut flowers etc. These 'small-scale' experiments have permitted great advances in the comprehension of plant – insect interactions. However, their relevance in the perspective of selection for increased crop resistance can be questioned. Indeed, the objective here is to manipulate the interaction to get substantial benefits in field conditions. Therefore, tests preferably need to be conducted in conditions that are as natural as possible. Major questions come up when applying 'small-scale' protocols in this context. For example, it is not always the case that insects respond in the same manner when facing a plant part, compared to a whole plant which architecture may play an important role in attractiveness or foraging strategy (*e.g.* Alonso & Herrera 1996 or Agerbirk *et al.* 2010). Furthermore, plants respond to wounding either physically and/or chemically (Gatehouse 2002; Howe & Jander 2008; Wu & Baldwin 2010). Therefore, the response induced by cutting a plant part could affect the behavior of the insect. Also, the wounding response could affect the nutritional quality of plant tissues and consequently insect survival, development or other physiological functions such as oogenesis. These issues would not be so problematic if induced responses were homogeneous among plant accessions. But it is known that induced responses, as well as constitutive resistance, vary with plant genotype (Walling 2000; Gatehouse 2002; Wu & Baldwin 2010).

Experiments conducted in the laboratory are mainly choice experiments, between two accessions to be compared directly or between a tested accession and a control. In the field, choice experiments are almost always performed. However, this may not be the most relevant protocol in a selection perspective. Crops consist most of the time of genetically identical plants. Therefore, all plants in a field are nearly homogeneous for their architectural, chemical or phenological characteristics (Tooker & Frank 2012). There is of course some variation between individual plants, but this variation is probably much reduced compared to intergenotypic differences. Hence, it is likely that in this context insects are not really facing a choice situation. They are confronted to one resource, and the questions are: how do they behave *in response to this sole resource*? How are they impacted *by the presence of this sole resource*? Choice tests are relevant when developing trap cropping strategies, where the insect is clearly confronted with a choice between plants to be protected and trap plants (Shelton & Badenes-Perez 2006). On the other hand, no-choice tests appear more relevant to the development of resistant cultivars. An important problem arises at this step: no-choice experiments are not feasible in the field as colonization cannot be controlled and it is often unrealistic to isolate accessions from each other.

ANOTHER APPROACH?

We discussed several issues that can explain why increasing crop resistance to insect pests remains a real challenge. All these barriers come from the phenotyping process needed when using insects. We therefore propose another approach where plant phenotyping for insect resistance could be carried out without insects.

General strategy

The central idea of the strategy is to *identify key plant traits that determine the intensity of the interaction* between the plant species to be protected and the insect pest to be controlled. If these traits are precisely identified and validated, it should theoretically be possible to perform further phenotyping (at any scale) on the sole basis of these traits. Most constraints imposed by insects would therefore disappear. The physical or biochemical markers would then be used for genetic resource screening or for assisted selection (as already proposed by Steinfath *et al.* 2010 or Kushalappa & Gunnaiah 2013).

The critical point of this approach is to identify the key plant traits to be targeted by genetic studies or selection. We propose a simple scheme to achieve such a goal in the plant – insect context:

(i) Select a small set of genotypes to be studied in depth, on the basis of the existing literature, previous experiments, contrasts that were found with other pests, genetic diversity of the crop species etc. The main advantage to work by comparing genotypes (and not species) is that if some intergenotypic variation is found, it proves that a basis is available for selection. The number of genotypes required is a compromise that depends on the study system. Indeed, the selection of 'candidate key traits' is based on comparisons between data obtained from the insect and physical/biochemical data obtained from the plant. The more genotypes in the panel, the more contrasts are likely to be observed and the more the correlations found are reliable. On the other hand, onerous experiments in the laboratory constrain the number of genotypes that can be studied simultaneously.

(ii) In strictly controlled conditions, test the effect of each genotype for one to four key step(s) of plant – insect interactions (these steps are discussed in the following subsections). Controlled conditions limit the variation observed among genotypes to mainly genetically determined variations, which is fundamental for selection. For reasons previously discussed, these tests should be performed on *entire plants* and in *no-choice experiments*. Protocols can be onerous, but a small number of genotypes keeps the task realistically feasible. Differences found among genotypes, for example in feeding intensity, would show that some genotypes are *by themselves* less stimulant than others, not *relatively to others*.

(iii) For interaction steps where intergenotypic differences were found, study in detail and *without any a priori* plant characteristics that could be responsible for the observed variation. Since Fraenkel (1959), plant – insect interactions are essentially seen through the prism of secondary metabolites. It is clear that they are an important part of the puzzle (Berenbaum & Zangerl 2008), but not the only part. Primary metabolites, which play a key role in the nutritional quality of plant tissues (Awmack & Leather 2002), are probably underestimated (Berenbaum 1995), as are physical traits (Clissold *et al.* 2009; Carmona *et al.* 2011). As many traits as possible should be considered simultaneously since plant resistance is likely to be based not only on a single trait but on a combination of several (Agrawal & Fishbein 2006; Agrawal 2011). From an analytical point of view, multivariate statistical analyses, especially Partial Least Squares - Discriminant Analysis (Barker & Rayens 2003) and Correspondence Discriminant Analysis (Perrière *et al.* 1996), are powerful tools to compare global profiles of plant genotypes and to identify discriminant variables, in a context where variables are often more numerous than individuals.

(iv) Correlate intergenotypic differences observed with the insect and intergenotypic differences in plant physical-biochemical characteristics. Candidate key traits are identified at this point.

(v) Test the validity of the candidate key traits in dedicated experiments. Methods of choice may be olfactometry with pure volatile compounds added to plant odor blends, supplementation of plant tissues with pure compounds, gene over-expression or silencing etc. The more experiments are performed on entire plants, the more they are likely to correlate to what happens in the field.

Four major steps of plant – insect interactions could theoretically be interesting to target: attraction, feeding intensity, egg production and oviposition, and larval development. We briefly discuss each of these steps below.

Attraction

Every crop season, insect pests of annual crops have to locate new host plants. Selecting for less attractive plants may then be beneficial in numerous cases. It would contribute to decreasing the number of individual insects colonizing the crop, especially if other attractive resources are available in the same landscape.

Genotypic variation of attractiveness has not received much attention yet. However, some studies already proved that such variation may exist (*e.g.* Broberg *et al.* 2005; Cook *et al.* 2006; Lopes Baldin & Beneduzzi 2010; Schlick-Souza *et al.* 2011; Rajabaskar *et al.* 2013).

Feeding intensity

Insect feeding intensity is influenced essentially by the chemical stimulation triggered by phagostimulant and phagodeterrent compounds present on the surface or inside plant tissues, but also by physical characteristics such as tissue toughness and trichome length/shape/density (Chapman 2003; Müller & Riederer 2005; Agrawal & Fishbein 2006; Clissold *et al.* 2009). Selecting for reduced feeding stimulation would contribute to a decrease in damage caused by individuals that are present on or in the plants.

Intergenotypic variation for feeding intensity was shown in many plant – insect systems (*e.g.* Glynn *et al.* 2004; Lyytinen *et al.* 2007; Niveyro *et al.* 2013; Ströcker *et al.* 2013 or Hervé *et al.* under revision [*Article 3*]). Using wide metabolic profiling, we have shown that our approach can lead to the identification of candidate key traits (Hervé *et al.* under revision [*Article 3*]).

Egg production and oviposition

In the short term, reducing the number of eggs laid by insect pests would be an interesting strategy in all cases where crop damage is caused by larvae (caterpillars for example). Indeed, the fewer damaging individuals that are present in the field, the less damage is likely to be caused. This may be particularly effective in combination with a lowered feeding stimulation. In the medium-long term, reducing the number of eggs laid may contribute to a decrease in the pest population size at the landscape scale.

Egg production is largely influenced by plant nutritional quality (Awmack & Leather 2002). For synovogenic insect species (*i.e.* species producing eggs during their adult life) feeding on the same plant where they lay eggs, reducing this quality may contribute to slow down egg production. Feeding stimulation, which is often neglected, sometimes also plays an important role by constraining the energy available for oogenesis (Hervé *et al.* 2014 [*Article 4*]). Finally, stimulation of oviposition can depend on immediate cues detected by females before or during egg laying. Selecting traits that reduce host 'acceptability' (Singer *et al.* 1992) could be relevant in cases where these types of cues are used.

Differences among genotypes or cultivars for the number of eggs they receive is a well-known phenomenon (*e.g.* Osier *et al.* 2000; Johnson 2008; Magalhães *et al.* 2008; Poelman *et al.* 2009; Mphosi & Foster 2010; Cheng et al. 2013; Hervé *et al.* 2014

[*Article 4*]), proving that a basis for selection really exists.

Larval development

Suitability for larval development is largely determined by the nutritional quality of the plant. Unlike the three previous steps, manipulating this one is more of an indirect strategy. Two main goals may be targeted. Firstly, egg production in proovogenic species (*i.e.* species not producing eggs at the adult stage but rather emerging with a fixed, finite egg load) depends on the food ingested at the larval stage. For these kinds of insect pests (*e.g.* many Lepidopteran species), reducing crop nutritional quality for larvae may constrain oogenesis to a larger extent. Therefore, it may contribute to decreasing the pest population size at the next generation. Secondly, larvae are exposed to natural enemies (predators and parasitoids) during their development. Extending the time needed for complete development, by acting on host nutritional quality, would increase the 'vulnerability window' to these enemies (Clancy & Price 1987). Selection based on plant traits may therefore directly favor biological control of the pest.

As for many traits we discussed, intergenotypic differences in suitability for larval development have been shown in a number of plant – insect systems (*e.g.* Glynn *et al.* 2004; Chen *et al.* 2009; Amin *et al.* 2011; Lehrman *et al.* 2012; Guo *et al.* 2013; Kher *et al.* 2013; Sandhyarani & Usha Rani 2013).

EFFICIENCY AND DURABILITY

At this point, it is of course hypothetical to predict the efficiency of the approach we propose. What is certain is that it would lead to quantitative, not qualitative, resistance which is generally considered to be more durable. Indeed, mechanisms preventing all damage (qualitative plant resistance or insecticides) exert a strong selection pressure on pest populations. Since any positive mutation provides an important fitness gain, these strategies are likely to be rapidly circumvented by targeted species. Numerous examples of insect resistance to insecticides are known, as well as pathogens overcoming R genes. Manipulating specific plant traits to decrease attractiveness will not prevent all insects to colonize the crop. In the same manner, decreasing feeding stimulation will not prevent all damage. However, such approaches would probably exert weak selection pressures, especially if the pest life span is long and crops are grown over extensive areas. If the kind of resistance we propose to select has to be circumvented, insect adaptation is likely to take a long time (especially if multiple resistant traits are combined).

A crucial point is to keep in mind the *agronomical benefit* of the strategy employed. Farmers and plant breeders are often used to dealing with insect pests by spraying insecticides. Their aim is to eradicate pests from the field. However, the crop may not be devastated even if insects are present. This is especially true for the great number of insect pests that cause damage at a specific, temporary vulnerable growth stage of the plant (*e.g.* sown seeds, seedlings or flower-bud stage). In that case, an interesting strategy could be not to avoid any attack – which is unrealistic – but rather to *delay* attacks. Insects can be present in the field, but if they cause less damage during the vulnerable stage of the crop, the agronomical benefit can be important. Attacks can be delayed using less attractive plants, which will slow down field colonization. Damage can be decreased at the susceptible stage using plants that are less stimulant for pest feeding. Insects will probably compensate during their life span (Behmer 2009), but it is not a problem if it occurs after the vulnerability period of the crop. Damage can also be reduced using less nutritive plants that constrain to a larger extent egg production of females. Again, they will probably compensate to maintain their fitness. But when larvae are the damaging individuals, if less eggs are laid during the susceptible stage the strategy may be valuable.

CONCLUSION AND PERSPECTIVES

Metabolic biomarkers are a powerful tool to predict plant phenotype and facilitate selection (Steinfath *et al.* 2010; Kushalappa & Gunnaiah 2013). In the case of plant – insect interactions, identifying such markers is a particularly challenging task. We think that the strategy we propose has the potential to address this challenge. However, it is only a first step. Since the objective is applied, it is necessary to test if physical/biochemical intergenotypic contrasts observed in controlled conditions are also found in the field. This comes with the usual constraint of growing plants in conditions that are as homogeneous as possible, to be able to perform unbiased screening and selection. Identifying biomarkers of resistance to insects would allow either breeding using biomarker-assisted selection, or genetic analyses to identify metabolic QTL (*e.g.* Wentzell *et al.* 2007; Sotelo *et al.* 2014). This second approach may even lead to a sort of 'marker-biomarker assisted selection' for resistance to insects. Finally, as it should be done for every targeted approach, it is necessary to verify if manipulating certain plant traits for resistance to one insect species does not increase susceptibility to other pests, or reduces benefits provided by the natural enemies of pests or pollinators. Although very difficult to achieve, multimodal resistance might be progressively constructed if detrimental effects are identified at each step of the pyramiding process.

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– CHAPTER 1 –

Attraction

Attractiveness of oilseed rape (*Brassica napus*) for the pollen beetle (*Meligethes aeneus*) varies with plant genotype but preference does not match performance

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Phytophagous insects locate their host plants essentially using volatile compounds emitted by these plants. Recognition and evaluation of the quality of a host is thought to be based either on specific ratios of ubiquitous volatiles, and on family-specific compounds. The preference – performance hypothesis in its larger sense states that insects should prefer plants that are of best quality for themselves or for their offspring. If this has been well studied at the plant interspecific scale, very few papers addressed this question at the intraspecific scale, by comparing attractiveness of plant genotypes of the same species. We tested the attraction of the pollen beetle (*Meligethes aeneus*) for six genotypes of oilseed rape (*Brassica napus*) in a no-choice olfactometer study. A gradient was shown, demonstrating that individuals prefer certain genotypes compared to others. We then tried to link this preference gradient with performance gradients obtained on the same plant genotypes in parallel studies: feeding intensity (which directly influences egg production), larval development and adult survival. No relationship was found with any measure of performance. We discuss the apparent lack of adaptive value of this result in the agronomical context where the interaction takes place.

INTRODUCTION

Host plant location by phytophagous insects generally relies on both olfactory and visual signals provided by the plant. Among these signals, Volatile Organic Compounds (VOCs) appear to be of primary importance (Bruce *et al.* 2005). Insects are able to detect the odor blend of host plants in a dense volatile background, and are thought to find these hosts by tracing back fine 'plant odor plumes' (Beyaert & Hilker 2014). Odor bouquets are usually composed of dozens, sometimes hundreds, of compounds emitted both by vegetative and floral organs (Visser 1986; Knudsen *et al.* 1993). Many of them are ubiquitous. Insect herbivores are thought to recognize host plants using specific ratios of these ubiquitous VOCs (Bruce *et al.* 2005; Bruce & Pickett 2011). Additionally, in plant families producing specific VOCs (*e.g.* isothiocyanates (ITCs), catabolites of glucosinolates which are themselves specific of a few families including Brassicaceae (Fahey *et al.* 2001)), these can be used by specialist insect species since they reliably indicate presence of plants of these particular families (Bruce *et al.* 2005; Raguso 2008).

According to the 'preference – performance' hypothesis (*e.g.* Thompson 1988), female phytophagous insects should lay eggs preferably on host plants that are more favorable for their offspring. Based on the founding results of Scheirs *et al.* (2000), Mayhew (2001) interestingly added that host choice can also be driven by female's performance; both mother and larval performance impacting on mother's fitness. Since an odor blend partly reflects the nutrient status or growth stage of the plant, and presence of homo/heterospecific competitors or predators, it is an element of host quality evaluation by host-seeking herbivores (Kesselmeier & Staudt 1999; Pichersky & Gershenzon 2002; Unsicker *et al.* 2009).

The pollen beetle (*Meligethes aeneus* F.; Coleoptera: Nitidulidae) is a major pest of oilseed rape (*Brassica napus* L.; Brassicaceae) (OSR) crops. Adults are generalist pollen feeders but females lay eggs only in closed flower buds of brassicaceous plants (Free & Williams 1978; Ekbom & Borg 1996; Carrié *et al.* 2012; Marques & Draper 2012). The species is univoltine. Adults overwinter in semi-natural habitats and their diapause ends when temperatures exceed 10 °C (Nilsson 1988). They then seek host plants where they feed and mate. Host location is achieved essentially through upwind anemotaxis driven by volatile compounds emitted by plants (Cook *et al.* 2002; Williams *et al.* 2007), although color can play a role (Giamoustaris & Mithen 1996; Blight & Smart 1999; Jönsson *et al.* 2007; Döring *et al.* 2012; Cook *et al.* 2013). Flights are induced when air temperature exceeds 10 °C but mass migrations above 15 °C (Free & Williams 1978; Ferguson *et al.* 2014). At the flight period, OSR fields are at the 'bud stage', *i.e.* flower buds are formed but still closed. Damage is caused by adults, which destroy buds to reach the pollen inside (Williams 2010). Yield losses up to 70 % have been recorded due to this pest (*e.g.* Nilsson 1987). Larval development takes place on the plant, and pupation in the soil. New generation beetles emerge in late spring, feed on many different plants families and seek overwintering sites in late summer (Williams & Free 1978; Williams 2010).

Influence of plant volatiles on pollen beetle behavior has been well studied at the plant interspecific scale, especially to design control strategies based on plants that are more attractive than OSR (Cook *et al.* 2006, 2007; Kaasik *et al.* 2014) and repellent compounds identified from other plant families (Mauchline *et al.* 2008, 2013; Pavela 2011). Additionally, influence of specific VOCs has been studied both in the laboratory and the field (Blight & Smart 1999; Smart & Blight 2000; Cook *et al.* 2007; Mauchline *et al.* 2008; Piesik *et al.* 2013). In comparison, few studies addressed the question of the intraspecific variation of OSR attractiveness. Only Cook *et al.* (2006) compared the influence of odors emitted by two cultivars differing in their glucosinolate leaf content. In a choice olfactometer test (as well as in semi-field and field experiments where

other factors can come into play), the pollen beetle was shown to prefer the cultivar that emitted more ITCs, confirming results obtained on pure compounds (Free & Williams 1978; Blight & Smart 1999).

The objective of this study was to assess, in a no-choice situation, the VOC-mediated attractiveness of six OSR genotypes for the pollen beetle. To do so, we developed a novel olfactometer bioassay. Potential intergenotypic differences were then compared with several other traits (feeding intensity, egg production, larval development and adult survival) determined on the same genotypes in parallel studies (Hervé *et al.* 2014 [*Article 4*]; Hervé *et al.* under revision [*Article 3*]; Hervé *et al.* in prep. [*Article 6*]), and discussed in the context of the preference – performance hypothesis.

MATERIALS AND METHODS

Plants

All genotypes used in this study were lines from the INRA OSR collection (BraCySol Center for Genetic Resources, INRA, Le Rheu, France). Plants were produced in controlled conditions as described in Hervé *et al.* (under revision) [*Article 3*] and used at BBCH stage 57 (Lancashire *et al.* 1991), *i.e.* the 'green bud stage'.

Insects

Overwintered pollen beetles were collected from an unsprayed winter OSR crop near Le Rheu (Brittany, France). Individuals were used for experiments 24 h after field collection. During this period, insects were starved and kept individually in small Petri dishes (diameter 3.5 cm) containing a moistened paper filter.

Olfactometer bioassay

Experimental setup – The linear tube olfactometer (Fig. 1) consisted of three parts. Part A was a plastic pot (length 10.5 cm, diameter 5.5 cm) to which a small plastic tube (length 4 cm, internal diameter 1.4 cm; hereafter named 'zone 0') was fixed. The tube was separated from the pot by a fine-mesh tulle. Part A was connected to a Capex 8C pump (Charles Austen) *via* a flowmeter. Part B was a glass tube (length 24 cm, internal diameter 1.5 cm), virtually divided into six 4 cm-zones (hereafter named 'zone 1' to 'zone 6'). Part B was connected to part C which was a second plastic pot. Parts B and C were separated by a fine-mesh tulle. Part C was connected to the top of a glass box (length 25 cm, width 25 cm, height 50 cm; D) containing the odor source. Finally, the pump and part D were connected to form a closed circuit. All connections were made with PTFE tubing (diameter 0.8 cm). The pump pushed air to part D

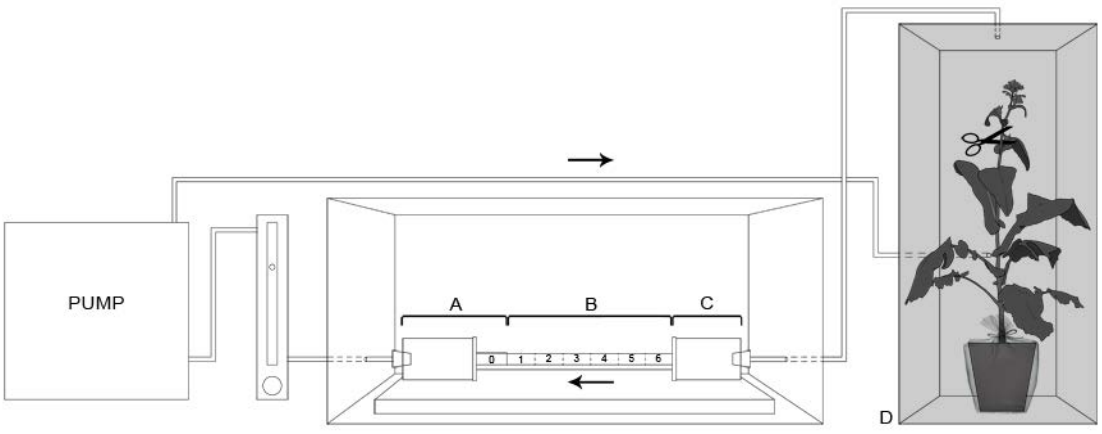

Fig. 1 Linear tube olfactometer used to study no-choice attraction of the pollen beetle (*M. aeneus*). The odor source is placed in part D (glass box). The insect is introduced into zone 0 (part A) and is able to move only in zones 0 to 6. Air circulation flows in a closed circuit and is generated by a laboratory pump. Arrows indicate direction of air flow

while pulling from part A, leading to an air movment from the odor source through the olfactometer. Air flow was adjusted to 800 ml.min^{-1}. To prevent any visual interaction between insects and plants, the olfactometer was placed in a white-painted cardboard box opened only at its front face, where behavior was observed.

Odor source – The odor source was an entire plant, with its pot enclosed just before experiment in a cooking bag (Fig. 1) to prevent any odor emission other than from the plant. To be closer to field conditions where stresses are multiple and no plant is hence likely to be completely undamaged, the stem was cut with scissors immediately before placing the plant in the glass vessel, between the third and the fourth inflorescence (from the top of the plant). Controls were performed with empty glass boxes. In all treatments, air circulation was left for 10 min before starting insect observations.

Experimental – An individual, unsexed, pollen beetle was placed in 'zone 0'. Insect movement in the seven zones of the system was then followed for 10 min, using the SequenceR interface (Hervé 2013). Based on the time spent in each zone, an attraction index was calculated:

$$Attraction\ index = \sum_{i=0}^{i_{max}} \frac{t_i}{t_{tot}} \times \frac{i}{i_{max}}$$

where i is the number of the zone, i_{max} the zone closest to the odor source (number six in our system), t_i the time spent in zone i and t_{tot} the total duration of the monitoring (600 sec in our study). Individuals were sexed after experiment following Ruther & Thiemann (1997).

Five individuals were used per plant (or with the same empty glass box for control individuals) and 12 plants were used per OSR genotype (idem for control individuals), resulting in 60 individuals per treatment. Replicates were performed randomly through time. Experiments took place between 8:00 am and 1:00 pm, in a controlled environment room maintained at 21 °C. The A, B, C and D parts of the system were changed after each series of five individuals, alcohol-cleaned and air-dried for at least 20 h.

Statistical analysis

Statistical analysis was performed using R software (R Core Team 2013). The value of the attraction index was analyzed using a Wald test on a Linear Mixed Model (function 'lmer', package 'lme4' (Bates *et al.* 2014)) considering as explanatory variables the treatment, the sex of individuals (fixed factor), the interaction between these two factors and the individual odor source (random factor). Pairwise comparisons of Least Squares Means were performed using the function 'lsmeans' (package 'lsmeans' (Lenth 2013)) and the False Discovery rate correction for *P*-values (Benjamini & Hochberg 1995).

RESULTS

The interaction between treatment and the sex of beetles had no significant effect (χ^2 = 4.56, *df* = 6, *P* = 0.602), neither did the sex factor taken alone (χ^2 = 0.067, *df* = 1, *P* = 0.796). A significant difference was found among treatments (χ^2 = 43.75, *df* = 6, *P* < 0.001; Fig. 2). Individuals exposed to the control odor source were less attracted than others (mean ± SE attraction index: 0.49 ± 0.03). Among OSR genotypes, 'Mar' was the most attractive (0.76 ± 0.03) whereas 'Darmor' and 'Markus' were at the other extreme of the gradient (0.62 ± 0.03 and 0.65 ± 0.03, respectively). The three other genotypes ('Liho', 'Yudal' and 'Express') were intermediate between these two groups.

DISCUSSION

In comparison with feeding intensity, oviposition or larval development/mortality, very few studies have addressed the question of a genotypic influence of VOC-mediated plant attractiveness toward phytophagous insects. However, as with all other traits, some intergenotypic variation was found (Broberg *et al.* 2005; Cook *et al.* 2006; Lopes Baldin & Beneduzzi 2010; Schlick-Souza *et al.* 2011; Rajabaskar *et al.* 2013). Most of these studies were based on choice tests. To our

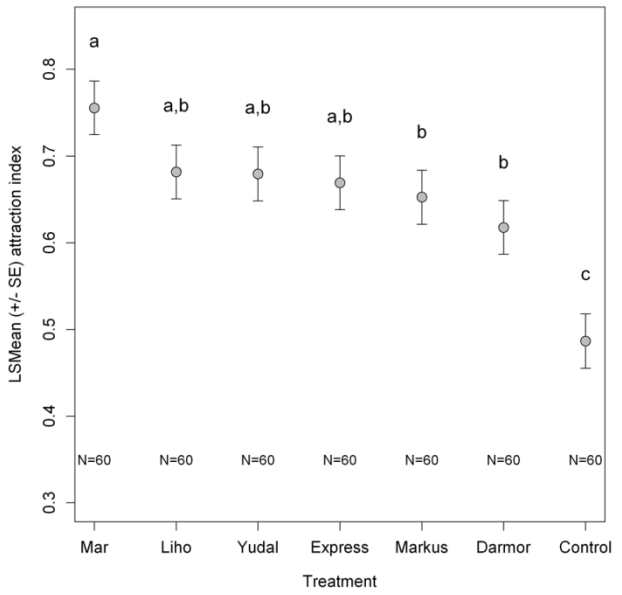

Fig. 2 Least Squares Mean (± SE) attraction index of six oilseed rape (*B. napus*) genotypes toward the pollen beetle (*M. aeneus*) (control: empty glass box). Different letters indicate statistically different LSMeans. N: number of individuals per treatment

knowledge, only Rajabaskar *et al.* (2013) showed that even in a no-choice situation, plant attractiveness can vary. In this case it was in the potato – green peach aphid system. Our results are in line with those obtained by Cook *et al.* (2006) who demonstrated that OSR attractiveness can vary with plant genotype, but are the first to show that it is still true in a no-choice context.

Attractiveness differences among odor blends emitted by the six OSR genotypes of this study could be based either on qualitative (presence/absence of specific compounds) and/or quantitative (total amount of VOCs emitted or ratio of the same compounds) variations (Raguso 2008). Both qualitative and quantitative differences were found in odor blends between 'Darmor' and 'Yudal' at an earlier plant growth stage (Kergunteuil 2013). It is likely that the same occurs at the bud stage.

Although volatile characterization is not achieved yet, two groups of VOCs were certainly present in odor bouquets. First, volatiles constitutively emitted by inflorescences and vegetative parts. Even if buds produce smaller amounts of compounds than flowers, they still emit enough to be detectable by the pollen beetle (Cook *et al.* 2002, 2007). Floral scents generally mainly consist of terpenoids and aromatic compounds (Schiestl 2010). Compounds released by vegetative parts are often dominated by terpenes (Pichersky & Gershenzon 2002; Unsicker *et al.* 2009). Secondly, volatiles induced in response to the injury we inflicted to the plant just before the experiment. At least three classes of metabolites are likely to be emitted: terpenes, fatty-acid derivatives called 'Green Leaf Volatiles' (GLVs) and ITCs (Pichersky & Gershenzon 2002; Ferry *et al.* 2004; Textor & Gershezon 2009; Unsicker *et al.* 2009). Either terpenes, aromatic compounds, GLVs and ITCs were identified in previous studies conducted on OSR (*e.g.* Tollsten & Bergström 1988; Blight *et al.* 1995; Jönsson *et al.* 2005; Cook *et al.* 2007).

Whether differences among the six OSR genotypes were due to attractive or repellent compounds is an unresolved question. It is probably partly both. Although floral volatiles are classically studied through the prism of the trade-off 'attracting pollinators – deterring herbivores' (Schiestl 2010; Muhlemann *et al.* 2014), a meta-analysis performed by Junker & Blüthgen (2010) suggested another point of view. Indeed, these authors rather showed that the greatest contrast was between 'obligate flower visitors' (which includes both pollinators and florivores), that are attracted by floral scents, and 'facultative flower visitors' that are repelled. The pollen beetle is an obligate flower visitor. Consistently with the hypothesis of Junker & Blüthgen (2010), it is attracted by most of the floral volatiles that were individually tested (Smart & Blight 2000; Cook *et al.* 2007). The function of terpenes and GLVs emitted after wounding (response to purely mechanical wounding and chewing-insect wounding is mostly the same (Ferry *et al.* 2004; Erb *et al.* 2012)) is thought to be defensive, by both deterring the attacking herbivore and attracting its natural enemies (Unsicker *et al.* 2009). Smart & Blight (2000) showed that several GLVs are effectively repellent for the pollen beetle. Finally, ITCs seem to be

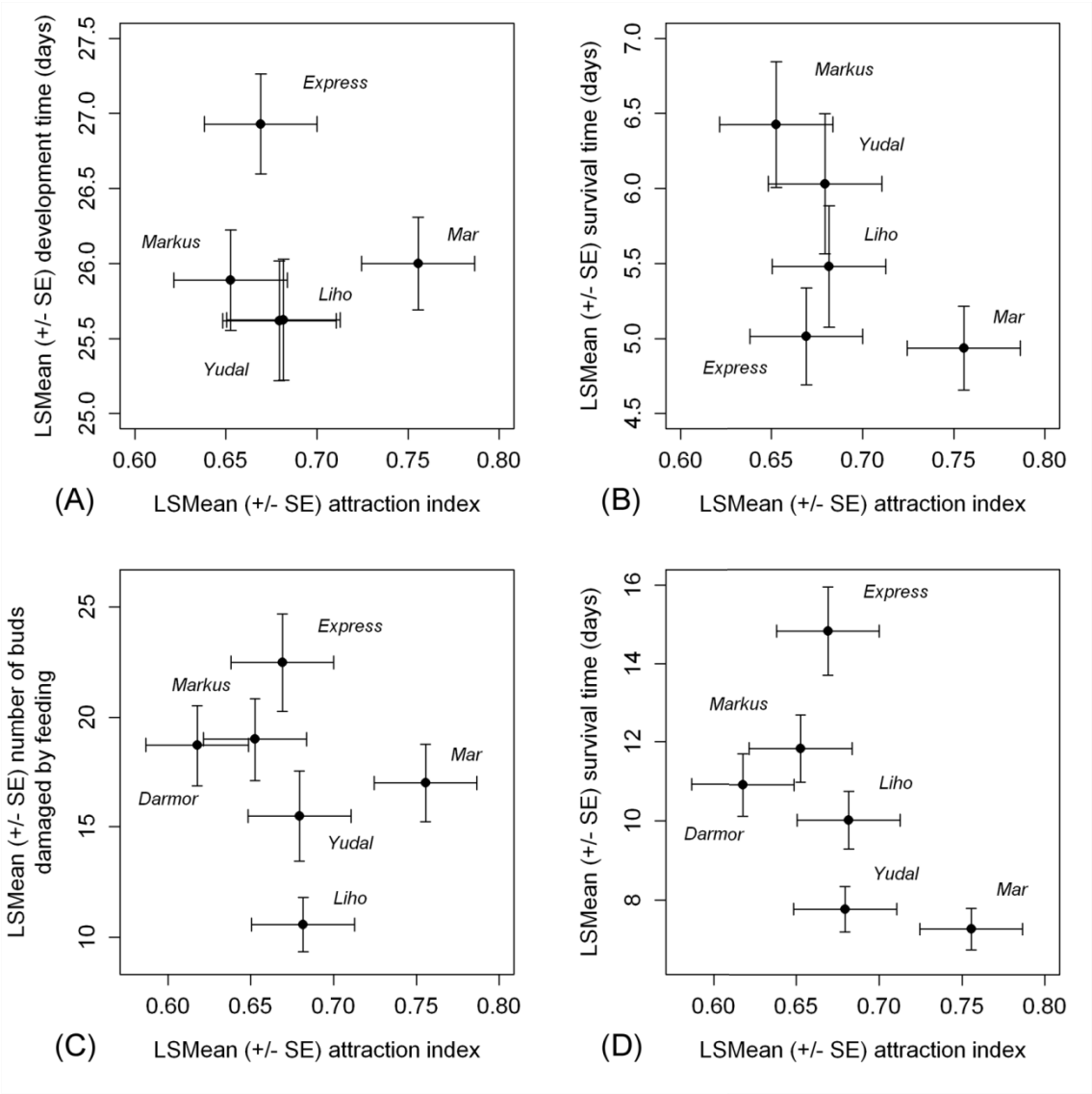

Fig. 3 Relationships between the mean attraction index of six oilseed rape (*B. napus*) genotypes for the pollen beetle (*M. aeneus*) and (A) the mean development time of larvae ($r^2 = 0.01$; data on 'Darmor' not available); (B) the mean survival time of emerging adults developed on the same genotypesunfed ($r^2 = 0.42$; data on 'Darmor' not available); (C) the mean feeding intensity of adults ($r^2 = 0.06$); (D) the mean survival time of adults sampled in the field and fed with the pollen of the same genotypes ($r^2 = 0.31$). Horizontal bars: N = 60 for all plant genotypes; vertical bars on the (A) and (B) graphs: N = 8 for 'Liho', 9 for 'Yudal', 13 for 'Express' and 'Markus', 14 for 'Mar'; vertical bars on the (C) graph: N = 10 for 'Express', 11 for 'Darmor' and 'Yudal', 12 for 'Liho', 'Mar' and 'Markus'; vertical bars on the (D) graph: N = 50 for all genotypes. Back-transformed Least Squares Means (± SE) are systematically represented. Data on development and survival come from Hervé *et al.* (in prep.) [*Article 6*]; data on feeding intensity come from Hervé *et al.* (under revision) [*Article 3*]

universally toxic (Wittstock *et al.* 2003). They are deterrent for the majority of phytophagous insects, but several specialists of brassicaceous plants adapted to these compounds and are attracted by them (*e.g.* the cabbage seedpod weevil *Ceutorhynchus assimilis* or the turnip sawfly *Athalia rosae* (Bruce 2014)). The pollen beetle is a specialist of Brassicaceae for oviposition. In coherence with this pattern it is attracted by several ITCs (Blight & Smart 1999; Cook *et al.* 2006).

Our results showed a gradient of preference, from the genotypes 'Darmor' and 'Markus' to the preferred 'Mar'. Since brassicaceous plants are the only oviposition sites of pollen beetle females, whereas adults can feed from many different plant families, this preference could be seen as a preference for oviposition sites. The

preference – performance hypothesis (*e.g.* Thompson 1988) states that a preference should reflect a gradient of larval performance. We estimated performance in another study conducted at the same OSR genotypes by measuring two traits: development time of larvae and survival of the emerging adults unfed (Hervé *et al.* in prep. [*Article 6*]). Although intergenotypic differences were found for both traits, no pattern linking preference and performance was apparent (Fig. 3A,B).

Following the hypothesis of Mayhew (2001), preference could be based on adult rather than larval performance. Egg production, an important component of female fitness, was shown to be essentially influenced by feeding intensity in the pollen beetle (Hervé *et al.* 2014 [*Article 4*]). Feeding intensity was studied on the same six OSR genotypes in another experiment (Hervé *et al.* under revision [*Article 3*]). Again, no pattern linking this intensity (thus, egg production) to preference appears (Fig. 3C). Finally, the preference gradient could be adaptive if it reflects the quality of the pollen provided by the plant for adult survival. This trait was also studied on the same six genotypes (Hervé *et al.* in prep. [*Article 6*]), but once again no pattern appears (Fig. 3D).

From an adaptive point of view, this situation makes no sense. However, it has already been observed at the interspecific scale with the white mustard, *Sinapis alba*. Indeed, this species is as attractive as OSR for the pollen beetle in the field (Ekbom & Borg 1996; Kaasik *et al.* 2014), whereas it is clearly a poor-quality host either for adult feeding (Ekbom & Borg 1996), oviposition (Borg & Ekbom 1996; Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Hopkins *et al.* 1998; Kaasik *et al.* 2014) and larval development (Ekbom 1998). The lack of link between preference and performance may, however, still make some sense in the agronomical context in which the OSR – pollen beetle interaction takes place. Oilseed rape is a species that results from the hybridization of *B. rapa* and *B. oleracea*, and no wild form is known (U 1935; Gómez-Campo & Prakash 1999; Doré & Varoquaux 2006; Allender & King 2010). Therefore, all OSR plants encountered by the pollen beetle come from the selective process conducted by plant breeders (or farmers). This selection, based exclusively on yield, may have considerably disturbed natural plant physiological functions. A striking example concerning plant defense is the lost ability of most of the North American maize varieties to emit *(E)*-β-caryophyllene (due to the shutdown of the expression of a *(E)*-β-caryophyllene synthase) in response to attacks of several phytophagous insects. This sesquiterpene is responsible for the attraction of natural enemies of these herbivores; plants are therefore less protected and suffer more damage (Rasmann *et al.* 2005; Köllner *et al.* 2008; Degenhardt *et al.* 2009). The loss of defensive traits during plant domestication is frequently postulated (Sotelo 1997). It can also be hypothesized that the breeding process would have broken genetic correlations between plant traits that are not involved in yield determination. In that case, volatile cues perceived by insects may, for example, not faithfully reflect host quality (for themselves or their offspring). This scenario may explain why preference is not related to any measure of performance. Moreover, varieties are regularly replaced by new ones. The breeding process from which these new varieties originated might have led to very different associations among plant phenotypic traits that are not involved in yield. This could considerably slow down adaptation of herbivores to their host plant.

Restoring plant defenses that have been broken by the breeding process was thought to be a promising strategy to protect crops against insect pests (Degenhardt *et al.* 2009). However, it appeared to be compromised by the fact that this restoration could come with considerable costs for the plant, overshadowing the potential benefits (Robert *et al.* 2013). Besides trap cropping (which is based on 'trap plants' that are more attractive than the culture to be protected (Shelton & Badenes-Perez 2006)), another valuable strategy may be to select plants to decrease their own attractiveness (not relatively to trap plants). Studies comparing varieties/genotypes in no-

choice tests are needed to test the feasibility of this method. This study (but also that of Rajabaskar *et al.* (2013)) suggests that it may be possible.

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– CHAPTER 2 –

Feeding intensity

Manipulating feeding stimulation to protect crops against insect pests?

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Enhancing natural mechanisms of plant defense against herbivores is one of the possible strategies to protect cultivated species against insect pests. Host plant feeding stimulation, which results from phagostimulant and phagodeterrent effects of both primary and secondary metabolites, could play a key role in levels of damage caused to crop plants. We tested this hypothesis by comparing the feeding intensity of the pollen beetle *Meligethes aeneus* on six oilseed rape (*Brassica napus*) genotypes in a feeding experiment, and by assessing the content of possible phagostimulant and phagodeterrent compounds in tissues targeted by the insect (flower buds). For this purpose, several dozens of primary and secondary metabolites were quantified by a set of chromatographic techniques. Intergenotypic variability was found both in the feeding experiment and in the metabolic profile of plant tissues. Biochemical composition of the perianth was in particular highly correlated to insect damage. Only a few compounds explained this correlation, among which was sucrose, known to be highly phagostimulant. Further testing is needed to validate the suggested impact of specific compounds we have identified. Nevertheless, our results open the way for a crop protection strategy based on artificial selection of key determinants of insect feeding stimulation.

INTRODUCTION

Plants defend themselves against herbivorous insects by a variety of mechanisms, including life history traits (*e.g.* escapement by shorter or earlier flowering phenology), architectural and structural characteristics (*e.g.* leaf toughness or trichome density) and chemical defenses (*e.g.* repellents, toxins or digestibility reducers) (Gatehouse 2002; Berenbaum & Zangerl 2008; Clissold *et al.* 2009; Carmona *et al.* 2011). These mechanisms that are under selection in natural plant populations, can also be favored by means of artificial selection to protect cultivated species against insect pests. Increasing trichome density of seedlings by transformation with a gene from a highly trichome-covered close species has, for example, led to the production of "hairy canola" (*Brassica napus*) in order to decrease damage caused by flea beetles, despite the fact that this species naturally harbors a low trichome density (Gruber *et al.* 2006).

We propose that another strategy could be used to protect crops against insect pests that cause damage by feeding: manipulation of chemical content of tissues targeted by the insect to reduce feeding stimulation, and therefore feeding damage. Although plant – herbivore interactions are often seen solely through the lens of secondary metabolites, feeding stimulation is greatly influenced by both primary and secondary compounds. It is the result of the integration – by the nervous system of the insect – of many sensory stimulations, triggered either by phagostimulant and phagodeterrent substances acting in synergistic, antagonistic or neutral ways and at precise concentrations (Chapman 2003).

Two conditions have to be met to seriously envisage the proposed strategy. Firstly, feeding intensity of the insect must differ among genotypes of the same host plant species. It is likely that this situation is common. Indeed, variation between different plant genotypes/varieties for feeding damage by the same insect species has already been shown in a number of plant families (see for example Glynn *et al.* (2004) in Salicaceae, Lyytinen *et al.* (2007) in Solanaceae, Niveyro *et al.* (2013) in Amaranthaceae or Ströcker *et al.* (2013) in Fabaceae). The second condition to satisfy is much more challenging, since it is to characterize the biochemical profile of the studied plant genotypes by assessing the balance between phagostimulant and phagodeterrent compounds in the eaten tissues, in order to identify key metabolites on which selection could be based. To our knowledge, no study has yet faced this challenge.

The pollen beetle (*Meligethes aeneus*) is a major insect pest of oilseed rape (OSR) crops. It is an univoltine specialist of brassicaceous plants for oviposition, but a generalist for feeding (Free & Williams 1978; Ekbom & Borg 1996; Carrié *et al.* 2012; Marques & Draper 2012). After diapausing in winter, adults feed on a wide range of plant families for about two weeks, which is necessary for ovary maturation, and migrate to brassicaceous plants for mating and ovipositing (Williams 2010). They colonize OSR fields when plants are at the bud stage (*i.e.* flowers not opened). This pollen-feeder destroys buds to reach the pollen inside, leading to potentially important yield losses (*e.g.* Nilsson 1987). This biological system is quite special because, although pollen beetles seem to dislike eating petals (Charpentier 1985), they have to eat the perianth (composed of sepals and petals) to reach the pollen in the anthers. Although adult feeding has important agronomical consequences, nothing is known about the chemical stimuli determining its intensity (including glucosinolates).

In the present study, we (i) tested for an intraspecific variation in OSR for pollen beetle feeding intensity and (ii) searched for key biochemical determinants of feeding stimulation through the screening of a large number of both primary and secondary compounds. Six OSR genotypes were compared in a feeding experiment in controlled conditions and the content of potentially phagostimulant and phagodeterrent compounds in plant tissues fed upon by the insect was assessed in each genotype. Several dozens of metabolites, belonging to

different chemical classes and potentially influencing feeding, were quantified separately in the tissue containing the food source (anthers) and in the tissue to be pierced to get the food (perianth): soluble carbohydrates/polyols, free amino acids, glucosinolates, flavonols and hydroxycinnamic acids. Soluble carbohydrates (*i.e.* sugars) were chosen because of their known major phagostimulant effect on insects (Chapman 2003). Free amino acids were chosen for the same reason, even though no data are available on the pollen beetle. Glucosinolates were chosen because of their well-studied action on phytophagous insects: they are mostly deterrent for generalists, whereas usually stimulant for specialists (reviewed *in* Hopkins *et al.* 2009). Flavonoids are long known to influence plant – insect interactions. Among them, flavonols were chosen because of the frequent influence they have on insect feeding, even if a general pattern cannot be easily described (Simmonds 2001, 2003; Treutter 2006). Finally, hydroxycinnamic acids were chosen because of their high concentration in brassicaceous plants (Francisco *et al.* 2009; Velasco *et al.* 2011), combined with the fact that some are suggested to influence insect feeding (Lin & Mullin1999; Kühnle & Müller 2009; Leiss *et al.* 2013).

MATERIALS AND METHODS

Plants

All genotypes used in this study are pure lines from an INRA OSR (*Brassica napus* L.; Brassicaceae) collection (BraCySol Genetic Resources, INRA, France). Both winter and spring OSR were used, exhibiting high/low erucic acid and high/low glucosinolate content in the seeds. No data are available on the interaction between the pollen beetle and any of these lines; genotypes used in this study were chosen from their known gradient of resistance to the pathogen *Plasmodiophora brassicae* (Wagner *et al.* 2012) (Table 1). Seeds were sown in individual propagation plugs (75 % peat, 13 % perlite, 12 % vermiculite; pH = 6; Fertiss) and placed in controlled conditions (photoperiod 13:11 L:D, temp. 20 : 17 °C) for 3 weeks. Plantlets were then vernalized between 3-10 weeks depending on the genotype (photoperiod 16:8 L:D, temp. 5 °C). After this period plantlets were transplanted in individual 2 l-pots (substrate: 85 % peat, 15 % perlite; pH = 6) and placed in controlled conditions (photoperiod 16:8 L:D, temp. 20 : 18 °C) until bud development (ca. 3 weeks). All plants used for experiments were at BBCH stage 55-57 (Lancashire *et al.* 1991), *i.e.* the 'green bud stage' which is the most susceptible to pollen beetles (Williams 2010).

Insects

Overwintered pollen beetles (*Meligethes aeneus* F.; Coleoptera: Nitidulidae) were collected in an unsprayed winter OSR crop (cv. Pollen) around the INRA station (Le Rheu, Brittany, France) and placed in controlled

Table 1 Previously known characteristics of the six genotypes used in this study and vernalization time. WOSR: winter oilseed rape; SOSR: spring oilseed rape. '0' genotypes were selected for a low concentration of erucic acid or glucosinolates in the seeds, whereas '+' genotypes contain high concentrations. Evaluation of resistance to *Plasmodiophora brassicae* come from Wagner *et al.* (2012). Vernalization is mandatory only for WOSR but was applied to SOSR to facilitate synchronization of growth stages among genotypes during experiments. The genotype 'Darmor' has to be vernalized for a longer duration to induce development of reproductive organs

		Seed profile			
Genotype	**Type**	**Erucic acid**	**Glucosinolates**	**Resistance to *P. brassicae***	**Vernalization (weeks)**
Darmor	WOSR	0	0	High	10
Express	WOSR	0	0	High	8
Liho	SOSR	+	+	Low	3
Mar	WOSR	0	0	Medium	8
Markus	WOSR	+	+	Low	8
Yudal	SOSR	+	+	Medium	3

conditions (photoperiod 16:8 L:D, temp. 20 °C) in cylindric plastic boxes (diameter 11.5 cm, height 8.5 cm) where they were fed with bee-collected pollen from organically grown plants. All insects were starved for 24 h before experiments and were kept individually in small Petri dishes (diameter 3.5 cm) containing a moistened paper filter. Individuals were used for experiments up to 5 days after field collection.

Feeding experiment

Four unsexed beetles were placed on the main inflorescence of an intact OSR plant, in a plastic pot (diameter 6.5 cm, height 9 cm) isolating this inflorescence from the rest of the plant. The pot was maintained by a nylon-thread fixed to the wall to avoid bending of the stem. Insects were able to feed from buds for four days. Plants were randomly placed in a climate room under the same controlled conditions as described above during this period.

After the four days, the individuals were sexed following Ruther & Thiemann (1997) and the inflorescence was carefully inspected. The total number of buds carried by the inflorescence and the number of buds damaged by feeding were counted to estimate resource availability and damage level of the plant, respectively. Buds damaged by feeding are easily distinguishable as they exhibit an irregular shape hole, often in the center of the bud, and anthers are damaged too. Due to technical constraints on plant production, an unequal number of plants were used per genotype ('Express': N = 10; 'Darmor', 'Yudal': N = 11; 'Liho', 'Mar', 'Markus': N = 12). Replicates were performed randomly through time.

Metabolic profiling

Twenty buds (length ca. 5 mm) from an intact plant were dissected under a binocular microscope. Sepals and petals (altogether named 'perianth') were isolated, as well as anthers, and immediately frozen in liquid nitrogen for fixation of the metabolism. Twenty plants were used per genotype, in five groups of four plants to gain enough material for biochemical analyses. Five samples per genotype were therefore used for metabolic profiling of the perianth, each sample comprising 80 buds. Samples were freeze-dried and ground to powder, then stored at -80 °C until analysis. Due probably to a greater water content, only four samples of the genotype 'Mar' contained enough dried material to perform all biochemical analyses. Exactly the same procedure was used for anthers (in this case all samples contained enough material for subsequent biochemical analyses).

Soluble carbohydrates, polyols and amino acids were extracted following Gravot *et al.* (2010), except that adonitol was used as the internal standard for soluble carbohydrates and polyols. Soluble carbohydrates and polyols were profiled by GC-FID according to Adams *et al.* (1999) and Lugan *et al.* (2009). Amino acids were profiled by UPLC-DAD according to Jubault *et al.* (2008) modified by Gravot *et al.* (2010). All compounds were identified from their retention time by comparison to external standards, and quantified based on the internal standard.

Glucosinolates, flavonols and hydroxycinnamic acids were extracted together according to the following protocol: 5 mg of dried powder was suspended in 1 ml of a MeOH-HForm (99:1) soln. and agitated for 30 s at room temperature. The extract was then ultra-sonicated for 5 min and rapidly centrifuged for sedimentation at room temperature. Then, 900 µl of the liquid phase was filtered through a 0.45 µm filter and transferred to a clean microtube for subsequent analyses. For glucosinolate profiling, a 10-fold dilution of the extract was made before analysis. An Acquity-TQD UPLC-PDA-MS (Waters) with electrospray ionization in a negative mode was used to analyse glucosinolates, flavonols and hydroxycinnamic acids. The chromatographic conditions were as follows: column Waters Acquity C18 (150 mm x 1.7 µm), flow rate 0.4 $ml.min^{-1}$, column oven temp. 25 °C, injection volume

2 µl. The A-eluent was H2O-HForm (99.9:0.01) and the B-eluent was MeCN-HForm (99.9:0.01). The applied gradient was: 0 to 0.2 min 2 % B, 0.2 to 3 min 62 % B, 3 to 8 min 90 % B; 8 to 9 min 90 % B; then return to initial conditions 2 % B in 1 min and re-equilibration for 1 min. Mass spectrometry was used to identify glucosinolates on the basis of their *m/z* response in negative electrospray mode, their retention time, and quantified by use of calibration curves obtained with commercially available standards (gluconapin was used to quantify glucobrassicanapin; progoitrin was used to quantify epiprogoitrin). Flavonols and hydroxycinnamic acids were detected using a photodiode array detector at specific wavelengths: 350 nm for flavonols and 320 nm for hydroxycinnamic acids. Their identification was based on their UV-visible spectra compared to pure compounds and bibliographic data, and their quantification was obtained using calibration curves of standards (aglycones were used to quantify glycosyl-derivatives).

Statistical analyses

All statistical analyses were performed using R software (R Core Team 2013).

Feeding experiment – The number of buds damaged was compared among the six genotypes using a likelihood ratio test on a Generalized Linear Model (GLM) (distribution: negative binomial, link function: log) (function 'glm.nb', package 'MASS' (Venables & Ripley 2002)) in which the total number of buds carried by the inflorescence and the number of females present in the replicate were introduced. Pairwise comparisons of Least Squares Means (LSMeans) were performed using the function 'lsmeans' (package 'lsmeans' (Lenth 2013)) and the False Discovery Rate correction for *P*-values (Benjamini & Hochberg 1995).

Metabolic Profiling – The following procedure was used for both perianth and anthers: a Principal Component Analysis was first performed on normalized (*i.e.* centered – unit variance-scaled) compound concentrations (function 'dudi.pca', package 'ade4' (Dray & Dufour 2007)) to detect possible outliers. Two samples from perianths of the genotype 'Mar' were excluded at this step because of biologically aberrant values caused by analytical issues. A Partial Least Squares – Discriminant Analysis (PLS-DA; Barker & Rayens 2003) was then performed on the normalized concentrations to discriminate genotypes based on their metabolic profiles (function 'plsda', package 'mixOmics' (González *et al.* 2011)). The significance of the discrimination was tested on each score plot using a MANOVA on the point coordinates. Pairwise comparisons were adjusted with the False Discovery Rate correction for *P*-values. Although metabolic profiling of the perianth of the genotype 'Mar' was based on only two samples, results including this genotype are presented since conclusions were identical whether or not it was included in the analysis.

RESULTS

Feeding experiment

The number of buds damaged was significantly different among OSR genotype ($\chi^2 = 28.113$, $df = 5$, $P < 0.001$; Fig. 1). 'Express' was the most damaged genotype (LSMean number of buds damaged ± SE: 22.49 ± 2.21), whereas 'Liho' was the least damaged (10.57 ± 1.23). An intermediate and homogeneous group was composed of the four other genotypes. The sex-ratio of beetles did not differ among genotypes (likelihood ratio test on a GLM comparing the proportion of females (distribution: binomial, link: logit): $\chi^2 = 1.163$, $df = 5$, $P = 0.948$; mean ± SE proportion of females: 0.57 ± 0.03). The number of buds damaged did not depend neither on total number of buds carried by the inflorescence ($\chi^2 = 0.0687$, $df = 1$, $P = 0.793$) nor on number of females ($\chi^2 = 0.008$, $df = 1$, $P = 0.930$).

Table 2 Compounds potentially detectable and detected in the perianth and anthers of oilseed rape buds. Values presented are means for all genotypes (minimum – maximum), in nmol.mg^{-1} dry wt. Abbreviations used in figures are given in brackets

Compound	Perianth	Anthers
Carbohydrates/polyols		
Cellobiose (Cel)	10.5 (7.1 - 15.2)	13.6 (8.0 - 25.9)
Fructose (Frc)	29.7 (4.4 - 66.7)	11.9 (20.0 - 30.4)
Galactose	-	-
Gentiobiose	-	-
Glucose (Glc)	29.0 (6.3 - 57.6)	10.7 (4.4 - 28.3)
Maltose (Mal)	0.08 (0 - 1.1)	-
Mannose (Mans)	4.4 (0 - 23.7)	0.8 (0 - 13.2)
Melibiose	-	-
Sucrose (Suc)	17.9 (6.3 - 32.6)	44.3 (29.4 - 65.0)
Trehalose	-	-
Galactinol	-	-
Mannitol (Manl)	-	0.04 (0 - 1.2)
Myo-inositol (Myo)	4.2 (2.7 - 6.4)	2.8 (1.6 - 6.7)
Sorbitol (Sor)	1.2 (0 - 3.2)	1.1 (0 - 2.7)
Free amino acids		
α-Alanine (a.Ala)	9.1 (5.6 - 14.2)	8.2 (5.4 - 12.7)
β-Alanine (b.Ala)	0.7 (0.4 - 1.1)	1.1 (0.4 - 3.5)
Arginine (Arg)	27.3 (4.1 - 44.0)	2.9 (0.6 - 7.1)
Asparagine (Asn)	31.5 (13.5 - 47.2)	13.4 (5.3 - 32.4)
Aspartic acid (Asp)	25.3 (17.6 - 39.4)	14.3 (6.8 - 26.0)
Cysteine (Cys)	0.4 (0 - 1.7)	0.7 (0.2 - 1.5)
S-methylcysteine (MeCys)	-	0.2 (0.02 - 2.8)
GABA	2.0 (0.8 - 4.0)	15.0 (2.2 - 52.4)
Glutamic acid (Glu)	40.0 (30.4 - 49.8)	23.3 (12.8 - 39.0)
Glutamine (Gln)	280.3 (142.2 - 433.5)	49.0 (27.6 - 95.1)
Glycine (Gly)	0.6 (0.2 - 1.7)	1.4 (0.7 - 3.9)
Histidine (His)	9.3 (3.0 - 18.0)	1.8 (0.2 - 4.0)
Isoleucine (Ile)	1.9 (1.0 - 4.3)	2.3 (0.5 - 6.0)
Leucine (Leu)	1.0 (0.6 - 1.7)	1.8 (0.5 - 4.3)
Lysine (Lys)	1.3 (0.6 - 2.5)	1.6 (0.5 - 3.5)
Methionine (Met)	0.3 (0.1 - 0.4)	0.4 (1.2 - 0.8)
Ornithine (Orn)	0.6 (0.04 - 1.7)	-
Phenylalanine (Phe)	0.7 (0.3 - 1.2)	1.2 (0.3 - 3.4)
Proline (Pro)	24.5 (7.0 - 79.8)	82.0 (34.4 - 152.3)
Hydroxyproline (Hpro)	-	0.3 (0.1 - 0.6)
Serine (Ser)	8.2 (4.9 - 15.5)	6.8 (4.3 - 13.0)
Threonine (Thr)	5.3 (3.5 - 7.3)	3.3 (1.9 - 6.2)
Tryptophan (Trp)	0.3 (0.1 - 1.4)	0.6 (0.07 - 1.7)
Tyrosine (Tyr)	0.4 (0.2 - 0.9)	0.9 (0.4 - 3.0)
Valine (Val)	3.9 (3.0 - 5.5)	5.4 (2.1 - 11.4)
Glucosinolates		
Glucobrassicanapin (Gbranap)	2.9 (0.2 - 9.0)	6.0 (0.3 - 16.3)
Glucobrassicin (Gbra)	0.4 (0 - 1.9)	0.2 (0 - 0.9)
4-Methoxyglucobrassicin	-	-
Glucoerucin (Geru)	-	0.2 (0 - 0.4)
Gluconapin (Gnap)	2.9 (0 - 10.5)	3.9 (0 - 12.1)
Gluconasturtiin (Gnas)	-	0.6 (0 - 2.4)
Glucotropaelin	-	-
Neoglucobrassicin	-	-
Progoitrin (Prog)	22.1 (1.5 - 85.7)	18.2 (1.1 - 69.3)
Epiprogoitrin (EProg)	-	2.9 (0 - 22.1)

Table 2 (continued)

Flavonols		
Kaempferol and derivatives (Kaempf)	0.1 (0 - 0.6)	5.7 (1.7 - 12.3)
Isorhamnetin and derivatives (Isorham)	3.2 (0.2 - 11.7)	6.9 (1.5 - 14.2)
Quercetin and derivatives (Querc)	0.8 (0.2 - 2.5)	6.6 (2.5 - 12.5)
Hydroxycinnamic acids		
Caffeic acid and derivatives	-	-
Coumaric acid and derivatives	-	-
Ferulic acid and derivatives	-	-
Sinapic acid and derivatives (AcSin)	4.3 (2.3 - 5.6)	1.4 (1.2 - 1.6)

Metabolic profiling

Among all detectable metabolites, 41 were found in the perianth and 45 in anthers (summary in Table 2; detail for perianth and anthers per genotype in Supplementary Tables S1 and S2, respectively). These metabolites were essentially similar in both types of tissues, with the exception of one sugar (maltose, only detected in the perianth), one polyol (mannitol, only detected in anthers), three free amino acids (ornithine only detected in the perianth, *S*-methylcysteine and hydroxyproline only detected in anthers) and three glucosinolates (glucoerucin, gluconasturtiin and epiprogoitrin, only detected in anthers). In both tissues, almost all compounds were present in the six OSR genotypes, leading to essentially quantitative differences in the metabolic profiles of these genotypes. Qualitative differences almost always involved metabolites present at concentrations close the detectability threshold of our equipment (maltose in the perianth; mannose, mannitol and sorbitol in anthers), with the exception of glucosinolates in anthers. In both tissues, the three components retained in the statistical analysis explained an important part of the intergenotypic variance (80.29 % in the PLS-DA on anthers, 76.23 % in the PLS-DA on the perianth).

Biochemical composition of anthers was dominated by proline, followed by a group composed of glutamine and sucrose, then a continuum from glutamic acid to minor compounds. Cellobiose, fructose and glucose were the most dominant sugars after sucrose. Progoitrin was clearly the glucosinolate and more generally the secondary metabolite with the highest concentration. The content of flavonols was quite equal and sinapic acid was the only hydroxycinnamic acid detected. The statistical analysis revealed that the metabolic profile of the six genotypes was quantitatively different, as shown by the good discrimination between them (Fig. 2). However, no pattern matching the gradient established from the feeding experiment was observed in any of the score plots.

Composition of the perianth was highly dominated by glutamine. Glutamic acid came after, followed by a group composed of asparagine, fructose, glucose, arginine and aspartic acid. Sucrose was the dominant

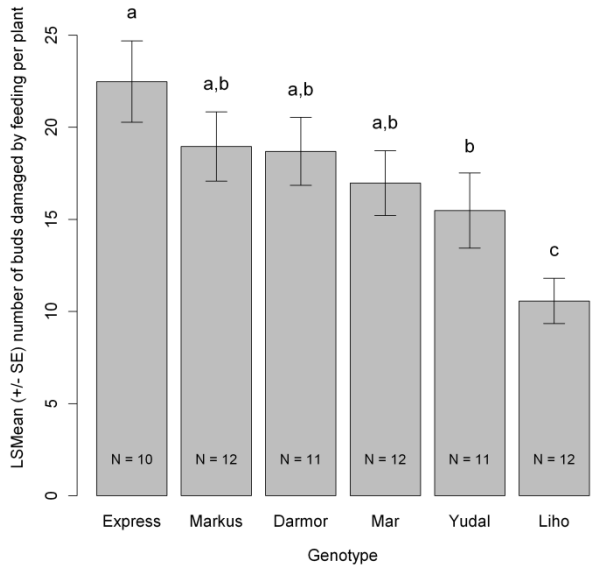

Fig. 1 Least Squares Mean (± SE) number of oilseed rape (*B. napus*) green buds damaged by the pollen beetle (*M. aeneus*) by feeding, per plant genotype (back-transformed values). Different letters indicate statistically different LSMeans. N: number of plants used per genotype

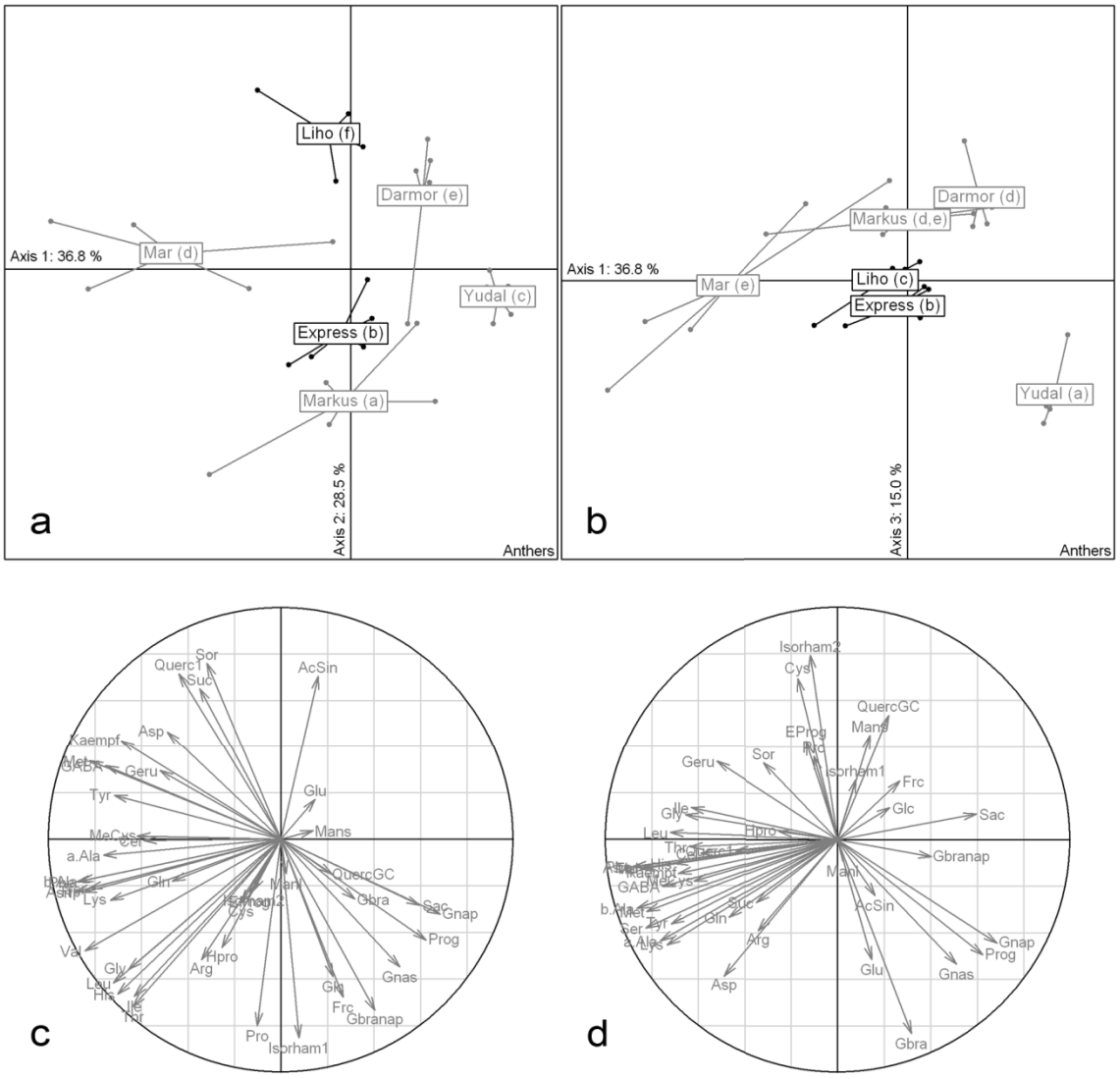

Fig. 2 Graphs of the Partial Least Squares – Discriminant Analysis performed on the biochemical composition of anthers of oilseed rape (*B. napus*) green buds. **a**: score plot 1-2 (65.3 % of the intergenotypic variance explained; results of the MANOVA testing for the discrimination of genotypes: *pseudo*-$F_{10,42}$ = 21.67, $P < 0.001$); **b**: score plot 1-3 (51.8 % of the intergenotypic variance explained; results of the MANOVA testing for the discrimination of genotypes: *pseudo*-$F_{10,42}$ = 10.436, $P < 0.001$); **c, d**: corresponding loading plots of metabolites. Different letters between brackets next to genotypes indicate statistically different biochemical profiles. Extreme plant genotypes in the feeding experiment are represented in black on the score plots

sugar after fructose and glucose. As in anthers, glucosinolate content was dominated by progoitrin and sinapic acid was the only hydroxycinnamic acid detected. Flavonol content consisted almost only of glysosyl-derivatives of isorhamnetin. The statistical analysis showed quantitative differences among genotypes in the metabolic profiles of their perianth (Fig. 3). In this case however, two interesting patterns were observed. On the first score plot (Fig. 3a), two groups were clearly separated: one composed only of the genotype 'Yudal', the second composed of the five other genotypes. The loading plot of metabolites (Fig. 3c) clearly indicated that glucosinolates explained this discrimination. Indeed, the four glucosinolates present in this tissue were much more concentrated in 'Yudal' compared to all other genotypes (ratio for the sum of all glucosinolates, 'Yudal' taken as the reference: 'Markus' 0.25, 'Express' 0.22, 'Darmor' 0.11, 'Mar' 0.07 and 'Liho' 0.04). On the second score plot (Fig. 3b), a gradient in the diagonal direction (from top-right to bottom-left) was observed. In this direction,

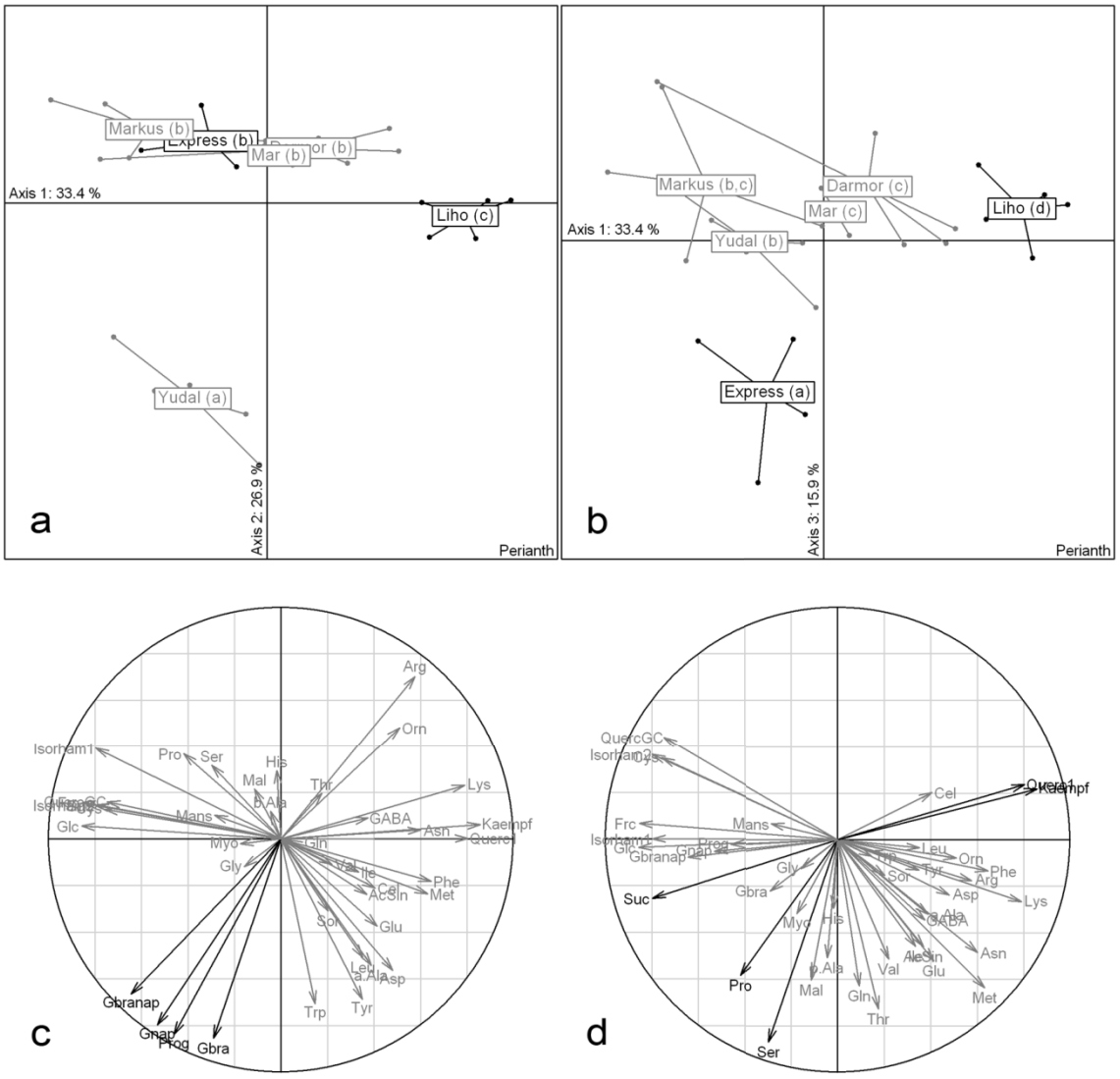

Fig. 3 Graphs of the Partial Least Squares – Discriminant Analysis performed on the biochemical composition of the perianth of oilseed rape (*B. napus*) green buds. **a**: score plot 1-2 (60.4 % of the intergenotypic variance explained; results of the MANOVA testing for the discrimination of genotypes: *pseudo*-$F_{10,48}$ = 17.56, $P < 0.001$); **b**: score plot 1-3 (49.3 % of the intergenotypic variance explained; results of the MANOVA testing for the discrimination of genotypes: *pseudo*-$F_{10,48}$ = 13.40, $P < 0.001$); **c, d**: corresponding loading plot of metabolites. Different letters between brackets next to genotypes indicate statistically different biochemical profiles. Extreme plant genotypes in the feeding experiment are represented in black on the score plots. Compounds discussed in the text are represented in black on the loading plots

the order of the six genotypes closely matches the gradient established from the feeding experiment. In contrast to the first score plot where the discrimination was due mostly to glucosinolates, the loading plot of the second score plot (Fig. 3d) revealed that metabolites from different biochemical classes were responsible for the discrimination. Based on the representativeness of the compounds on this map (*i.e.* the length of the corresponding arrows), the discrimination in this diagonal direction was mostly due to sucrose (a sugar), proline, serine (two free amino acids), quercetin-3-*O*-sophoroside and kaempferol-3-*O*-sophoroside (two flavonols). Sucrose, proline and serine were positively correlated to the observed gradient (*i.e.* samples in the bottom-left section of the circle are more concentrated), whereas the two flavonols were negatively correlated with the gradient (*i.e.* samples in the top-right section of

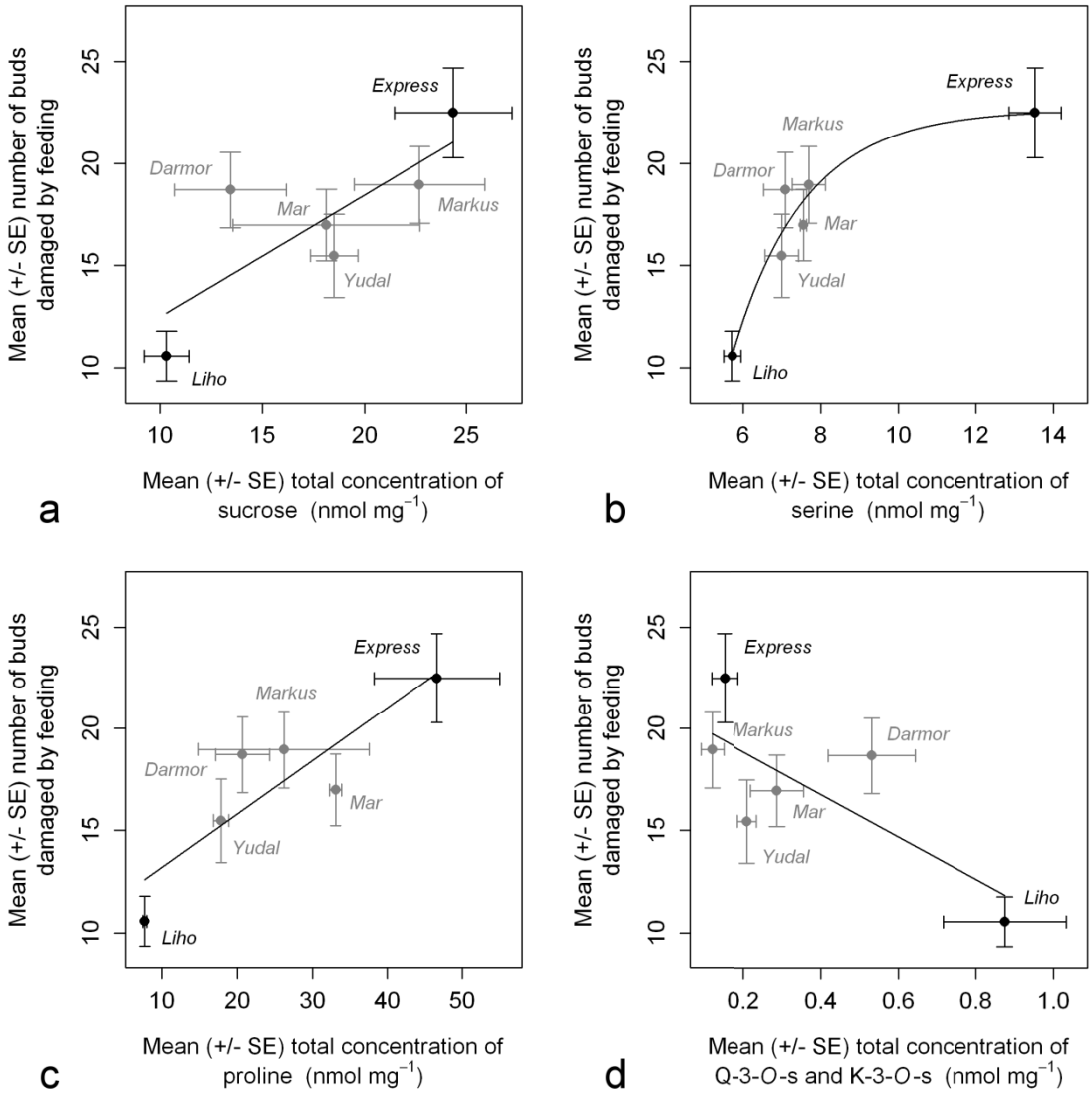

Fig. 4 Relationships between the mean number of oilseed rape (*B. napus*) green buds damaged by the pollen beetle (*M. aeneus*) by feeding and **a** the mean concentration of sucrose in the perianth ($r^2 = 0.63$); **b** the mean concentration of serine in the perianth (*pseudo*-$r^2 = 0.84$); **c** the mean concentration of proline in the perianth ($r^2 = 0.76$); **d** the mean total concentration of quercetin-3-*O*-sophoroside (Q-3-*O*-s) and kaempferol-3-*O*-sophoroside (K-3-*O*-s) in the perianth ($r^2 = 0.57$). Horizontal bars: N = 5 for all plant genotypes except 'Mar' (N = 2); vertical bars: N = 10 for 'Express', 11 for 'Darmor' and 'Yudal', 12 for 'Liho', 'Mar' and 'Markus'. Extreme genotypes in the feeding experiment are represented in black

the circle are more concentrated) (Fig. 4). Interestingly, sucrose and the two flavonols were particularly highly and negatively correlated to each other.

DISCUSSION

Cultivars containing reduced amounts of known feeding stimulants could be a possible strategy to protect cultivated plant species against insect pests that cause damage by feeding. Before such a control strategy based on artificial selection of less phagostimulant plants can be envisaged, two conditions have to be met. We demonstrated that both these conditions were satisfied in the interaction between OSR and the pollen beetle.

Feeding intensity on different host plant genotypes

Results of the feeding experiment clearly showed that intraspecific variation for feeding stimulation toward the

pollen beetle exists in OSR. Although new for this insect, this result is not surprising as it has already been shown with other specialists of this plant species (*e.g.* the cabbage stem weevil *Ceutorhynchus pallidactylus* (Eickermann & Ulber 2010) and the cabbage stem flea beetle *Psylliodes chrysocephala* (Giamoustaris & Mithen 1995)), and more generally in a great variety of plant-insect systems (*e.g.* Barrett & Agrawal 2004; Glynn *et al.* 2004; Lyytinen *et al.* 2007; Henery *et al.* 2008; El Bouhssini *et al.* 2013; Jackson & Harrison 2013; Knutson *et al.* 2013; Niveyro *et al.* 2013; Ströcker *et al.* 2013).

Key determinants of feeding stimulation

Our results showed that, regarding only the perianth and without any *a priori* on the insect behavior, genotypes are distributed along a gradient which is very close to the gradient observed in the feeding experiment. This suggests that the perianth is the key tissue determining OSR feeding stimulation in the pollen beetle.

Among the 41 compounds found in the perianth, only five mainly contributed to the link between the biochemical data and results of the feeding experiment: sucrose, proline, serine, quercetin-3-*O*-sophoroside and kaempferol-3-*O*-sophoroside. This small proportion suggests that a few compounds could play a crucial role in feeding stimulation for this insect species. To date, no data are available on the effect of these metabolites on the pollen beetle. However, hypotheses can still be drawn from the literature on other phytophagous or omnivorous Coleopteran species.

All insects studied so far have gustatory cells responding to sugars, which can be considered as the most phagostimulant compounds for them (Chapman 2003). Among these compounds, sucrose is always stimulating when tested alone in artificial feeding experiments, and consistently appears as one of the most stimulating sugars (Mitchell & Gregory 1979; Bartlet *et al.* 1994; Isidoro *et al.* 1998; Merivee *et al.* 2008, 2012; Hori *et al.* 2010; Tooming *et al.* 2012). This is in agreement with our results showing that the more sucrose in the perianth of OSR genotypes, the more they are attacked by the pollen beetle. This sole result strongly supports the hypothesis of a direct relationship between pollen beetle damage and perianth biochemical composition.

No general pattern in insect feeding has ever been observed with free amino acids, and insects seem to respond to them in a specific manner. However studies generally show that they are phagostimulant, although much less so than sugars (Chapman 2003). Considering only phytophagous and omnivorous Coleopteran species, two compounds are consistently found to be stimulant: alanine and serine (Mitchell & Schoonhoven 1974; Mitchell & Gregory 1979; Hollister & Mullin 1998; Kim & Mullin 1998; Merivee *et al.* 2008). In our study, OSR genotypes having more serine in their perianth were more damaged by pollen beetles, which is in agreement with results of studies conducted on other species. We found the same pattern with proline. However, if proline was shown as highly phagostimulant in some studies (Mitchell & Schoonhoven 1974; Mitchell & Gregory 1979; Hollister & Mullin 1998), no effect was observed in others (Kim & Mullin 1998; Merivee *et al.* 2008). From their high correlations with feeding intensity, it is likely that serine and proline played an additive phagostimulant effect in our biological system.

The situation is more ambiguous with quercetin-3-*O*-sophoroside and kaempferol-3-*O*-sophoroside. Indeed, the gustatory effect of this class of compounds is highly species-specific (Simmonds 2001, 2003). Among the few studies available on phytophagous Coleopteran species, the aglycone quercetin was shown to be phagodeterrent for *Epilachna paenulata* (Diaz Napal *et al.* 2009, 2010) whereas quercetin-3-*O*-glucoside was phagostimulant for *Diabrotica virgifera virgifera* (Lin & Mullin 1999). No data are available in the literature for the glycoside derivative we found, quercetin-3-*O*-sophoroside. In the same manner, kaempferol-3-*O*-xylosylgalactoside was

shown to be phagostimulant for *Phyllotreta armoraciae* (Nielsen *et al.* 1979), but no data are available for the glycoside derivative we found, kaempferol-3-*O*-sophoroside. It is unknown if these two flavonols were phagodeterrent or not, as their very high negative correlation with sucrose may be a confounding effect. This correlation is itself intriguing, but experiments in our study cannot identify its origin. At least we may suggest that since glucose is the starting point of the biosynthesis of both sucrose and flavonols (*via* the shikimic acid pathway (Ali *et al.* 2009)), some OSR genotypes may invest more in one pathway compared to the other.

Further investigations, especially artificial feeding experiments on single and mixed diets, are needed to give solid conclusions about the hypothesis we made for each of these metabolites. However the literature strongly supports the idea of a crucial role of sucrose and serine.

Influence of glucosinolates in stimulation of pollen beetle feeding

Although the genotype 'Yudal' contained much higher levels of glucosinolates in its perianth than all other genotypes, it suffered an intermediate level of damage by the pollen beetle. The variation in glucosinolate content we observed (maximum – minimum ratio: 23.2) hence neither stimulated, nor deterred insect feeding. This result seems to indicate that pollen beetle adults do not behaviorally avoid glucosinolates, and consequently are probably able to deal with them after ingestion. Further studies are needed to identify the underlying mechanism, which could be detoxification, sequestration, target-site mutation or excretion (Després *et al.* 2007). Whatever the mechanisms involved, glucosinolates do not seem to play a determinant role in the intensity of feeding damage caused by pollen beetle adults. This contrasts with other OSR pests such as *P. chrysocephala* (Bartlet & Williams 1991; Bartlet *et al.* 1994), and with the well-demonstrated influence of some glucosinolate-catabolites (isothiocyanates) in attracting the pollen beetle at distance (Blight & Smart 1999; Smart & Blight 2000; Cook *et al.* 2006). More generally, it highlights the importance of not focusing only on family-specific defense compounds in plant – herbivore studies, as Berenbaum argued some years ago (Berenbaum 1995).

Evolutionary perspective

It appears surprising that pollen beetle feeding intensity is correlated with perianth and not anther chemical composition. Anthers contain the food source (*i.e.* pollen) whereas the perianth is just a tissue beetles have to pierce (and consume) to reach the food. It could have been hypothesized that beetles' feeding behavior should be selected based on pollen chemistry, as seems the case for other pollinators such as honey bees (Cook *et al.* 2003). A possible explanation of this unexpected result is that, although the pollen beetle is a specialist of brassicaceous plants for oviposition, it is a generalist for adult feeding. Indeed, pollen beetles are found in crops of many plant families (Free & Williams 1978; Carrié *et al.* 2012; Marques & Draper 2012). Thus, it is possible that their behavior is not selected to discriminate at a fine, intraspecific, biochemical scale as they usually forage on a great variety of flowers with different pollen compositions. Moreover, as soon as blossoming starts, pollen beetles prefer to feed on open flowers – where the pollen is directly accessible – rather than on closed buds (Williams & Free 1978). The period during which they have to eat the perianth is then quite short compared to the total time eating pollen from open flowers (a few weeks *vs.* 3-6 months). It can therefore be hypothesized that the biochemical composition of the perianth exerts a weak selection pressure on the feeding behavior of these beetles. Consequently, the insect could be poorly adapted to proceed with feeding when this tissue is not gustatory stimulant, and would therefore exert no particular selection pressure on the chemical composition of the perianth. Thus, no coevolution may occur on plant

feeding stimulation in this system. If confirmed, this fact would be of valuable interest for designing sustainable plant protection strategies.

Prospects for crop protection

We worked in strict controlled conditions to focus on the genetic variation of traits we observed. Moreover, despite the relatively small size of our samples (between 10-12 plants per genotype), the intragenotypic variation in the number of buds damaged was much reduced compared to the intergenotypic variation. This strongly suggests that differences in damage levels were influenced essentially by plant genotype. We cannot exclude the possibility that vernalization indirectly influenced feeding stimulation, since OSR (as well as many other species from different families) accumulates sugars and proline in cold condition (Sasaki *et al.* 1996; Rapacz 1998) and all genotypes were not vernalized for the same duration. Differences we observed could therefore at least partly depend on a genotypic response to cold temperatures. However, the fact that the genotype vernalized for the longest duration ('Darmor') was intermediate in the feeding gradient and was even not different from one of the two genotypes vernalized for the shortest duration ('Yudal') does not support this hypothesis.

Our study opens the way for selecting cultivated plants with reduced feeding stimulants in order to decrease insect damage. In our system, it is likely that a substantial reduction of feeding damage caused by the insect could be achieved through a few key compounds. Parallel to classical varietal selection, based on phenotyping dozens of plant genotypes for their resistance to the insect (*e.g.* Alagar *et al.* (2007) and Tefera *et al.* (2013) in rice and maize productions, respectively), this result supports the encouraging possibility to test the feasibility of a new selection process based on targeted key metabolites.

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Table S1 Mean (SE) concentration (nmol.mg-1 dry wt) of all compounds detected in the perianth of each genotype

	Darmor	Express	Liho	Mar	Markus	Yudal
Carbohydrates/polyols						
Cellobiose	8.0 (0.2)	8.4 (0.4)	13.8 (0.8)	12.2 (0.5)	11.0 (0.5)	10.5 (1.2)
Fructose	18.5 (8.6)	36.0 (5.4)	15.8 (3.5)	23.9 (1.8)	50.2 (8.2)	30.3 (4.3)
Glucose	17.1 (6.1)	36.0 (5.0)	17.4 (3.2)	25.9 (2.3)	43.4 (6.4)	32.6 (3.9)
Maltose	0 (0)	0.4 (0.3)	0 (0)	0 (0)	0 (0)	0 (0)
Mannose	6.6 (4.3)	5.8 (0.6)	2.6 (0.7)	6.5 (0.2)	2.5 (0.6)	3.5 (0.4)
Sucrose	13.4 (2.7)	24.4 (2.9)	10.3 (1.1)	18.1 (4.6)	22.7 (3.2)	18.5 (1.2)
Myo-inositol	3.7 (0.4)	4.9 (0.3)	4.2 (0.6)	3.1 (0.1)	4.3 (0.6)	4.4 (0.3)
Sorbitol	0.9 (0.2)	1.4 (0.2)	1.8 (0.6)	1.3 (0.3)	0.3 (0.2)	1.4 (0.2)
Free amino acids						
α-Alanine	7.4 (0.7)	8.5 (0.6)	11.1 (1.0)	8.1 (0.6)	8.3 (0.8)	10.5 (0.5)
β-Alanine	0.5 (0.03)	0.8 (0.02)	0.7 (0.1)	0.6 (0.2)	0.8 (0.1)	0.6 (0.03)
Arginine	30.3 (5.5)	31.7 (2.4)	37.0 (2.7)	24.7 (1.0)	31.7 (3.3)	6.8 (0.8)
Asparagine	32.2 (5.6)	34.4 (3.4)	35.2 (3.3)	26.5 (1.7)	28.8 (2.8)	28.8 (3.0)
Aspartic acid	23.4 (1.5)	24.2 (0.7)	29.8 (2.5)	20.8 (2.9)	21.9 (1.8)	29.1 (1.5)
Cysteine	0.4 (0.3)	0.3 (0.1)	0.1 (0.02)	0.2 (0.1)	1.0 (0.3)	0.4 (0.1)
GABA	1.6 (0.2)	2.2 (0.2)	2.3 (0.4)	2.4 (0.3)	1.8 (0.3)	1.8 (0.2)
Glutamic acid	38.7 (2.7)	40.9 (1.7)	42.9 (2.4)	35.8 (1.8)	36.7 (2.6)	42.0 (2.0)
Glutamine	281.5 (43.0)	332.7 (27.2)	218.2 (14.1)	368.5 (41.5)	245.3 (29.7)	288.5 (23.8)
Glycine	0.3 (0.1)	0.7 (0.2)	0.6 (0.1)	0.8 (0.2)	0.8 (0.2)	0.7 (0.1)
Histidine	9.8 (1.9)	9.8 (0.8)	5.4 (0.5)	16.1 (2.0)	11.0 (1.3)	7.7 (1.3)
Isoleucine	2.2 (0.4)	2.1 (0.2)	1.7 (0.2)	2.1 (0.1)	1.3 (0.1)	2.0 (0.6)
Leucine	1.0 (0.1)	0.8 (0.1)	1.0 (0.1)	1.1 (0.04)	0.9 (0.1)	1.2 (0.2)
Lysine	1.2 (0.2)	1.4 (0.1)	1.8 (0.2)	1.4 (0.1)	1.1 (0.1)	0.9 (0.1)
Methionine	0.3 (0.04)	0.4 (0.02)	0.4 (0.02)	0.3 (0.02)	0.2 (0.03)	0.3 (0.03)
Ornithine	1.0 (0.3)	0.6 (0.1)	0.7 (0.1)	0.8 (0.3)	0.7 (0.3)	0.1 (0.04)
Phenylalanine	0.7 (0.1)	0.6 (0.04)	0.8 (0.1)	0.7 (0.03)	0.6 (0.05)	0.6 (0.2)
Proline	20.7 (3.6)	46.6 (8.4)	7.7 (0.3)	33.1 (0.3)	26.2 (11.3)	17.8 (1.0)
Serine	7.1 (0.6)	13.5 (0.7)	5.7 (0.2)	7.6 (0.1)	7.7 (0.4)	7.0 (0.4)
Threonine	5.4 (0.6)	6.3 (0.3)	5.3 (0.3)	4.5 (0.1)	5.0 (0.3)	4.7 (0.4)
Tryptophan	0.2 (0.04)	0.1 (0.03)	0.3 (0.1)	0.2 (0.01)	0.4 (0.01)	0.7 (0.3)
Tyrosine	0.4 (0.05)	0.4 (0.04)	0.5 (0.03)	0.4 (0.1)	0.3 (0.04)	0.6 (0.1)
Valine	3.9 (0.4)	4.1 (0.1)	3.7 (0.2)	3.9 (0.2)	3.7 (0.4)	3.9 (0.4)
Glucosinolates						
Glucobrassicanapin	0.7 (0.3)	2.9 (0.4)	0.5 (0.1)	0.4 (0.03)	3.8 (0.2)	7.5 (0.5)
Glucobrassicin	0.1 (0.02)	0.4 (0.02)	0.1 (0.02)	0.3 (0.05)	0.1 (0.02)	1.2 (0.2)
Gluconapin	1.5 (0.5)	2.5 (0.3)	0.2 (0.1)	1.4 (0.01)	2.2 (0.1)	8.9 (0.5)
Progoitrin	7.9 (2.4)	14.9 (2.8)	3.2 (0.7)	4.5 (0.2)	16.0 (1.0)	75.8 (4.2)
Flavonols						
Kaempferol-3-*O*-sophoroside	0.2 (0.04)	0.04 (0.01)	0.4 (0.1)	0.1 (0.04)	0.03 (0.01)	0.04 (0.01)
Isorhamnetin and derivatives	2.9 (2.2)	3.2 (0.6)	0.4 (0.1)	2.0 (0.3)	6.7 (1.6)	3.2 (0.7)
Quercetin and derivatives	0.9 (0.4)	0.5 (0.1)	0.5 (0.1)	0.4 (0.1)	1.4 (0.3)	0.7 (0.1)
Hydroxycinnamic acids						
Sinapic acid	3.4 (0.3)	5.1 (0.2)	5.2 (0.3)	3.7 (0.4)	3.6 (0.4)	4.4 (0.2)

Table S2 Mean (SE) concentration (nmol.mg-1 dry wt) of all compounds detected in anthers of each genotype

	Darmor	Express	Liho	Mar	Markus	Yudal
Carbohydrates/polyols						
Cellobiose	10.6 (1.6)	9.6 (0.3)	12.9 (0.6)	21.0 (1.6)	15.0 (1.1)	12.7 (0.2)
Fructose	10.7 (4.6)	13.0 (1.3)	7.0 (0.6)	7.8 (1.6)	19.3 (4.1)	13.5 (1.1)
Glucose	9.2 (2.3)	10.1 (0.7)	7.5 (0.7)	9.4 (1.3)	14.9 (3.8)	12.9 (0.5)
Mannose	4.5 (2.3)	0 (0)	0 (0)	0.2 (0.2)	0 (0)	0 (0)
Sucrose	55.7 (3.8)	44.7 (0.9)	33.7 (1.5)	35.5 (1.7)	44.8 (4.1)	51.2 (1.2)
Mannitol	0 (0)	0.2 (0.2)	0 (0)	0 (0)	0 (0)	0 (0)
Myo-inositol	2.8 (0.4)	2.1 (0.3)	4.2 (0.7)	3.1 (0.6)	2.0 (0.3)	2.5 (0.3)
Sorbitol	1.8 (0.3)	1.0 (0.3)	2.3 (0.1)	1.7 (0.2)	0 (0)	0 (0)
Free amino acids						
α-Alanine	6.8 (0.4)	8.1 (0.9)	8.3 (0.6)	10.4 (1.1)	8.0 (0.8)	7.9 (0.7)
β-Alanine	0.5 (0.1)	1.1 (0.1)	0.8 (0.1)	2.2 (0.5)	1.2 (0.1)	0.8 (0.1)
Arginine	0.9 (0.1)	5.9 (0.5)	2.0 (0.2)	2.8 (0.6)	3.0 (0.7)	2.6 (0.2)
Asparagine	10.5 (1.5)	15.6 (0.8)	11.5 (0.9)	21.3 (4.9)	15.0 (3.5)	7.4 (0.7)
Aspartic acid	12.4 (1.2)	15.6 (1.3)	16.4 (2.3)	19.7 (2.8)	9.2 (0.7	15.6 (2.1)
Cysteine	0.9 (0.04)	0.5 (0.1)	0.3 (0.02)	1.0 (0.2)	1.1 (0.1)	0.3 (0.04)
S-methylcysteine	0.05 (0.01)	0.03 (0)	0.1 (0.01)	0.9 (0.5)	0.1 (0.01)	0.04 (0.01)
GABA	12.2 (2.6)	10.0 (1.0)	17.2 (3.7)	34.9 (8.6)	8.6 (3.4)	7.3 (1.1)
Glutamic acid	22.5 (1.6)	23.7 (3.2)	23.1 (1.7)	23.2 (2.7)	18.4 (1.9)	29.1 (3.4)
Glutamine	39.9 (2.9)	69.5 (9.2)	39.1 (1.9)	64.2 (4.9)	36.8 (2.6)	44.8 (4.8)
Glycine	1.0 (0.3)	1.3 (0.04)	1.1 (0.1)	1.9 (0.2)	2.1 (0.5)	1.0 (0.1)
Histidine	0.7 (0.3)	2.4 (0.2)	1.1 (0.2)	2.8 (0.4)	2.5 (0.4)	1.3 (0.1)
Isoleucine	1.3 (0.6)	3.1 (0.1)	1.6 (0.2)	3.1 (0.4)	3.6 (0.7)	1.2 (0.2)
Leucine	0.9 (0.3)	2.1 (0.1)	1.4 (0.1)	2.6 (0.3)	2.6 (0.5)	1.1 (0.2)
Lysine	0.7 (0.1)	2.1 (0.2)	1.8 (0.2)	2.1 (0.4)	1.7 (0.3)	1.3 (0.1)
Methionine	0.3 (0.02)	0.4 (0.1)	0.6 (0.1)	0.6 (0.1)	0.3 (0.1)	0.3 (0.02)
Phenylalanine	0.4 (0.1)	1.1 (0.1)	1.4 (0.3)	2.1 (0.5)	1.7 (0.4)	0.5 (0.1)
Proline	66.9 (7.5)	108.5 (14.9)	51.2 (3.9)	77.3 (13.2)	126.2 (7.3)	61.8 (3.1)
Hydroxyproline	0.3 (0.04)	0.4 (0.1)	0.2 (0.02)	0.3 (0.1)	0.4 (0.1)	0.2 (0.03)
Serine	5.2 (0.2)	7.5 (1.0)	5.7 (0.4)	9.8 (1.4)	6.3 (0.8)	6.0 (0.3)
Threonine	2.4 (0.4)	3.8 (0.3)	2.3 (0.1)	4.0 (0.4)	4.3 (0.6)	2.7 (0.1)
Tryptophan	0.2 (0.1)	0.7 (0.1)	0.7 (0.2)	1.0 (0.3)	0.8 (0.2)	0.2 (0.03)
Tyrosine	0.7 (0.1)	0.8 (0.1)	0.8 (0.2)	1.7 (0.4)	0.6 (0.1)	0.7 (0.04)
Valine	3.1 (0.7)	6.7 (0.4)	4.2 (0.4)	8.1 (1.2)	6.6 (1.0)	3.5 (0.2)
Glucosinolates						
Glucobrassicanapin	4.0 (0.3)	6.2 (1.7)	1.2 (0.05)	0.8 (0.3)	14.0 (1.1)	10.0 (0.9)
Glucobrassicin	0.02 (0.02)	0.3 (0.1)	0 (0)	0.2 (0.1)	0.05 (0.03)	0.7 (0.1)
Glucoerucin	0.3 (0.01)	0.3 (0.04)	0.3 (0)	0.3 (0.03)	0.2 (0.1)	0.1 (0)
Gluconapin	4.4 (0.6)	3.1 (0.9)	0 (0)	1.0 (0.5)	4.0 (0.5)	11.2 (0.4)
Gluconasturtiin	0.1 (0.02)	0.5 (0.1)	0 (0)	0.03 (0.02)	1.0 (0.2)	2.0 (0.1)
Progoitrin	9.1 (1.2)	12.8 (3.2)	3.6 (0.2)	4.6 (1.9)	24.3 (4.5)	55.0 (4.0)
Epiprogoitrin	8.7 (0.7)	9.9 (4.0)	3.7 (0.2)	4.6 (2.0)	8.6 (3.6)	0 (0)
Flavonols						
Kaempferol-3-*O*-sophoroside	4.1 (0.6)	3.3 (0.6)	8.1 (0.5)	8.8 (1.0)	5.5 (0.1)	4.4 (0.3)
Isorhamnetin and derivatives	7.4 (0.5)	6.3 (0.5)	2.6 (0.3)	8.4 (2.0)	12.1 (0.8)	4.3 (0.3)
Quercetin and derivatives	7.8 (1.4)	4.1 (0.5)	8.9 (0.9)	8.3 (1.2)	5.4 (0.4)	5.0 (0.4)
Hydroxycinnamic acids						
Sinapic acid	1.4 (0.04)	1.3 (0.04)	1.5 (0.03)	1.3 (0.03)	1.3 (0.02)	1.4 (0.04)

– CHAPTER 3 –

Egg production and oviposition

How oilseed rape (*Brassica napus*) genotype influences pollen beetle (*Meligethes aeneus*) oviposition

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ABSTRACT

Oviposition of phytophagous insects is determined either by adaptive behaviors allowing evaluation and response to host plant quality and/or by nutritional constraints occurring during oogenesis. Besides differences found among host plant species, plant intraspecific diversity can also affect insect oviposition. However, to date few studies have extensively investigated the factors accounting for the effect of this intraspecific variation. We addressed this question using oilseed rape (*Brassica napus*) and the pollen beetle (*Meligethes aeneus*), a phytophagous insect that uses the same plants and plant organs both for feeding and laying eggs. Our objectives were to test for a genotypic effect of oilseed rape on pollen beetle oviposition and identify the origin of the possible intergenotypic differences. We tested three hypotheses: oviposition is directly linked to (i) the amount of food eaten; (ii) the nutritional quality of the food eaten; (iii) a preference of females for certain plant genotypes. Results showed intergenotypic differences in both the number and the size of eggs laid. The factor that best accounted for most of these differences was the amount of food eaten. Nutritional quality of the pollen was of minor importance and females exhibited no preference among genotypes. These results reveal the importance of adult feeding on subsequent oviposition in phytophagous insects, an often neglected factor which partly determines the amount of energy available for oogenesis. Taking into account this factor may be of crucial importance in studies conducted on synovogenic insect species feeding on the same plant on which they lay eggs.

INTRODUCTION

Oviposition of phytophagous insects is a complex trait that may be influenced by a many physiological, behavioral and ecological variables. It has long been known that insects generally show a preference for certain plant species. This preference is assumed to be modelled by natural selection to match plant quality for offspring development (the classical 'preference – performance' hypothesis (*e.g.* Thompson 1988), globally validated by experimental studies (Gripenberg *et al.* 2010)). This preference can be expressed through the number of eggs laid, but also through their size as egg size is generally correlated to progeny performance (Fox & Czesak 2000). A trade-off often occurs between egg number and egg size (Fox & Czesak 2000), but no correlation (*e.g.* Elkin & Reid 2005; Bauerfeind & Fischer 2009), or even positive correlations (*e.g.* Ekbom & Popov 2004) are sometimes found. Oviposition preference depends on a variety of factors, some innate and others linked to the environmental context. Diet specialization seems to be an important, innate, factor: the more an insect is specialized, the more it expresses a fine preference (Gripenberg *et al.* 2010). Environmental factors can also modulate oviposition preference, mainly by influencing host encounter rate. Indeed, a low encounter rate of the most preferred host(s) often leads to a higher 'motivation' to lay eggs, and oviposition on less preferred species (Singer *et al.* 1992). However, egg maturation is not a completely blind process and can be influenced by external factors such as feedback from oviposition, host feeding and host sensory cuing (Papaj 2000).

In parallel to the 'behavioral determinants', oviposition can also be greatly influenced by nutritional constraints imposed by the host plant. Oogenesis is indeed directly linked positively to nutritive substances provided by the plant (*e.g.* polysaccharides or essential amino acids), but also often negatively impacted by antibiotic compounds such as secondary defensive metabolites (Awmack & Leather 2002).

Besides differences observed among plant species, different plant populations, cultivars or genotypes also showed significant variations in the number of eggs laid by insects, in all plant families tested (*e.g.* Osier *et al.* 2000; Johnson 2008; Magalhães *et al.* 2008; Poelman *et al.* 2009; Mphosi & Foster 2010; Cheng *et al.* 2013). Studies generally focus on cultivated species, aiming at introducing resistance factors into new varieties, *e.g.* tomato (de Sena Fernandes *et al.* 2011) and wheat (Beres *et al.* 2013). When factors decreasing insect oviposition are looked for, studies often focus on secondary metabolites, as they are considered major drivers of the coevolution between plants and insects (Fraenkel 1959; Ehrlich & Raven 1964; Berenbaum & Zangerl 2008). These so-called defensive compounds are mostly toxic and deter generalist phytophagous species, whereas they are often neutral or even stimulants for specialists (Després *et al.* 2007). However, if this is sometimes confirmed (*e.g.* Magalhães *et al.* 2008; Cheng *et al.* 2013), it is only part of the story. Oviposition (in terms of either number or size of eggs) is influenced by the amount of energy available for oogenesis, by possible trade-offs in allocation of this energy, by antibiotic compounds reducing oogenesis, by factors stimulating or inhibiting oviposition and by the motivational state of ovipositing females. To our knowledge, no study has so far taken into account all of these factors to explain how plant intraspecific variation influences insect oviposition.

The pollen beetle (*Meligethes aeneus* F.; Coleoptera: Nitidulidae) is a major insect pest of oilseed rape (*Brassica napus* L.; Brassicaceae) (OSR) crops. It is an univoltin specialist of brassicaceous plants for oviposition (Free & Williams 1978; Ekbom & Borg 1996). After diapausing in winter, adults look for brassicaceous plants for mating and ovipositing. They colonize OSR fields when plants are at the bud stage (*i.e.* flowers not open). This pollen feeder destroys buds to reach the pollen inside (which can drastically reduce crop yields (*e.g.* Nilsson 1987)). Oviposition takes place inside buds, but does not provoke bud destruction as

larvae develop inside until flowering. Previous studies at the interspecific level showed that among the Brassicaceae, some species receive different numbers of eggs (Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Hopkins *et al.* 1998; Kaasik *et al.* 2014). In particular, a constant contrast appeared between OSR and the white mustard, *Sinapis alba*, with more eggs being laid on the former. These interspecific differences seem to be partly linked to female behavior, the 'acceptability' (Singer *et al.* 1992) of *S. alba* being clearly inferior to that of *B. napus* (Borg & Ekbom 1996). On the other hand, egg production is reduced or even stopped when pollen beetles are placed on lower-quality hosts (Hopkins & Ekbom 1996), suggesting nutritional constraints. However, the link between quantity/quality of food eaten and oogenesis remains unexplored. It is only known that oogenesis is a short process (approximately two days) in *M. aeneus* (Ekbom & Ferdinand 2003). Eggs are also shorter when they are laid on *S. alba* compared to *B. napus* (Ekbom & Popov 2004). Antibiosis has been suggested to explain this pattern, but not tested. At the intraspecific level and to date, no study has aimed at comparing different OSR genotypes for pollen beetle oviposition.

The objectives of this study were to investigate whether plant genotype influences oviposition (in terms of both number and size of eggs) of the pollen beetle and to determine which factors are responsible for this potential variation. We tested three hypotheses: (i) oviposition is directly linked to the amount of food eaten, *i.e.* the amount of energy available for oogenesis; (ii) oviposition is directly linked to the nutritional quality of the food eaten; (ii) oviposition is directly linked to a preference of females for certain plant genotypes. Three experiments were conducted. The first one assessed the variation among OSR genotypes and specifically tested for an effect of the amount of food eaten. The second one assessed the quality of the pollen of each genotype, by quantifying compounds known to possibly influence (positively or negatively) nutritional quality for insects. The third one specifically tested for a preference among genotypes, by excluding effects of food and female motivation to lay eggs. Indeed, pollen beetles express a typical behavioral oviposition sequence during which host quality is likely to be assessed (Borg & Ekbom 1996). Since different host plant species are discriminated during this sequence, immediate cues obtained from different plant genotypes may lead to a preference for certain genotypes. To keep the interaction as natural as possible, all experiments were performed on entire, intact plants.

MATERIALS AND METHODS

Plants

All genotypes used in this study are lines coming from the INRA OSR collection (BraCySol Center for Genetic Resources, INRA, Le Rheu, France). Both winter and spring OSR were used. For all experiments, plants were raised cultivated in controlled conditions as described previously (Hervé *et al.* submitted [*Article 3*]) and used at BBCH stage 55-57 (Lancashire *et al.* 1991), *i.e.* the 'green bud stage'.

Intergenotypic variation and effect of the amount of food eaten

Method – This experiment was described in a previous paper (Hervé *et al.* submitted [*Article 3*]). Briefly, four pollen beetles sampled in an unsprayed winter OSR crop were maintained for four days on the main inflorescence of an entire OSR plant, in a plastic pot (diameter 6.5 cm, height 9 cm) isolating this inflorescence from the rest of the plant. Plants were placed randomly in controlled conditions (photoperiod 16 h: 8 h light: dark, temperature 20 °C) during this period. After four days, the inflorescence was carefully inspected and the number of buds damaged by feeding and oviposition was counted. The two kinds of damage can be distinguished easily, as feeding holes are very large and the bud is blown up, whereas oviposition holes

are small and systematically localized at the basis of the bud (which continues its normal development) (Ekbom & Borg 1996). All buds presenting oviposition holes were dissected under a stereomicroscope (Leica MZ8, 20x magnification). Eggs were then counted and their length was measured (50x magnification). Ten to 12 replicates performed randomly through time were achieved per genotype. Beetles were sexed after the experiment following Ruther & Thiemann (1997). Zero to four females were present in each replicate, but the sex ratio was not different among OSR genotypes (likelihood ratio test on a Generalized Linear Model (GLM) comparing the proportion of females (distribution: binomial, link: logit): $\chi^2 = 1.163$, $df = 5$, $P = 0.95$; mean ± SE proportion of females: 0.57 ± 0.03). The effect of OSR genotype on pollen beetle feeding was analyzed in another article, in relation to the biochemical composition of several bud tissues (Hervé *et al.* submitted [*Article 3*]). Herein the data on number of buds damaged by feeding were analyzed in relation to the number and the size of eggs laid.

Analysis – All statistical analyses were performed using R software (R Core Team 2013). Number of eggs laid per plant was analyzed using a likelihood ratio test on a GLM (distribution: Poisson, link function: log). Explanatory variables included in the model were the number of buds damaged by feeding, the OSR genotype and the number of females present in the replicate. Length of eggs was analyzed using a Wald test on a Linear Mixed Model (LMM; function 'lmer', package 'lme4' (Bates *et al.* 2014)) considering as explanatory variables the number of buds damaged by feeding, the OSR genotype (fixed factor), the number of females present in the replicate, the individual plant (random factor) and the individual buds in which eggs were found (random factor nested into the plant factor). When needed, pairwise comparisons of Least Squares Means (LSMeans) were performed using the function 'lsmeans' (package 'lsmeans' (Lenth 2013)) and the False Discovery Rate correction for *P*-values (Benjamini & Hochberg 1995).

Nutritional quality of the food

Method – A number of biochemical variables possibly affecting the nutritional quality of the pollen were quantified in bud anthers (*i.e.* the tissue from which pollen beetles feed upon). These variables belonged to seven classes: total essential amino acids, starch, glucosinolates, flavonols, hydroxycinnamic acids, *S*-methylcysteine sulfoxide (SMCO) and the C:N ratio. Within each class, all detectable compounds were individually quantified.

In a previous experiment (Hervé *et al.* submitted [*Article 3*]), glucosinolates, flavonols and hydroxycinnamic acids were quantified in anthers from dissected flower buds of the same six OSR genotypes. Five samples were used per genotype, each one being a mixture of anthers of 80 buds coming from four plants (20 buds per plant). For the present study and from the same material, (i) starch was quantified according to Musse *et al.* (2013); (ii) SMCO was extracted following Gravot *et al.* (2010) and quantified according to Jubault *et al.* (2008) modified by Gravot *et al.* (2010); (iii) the C:N ratio was determined using a Flash EA1112 CHNS/O (Thermo) elemental analyzer; (iv) total amino acid concentrations were obtained according to the following protocol: 5 mg of dried powder was suspended in 1 mL of HCl 6 N in a 10 mL glass-tube. The atmosphere of the tube was saturated with nitrogen gas, and the tube was placed for 30 h in a dry bath at 110 °C for protein hydrolysis (note that at this step, cysteine, methionine and tryptophan are partly or totally degraded). After that delay, 200 µL of the liquid phase was evaporated for 2 h at 30 °C. The pellet was then diluted in 200 µL of ultrapure water and evaporated for 2 h at 30 °C. This step was repeated twice. The final pellet was diluted in 1 mL of ultrapure water and centrifuged for 10 min at 15 °C (12,000 *g*) for sedimentation. From the supernatant, amino acids were

then profiled by Ultra Performance Liquid Chromatography – Diode Array Detector according to Jubault *et al.* (2008) modified by Gravot *et al.* (2010). Essential amino acids retained in the analysis were: arginine, histidine, isoleucine, leucine, lysine, phenylalanine and threonine (Chapman 2013).

Analysis – A Partial Least Squares – Discriminant Analysis (PLS-DA; Barker & Rayens 2003) was performed on the normalized variables to discriminate genotypes based on the biochemical composition of their anthers (function 'plsda', package 'mixOmics' (González *et al.* 2011)). The significance of the discrimination was tested using a MANOVA on the point coordinates on the factorial map. Pairwise comparisons were adjusted with the False Discovery Rate correction for *P*-values.

Female oviposition preference

Method – Pollen beetles were sampled in the same unsprayed winter OSR crop and sexed by observing mating behavior. Each female was immediately and individually placed for two hours on the main inflorescence of an intact OSR plant, in a plastic pot as described above. Previous observations revealed that this time period allows the female to perform a single oviposition sequence, but not to feed. Plants were kept in the same conditions as described above during this period. After this delay, the bud in which the female oviposited was dissected. Eggs were then counted and their length was measured as previously described. All experiments started at 9:30 a.m. A few females did not go up on the inflorescence during the two hours. They were considered as 'non-motivated' and excluded from the experiment. All females that went up on the inflorescence laid eggs. Twenty 'motivated' females (corresponding to 20 different plants) were used per genotype. Replicates were performed randomly through time and space. No female ever fed during the experiment.

Analysis – The number of eggs laid per female (*i.e.* per plant) was compared among the six genotypes using a likelihood ratio test on a GLM (distribution: Poisson, link function: log). Length of eggs was compared among genotypes using a Wald test on a LMM including genotype as a fixed factor and the individual female as a random factor.

RESULTS

Intergenotypic variation and effect of the amount of food eaten

The number of females present on the plant significantly influenced the number of eggs laid (more eggs were laid when more females were present) but not the length of these eggs (Table 1). The number of buds damaged by feeding was different among OSR genotypes (likelihood ratio test on a GLM (distribution: negative binomial, link function: log): $\chi^2 = 28.11$, $df = 5$, $P < 0.001$). However, independently of plant genotype, the number of buds damaged by feeding had a significant effect both on the number of eggs laid and on their length (Table 1). Both effects were positive, meaning that (i) more eggs were laid and (ii) these eggs were bigger

Table 1 Influence of number of pollen beetle females, number of buds fed upon and oilseed rape genotypes on number and length of laid eggs. Number of eggs laid was analyzed with a likelihood ratio test applied on a GLM, length of eggs was analyzed with a Wald test applied on a LMM. *P*-values inferior to $\alpha = 0.05$ are represented in bold

	Number of females			**Number of buds damaged by feeding**			**Genotype**		
	χ^2	*df*	*P*	χ^2	*df*	*P*	χ^2	*df*	*P*
Number of eggs laid	58.73	1	**< 0.001**	4.98	1	**0.026**	0.88	5	0.97
Length of eggs	0.02	1	0.90	7.98	1	**0.005**	16.29	5	**0.006**

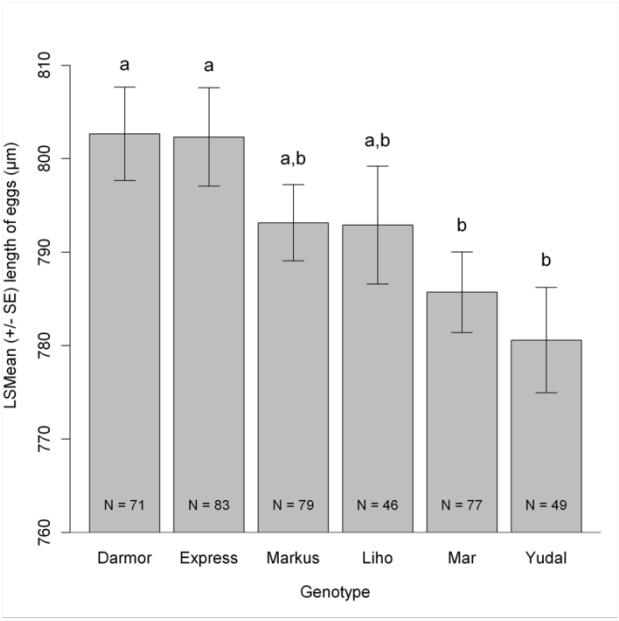

Fig. 1 Least Squares Mean (± SE) length of eggs laid by the pollen beetle (*M. aeneus*) on the six oilseed rape (*B. napus*) genotypes (back-transformed values). Different letters indicate statistically different LSMeans. N: number of eggs laid per genotype

when the number of buds damaged by feeding increased. After controlling for the effect of the number of buds damaged by feeding, genotype had a significant effect only on egg length (Table 1). Pairwise comparisons showed a separation between two groups (Fig. 1): eggs laid were longer on the genotypes 'Darmor' and 'Express', compared with the genotypes 'Mar' and 'Yudal'. The two other genotypes, 'Liho' and 'Markus', were intermediate between these two groups.

Nutritional quality of the food

In agreement with classical data on plant physiology and on the basis of screened compounds, primary metabolites (essential amino acids, starch and SMCO) were more concentrated than secondary compounds (glucosinolates, flavonols and hydroxycinnamic acids) (mean ± SE: 677.8 ± 41.9 nmol.mg^{-1} *vs.* 55.7 ± 4.7 nmol.mg^{-1}; Table 2). All detectable compounds were present in the six OSR genotypes except some glucosinolates, for which qualitative intergenotypic differences were found (Table 2).

The PLS-DA and the associated MANOVA (Fig. 2, detailed concentrations in Table 2) revealed that the biochemical profile of anthers was different enough to discriminate plant genotypes. On the first factorial map, which explained 60.2 % of the intergenotypic variance (Fig. 2, left), an interesting pattern appeared. Indeed, four genotypes ('Markus', 'Express', 'Darmor' and 'Liho') were very close to each other whereas the other two ('Yudal' and 'Mar') were clearly separated. These two genotypes were those on which the smallest eggs were laid (Fig. 1). The loading plot of biochemical variables (Fig. 2, right) indicated that these two genotypes were separated from the group for partly identical and partly different reasons. Common reasons were a smaller concentration of all essential amino acids (concentrations of all these amino acids were highly correlated, explaining why the corresponding arrows overlap in the upper part of the circle) and a higher concentration of SMCO. Additionally, 'Yudal' (i) exhibited a higher concentration of most of the glucosinolates (gluconasturtiin, progoitrin, gluconapin and glucobrassicin) compared to all other genotypes and (ii) shared with 'Markus' a higher concentration of glucobrassicanapin (a glucosinolate), a higher concentration of an undetermined glycosyl derivative of isorhamnetin (a flavonol) and a higher C:N ratio in comparison with other genotypes. In addition to essential amino acids and SMCO, 'Mar' (i) was characterized by a smaller starch concentration compared to all other genotypes, and (ii) shared with 'Liho' a higher concentration of glucoerucin (another glucosinolate), kampferol-3-*O*-sophoroside and quercetin-3-*O*-sophoroside (two flavonols) in comparison with other genotypes.

Female oviposition preference

No difference among the six genotypes was observed, neither for the number of eggs laid (χ^2 = 3.27, *df* = 5, *P* = 0.66) nor for their length (χ^2 = 2.79, *df* = 5, *P* = 0.73). The mean (± SE) number of eggs laid per female was

Table 2 Mean (SE) (i) concentration of compounds and (ii) C:N ratio quantified in anthers of each oilseed rape genotype. Values are in nmol.mg^{-1} dry weight for total essential amino acids, glucosinolates, flavonols and hydroxycinnamic acids; in nmol of glucose equivalents.mg^{-1} dry weight for starch; no unit for C:N. Glucosinolate, flavonol and hydroxycinnamic acid concentrations come from Hervé *et al.* (submitted [*Article 3*])

	Genotype					
	Darmor	**Express**	**Liho**	**Mar**	**Markus**	**Yudal**
Total essential amino acids						
Arginine	20.6 (2.8)	26.1 (5.7)	24.4 (3.1)	14.6 (2.3)	22.1 (4.2)	17.7 (1.3)
Histidine	10.4 (1.2)	12.2 (2.7)	11.7 (1.4)	7.2 (1.1)	10.9 (2.2)	8.3 (0.7)
Isoleucine	24.4 (2.9)	25.8 (6.1)	27.3 (3.3)	16.2 (2.5)	25.3 (5.0)	19.4 (1.5)
Leucine	40.6 (4.9)	46.5 (10.2)	45.9 (5.6)	27.0 (4.1)	40.6 (8.1)	32.1 (2.5)
Lysine	36.1 (4.4)	43.3 (9.4)	41.1 (4.7)	23.6 (3.3)	37.8 (7.6)	29.0 (2.3)
Phenylalanine	19.2 (2.4)	22.2 (4.9)	21.9 (2.7)	12.9 (2.0)	19.5 (3.8)	15.3 (1.2)
Threonine	25.7 (3.0)	29.0 (6.4)	28.5 (3.4)	16.7 (2.5)	25.9 (5.2)	20.1 (1.6)
Starch	561.5 (93.2)	413.3 (118.1)	326.6 (32.4)	253.8 (71.8)	527.3 (91.0)	412.8 (20.5)
***S*-methylcysteine sulfoxide**	83.7 (3.3)	106.8 (23.5)	20.8 (2.8)	149.2 (6.1)	65.7 (0.6)	117.2 (4.6)
Glucosinolates						
Glucobrassicanapin	4.0 (0.3)	6.2 (1.7)	1.2 (0.05)	0.8 (0.3)	14.0 (1.1)	10.0 (0.9)
Glucobrassicin	0.02 (0.02)	0.3 (0.1)	0 (0)	0.2 (0.1)	0.05 (0.03)	0.7 (0.1)
Glucoerucin	0.3 (0.01)	0.3 (0.04)	0.3 (0)	0.3 (0.03)	0.2 (0.1)	0.1 (0)
Gluconapin	4.4 (0.6)	3.1 (0.9)	0 (0)	1.0 (0.5)	4.0 (0.5)	11.2 (0.4)
Gluconasturtiin	0.1 (0.02)	0.5 (0.1)	0 (0)	0.03 (0.02)	1.0 (0.2)	2.0 (0.1)
Progoitrin	9.1 (1.2)	12.8 (3.2)	3.6 (0.2)	4.6 (1.9)	24.3 (4.5)	55.0 (4.0)
Epiprogoitrin	8.7 (0.7)	9.9 (4.0)	3.7 (0.2)	4.6 (2.0)	8.6 (3.6)	0 (0)
Flavonols						
Kaempferol-3-*O*-sophoroside	4.1 (0.6)	3.3 (0.6)	8.1 (0.5)	8.8 (1.0)	5.5 (0.1)	4.4 (0.3)
Isorhamnetin and derivatives	7.4 (0.5)	6.3 (0.5)	2.6 (0.3)	8.4 (2.0)	12.1 (0.8)	4.3 (0.3)
Quercetin and derivatives	7.8 (1.4)	4.1 (0.5)	8.9 (0.9)	8.3 (1.2)	5.4 (0.4)	5.0 (0.4)
Hydroxycinnamic acids						
Sinapic acid	1.4 (0.04)	1.3 (0.04)	1.5 (0.03)	1.3 (0.03)	1.3 (0.02)	1.4 (0.04)
C:N	8.90 (0.10)	8.63 (0.07)	8.95 (0.03)	8.44 (0.21)	9.02 (0.10)	8.97 (0.07)

2.6 ± 0.1 and the mean length of these eggs was 780.7 ± 4.6 µm.

DISCUSSION

Comparative studies are an essential tool to understand the ecological and evolutionary significance of specific plant and/or insect traits in plant – insect interactions (Rasmann & Agrawal 2009). These studies were historically conducted at the interspecific scale, but the focus has also progressively shifted towards the intraspecific scale. This allows plant – insect interactions to be assessed in a finer way as soon as it becomes obvious that different plant and/or insect genotypes may 'behave' differently. In an applied perspective, plant intraspecific variation in resistance to insect pests also offers new opportunities to protect cultivated species (*e.g.* de Sena Fernandes *et al.* 2011; Beres *et al.* 2013).

In the present study, we focused on the interaction between OSR and the pollen beetle *M. aeneus*, and found that oviposition of the insect is differently impacted by plant genotype, for the number of eggs laid and for the length of these eggs. Although this is the first time that such an influence has been shown in this biological system, it was already observed in many others (*e.g.* Osier *et al.* 2000; Johnson 2008; Magalhães *et al.* 2008; Poelman *et al.* 2009; Mphosi & Foster 2010; Cheng *et al.* 2013). However, our study is the first to take into account a large set of factors to clearly identify which ones explain the influence of plant genotype.

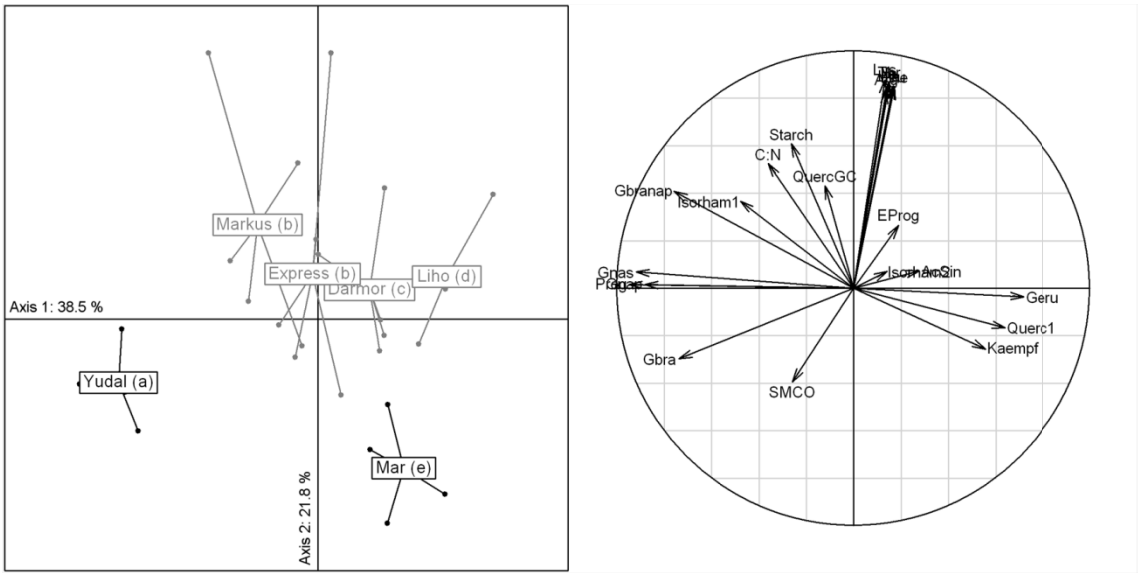

Fig. 2 Factorial map (left) and loading plot (right) of the Partial Least Squares – Discriminant Analysis performed on the biochemical composition of anthers of six oilseed rape (*B. napus*) genotypes (Table 2). The factorial map explained 60.2 % of the intergenotypic variance. Different letters in brackets indicate statistically different biochemical profiles (results of the MANOVA testing for the discrimination of genotypes: *pseudo*-$F_{10,48}$ = 13.533, $P < 0.001$). Genotypes on which the smallest eggs were laid (Fig. 1) are represented in black

We tested three hypotheses: (i) oviposition (*i.e.* number and size of eggs) is directly linked to the amount of food eaten, *i.e.* the amount of energy available for oogenesis; (ii) oviposition is directly linked to the nutritional quality of the food eaten; (iii) oviposition is directly linked to a preference of females for certain plant genotypes.

Is oviposition directly linked to the amount of food eaten?

Insect fecundity is classically interpreted from two angles: adaptation of the insect to adjust egg production and/or laying depending on host quality, and effect of nutritional quality of the food on oogenesis (reviewed *in* Papaj (2000) and Awmack & Leather (2002)). However, for a given food quality, quantity of food eaten, a simple but often neglected variable, may also play an important role. As more food ingested is necessarily correlated to a greater amount of energy available for oogenesis, it seems essential to take this parameter into account especially when studying oviposition patterns of insects that feed and oviposit on the same host plant. Moreover, insects are known to adjust their food intake to increase their performance when facing complementary food sources differing in their quality (reviewed *in* Behmer 2009). However, feeding is rarely controlled or even assessed in experiments on insect oviposition.

Results of the first experiment showed that, independently of plant genotype, the number of eggs laid by the pollen beetle, as well as the length of these eggs, was directly linked to the number of buds damaged by feeding. The pollen beetle does not accumulate eggs and its egg production is highly plastic, as it can be stopped, slowed down or increased depending on availability and quality of the host plant (Hopkins & Ekbom 1996, 1999). Our results clearly demonstrate a relationship between feeding and oviposition: the more buds were damaged, the more eggs were produced and eggs were longer. Contrary to the general theory (Fox & Czesak 2000), we found no negative correlation between these two traits, suggesting that no trade-off occurs in *M. aeneus*. This is in agreement with a study conducted at the interspecific level, in which more and longer eggs were laid on OSR than on the white mustard *S. alba* (Ekbom & Popov

2004). The absence of such a trade-off can be seen as an adaptive strategy in a species such as this one with a low adult mortality and a long oviposition period (several months) (Hopkins & Ekbom 1996). In a previous study (Hervé *et al.* submitted [*Article 3*]), pollen beetle feeding was shown to be correlated to the biochemical composition of the perianth (*i.e.* sepals and petals that beetles have to eat in order to reach the pollen inside the bud). In particular, the concentration of sucrose in this flower part was a good predictor of the number of buds damaged. Results of the present study suggest that plant feeding stimulation was, *via* a domino effect, at the origin of the intraspecific variation in the number of eggs laid by constraining the amount of energy available to produce these eggs.

Considering the size of eggs, things seem more complex. This potentially important parameter in larval development and survival (Fox & Czesak 2000) was independently influenced both by the amount of food eaten and by plant genotype. Beyond a 'simple' effect of the quantity of energy available for oogenesis, the genotypic effect suggests either (i) that the nutritional quality of the pollen of 'Mar' and 'Yudal' is reduced, exerting a larger constraint on oogenesis, and/or (ii) that pollen beetle females prefer to lay shorter eggs on these two genotypes. Indeed, since the duration of the experiments (four days) was longer than the time needed to produce an egg (two days (Ekbom & Ferdinand 2003)), the two factors cannot be distinguished at this point.

Is oviposition directly linked to the nutritional quality of the food eaten?

Plant nutritional quality is a complex trait, influenced by both positive and negative compounds. Positive compounds are mostly sources of energy (*e.g.* polysaccharides) or metabolites that insects are not able to produce (*e.g.* essential amino acids), whereas negative compounds are essentially antifeedants (*e.g.* tannins) or antibiotics (*e.g.* alkaloids or cyanogenic compounds) (reviewed *in* Awmack & Leather 2002). We tried to assess the nutritional quality of the pollen of the six OSR genotypes tested by quantifying a variety of biochemical variables.

An interesting pattern appeared when comparing biochemical data to egg length: the two genotypes on which the shortest eggs were laid were 'Mar' and 'Yudal'; anthers of these genotypes also had a biochemical profile clearly different from the four other genotypes. They were characterized by a smaller concentration of all essential amino acids (and of starch for 'Mar'), unambiguously showing that their pollen was of reduced nutritive value. They also exhibited the highest concentrations of SMCO, a non-proteinogenic amino acid known to be an antinutritional metabolite for mammalian herbivores (Paul *et al.* 1986), and the highest concentrations of three flavonols (one specific to 'Yudal', the two others specific to 'Mar'), a class of compounds often acting as digestibility reducers or toxins (Treutter 2006). Additionally, anthers of 'Yudal' were more concentrated in most of the glucosinolates. If some specialists of brassicaceous plants are able to detoxify them (Després *et al.* 2007), the products of their degradation when the plant is wounded are still toxic for others (*e.g.* Agrawal & Kurashige 2003). The higher concentration of these products in 'Yudal' could hence alter oogenesis of female pollen beetles, even if this species only lays eggs on brassicaceous plants. Finally, anthers of 'Yudal' exhibited a higher C:N ratio compared to most of other genotypes, also suggesting a reduced nutritional quality (Awmack & Leather 2002).

Altogether, these results strongly suggest that the pollen of 'Mar' and 'Yudal' is of reduced nutritional quality compared to the other genotypes. This is supported by data on adult survival, showing that pollen beetle individuals live for a shorter time when feeding exclusively from the pollen of 'Mar' or 'Yudal', compared to the other four genotypes (Hervé *et al.* in prep. [*Article 6*]). Further investigations are needed to test for an antibiotic or antidigestive effect of SMCO,

flavonols and glucosinolates on the pollen beetle, but our study argues for a direct link between food quality and length of eggs. However, whether the reduction of egg length we observed has an impact on larval development or survival (*i.e.* on female fitness) remains uncertain.

Is oviposition directly linked to female preference?

Results of the third experiment showed that pollen beetle females have no preference for any of the six OSR genotypes tested. This indicates that influence of plant genotype is not linked to a greater stimulation of oviposition, in contrast to what was shown at the interspecific level with different potential host species (Borg & Ekbom 1996).

Behavior of specialist phytophagous insects is generally interpreted in relation to the concentration of secondary metabolites that are typical of the plant family they attack. Although we used a limited set of genotypes, buds from these genotypes exhibited marked differences in their glucosinolate content. These secondary metabolites are known to influence many insect species in a quite clear pattern: they are mostly deterrents for generalist phytophagous insects, whereas they are neutral or even stimulants for specialist species (reviewed *in* Hopkins *et al.* 2009). In a previous study conducted on *S. alba*, the pollen beetle showed no preference for lines containing more glucosinolates in their flower buds (Hopkins *et al.* 1998). However, *S. alba* is known to be a low-quality host for *M. aeneus* (Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Hopkins *et al.* 1998), possibly biasing this result. In addition, the glucosinolate content of *S. alba* is dominated by sinalbin (Hopkins *et al.* 1998) which was absent from our samples. In the present study conducted on a high-quality host, we clearly confirm that variation of total glucosinolate content (maximum – minimum ratio: 9.0), as well as variation in individual compound concentrations, do not influence pollen beetle oviposition behavior. Hervé *et al.* (submitted) [*Article 3*] previously showed that even greater variations does not stimulate feeding. This situation is not so common for a specialist of brassicaceous species, where glucosinolates were shown to stimulate oviposition and/or feeding in about 25 specialist insects (Hopkins *et al.* 2009). Despite the apparent lack of a major influence of glucosinolates once females are on the plant, some of their volatile breakdown products (like isothiocyanates) could still play an important role by attracting *M. aeneus* to the host plant (Blight & Smart 1999; Smart & Blight 2000; Cook *et al.* 2006).

Overall, our results show that OSR genotype has a clear and direct influence on pollen beetle oviposition, by constraining egg production (in terms of number and size of eggs). The quantity of food eaten, a very simple but generally overlooked factor, explained most of the variation in oviposition levels and quality. Nutritional quality of the food also played a role, but to a lesser extent than food quantity.

Therefore, feeding stimulation – especially through plant biochemistry – may be an important determinant of oviposition in phytophagous insects, especially in synovogenic species feeding on the same plant where they lay eggs. Host availability and nutritional quality of the host plant play a crucial role on ovarian dynamics (Papaj 2000; Awmack & Leather 2002), but the possible impact of host feeding stimulation may have been insufficiently considered. We plead for a better consideration of this plant trait. Although challenging to assess, it may at least partly explain some observed patterns or worst, be a confounding factor in experiments on oviposition of phytophagous insects conducted both in the laboratory and in the field.

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Oviposition behavior of the pollen beetle (*Meligethes aeneus*): a functional study

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The recognition by female phytophagous insects of a plant as a 'good' or 'bad' host for egg laying is based on a variety of cues (either visual, physical or chemical). Specific cues are often looked for during stereotypic oviposition behaviors, composed of several phases having their own function(s). In this study the oviposition behavior of the pollen beetle *Meligethes aeneus*, a pest which lays eggs in flower buds of only some brassicaceous plants, was described in detail on five oilseed rape (*Brassica napus*) genotypes. In parallel, setae borne by the ovipositor were characterized by scanning electron microscopy. Observations showed that the stereotypic oviposition sequence is functionally divided into three independent phases: external inspection, internal inspection and egg laying. The ovipositor plays a role in all phases by getting information about external and internal bud parts. This role appears to be only physical since all the setae it bears are mechanoreceptors. Despite the fact that the pollen beetle is a specialist for oviposition, important variations in secondary metabolites that are typical of its host plant family (*i.e.* glucosinolates) on the bud did not influence clutch size. The crucial phase in the oviposition sequence seems to be the external inspection, during which poor and high-quality host plants are probably discriminated. Chemical information on bud surface is likely to be determinant in this process.

INTRODUCTION

The classical 'preference – performance' hypothesis (*e.g.* Thompson 1988) predicts that female insects should be selected to lay eggs preferentially in oviposition sites that are favorable for offspring development. In phytophagous species, this hypothesis has been supported by a recent meta-analysis (Gripenberg *et al.* 2010). The recognition of a plant as a host or non-host, and more precisely of the quality of a host plant, is based on cues (*i.e.* plant traits) used by females. These can be either chemical and/or visual, acting at distance (*e.g.* volatile compounds, size or architecture) and/or at contact (*e.g.* primary and secondary metabolites inside and on surface of plant tissues, trichome density, tissue thickness or toughness).

In many insect species, females show a stereotypic oviposition behavior (*e.g.* the cabbage root fly *Delia radicum* (Städler & Schöni 1990), the mustard leaf beetle *Phaedon cochleariae* (Müller & Rosenberger 2006) or the cabbage seedpod weevil *Ceutorhynchus obstrictus* (Ulmer & Dosdall 2006)). Such behavioral sequence is often divided in several phases, each of them having one or several precise function(s). Specific cues are detected during these phases, by means of sensory organs such as mechano- or chemoreceptors borne by antennae, tarsi or the ovipositor. Describing stereotypic oviposition behaviors, deciphering their function(s) and characterizing sensory organs that are used by females is a first step before identifying specific cues used by females to evaluate plant quality.

The pollen beetle (*Meligethes aeneus* F.; Coleoptera: Nitidulidae) is one of the major insect pests of oilseed rape (*Brassica napus* L.; Brassicaceae) (OSR) crops. Females lay eggs only on brassicaceous plants (Free & Williams 1978; Ekbom & Borg 1996). Adults colonize OSR fields after their winter diapause, when plants are at the bud stage. They destroy flower buds to reach the pollen inside, from which they feed. This destruction sometimes leads to important yield losses (Nilsson 1987). Mating occurs on the plant and females oviposit inside buds, after having made a small hole at its base. Reproduction goes on until death of the individuals, in summer. Buds are not destroyed during oviposition and continue their normal development. Larvae hatch inside the bud and feed from the pollen contained in anthers during their first instar. Transition to the second (and last) instar occurs approximately at bud flowering. Second-instar larvae move from one flower to another, still feeding on pollen. They finally drop from the plant at the end of their development and pupate into the soil (Williams 2010).

Not all brassicaceous plants are accepted for oviposition by females even when they are attractive at distance (Cook *et al.* 2004; Veromann *et al.* 2012; Kaasik *et al.* 2014a, 2014b). Cues present on the buds and acting upon contact play a key role in determining oviposition. White mustard *Sinapis alba* is, for example, especially known to be a low-quality host for the pollen beetle (Ekbom and Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Hopkins *et al.* 1998). Borg & Ekbom (1996) characterized for the first time the oviposition behavior of pollen beetle females and proved that *S. alba* is of inferior acceptability (in the strict sense of Singer (2000)) compared to *Brassica* spp. They showed that the flower bud is inspected in a stereotypic sequence before oviposition, and that this inspection can lead to females stopping the sequence and leaving the plant. Although contact cues appear essential in determining plant acceptability, these authors were not able to identify the cues females use to make their decision.

The aims of this study were (i) to describe in more detail the oviposition behavior of the pollen beetle, (ii) to give a functional interpretation of the different steps of the behavioral sequence, and (iii) to identify the critical step(s) of this sequence determining the acceptance of the plant. For this purpose, five OSR genotypes for which the biochemical composition of buds is known (Hervé *et al.* under revision [*Article 3*]) were compared in a no-choice experiment, as precisely as possible. As the ovipositor is known to bear setae in its distal part in

the Meligethinae subfamily (Audisio *et al.* 2009), morphology of these setae was also characterized by scanning electron microscopy (SEM) to understand their function.

MATERIALS AND METHODS

Plants

All genotypes used in this study were lines from the INRA OSR collection (BraCySol Center for Genetic Resources, INRA, Le Rheu, France). Both winter (genotypes 'Darmor', 'Express' and 'Mar') and spring (genotypes 'Liho' and 'Yudal') OSR genotypes were used. Plants were produced in controlled conditions as described in Hervé *et al.* (under revision) [*Article 3*] and used at BBCH stage 55-57 (Lancashire *et al.* 1991), *i.e.* the 'green bud stage'. To keep the interaction natural, entire intact plants were used.

Insects

Overwintered pollen beetle females were collected from an unsprayed winter OSR crop near Le Rheu (Brittany, France), in April-May. Females were identified by observing mating behavior. Experiments took place within 3 h after collection.

Oviposition behavior characterization

One female was placed on the main inflorescence of an intact OSR plant, in a plastic pot (diameter 6.5 cm, height 9 cm) isolating this inflorescence from the rest of the plant. As described by Borg & Ekbom (1996), the beginning of the oviposition sequence is visible when, after walking on several buds, a female walks circuitously on the same bud. Observations were carried out by constantly following the female with a hand magnifier (x 5), recording the sequence and the duration of each behavior with a handheld recorder, and transcribing it later with the interface SequenceR (Hervé 2013). Based on the previous characterization of Borg & Ekbom (1996) and our preliminary observations, six behaviors were considered (Table 1). After the end of the sequence, the bud in which the female oviposited was measured and dissected to count the number of eggs laid. A different plant was used for each female. Thirty different individuals were recorded per OSR genotype. Replicates were conducted randomly through time (total study period: about one month) and experiments took place at 20 °C.

Scanning electron microscopy of the ovipositor

About 40 ovipositors were dissected, dehydrated by successive alcohol-bath (70 %, 80 %, 90 %, 96 % and 100 %), critical-point dried and coated with gold-paladium. Observations were then performed with a JSM-7100F (Jeol) microscope.

Statistical analysis

All statistical analyses were performed using R software (R Core Team 2013). The proportion of females completing their oviposition sequence was compared

Table 1 Behaviors used to characterize the oviposition sequence of *M. aeneus*

Behavior	Description
Walking	All locomotion except for "Walking with ovipositor"
Walking with ovipositor	Walking with ovipositor tapping on the bud surface
Resting	No locomotion
Biting	Female stands still and chews on the bud; once a hole is initiated all biting behavior occurs at the same location
Ovipositor inside hole	Female stands still, inserts her ovipositor inside the hole bitten and taps on internal bud organs. The female is wiggling and antennae are constantly agitated
Oviposition	Ovipositor is inserted into the hole and egg(s) laid. The female is completely immobile, including antennae

among genotypes using a likelihood ratio test on a Generalized Linear Model (GLM) (distribution: binomial, link function: logit). Only females that completed their sequence were included in subsequent analyses. Number of eggs laid was analyzed using a likelihood ratio test on a GLM (distribution: Poisson, link function: log) taking into account OSR genotype, size of the bud in which oviposition took place and duration of each behavior. ANOVAs were used to compare genotypes for the size of the bud in which the female oviposited, the duration of each behavior (durations of 'Walking with ovipositor' and 'Resting' had to be log-transformed for a better model fit) and the total duration of the sequence. When needed, pairwise comparisons of Least Squares Means were performed using the function 'lsmeans' (package 'lsmeans' (Lenth 2013)) and the False Discovery Rate (FDR) correction for *P*-values (Benjamini & Hochberg 1995). Pearson's correlation tests were used to assess the relationship between duration of all pairs of behaviors. The *P*-value of each test was adjusted with the FDR correction.

RESULTS

Oviposition behavior characterization

The mean length (± SE) of the bud in which pollen beetle females laid eggs was 4.0 (± 0.07) mm. Although the size of all buds forming an OSR raceme are quite variable, ranging between less than 1 mm and 7-8 mm long, only a narrow size range was used by females (90 % of chosen buds had a length between 3 and 5.5 mm). No difference was observed among the five OSR genotypes for the length of the chosen bud ($F_{4,128} = 0.80$, $P = 0.525$).

The observed sequences were consistent with the description of Borg & Ekbom (1996), but we recorded finer details during two particular steps. Firstly, Borg & Ekbom (1996) described a behavior of "walking with the abdomen touching the bud surface". Our observations showed that during this step, the abdomen is very close to the bud surface but does not touch it, and the ovipositor is partly extruded, tapping for a few seconds with its distal end on the bud surface. Secondly, Borg & Ekbom (1996) described a behavior consisting of "placing the abdomen over the bite hole". Our observations showed that the abdomen is not only placed over the hole, but that the ovipositor is fully extruded and inserted inside the hole, tapping on bud organs we were not able to identify. Finally, we add that antennae were

Table 2 Results of (i) ANOVAs comparing durations of each behavior and total oviposition sequence of *M. aeneus* on oilseed rape; (ii) likelihood ratio tests comparing number of eggs and number of females having completed their oviposition sequence. Mean (SE) values are given. For significant ANOVAs, different letters show statistically different means. *P*-values less than α = 0.05 are represented in bold

			Genotype				
	$F_{4,128}$	*P*	Darmor	Express	Liho	Mar	Yudal
Behavior duration (s)							
Walking	0.844	0.500	456.2 (50.4)	363.5 (46.8)	369.9 (39.5)	382.1 (54.3)	447.1 (50.7)
Walking with ovipositor	1.440	0.225	39.1 (9.3)	22.5 (5.0)	37.1 (3.9)	42.3 (11.9)	33.7 (5.4)
Resting	1.503	0.205	22.3 (7.1)	8.4 (3.9)	17.9 (5.3)	21.8 (8.7)	26.2 (12.4)
Biting	8.780	**< 0.001**	1256.1 (128.2) a	705.8 (83.9) b	629.0 (84.3) b	1181.9 (126.6) a	671.1 (83.8) b
Ovipositor inside hole	5.636	**< 0.001**	541.2 (73.7) a	285.0 (51.0) b	266.1 (34.0) b	461.4 (59.6) a	280.1 (42.4) b
Oviposition	1.035	0.392	425.7 (40.4)	452.6 (47.6)	366.6 (41.8)	358.2 (46.7)	461.4 (57.7)
Total sequence (s)	7.516	**< 0.001**	2741.2 (200.1) a	1837.6 (144.1) b	1686.7 (137.1) b	2450.3 (163.1) a	1919.7 (167.6) b
	χ^2_4	*P*					
Number of eggs	3.066	0.547	3.1 (0.2)	2.9 (0.3)	3.1 (0.3)	2.4 (0.2)	2.9 (0.2)
Proportion of females completing sequence	4.183	0.382	25/30	27/30	29/30	25/30	27/30

constantly used to tap the bud surface throughout the 'Walking' and 'Walking with ovipositor' behaviors.

No difference was found among OSR genotypes for the duration of 'Walking', 'Walking with ovipositor', 'Resting' and 'Oviposition' (Table 2). On the contrary, the mean duration of 'Biting' and 'Ovipositor inside hole' was significantly longer in genotypes 'Darmor' and 'Mar' compared to 'Express', 'Liho' and 'Yudal'. Consequently, the mean total time of the sequence was greater in 'Darmor' and 'Mar' than in the three other genotypes. Pairwise correlations between durations of each behavior (Table 3) revealed that all durations were independent, except for two pairs. First, the time spent walking was highly positively correlated to the time spent walking with the ovipositor tapping on the bud surface. Secondly, the time spent biting the perianth was highly positively correlated to the time spent with the ovipositor tapping inside the bud.

A high proportion of females completed their oviposition sequence (overall proportion [95 % CI]: 0.89 [0.82 - 0.93]). This proportion was not statistically different among OSR genotypes (Table 2). Altogether, 17 females left the bud before laying eggs. Six left it before biting any hole, after a mean time (± SE) of 561.2 (± 94.0) s (26.6 % of the mean total time of completed sequences). The other eleven females left the bud after at least starting biting, after a mean time of 1,250.4 (± 269.2) s (59.4 % of the mean total time of completed sequences). Between one-six eggs were laid, with a mean (± SE) of 2.88 (± 0.11). The number of eggs laid was not influenced by any variable except the duration of the 'Oviposition' behavior (Genotype: $\chi^2 = 3.07$, $df = 4$, $P = 0.547$; Size of the bud in which eggs were laid: $\chi^2 = 0.02$, $df = 1$, $P = 0.890$; 'Walking': $\chi^2 = 0.001$, $df = 1$, $P = 0.971$; 'Walking with abdomen': $\chi^2 = 0.88$, $df = 1$, $P = 0.349$; 'Resting': $\chi^2 = 0.03$, $df = 1$, $P = 0.866$; 'Biting': $\chi^2 = 0.24$, $df = 1$, $P = 0.625$; 'Abdomen over hole': $\chi^2 = 0.50$, $df = 1$, $P = 0.481$; 'Oviposition': $\chi^2 = 4.37$, $df = 1$, $P = 0.037$). The more time a female spent laying eggs, the more eggs were laid.

Scanning electron microscopy of the ovipositor

About 15 setae were found on each half of the ovipositor. They were located at the end of the gonostyloid (*i.e.* the distal end of the gonocoxites), for one part on the gonostyloid themselves and for the other part on two cylindrical styli (Fig. 1b, c). Two types of sensilla were observed: long trichoid sensilla (between 10-15 µm long; Fig. 1d) and short basiconic sensilla (2-3 µm long; Fig. 1e, f). Both types were strictly aporous.

DISCUSSION

Functional organization of the oviposition sequence

Borg & Ekbom (1996) made the first, broad, description of the oviposition behavior of the pollen beetle. Based on our results on the correlation and the transitional frequencies between behaviors, we were able to go further and draw a general functional view of the oviposition sequence of this species (Fig. 2). This sequence is divided into three independent steps: the first

Table 3 Pearson's correlation coefficients between durations of behaviors of the oviposition sequence of *M. aeneus* on oilseed rape (*df* = 131 in each case). No symbol: P > 0.05; ******* P < 0.001

	Walking with ovipositor	**Resting**	**Biting**	**Ovipositor inside hole**	**Oviposition**
Walking	0.598 ***	0.052	- 0.012	0.149	0.011
Walking with ovipositor	—	0.044	0.021	0.107	- 0.032
Resting	—	—	0.066	0.044	- 0.071
Biting	—	—	—	0.700 ***	0.039
Ovipositor inside hole	—	—	—	—	0.043

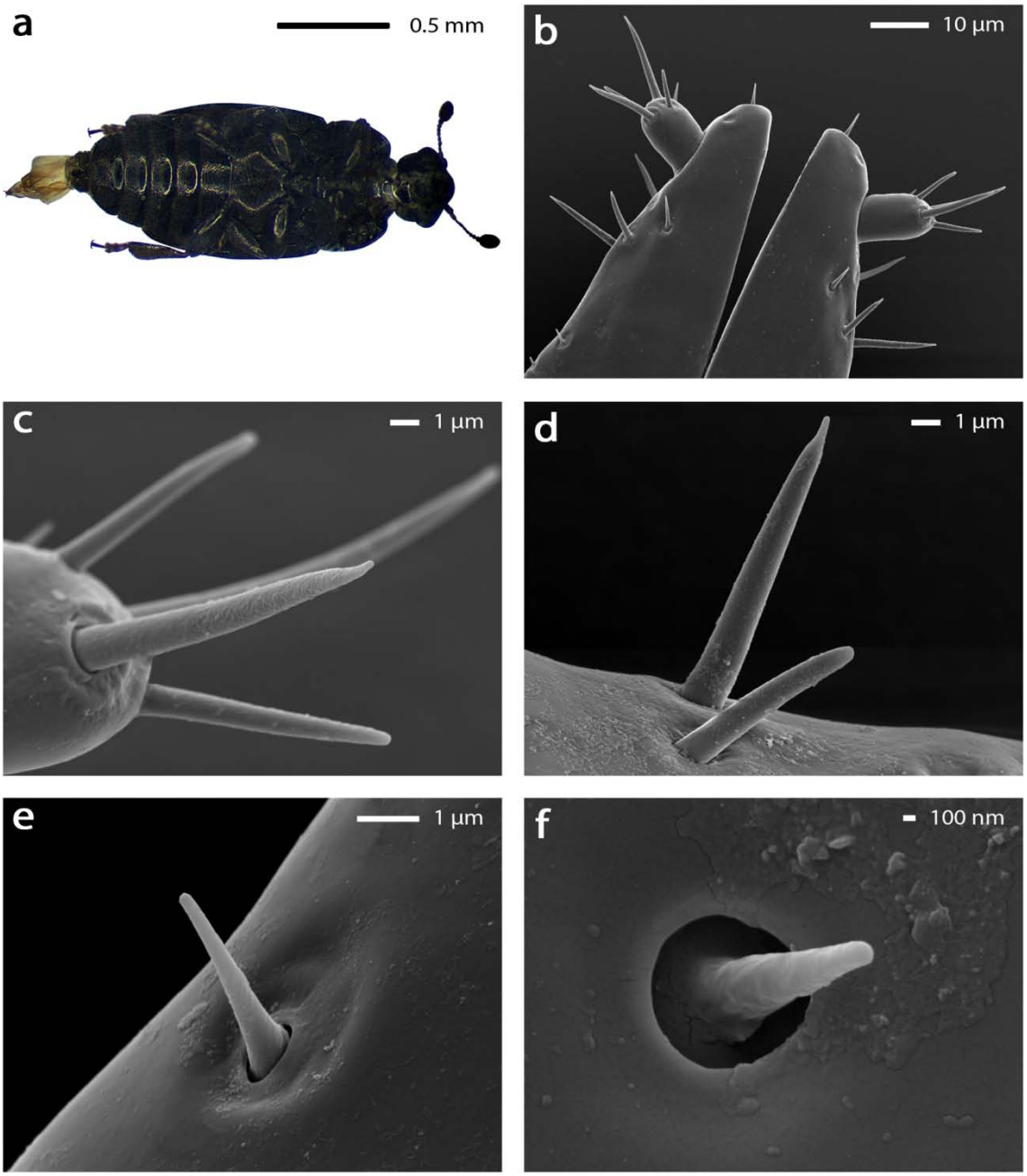

Fig. 1 Stereomicroscope observation: **a** - pollen beetle female (*M. aeneus*) with fully extruded ovipositor (x 4). SEM observation of the ovipositor: **b** - distal end of the ovipositor (gonostyloid) (x 1,400); **c** - Stylus born by the gonostylus (x 7,000); **d** - trichoid sensilla (x 7,000); **e** - basiconic sensillum (x 15,000); **f** - basiconic sensillum (x 30,000)

comprising alternate walking and walking with the ovipositor tapping on the bud surface. This probably represents 'external inspection' of the bud. If the female did not leave the bud during this first step, the second step started. This comprised alternate biting of the oviposition hole and, after a U-turn, placing the ovipositor inside this hole and tapping on internal organs of the bud. We called this phase 'internal inspection'. Finally, if the female did not leave the bud during the second step, the third and final step (consisting of laying eggs) started.

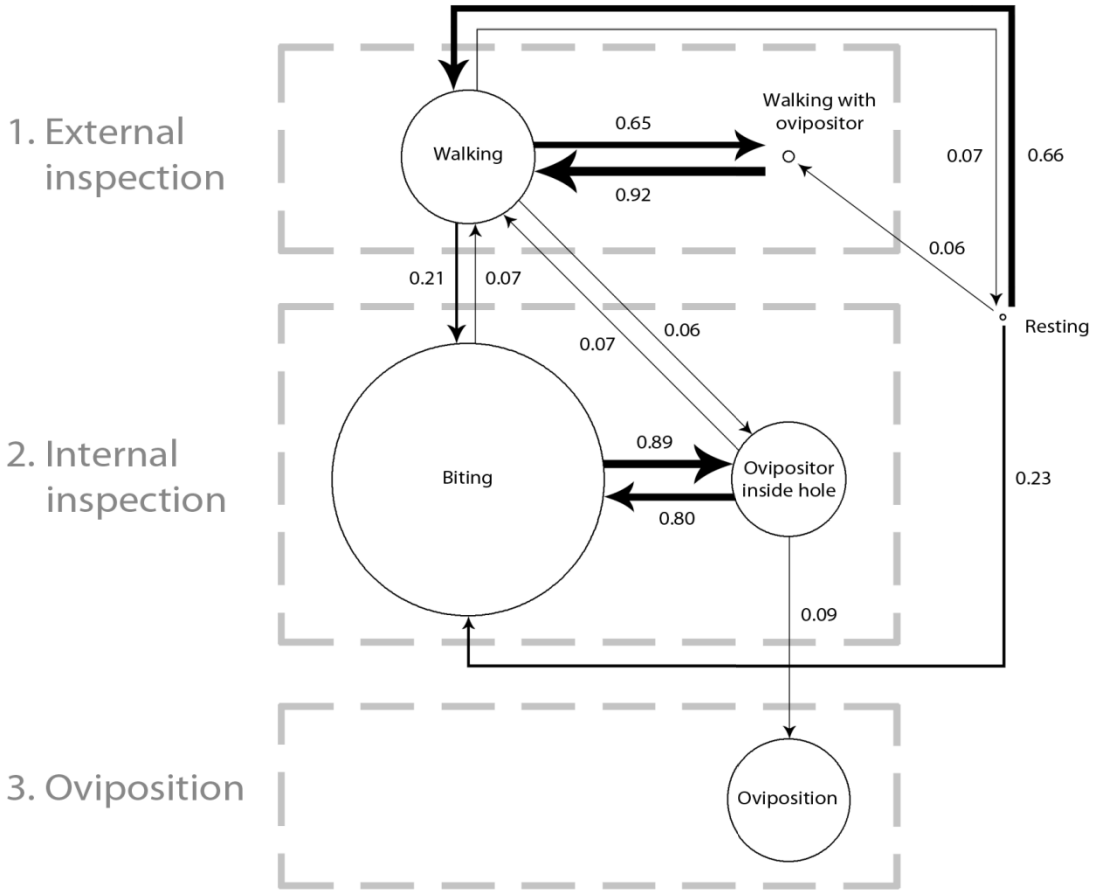

Fig. 2 Transitional diagram of behaviors of the oviposition sequence of the pollen beetle (*M. aeneus*) on oilseed rape (*B. napus*). Size of circles is proportional to the mean duration of the corresponding behavior. Size of arrows is proportional to the corresponding transition rate between the two linked behaviors. Transition rates ≥ 0.05 are represented

External inspection

It is very likely that external inspection of the bud surface has several functions. Pollen beetle females oviposited only in a narrow bud size range, which supports previous results (Nilsson 1989; Ekbom & Borg 1996; Ferguson *et al.* 2014). This bud selection is considered as an adaptive compromise between the protection of larvae against natural enemies until bud opening, and the amount of food (*i.e.* pollen) available for these larvae during the first part of their development (Ekbom & Borg 1996). The external inspection, as it is the first step of the oviposition sequence, probably plays a crucial role in evaluating the size of the bud. The process by which the female assesses this size is unknown, but it might be based on a comparison with its own size.

The majority of females oviposited on the five studied OSR genotypes, confirming that OSR is a host species of high acceptability. Borg & Ekbom (1996) showed that during the external inspection, *S. alba*, a known low-quality host plant species for the pollen beetle (Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998) was systematically rejected after a very short walk on the bud surface. A second function of this phase of the oviposition sequence could hence be to discriminate between poor and high-quality host plants. Cues that are used by the female to perform such discrimination are likely to be multiple. Indeed, phytophagous insects and plant surfaces interact in a complex manner, as both physical and chemical parameters can influence insect behavior (reviewed *in* Müller & Riederer (2005)). However, as the pollen beetle

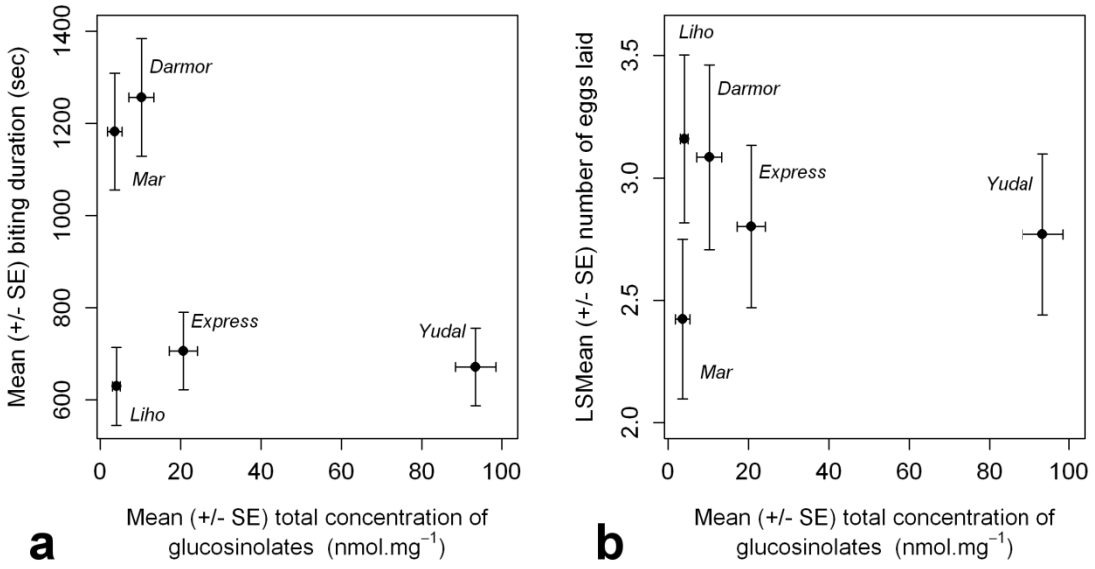

Fig. 3 Relationships between the mean total concentration of glucosinolates in the perianth of five oilseed rape (*B. napus*) genotypes and **a** - the mean biting duration of ovipositing pollen beetle (*M. aeneus*) females ($r^2 = 0.18$); **b** - the mean number of eggs laid ($r^2 = 0.03$). Horizontal bars: N = 4 for 'Mar', N = 5 for other genotypes; vertical bars: N = 25 for 'Darmor' and 'Mar', 27 for 'Express' and 'Yudal', 29 for 'Liho'. Data on glucosinolate concentration come from Hervé *et al.* (under revision) [*Article 3*]

oviposits only on certain brassicaceous plant species, it is likely that chemical cues (*e.g.* surface metabolites), which are more specific than physical ones (*e.g.* trichome density), are of primary importance. Interestingly, we observed that female's antennae are constantly used to tap the bud surface throughout the external inspection. This suggests that surface compounds are sampled during this phase of the oviposition sequence.

Our results revealed that female's ovipositor plays an active role in the external inspection, by tapping on the bud surface. This organ is known to bear sensilla at its distal end (Audisio *et al.* 2009), although their nature remains unknown. Our observations showed that all of them are totally aporous, clearly indicating that they have a sole, mechanosensory role (Chapman 2013). The function of the behavior 'Walking with ovipositor' is probably therefore to get physical information from the bud. It may either be the toughness or the thickness of the perianth which has to be pierced to bite the oviposition hole.

Internal inspection

The internal inspection consists of alternating biting of the oviposition hole and tapping inside the bud with the ovipositor. During biting, it is possible that the female would be influenced by cues from the perianth, which could either be physical or chemical. Although often neglected, plant toughness can negatively influence chewing insects by reducing consumption rate (Clissold *et al.* 2009). On the other hand, mouth parts of insects are a 'hot spot' of chemosensory receptors (Chapman 2013) and the pollen beetle is not an exception (Błażejewicz-Zawadziska & Błażejewski 2002). Interestingly, clear differences appeared among OSR genotypes, dividing them into two groups. The biochemical composition of the perianth of all of these genotypes has previously been characterized by Hervé *et al.* (under revision) [*Article 3*] and showed two clear results. Firstly, 'Yudal' is much more concentrated in total glucosinolates – secondary metabolites typical of a few plant families including Brassicaceae (Fahey *et al.* 2001) – than the other genotypes (in descending order and relatively to 'Yudal': 'Express' 0.22, 'Darmor' 0.11, 'Liho' 0.04 and 'Mar' 0.04). Glucosinolate profiles are similar; differences are

essentially quantitative. Biting duration being equivalent on 'Yudal', 'Express' and 'Liho', this suggests that there is no link between glucosinolate content of the perianth and biting duration (Fig. 3a). Moreover, the number of eggs laid was not different among genotypes, which also indicates the absence of link between glucosinolate content of the perianth and clutch size (Fig. 3b). These results confirm those of Hervé *et al.* (2014) [*Article 4*] that an important increase in glucosinolate content (maximum – minimum ratio: 23.4) does not further stimulate oviposition of the pollen beetle. Such a pattern was previously reported for another insect specialized on brassicaceous plants, the cabbage seedpod weevil *Ceutorhynchus obstrictus* (Ulmer & Dosdall 2006). This conclusion is rather unexpected, as increased amounts of glucosinolates generally stimulate feeding and oviposition of phytophagous insects specialized on brassicaceous plants (reviewed *in* Hopkins *et al.* 2009). It has to be noted that since we did not use glucosinolate-free plants, it cannot be concluded that these compounds do not stimulate oviposition at all. Secondly, Hervé *et al.* (under revision) [*Article 3*] showed a gradient of feeding stimulation among the same five genotypes: 'Express' is the most stimulant, 'Liho' the least and the three others are intermediate. Again, biting duration does not seem to be linked with perianth biochemistry. All of these results suggest that biting duration could be independent of the biochemical composition of the perianth and is possibly influenced only by its structural characteristics. Finally, since no difference in the acceptability or in the number of eggs laid has been observed among the five OSR genotypes, it seems that oviposition is not determined by cues present in the perianth of this host plant. Further studies on a greater number of genotypes are needed to confirm this hypothesis.

During the internal inspection, pollen beetle females tap inside the flower bud with their ovipositor. In a study comparing two OSR genotypes producing male-fertile or male-sterile flowers (Cook *et al.* 2004), pollen beetle females made the same number of oviposition holes on buds from the two types of plants. However, oviposition took place more often on male-fertile plants (*i.e.* the likelihood of laying eggs after having making a hole was greater in pollen-containing buds). These results, combined with our observations on the mechanosensory function of ovipositor's sensilla, suggest that the behavior 'Ovipositor inside hole' is likely to be no more than a simple assessment of the presence (and possibly the size) of anthers.

Finally, it seems that during the second phase of the oviposition sequence of the pollen beetle, chemical (*i.e.* specific) information provided by the bud does not play an important role. It suggests that this phase is not decisive in the assessment of host quality. This is concurrent with the results of Borg & Ekbom (1996) who found that even on the low-quality host *S. alba*, once females started this phase they did not stop the sequence until its end. In our study, it is likely that the few interruptions that occurred after females started biting their oviposition hole were accidental and not purposeful. Some disturbance in our experimental conditions may have occurred.

Oviposition

The third and last phase of the oviposition sequence consists of laying eggs. We found no difference among OSR genotypes in terms of number of eggs laid. Borg & Ekbom (1996) showed that for females completing their sequence, there was no difference among host species differing in their quality. In the same manner, Cook *et al.* (2004) showed that for females having oviposited, the same number of eggs was laid in male-fertile and male-sterile buds. These results suggest that clutch size of the pollen beetle is not influenced by immediate cues obtained during the oviposition sequence. The daily egg load of individual pollen beetle females has previously been shown to be comprised between one and five eggs (Hopkins & Ekbom 1996; Ekbom & Ferdinand 2003; Ferguson *et al.* 2014). Our

results suggest that, if a host plant is accepted, all available mature eggs are laid.

Conclusion

Our observations have resulted in a more detailed characterization of the behavioral sequence of oviposition in pollen beetle, and highlighted the importance of females' ovipositor in gaining information from the oviposition site. Combined with results of previous studies, we were able to draw a functional scheme of the oviposition sequence, divided into three phases. Oviposition starts with an external inspection of the flower bud, in which the acceptability of the plant is assessed. This assessment is likely to be influenced mainly by chemical information, *i.e.* surface metabolites. This is likely to be the critical step, discriminating low and high-quality host plants. The second phase is an internal inspection of the bud, probably essentially influenced by structural parameters of the perianth and during which the presence (and possibly the size) of anthers (*i.e.* food for larval development) is assessed. Finally, the third phase consists of laying probably all the mature eggs the female is carrying.

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– CHAPTER 4 –

Larval development

Plant genotype affects nutritional quality of oilseed rape (*Brassica napus*) for adults and larvae of the pollen beetle (*Meligethes aeneus*)

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Plant nutritional quality is one of the main factors influencing the fitness of phytophagous insects. This quality can vary among genotypes of the same host plant species, but also relative to the insect sex or its life stage. We compared the nutritional quality of six oilseed rape (*Brassica napus*) genotypes for larvae and adults of a major insect pest of oilseed rape crops, the pollen beetle (*Meligethes aeneus*). All traits measured varied among genotypes: larval development duration, life span of unfed emerging adults and survival time of field-sampled adults fed with pollen from the different genotypes. One plant genotype in particular ('Mar') was detrimental for both life stages. Based on a previous biochemical characterization of pollen from the same oilseed rape genotypes, starch seemed to be an important limiting factor of plant nutritional quality for this insect, especially for larvae. A high concentration of glucosinolates in the pollen affected adult survival but not larval development. The hypothesis of a detoxification mechanism occurring in this species and over-expression in larvae is proposed. Finally, the potential for a strategy involving manipulating plant quality to increase pollen beetle development time and favor biological control by natural enemies of this pest is discussed.

INTRODUCTION

A crucial determinant of insect herbivore fitness is the nutritional quality of their host plant(s). This quality is a complex trait resulting from many different biochemical variables having additive, antagonistic or even synergistic effects. Briefly, it depends either on compounds insects are not able to produce by themselves (*e.g.* essential amino acids or vitamins), sources of energy (*e.g.* carbohydrates or lipids) but also digestibility reducers (*e.g.* tannins) or toxic metabolites (*e.g.* alkaloids or terpenoids) (reviewed *in* Awmack & Leather 2002). Plant quality for insect herbivores is always relative since dietary requirements frequently vary between females and males of the same species, or between larval and adult life stages (Scriber & Slansky 1981).

Foraging strategies of phytophagous insects are partly based on the nutritional quality of the plants they encounter. It has long been known that insects are able to compensate for poor plant nutritional quality, primarily by mixing complementary food sources (reviewed *in* Behmer 2009). In this context, agrosystems present a major characteristic that differentiates them from natural ecosystems: plant diversity is generally greatly reduced and crops represent, most of the time, patches of nearly genetically identical host plants (Tooker & Frank 2012). Consequently, nutritional quality is quite homogeneous among plants on which many herbivorous insects stay for major periods of their life.

Monocultures are generally seen as more prone to pest attacks than polycultures because of their uniform susceptibility to these pests (Tooker & Frank 2012). However, one may also consider that if the cultivated plant line is of poor quality for an insect pest with low mobility, this insect will have more difficulty in compensating and in reaching its optimal dietary intake. Its fitness may hence be affected. This is sort of an applied version of a plant defense strategy proposed by Berenbaum (1995), which unfortunately did not seem to receive much attention (but see Wright *et al.* (2003) and Zangerl & Berenbaum (2004)).

The pollen beetle (*Meligethes aeneus* F., Coleoptera: Nitidulidae) is an univoltine pollen-feeding insect. Adults are generalist and commonly found in a great diversity of plant families (Free & Williams 1978; Carrié *et al.* 2012; Marques & Draper 2012). However, oviposition takes place only on brassicaceous plants (Free & Williams 1978; Ekbom & Borg 1996). Adults overwinter in semi-natural habitats and have to disperse after their diapause to locate host plants. Females are sexually mature only after about two weeks of post-diapause feeding (Williams 2010). They mate and oviposit on the same plant, laying eggs inside flower buds. Egg production is directly impacted by the host plant, since females feed on the same plant where they lay eggs and oogenesis is a short continuous process in this species (Hopkins & Ekbom 1999; Ekbom & Ferdinand 2003; Ekbom & Popov 2004; Hervé *et al.* 2014 [*Article 4*]). Larvae are *de facto* specialists of the Brassicaceae family. They feed essentially on pollen, although it is not completely obligatory for their development (Cook *et al.* 2004). First instar larvae develop inside a closed bud, usually reaching the second (and last) instar when the flower opens; then they feed on pollen of opened flowers on the same plant (Williams & Free 1978). Pupation takes place in the surrounding soil and new-generation adults emerge at the end of spring (Williams 2010). The great difference between adults and larvae in dispersal ability and diet breadth seems to have resulted in contrasted investment in certain physiological functions. Indeed, genes related to olfactory and visual signal perception are over-expressed in adults, while genes encoding some catabolism enzymes are over-expressed in larvae (Vogel *et al.* 2014).

Oilseed rape (*Brassica napus* L., Brassicaceae) (OSR) crops represent an enormous number of host plants in spring. The pollen beetle has adapted to this resource, probably within a short period of time (Hokkanen 2000). Nowadays it is clear that OSR is one the best-quality host species for this insect, for both egg production and larval performance (Ekbom & Borg

1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Ekbom & Popov 2004; Kaasik *et al.* 2014). For this reason, the polle, beetle has become a major pest of OSR. Indeed, before blossoming starts, adults destroy flower buds to reach the pollen they contain, leading to potentially important yield losses (*e.g.* Nilsson 1987).

Our aim was to study whether OSR genotype can differentially affect pollen beetle larvae and adults through its nutritional quality, and determine whether effects on pollen beetle are similar between adults and larvae. Adult survival and larval development were studied in controlled conditions. To keep the interaction as natural as possible, development was assessed on entire, intact plants. The amount of food available for larvae during their development was also assessed to take into account this possibly confounding factor. Finally, we aimed to identify the major determinants of plant quality for pollen beetle adults and larvae by linking the present results to a previous biochemical characterization of the pollen of the same six OSR genotypes (Hervé *et al.* 2014 [*Article 4*]).

MATERIALS AND METHODS

Plants

All genotypes used in this study are lines from the INRA OSR collection (BraCySol Center for Genetic Resources, INRA, Le Rheu, France). Both winter (genotypes 'Darmor', 'Express', 'Mar' and 'Markus') and spring (genotypes 'Liho' and 'Yudal') OSR genotypes were used. Plants were produced in controlled conditions as described in Hervé *et al*. (under revision [*Article 3*]), except for the experiment on larval development where 0.8 l-pots were used.

Insects

Overwintered pollen beetles were collected from an unsprayed winter OSR crops near Le Rheu (Brittany, France). Individuals were used for experiments immediately. For the experiment on larval development, females were identified by observing mating behavior.

Amount of food available for larvae

Three flower buds (between 6-8 mm long) of an intact plant (BBCH stage 59 (Lancashire *et al*. 1991), *i.e.* the 'yellow bud stage') were dissected. Since a small proportion of anthers naturally abort during their development, three anthers were randomly chosen among the six potentially available per bud. They were then weighed together using a XS105 Dual Range (Metler Toledo) scale. Ten plants were used per OSR genotype, randomly chosen through time.

Larval development and survival of emerging adults

One female was individually placed for two hours on the main inflorescence of an intact plant (BBCH stage 55-57, *i.e.* the 'green bud stage'), in a plastic pot (diameter 6.5 cm, height 9 cm) isolating this inflorescence from the rest of the plant. Previous observations revealed that this time period allows the female to perform a single oviposition sequence, but not to feed. The different genotypes were randomly alternated on shelves placed in a controlled room (LD 16: 8 h photoperiod, temperature 20 °C) during this period and for the rest of the experiment. After two hours the female was removed and the whole plant was placed in a microperforated plastic bag (diameter 19 cm, height 69 cm). All experiments started at 9:30 am and 20 plants were used per genotype. All replicates were performed randomly through time. No female ever fed during the experiment. It must be noted that oviposition can only be supposed in this experimental design, since the only way to verify it is to dissect flower buds (therefore precluding any further larval development). However previous experiments showed that females lay equivalent numbers of eggs on the six genotypes tested in the present study (Hervé *et al.* 2014 [*Article 4*], Hervé *et al.* under revision [*Article 5*]).

Fourteen days after placing females on the inflorescence, *i.e.* during the pupation period at this temperature (Cook *et al*. 2004), access to any food source for emerging adult pollen beetles was avoided by cutting all of the aerial part of the plant and removing from the pot all sepals, petals and anthers that had naturally dropped down during plant growing. Presence of larvae was checked at this time. No living or dead larvae were observed. When adults emerged about 10 days later, they were individually placed in small Petri dishes (diameter 3.5 cm) containing a moistened paper filter (moisturized daily) until death. Adult emergence and subsequent survival were checked daily. Individuals were sexed after their death following Ruther & Thiemann (1997).

Survival of field adults

Pollen beetle adults sampled in the field were individually placed in small Petri dishes as described above and starved for three days. Previous observations showed that mortality was induced after three days of starvation, indicating that energy reserves were reduced to a low level. Individuals were then fed *ad libitum* for two days with two flowers abscissed from the same test OSR genotype, collected from greenhouse-grown plants. After this period, flowers were removed and insect survival was checked daily. Individuals not fed during these two days were used as control. The sexes of beetles were determined after their death as described above. Fifty replicates were performed per treatment (OSR genotype or control), randomly through time and space and in controlled conditions as described above.

Statistical analysis

All statistical analyses were performed using R software (R Core Team, 2013).

Amount of food available for larvae – Mass of anthers was compared among OSR genotypes using a Wald test on a Linear Mixed Model (function 'lmer', package 'lme4' (Bates *et al.* 2014)) taking into account the plant genotype (fixed factor) and the individual plant (random factor). Pairwise comparisons of Least Squares Means (LSMeans) were performed using the function 'lsmeans' (package 'lsmeans' (Lenth 2013)) and the False Discovery Rate (FDR) correction for *P*-values (Benjamini & Hochberg 1995).

Larval development and survival of emerging adults – Numbers of emerging adults per plant were compared among genotypes using a likelihood ratio test on a Generalized Linear Model (GLM; distribution: negative binomial, link function: log) (function 'glm.nb', package 'MASS' (Venables & Ripley 2002)). Development time was analyzed using a likelihood ratio test on a GLM (distribution: Gamma, link function: inverse) taking into account the OSR genotype and the sex of individuals. Survival time of emerging adults was analyzed using a likelihood ratio test on a parametric survival regression (distribution: Weibull, link function: log) (function 'survreg', package 'survival' (Therneau & Grambsch 2000)) considering as explanatory variables the development time, the OSR genotype and the sex of individuals. Pairwise comparisons of LSMeans were then

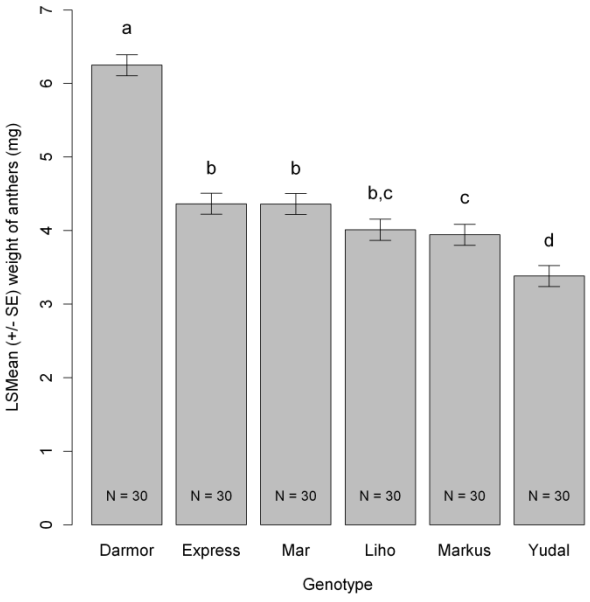

Fig. 1 Least Squares Mean (± SE) weight of three anthers from flower buds of six oilseed rape (*B. napus*) genotypes. Different letters indicate statistically different LSMeans. N: number of triplets of anthers weighed per genotype

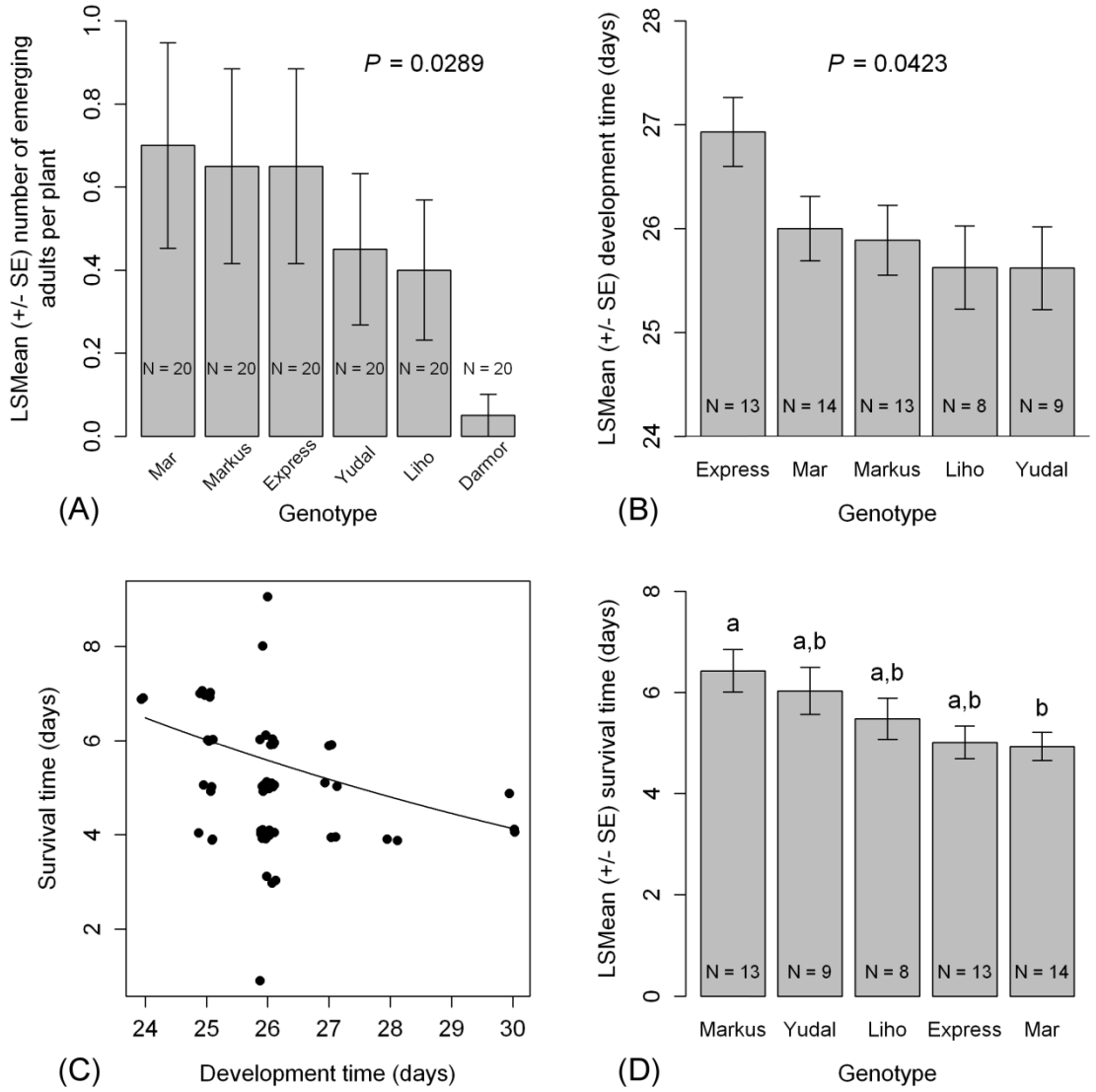

Fig. 2 (A) Least Squares Mean number of pollen beetles (*M. aeneus*) emerging from six oilseed rape (*B. napus*) genotypes after completing development on the plant (N: number of plants per genotype). (B) LSMean development time of emerging pollen beetles per plant genotype (N: number of individuals per genotype). (C) Relationship between development time and survival time after emergence (a small amount of noise was added to show points located at the same coordinates). (D) LSMean survival time of emerging pollen beetles per plant genotype (N: number of individuals per genotype). Back-transformed LSMeans (± SE) are systematically represented. Different letters indicate statistically different LSMeans

computed, adjusted with the FDR correction.

Survival of field adults – Survival time of field-sampled adults was analyzed using a likelihood ratio test on a parametric survival regression (distribution: Weibull, link function: log) taking into account the treatment (OSR genotype or control) and the sex of individuals. Pairwise comparisons of LSMeans were then computed, adjusted with the FDR correction.

RESULTS

Amount of food available for larvae

The mass of anthers was significantly different among OSR genotypes ($\chi^2 = 233.87$, $df = 5$, $P < 0.001$; Fig. 1). 'Darmor' exhibited the highest mass (mean ± SE: 6.25 ± 0.14 mg) whereas 'Yudal' exhibited the lowest (3.38 ± 0.14 mg). The other four genotypes were intermediate between these two extremes.

Larval development

One to 14 individuals were obtained per OSR genotype. A significant variation was found among these genotypes for the number of adults produced per plant (χ^2 = 12.47, *df* = 5, *P* = 0.0289; Fig. 2A). However, pairwise comparisons showed no significant differences even if several contrasts involving the genotype 'Darmor' showed a *P* < 0.10 (*vs.* 'Express', 'Mar' and 'Markus'). Since only one individual was obtained on 'Darmor', this genotype was excluded from further analyses. Other genotypes produced at least eight individuals.

Development time was not influenced by the sex of individuals (χ^2 = 0.26, *df* = 1, *P* = 0.611) but differed significantly among OSR genotypes (χ^2 = 9.89, *df* = 4, *P* = 0.0423; Fig. 2B). Pairwise comparisons showed no differences at the 0.05 significance threshold, but development was nearly significantly longer on the genotype 'Express' (mean ± SE: 26.93 ± 0.33 days) compared to 'Liho' and 'Yudal' (25.62 ± 0.40 days and *P* = 0.0673 in each case).

Survival of emerging adults

Survival of adults emerging from the different genotypes was significantly influenced by development duration (χ^2 = 4.18, *df* = 1, *P* = 0.0408; Fig. 2C): the longer the time needed for development, the shorter the adult life span. Plant genotype had a significant additive effect (χ^2 = 11.47, *df* = 4, *P* = 0.0218; Fig. 2D): survival time was longer when individuals developed on 'Markus' (6.43 ± 0.42 days) compared to 'Mar' (4.93 ± 0.28 days). Other genotypes were intermediate between these two extremes, but the difference between 'Markus' and 'Express' (5.01 ± 0.32 days) borders on significance (*P* = 0.0645). No difference was observed between females and males (χ^2 = 0.0068, *df* = 1, *P* = 0.934).

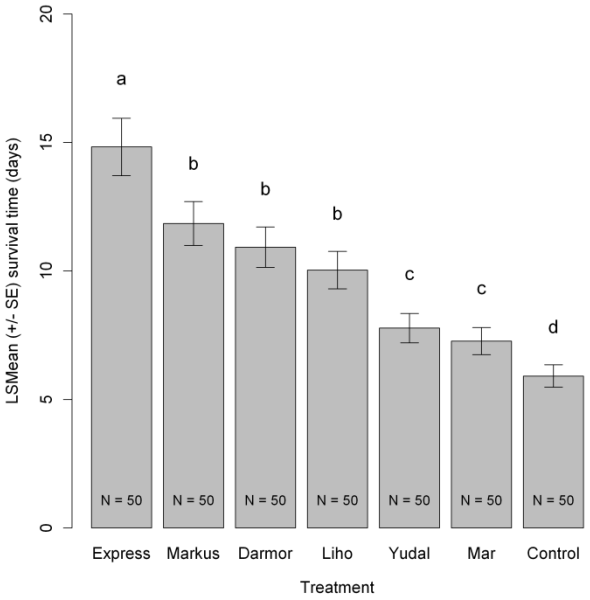

Fig. 3 Least Squares Mean (± SE) survival time of adult pollen beetles (*M. aeneus*) fed on six oilseed rape (*B. napus*) genotypes (control individuals not fed) (back-transformed values). Different letters indicate statistically different LSMeans. N: number of individuals per treatment

Survival of field adults

The survival time was significantly different among treatments (χ^2 = 96.98, *df* = 6, *P* < 0.001; Fig. 3). Control individuals had the shortest survival time (5.91 ± 0.44 days). Considering survival on OSR genotypes, individuals fed on 'Express' had the longest survival time (14.82 ± 1.12 days) and individuals fed on 'Yudal' and 'Mar' the shortest (7.78 ± 0.57 and 7.27 ± 0.53 days, respectively). Sex of individuals also had a significant effect on survival time (χ^2 = 5.77, *df* = 1, *P* = 0.0163): males survived for a longer duration than females (10.05 ± 0.41 *vs.* 8.78 ± 0.35 days, respectively).

DISCUSSION

The present study aimed test at whether or not the nutritional quality of OSR for both larval and adult stages of the pollen beetle varies with plant genotype. We found a genotypic effect for all traits we observed. At the interspecific scale, it has already been shown that larval performance differs among some brassicaceous plant species (Ekbom 1998). At the intraspecific scale, our

study is the first to demonstrate that OSR genotype impacts both life stages of the pollen beetle. This conclusion is not really surprising since such plant intraspecific variation is known in a variety of plant – insect interactions (*e.g.* Glynn *et al.* 2004; Chen *et al.* 2009; Amin *et al.* 2011; Lehrman *et al.* 2012; Guo *et al.* 2013; Sandhyarani & Usha Rani 2013).

Plant genotype and number of offspring produced

The number of offspring produced was not different among OSR genotypes, except for 'Darmor' from which only one individual emerged. We showed in a previous study that in the same experimental conditions, clutch size of pollen beetle females does not differ among the genotypes we used (Hervé *et al.* under revision [*Article 5*]). Although we cannot rule out the possibility that for some reason fewer eggs were laid in buds of 'Darmor', these results suggest that 'Darmor' has detrimental effects on larval development. Further studies conducted on this genotype in more controlled conditions would be necessary to confirm if it does really not support larval development – and determine why.

Factors determining plant nutritional quality for larvae

We measured two traits to estimate the impact of the plant on larvae: the time needed to complete development to adulthood and the survival of emerging (unfed) adults. We found intergenotypic differences for both traits. The quantity of the main food source of larvae (*i.e.* pollen) differed among OSR genotypes but was clearly not related to larval performance. This result shows that food quantity was not a limiting factor in our experiments and consequently that plant nutritional quality was determinant. The negative relationship between development time and adult life span supports this conclusion. Indeed, a longer period needed, infers complete development suggests either a slower accumulation of energy and/or a greater accumulation of toxins, whereas a shorter survival of unfed adults infers that less energy was accumulated during development.

The nutritional quality of the pollen of the six genotypes used in this study was estimated in a previous paper through the quantification of a number of biochemical parameters (Hervé *et al*. 2014 [*Article 4*]). In the light of the present results, some hypotheses can be drawn about determinant components of pollen quality for pollen beetle larvae. Other than 'Darmor', the least profitable genotype was clearly 'Express', on which development was the longest and adult life span among the shortest (even after having taken into account the effect of development duration). Unfortunately, the biochemical profiling of the pollen does not help to understand the origin of this detrimental effect since no compounds differentiated this genotype from the others (Hervé *et al.* 2014 [*Article 4*]). However, an interesting pattern appears with the other four genotypes. Development duration was similar on these genotypes, but not adult life span. A gradient was shown from 'Mar' to 'Markus', the former being the least profitable. Among all compounds quantified in Hervé *et al.* (2014) [*Article 4*], this gradient showed a very high correlation with the concentration of starch in the pollen (Fig. 4A). As a polysaccharide, this compound is an important source of energy for insects (Chapman 2013). Although it is unlikely that only one compound was responsible for the variation in adult survival, it seems that pollen starch concentration may be an important determinant of nutritional quality for pollen beetle larvae. It is not the first time that a positive correlation between plant genotypic variation of starch content and insect herbivore performance has been shown (Osier & Lindroth 2004). To our knowledge, however, our study is the first to show such a high correlation. This result argues the need for better consideration of plant primary metabolites in studies of plant – insect interactions, as Berenbaum (1995) pointed our some years ago.

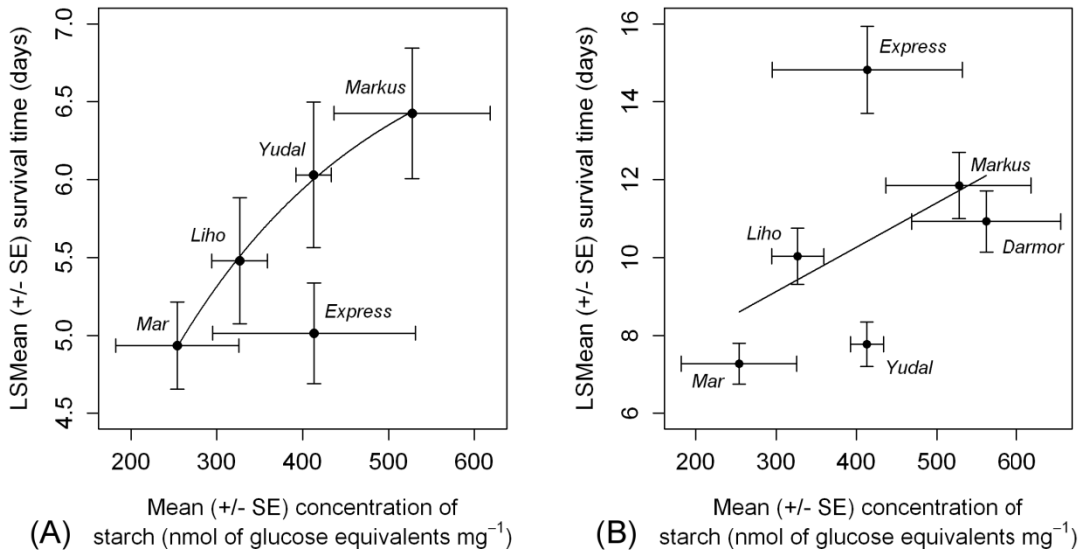

Fig. 4 Relationships between the mean concentration of starch in the pollen of six oilseed rape (*B. napus*) genotypes and the mean survival time of (A) adult pollen beetles (*M. aeneus*) reared on the same genotypes (*pseudo*-r^2 = 1.00; 'Express' excluded from the relationship); (B) adults sampled in the field and fed with the pollen of the same genotypes (r^2 = 0.23). Horizontal bars: N = 5 for all plant genotypes; vertical bars on the left-graph: N = 8 for 'Liho', 9 for 'Yudal', 13 for 'Express' and 'Markus', 14 for 'Mar'; vertical bars on the right-graph: N = 50 for all genotypes. Data on starch concentration come from Hervé *et al.* (2014) [*Article 4*]

Factors determining plant nutritional quality for adults

Starved individuals were fed *ad libitum* for two days before being starved again until death. The variation we observed among OSR genotypes was hence likely to be due to differences in nutritional quality of the pollen provided. This hypothesis is supported by results of a previous study conducted on the same six genotypes that showed that females fed with the two genotypes on which the survival time was the shortest ('Yudal' and 'Mar') also produced the smallest eggs (Hervé *et al.* 2014) [*Article 4*].

The genotype 'Express' showed a surprising pattern: it was clearly the most profitable for adults, whereas it was the least profitable for larvae. This intriguing paradox is not explained by biochemical data obtained by Hervé *et al.* (2014) [*Article 4*]. Further studies are needed to explain this finding. Interestingly, 'Mar' was detrimental for both larvae and adults. Starch might therefore also be limiting for adults, but the relationship between survival and pollen starch content is much less clear in adults compared with larvae (Fig. 4B). Finally, a unique characteristic differentiated the pollen of 'Yudal' in comparison to all other genotypes: it is much more concentrated in glucosinolates (Hervé *et al.* 2014) [*Article 4*]. Our results suggest that pollen beetle adults would be affected by these secondary metabolites, whereas larvae would not. This point is specifically discussed in the next section.

Pollen beetles and glucosinolates: a detoxification story?

Glucosinolates are secondary metabolites that are typical of a few plant families including Brassicaceae (Fahey *et al.* 2001). Some of their catabolites (isothiocyanates), produced when the plant is wounded, seem to be universally toxic (Wittstock *et al.* 2003). Although not all glucosinolates systematically have the same effect, a general pattern emerged from many studies: they are mostly deterrents for generalist insect species whereas they are neutral or even stimulant for insects specialized on brassicaceous plants (reviewed *in* Hopkins *et al.* 2009). These specialist species are able to cope with glucosinolates, for example by avoiding the

formation of isothiocyanates or by detoxifying them (reviewed *in* Winde & Wittstock 2011). Although nothing has been experimentally demonstrated to date for the pollen beetle, elements from different studies can lead to draw a coherent scheme.

Pollen beetle adults are generalist pollen feeders (Free & Williams 1978; Carrié *et al.* 2012; Marques & Draper 2012) whereas larvae are specialized on Brassicaceae (Cook *et al.* 2004). It was previously shown that adult feeding is not deterred by glucosinolates (Hervé *et al.* under revision [*Article 3*]), suggesting that adults may be able to deal with them. The classical system by which insects deal with toxic secondary metabolites is metabolic resistance, *i.e.* detoxification. Three super-families of enzymes can perform such reactions: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxylesterases (Després *et al.* 2007). Previous studies aiming to decipher how the pollen beetle has become non-susceptible to widely-used insecticides (pyrethroids) revealed that more than 70 P450s are expressed in adults (Zimmer *et al.* 2014) and that two GSTs are also highly expressed (Erban & Stara 2014). These findings encourage detailed studies of if (and how) pollen beetle adults are really able to detoxify glucosinolates. Whatever the system involved, it seems, however, not completely effective. Indeed, the most glucosinolate-rich genotype of this study ('Yudal') negatively impacted both adult survival and egg production (Hervé *et al.* 2014) [*Article 4*]. These results do not argue against the hypothesis of a glucosinolate detoxification mechanism in adults, since even specialized insects can be affected by isothiocyanates (Agrawal & Kurashige 2003).

Pollen beetle larvae seem not to be affected by glucosinolates, as both development and survival were as good on 'Yudal' as on less glucosinolate-rich genotypes. This is consistent with the fact that larvae are more specialized than adults on this plant family. It also suggests that if a detoxification mechanism exists in this species, it would be more effective in larvae. Results of a recent study comparing the transcriptome of both larvae and adult pollen beetles confirm this hypothesis. Indeed, genes encoding P450s and GSTs were more than eight times over-expressed in larvae compared to adults (Vogel *et al.* 2014).

Finally, β-glucosidases and UDP-glycosyltransferases were also found to be over-expressed in larvae (Vogel *et al.* 2014). These enzymes are typical of another mechanism of resistance to certain secondary metabolites. Toxic compounds are often deactivated in the plant by addition of a sugar-moiety (Morant *et al.* 2008). This is the case of glucosinolates, which are activated by glycosylation. The aglycone product is highly instable and spontaneously rearranges into isothiocyanates (Winde & Wittstock 2011). A strategy that evolved in some species (*e.g.* the Lepidopteran *Spodoptera frugiperda* (Maag *et al.* 2014)) is to deactivate toxic compounds produced by their host plant using UDP-glycosyltransferases, and to further reuse them against their own natural enemies (by reactivation using β-glucosidases). The presence of such a mechanism in pollen beetle larvae is strongly suggested by transcriptomic data and could partly explain why they seem less impacted than adults by glucosinolates. Moreover, reuse of deactivated isothiocyanates may be a valuable strategy for pollen beetle larvae to defend themselves against parasitoids, which are known to be responsible of high larval mortality in the field (Ulber *et al.* 2010).

Applied perspective

Clancy & Price (1987) proposed a mechanism by which reduced nutritional quality may be selected as a defense strategy against insect herbivores in plants. Their 'slow growth – high mortality' hypothesis (SGHMH) states that longer larval development duration should be associated with a longer vulnerability period to natural enemy attack (Clancy & Price 1987). The SGHMH received some experimental support (reviewed *in* Williams 1999). Most of the OSR genotypes we used in

this study are not cultivated. Differences we observed for the development duration of pollen beetle larvae are hence not the resultant of a selective process that occurred in defense to this species. However, the SGHMH principle may be put into practice to enhance crop protection against this pest. Indeed, parasitism rate of pollen beetle larvae was reported to regularly exceed 50 % in the field (reviewed *in* Ulber *et al.* 2010). Extending the vulnerability window of larvae may result in even greater population control by natural enemies. In the medium-term, this strategy may contribute to reduce pollen beetle population size. Further studies on the genotype 'Express', which for an unknown reason prolonged the development duration of larvae, may identify plant biochemical/physical traits that could be manipulated to consider such a strategy.

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– GENERAL DISCUSSION –

SUMMARY OF RESULTS

The first objective of this PhD thesis was to test if attraction, feeding intensity, egg production, oviposition and larval development of the pollen beetle could vary with OSR genotype. We also aimed to identify candidate key traits determining the potential observed variation. Results are summarized in Table 1.

Attraction

Article 2 showed that variation exists in plant attractiveness to pollen beetles. However, whether or not the differences we observed in attraction index would result in a decreased (or slowed down) colonization in the field remains to be tested. A semi-field experiment in which a known number of beetles are released into a cage (or tunnel) containing potted plants (as in Cook *et al.* 2006 but in a no-choice situation) could give interesting data to address this question. Moreover, this kind of experiment does not test only the VOC-mediated attraction since plant size and architecture also provide visual cues to the insect. Hence, it is closer to what is likely to happen in the field.

The characterization of the odor bouquet emitted by the six OSR genotypes of this study is not performed yet (but will be available in the next few months). Considering intergenotypic comparisons, the only known effect in the literature was found by Cook *et al.* (2006) who showed that pollen beetles confronted with two cultivars preferred the one emitting more ITCs. This result confirmed those of Blight & Smart (1999) who proved that in the field, pollen beetles were attracted by lures of all eight pure ITCs tested, and by a mixture of allyl-, 3-butenyl-, 4-pentenyl- and 2-phenylethyl-ITCs. Additionally, Cook *et al.* (2007a) found that phenylacetaldehyde and indole (two aromatic floral compounds) are attractive to the pollen beetle when tested alone in olfactometer experiments. It is difficult to draw hypotheses about the compounds explaining the differences we found. Flower buds, as reproductive tissues, are likely to contain high amounts of glucosinolates (GSLs) (precursors of ITCs) compared to vegetative organs (Hopkins *et al.* 2009). From the sole point of view of inflorescences, our result does not correlate with the known effect of ITCs. Indeed, the two

Table 1 Summary of results found during this thesis. Primary metabolites are colored in blue, secondary metabolites in orange. **++** : major role; **+** : minor role. ↗ : candidate traits having a positive effect on the insect; ↘ : candidate traits having a negative effect on the insect. In brackets are minor candidate traits (see text)

	Intergenotypic variation	Putative origin	Candidate key traits
Attraction *Article 2*	✓	Volatile compounds	?
Feeding intensity *Article 3*	✓	Biochemical composition of the perianth	↗ : sucrose, proline, serine (↘ : quercetin-3-*O*-sophoroside, kaempferol-3-*O*-sophoroside)
Egg production *Articles 4 & 6*	✓	**++** : amount of food eaten **+** : nutritional quality of the food	Traits triggering feeding stimulation ↗ : starch ↘ : glucosinolates [gluconapin, gluconasturtiin, progoitrin] (SMCO)
Oviposition *Articles 4 & 5*	✗		
Larval development *Article 6*	✓	Nutritional quality of the food	↗ : starch

extreme genotypes for GSL bud content ('Yudal' and 'Liho') showed equal attractiveness whereas extreme genotypes for attractiveness ('Mar', 'Markus' and 'Darmor') were intermediate for GSL bud content (*Article 3*). However, we did not quantify GSLs in leaves, organs that are likely to emit the highest amounts of VOCs due to their large area. Because GSL profile of different plant organs possibly show qualitative and quantitative variations (Hopkins *et al.* 2009), leaf content cannot be inferred from bud content. Odor bouquet characterization will bring crucial elements to go further into the identification of key attractive/repulsive compounds (or ratios of compounds).

Feeding intensity

It is very intriguing that feeding behavior has not been more studied, since it is clearly the most damaging behavior from an agronomical point of view. To our knowledge, only Ekbom & Borg (1996) compared several brassicaceous species for this trait. Some differences were found, but no studies were further conducted. In *Article 3* we showed a gradient of feeding intensity among the six OSR genotypes. This result may be very promising for crop protection (see section 'Perspectives for crop protection'). Through detailed metabolic profiling, we hypothesized that the perianth is the key tissue determining feeding intensity. From the correlation between feeding data and biochemical composition of the perianth, we then identified three compounds that may be phagostimulant (sucrose, proline and serine) and two others that may be phagodeterrent (quercetin-3-*O*-sophoroside and kaempferol-3-*O*-sophoroside). The phagostimulant power of sugars, especially sucrose, is well-established in the literature (*e.g.* Mitchell & Gregory 1979; Bartlet *et al.* 1994; Isidoro *et al.* 1998; Chapman 2003; Merivee *et al.* 2008, 2012; Hori *et al.* 2010; Tooming *et al.* 2012). We strongly believe that sucrose is the key metabolite explaining the gradient we found, whereas the two free amino acids (proline and serine) may play a reinforcement role. The strong correlation between concentrations of sucrose and the two flavonols (quercetin-3-*O*-sophoroside and kaempferol-3-*O*-sophoroside) suggests that these two compounds are more likely to play a confounding effect than to have a real phagodeterrent effect. However, all five compounds have now to be tested (individually and in mixtures) to support the importance of their influence on pollen beetle feeding. We set up a supplementation protocol on entire plants, in which an aqueous solution containing the substances to be tested was sprayed on flower buds. After 16 hours, four pollen beetles were placed on the inflorescence and allowed to feed for 24 hours, after which time the number of buds damaged by feeding was assessed. Only preliminary results have been obtained to date, using a solution of sucrose 40 mM (a usual concentration in artificial feeding experiments, *e.g.* Bartlet *et al.* 1994). They indicated a significant phagostimulant effect of sucrose (20.8 ± 4.2 *vs.* 12.3 ± 3.0 % of buds damaged, N = 6 plants per treatment, $P = 0.0289$), which is very encouraging to go further.

Egg production

It was already known that oogenesis is relatively short in the pollen beetle (around two days; Ekbom & Ferdinand 2003). Since oogenesis is directly influenced by the nutritional quality of the food eaten (Awmack & Leather 2002) and pollen beetle females feed on the same plant where they lay eggs, we hypothesized that the plant would affect egg production through the food it provides. *Article 4* demonstrated such a relationship. We showed that the number of eggs produced was positively related to the amount of food eaten, estimated by the number of buds damaged by feeding. Hence, through a domino effect, feeding stimulation seems to directly influence egg production. This has been previously shown with other phytophagous insects (*e.g.* Sisterson 2012), but not with the pollen beetle. It highlights the need for better consideration of feeding patterns, which are often ignored when working on herbivore egg production. It

further puts into perspective a hypothesis proposed a few years ago on this insect species. Hopkins & Ekbom (1996, 1999) showed that egg production of the pollen beetle is reduced depending on host availability and host species. This result was interpreted as a consequence of a variation in oviposition stimuli between these species, reflecting different host qualities. This is likely, but could be only part of the picture. Indeed, the current experiments lasted for five days, which is longer than oogenesis time, and insects were fed with the same plant on which they oviposited. Therefore the possibility cannot be excluded that reduction of egg production in these studies was at least partly due to a diminution in food consumption.

Results of *Article 4* also suggested that pollen nutritional quality plays a role in egg production, since biochemical profiles of anthers partly matched the intergenotypic gradient observed for egg length. Results obtained in *Article 6* on survival of field-sampled adults support this hypothesis since the same two genotypes ('Mar' and 'Yudal') were detrimental for both oogenesis and survival (Fig. 1).

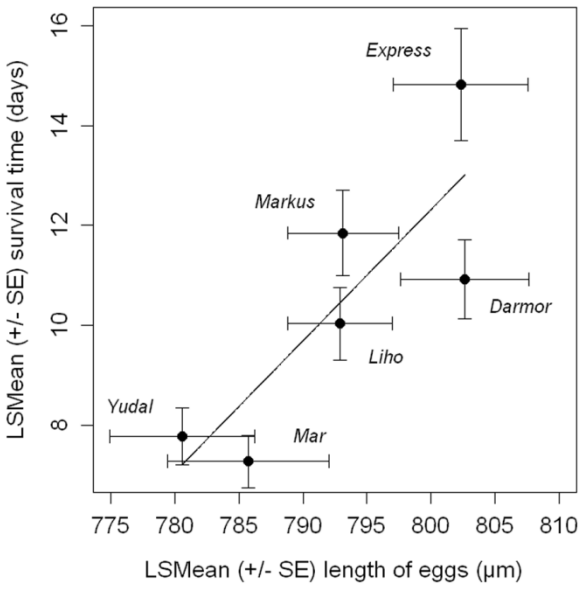

Fig. 1 Relationship between the genotypic effects observed on egg length (*Article 4*) and survival of field-sampled adults (*Article 6*) ($r^2 = 0.69$)

Determinants of pollen nutritional quality that we suggested in *Article 4* were possible nutritional compounds having a positive effect (starch and total essential amino acids (TEAAs)) and possible antinutritional or antibiotic compounds having a negative effect (some GSLs and SMCO). Data obtained in *Article 6* allow refining of these hypotheses. Indeed, starch was also found to be highly correlated with larval performance. Therefore it remains a good candidate key trait determining pollen nutritional quality. On the other hand, larvae developed equally well on 'Yudal' (which shared with 'Mar' the lowest concentration of TEAAs in its pollen) than on genotypes showing higher TEAA concentrations. It is hence unlikely that TEAAs were a limiting factor. 'Yudal' was clearly the most GSL-rich. We suggested that GSLs could affect pollen beetle adults but not larvae. Results seem coherent enough to consider GSLs as candidate key traits determining pollen nutritional quality, but only for adults. In more detail, the pollen of 'Yudal' was characterized by a greater concentration of gluconapin, gluconasturtiin and progoitrin compared with other genotypes. These GSLs, which are all catabolized into toxic ITCs (Hopkins *et al.* 2009), appear therefore as particularly good candidate key traits. 'Yudal' and 'Mar' were also the most SMCO-rich, but 'Yudal' was not detrimental for larvae. We may have hypothesized that only larvae are able to deal with SMCO, as we did for GSLs. However, the impact of SMCO on insects is itself hypothetical since it was only shown in mammalian herbivores (Paul *et al.* 1986). Further tests are needed to decide whether or not SMCO should be considered as a candidate key trait. Finally, although we performed quite a large metabolic profiling to estimate pollen nutritional quality, it is certain that we missed important variables. Indeed, it was shown in *Article 6* that 'Express' was more favorable than any other genotype for field-sampled adult survival. However, nothing in our analyses differentiated this genotype from others from a biochemical point of view.

Oviposition

Oviposition, in the sense of a behavioral preference of females for laying eggs in certain genotypes, is the only trait for which we did not find any intergenotypic variation. In both *Articles 4* and *5*, an equal number of eggs were laid on all OSR genotypes by motivated females not having experienced these genotypes before. In *Article 5*, we observed differences in the oviposition behavior, but these did not lead to different numbers of eggs being laid. It is clear from Borg & Ekbom (1996) and *Article 5* that pollen beetle females use plant cues during their oviposition sequence. However, the absence of a contrast among OSR genotypes does not allow hypotheses to be proposed about the cues that are determinant. At least can we suggest that surface chemicals are likely to play a major role in the interspecific contrasts that were observed (Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998) since they provide more specific information than physical traits.

Larval development

Article 6 showed that OSR genotypes differentially impacted larval development of the pollen beetle. We used two measures of larval performance: the time needed for complete development from egg to adulthood and the survival time of unfed emerging adults. Both were more likely to be influenced by pollen nutritional quality than quantity. Further studies are needed to assess the ecological significance of the differences we found. In particular, the question remains if increased development time observed on some genotypes would lead to an increased parasitism/predation rate by natural enemies. It may be the case if the longer development time we observed was caused by prolonged larval instars, which are the vulnerable stage to these natural enemies (especially parasitoids). Cook *et al.* (2004a) showed that larvae needed longer to develop on male-sterile compared with male-fertile flowers, and that the increased duration came only from a longer first instar. This is encouraging for performing more detailed measurements on entire plants, even if it comes with logistical constraints.

'Express' was clearly the most detrimental genotype for larvae, by both increasing development time and reducing survival after emergence. As for adults, the biochemical profiling we performed in *Article 4* did not reveal anything that could explain this negative effect. However, we showed a very high correlation between pollen starch content and survival of emerging adults when considering the four genotypes on which development was equally long. Therefore, starch seems to be a good candidate key trait determining pollen nutritional quality for larvae.

Cross-referencing results on development and survival with enzymatic and transcriptomic data from the literature enabled us to propose the hypothesis that pollen beetles detoxify GSLs, but that larvae are more efficient in doing so than adults. This remains to be confirmed but would not be surprising since detoxification is a very common way of dealing with toxic plant secondary metabolites in phytophagous insects (Després *et al.* 2007). Further, it is possible that larvae are able to deactivate/reactivate GSLs using a couple of enzymes (β-glucosidases and UDP-glycosyltransferases) that is typical of a system of defense/reuse of plant toxic chemicals. Although not much studied, occurrence of this system in herbivorous insects is likely to be more frequent than previously thought (Erb pers. comm.). Testing the existence of such mechanism would be of great interest in the ecological context of interactions between pollen beetle larvae and their natural enemies.

A global conclusion emerges from results presented in this section: a small panel of plant genotypes (only six in this thesis, the maximum that we were able to simultaneously study) can be sufficient to identify candidate key traits. At this point, the approach we proposed in *Article 1* is largely supported – except maybe for oviposition cues. Of course, these traits have now to

be validated. We think that the main strength of this approach is to stay as close as possible to what happens in nature, by working on entire plants and in no-choice situations. In *Article 1*, we stated that laboratory tests should preferably be conducted in these conditions. Additional data we obtained, not presented in other chapters of this thesis, show the importance of these two points.

THE IMPORTANCE OF WORKING WITH ENTIRE PLANTS AND USING NO-CHOICE TESTS

Plant parts or entire plants?

Since it is certain that cutting plant parts induces reactions in plant tissues, it may not be the most relevant method to infer what happens in nature on entire plants. In parallel to the experiment conducted on feeding intensity (*Article 3*), we also compared the same six OSR genotypes using only one bud per beetle. One hundred beetles were individually isolated per genotype, in small Petri dishes. Each one was given an individual flower bud for 24 hours. After this delay, buds were simply noted as 'intact' or 'damaged by feeding'. Results are shown in Fig. 2. They are drastically different from what we obtained on entire plants, and prove that high throughput phenotyping protocols on plant parts should be used with caution.

Choice or no-choice tests?

At the beginning of this PhD, a field experiment was set up to screen 17 OSR genotypes for pollen beetle colonization. To avoid as much as possible the known effect of flowering phenology, plants were sown and vernalized in the greenhouse. Plantlets were then individually transplanted in the field (randomized complete 3-block design, 72 plants per genotype). At one sampling date, almost all plants were at the bud stage (a small proportion of plants already had a few flowers). A clear intergenotypic gradient was found (Fig. 3). However, this gradient is again completely different from what we found in no-choice tests in the laboratory. For example, no individual was found on 'Mar' in the field whereas it is the most attractive in olfactometer experiments, when 'Markus' is one of the least attractive in the laboratory but was one of the most colonized in the field. Additionally, we showed that independently of the genotype, plant size was positively related to insect colonization (Fig. 4A). The following weeks, effects of flowering phenology (not shown) and plant height (Fig. 4B) were more and more marked. We did not try to explain all differences we observed, but these results show the difficulty of screening for resistance to insects in field experiments in the choice-test situation. For example, the effect of plant height proves that insect choice is not only related to the intrinsic attractiveness of a plant (which depends partly on plant size, since taller

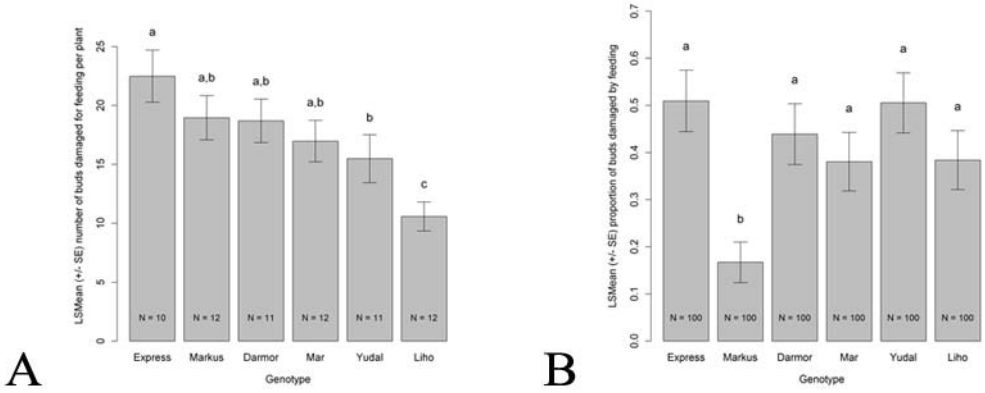

Fig. 2 A: Intergenotypic gradient obtained for feeding intensity on entire plants (*Article 3*). N: number of plants per genotype. B: Gradient obtained on single detached buds (Wald test on a GLMM (family: binomial, link: logit)). N: number of beetles per genotype

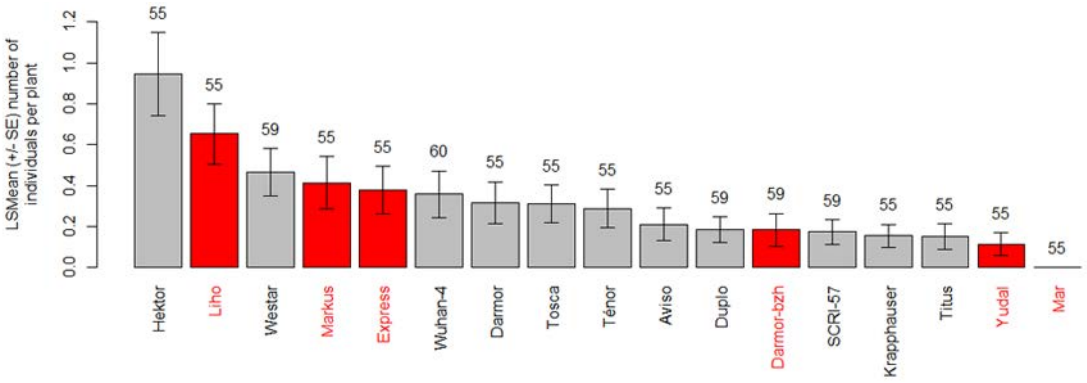

Fig. 3 Intergenotypic gradient found in the field experiment (GLMM (family: Poisson, link: log); genotype effect: $P < 0.001$). Numbers above bars are median BBCH stage (< 60: bud stage, no flower; 60: first flowers open). N = 48 plants per genotype. Genotypes in red are the six used in this thesis

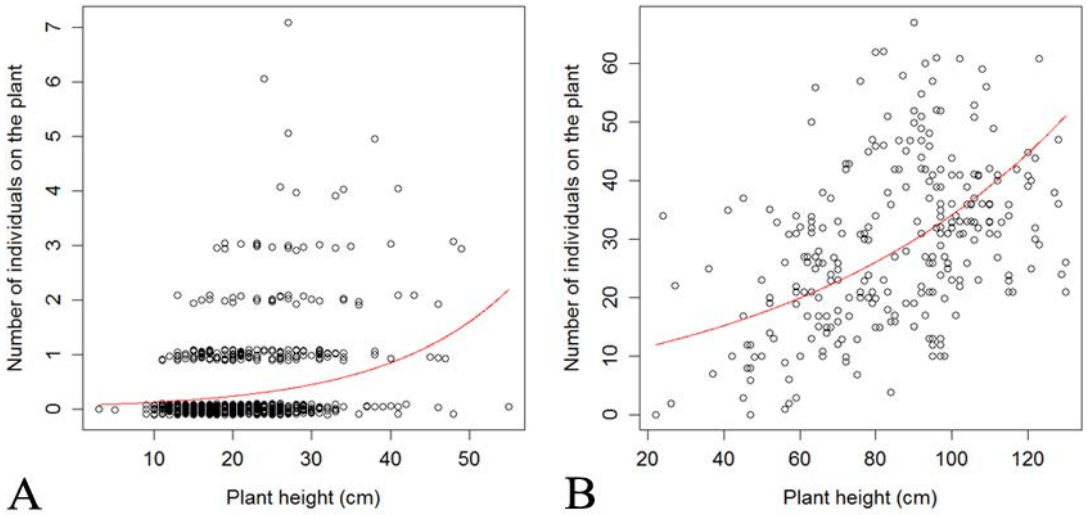

Fig. 4 Relationship between plant height and colonization by *M. aeneus* in the field experiment. A: Plants at the bud stage (same data and model as Fig. 3; height effect: $P < 0.001$). B: Four weeks later, all plants flowering (height effect: $P < 0.001$)

plants are more visible), but also to a comparison with surrounding plants. This may be a dramatic confounding effect when trying to identify sources of resistance.

From our point of view, these two illustrations support the approach that we propose. Identification of plant traits increasing resistance is likely to be much less biased when studied in the laboratory, with entire plants and in no-choice experiments. It requires working with a small panel of plant genotypes and needs further validation steps. However, this approach might be worthwhile in a context where methods to protect crops against insect pests are not sufficiently efficient and new ones are needed.

HYPOTHETICAL EVOLUTIONARY AND ECOLOGICAL BASES OF THE OILSEED RAPE – POLLEN BEETLE INTERACTION

Our results combined with the existing literature allow us to make assumptions about the bases of the interaction between OSR and the pollen beetle, both from

an evolutionary and an ecological point of view. The following sections are only hypothetical and would need further experimental testing.

Ancestral host, host shifting and adaptation to oilseed rape

At the evolutionary scale, OSR is a very recent species which is likely to have resulted from the hybridization of turnip rape (*Brassica rapa*) and cabbage (*B. oleracea*) that were cultivated in the same gardens in the Middle Ages (U 1935; Doré & Varoquaux 2006; Allender & King 2010). The pollen beetle, which is especially known in OSR crops nowadays, necessarily shifted from one or several ancestral host(s). Based on populations of pollen beetles sampled in areas of Finland where OSR is cultivated for different times, Hokkanen (2000) showed that pollen beetle fitness increases linearly and very rapidly with the length of OSR cultivation (Fig. 5A). We showed that egg production is directly influenced by feeding intensity. The enormous amount of feeding resources that represent OSR crops, in comparison with wild plants, is hence likely to have 'mechanically' increased pollen beetle fitness. However, the effect of cultivation time on fitness clearly demonstrates that the insect has adapted to this new resource. Another finding of Hokkanen (2000) supports this hypothesis: tolerance to intraspecific competition (estimated from the number of new-generation individuals produced depending on beetle density at oviposition) decreases with years of OSR cultivation. Additionally, Hokkanen (2000) showed that females lay many more eggs than can be supported by a plant: around 90 % of buds contained more than one egg, whereas only about 40 % of flowers had more than one second-instar larva (Fig. 5B). Nilsson (1988) reported that in the field, between 16-48 % of larvae found on the soil were dead, immature larvae. We also found that although females laid on average between 2.5-3 eggs on the genotypes we studied and in our experimental conditions (*Articles 4* and *5*), only 0.5 emerging individuals were produced per female in *Article 6*. Together, these three results strongly suggest that larval mortality is high in natural conditions, even without taking into account natural enemies. Finally, all elements presented in this section support a scenario that Hokkanen (2000) was the first to propose: during the last

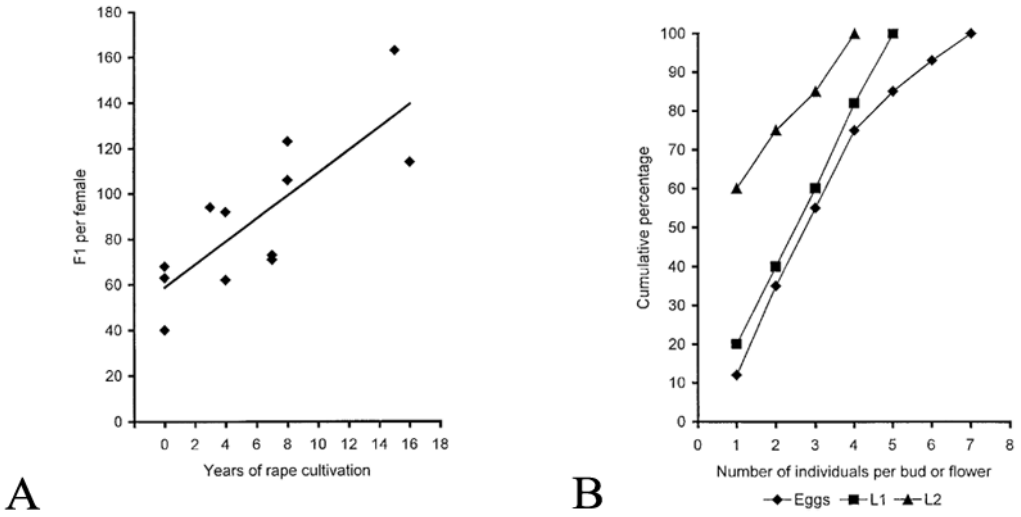

Fig. 5 A: Mean production of new-generation (F1) adult beetles per female in populations of *M. aeneus* from reference areas and areas with different durations of OSR cultivation (two-year field cage experiments with individuals from different populations collected just after their diapause, confronted to the same plant cultivar and at the same density). B: Intraspecific competition at the larval stage: cumulative distribution of the individual buds or flowers containing 1, 2, 3 etc eggs or larvae of different instars. From Hokkanen (2000)

few centuries, the availability of a new host plant (OSR) which represented an unlimited amount of feeding resources and oviposition sites made the pollen beetle switch from wild plants to this plant species; on the *r*/*K* continuum (Pianka 1970), this adaptation moved the cursor toward the pure *r* strategy, *i.e.* producing more offspring but with a lower survival probability for each of them. From this point of view, it seems inevitable that as soon as OSR area increased to bigger and bigger fields, the pollen beetle became a pest.

One question remains open: which species was (or were) the ancestral host plant(s) of the pollen beetle? The very quick adaptation to OSR showed by Hokkanen (2000) suggests that it should be a close relative of *B. napus*. When looking at results of studies having compared different brassicaceous species, it appears that the best-quality hosts are closely genetically related (Fig. 6). It seems easy to suggest *B. rapa* as a good candidate. However, this is probably too easy for at least three reasons. First, very few plant species outside the Brassiceae tribe were studied. The figure may suggest that the more plants are genetically divergent from *B. napus*, the less they are suitable for the pollen beetle. Studies with hosts belonging to the Brassicaceae family (*i.e.* potential hosts) but from tribes other than Brassiceae are necessary to confirm this hypothesis. Secondly, we are missing crucial data on wild types of *B. oleracea* (the second progenitor of OSR) and its close relative (Fig. 7). These species might be better hosts than *B. rapa* or even *B. napus*. Thirdly, all studies conducted in the laboratory used pollen beetles collected in OSR crops, and field studies were conducted in area where OSR is commonly cultivated (pollen beetles colonizing field experiments are therefore likely to have developed on OSR). At the short scale, it is well-known that insects can perform better on the host from which they originated

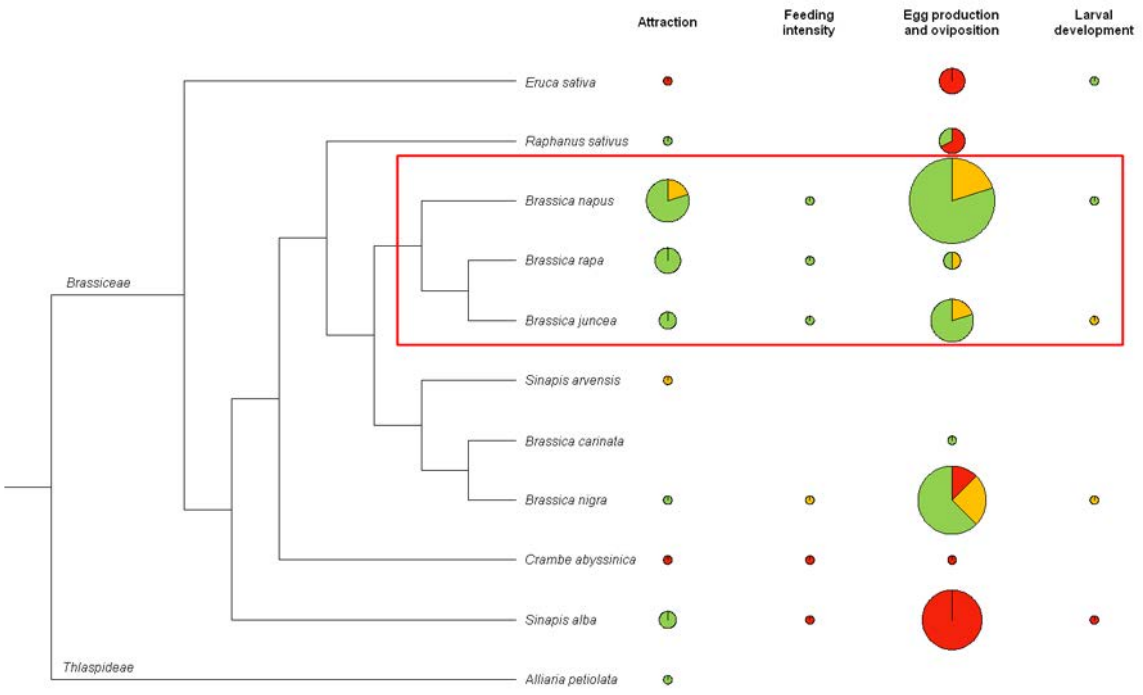

Fig. 6 Summary of results obtained in interspecific comparisons of brassicaceous species for their suitability for the pollen beetle (Free & Williams 1978; Borg & Ekbom 1996; Ekbom & Borg 1996; Hopkins & Ekbom 1996, 1999; Ekbom 1998; Hopkins *et al.* 1998; Cook *et al.* 2007a; Veromann *et al.* 2012; Kovács *et al.* 2013; Kaasik *et al.* 2014a, 2014b). For each paper, studied species were broadly classified as 'good host' (green), 'medium host' (orange) or 'bad host' (red). Size of circles is proportional to the number of studies having tested the corresponding species (N = 1 minimum, 10 maximum). Phylogenetic relationships of studied species are shown on the left of the diagram (Brassibase)

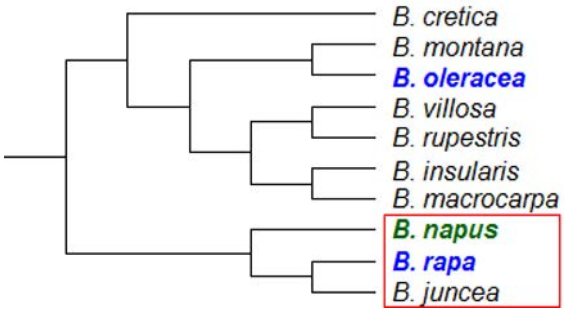

Fig. 7 Phylogenetic relationships of OSR and its closest relative species (Brassibase). OSR progenitors are colored in blue. Species on which the pollen beetle was studied are surrounded by a red rectangle

(Jaenike 1990). At a longer scale, adaptation of the pollen beetle to OSR might have led to a lower adaptation to its ancestral host(s). These two factors possibly bias interspecific comparisons.

The 'generalist – specialist ambiguity'

The pollen beetle is almost always presented as a 'specialist of the Brassicaceae family'. This is true for oviposition, but it does not take into account the feeding aspect of the interaction. As with many lepidopteran species for example, the pollen beetle is specialist as larvae and generalist as adults. However, most Lepidoptera that are commonly studied (*e.g. Danaus plexippus*, *Manduca sexta*, *Ostrinia nubilalis*, *Pieris rapae*, *P. brassicae*, *Plutella xylostella*…) differ from the pollen beetle in one important point: the damaging stage for the plant is the specialist larva, not the generalist adult. Since damage that can affect OSR fitness (thus, yield) is caused only by adult feeding on flower buds, it may be interesting to sometimes consider the pollen beetle more as a generalist than a specialist species, especially in studies aiming to improve crop protection by decreasing feeding intensity.

The 'generalist – specialist ambiguity' is well illustrated by the relationship between the pollen beetle and GSLs. We showed that great variations in GSL content of both perianth (maximum ('Yudal') - minimum ('Liho') ratio: 23.2) and anthers (maximum ('Yudal') - minimum ('Liho') ratio: 9.0) do not affect adult feeding (*Article 3*). This finding is not common. Indeed, a general pattern is often found with GSLs: they are mostly deterrent for generalist insects whereas they are stimulant for many specialists (Hopkins *et al.* 2009). However, this absence of relationship may be understood by considering that the pollen beetle is a 'generalist, feeding most of the time on Brassicaceae'. Since adults feed on the pollen of many plant families, they are likely to face very different pollen biochemical compositions, including different specific secondary compounds (such as GSLs) or different proportions of common secondary compounds (*e.g.* alkaloids or terpenes). The main way by which generalists deal with this diversity of biochemical profiles is detoxification. This strategy has the great advantage to be based on a few enzyme families that are able to use a great variety of compounds as substrates (Després *et al.* 2007). The detoxification ability of pollen beetle adults is likely to be effective against a large diversity of chemicals. Having said that, it is not surprising that pollen beetle resistance to pyrethroids (widely-used insecticides), which is achieved essentially by detoxification (Philippou *et al.* 2010), emerged so rapidly in the 1970's (Lakocy 1977). Moreover, since adults feed on brassicaceous plants throughout their reproductive period (which lasts for about four months, when the total activity period (without diapause) is about six months), they are most of the times confronted to a food containing GSLs. In this context, it would be adaptive that detoxification is particularly efficient against GSLs. This may explain why pollen beetle adults are not deterred by GSLs. On the other hand, being stimulated by specific secondary metabolites does not make any adaptive sense in a generalist strategy. This may explain why pollen beetle adults were not stimulated by increased amounts of GSLs in our study (it cannot be concluded that adults are not stimulated at all by GSLs since we did not test them on GSL-free plants).

Our results suggest that pollen beetle adults are affected in oogenesis and survival by high amounts of

GSLs (represented by the genotype 'Yudal'), whereas larvae are not (*Articles 4* and *6*). This is also logical if thinking of the pollen beetle not only as a specialist, but as a combination between a generalist adult and specialist larva. The generalist – specialist paradigm states that the former should tolerate a greater diversity of plant defense compounds but be affected by high amounts of specific ones, whereas the latter should tolerate a smaller diversity of substances but at higher levels (Ali & Agrawal 2012). This is exactly what emerged from our results: generalist adults seem affected by GSL-rich pollen (both in oogenesis and survival), but larvae were obviously not. Additionally, Cook *et al.* (2004a) showed that larval development is highly negatively affected when provided with the Fabaceae *Vicia faba*, which contains specific furanocoumarins and high amounts of toxic non proteogenic amino acids such as canavanine (Wink 2013). Finally, it is not surprising that larvae over-express β-glucosidases and UDP-glycosyltransferases compared with adults. Since it requires enzymes able to recognize specific compounds as substrates, this system of deactivation/reactivation of plant defenses is known especially in specialist herbivores (*e.g.* the rice armyworm *Mythimna separata* (Sasai *et al.* 2009) or the Western corn rootworm *Diabrotica virgifera virgifera* (Erb & Robert pers. comm.)). If this hypothesis is confirmed by further experiments, the pollen beetle would be, to our knowledge, the first species discovered that is able to deactivate ITCs by glycosylation.

Apparent adaptive nonsense makes sense in an agronomical context

Several results found during this thesis seem counterintuitive at first. We already discussed the apparent absence of a link between GSL variation and adult feeding. Two other findings are intriguing.

First, the fact that adult feeding intensity seems more influenced by perianth than anther biochemical composition (*Article 3*). Since the pollen beetle is a pollen feeder, it could have been hypothesized that its feeding behavior is selected to respond to pollen composition (as it is the case for honey bees for example (Cook *et al.* 2003)). Our results suggest that adults would stop feeding if the perianth is not stimulant enough, even if the real food source (*i.e.* pollen) could be of good quality. This looks like adaptive nonsense. However, it has to be kept in mind that (i) egg production depends mainly on feeding intensity, (ii) OSR fields represent unlimited amounts of feeding resources, (iii) the egg-laying period of females is much longer (around four months) than the period where only buds are available (around two-three weeks) and (iv) pollen beetles stop feeding from buds as soon as blossoming starts. Taken together, these elements suggest that females are not egg-limited. Indeed, the amount of feeding resources is so important that eggs can be continuously produced. Moreover, these eggs can be produced and laid during a long period. Therefore, is it likely that the slight reduction in egg production that perianth composition may cause during a few weeks has only a minor impact on female fitness. The probable high egg and larval mortality that we previously discussed may even lower this impact. Finally, perianth composition is likely to exert a very low selection pressure on pollen beetle adults, maybe even none. The agronomical importance of bud-feeding makes people focus on this particular point of the OSR – pollen beetle interaction, but in fact it may be nearly insignificant in the evolution of the behavior of this beetle.

A second surprising result of this thesis is that attraction (thus, long-distance preference) of pollen beetle adults to plant volatiles does not correlate to any measure of performance (egg production through feeding intensity, survival of field-sampled adults, development time or survival of unfed emerging adults) (*Article 2*). It is possible that we did not use appropriate measures of preference/performance. However, intergenotypic gradients are so different that we think that there really is no correlation. In particular, 'Mar' was the most

attractive genotype whereas it was clearly detrimental for oogenesis and survival, and only intermediate for feeding intensity and development time. The classical preference – performance framework seems not to apply in this biological system. But again, placing the interaction into its agronomical context may explain this apparent adaptive nonsense. The basis of the preference – performance hypothesis is that phytophagous insects should prefer to use host plants that are the most profitable for their fitness. It requires that selection favors individuals that recognize best-quality hosts, by means of cues such as volatiles. Although we are not aware of any study having tested this hypothesis with the pollen beetle and wild plants, there is no reason to think that it does not apply. However, it is entirely possible that it is not the case with OSR. Indeed, no wild form of OSR is known (Gómez-Campo & Prakash 1999). Therefore, all plants encountered by the pollen beetle come from the selective process conducted by breeders and farmers. Since this process is focused only on associating plant traits that increase yield (most of the time specifically oil yield), it would not be surprising that at the same time, it has broken associations of other traits that correlate in wild relative species. There are known examples of breeding processes that made plants unable to express ecologically fundamental functions, such as response to herbivore attacks (Sotelo 1997). Hence, it is possible that volatiles meaning 'good-quality host' in wild hosts of the pollen beetle do not correlate at all with OSR quality for the beetle. Moreover, pollen beetles can adapt to the combination of traits present in OSR only if this combination is stable over time. Since cultivars are regularly replaced by new ones that are more efficient (in yield, resistance to a particular abiotic stress or a disease etc), it is possible that these new cultivars come with different combinations of traits that are not directly involved in yield. Insect adaptation seems hardly achievable in this context.

All elements we presented and discussed in this section lead to a global conclusion: the interaction between OSR and the pollen beetle cannot be seen in the same light as other plant – insect interactions that occur in wild ecosystems. The agronomical context in which this interaction takes place may have disturbed basic ecological processes such as host plant selection, and relaxed important evolutionary constraints by providing unlimited amounts of food and oviposition sites. It would be interesting to test if the same pattern is found with other pests of cultivated plant species. Moreover, the gap is probably huge between the agronomical impact of the bud-feeding behavior of the pollen beetle and its evolutionary significance for the insect. These conclusions might be a chance for crop protection.

PERSPECTIVES FOR CROP PROTECTION

Genetically determined intraspecific variability is the required basis to start a selective process. An interesting finding of this thesis is that we found intergenotypic differences for almost all traits we studied. Since we used plants that were grown in strictly controlled conditions, the contrasts we observed were likely to be mostly genetically determined. An element supports this hypothesis: throughout all experiments we conducted, the intragenotypic variation was very often much reduced compared with the intergenotypic variation, even when sometimes using low numbers of individual plants. It is highly improbable that we observed the greatest differences that exist in all OSR genetic diversity. Thus there is promise into going more depth for screening for resistance to the pollen beetle.

A second interesting point is that the intergenotypic gradients we observed were often different (Fig. 8). All six genotypes were extreme in at least one gradient. Some genotypes ('Express' and 'Mar') were even at opposite extremes depending on the trait studied. This result may show that plant characteristics that caused

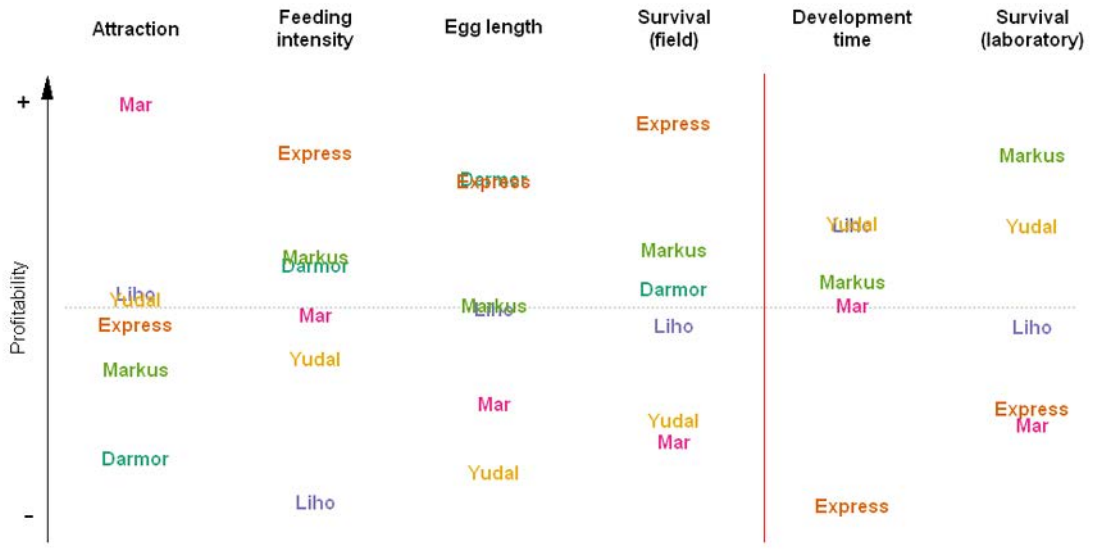

Fig. 8 Intergenotypic gradients of profitability found for each trait studied ('Survival (field)': pollen beetles sampled in the field and fed on the six OSR genotypes; 'Survival (laboratory)': unfed pollen beetles emerging from these genotypes). The red line separates traits related to adults (left) and larvae (right). Raw data were normalized (*i.e.* centered and unit-variance scaled) to be shown on the same scale. The grey line represents the mean of all gradients. A longer development time showing a lower profitability of the plant, the development time gradient was reversed

these gradients are at least partly independent. In an applied perspective and if true, this may facilitate pyramiding several sources of resistance into the same cultivars.

Based on the results of this thesis, the most promising way to decrease damage caused by the pollen beetle is certainly to manipulate bud biochemical content to lower feeding stimulation. With only six plant genotypes, the contrast we showed is already great: two times more buds were damaged on 'Express' compared to 'Liho' (*Article 3*). From an agronomical point of view, this difference is promising. It is even more interesting when bearing in mind that pollen beetle adults stop feeding on buds as soon as blossoming starts and are not considered as a pest afterwards. Indeed, insecticide application is recommended to farmers only at the bud stage. It is not necessary when plants are flowering. The vulnerability window of the crop is therefore very short, up to three weeks maximum. If feeding stimulation is reduced during this short period of time, the agronomical benefit may be important whereas the impact on the pollen beetle (through egg production) may be nearly negligible. Further experiments are needed to explore the feasibility of such manipulation. If verified, this strategy might be both efficient and sustainable.

Manipulating plant attraction may also be valuable. Our olfactometer experiment is not sufficient alone to predict what would happen in the field, but confirms that attractiveness can vary with plant genotype. If it is validated by further experiments and if determinants of this attraction are identified, this trait may be manipulated in two ways. First, by delaying colonization of the field when plants are at the bud stage. The less plants are attractive, the more pollen beetles would take time to colonize them and the more damage would be reduced during the susceptible stage of the crop. However, it is not certain that a slightly lower attractiveness would really prevent crop colonization. Decreasing attractiveness could be more effective in a second way: by enhancing the efficiency of push-pull strategies (Cook *et al.* 2007b). In this situation, where insects have to make a choice between plants to be protected and trap plants, the aim is to create the greatest attractiveness contrast that is possible between these two

types of plants. Using plants that flower earlier than those to be protected is the basis of the existing method (Cook *et al.* 2004b, 2006, 2007a; Nerad *et al.* 2004; Nilsson 2004; Frearson *et al.* 2005). It has been proposed to enhance the attractiveness contrast by diffusing repellent odors into the crop. Lavender essential oil seemed effective in doing so (Mauchline *et al.* 2005, 2013). Selecting crop plants to be less attractive, while selecting trap plants to be more attractive, may add to the effect of flowering phenology.

A third option might be to slow down larval development. This is much more hypothetical since it has first to be verified if one or two days more development really leads to greater biological control by the pollen beetle's natural enemies. Among these, ichneumonid parasitoids are the most specific. Parasitism rate of pollen beetle larvae was reported to regularly exceed 50 % in the field (Ulber *et al.* 2010). However, although biological control is one of the bases of IPM, it is almost certain that breeders would not deeply invest into this indirect way.

Whatever the option chosen, an interesting next step could be to screen OSR genetic diversity for the candidate key traits we identified. Genotypes showing greater contrasts than our panel of six could then be used to validate these traits. If validated, key traits may be the basis of a selective process or of genetic studies aiming to identify QTL by which they are controlled. It also has to be tested if manipulating these plant traits does not increase susceptibility to other pests, or reduce benefits provided by pest natural enemies or pollinators.

CONCLUSION

Increasing resistance of cultivated plants to insect pests may be a valuable strategy to protect crops, together with other tactics of IPM. One possibility consists of introgressing resistance factors from relative species. For example, canola (*B. napus*) lines resistant to the cabbage seedpod weevil (*Ceutorhynchus obstrictus*) were obtained by crossing this plant with the resistant white mustard, *Sinapis alba* (*e.g.* Dosdall & Kott 2006; Ulmer & Dosdall 2006; Tansey *et al.* 2010a, 2010b). Another possibility is to promote resistant factors that are already present in the plant species to be protected. In this thesis we proposed an approach to identify key plants traits that can favor resistance of cultivated plants to insect pests. Using the OSR – pollen beetle system, we proved that the first half of the way, *i.e.* identifying candidate key traits, is feasible. Indeed, starting from only six plant genotypes but *via* comprehensive biochemical profiling, we identified a few compounds that may have a key role. This is only a first step, since the second half of the way is to validate candidate key traits. We do not claim that the interaction is dependent on only a few compounds. Plant – insect interactions are complex, in wild ecosystems but also in agrosystems. However, we believe that from an agronomical point of view, manipulating a few key plant traits may bring substantial benefits.

Surprisingly, most of the traits we identified were primary, not secondary metabolites (Table 1). This result does not fit the classical assumption that plant – insect interactions are driven essentially by secondary substances (especially specific ones). However, examples showing no link between specific secondary compounds and herbivore preference/performance are already known (*e.g.* Barrett & Agrawal 2004; Henery *et al.* 2008; Poelman *et al.* 2008). Results obtained throughout this thesis highlight the need for better consideration of primary metabolites in studies on plant – insect interactions, in accordance with the 20 year-old, yet often neglected, claim of Berenbaum (1995).

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– FRENCH SUMMARY –

INTRODUCTION

La course aux armements entre plantes et insectes phytophages

L'histoire évolutive que partagent les plantes et les insectes phytophages dure depuis environ 415 millions d'années. Au cours de ce processus coévolutif, les plantes ont développé de multiples systèmes de défense contre ces ennemis tandis que ceux-ci ont répondu par d'efficaces contre-adaptations.

La résistance des plantes aux insectes est le plus souvent complexe, impliquant de nombreux traits. Parmi ceux-ci, les métabolites secondaires – qui peuvent être répulsifs, toxiques ou bloquer la digestion – sont vus comme déterminants. Beaucoup d'entre eux sont spécifiques d'une ou de quelques familles végétales. Ces composés spécifiques sont considérés comme étant à l'origine du degré élevé de spécialisation (pour les ressources trophiques ou les sites de ponte) dont font preuve la majorité des insectes – seuls 10 % de ceux-ci sont de vrais généralistes. Une théorie majeure de l'écologie chimique dit que les insectes généralistes toléreraient une plus grande diversité de métabolites secondaires mais à des concentrations peu importantes, tandis que les spécialistes tolèreraient une plus faible diversité de composés (ceux produits par la famille végétale sur laquelle ils sont spécialisés) mais à des concentrations élevées. Ces composés spécifiques sont même parfois attractifs ou stimulants (pour l'alimentation ou la ponte) vis-à-vis des spécialistes.

Interpréter les interactions plante – insecte par le seul prisme des métabolites secondaires est probablement trop réducteur. Ces interactions sont largement influencées par la qualité nutritionnelle de la plante pour le phytophage, qui elle-même dépend en grande partie de composés primaires. Plus généralement, les caractéristiques physiques de la plante (couleur, taille, architecture...) tout comme sa phénologie (de floraison par exemple) peuvent également jouer un grand rôle. Enfin, la résistance n'est pas le seul moyen de défense des plantes face aux herbivores. Une autre stratégie, bien moins étudiée, est la tolérance, *i.e.* la capacité à compenser (voire surcompenser) les dommages subis.

Les insectes ne sont pas en reste dans cette course aux armements. Plusieurs mécanismes aujourd'hui bien connus leur permettent d'éviter, de tolérer ou même de réutiliser à leur profit les composés de défense produits par les plantes. Ils peuvent par exemple éviter les plantes (ou les organes végétaux) les plus riches en composés toxiques, excréter ces composés très rapidement ou les détoxifier à l'aide d'enzymes spécialisées. Plusieurs mécanismes peuvent d'ailleurs s'exprimer simultanément.

Protéger les cultures contre les insectes ravageurs

Les insectes ravageurs sont considérés comme étant responsables d'environ 10 à 15 % des pertes de rendement au niveau mondial. Les méthodes de lutte classiques (insecticides et plantes Bt-transformées), que l'on pourrait appeler « qualitatives », sont efficaces à court terme mais sont assez rapidement contournées par les insectes. Une alternative à ces méthodes est l'emploi de stratégies « quantitatives », qui cherchent avant tout à diminuer l'impact des ravageurs sur les cultures et non à les éradiquer. La protection intégrée des cultures, et tous les leviers sur lesquels elle s'appuie, s'inscrit pleinement dans ce cadre. Parmi les méthodes envisageables, l'une pourrait être efficace mais est très peu développée : améliorer, au moyen de la sélection, la résistance naturelle des plantes aux insectes ravageurs.

– Article 1 –

Protecting crops against insect pests by selecting for increased plant resistance: major brakes and alternative approach

In prep. for *Pest Management Science*

Dans cet article, nous détaillons les freins à l'utilisation de cette méthode et proposons une démarche alternative à celle classiquement employée.

La sélection est basée sur de multiples étapes de phénotypage des plantes, avec des effectifs les plus

grands possibles. L'appliquer à la résistance aux insectes pose de sérieux problèmes, tous liés à ce phénotypage : de très nombreux insectes sont nécessaires mais sont parfois impossibles à produire en masse en élevage ; les protocoles et prises de données sont souvent lourds ; le screening au champ peut être biaisé par la direction du vent dominant, la taille des unités expérimentales ou des différences morphologiques/phénologiques entre accessions. De plus, le fait même de phénotyper simultanément plusieurs accessions en laissant les insectes choisir celles qu'ils préfèrent n'est pas forcément ce qu'il y a de plus pertinent dans une optique d'amélioration de la résistance. En effet, en situation naturelle les cultures sont très majoritairement composées de variétés lignées. Les plantes sont ainsi toutes relativement homogènes et l'insecte est plus dans une situation de non-choix que de choix. Le phénotypage serait plus proche de la réalité du champ s'il était réalisé en conditions de non-choix. Enfin, la comparaison de multiples accessions au laboratoire est généralement conduite en utilisant des protocoles simplifiés basés sur des organes végétaux isolés (disques foliaires, fleurs…). Toute blessure induisant une réponse métabolique de la plante, il est probablement plus pertinent de travailler sur plantes entières plutôt que sur organes isolés lorsque l'objectif est d'être le plus proche possible de l'interaction en milieu naturel.

Nous proposons une approche qui permettrait de lever les verrous les plus importants des méthodes de sélection classiques appliquées à la résistance aux insectes. Celle-ci consiste à identifier une série de traits-clés de la plante qui modulent son interaction avec le phytophage. Plus précisément, l'objectif est de comprendre ce qui détermine à quel point l'insecte (i) est attiré par la plante, (ii) s'en nourrit, (iii) produit et pond des œufs dessus, et (iv) s'y développe. Si de tels traits-clés sont identifiés, *i.e.* s'ils permettent de prédire le niveau de résistance au ravageur de façon fiable, une approche de sélection peut ensuite être envisagée sur leur seule base (sans nécessiter d'insecte). L'identification de tels traits est conduite au laboratoire, en comparant (sur plantes entières et en situation de non-choix) la préférence/performance de l'insecte sur un panel réduit de génotypes de la plante à protéger, et en corrélant cette préférence/performance avec un ensemble de paramètres physico-chimiques mesurés sans *a priori* sur ces mêmes génotypes. De ces corrélations devraient émerger des traits-clés candidats, qui devront être validés dans un second temps.

Cette approche résolument quantitative pourrait être efficace contre de nombreux insectes ravageurs, en particulier ceux causant des dommages à un stade temporairement sensible de la culture (les très jeunes plantules par exemple). Elle pourrait permettre de réduire les attaques à ce stade particulier, notamment en les décalant à un stade ultérieur où la plante est moins sensible.

OBJECTIFS DE LA THÈSE ET SYSTÈME D'ÉTUDE

L'objectif de cette thèse est d'identifier des traits-clés candidats modulant l'interaction entre le colza (*Brassica napus*, Brassicaceae) et le méligèthe du colza (*Meligethes aeneus*, Coleoptera : Nitiduliae). Le colza est l'une des cultures oléagineuses majeures dans le monde. Le méligèthe en est l'un des principaux insectes ravageurs.

Les dégâts agronomiques (potentiellement très importants) causés par le méligèthe sont dus aux adultes, des pollinivores généralistes qui colonisent les champs après leur diapause hivernale, au moment où les plantes sont au stade boutons floraux (*i.e.* aucune fleur n'est encore ouverte). Ces adultes détruisent les boutons floraux pour se nourrir du pollen qu'ils contiennent. La ponte a lieu dans des boutons floraux des mêmes plantes, mais n'endommage pas ceux-ci. Du point de vue de l'oviposition, les méligèthes sont spécialistes des brassicacées. Les larves se développent en partie dans le bouton floral, puis sur des fleurs ouvertes. Comme les

adultes, elles se nourrissent essentiellement de pollen. La métamorphose a lieu dans le sol, et les adultes de nouvelle génération apparaissent au début de l'été. Après quelques semaines, ils rejoignent leur site d'hivernation d'où ils ressortiront l'année suivante.

La lutte contre le méligèthe est essentiellement basée sur des insecticides, mais le niveau de résistance du ravageur augmente rapidement depuis la fin des années 1970. De nouvelles méthodes sont développées depuis une quinzaine d'années, dans le cadre de la protection intégrée. Cette thèse a pour but de jeter les bases d'une nouvelle approche permettant d'utiliser une méthode originale vis-à-vis des insectes : la sélection pour une meilleure résistance naturelle du colza.

Suivant la démarche présentée dans l'Article 1, quatre étapes majeures de l'interaction colza – méligèthe sont étudiées : l'attraction à distance (Chapitre 1), l'alimentation des adultes (Chapitre 2), la production et la ponte des œufs (Chapitre 3) et le développement larvaire (Chapitre 4). Le panel est composé de six génotypes : 'Darmor', 'Express', 'Liho', 'Mar', 'Markus' et 'Yudal'.

CHAPITRE 1 : ATTRACTION

– Article 2 –

*Attractiveness of oilseed rape (*Brassica napus*) for the pollen beetle (*Meligethes aeneus*) varies with plant genotype but preference does not match performance*

In prep. for *Behavioral Ecology*

Dans cet article, nous comparons l'attractivité des six génotypes pour le méligèthe en olfactométrie. Le dispositif expérimental est un olfactomètre tubulaire dans lequel les individus sont observés seuls, pendant 10 minutes. La source d'odeur est une plante entière, dont la tige est coupée sous la troisième inflorescence juste avant l'expérimentation afin de mimer les conditions naturelles où aucune plante n'est probablement saine.

Un gradient d'attractivité a été montré, depuis les génotypes 'Darmor' et 'Markus' (les moins attractifs) jusqu'au génotype 'Mar' (le plus attractif). La caractérisation des bouquets d'odeurs émis par ces génotypes est en cours.

Nous discutons des origines possibles de cette variation d'attractivité. En parallèle, la mise en relation du gradient d'attractivité (assimilable à un gradient de préférence) avec les différentes mesures de performance réalisées dans les autres articles ne montre aucune corrélation. Nous proposons que ce non-sens sur le plan adaptatif puisse être expliqué par le contexte agronomique dans lequel l'interaction a lieu. Il est en effet possible que le processus de sélection qui est continuellement appliqué sur le colza (pour augmenter son rendement) ait rompu des associations avec d'autres traits qui sont présentes dans les espèces sauvages. Un message olfactif interprété comme de bonne qualité par le méligèthe pourrait de ce fait n'avoir plus aucun rapport avec la qualité réelle de la plante (en termes nutritifs par exemple).

CHAPITRE 2 : ALIMENTATION DES ADULTES

– Article 3 –

Manipulating feeding stimulation to protect crops against insect pests?

Submitted to *Journal of Chemical Ecology*

Dans cet article, nous comparons le nombre de boutons floraux attaqués pour alimentation par quatre méligèthes adultes, placés sur l'inflorescence d'une plante entière pendant quatre jours. En parallèle, nous quantifions par chromatographie cinq classes de composés primaires et secondaires (sucres, acides aminés libres, glucosinolates, flavonols et acides hydroxycinnamiques) pouvant avoir un effet phagostimulant ou dissuasif sur l'insecte, séparément dans les anthères (qui contiennent la source de nourriture, *i.e.* le pollen) et le périanthe (le tissu que les méligèthes doivent traverser, et ingérer, pour atteindre les anthères).

Un gradient d'attaque important a été montré, depuis le génotype 'Liho' (le moins attaqué) jusqu'au génotype 'Express' (le plus attaqué). Des différences de profil biochimique, essentiellement quantitatives, ont également été mises en évidence dans les deux tissus étudiés. Aucune corrélation n'est observée entre le gradient d'attaque et la biochimie des anthères. À l'inverse, une forte corrélation est observée avec la biochimie du périanthe. Parmi la quarantaine de composés quantifiés, seuls cinq expliquent cette corrélation (saccharose, sérine, proline et deux flavonols). Les glucosinolates, métabolites secondaires typiques des brassicacées, ne semblent pas avoir d'influence contrairement à ce qui est généralement observé.

Nous discutons les causes pouvant expliquer que seul le périanthe semble déterminant dans la stimulation de l'alimentation des méligèthes adultes. D'après la bibliographie, trois des cinq composés identifiés apparaissent comme de bons traits-clés candidats, en particulier le saccharose dont l'effet sur les insectes est systématiquement phagostimulant.

CHAPITRE 3 : PRODUCTION ET PONTE DES ŒUFS

– Article 4 –

*How oilseed rape (*Brassica napus*) genotype influences pollen beetle (*Meligethes aeneus*) oviposition*

Published in *Arthropod-Plant Interactions*

Dans cet article, nous cherchons à savoir si le génotype de colza peut influencer le nombre et la qualité (estimée par la longueur) des œufs pondus par le méligèthe, et à expliquer les causes d'un possible effet. Trois hypothèses sont testées : (i) l'oviposition (dans sons sens large, *i.e.* à la fois l'ovogenèse et la ponte en elle-même) dépend de la quantité de nourriture ingérée. Cette hypothèse est testée grâce à l'expérimentation présentée dans l'*Article 3*, dans laquelle les œufs pondus pendant les quatre jours ont également été dénombrés et mesurés. (ii) L'oviposition dépend de la qualité nutritionnelle de la nourriture ingérée. Cette qualité est estimée par profilage métabolique du pollen, dans lequel une vingtaine de composés pouvant avoir un impact positif ou négatif sur l'insecte sont quantifiés (acides aminés essentiels totaux, amidon, SMCO, glucosinolates, flavonols, acides hydroxycinnamiques [les données de ces trois dernières classes venant de l'*Article 3*]), ainsi qu'une variable de synthèse, le rapport C:N. (iii) L'oviposition dépend d'une préférence comportementale des femelles pour certains génotypes. Cette hypothèse est testée en confrontant des femelles prêtes à pondre avec les six génotypes étudiés, mais sans qu'elles les aient jamais rencontrés auparavant.

Une variation a été montrée pour le nombre d'œufs pondus. Le seul facteur explicatif est la quantité de nourriture ingérée, estimée par le nombre de boutons attaqués pour alimentation sur la même plante. La longueur des œufs varie également, mais est influencée à la fois par la quantité de nourriture ingérée et le génotype. Cet effet génotypique s'explique uniquement par une différence de qualité nutritionnelle, et non par une préférence comportementale des femelles. Plusieurs traits-clés candidats pour la qualité nutritionnelle sont proposés : acides aminés essentiels totaux, amidon (effets positifs) et glucosinolates (effet négatif).

Nous discutons le fait que la quantité de nourriture ingérée soit le facteur déterminant de l'oviposition du méligèthe, et plaidons pour que ce facteur souvent négligé soit davantage pris en compte dans les études sur la ponte des insectes phytophages. Il pourrait en effet contraindre l'ovogenèse d'une manière importante, et même possiblement biaiser certains résultats lorsque l'espèce étudiée s'alimente sur la même plante où elle pond ses œufs. Nous discutons également l'absence de préférence des femelles malgré de grandes variations de concentration en glucosinolates dans les boutons floraux, ceux-ci étant généralement stimulants pour les insectes spécialistes des brassicacées.

– Article 5 –

*Oviposition behavior of the pollen beetle (*Meligethes aeneus*): a functional study*

Submitted to *Journal of Insect Behavior*

Dans cet article, nous décrivons en détail le comportement de ponte des femelles méligèthes, et le comparons sur cinq génotypes de colza. En parallèle, nous décrivons pour la première fois les sensilles présentes sur l'ovipositeur, par microscopie électronique à balayage.

Par rapport à ce qui était déjà connu, un niveau de précision supplémentaire a été atteint dans la description de la séquence comportementale de ponte. Une caractérisation fonctionnelle a été réalisée, mettant en évidence la division de la séquence en trois phases relativement indépendantes : une inspection externe de la surface du bouton floral, une inspection interne et la ponte en elle-même. Le rôle de l'ovipositeur a été montré dans les deux phases d'inspection. La caractérisation morphologique des sensilles qu'il porte a montré deux types de récepteurs (trichoïdes et basiconiques). Tous deux ne présentent aucun pore, indiquant une unique fonction mécanoréceptrice. Malgré un contraste entre génotypes pour la durée de certains comportements, aucune différence n'a été observée ni dans le taux d'acceptation de la plante, ni dans le nombre d'œufs pondus.

Nous discutons les fonctions possiblement exercées par les deux phases d'inspection, ainsi que le type d'information recherché par les femelles (essentiellement à l'aide de leurs antennes et de leur ovipositeur).

CHAPITRE 4 : DÉVELOPPEMENT LARVAIRE

– Article 6 –

*Plant genotype affects nutritional quality of oilseed rape (*Brassica napus*) for adults and larvae of the pollen beetle (*Meligethes aeneus*)*

In prep. for *Journal of Insect Physiology*

Dans cet article, nous cherchons à savoir si la qualité nutritionnelle du colza pour les larves de méligèthes peut varier avec le génotype de la plante, et expliquer pourquoi. Pour cela, la durée du développement larvaire est comparée entre génotypes, ainsi que la quantité de réserves énergétiques accumulées par ces larves pendant leur développement (estimée par la survie des adultes, non nourris, après émergence). En parallèle, nous menons une expérimentation comparative de survie sur des adultes prélevés au champ, afin de confirmer qu'ils sont impactés par la qualité nutritionnelle du pollen, une hypothèse émise dans l'*Article 4*.

Il a été montré que les larves sont différemment impactées par le génotype de la plante. Le développement est plus long sur le génotype 'Express' comparé aux autres, et un gradient de survie a été obtenu, depuis les génotypes 'Mar' et 'Express' (les moins favorables) jusqu'au génotype 'Markus' (le plus favorable). Un gradient a également été montré pour la survie des adultes prélevés au champ, depuis les génotypes 'Mar' et 'Yudal' (les moins favorables) jusqu'au génotype 'Express' (le plus favorable).

Nous discutons de l'origine possible des contrastes observés et de leur impact écologique. L'hypothèse d'une différence de qualité nutritionnelle est validée, tant pour les larves que pour les adultes (les deux mêmes génotypes sont les moins favorables pour l'ovogenèse et la survie des adultes prélevés au champ). Par rapport aux traits-clés candidats suggérés dans l'Article 4, certains apparaissent comme particulièrement intéressants : l'amidon (effet positif sur les larves et vraisemblablement sur les adultes) et les glucosinolates (effet négatif uniquement sur les adultes). Sur la base de

nos résultats et de données de la bibliographie, nous proposons l'hypothèse que les méligèthes détoxifient les glucosinolates, mais que les larves sont plus efficaces que les adultes dans cette tâche. Cette hypothèse est soutenue par le fait que les adultes sont généralistes pour leur alimentation, tandis que les larves sont spécialistes.

DISCUSSION GÉNÉRALE

Cette thèse visait à tester l'approche que nous proposons pour améliorer la résistance naturelle des plantes cultivées aux insectes ravageurs. En bref : revenir au laboratoire et étudier en détail l'effet d'un petit nombre de génotypes sur les phases majeures de l'interaction, afin d'identifier des traits-clés de la plante sur la base desquels la sélection pourrait ensuite être conduite.

Nous avons obtenu des contrastes entre génotypes pour quasiment tous les traits que nous avons étudiés (attraction, alimentation, survie, développement...). À l'aide de profilages métaboliques relativement larges, nous avons identifié quelques composés qui pourraient jouer un rôle clé dans l'interaction colza – méligèthe : les concentrations en saccharose, sérine et proline libres dans le périanthe des boutons floraux ; les concentrations en amidon et en glucosinolates dans le pollen. Il ressort que la plupart de ces composés sont primaires, ce qui plaide pour une meilleure prise en compte de ces métabolites dans l'étude des interactions plante – insecte (qui sont généralement vues par le seul prisme des métabolites secondaires).

D'un point de vue fondamental, l'ensemble des données obtenues combiné à la bibliographie existante permet de construire un cadre théorique global dans lequel l'interaction a lieu. Nous discutons en particulier de comment le méligèthe s'est adapté au colza, une plante très récente sur le plan évolutif mais présente en très grande quantité là où elle est cultivée ; de la relation entre le méligèthe et les glucosinolates (des métabolites secondaires à l'influence souvent majeure dans les interactions entre brassicacées et insectes phytophages) et du caractère potentiellement adaptatif des résultats que nous avons obtenus ; du fait que le périanthe, et non le pollen, semble jouer un rôle déterminant dans la stimulation de l'alimentation des adultes, alors que ceux-ci sont pollinivores. Nous proposons que le contexte agronomique dans lequel l'interaction a lieu aujourd'hui ait largement modifié la relation historique qui lie le méligèthe et les brassicacées sauvages sur le plan évolutif.

D'un point de vue appliqué, le fait que nous ayons presque toujours obtenu des contrastes entre génotypes montre qu'une base de variabilité est probablement disponible pour la sélection. De plus, les gradients intergénotypiques sont souvent assez différents, suggérant que les traits déterminants de la plante pourraient être indépendamment contrôlés. Ces deux constats sont prometteurs pour approfondir la démarche que nous proposons. Plus précisément, manipuler la composition chimique du périanthe pour diminuer la stimulation de l'alimentation par les méligèthes adultes pourrait s'avérer être une stratégie efficace et durable. Manipuler l'attractivité des plantes, sous réserve d'avoir préalablement identifié les composés volatils (ou ratios de composés) déterminants, pourrait également être une piste intéressante, mais probablement davantage dans un contexte de « push and pull ».

En conclusion, cette thèse a montré qu'une nouvelle voie était peut-être envisageable pour contribuer à protéger les cultures de façon durable contre les insectes ravageurs, en particulier pour les systèmes agronomiques où le phénotypage classique est irréalisable et où les dégâts sont causés à un stade temporairement sensible de la culture. Nous n'avons cependant réalisé que la moitié du chemin : identifier quelques traits-clés candidats. Il reste maintenant à mener l'étape déterminante de validation de ces traits. Et bien sûr, à vérifier qu'ils ne favorisent pas d'autres ravageurs ou au contraire n'ont pas un impact négatif sur les organismes bénéfiques tels les pollinisateurs.

– SCIENTIFIC COMMUNICATIONS –

Books (general audience)

Hervé M, Poinsot D (2013) L'évolution des espèces – 1. Les preuves. Apogée, Rennes, 64p [*in French*]

Hervé M, Poinsot D (2013) L'évolution des espèces – 2. Les mécanismes. Apogée, Rennes, 64p [*in French*]

Scientific articles (international peer-reviewed journals)

Submitted

Hervé MR, Delourme R, Gravot A, Marnet N, Berardocco S, Cortesero AM. Manipulating feeding stimulation to protect crops against insect pests? (under revision for *Journal of Chemical Ecology*)

Hervé MR, Garcia N, Trabalon M, Le Ralec A, Delourme R, Cortesero AM. Oviposition behaviour of the pollen beetle (*Meligethes aeneus*): a functional study (under revision for *Journal of Insect Behavior*)

Published

Hervé MR, Delourme R, Leclair M, Marnet N, Cortesero AM (2014) How oilseed rape (*Brassica napus*) genotype influences pollen beetle (*Meligethes aeneus*) oviposition. Arthr Plant Interact, DOI: 10.1007/s11829-014-9321-4

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Hervé MR, Marnet N, Leclair M, Delourme R, Cortesero AM (2013) Differential rates of attack of oilseed rape genotypes by the pollen beetle: the cues may be in the bud wall. *15th meeting of the IOBC-WPRS working group "Integrated Control in Oilseed Crops"*, Belvaux, Luxembourg

Hervé MR, Marnet N, Leclair M, Delourme R, Cortesero AM (2013) What leads the pollen beetle to attack its host plant? *1st meeting of the National Association of Young Researchers in Chemical Ecology, Montpellier*, France. [*In French*]

Andrade T, Hervé MR, Outreman Y, Krespi L, van Baaren J (2012) Winter host selection influences fitness traits in an aphid parasitoid. *XXIVth International Congress of Entomology*, Daegu, Korea

Thieltges H, Du Bosq S, Hervé MR, Biquand V, Henry L, Deleporte P (2012) Temporal evolution in short songs of yellow-rumped and red-rumped caciques (*Cacicus cela* and *Cacicus haemorrhous*). *VIth European Conference on Behavioural Biology*, Essen, Germany

Posters

Hervé MR, Outreman Y, Krespi L, Van Baaren J (2011) The optimality of compromise: parasitic strategy in limiting host resource conditions. *7th meeting "Ecology and Behaviour"*, Rennes, France

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RÉSUMÉ

Au cours du processus coévolutif qui lie les plantes et les insectes phytophages depuis plus de 400 millions d'années, les plantes ont développé de multiples systèmes de défense contre ces ennemis. Dans un contexte agronomique, manipuler ces systèmes au moyen de la sélection pourrait contribuer à réduire les dommages causés par les insectes ravageurs, en augmentant la résistance naturelle des plantes. Cette stratégie se heurte cependant à des contraintes très fortes – à la fois logistiques et conceptuelles – lorsqu'il s'agit de l'appliquer aux insectes.

Après avoir détaillé ces contraintes, qui relèvent du processus de phénotypage nécessaire à toute sélection, nous proposons une démarche alternative aux méthodes classiques. Celle-ci vise à identifier des traits-clés de la plante qui modulent son interaction avec le ravageur. Si de tels traits sont identifiés et validés expérimentalement, ils permettront ensuite de conduire la sélection sans nécessiter d'insecte.

Nous avons testé cette démarche dans un système composé du colza (*Brassica napus*), l'une des principales cultures oléagineuses au niveau mondial, et du méligèthe du colza (*Meligethes aeneus*), un ravageur majeur de cette plante. Le méligèthe est un coléoptère pollinivore univoltin dont les adultes sont généralistes, mais qui ne pondent que sur certaines plantes de la famille des brassicacées (les larves sont donc spécialistes). Les dégâts agronomiques sont causés par les adultes qui, avant que la floraison ne démarre, détruisent les boutons floraux pour atteindre le pollen qu'ils contiennent. Les femelles pondent leurs œufs également dans des boutons floraux.

Quatre étapes cruciales de l'interaction ont été étudiées : l'attraction à distance, l'alimentation des adultes, la production et la ponte des œufs, et le développement larvaire. Pour ce faire, six génotypes de colza ont été comparés dans une série d'expérimentation au laboratoire. La mise en relation des résultats de préférence/performance de l'insecte avec des profilages métaboliques larges de tissus floraux cibles a permis d'identifier des traits-clés candidats. Les conclusions principales de ce travail sont (i) que la composition biochimique du périanthe est déterminante dans la stimulation de l'alimentation des adultes, et que cette stimulation pourrait être largement sous l'influence d'un petit nombre de composés dont le saccharose ; (ii) que cette stimulation détermine de façon majeure, par un effet domino, la production d'œufs en contraignant l'ovogenèse ; (iii) que la qualité nutritionnelle du pollen impacte à la fois les larves et les adultes, et que cette qualité pourrait être déterminée en bonne partie par la concentration en amidon et en certains glucosinolates (des métabolites secondaires typiques de quelques familles végétales dont les brassicacées). La combinaison des différents résultats obtenus permet également de proposer des hypothèses plus générales, parmi lesquelles le fait que le contexte agronomique dans lequel l'interaction a lieu ait largement influencé, voire perturbé, l'interaction qui liait le méligèthe et les brassicacées sauvages avant que les cultures de colza ne se généralisent ; et le fait que les méligèthes détoxifient les glucosinolates, mais moins efficacement chez les adultes (généralistes) que chez les larves (spécialistes).

En conclusion, cette thèse a montré qu'une nouvelle voie était peut-être envisageable pour contribuer à protéger les cultures de façon durable contre les insectes ravageurs, en particulier pour les systèmes agronomiques où le phénotypage classique est irréalisable et où les dégâts sont causés à un stade temporairement sensible de la culture. Nous n'avons cependant réalisé que la moitié du chemin : identifier quelques traits-clés candidats. Il reste maintenant à mener l'étape déterminante de validation de ces traits.