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Consequences of combined herbivore feeding and pathogen infection for fitness of Barbarea vulgaris plants

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Consequences of herbivore feeding and pathogen infection for fitness of *Barbarea vulgaris* plants

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Declaration of Authorship: TvM and TPH conceived and designed the experiments. TvM performed all the experiments with help from VK, KRM, TS and NMvD. TvM, VKP, and TS analysed the data. TvM and TPH wrote the manuscript; other authors provided editorial advice.

Abstract

Plants are often attacked by pathogens and insects. Their combined impact on plant performance and fitness depends on complicated three-way interactions and the plants ability to compensate for resource losses. Here, we evaluate the response of *Barbarea vulgaris*, a wild crucifer, to combined attack by the oomycete *Albugo* sp., causing white rust, and a flea beetle, *Phyllotreta nemorum.* Plants from two *B. vulgaris* types that differ in resistance to *P. nemorum* were exposed to *Albugo* and *P. nemorum* alone and in combination, and monitored for pathogen infection, herbivore damage, defence compounds, nutritional quality, biomass and seed production.

Albugo developed strong infections in the insect-resistant plants, whereas insect-susceptible plants were hardly infected. Concentrations of *Albugo* DNA were higher in plants also exposed to herbivory; likewise, flea beetle larvae caused more damage on *Albugo*-infected plants. Concentrations of saponins and glucosinolates strongly increased when plants were exposed to *P. nemorum*, when insect-susceptible plants were exposed to *Albugo*, and sometimes even more in the combined treatment. The biomass of young insect-susceptible plants was lower when exposed to flea beetles, and the number of leaves of both plant types was negatively affected by combined exposure. After flowering, however, adult plants produced similar numbers of viable seeds, irrespective of treatment.

Our study supports that pathogens and herbivores can benefit from each others presence on a host plant and that the plant reacts by inducing specific and general defences. However, plants may be able to compensate for biomass loss of single and combined attacks over time.

Key-words: *Albugo sp.*; defence reactions, plant vigour; *Phyllotreta nemorum*; three-way interactions.

Introduction

Plants are often attacked simultaneously by phytopathogens and insect herbivores, and interactions between them are therefore common (Hatcher 1995; Hauser et al. 2013). The pathogens and insects may interact directly, e.g. if pathogen spores are transported by insects to suitable plant tissues, or indirectly through changes in the plant induced by one antagonist that also affect the other. Thus, pathogen infections can modify attractiveness of the host plant to herbivorous insects (Stout et al. 2006; van Molken et al. 2012) as well as their consumption, growth rate, survival and fitness (Hatcher 1995; Hatcher and Paul 2000; Paul et al. 2000; Rostas and Hilker 2002; Stout et al. 2006; Mouttet et al. 2011; Tack and Dicke 2013).

Indirect interactions between plant antagonists may be caused by defence compounds induced by one antagonist that also affect the other; alternatively, one antagonist may suppress plant defence levels to the benefit of the other. Complex cross-talk between defence signalling pathways in the plant may also contribute to such interactions, as different functional groups of herbivores and pathogens induce different signal pathways that may interfere with each other (Pieterse and Dicke 2007; Koornneef and Pieterse 2008; Thaler et al. 2012). Other causes of interactions may involve changes in resource partitioning or allocation as a consequence of attack (Hatcher 1995). Thus, the combined impact of pathogens and insect herbivores may differ significantly from the sum of impacts of each antagonist on its own (Hatcher 1995; Hauser et al. 2013).

The immediate resource losses incurred by combined attacks by pathogens and herbivores may to some extent be compensated for by re-growth, depending on the amount and distribution of losses, stored resources, integration and mobility among compartments, architecture, and environmental conditions (Paul et al. 2000; Nunez-Farfan et al. 2007; Fornoni 2011). Unfortunately, only few studies have evaluated how the proximate impacts of

pathogen-herbivore interactions translate into effects on plant performance and fitness (Morris et al. 2007; Hauser et al. 2013). Such knowledge is crucial for understanding the evolution of complex plant defences and for integrated pest management.

Here, we analyse interactions between the wild herbaceous crucifer *Barbarea vulgaris* (Brassicaceae), a flea beetle, and a pathogen. The subspecies ssp. *arcuata* (Opiz.) Simkovics of *Barbarea vulgaris* contains two divergent evolutionary types (Agerbirk et al. 2003b; Toneatto et al. 2010; Hauser et al. 2012; Toneatto et al. 2012) that differ in resistance to the flea beetle *Phyllotreta nemorum* and other important specialist herbivores (Nielsen 1997; Renwick 2002). One plant type is susceptible to all known *P. nemorum* genotypes and has Pubescent rosette leaves (and therefore designated P-type (Nielsen 1997)), whereas the other is resistant to most genotypes of *P. nemorum* and has Glabrous leaves (G-type). The two plant types co-exist in Denmark but predominantly in separate populations (Nielsen, unpublished).

Barbarea vulgaris is also attacked by an oomycete pathogen *Albugo* sp. (van Mölken, unpublished). *Albugo* (as it will be called here) can be observed in natural *B. vulgaris* populations in Denmark (van Mölken et al., unpublished), has frequently affected our experimental plants at the University, and has been detected in historical herbarium sheets (Choi et al. 2011). The P- and G-type of *B. vulgaris* have been suggested to differ also in susceptibility to *Albugo* (Toneatto 2009), based on observations of spontaneously infected plants in a greenhouse.

Possible interactions between *B. vulgaris*, flea beetles and *Albugo* could be caused by several different mechanisms. The resistance against flea beetles is caused especially by the saponin hederagenin cellobioside (3-O-cellobiosyl-hederagenin) (Shinoda et al. 2002; Kuzina et al. 2009; Nielsen et al. 2010; Augustin et al. 2011; Augustin et al. 2012), which is present in G-plants from spring to autumn (Agerbirk et al. 2003a). Putative saponins have also been discovered in P-plants (Kuzina et al. 2011), however it is unknown if these have a resistance

function against any antagonists. Saponins are known to affect many different herbivores and pathogens (Osbourn 1996; Augustin et al. 2011), and *Albugo* may possibly be sensitive to P-type saponins. If so, interactions between flea beetles and *Albugo* could result from increased production of saponins when both are present.

The same mechanism of interaction could potentially arise from induction of glucosinolates. The P-type mainly contains glucosibarin (the optical R-isomer of 2-hydroxy-2-phenylethyl-glucosinolate: 2R) whereas the G-type contains glucobarbarin (the optical S isomer: 2S) (Agerbirk et al. 2003a; Agerbirk and Olsen 2011). Glucosinolates are often toxic or deterrent to non-crucifer specialist insects, and play a role in host selection by crucifer specialists (Fahey et al. 2001; Griffiths et al. 2001; Renwick 2002). Glucosinolates may affect fungi and microorganisms (Fahey et al. 2001), including oomycete pathogens (Schlaeppi et al. 2010; Wang et al. 2013), and possibly also *Albugo* species (Mathur et al. 2013). The strong resistance of G-type plants against flea beetles is not caused by their specific glucosinolates (Agerbirk et al. 2001, 2003b); however, glucosinolates may still affect the flea beetles to a lesser degree.

Albugo infection of *B. vulgaris* may affect oviposition preference of flea beetles, as has been shown for the specialist herbivorous butterfly *Pieris rapae* on the related crucifer *Lepidium oleraceum* (Hasenbank et al. 2011). *Albugo sp.* is able to suppress defences of *Arabidopsis thaliana* and *Brassica juncea*, which enables otherwise incompatible downy mildew strains to infect the plants (Cooper et al. 2008); it is not known if this suppression also affects defence compounds active against herbivores.

Finally, antagonistic interactions may occur in *B. vulgaris* between the presumed salicylic acid-based signals triggered by the biotroph *Albugo* and jasmonic acid-based signals triggered by the chewing and mining flea beetles (Pieterse and Dicke 2007; Koornneef and Pieterse 2008; Thaler et al. 2012). However, the specificity of signals in response to these two

antagonists has never been tested to our knowledge.

Here, we tested whether *Albugo* infection of the two *B. vulgaris* plant types modifies their interaction with flea beetles, and *vice versa*, and whether this leads to interactive impacts on plant growth and reproduction. We experimentally applied *Albugo* and *P. nemorum*, alone and in combination, to P- and G-plants in a glasshouse and analysed (i) the degree of herbivory and pathogen infection, (ii) levels of defence related compounds and nutritional quality of the plants, (iii) biomass accumulation and iv) production of viable seeds.

Materials and methods

Experimental design

Barbarea vulgaris plants for this experiment originated from a G-type population from Kværkeby and a P-type from Tissø, both Zealand, Denmark. Both populations are well studied and typical for the two plant types with respect to resistance, saponin and glucosinolate content (Agerbirk et al. 2001; de Jong et al. 2001; Agerbirk et al. 2003b).

In March 2010, seeds were sown in a greenhouse with 18 hours light and 6 hours dark. Two hundred seedlings of each plant type were one week later transplanted individually into plastic pots with standard potting soil. Metal halide lamps (Philips HPI-T plus 400W) supplemented daylight, as saponin production in *B. vulgaris* depends on light quality. When plants were three weeks old and had four to five true leaves, they were transferred to a 15 °C dark chamber and covered with plastic to keep a high humidity.

Next day, half of the plants were inoculated with *Albugo*, using a field isolate originating from naturally infected *B. vulgaris* G-plants from the university campus. A fresh source of inoculum was maintained through serial passage in G-plants. We have never found naturally infected P-plants, and strains adapted to this plant type (if any) thus could not be included. *Albugo* sporangia were collected by tapping leaves with mature pustules onto a glass slide.

Inoculum was prepared based on a protocol by Dangl et al. (1992): sporangia were hydrated for 90 min in deionised H_2O at 15 °C, and adjusted to 7 x 10^4 sporangia per ml. The plants to be infected by *Albugo* were inoculated with 5 separate drops of 10 µl inoculum on each of their four youngest leaves, and were subsequently kept in plastic bags in darkness at 15 °C. The other half of the plants were given the same treatment, but without sporangia. After three days, all plants were transferred to 18 hours light, 6 hours dark at 17 °C. White rust began to develop ten days post inoculation (dpi); at 14 dpi the number of leaves with rust was counted (Fig 1d).

At 17 dpi, the plants were divided in two sets with pairwise matching individual sizes, and for infected plants with the same number of leaves with white rust; these sets were used for the first and second harvest, respectively (see below); each set included 66 P- and G-plants. Infected and non-infected plants were further assigned to the flea beetle treatment and a control. *Albugo* and *P. nemorum* were thus applied in a fully factorial design with 14 replicate plants per plant type, treatment, and harvest. For logistic reasons we did not include a treatment where flea beetles were added before *Albugo.*

All plants were individually covered with mesh bags, and the first portion of flea beetles were added to the assigned plants. The flea beetles were taken from a susceptible line maintained at the university as described by Nielsen (1999). Adults used in our experiment were not older than seven days, and were not sexed before used. A total of nine beetles were added in three portions over 20 days. One month after the first beetles was added (56 dpi), mesh bags and beetles were removed, and the number of leaves counted. At this time, some of the beetles had mated and larval mines were observed in leaves of 91 % of the P-plants.

One of the two sets of plants was then used to analyse biomass and chemical composition (*first harvest*). A leaf disk (8 mm Ø) from the 5^{th} youngest rosette leaf was frozen in liquid nitrogen, stored at -70 °C, and used for saponin analysis (see below). Five leaf disks (22-29

mm Ø) from the 4th, 8th and 12th youngest and the 4th and 7th oldest leaves were scanned on a flat bed scanner to quantify beetle damage, and analysed for *Albugo* infection and glucosinolate content; a disk from the 6th youngest leaf was analysed for carbon/nitrogen content (see below). The dry weight of leaves (including leaf disks) and roots were measured separately.

The other set of plants (for second harvest) was vernalized at 4 °C for three months, and transferred to a heated greenhouse with natural light in the beginning of August. When plants had started flowering after 4 weeks, plants from each treatment and plant type were placed in separate mesh tents, and male bumble bees were added as pollinators. A minimum of five bumble bees were present in each tent for one month, at which time flowering had ended. Plants were then transferred to a climate chamber for siliques to ripen.

At the *second harvest*, flowers, siliques, and seeds were dried and counted, and the flowering stalks weighed. Seed germination of 200 seeds per plant was tested on moist filter paper in two Petri dishes per plant at 14 h light/20°C, 10 h dark/ 12 °C. Seeds were considered as germinated if cotyledons emerged within 20 days.

Albugo symptoms, infection and herbivore feeding

The development of white blister rust was visually estimated at the time of first harvest (56 dpi) as the percentage leaf area covered by pustules. Each leaf was assigned to one of five damage categories: 0: no damage; 1: ≥ 0-20%; 2: 21-40%; 3: 41-60%; 4: 61-80%; and 5: 81-100% damage. The total percentage of leaf area with rust was estimated as the sum of the multiplum of the percentage of leaves of each damage class with the mean percentage damage of that class.

Albugo infection was estimated by quantitative PCR, using specific primers targeting the internal transcribed spacer (ITS) region (Ac_F2: GCTTCGGCTTGACACATTAG; Ac_R1:

197 TCCGTCTCCTTGATGACCTT; Van Mölken et al., in preparation). Briefly, the five dried
198 leaf disks scanned for herbivore consumption (see below) were ground with a mixer mill
199 (Tissuelyser II, Retsch GmbH) and the mix used for DNA and glucosinolate analyses. DNA
200 was extracted with a DNeasy Plant Mini Kit (Qiagen), and quantitative PCR performed on a
201 Mx3000P machine (Stratagene). The PCR reaction was set up in duplicate for each sample
202 using Brilliant II SYBR Green QPCR mastermix (Agilent Technologies) following the
203 manufacturer's instructions. Standards of serially diluted *Albugo* DNA in water of known
204 concentrations were included. Another standard series was used to estimate the minimum
205 amount of pure *Albugo* DNA that could be detected. After amplification, a melting curve
206 analysis ensured that only one PCR product was amplified.
207 *P. nemorum* feeding was estimated as the average percentage of the area of the five leaf
208 discs per plant consumed by adults (holes in leaves) and larvae (leaf-mines), using the
209 software ImageJ (http://rsbweb.nih.gov/ij/).

210 Plant biochemical composition

211 Saponins were extracted from the 5th youngest leaf disk by the methods of Kuzina et al.
212 (2009). They were then analysed by liquid chromatography-mass spectrometry (LC-MS) on
213 an Agilent 1100 Series LC (Agilent Technologies) coupled to a Bruker HCT-Ultra ion trap
214 mass spectrometer (Bruker Daltonics). A Gemini-NX column (Phenomenex; 3 µM, C18,
215 110A, 2 x 150 mm) was used at a flow rate of 0.2 ml · min^{-1}, proceeded by a SecurityGuard
216 (Phenomenex Gemini-NX C18 4x20 mm). Oven temperature was maintained at 35 °C. The
217 mobile phases were: A: water with 0.02 % (v/v) trifluoro acetic acid (TFAA); B: acetonitrile
218 with 0.02 % (v/v) TFAA. The gradient program was: 0 to 1 min, isocratic 12 % B; 1 to 33
219 min, linear gradient 12 to 80 % B; 33 to 35 min, linear gradient 80 % to 99 % B; 35 to 38
220 isocratic 99 % B; 38 to 45 min, isocratic 12 % B. The mass spectrometer was run in negative

electrospray mode, and the mass range m/z 400-1400 acquired.

Five saponins were scored in G-plants: 3-O-cellobiosyl-hederagenin (*m/z* [M+TFA-] 909, RT 21.5), 3-O-cellobiosyl-oleanoic acid (*m/z* [M+TFA-] 893, RT 24.4), 3-O-cellobiosyl-gypsogenin (*m/z* [M+TFA-] 907, RT 22.5), 3-O-cellobiosyl-4-epihederagenin (*m/z* [M+TFA-] 909, RT 22.6), and 3-O-cellobiosyl-cochalic acid (*m/z* [M+TFA-] 909, RT 20.7). Three putative saponin compounds were scored in P-plants; these correspond to the putative P-type saponins in Kuzina et al. (2011), based on their mass-to-charge (m/z) ([M+TFAA-]=1073 for saponins 1 (RT 14.8) and 3 (RT 15.5) ; [M+TFAA-]=1159 for saponin 2 (RT 16.2). Peak areas of the saponins were used as estimates of relative saponin content, as exact concentrations could not be determined.

Glucosinolates

Glucosinolates were extracted from 50-100 mg of ground leaf discs (same as used for estimation of herbivore consumption; see above), and analysed as described in van Leur et al. (2008). Additional standards of progoitrin, gluconapin, glucoiberin, glucobrassicanapin, glucotropeaolin, gluconasturtiin, glucoraphanin, glucocoerucin, glucobrassicin, and sinalbin (Phytoplan, Heidelberg, Germany) were used. To calculate concentrations, the glucosinolate measurements were divided by the dry weight of the sample.

Total nitrogen and carbon was measured by mass spectrometry of 3.5 to 4.5 mg of leaf tissue, which was combusted in tin capsules, and analysed with an elemental analyser (20–20; Europa Scientific, Crewe, UK) according to the Dumas method (Schjoerring et al. 1993).

Data analysis

Effects of *P. nemorum*, *Albugo* and their combination on the measured traits were analysed by ANOVA (proc GLM); all analyses were done for P- and G-plants separately, due to their difference in resistance to *P. nemorum*. If assumptions of normality and homoscedasticity did

not hold, data were transformed; otherwise we used GEE analysis (proc GENMOD) after tests of "Goodness of fit" in GENMOD to determine the appropriate distribution. Seed germination was analysed using events/trials data. Multiple comparisons were tested both in proc GLM (tdiff) and proc GENMOD (diff).

The leaf area with white rust was correlated to *Albugo* DNA levels (log); this was only done for inoculated G-plants, since P-plants hardly developed rust. Herbivore consumption was only analysed in treatments where flea beetles were applied, and only for P-plants (G-plants are resistant). Some non-inoculated plants developed white rust during the experiment and were excluded from all analyses. All tests were carried out with SAS, version 9.1 (SAS Institute Inc., Cary, USA).

Results

Albugo and flea beetle interactions

White blister rust developed on only 17 % of the P-plants as compared to 81 % of the G-plants (14 dpi; Fig. 1); a similar difference was found at first harvest (56 dpi; results not shown). Likewise, only very low levels of *Albugo* DNA were detected in leaf extracts of inoculated P-plants (without flea beetles), while the content in G-plants was much higher (Fig. 2).

Extracts of inoculated plants that were also exposed to flea beetles contained more *Albugo* DNA than inoculated plants without beetles (77 and 2.6 times more DNA in P- and G-plants, respectively, Fig. 2; Online Resource 1). Inoculated G-plants with flea beetles also developed more rust in younger parts of the plants (results not shown). The leaf area covered with rust was positively correlated with *Albugo* DNA content in G-plants (N= 28; r= 0.658; p= 0.0001).

Flea beetle larvae consumed 56 % more leaf tissue of pathogen-exposed P-plants than of

non-inoculated plants (Fig. 2). However, the area consumed by adult flea beetles was not influenced by *Albugo* infection (Fig. 2, Online Resource 1).

G-plants were highly resistant to flea beetles, as expected, and only 10 out of 25 G-plants had more than 1 % leaf area damaged by adults, and only on old leaves. Only three plants were damaged by larvae, and this never exceeded 0.1 % of leaf area (data not shown).

Biochemical changes in plants

The content of saponin 1 in P-plants increased with herbivore exposure and with pathogen infection (Fig. 3; Online Resource 1); there was a trend towards an even higher expression in the combined treatment (Table 1). Similar results were obtained for the other saponin compounds produced by P-plants (Table 1).

The resistance-causing saponin of G-plants, hederagenin cellobioside, increased in plants exposed to herbivores and even more in plants exposed also to *Albugo* (Fig. 3; Online Resource 1). In contrast, this saponin was not affected by pathogen infection alone. Similar results were obtained for the other saponins tested (Table 1; Online Resource 1).

Glucosinolates increased strongly in both P- and G-plants when exposed to flea beetles (Fig. 3). In P-plants there was an additional increase when also exposed to *Albugo*; in G-plants the glucosinolate concentration was lower in the combined treatment than when only exposed to herbivory (Online Resource 1; Fig. 3). The glucosinolates were not much affected by the pathogen treatment alone.

The carbon-nitrogen ratio of P-plants was negatively affected by flea beetles and decreased by 26 % and 29 % in the herbivore and combined treatments, respectively; this was caused by increased nitrogen concentrations (Table 1); pathogen infection did not affect the carbon-nitrogen ratio. In G-plants, the ratio was positively affected by pathogen infection, due to a decreased nitrogen concentration (Table 1); the other treatments had no effect.

293 Plant size after the herbivory and pathogen treatment

294 At the *first harvest*, herbivory had decreased both the root and shoot biomass of P- and G-
295 plants (Table 1; 28 and 16% decrease in total weight, respectively), whereas there was no
296 effect of *Albugo* or any interaction between flea beetles and *Albugo* (Table 1). Biomass
297 allocation to shoots and roots did not differ between treatments (Table 1).
298 Plants exposed to both flea beetles and *Albugo* had a lower number of leaves, whereas
299 there was only little, or no, effect of the herbivore and the pathogen on their own (Fig. 3). The
300 reduction in number of leaves for G-plants was significant although the magnitude was small
301 (Table 1; Online Resource 1).

302 Plant reproduction

303 At the *second harvest*, the number of flowers, siliques, and seeds did not differ between
304 treatments (Table 1). There was a small positive effect of flea beetles on seed weight of P-
305 plants, and a slightly negative effect of *Albugo* on seed weight of G-plants. Seed germination
306 was higher for P-plants exposed to herbivory and for G-plants exposed to the pathogen; in
307 addition, there was a significant interaction between the effects of herbivores and the
308 pathogen in both plant types. However, the number of viable seeds per plant (number of seeds
309 multiplied by germination rate) did not differ between treatments for neither P- or G-plants
310 (Table 1).

311 ## Discussion

312 Our results show that the insect-resistant G-plants of *Barbarea vulgaris* are much more prone
313 to *Albugo sp*. infection than the insect-susceptible P-plants. *Albugo* and flea beetles clearly
314 affect each others performance on the plant, and induce enhanced levels of plant defence
315 compounds in some of the combined treatments. However, plant biomass was affected only

by flea beetles in P-plants, and overall reproduction was not affected by any of the treatments, indicating that plants were able to compensate for resource losses to the pathogen and herbivores.

Different responses to Albugo of the two plant types

A difference in susceptibility to *Albugo* between P- and G-plants was originally suggested by Toneatto (2009), based on spontaneously infected plants in a crossing experiment. Here we found the same difference between plant types: less than 20% of the P-plants developed white rust and hardly any plants contained *Albugo* DNA, while more than 80% of the G-plants developed white rust and contained *Albugo* DNA. Other inoculation experiments by our group have shown equivalent differences in white rust development, using other P- and G-type populations of *B. vulgaris* (Christensen, Heimes, Laybourn, Van Mölken and Hauser, unpublished). Furthermore, we have found white blister rust in natural populations of G-plants, but never in P-populations (Van Mölken et al., in prep). The difference in susceptibility to *Albugo* between P- and G-plants thus seems to be associated with the overall divergence between the two plant types of *B. vulgaris* (Agerbirk et al. 2003a; Hauser et al. 2012).

Leaf extracts of a few P-plants contained *Albugo* DNA but no white rust was observed on the plants. This may be caused by asymptomatic endophytic infections of the plants by *Albugo*, as suggested by Jacobson et al. (1998) and Ploch and Thines (2011).

Interactions between *Albugo* and flea beetles

Albugo and flea beetles clearly facilitated each other, with more white rust and *Albugo* DNA in plants also exposed to flea beetles and a higher consumption of larvae in plants also exposed to *Albugo*. Flea beetles probably spread sporangia among leaves and thereby enhanced dispersal and reinfection. *Albugo* usually enter the plant via the stomata and has to

grow actively through plant tissue to spread beyond the point of initial infection. Physical movement of inoculum could thus increase spread and infection success.

The higher consumption by larvae in *Albugo*-infected P-plants, despite the low infection success of *Albugo* in these plants, could be caused by a lower food quality or palatability that forces larvae to feed more to obtain the necessary nutrients. Especially glucosinolates increased in plants exposed to both *P. nemorum* and *Albugo*, and even though the P-type glucosinolates (and saponins) do not confer resistance to the beetles they may decrease digestibility. Alternatively, *Albugo* could have suppressed unknown defence component that otherwise restricts larval feeding.

Changes in plant biochemistry

Saponins and glucosinolates were strongly upregulated upon flea beetle attack. Induction of the saponins is new to us, as we have so far considered them to be constitutively produced during the growing season (but see van Leur et al. (2006)). The increased production of hederagenin cellobioside upon flea beetle attack, as well as other G-type saponins, fit their function in resistance against these (Nielsen 1997; Agerbirk et al. 2003a; Kuzina et al. 2009; Kuzina et al. 2011). However, the increased production of saponins by P-plants exposed to flea beetles does not seem adaptive as these saponins clearly do not impede flea-beetle feeding.

Saponins were also upregulated by *Albugo* exposure in P-plants and in the combined treatments of both P- and G- plants (however, only significant in G-plants). As most P-plants did not develop white rust upon *Albugo* inoculation, this could suggest a role of P-type saponins in resistance. Indeed, some saponins are known to confer resistance against pathogens (Osbourn 1996). Preliminary results, however, suggest that this is not the case (Christensen et al, unpublished). Szakiel et al. (2011) suggested that induction of saponins is

part of an overall plant defence system, and especially P-type saponins may thus be induced inspecifically by pathogens and herbivores, even by species on which they have no effect.

The strong induction of glucosinolates by flea beetles is in agreement with several other studies (reviewed by Hopkins et al. (2009)); however, another study of *B. vulgaris* did not find increased concentrations when exposed to the root fly *Delia radicum*, even though glucosinolates were induced by jasmonic acid application (van Leur et al. 2006; van Leur et al. 2008), and concentrations in *Brassica nigra* were not increased by the flea beetle *P. cruciferae* (Traw 2002; Traw and Dawson 2002). Glucosinolate induction by herbivores may thus depend on the species pairs involved. However, glucosinolates are not responsible for the strong resistance of *B. vulgaris* G-plants against flea-beetles (Agerbirk et al. 2003b; Kuzina et al. 2011), suggesting that their induction is triggered as part of a general response to insect damage, as for the saponins.

In P-plants, *Albugo* induced higher glucosinolate concentrations when together with beetles, but not on its own. Glucosinolates may protect plants against fungal pathogens (Halkier and Gershenzon 2006), but to our knowledge it has not been studied if they also affect oomycetes. The defensive effect of glucosinolates requires cell damage, and *Albugo* infection may not cause enough damage to trigger this.

In most of the combined treatments the content of saponins and glucosinolates was approximately additive (i.e. equal to the sum of induced concentrations of the single treatments), or perhaps slightly synergistic. This does not support recent hypotheses on antagonistic interactions between different plant defence signalling systems (Koornneef and Pieterse 2008; Thaler et al. 2012). *Albugo*, as a biotroph pathogen, is expected to trigger a salicylic acid-based defence signalling, which may antagonise the jasmonic acid-based signalling triggered by the cell-damaging flea beetles. Only for glucosinolates in G-plants did we find an antagonistic interaction, where the content of glucobarbarin was significantly

lower in the combined treatment than in the treatment with only flea beetles. We have no reasonable explanation for why the plant types differ in this respect.

Flea-beetles increased the amount of nitrogen relative to carbon in P-plants, but not in G-plants. This was measured as total nitrogen, and may reflect the increase in glucosinolate content when exposed to herbivory. Gomez et al. (2010) have shown that nitrogen may be re-allocated to other parts of the plant upon herbivory as a strategy to preserve nitrogen for re-growth. Whether this was the case for *B. vulgaris* we cannot determine as roots were not analysed.

Plant performance and reproduction

Plants that had been exposed to both *Albugo* and flea beetles had fewer leaves at first harvest than those exposed to only one of them. This may be explained by the increased damage by larvae in *Albugo* infected plants and the increased *Albugo* infection in plants with flea beetles. Surprisingly, *Albugo* has a negative effect on the number of leaves in the P-plants, even though most of these plants are resistant to *Albugo* (i.e. do not develop white blister rust upon inoculation), but only if the plants were also affected by flea beetles. Similarly, flea beetles decreased biomass also in the flea-beetle resistant G-plants, and had a negative impact on the number of leaves in those plants when together with *Albugo*. This indicates that even when plants are resistant they have to spend resources on defences (Agrawal et al. 1999), which may otherwise have been used for producing leaves and biomass. Our observation that saponins and glucosinolates were strongly upregulated by flea beetles in both resistant and susceptible plants supports this.

At maturity, plants did not differ in reproductive output among the four treatments, despite the differences in pathogen infection, herbivore feeding, leaf number and biomass at first harvest. Likewise, there were no differences in total biomass of the flowering stalks.

Between first and second harvest, plants grew enormously, branched prolifically, and have most likely outgrown the earlier differences. Thus, plasticity in growth-related traits may allow plants to compensate for resource losses from an early attack (Paul et al. 2000; Nunez-Farfan et al. 2007; Fornoni 2011). This is in agreement with recent meta-analyses that pathogens and herbivores can strongly influence each other and the plant parts they attack, but that plant biomass and reproduction is on average less affected by such interactive impacts (Morris et al. 2007; Hauser et al. 2013).

Implications

A growing number of studies have shown that arthropod herbivores and plant-associated microorganisms can seriously affect each other while on the same plant, either directly or mediated by the plant (Hatcher 1995; Hatcher and Paul 2000; Mouttet et al. 2011; Paul et al. 2000; Rostas and Hilker 2002; Stout et al. 2006; Tack and Dicke 2013). Our study shows clear examples this, both for antagonist success and induced changes in the plant that may subsequently affect both (and other) antagonists. However, while these immediate plant-pathogen-herbivore interactions may be interesting and important, their impact on plant performance, fitness and yield may be strongly moderated by compensatory growth (Fournier et al. 2006; Hauser et al. 2013), as also shown by our results. Unfortunately, very little is known about this; in the meta-analysis of Hauser et al. (2013), only 35 data sets could be found that had estimated combined impacts of herbivores and pathogens on plant performance, despite its clear relevance for ecologists and agronomists alike.

An interesting aspect from our study is that plant chemical defences may be upregulated upon combined attack by pathogens and herbivores, even when plants are resistant to one of the antagonists. In *B. vulgaris* this may be due to the induction of a generalized defence response by both *Albugo* and flea beetles, but this may however differ among plant species,

specificity of the defence systems, and which antagonist they encounter.

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References

Agerbirk N, Olsen CE, Nielsen JK (2001) Seasonal variation in leaf glucosinolates and insect resistance in two types of *Barbarea vulgaris* ssp *arcuata*. Phytochemistry 58:91-100.

Agerbirk N, Olsen CE, Bibby BM, Frandsen HO, Brown LD, Nielsen JK, Renwick JAA (2003a) A saponin correlated with variable resistance of *Barbarea vulgaris* to the diamondback moth *Plutella xylostella*. Journal of Chemical Ecology 29:1417-1433.

Agerbirk N, Ørgaard M, Nielsen JK (2003b) Glucosinolates, flea beetle resistance, and leaf pubescence as taxonomic characters in the genus Barbarea (Brassicaceae). Phytochemistry 63:69-80.

Agerbirk N, Olsen CE (2011) Isoferuloyl derivatives of five seed glucosinolates in the crucifer genus Barbarea. Phytochemistry 72:610-623.

Agrawal AA, Strauss SY, Stout MJ (1999) Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. Evolution 53:1093-1104.

Augustin JM, Kuzina V, Andersen SB, Bak S (2011) Molecular activities, biosynthesis and evolution of triterpenoid saponins. Phytochemistry 72:435-457.

Augustin JM, Drok S, Shinoda T, Sanmiya K, Nielsen JK, Khakimov B, Olsen CE, Hansen EH, Kuzina V, Ekstrøm CT, Hauser T, Bak S (2012) UDP-glycosyltransferases from the UGT73C subfamily in *Barbarea vulgaris* catalyze sapogenin 3-O-glucosylation in saponin-mediated insect resistance. Plant Physiology 160:1881-1895.

Choi Y-J, Shin H-D, Ploch S, Thines M (2011) Three new phylogenetic lineages are the closest relatives of the widespread species *Albugo candida*. Fungal Biology 115:598-607.

Cooper AJ, Latunde-Dada AO, Woods-Tor A, Lynn J, Lucas JA, Crute IR, Holub EB (2008) Basic compatibility of *Albugo candida* in *Arabidopsis thaliana* and *Brassica juncea* causes broad-spectrum suppression of innate immunity. Molecular Plant-Microbe Interactions 21:745-756.

Dangl J, Holub E, Debener T, Lehnackers H, Ritter C, Crute I, Koncz C, Chua N, Schell J (1992) Genetic definition of loci involved in Arabidopsis-pathogen interactions. In: Koncz CC, N. H.;Schell, J. (ed) Methods in Arabidopsis research. World Scientific Publishing Co, Singapore, pp 393-418

de Jong PW, de Vos H, Nielsen JK (2001) Demic structure and its relation with the distribution of an adaptive trait in Danish flea beetles. Molecular Ecology 10:1323-1332.

Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56:5-51.

Fornoni J (2011) Ecological and evolutionary implications of plant tolerance to herbivory. Functional Ecology 25:399-407.

Fournier V, Rosenheim JA, Brodeur J, Diez JM, Johnson MW (2006) Multiple plant exploiters on a shared host: Testing for nonadditive effects on plant performance. Ecological Applications 16:2382-2398.

Gomez S, Ferrieri RA, Schueller M, Orians CM (2010) Methyl jasmonate elicits rapid changes in carbon and nitrogen dynamics in tomato. New Phytologist 188:835-844.

Griffiths DW, Deighton N, Birch ANE, Patrian B, Baur R, Stadler E (2001) Identification of glucosinolates on the leaf surface of plants from the Cruciferae and other closely related species. Phytochemistry 57:693-700.

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annual

494 Review of Plant Biology, vol 57, pp 303-333
495 Hasenbank M, Brandon A, Hartley S (2011) White butterfly (*Pieris rapae*) and the white rust
496 *Albugo candida* on Cook's scurvy grass (*Lepidium oleraceum*). New Zealand Journal
497 of Ecology 35:69-75.
498 Hatcher PE (1995) Three-way interactions between plant-pathogenic fungi, herbivorous
499 insects and their host plants. Biological Reviews 70:639-694.
500 Hatcher PE, Paul ND (2000) On integrating molecular and ecological studies of plant
501 resistance: variety of mechanisms and breadth of antagonists. Journal of Ecology
502 88:702-706.
503 Hauser T, Toneatto F, Nielsen J (2012) Genetic and geographic structure of an insect resistant
504 and a susceptible type of *Barbarea vulgaris* in western Europe. Evolutionary Ecology
505 26:611-624.
506 Hauser TP, Christensen S, Heimes C, Kiær LP (2013) Combined effects of arthropod
507 herbivores and phytopathogens on plant performance. Functional Ecology in press.
508 Hopkins RJ, van Dam NM, van Loon JJA (2009) Role of glucosinolates in insect-plant
509 relationships and multitrophic interactions. Annual Review of Entomology, vol 54, pp
510 57-83
511 Jacobson DJ, LeFebvre SH, Ojerio RS, Berwald N, Heikkinen E, Ql (1998) Persistent,
512 systemic, asymptomatic infections of *Albugo candida*, an oomycete parasite, detected
513 in three wild crucifer species. Canadian Journal of Botany-Revue Canadienne De
514 Botanique 76:739-750.
515 Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiology 146:839-
516 844.
517 Kuzina V, Ekstrom CT, Andersen SB, Nielsen JK, Olsen CE, Bak S (2009) Identification of
518 defense compounds in *Barbarea vulgaris* against the herbivore *Phyllotreta nemorum*
519 by an ecometabolomic approach. Plant Physiology 151:1977-1990.
520 Kuzina V, Nielsen JK, Augustin JM, Torp AM, Bak S, Andersen SB (2011) *Barbarea*
521 *vulgaris* linkage map and quantitative trait loci for saponins, glucosinolates, hairiness
522 and resistance to the herbivore *Phyllotreta nemorum*. Phytochemistry 72:188-198.
523 Mathur P, Sharma E, Singh SD, Bhatnagar AK, Singh VP, Kapoor R (2013) Effect of
524 elevated CO2 on infection of three foliar diseases in oilseed *Brassica juncea*. Journal
525 of Plant Pathology 95:135-144.
526 Morris WF, Hufbauer RA, Agrawal AA, Bever JD, Borowicz VA, Gilbert GS, Maron JL,
527 Mitchell CE, Parker IM, Power AG, Torchin ME, Vazquez DP (2007) Direct and
528 interactive effects of enemies and mutualists on plant performance: A meta-analysis.
529 Ecology 88:1021-1029.
530 Mouttet R, Bearez P, Thomas C, Desneux N (2011) Phytophagous arthropods and a pathogen
531 sharing a host plant: evidence for indirect plant-mediated interactions. PLoS One
532 6:e18840.
533 Nielsen J, Nagao T, Okabe H, Shinoda T (2010) Resistance in the plant, *Barbarea vulgaris*,
534 and counter-adaptations in flea beetles mediated by saponins. Journal of Chemical
535 Ecology 36:277-285.
536 Nielsen JK (1997) Variation in defences of the plant *Barbarea vulgaris* and in
537 counteradaptations by the flea beetle *Phyllotreta nemorum*. Entomologia
538 Experimentalis et Applicata 82:25-35.
539 Nielsen JK (1999) Specificity of a Y-linked gene in the flea beetle *Phyllotreta nemorum* for
540 defences in *Barbarea vulgaris*. Entomologia Experimentalis et Applicata 91:359-368.
541 Nunez-Farfan J, Fornoni J, Valverde PL (2007) The evolution of resistance and tolerance to
542 herbivores. Annual Review of Ecology Evolution and Systematics 38:541-566.

543 Osbourn A (1996) Saponins and plant defence - A soap story. Trends in Plant Science 1:4-9.
544 Paul ND, Hatcher PE, Taylor JE (2000) Coping with multiple enemies: an integration of
545 molecular and ecological perspectives. Trends in Plant Science 5:220-225.
546 Pieterse CMJ, Dicke M (2007) Plant interactions with microbes and insects: from molecular
547 mechanisms to ecology. Trends in Plant Science 12:564-569.
548 Ploch S, Thines M (2011) Obligate biotrophic pathogens of the genus Albugo are widespread
549 as asymptomatic endophytes in natural populations of Brassicaceae. Mol Ecol
550 20:3692-3699.
551 Renwick JAA (2002) The chemical world of crucivores: lures, treats and traps. Entomologia
552 Experimentalis et Applicata 104:35-42.
553 Rostas M, Hilker M (2002) Asymmetric plant-mediated cross-effects between a herbivorous
554 insect and a phytopathogenic fungus. Agricultural and Forest Entomology 4:223-231.
555 Schjoerring JK, Kyllingsbaek A, Mortensen JV, ByskovNielsen S (1993) Field investigations
556 of ammonia exchange between barley plants and the atmosphere. 1. Concentration
557 profiles and flux densities of ammonia. Plant Cell Environ. 16:161-167.
558 Schlaeppi K, Abou-Mansour E, Buchala A, Mauch F (2010) Disease resistance of
559 Arabidopsis to *Phytophthora brassicae* is established by the sequential action of
560 indole glucosinolates and camalexin. Plant Journal 62:840-851.
561 Shinoda T, Nagao T, Nakayama M, Serizawa H, Koshioka M, Okabe H, Kawai A (2002)
562 Identification of a triterpenoid saponin from a crucifer, *Barbarea vulgaris*, as a
563 feeding deterrent to the diamondback moth, *Plutella xylostella*. Journal of Chemical
564 Ecology 28:587-599.
565 Stout MJ, Thaler JS, Thomma BPHJ (2006) Plant-mediated interactions between pathogenic
566 microorganisms and herbivorous arthropods. Annual Review of Entomology 51:663-
567 689.
568 Szakiel A, Paczkowski C, Henry M (2011) Influence of environmental biotic factors on the
569 content of saponins in plants. Phytochemistry Reviews 10:493-502.
570 Tack AJM, Dicke M (2013) Plant pathogens structure arthropod communities across multiple
571 spatial and temporal scales. Functional Ecology 27:633-645.
572 Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal
573 crosstalk. Trends in Plant Science 17:260-270.
574 Toneatto F (2009) Genetics and distribution of insect resistance polymorphism in *Barbarea*
575 *vulgaris*. PhD, University of Copenhagen, Frederiksberg
576 Toneatto F, Nielsen JK, Ørgaard M, Hauser TP (2010) Genetic and sexual separation between
577 insect resistant and susceptible *Barbarea vulgaris* plants in Denmark. Molecular
578 Ecology 19:3456-3465.
579 Toneatto F, Hauser TP, Nielsen JK, Ørgaard M (2012) Genetic diversity and similarity in the
580 *Barbarea vulgaris* complex (Brassicaceae). Nordic Journal of Botany 30:506-512.
581 Traw MB (2002) Is induction response negatively correlated with constitutive resistance in
582 black mustard? Evolution 56:2196-2205.
583 Traw MB, Dawson TE (2002) Reduced performance of two specialist herbivores
584 (Lepidoptera : Pieridae, Coleoptera : Chrysomelidae) on new leaves of damaged black
585 mustard plants. Environmental Entomology 31:714-722.
586 van Leur H, Raaijmakers CE, van Dam NM (2006) A heritable glucosinolate polymorphism
587 within natural populations of *Barbarea vulgaris*. Phytochemistry 67:1214-1223.
588 van Leur H, Raaijmakers CE, van Dam NM (2008) Reciprocal interactions between the
589 cabbage root fly (*Delia radicum*) and two glucosinolate phenotypes of *Barbarea*
590 *vulgaris*. Entomologia Experimentalis et Applicata 128:312-322.
591 van Molken T, de Caluwe H, Hordijk CA, Leon-Reyes A, Snoeren TAL, van Dam NM,

592 Stuefer JF (2012) Virus infection decreases the attractiveness of white clover plants
593 for a non-vectoring herbivore. Oecologia 170:433-444.
594 Wang Y, Bouwmeester K, Van de Mortel JE, Shan WX, Govers F (2013) A novel
595 Arabidopsis-oomycete pathosystem: differential interactions with *Phytophthora*
596 *capsici* reveal a role for camalexin, indole glucosinolates and salicylic acid in defence.
597 Plant Cell Environ. 36:1192-1203.
598
599
600

Figure legends

Fig. 1. Species used in this study: (a) *Barbarea vulgaris* in the rosette and flowering stage. Inserts show a pubescent P-type leaf in the lower left corner and a glabrous G-type leaf in the lower middle part; (b) *Phyllotreta nemorum* adult (left) and larva (right); (c) white blister rust (pustules) caused by the *Albugo sp.*; (d) number of P- and G-plants showing symptoms 14 days post inoculation.

Fig. 2. *Albugo* DNA content and flea beetle damage after infestation with flea beetles (Herb), *Albugo* (Path) and both (H&P). Mean values ± SE are shown for (a) ng *Albugo* DNA (out of 10 ng total) in leaves of P- and G-plants; insert shows a DNA melting curve (temperature (°C) x fluorescence) demonstrating that only one PCR product was amplified; (b) percentage of leaf area consumed by beetles and larvae in flea beetle susceptible P-plants; G-plants are resistant and not damaged. Columns with different letters of the same case are significantly different at $p < 0.05$.

Fig. 3. Plant traits affected by interactions between flea beetles (Herbivore) and *Albugo* (Pathogen): (a) saponin content, (b) glucosinolate concentrations (micromoles per gram dry mass); (c) number of leaves; shown for P- and G-plants separately. Columns indicate mean values ± SE; note that y-axes differ. Columns with different letters are significantly different at $p < 0.05$.

Tables

Table 1. Mean values (± SE) of traits measured on plant of the two plant types in the control, herbivore, pathogen, and combined treatments. Significant differences between a treatment and the control are indicated by bold types; significance levels for interaction terms are indicated by asterisks ((*): p<0.1; *: p<0.05; **: p<0.01;***: p<0.001). Results from statistical analyses in Online Resource 1.

	P- plants				G-plants			
Traits	Control	Herbivore	Pathogen	Herbivore + pathogen	Control	Herbivore	Pathogen	Herbivore + pathogen
Percentage adult flea beetle damage		2.8 ± 0.6		3.0 ± 0.6				
Percentage flea beetle larvae damage		7 ± 3		**12 ± 3 ***				
A. candida DNA (ng)	0.000 ± 0.0000	0.000 ± 0.0001	0.006 ± 0.0014	**0.461 ± 0.4474 ***	0.000 ± 0.0001	0.000 ± 0.0001	0.626 ± 0.3518	**1.662 ± 0.6611 ****
Saponin 1 | Hederagenin cellobioside [a]	138 ± 26	**278 ± 46**	**283 ± 65**	361 ± 66 (*)	134 ± 6	**183 ± 8**	128 ± 5	**206 ± 8 ***
Saponin 2 | Cochalic acid cellobioside [a]	225 ± 36	**403 ± 52**	**451 ± 102**	496 ± 76 (*)	15 ± 3	**50 ± 7**	**12 ± 4**	**73 ± 6 ***
Saponin 3 | Oleanolic acid cellobioside [a]	93 ± 23	**214 ± 41**	**242 ± 61**	355 ± 73 (*)	78 ± 5	**134 ± 8**	78 ± 7	165 ± 11 (*)
Glucosinolates ($\mu mol \cdot g^{-1}$) [b]	19 ± 2	**39 ± 5**	**26 ± 3**	56 ± 3	25 ± 4	**56 ± 3**	**25 ± 2**	**40 ± 2 ****
Percentage nitrogen	2.0 ± 0.2	**2.4 ± 0.2**	2.4 ± 0.3	2.7 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	**1.8 ± 0.2**	1.9 ± 0.1
Percentage carbon	42.0 ± 0.4	**40.9 ± 0.4**	**43.2 ± 0.3**	41.6 ± 0.4	42.2 ± 0.2	42.0 ± 0.3	42.0 ± 0.3	42.4 ± 0.4
Carbon-nitrogen ratio	23.5 ± 1.7	**17.5 ± 1.0**	21.6 ± 2.8	16.6 ± 1.3	21.4 ± 1.4	20.8 ± 1.2	**26.1 ± 2.2**	23.5 ± 1.1
Root biomass (g)	1.96 ± 0.08	**1.35 ± 0.11**	**2.24 ± 0.09**	1.46 ± 0.15	1.66 ± 0.11	**1.48 ± 0.11**	1.66 ± 0.09	1.40 ± 0.07
Shoot biomass (g)	6.32 ± 0.16	**4.55 ± 0.27**	6.81 ± 0.15	4.53 ± 0.39	6.61 ± 0.22	**5.90 ± 0.27**	6.88 ± 0.28	6.05 ± 0.23
Root-shoot ratio	0.31 ± 0.01	0.29 ± 0.02	0.33 ± 0.01	0.32 ± 0.02	0.25 ± 0.02	0.25 ± 0.02	0.24 ± 0.01	0.23 ± 0.01
Number of leaves	37.4 ± 1.8	**35.7 ± 1.6**	37.6 ± 1.1	34.1 ± 1.1	40.6 ± 1.7	**40.8 ± 1.7**	41.8 ± 2.0	**36.5 ± 1.4 ***
Biomass per leaf (g)	0.18 ± 0.01	**0.13 ± 0.01**	0.19 ± 0.01	0.13 ± 0.01	0.17 ± 0.01	**0.14 ± 0.01**	0.17 ± 0.01	0.17 ± 0.01
Total number of flowers	719 ± 81	819 ± 92	803 ± 77	851 ± 98	599 ± 132	676 ± 113	533 ± 63	470 ± 53
Total number of seed pods	230 ± 30	286 ± 40	278 ± 26	265 ± 29	404 ± 75	379 ± 63	319 ± 30	322 ± 33
Number of seeds	1596 ± 290	1970 ± 495	2221 ± 356	1692 ± 200	4252 ± 745	3899 ± 828	3550 ± 325	3135 ± 377
Biomass per seed (mg)	0.32 ± 0.02	**0.38 ± 0.02**	0.32 ± 0.02	0.34 ± 0.03 (*)	0.52 ± 0.02	0.51 ± 0.02	**0.51 ± 0.01**	0.48 ± 0.02
Biomass flowering stalks (g)	6.4 ± 0.4	7.1 ± 0.6	7.0 ± 0.4	5.8 ± 0.4	6.5 ± 0.9	6.9 ± 0.8	6.1 ± 0.5	5.5 ± 0.5
Seed germination (%)	79 ± 5	**87 ± 3**	82 ± 4	**87 ± 3 ***	73 ± 11	66 ± 7	**76 ± 5**	**79 ± 7 ****
Total number of germinating seeds	1311 ± 268	1765 ± 482	1792 ± 330	1455 ± 187	3407 ± 814	2504 ± 465	2887 ± 321	2838 ± 445

[a] P-plants produce saponins 1-3, only, G-plants produce hederagenin, cochalic acid, and oleanolic acid cellobiocide; values show peak areas. [b] P-plants produce mainly glucosibarin, G-plants glucobarbarin.

Figs

Figure 1

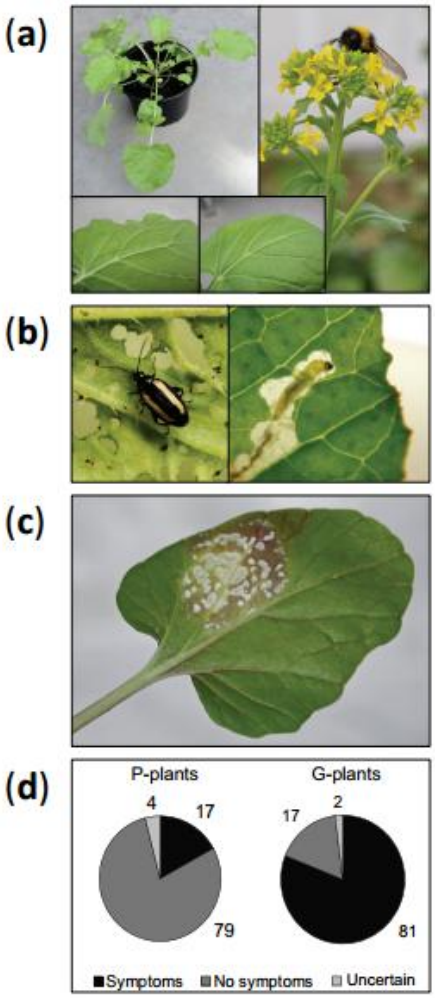


Figure 2

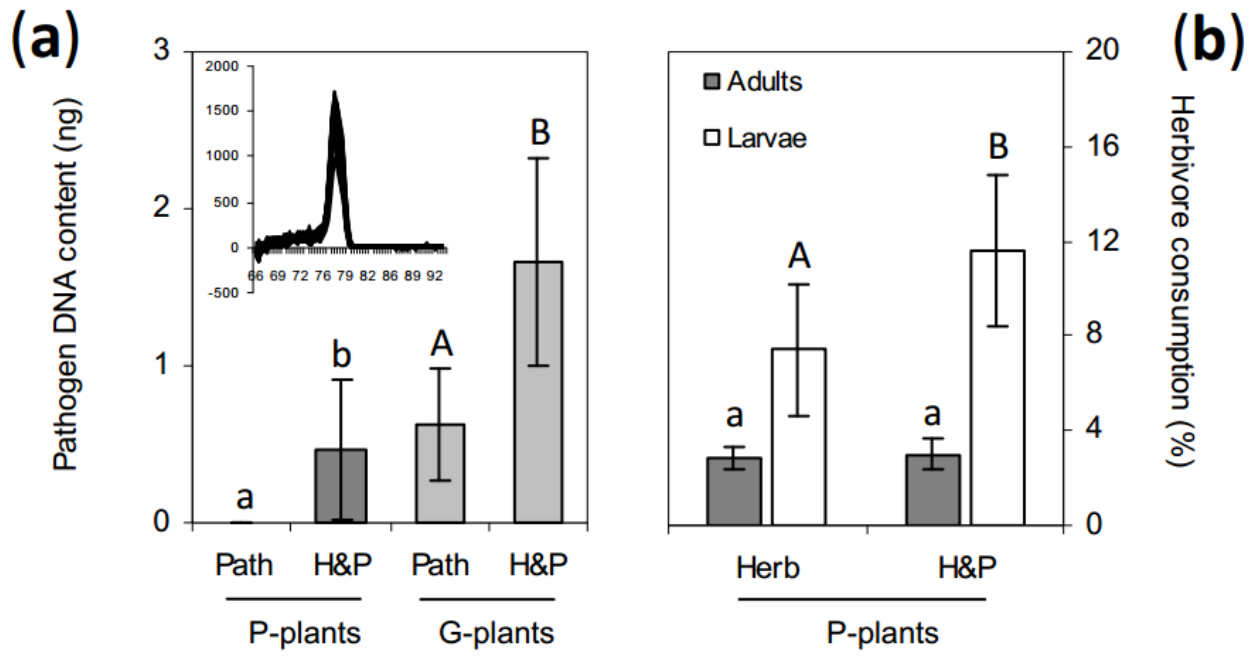
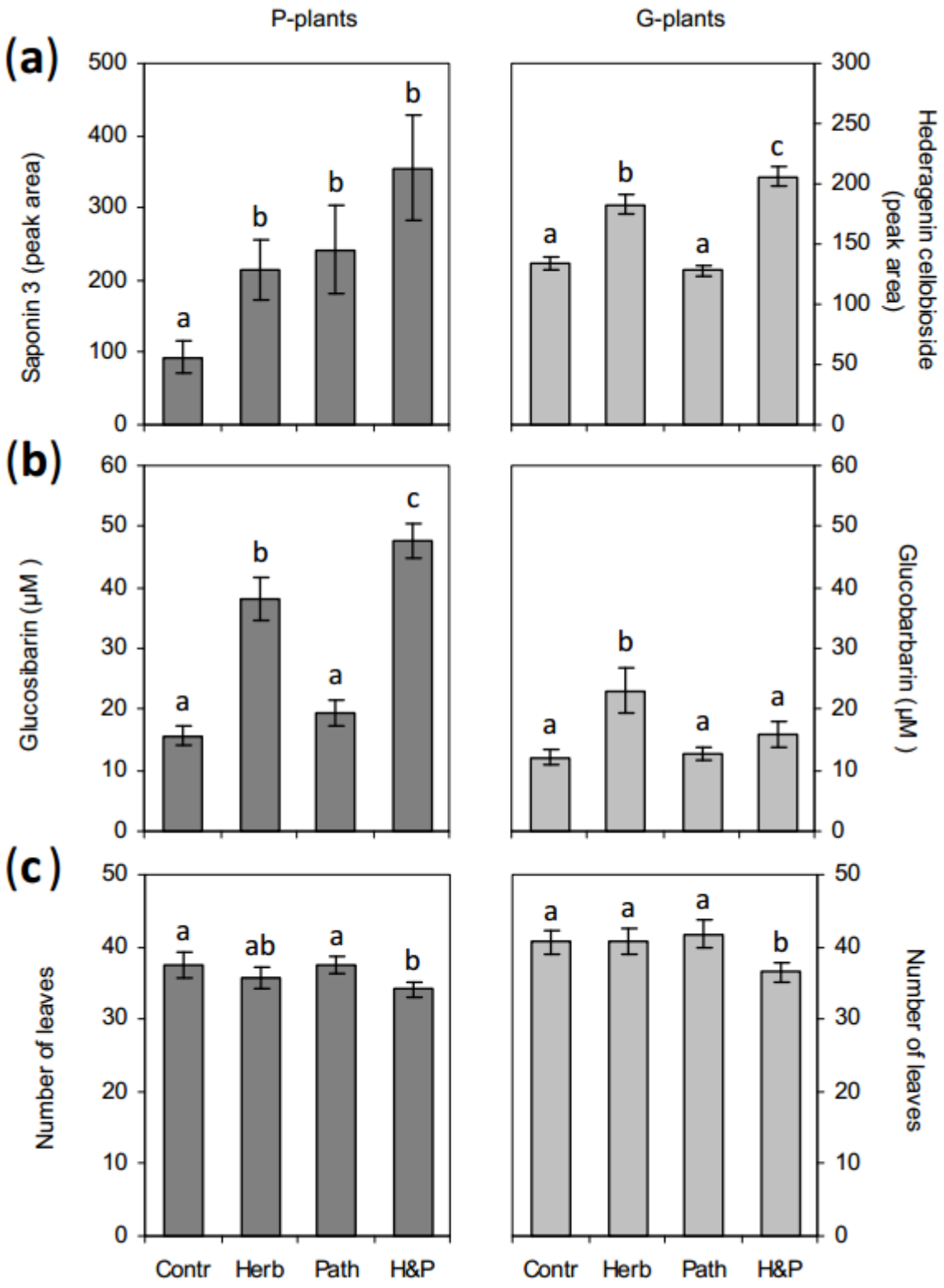

(a)
Pathogen DNA content (ng)
3
2
1
0
2000
1500
1000
500
0
-500
66 69 72 76 79 82 86 89 92
a
b
A
B
Path
H&P
Path
H&P
P-plants
G-plants
(b)
Herbivore consumption (%)
20
16
12
8
4
0
Adults
Larvae
a
A
a
B
Herb
H&P
P-plants

Figure 3


P-plants
G-plants
(a)
Saponin 3 (peak area)
Hederagenin cellobioside (peak area)
(b)
Glucosibarin (µM)
Glucobarbarin (µM)
(c)
Number of leaves
Number of leaves
Contr
Herb
Path
H&P
Contr
Herb
Path
H&P

Supplementary Table 1. Statistical analysis (ANOVA: F, or Genmod: X^2) of the consumption by flea beetles (herbivore), *Albugo candida* (pathogen) and their combination, and their effects on biochemical, growth, and reproductive traits in P- and G-plants of *Barbarea vulgaris*. Traits with a mean square for the error term were tested by ANOVA, traits without by proc GENMOD. Significant effects are indicated in bold.

P-plants	Error		Herbivore				Pathogen				Herbivore x pathogen			
Traits	df*	MS†	df*	MS†	F/ X^2 ‡	*p*	df*	MS†	F/ X^2 ‡	*p*	df*	MS†	F/ X^2 ‡	*p*
% Adult flea beetle consumption							1		0.00	0.9802				
% Flea beetle larvae consumption							1		4.54	**0.0331**				
% Total flea beetle consumption							1		0.69	0.4050				
A. candida DNA			1		7.58	**0.0059**								
P-type saponin 1			1		6.36	**0.0117**	1		8.65	**0.0033**	1		3.65	0.0561
P-type saponin 2			1		4.94	**0.0262**	1		7.74	**0.0054**	1		3.26	0.0711
P-type saponin 3			1		6.95	**0.0084**	1		9.57	**0.0020**	1		2.77	0.0959
Glucosibarin			1		39.8	**<0.0001**	1		13.71	**0.0002**	1		1.75	0.1859
% Nitrogen	50	0.020	1	0.110	5.55	**0.0224**	1	0.038	1.91	0.1730	1	0.004	0.20	0.6547
% Carbon	50	1.832	1	26.74	14.6	**0.0004**	1	12.61	6.88	**0.0115**	1	0.715	0.39	0.5349
Carbon-nitrogen ratio	50	0.019	1	0.148	7.97	**0.0068**	1	0.025	1.33	0.2542	1	0.003	0.16	0.6885
Root biomass	50	5.401	1	223.3	41.4	**<0.0001**	1	33.85	6.27	**0.0156**	1	7.998	1.48	0.2294
Shoot biomass			1		41.5	**<0.0001**	1		0.85	0.3553	1		0.98	0.3213
Total biomass			1		42.1	**<0.0001**	1		1.58	0.2085	1		0.97	0.3238
Root-shoot ratio	50	0.003	1	0.003	0.81	0.3729	1	0.006	1.74	0.1936	1	0.000	0.02	0.8753
% Biomass allocation to shoot			1		0.15	0.6938	1		0.24	0.6222	1		0.00	0.9606
% Biomass allocation to roots			1		0.50	0.4779	1		0.79	0.3751	1		0.01	0.9111
Number of leaves			1		4.86	**0.0275**	1		0.43	0.5136	1		0.63	0.4286
Biomass per leaf	50	0.002	1	0.040	26.2	**<0.0001**	1	0.001	0.36	0.5527	1	0.000	0.15	0.7004
Total number of flowers	51	108372	1	85958	0.79	0.3773	1	38844	0.36	0.5520	1	5914	0.05	0.8162
Total number of seed pods	51	14185	1	8096	0.57	0.4534	1	1574	0.11	0.7404	1	13622	0.96	0.3317
Total seed weight			1		0.49	0.4855	1		0.00	0.9443	1		2.22	0.1361
Number of seeds			1		0.00	0.9662	1		0.10	0.7497	1		1.37	0.2418
Biomass per seed	51	0.006	1	0.027	4.44	**0.0400**	1	0.009	1.41	0.2399	1	0.003	0.47	0.4942
Biomass flowering stalks	51	2.692	1	0.206	0.08	0.7831	1	3.298	1.22	0.2736	1	9.396	3.49	0.0675
Seed germination			1		70.5	**<0.0001**	1		1.76	0.1851	1		4.10	**0.0428**
Total number of germinating seeds			1		0.06	0.8052	1		0.25	0.6202	1		1.01	0.3145

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G-plants	Error		Herbivore				Pathogen				Herbivore x pathogen			
Traits	df*	MS†	df*	MS†	F/ X^2 ‡	*p*	df*	MS†	F/ X^2 ‡	*p*	df*	MS†	F/ X^2 ‡	*p*
A. candida DNA			1		6.81	**0.0091**								
Hederagenin cellobioside	50	561.4	1	53540	95.4	**<0.0001**	1	975.6	1.74	0.1934	1	2821	5.02	**0.0295**
Cochalic acid cellobioside	50	320.7	1	30462	95.0	**<0.0001**	1	1375	4.29	**0.0436**	1	2221	6.93	**0.0113**
Oleanolic acid cellobioside	50	0.011	1	1.089	103.0	**<0.0001**	1	0.021	1.95	0.1684	1	0.031	2.95	0.0923
Gypsegenin cellobioside	50	169.7	1	11389	67.1	**<0.0001**	1	48.19	0.28	0.5964	1	330.4	1.95	0.1691
4-Ephihederagenin cellobioside	50	66.34	1	6742	101.6	**<0.0001**	1	191.3	2.88	0.0957	1	273.5	4.12	**0.0476**
Glucobarbarin	47	62.76	1	6745	107.5	**<0.0001**	1	784.5	12.5	**0.0009**	1	753.5	12.0	**0.0011**
% Nitrogen	50	0.013	1	0.006	0.46	0.5021	1	0.059	4.59	**0.0370**	1	0.003	0.24	0.6234
% Carbon	50	1.141	1	0.246	0.22	0.6444	1	0.246	0.22	0.6444	1	1.380	1.21	0.2768
Carbon-nitrogen ratio	50	0.012	1	0.005	0.44	0.5086	1	0.061	5.30	**0.0256**	1	0.002	0.17	0.6851
Root biomass	50	0.251	1	0.680	5.43	**0.0239**	1	0.022	0.17	0.6782	1	0.020	0.16	0.6902
Shoot biomass	50	0.831	1	7.835	9.42	**0.0035**	1	0.572	0.69	0.4108	1	0.041	0.05	0.8262
Total biomass	50	1.274	1	13.13	10.3	**0.0023**	1	0.370	0.29	0.5921	1	0.118	0.09	0.7624
Root-shoot ratio	50	0.004	1	0.001	0.28	0.5978	1	0.006	1.38	0.2458	1	0.000	0.11	0.7441
% Biomass allocation to shoot			1		0.02	0.8746	1		0.13	0.7226	1		0.02	0.8856
% Biomass allocation to roots			1		0.11	0.7419	1		0.53	0.4684	1		0.09	0.7631
Number of leaves			1		4.66	**0.0309**	1		1.84	0.1749	1		5.16	**0.0232**
Biomass per leaf	49	0.001	1	0.003	4.27	**0.0440**	1	0.005	5.66	**0.0213**	1	0.001	1.75	0.1915
Total number of flowers			1		0.00	0.9649	1		1.00	0.3171	1		0.58	0.4470
Total number of seed pods	35	21862	1	477.1	0.02	0.8834	1	19487	0.89	0.3516	1	2819	0.13	0.7217
Total seed weight	35	0.781	1	0.562	0.72	0.4018	1	1.143	1.46	0.2343	1	0.000	0.00	0.9865
Number of seeds			1		0.29	0.5918	1		0.82	0.3640	1		0.00	0.9739
Biomass per seed	35	0.003	1	0.003	1.07	0.3090	1	0.013	4.27	**0.0463**	1	0.001	0.38	0.5430
Biomass flowering stalks	35	4.737	1	0.089	0.02	0.8916	1	1.845	0.39	0.5366	1	1.862	0.39	0.5348
Seed germination			1		1.97	0.1604	1		52.8	**<0.0001**	1		18.7	**<0.0001**
Total number of germinating seeds			1		0.76	0.3838	1		0.01	0.9248	1		0.55	0.4579

Degrees of freedom. † Mean square. ‡ F-statistics or Chi-square statistics respectively.