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Screening the variability in oilseed rape resistance to pollen beetle attacks in the field and assessment of biochemical biomarkers

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Abstract

The pollen beetle (*Brassicogethes aeneus*) is one of the main insect pests affecting oilseed rape crops. Efficiency of insecticides used to control this pest is decreasing due to the development of resistance to compounds such as pyrethroids in many populations. Breeding oilseed rape for resistance to pollen beetle attacks could be an interesting strategy to find alternative control methods but has not been really developed in this crop yet. However, screening plants for insect resistance remains complicated as it often involves field tests on large genotype collections which are complicated to carry out without biases. Current knowledge on the chemical ecology of interactions between oilseed rape and pollen beetles could help finding biochemical markers of this resistance and bypass this problematic field screening phase, thus allowing an indirect breeding approach. Previous laboratory tests have shown that variations in attack levels among a small set of oilseed genotypes could be explained by the biochemistry of bud tissues. The present study aimed at validating this link under field conditions. For that purpose, we conducted a multi-site experiment in France with 19 genotypes exposed to pollen beetle attacks. We phenotyped pollen beetle damage and sampled buds in the field to assess their chemical composition. Large variability in pollen beetle attacks was observed over the genotypes. These attack levels were consistent between locations. Bud chemistry was highly variable, but most compounds were well correlated between locations. Potential biomarkers previously identified in laboratory experiments were not confirmed to be correlated with resistance to pollen beetles in the field, but new compounds which may be considered interesting markers for resistance screening against the pollen beetle emerged.

Keywords Phenotyping · Metabolite · Plant resistance · *Brassicogethes aeneus* · *Brassica napus*

Key message

- Variability in resistance to pollen beetle feeding exists in oilseed rape and can be screened in the field.
- The effect of potential biochemical biomarkers previously identified is not confirmed. The concentration of two compounds in the perianth is correlated with expression of resistance.
- Important environmental variability in the plant chemistry is observed and challenges our capacity to efficiently identify biochemical biomarkers.

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Introduction

Insect pests are one of the main causes of agricultural yield losses, destroying 13–16% of annual crop production worldwide (Culliney 2014). These pests are mainly managed through the use of synthetic insecticides which are increasingly recognized as harmful for human health and the environment (Devine and Furlong 2007). This management strategy needs to be replaced by more sustainable alternatives.

Breeding plants for insect resistance could be an interesting approach to reduce agriculture dependency on pesticides (Wiseman 1994; Singh and Schwartz 2010). This strategy has already been implemented in the past and has shown its efficiency in controlling some insect populations (Smith 2005). Interest in this management strategy is growing as plant resistances to insects are easy to use and mostly compatible with other management practices such as biocontrol (Broekgaarden et al. 2008; VanDoorn and de Vos 2013; Stenberg et al. 2015; Tamiru et al. 2015; Mitchell et al. 2016). Recent developments in genetics through genome sequencing and marker-assisted breeding have considerably facilitated plant breeding, allowing more rapid and easier achievement of breeders' goals (Smith and Clement 2012). However, breeding plants for insect resistance remains challenging. Plant phenotyping is becoming the rate-limiting step of breeding programs, and this is especially true for developing plants resistant to insects (Barah and Bones 2015; Goggin et al. 2015; Mitchell et al. 2016). Most of the time, to identify resistant genotypes, phenotyping is addressed through direct confrontation of insects and plants (Tingey 1986), but this approach is time-consuming and can be highly complex for insect species that cannot be reared (Stout and Davis 2009). Technical advances in phenotyping such as image processing and metabolomics bring new opportunities to develop more efficient screening methods circumventing the phenotyping bottleneck (Furbank and Tester 2011; Goggin et al. 2015).

Basic researches demonstrated the importance of plant chemistry as a mediator of plant–insect interactions (Berenbaum and Zangerl 2008). Secondary metabolites such as alkaloids and glucosinolates are used by plants to protect themselves against insects (Mithöfer and Boland 2012). Other compounds such as primary metabolites are major components of insect choice and use of plants (Berenbaum 1995; Awmack and Leather 2002). While the former deter, intoxicate or limit the feeding ability of insects, the latter have a nutritional value and are essential to their survival, growth and development. Therefore, plant metabolites could be used as markers of plant–insect relationships. Identification of compounds related to plant susceptibility

or resistance to insects could allow large screenings of genotypes based on plant biochemistry rather than on direct confrontation of plants and insects (Smith 2005). With the reduction in costs and increase in accuracy of chemical analyses, it is becoming easier to analyse large numbers of samples and target such approach (Fernie and Schauer 2009).

Oilseed rape (OSR, *Brassica napus*) is the most cultivated oilseed crop in Europe with 6.32 M ha grown in 2016 (Eurostat 2016). Winter OSR is attacked by a vast array of insects (Williams 2010), and thus, it is one of the most insecticide-demanding field crops in Europe (AGRESTE 2013). The pollen beetle (*Brassicogethes aeneus*, formerly *Meligethes aeneus*) is a major pest of OSR in Europe (Gagic et al. 2016). This insect overwinters in the soil and leaf litter of forests and emerges when mean temperatures rise above 10–12 °C (Ferguson et al. 2015). It migrates to OSR fields when plants are at the green-bud stage and feeds on pollen by destroying flower buds, leading to decreased yields when populations are high (Williams and Free 1978). Intraspecific variability in damage caused by the pollen beetle in OSR has already been observed. Early flowering genotypes, for example, can escape pollen beetle attacks by flowering before insect arrival (Kleine 1921), but these genotypes can also be highly affected by frost damage. Variability in OSR resistance to pollen beetle feeding not related to the plant phenology has been found in experiments conducted under controlled conditions (Hervé et al. 2014). Field screening of pollen beetle resistance is challenging as insect infestation is strongly driven by plant phenology through the flowering period (Frearson et al. 2005; Cook et al. 2007), and as explained above, early flowering plants can escape attacks. Testing a large number of genotypes with diverse flowering periods for resistance to pollen beetle in the field could lead to biased estimations of resistance (Hervé et al. 2017). Furthermore, estimating resistance to pollen beetle is not trivial, and previous studies have quantified infestations using two methods. The first one is based on counting adult pollen beetles present on the plant (Rusch et al. 2013; Kaasik et al. 2014). This measure of abundance is easy to do but is not adapted to screen for resistance as pollen beetles are highly mobile and their abundance depending on the weather can strongly vary between sampling days or even hours. Abundance and damage of pollen beetles have been shown to be weakly correlated (Hansen 2003; Rusch et al. 2013; Gagic et al. 2016) which confirms that this metric is not the best estimator of plant resistance. A second method to estimate damage has been proposed by counting podless stalks (Free and Williams 1978; Gagic et al. 2016). This method has been criticised as podless stalks related to pollen beetle attacks can be confounded with abortion due to physiological factors or damage caused by the brassica pod midge (*Dasineura brassicae*) (Free and Williams 1978; Tatchell 1983). New

methods need to be developed before resistance of different genotypes can be assessed in the field. Chemical analyses performed on OSR buds identified five potential biochemical biomarkers present in the perianth and correlated with the pattern of resistance to pollen beetles under controlled laboratory conditions (Hervé et al. 2014). These compounds are both primary metabolites (i.e. serine, proline and sucrose) and secondary metabolites (i.e. flavonols such as quercetin-3-O-sophoroside and kaempferol-3-O-sophoroside). Primary metabolites of the perianth increased bud attacks, while secondary metabolites decreased these attacks. Surprisingly, glucosinolates seemed not to be involved. While these results are encouraging, they need to be confirmed under field conditions. The aims of the present study were: (1) to develop a screening method allowing to compare resistance of different OSR genotypes to pollen beetle damage in the field; (2) to validate the biomarkers identified by Hervé et al. (2014) on a new set of genotypes and under field conditions; (3) to identify new potential biomarkers; and (4) to set the grounds for a future biomarker-assisted screening method for pollen beetle resistance in OSR.

Materials and methods

Plant materials

The set of genotypes used by Hervé et al. (2014) contained genotypes with contrasted flowering periods, mixing spring and winter types, and could not be tested in field screening (Hervé et al. 2017). The genotypes used in our experiments were all winter types selected among the Biogemma collection. These accessions were obtained from different genetic resource centres, and their phenology was observed in previous years in the field (Table S1). Accession choice was based on homogeneous phenology and maximum genetic diversity as observed after genotyping with the illumina 60k infinium Brassica chip (Clarke et al. 2016) (data not shown).

In 2015–2016, twenty genotypes of oilseed rape comprising ten early flowering genotypes and ten late-flowering genotypes were selected for the trials (Table S1). This was done to maximize the chances of the presence of material at the susceptible stage when the pollen beetle flight occurred. In 2016–2017, another set of 20 genotypes was selected with six accessions in common with the 2015–2016 trials (Table S1). This time, the set spanned only the late-flowering window, but maximized the genetic diversity.

Field trials

Field trials were conducted in two locations for two consecutive years. They were set up in Cornebarrieu (43°39′41.8″N 1°18′27.4″E, Occitanie, France) and Liverdy-en-Brie (48°40′09.1″N 2°46′28.6″E, Ile de France, France) in 2015–2016 and in Mondonville (43°40′35.8″N 1°17′28.3″E, Occitanie, France) and Liverdy-en-Brie in 2016–2017. In trials conducted in Occitanie, the median of the maximum and minimum temperature during the growing season (September–April) was 14.9 °C and 7.2 °C in 2015–2016 season and 15.1 °C and 6.25 °C in 2016-2017. The total amount of precipitation during the season was 375 mm in 2015–2016 and 314 mm in 2016–2017. In Liverdy-en-Brie, the median of the maximum and minimum temperature was 12.5 °C and 5.7 °C in 2015–2016 season and 11.9 °C and 4.5 °C in 2016–2017. The total amount of precipitation during the season was 412 mm in 2015–2016 and 291 mm in 2016–2017. Other climatic data can be found on Agri4Cast [http://agri4cast.jrc.ec.europa.eu (14/11/2019)]. Field trials were designed in a randomized complete block design with three blocks. Each genotype was sown in plots of six rows of 4 m length, spaced by 50 cm. To reduce border effects, six rows of a late-flowering genotype (Licorne in 2015–2016 and Ariana in 2016–2017) were sown around the experimental area.

Pollen beetle and plant growth monitoring

Pollen beetle arrival on the field was monitored using four yellow water traps filled with water and detergent and placed at each corner of the fields. In the spring 2016, pollen beetles arrived at Cornebarrieu on February 25 and on March 23 at Liverdy-en-Brie. In 2017, insects arrived at Mondonville on February 16 and in Liverdy-en-Brie on March 14. Oilseed rape plants were checked once a week to monitor growth stage of each plot using the universal BBCH-scale (Lancashire et al. 1991) to ensure that damage sampling occurred between the "green buds" stage (BBCH growth stage 51) and the beginning of the flowering (BBCH growth stage 60) when plants are most susceptible to pollen beetle attacks. Damage sampling started once pollen beetles colonized experimental plots, and most genotypes had individual flower buds visible and closed (BBCH growth stage 55–57).

Pollen beetle damage estimation

The number of attacked buds was counted on the first raceme. All buds with one or more holes through the perianth caused by chewing, fading buds with oviposition hole as well as abscised buds were considered as pollen beetle damage. Pollen beetle damage was estimated by randomly sampling 15 plants with visible flower buds (BBCH growth stage 51–60) in each plot (45 plants/genotype). Plant size and growth stage were recorded for each plant. Plant growth stage was pooled to allow easier measurement: flower buds visible and under or at the level of leaves (BBCH 51–53), flower buds above leaves 55–57, first petals visible and

flower bud still enclosed (BBCH 59), beginning of the flowering (BBCH 60–61).

In 2016, plant sampling was carried out on April 6 at Cornebarrieu and on April 18 at Liverdy-en-Brie. In 2017, it was done on March 28 at Mondonville and between April 10 and 11 at Liverdy-en-Brie.

Plant sampling for chemical analysis

Plants were sampled in 2017 to analyse perianth chemistry. All plants were collected at the same growth stage ("yellow buds", BBCH 59) to avoid potential variations in chemistry caused by differences in growth stage. Sampling occurred between March 22 and April 6 at Mondonville and between April 10 and April 13 at Liverdy-en-Brie. Two samples were prepared in each plot to collect a total of six samples for each genotype and location. To complete one sample, four plants were harvested in one plot and 20 buds (length > 3 mm) on the first raceme of each plant were collected. These buds were then dissected to sample the perianths and immediately frozen in dry ice. Perianths were stored at − 20 °C before being freeze-dried and ground to powder before analysis.

Metabolic profiling

Free amino acids (AA), non-structural carbohydrates, polyols and organic acids (CPOA), glucosinolates (GSL) and flavonols (FO) were analysed on sampled perianths.

Quantification of AA and CPOA was based on Gravot et al. (2010). Extraction of these compounds from freeze-dried perianth powder was performed on 10 mg of powder, with a methanol–chloroform–water-based extraction. Plant powder was suspended in 500 µl of methanol containing two internal standards: 200 µM of 3-aminobutyric acid (BABA) (for quantification of AA) and 400 µM of adonitol (for quantification of CPOA). This suspension was agitated for 15 min at room temperature, and 250 µl of chloroform was added followed by 10 min agitation. Five hundred microlitres of water was added, and samples were vortexed and centrifuged at 12,000*g* for 5 min to induce phase separation. The upper phase was transferred to a clean microtube and used for subsequent analysis.

CPOA were analysed by GC-FID according to Adams et al. (1999) and Lugan et al. (2009). The online derivatization was realized with a Trace 1300 GC-FID (Thermo Scientific) equipped with a TriPlus RSH (Thermo Scientific). Fifty microlitres of the extract was dried under vacuum. The residue was redissolved in 50 µl of pyridine containing 20 mg ml^{-1} methoxyamine hydrochloride, under orbital shaking at 40 °C for 90 min. Fifty microlitres of MSTFA (N-methyl-trimethylsilyltrifluoroacetamide) was added before incubation at 40 °C for 30 min. One microlitre of the mixture was injected into the GC-FID with a split/splitless injector (split mode set to 1:20) at 260 °C, on a TG-5MS column (30 m × 0.32 mm × 0.25 mm, Thermo Scientific) connected to a flame ionization detector at 300 °C. The temperature gradient of the GC oven was set as follows: 4 min at 100 °C followed by an increase of 10 °C min^{-1} up to 198 °C and maintained at this temperature for 2 min; an increase of 1 °C min^{-1} up to 202 °C; then an increase of 15 °C min^{-1} ramp up to 268 °C and held for 3 min followed by an increase of 1 °C min^{-1} up to 272 °C and raised 210 °C at 10 °C min^{-1} maintained for 7 min. Adonitol was used as internal standard.

For AA profiling, 50 µl of methanol–water extract was dried under vacuum. The dry residue was suspended in 50 µl of ultrapure water, and 5 µl of this suspension was used for AccQ-Tag Ultra Derivatization (Waters). Derivatized amino acids were analysed using an Acquity UPLC-DAD system (Waters) according to Jubault et al. (2008) except that the column used for analyses was heated at 53 °C and that amino acids were detected at 265 nm using a photodiode array detector. BABA was used as internal standard.

Extraction and analysis of GSL and FO were based on Hervé et al. (2014). Ten milligrams of freeze-dried powder of perianths was suspended in 1 ml of methanol–formic acid (99: 1) and agitated using a vortex for 30 s at room temperature. The tubes were placed in an ultra-sonicated bath for 5 min and centrifuged for sedimentation. Six hundreds microlitres of the liquid phase was then transferred to a clean microtube and directly used for analyse. An Acquity-TQD UPLC-PDA-MS system (Waters) with electrospray ionization in a negative mode was used to analyse GSL and FO. Chromatographic conditions were as follows: column Water Acquity C18 (150 mm × 2.1 mm × 1.7 µm), flow rate 0.4 ml min^{-1}, column oven temperature of 25 °C, injection volume of 2 µl. The A eluent was water–formic acid solution (99.9: 0.01), and the B eluent was acetonitrile–formic acid solution (99.9:0.01). The applied gradient was 0–0.2 min 2% B, 0.2–3 min 62% B, 3–8 min 90% B, 8–9 min 90% B and then returned to initial conditions 2% B in 1 min and re-equilibration for 1 min.

Mass spectrometry was used to identify GSL based on the m/z response in negative electrospray mode and their retention time. For quantifications, calibration curves were obtained from a stock solution at 250 µmol l^{-1} of three commercially available standards (glucoerucin, gluconasturtiin and glucobrassicin) with four different dilutions (3-, 5-, 10- and 50-fold). Glucoerucin, gluconasturtiin and glucobrassicin calibration curves were, respectively, used to quantify aliphatic, aromatic and indolyl GSL.

FO were analysed using a photodiode array detector at 350 nm. Identification was made based on UV spectra and m/z response (Velasco et al. 2011). Quantification of FO was made with UV calibration curves from a stock solution at 200 µmol l^{-1} of three commercially available compounds

(isorhamnetin-3-O-glycoside, quercetin-3-O-glycoside and kaempferol-3-O-glycoside) at three different dilutions (10, 20 and 50-fold). These curves were used to quantify compounds structurally related to isorhamnetin, quercetin and kaempferol.

Data analysis

All statistical analyses were performed using R software (R Core Team 2016).

Pollen beetle infestation of OSR on different sampling occasions

An ANOVA was performed to compare the damage level of OSR at each sampling occasion. The number of damaged buds of the six genotypes sampled in every location and year (i.e. Bolko, G28, Grizzly, Sarepta, Lembke, Lira) was accounted to allow comparison of pollen beetle infestation. Pairwise comparisons of least squares means (LSM) were performed on this ANOVA (package "lsmeans"; Lenth 2016) with false discovery rate correction for p values (Benjamini and Hochberg 1995).

Genotype resistance to pollen beetle

To test differences between genotypes for resistance to pollen beetle, different models were built for each location and year. Models were made as follows:

$$Y_{ijk} = \mu + B_j + Sa_{ijk} + D_{ijk} + G_i + \underline{P_{ij}} + \underline{E_{ijk}} \quad \text{(model 1)}$$

With random and fixed terms symbolized by underlined and non-underlined letters, respectively, Y_{ijk} is the number of damaged buds of the plant k, in the plot of the genotype i in the block j; μ is the overall mean; B_j is the block effect; S_{ijk} is the size of the plant considered; a is the regression coefficient of the plant size effect; D_{ijk} is the effect of growth stage of the plant; G_i is the genotypic effect; P_{ij} is the plot effect; and E_{ijk} is the residual error following a normal distribution $N(0, \sigma_e^2)$. Wald Chi-square tests were performed on these models to test significant effect of each fixed term. Pairwise comparisons of least squares means (LSM) were performed as described in the previous section. Pearson's correlation tests were then performed to obtain correlations between LSM of each genotype from different locations and years.

Differences in perianth chemistry

To investigate differences in perianth chemistry between genotypes and locations for 2017 data, the following model was used for each compound:

$$Y_{ijl} = \mu + L_l + B_{jl} + G_i + GL_{il} + E_{ijl} \quad \text{(model 2)}$$

where Y_{ijl} is the mean concentration of samples in the block j, the genotype i and the location l; μ is the overall mean; L_l is the effect of the location; B_{jl} is the effect of the block nested in the location; G_i is the effect of the genotype i; GL_{il} is the effect of the interaction between the genotype and the location; and E_{ijl} is the residual error following a normal distribution $N(0, \sigma_e^2)$. F tests were performed on these models to test significant effect of each variable.

A model similar to model 2 was built with $L_l, B_{jl}, G_i, GL_{il}$ considered as random effects to obtain the percentage of variance of the concentration of each compound explained by the effects of the genotype, the location, the effect of the block nested in the location, the interaction between location and genotype and the residual variance. Variance components of each factor were extracted using "VarCorr" function (package "lme4"; Bates et al. 2014).

Relationship between perianth chemistry and pollen beetle resistance

To establish a relationship between genotype chemistry and resistance, LSM of the concentration of each compound and genotype were extracted from model 2. To compare these LSM to similar values of pollen beetle susceptibility, a model based on average plot values and accounting for the two locations at the same time was needed. To do so, the first model was built to obtain damage level for each plot:

$$Y_k = \mu + D_k + Sa_k + E_k \quad \text{(model 3)}$$

where Y_k is the number of damaged buds on the plant k; μ is the overall mean; D_k is the effect of growth stage; S_k is the size of the plant considered; a is the regression coefficient of the plant size effect; and E_k is the residual error following a normal distribution $N(0, \sigma_e^2)$.

The second model was made to compute LSM of the number of damaged buds per genotype over all locations:

$$Y'_{ijl} = \mu + L_l + B_{jl} + G_i + GL_{il} + E_{ijl} \quad \text{(model 4)}$$

where Y'_{ijl} is the mean value of residuals from model 3 in a plot j of the block j plus the mean number of damaged buds over all genotypes in the location l; μ is the overall mean; L_l is the effect of the location; B_{jl} is the effect of the block nested in the location; G_i is the effect of the genotype; GL_{il} is the effect of the interaction between the location and the genotype; and E_{ijk} is the residual error following a normal distribution $N(0, \sigma_e^2)$. LSM were then computed based on model 4 as explained above.

To investigate relationships between compounds and resistance, Pearson's correlations between LSM of the number of damaged buds for each genotype (model 4) and LSM of concentration of each compound for each genotype

(model 2) were computed. A sparse partial least squares (sPLS) regression was also done to identify more complex associations between plant resistance and chemistry. This analysis allows finding variables that contribute the most to the association between the number of damaged buds per genotype and the chemistry (Lê Cao et al. 2008). Selection of variables with the sPLS was limited to five compounds using three components.

Results

Pollen beetle infestation of OSR on different sampling occasions

The number of damaged buds due to pollen beetles varied between sampling sessions (Fig. 1, $F_{3, 1321} = 562.99$, $P < 0.001$). The greatest damage level was observed during the two years of sampling in Occitanie (Cornebarrieu 2016 and Mondonville 2017). The damage level in 2017 was more than that of 2016 at this location (Fig. 1). No differences were observed between years in Liverdy-en-Brie (Fig. 1).

Genotype resistance to pollen beetle

In 2016, pollen beetles colonized the field trials late in the season. Early flowering genotypes passed the susceptible bud stage before the main migration of beetles and thus were not sampled (Table S1). Due to time constraints, only six
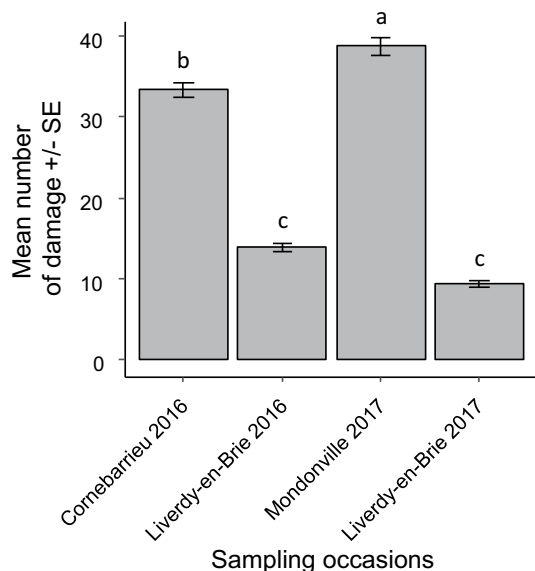
genotypes could be sampled for damage in 2016 at the two locations (Table S1). In 2017, twenty genotypes were sampled at each location (Table S1). However, Goeland had a particular morphology with its primary racemes aborting during plant growth. Thus, this genotype was not included into analyses.

Plant size had a significant positive effect on the damage level caused by pollen beetles at every sampling session (Table 1). Plant growth stage had a significant effect on the number of damaged buds for each sampling except at Liverdy-en-Brie in 2017 (Table 1). The number of damaged buds seemed to increase as plants developed. Significant differences were also observed between blocks in Cornebarrieu 2016 and Mondonville 2017 (Table 1). Genotype had a significant effect on insect attack during each year and at each location except at Liverdy-en-Brie during the spring 2016 (Tables 1, 2). Strong correlations were found between average level of damage of different years and locations (Table 3).

Differences in perianth chemistry

Chemical analysis led to quantification of 38 compounds: 18 AA, 7 CPOA, 7 FO and 6 GSL (Table 4, Table S2). To ensure quality of analyses, correlations of LSM values of each genotype between two blocks in each site and year were checked. For most of the compounds, correlations were high

Fig. 1 Mean (± SE) number of damaged OSR buds caused by the pollen beetle in all sites and years sampled. Means are based on data from the six genotypes in common each year and at each location. ANOVA: $F_{3, 1321} = 562.99$, $P < 0.001$. Different letters indicate significant differences

Table 1 Results of type II Wald Chi-square tests conducted on linear mixed models explaining the number of pollen beetle attacks on OSR inflorescences by the fixed effects of the block, the plant size, the plant growth stage and the plant genotype

Years	Locations	Variables	χ^2	df	P
2016	Liverdy-en-Brie	Block	5.51	2	0.064
		Plant size	30.55	1	**< 0.001**
		Growth stage	0.21	1	0.647
		Genotype	7.63	5	0.178
	Cornebarrieu	Block	8.02	2	**0.018**
		Plant size	45.13	1	**< 0.001**
		Growth stage	11.88	2	**0.003**
		Genotype	21.91	5	**< 0.001**
2017	Liverdy-en-Brie	Block	1.76	2	0.416
		Plant size	67.38	1	**< 0.001**
		Growth stage	6.26	1	**0.012**
		Genotype	85.11	18	**< 0.001**
	Mondonville	Block	11.16	2	**0.004**
		Plant size	214.41	1	**< 0.001**
		Growth stage	169.52	3	**< 0.001**
		Genotype	71.06	18	**< 0.001**

The plot was accounted as random effect. Different models were computed for each sampling session. Significant effects are presented in bold

Table 2 Least squares means (LSM) (± SE) of the number of damage caused by pollen beetles for each genotype sampled at the two locations in 2016 and 2017

Genotypes	Liverdy-en-Brie 2016		Cornebarrieu 2016		Liverdy-en-Brie 2017		Mondonville 2017	
	LSM	Groups	LSM	Groups	LSM	Groups	LSM	Groups
Akamar					12.21 (1.15)	cde	41.49 (3.71)	bcd
Bolko	12.60 (2.31)	a	27.33 (2.97)	ab	8.70 (1.21)	abcd	33.82 (3.69)	abcd
G5					5.35 (1.21)	ab	18.01 (3.76)	a
Cresus					11.21 (1.14)	cde	40.16 (3.74)	bcd
Debruder Dippes					6.91 (1.14)	abc	40.60 (3.8)	bcd
G10					5.26 (1.17)	a	34.87 (3.88)	abcd
Grizzly	17.14 (1.81)	a	36.41 (2.69)	b	14.56 (1.15)	e	47.73 (3.74)	d
Kombi					11.05 (1.19)	bcde	43.35 (3.74)	bcd
G14					8.12 (1.15)	abcd	34.04 (3.74)	abcd
Lembke	11.03 (2.23)	a	28.07 (2.77)	ab	8.53 (1.17)	abcd	32.74 (3.77)	abcd
Lira	15.96 (1.88)	a	32.12 (2.76)	ab	11.26 (1.14)	cde	37.35 (3.73)	bcd
G18					8.30 (1.14)	abcd	25.55 (3.76)	ab
G19					10.41 (1.15)	abcde	39.53 (3.75)	bcd
Pollux					10.29 (1.17)	abcde	46.09 (3.74)	cd
Quedlinburger Platzester					8.00 (1.14)	abcd	37.58 (3.76)	bcd
G28	9.96 (2.26)	a	22.63 (2.75)	a	8.69 (1.16)	abcd	27.97 (3.83)	abc
Rasant					7.93 (1.13)	abcd	34.59 (3.77)	abcd
Sarepta	15.16 (1.83)	a	37.57 (2.78)	b	7.04 (1.17)	abc	34.93 (3.76)	abcd
Tor					12.93 (1.14)	de	44.86 (3.76)	cd

Different letters indicate significant differences in the number of damaged buds between genotypes computed independently according to years and locations

Table 3 Pearson's correlation coefficients comparing LSM of the number of damage on OSR caused by pollen beetles between various locations and years

		2016		2017	
		Cornebarrieu	Liverdy-en-Brie	Mondonville	Liverdy-en-Brie
2016	Cornebarrieu	–	0.89	0.75	0.34
	Liverdy-en-Brie	–	–	0.87	0.63
2017	Mondonville	–	–	–	0.71
	Liverdy-en-Brie	–	–	–	–

(Table S3). The mean correlation was $r = 0.68$ (SE = 0.03) for Mondonville and $r = 0.54$ (SE = 0.03) for Liverdy-en-Brie. Mean concentrations by genotype were stable between locations as average correlation coefficient was high (mean $r = 0.46$, SE of $r = 0.05$), but correlations were variable depending on compounds (Table S4).

Significant differences between genotypes were found for most of the compounds (Table 4). Only neoglucobrassicin, methionine, isoleucine and phenylalanine did not vary according to genotype (Table 4). Twenty-eight out of the 38 compounds varied according to location, and 19 varied according to interactions between genotypes and locations (Table 4). The average variance explained was highest for the location effect which accounted a mean of 44% of variation in concentration (Table 4). Genotype explained an average of 19% of the variation, but variability was high

(min = 0%, max = 64%). The interaction between genotypes and locations explained an average of 15%, and variability was also high (min = 0%, max = 54%). The interaction between location and block was marginal and explained only 1% of this variability.

Relation between perianth chemistry and pollen beetle resistance

To link compounds to plant resistance, correlations between LSM of damage level and LSM of concentrations were computed (Table 5). Compounds identified by Hervé et al. (2014) as potential biomarkers of resistance to pollen beetle had low levels of correlations: quercetin-3-O-sophoroside = 0.03, kaempferol-3-O-sophoroside = 0.11, proline = 0.10, serine = 0.30, sucrose = 0.03. Only two compounds had

Table 4 P values of the effect of the genotype, the location, the interaction between genotype and location and the block effect nested in the location on perianth chemistry and variance explained by these variables

Compounds	Classes	P				Variance explained				
		Genotype	Location	Genotype×location	Block	Genotype (%)	Location (%)	Genotype×location (%)	Block (%)	Residuals (%)
S-Methylcysteine sulfoxide (SMCSO)	AA	0	0.001	0.449	0	64	4	7	8	18
γ-Aminobutyric acid (GABA)	AA	0	0	0.066	1	3	68	11	0	18
Arginine	AA	0	0	0	1	5	51	25	1	18
Asparagine	AA	0	0	0	1	4	66	21	0	9
Aspartic acid	AA	0	0.001	0	1	0	24	52	0	24
Glutamic acid	AA	0	0.903	0	1	4	9	54	0	32
Glutamine	AA	0	0	0	1	11	46	29	0	14
Histidine	AA	0.005	0	0.004	0.11	0	61	16	4	20
Isoleucine	AA	0.81	0	1	1	1	79	3	0	17
Methionine	AA	1	0	1	1	1	83	1	0	16
Phenylalanine	AA	0.079	0	0.335	1	1	80	6	0	13
Proline	AA	0	0.001	0.171	1	45	20	12	0	24
Serine	AA	0	0.154	1	1	22	33	4	0	41
Threonine	AA	0	0	0.25	1	9	66	8	0	17
Tryptophan	AA	0.021	0	0.005	1	0	77	10	0	13
Valine	AA	0	0	0.002	1	2	79	9	0	10
α-Alanine	AA	0	1	1	1	21	12	15	0	51
β-Alanine	AA	0	0	0	0.083	10	71	10	1	8
Fructose	CPOA	0	0	0.001	1	5	88	4	0	4
Glucose	CPOA	0	0	0.074	1	11	81	3	0	5
Glyceric acid	CPOA	0	0	0.002	1	46	35	9	0	10
Malic acid	CPOA	0	0	0	1	11	55	17	1	17
myo-Inositol	CPOA	0	1	0.001	1	48	0	26	0	26
Quinic acid	CPOA	0	0.033	0.39	1	45	13	13	0	30
Sucrose	CPOA	0	0	0	1	11	54	23	0	13
Unk.FO1	FO	0.001	1	0.052	1	12	0	32	0	56
Unk.FO2	FO	0	1	1	1	61	2	7	0	31
Isorhamnetin-di-glucoside	FO	0	1	1	1	13	78	0	0	9
Kaempferol-3-O-sophoroside	FO	0	0.001	1	0.12	26	49	4	3	18
Quercetin-3-O-caffeoyl-sophoroside-7-O-glucoside	FO	0	0	1	1	27	55	1	1	16
Quercetin-3-O-sophoroside	FO	0	0	0	0.025	13	62	11	2	11
Quercetin-3-O-sophoroside-7-O-glucoside	FO	0	0.008	1	1	30	23	11	0	37

Table 4 (continued)

Compounds	Classes	P				Variance explained				
		Genotype	Location	Genotype×location	Block	Genotype (%)	Location (%)	Genotype×location (%)	Block (%)	Residuals (%)
Epiprogoitrin	GSL	0	0	0	1	19	50	16	0	16
Glucobrassicin	GSL	0	0.364	0	0.01	7	25	33	7	27
Gluconapin	GSL	0	0	0	1	18	51	24	0	8
Gluconasturtiin	GSL	0	0.938	0.022	1	47	4	20	0	29
Neoglucobrassicin	GSL	0.075	1	1	1	16	10	5	1	68
Progoitrin	GSL	0	0.726	0.497	1	46	12	12	0	30

Classes: free amino acids (AA), carbohydrates, polyols, organic acids (CPOA), flavonols (FO), glucosinolates (GSL). Unk.FO1 and Unk.FO2 are two unidentified flavonols

significant correlations (Fig. 2): quinic acid ($r = -0.51$, $P = 0.03$) and arginine ($r = 0.50$, $P = 0.03$) (Fig. 2).

The sPLS analysis performed on our dataset did not find an overall relationship between plant chemistry and the number of damaged buds as none of the PLS components had Q2 values higher than 0.0975 (Q2 component 1 = −0.108, Q2 component 2 = −0.295, Q2 component 3 = −0.624).

Discussion

Previous studies showed that substantial differences in resistance to pollen beetle feeding are present in OSR plants when tested in controlled conditions (Hervé et al. 2014). The present experiments revealed that this intraspecific variability also exists among OSR genotypes grown and exposed to pollen beetles in the field. Differences between genotypes in such conditions were also substantial as the most susceptible genotype suffered more than twice as much damage as the most resistant one. These differences were verified over two consecutive years and in different locations, indicating that our observations are reliable and that the gradient of resistance observed is stable in different environments and growing conditions. When screening for resistance to pollen beetle, plant earliness is a major issue as insects are more attracted to the most developed plants (Hervé et al. 2017). Choosing OSR genotypes with simultaneous susceptibility periods in the present study allowed to reduce this bias. Even if we used genotypes with a limited flowering period in our experiments, variations in phenology still existed among genotypes. These variations were integrated in the statistical analyses by taking plant growth stage and size into account in the tests. Screening larger collections of genotypes for pollen beetle resistance seems to be feasible as long as plants with simultaneous susceptible periods are used. Several differences among genotypes could explain the damage gradient observed in our experiments. Genotypes could differ in attractiveness or appetability to pollen beetles. Previous laboratory experiments did not find large variability in OSR attraction to pollen beetle but demonstrated a variability in feeding intensity that could be related to perianth composition (Hervé et al. 2017). Therefore, differences between genotypes observed in our field experiments could be more related to feeding stimulation than to attraction. Semi-field experiments where insects would be introduced into OSR caged plots could help confirming which one of these two mechanisms is involved in the gradient we observed in our field trials (Smith 2005).

Oilseed rape perianths were collected in 2017 at two locations, and their composition in different classes of compounds was analysed. High correlations of concentrations were found between blocks on a single location for all compounds. This indicates that quantification of

Table 5 Pearson's correlations between the LSM concentration of each compound quantified in OSR perianths of floral buds and the number of damaged buds

Compounds	Classes	r	P
S-Methylcysteine sulfoxide (SMCSO)	AA	−0.01	0.98
4-Aminobutanoic acid (GABA)	AA	0.15	0.53
Arginine	AA	0.50	**0.03**
Asparagine	AA	0.35	0.15
Aspartic acid	AA	0.38	0.11
Glutamic acid	AA	0.17	0.50
Glutamine	AA	0.24	0.32
Histidine	AA	0.24	0.32
Isoleucine	AA	0.00	0.98
Methionine	AA	0.01	0.98
Phenylalanine	AA	−0.06	0.82
Proline	AA	0.10	0.70
Serine	AA	0.30	0.22
Threonine	AA	0.22	0.37
Tryptophan	AA	−0.01	0.95
Valine	AA	0.09	0.72
α-Alanine	AA	0.13	0.60
β-Alanine	AA	0.20	0.40
Fructose	CPOA	0.06	0.82
Glucose	CPOA	0.04	0.86
Glyceric acid	CPOA	−0.27	0.26
Malic acid	CPOA	0.15	0.54
myo-Inositol	CPOA	−0.08	0.74
Quinic acid	CPOA	−0.51	**0.03**
Sucrose	CPOA	0.03	0.89
Unk.FO1	FO	−0.01	0.98
Unk.FO2	FO	0.01	0.97
Isorhamnetin-di-glucoside	FO	−0.09	0.70
Kaempferol-3-O-sophoroside	FO	0.11	0.65
Quercetin-3-O-caffeoyl-sophoroside-7-O-glucoside	FO	0.15	0.53
Quercetin-3-O-sophoroside	FO	0.03	0.92
Quercetin-3-O-sophoroside-7-O-glucoside	FO	−0.15	0.54
Epiprogoitrin	GSL	0.31	0.20
Glucobrassicin	GSL	0.05	0.85
Gluconapin	GSL	0.14	0.57
Gluconasturtiin	GSL	0.28	0.25
Neoglucobrassicin	GSL	−0.11	0.65
Progoitrin	GSL	0.34	0.16

Significant correlations are presented in bold

metabolites was consistent within a location. Differences in perianth chemistry between genotypes were observed for almost all compounds (34 out of 38). This result shows that chemical composition of perianths is different among the genotypes tested here. Two compounds were shown to be related to the number of damaged bud caused by pollen beetles (i.e. quinic acid and arginine with a negative and positive relationship, respectively). The strongest relationship was found with quinic acid, but this association is mainly driven by genotype G5. While high

concentrations of quinic acid in perianths of this genotype have been observed in both locations sampled, other genotypes expressing high concentration of quinic acid in their perianths need to be identified to confirm the role of this compound. The two compounds identified are not known to be directly related to plant defence (Winter et al. 2015). However, they could be involved in physiological processes correlated with the expression of resistance. Whether such compounds can be used as biomarkers of resistance against the pollen beetle remains to be studied.

Fig. 2 Relationship between LSM of the number of OSR buds damaged by pollen beetles and concentrations of compounds having significant correlations. Lines and shaded areas show linear models predictions $\pm$ SE
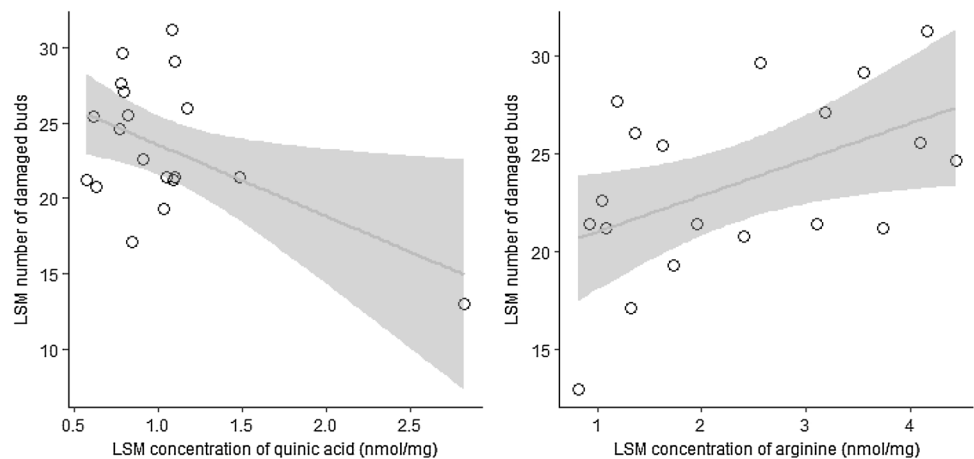
Concentrations of previously identified biomarkers (i.e. serine, proline, sucrose, quercetin-3-O-sophoroside and kaempferol-3-O-sophoroside) were not correlated with pollen beetle damage in our experiments. This lack of relation does not imply the absence of effect of these molecules, but their effect may be minimized in field conditions. Furthermore, differences in biomarkers identified could arise from differences in genotypes tested as, due to their phenology, none of the genotypes used by Hervé et al. (2014) could be screened in the present study.

To our knowledge, most attempts to identify biochemical biomarkers of plant resistance to insects have been conducted in laboratory or greenhouse bioassays (Wang et al. 2005; Omoloye and Vidal 2007; Leiss et al. 2009; Elek et al. 2013; Wang et al. 2017). Such bioassays allow for experimental conditions to be standardised and helped identifying some biochemical biomarkers that were used for screening plant resistance to insects (Shaw et al. 2009). However, whether such biomarkers can also influence levels of attack in the field remains mostly undocumented. Indeed, experimental and environmental conditions can strongly affect other plant traits such as plant morphology or pigmentation (Mishra et al. 2012) that may influence damage levels by herbivorous insects. Also, light intensity, temperature and soil conditions can affect plant tissue composition (Jänkänpää et al. 2012; Sun et al. 2016, 2017). Furthermore, most plant resistances to insects have a polygenic basis and are prone to environmental variations (Smith and Clement 2012). Experiments conducted in laboratory and field conditions may thus lead to different results (Alexandersson et al. 2014). Cysteine proteinase inhibitor, for example, was shown to be involved in induced defences of soybean against the Mexican bean beetle in greenhouse bioassays but was not related to insect performance in the field (Underwood et al. 2002). Consequently, conducting experiments in realistic cropping conditions seems essential to identify robust biomarkers of insect resistance.

Even if reliable quantifications of chemical compounds were performed in our experiments, perianth chemistry was greatly affected by the environment. Correlations of mean concentration per genotype between locations strongly differed according to compounds considered. Some compounds showed a very stable gradient of concentrations, while others did not correlate between locations. Even if significant differences between genotypes were observed for most compounds, a large part of the variability is explained by the environment and its interaction with the genotype. These observations are consistent with data from other studies. Indeed, several field experiments showed that the environment and interactions between the environment and the genotype explained most of chemical variability, whereas the genotype only accounted for a small proportion of it (Lee et al. 2003; Davies et al. 2010; Chen et al. 2016; Matros et al. 2017). In a study on durum wheat grains for example, most of the variability in primary metabolite composition was explained by the interaction between the genotype and the environment (54%), followed by the environment (42%), and finally, the genotype accounted for only 4% of this variability (Beleggia et al. 2013). The environmental variability in plant chemistry greatly affects the potential utilisation of biochemical biomarkers in breeding processes and thus should be accounted for to develop a reliable approach (Guo et al. 2016). To our knowledge, experiments meeting these criteria are rare (but see, for example, Abdel-Aal et al. 2001 and Anyanga et al. 2017) and most field screenings are performed one year at one location (Malchev et al. 2010; Schaefer-Koesterke et al. 2017; Schaefer et al. 2017).

As we have seen, the screening method presented here allowed the identification of genotypes with an interesting level of resistance to the pollen beetle (e.g. G5 and G10), and new genotypes with a resistance to this insect could be found in further trials. Interactions between OSR and other insect pests have not been studied here, but for further research, it will be important to consider the susceptibility of interesting

genotypes to other pests. Even if there is no evolutionary trade-off between resistances to different insects, several studies point out that these trade-offs could exist (Koricheva et al. 2004). Consequently, it will be important to check how OSR genotypes behave under multiple attacks in order to avoid breeding plants with resistance to the pollen beetle, but that are highly susceptible to other insects. Using biochemical biomarkers to predict plant resistance has been less successful in our study than identification of resistant plants. However, most of the genotypes screened here were susceptible or even highly susceptible to pollen beetle attacks. The small number of resistant genotypes in our experiment challenged our capacity to understand the specificity of these plants. Identifying biochemical biomarkers may require the use of additional genotypes displaying some resistance to the pollen beetle that can be chemically compared to susceptible ones.

Author contributions

GSC, DR, LE and JO participated in plant sampling, damage assessments and chemical analyses. GSC and AL performed the data analyses. GSC, DR, SF and AMC conceived and designed the research. GSC, SF and AMC wrote the paper. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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