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Oecologia

Consequences of combined herbivore feeding and pathogen infection for fitness of *Barbarea vulgaris* plants --Manuscript Draft--

Manuscript Number:	
Full Title:	Consequences of combined herbivore feeding and pathogen infection for fitness of <i>Barbarea vulgaris</i> plants
Article Type:	Plant-microbe-animal interactions – original research
Corresponding Author:	Thure Pavlo Hauser, Ph.D. DENMARK
Order of Authors:	Tamara van Mølken Vera Kuzina Karen Rysbjerg Munk Carl Erik Olsen Thomas Sundelin Nicole van Dam Thure Pavlo Hauser, Ph.D.
Suggested Reviewers:	Arjen Biere A.Biere@nioo.knaw.nl One of the few researchers focusing on interactions of plants with both phytopathogens and arthropod herbivores. Guest editor on the subject in an recent volume of <i>Funct Ecol</i> Ayco Tack ayco.tack@helsinki.fi another ecologist who has published on three-way interactions Valerie Fournier valfourn@rci.rutgers.edu has one of the best analyses of three way plant-microbe-arthropod interactions
Opposed Reviewers:	

24 **Abstract**

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

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

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

45
46 **Key-words:** *Albugo* sp.; defence reactions, plant vigour; *Phyllotreta nemorum*; three-way
47 interactions.

48

49 **Introduction**

50 Plants are often attacked simultaneously by phytopathogens and insect herbivores, and
51 interactions between them are therefore common (Hatcher 1995; Hauser et al. 2013). The
52 pathogens and insects may interact directly, e.g. if pathogen spores are transported by insects
53 to suitable plant tissues, or indirectly through changes in the plant induced by one antagonist
54 that also affect the other. Thus, pathogen infections can modify attractiveness of the host plant
55 to herbivorous insects (Stout et al. 2006; van Molken et al. 2012) as well as their
56 consumption, growth rate, survival and fitness (Hatcher 1995; Hatcher and Paul 2000; Paul et
57 al. 2000; Rostas and Hilker 2002; Stout et al. 2006; Mouttet et al. 2011; Tack and Dicke
58 2013).

59 Indirect interactions between plant antagonists may be caused by defence compounds
60 induced by one antagonist that also affect the other; alternatively, one antagonist may
61 suppress plant defence levels to the benefit of the other. Complex cross-talk between defence
62 signalling pathways in the plant may also contribute to such interactions, as different
63 functional groups of herbivores and pathogens induce different signal pathways that may
64 interfere with each other (Pieterse and Dicke 2007; Koornneef and Pieterse 2008; Thaler et al.
65 2012). Other causes of interactions may involve changes in resource partitioning or allocation
66 as a consequence of attack (Hatcher 1995). Thus, the combined impact of pathogens and
67 insect herbivores may differ significantly from the sum of impacts of each antagonist on its
68 own (Hatcher 1995; Hauser et al. 2013).

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

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

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

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

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

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

103 The same mechanism of interaction could potentially arise from induction of
104 glucosinolates. The P-type mainly contains glucosibarin (the optical R-isomer of 2-hydroxy-
105 2-phenylethyl-glucosinolate: 2R) whereas the G-type contains glucobarbarin (the optical S
106 isomer: 2S) (Agerbirk et al. 2003a; Agerbirk and Olsen 2011). Glucosinolates are often toxic
107 or deterrent to non-crucifer specialist insects, and play a role in host selection by crucifer
108 specialists (Fahey et al. 2001; Griffiths et al. 2001; Renwick 2002). Glucosinolates may affect
109 fungi and microorganisms (Fahey et al. 2001), including oomycete pathogens (Schlaeppli et al.
110 2010; Wang et al. 2013), and possibly also *Albugo* species (Mathur et al. 2013). The strong
111 resistance of G-type plants against flea beetles is not caused by their specific glucosinolates
112 (Agerbirk et al. 2001, 2003b); however, glucosinolates may still affect the flea beetles to a
113 lesser degree.

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

120 Finally, antagonistic interactions may occur in *B. vulgaris* between the presumed salicylic
121 acid-based signals triggered by the biotroph *Albugo* and jasmonic acid-based signals triggered
122 by the chewing and mining flea beetles (Pieterse and Dicke 2007; Koornneef and Pieterse
123 2008; Thaler et al. 2012). However, the specificity of signals in response to these two

124 antagonists has never been tested to our knowledge.

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

131 **Materials and methods**

132 Experimental design

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

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

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

148 Inoculum was prepared based on a protocol by Dangl et al. (1992): sporangia were hydrated
149 for 90 min in deionised H₂O at 15 °C, and adjusted to 7 x 10⁴ sporangia per ml. The plants to
150 be infected by *Albugo* were inoculated with 5 separate drops of 10 µl inoculum on each of
151 their four youngest leaves, and were subsequently kept in plastic bags in darkness at 15 °C.
152 The other half of the plants were given the same treatment, but without sporangia. After three
153 days, all plants were transferred to 18 hours light, 6 hours dark at 17 °C. White rust began to
154 develop ten days post inoculation (dpi); at 14 dpi the number of leaves with rust was counted
155 (Fig 1d).

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

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

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

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

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

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

188 *Albugo* symptoms, infection and herbivore feeding

189 The development of white blister rust was visually estimated at the time of first harvest (56
190 dpi) as the percentage leaf area covered by pustules. Each leaf was assigned to one of five
191 damage categories: 0: no damage; 1: ≥ 0-20%; 2: 21-40%; 3: 41-60%; 4: 61-80%; and 5: 81-
192 100% damage. The total percentage of leaf area with rust was estimated as the sum of the
193 multiplum of the percentage of leaves of each damage class with the mean percentage damage
194 of that class.

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

197 TCCGTCTCCTTGATGACCTT; Van Mólken 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 μ M, C18,
215 110A, 2 x 150 mm) was used at a flow rate of 0.2 ml \cdot min⁻¹, preceded 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

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

222 Five saponins were scored in G-plants: 3-O-cellobiosyl-hederagenin (m/z [M+TFA-] 909,
223 RT 21.5), 3-O-cellobiosyl-oleanoic acid (m/z [M+TFA-] 893, RT 24.4), 3-O-cellobiosyl-
224 gypsogenin (m/z [M+TFA-] 907, RT 22.5), 3-O-cellobiosyl-4-epihederagenin (m/z [M+TFA-]
225 909, RT 22.6), and 3-O-cellobiosyl-cochalic acid (m/z [M+TFA-] 909, RT 20.7). Three
226 putative saponin compounds were scored in P-plants; these correspond to the putative P-type
227 saponins in Kuzina et al. (2011), based on their mass-to-charge (m/z) ([M+TFAA-]=1073 for
228 saponins 1 (RT 14.8) and 3 (RT 15.5) ; [M+TFAA-]=1159 for saponin 2 (RT 16.2). Peak
229 areas of the saponins were used as estimates of relative saponin content, as exact
230 concentrations could not be determined.

231 Glucosinolates

232 Glucosinolates were extracted from 50-100 mg of ground leaf discs (same as used for
233 estimation of herbivore consumption; see above), and analysed as described in van Leur et al.
234 (2008). Additional standards of progoitrin, gluconapin, glucoiberin, glucobrassicinapin,
235 glucotropeolin, gluconasturtiin, glucoraphanin, glucocoerucin, glucobrassicin, and sinalbin
236 (Phytoplan, Heidelberg, Germany) were used. To calculate concentrations, the glucosinolate
237 measurements were divided by the dry weight of the sample.

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

241 Data analysis

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

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

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

255 **Results**

256 *Albugo* and flea beetle interactions

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

262 Extracts of inoculated plants that were also exposed to flea beetles contained more
263 *Albugo* DNA than inoculated plants without beetles (77 and 2.6 times more DNA in P- and G-
264 plants, respectively, Fig. 2; Online Resource 1). Inoculated G-plants with flea beetles also
265 developed more rust in younger parts of the plants (results not shown). The leaf area covered
266 with rust was positively correlated with *Albugo* DNA content in G-plants (N= 28; $r= 0.658$;
267 $p= 0.0001$).

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

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

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

274 Biochemical changes in plants

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

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

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

288 The carbon-nitrogen ratio of P-plants was negatively affected by flea beetles and
289 decreased by 26 % and 29 % in the herbivore and combined treatments, respectively; this was
290 caused by increased nitrogen concentrations (Table 1); pathogen infection did not affect the
291 carbon-nitrogen ratio. In G-plants, the ratio was positively affected by pathogen infection, due
292 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

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

319 Different responses to *Albugo* of the two plant types

320 A difference in susceptibility to *Albugo* between P- and G-plants was originally suggested by
321 Toneatto (2009), based on spontaneously infected plants in a crossing experiment. Here we
322 found the same difference between plant types: less than 20% of the P-plants developed white
323 rust and hardly any plants contained *Albugo* DNA, while more than 80% of the G-plants
324 developed white rust and contained *Albugo* DNA. Other inoculation experiments by our
325 group have shown equivalent differences in white rust development, using other P- and G-
326 type populations of *B. vulgaris* (Christensen, Heimes, Laybourn, Van Mólken and Hauser,
327 unpublished). Furthermore, we have found white blister rust in natural populations of G-
328 plants, but never in P-populations (Van Mólken et al., in prep). The difference in
329 susceptibility to *Albugo* between P- and G-plants thus seems to be associated with the overall
330 divergence between the two plant types of *B. vulgaris* (Agerbirk et al. 2003a; Hauser et al.
331 2012).

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

335 Interactions between *Albugo* and flea beetles

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

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

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

349 Changes in plant biochemistry

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

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

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

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

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

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

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

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

397 Plant performance and reproduction

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

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

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

420 Implications

421 A growing number of studies have shown that arthropod herbivores and plant-associated
422 microorganisms can seriously affect each other while on the same plant, either directly or
423 mediated by the plant (Hatcher 1995; Hatcher and Paul 2000; Mouttet et al. 2011; Paul et al.
424 2000; Rostas and Hilker 2002; Stout et al. 2006; Tack and Dicke 2013). Our study shows
425 clear examples this, both for antagonist success and induced changes in the plant that may
426 subsequently affect both (and other) antagonists. However, while these immediate plant-
427 pathogen-herbivore interactions may be interesting and important, their impact on plant
428 performance, fitness and yield may be strongly moderated by compensatory growth (Fournier
429 et al. 2006; Hauser et al. 2013), as also shown by our results. Unfortunately, very little is
430 known about this; in the meta-analysis of Hauser et al. (2013), only 35 data sets could be
431 found that had estimated combined impacts of herbivores and pathogens on plant
432 performance, despite its clear relevance for ecologists and agronomists alike.

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

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

438 **Acknowledgements**

439 We are grateful to the Barbarea group at University of Copenhagen for fruitful discussions
440 and support. We thank L. Munck and E.B. Holub for advice on *Albugo* inoculation. M-B.
441 Sauer, C. Heimes, S. Christensen and H. Olsen helped with practical work, J.K. Schjoerring
442 and A.H. Ivø with nitrogen analyses, and R.A. de Graaf with glucosinolate analyses. This
443 work was funded by the Danish Agency for Science, Technology and Innovation (grant no.
444 274-08-0462); the authors declare that they have no conflict of interest with this Agency. All
445 experiments comply with current Danish laws.

446

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598
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600

601 **Figure legends**

602 **Fig. 1.** Species used in this study: (a) *Barbarea vulgaris* in the rosette and flowering stage.

603 Inserts show a pubescent P-type leaf in the lower left corner and a glabrous G-type leaf in the
604 lower middle part; (b) *Phyllotreta nemorum* adult (left) and larva (right); (c) white blister rust
605 (pustules) caused by the *Albugo sp.*; (d) number of P- and G-plants showing symptoms 14
606 days post inoculation.

607

608 **Fig. 2.** *Albugo* DNA content and flea beetle damage after infestation with flea beetles (Herb),
609 *Albugo* (Path) and both (H&P). Mean values \pm SE are shown for (a) ng *Albugo* DNA (out of
610 10 ng total) in leaves of P- and G-plants; insert shows a DNA melting curve (temperature ($^{\circ}$ C)
611 x fluorescence) demonstrating that only one PCR product was amplified; (b) percentage of
612 leaf area consumed by beetles and larvae in flea beetle susceptible P-plants; G-plants are
613 resistant and not damaged. Columns with different letters of the same case are significantly
614 different at $p < 0.05$.

615

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

Tables

Table 1. Mean values (\pm 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.

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

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

Figs

Figure 1

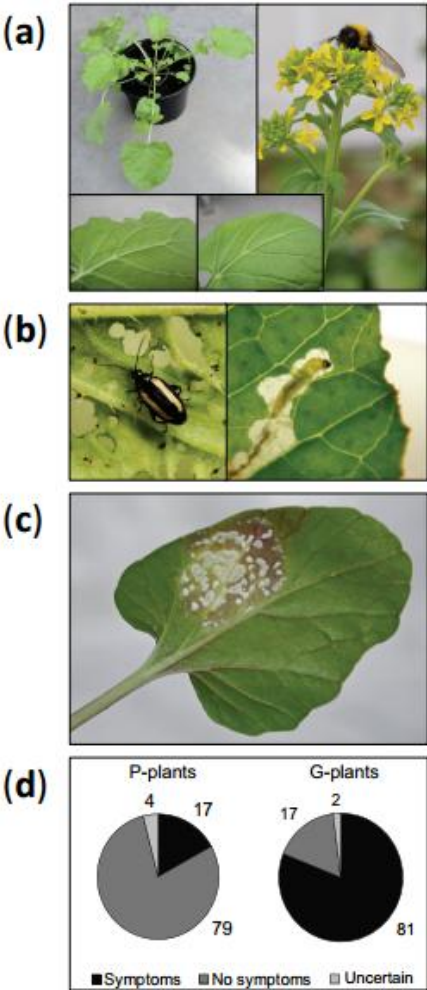


Figure 2

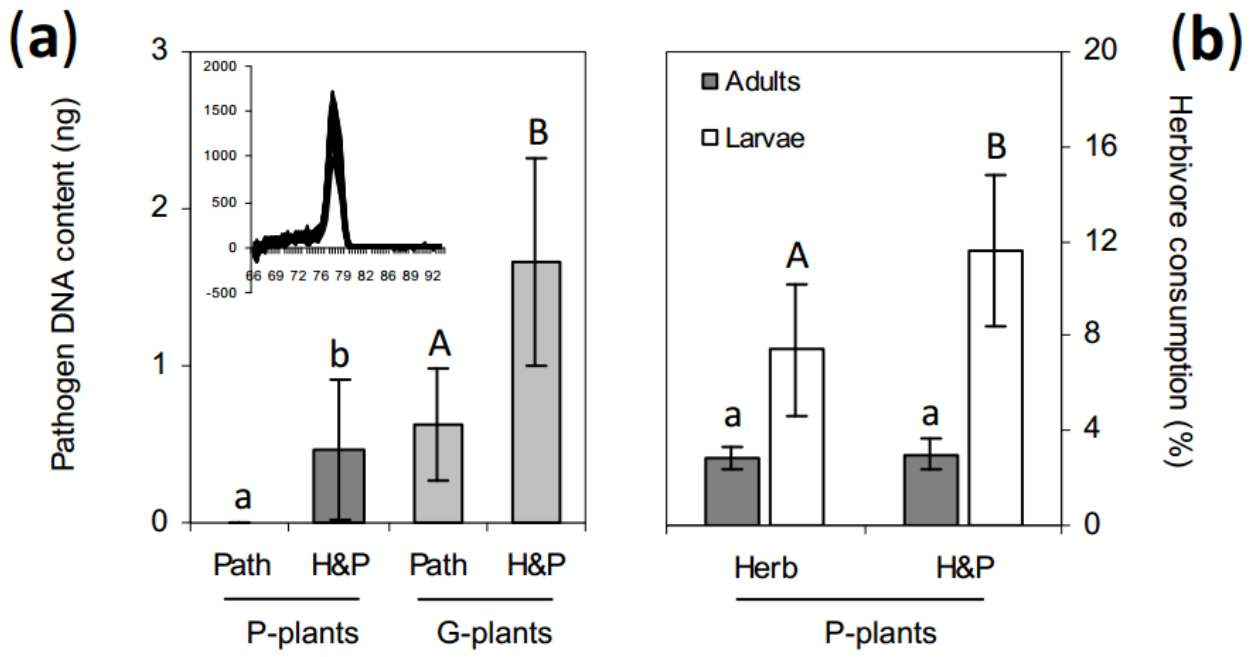
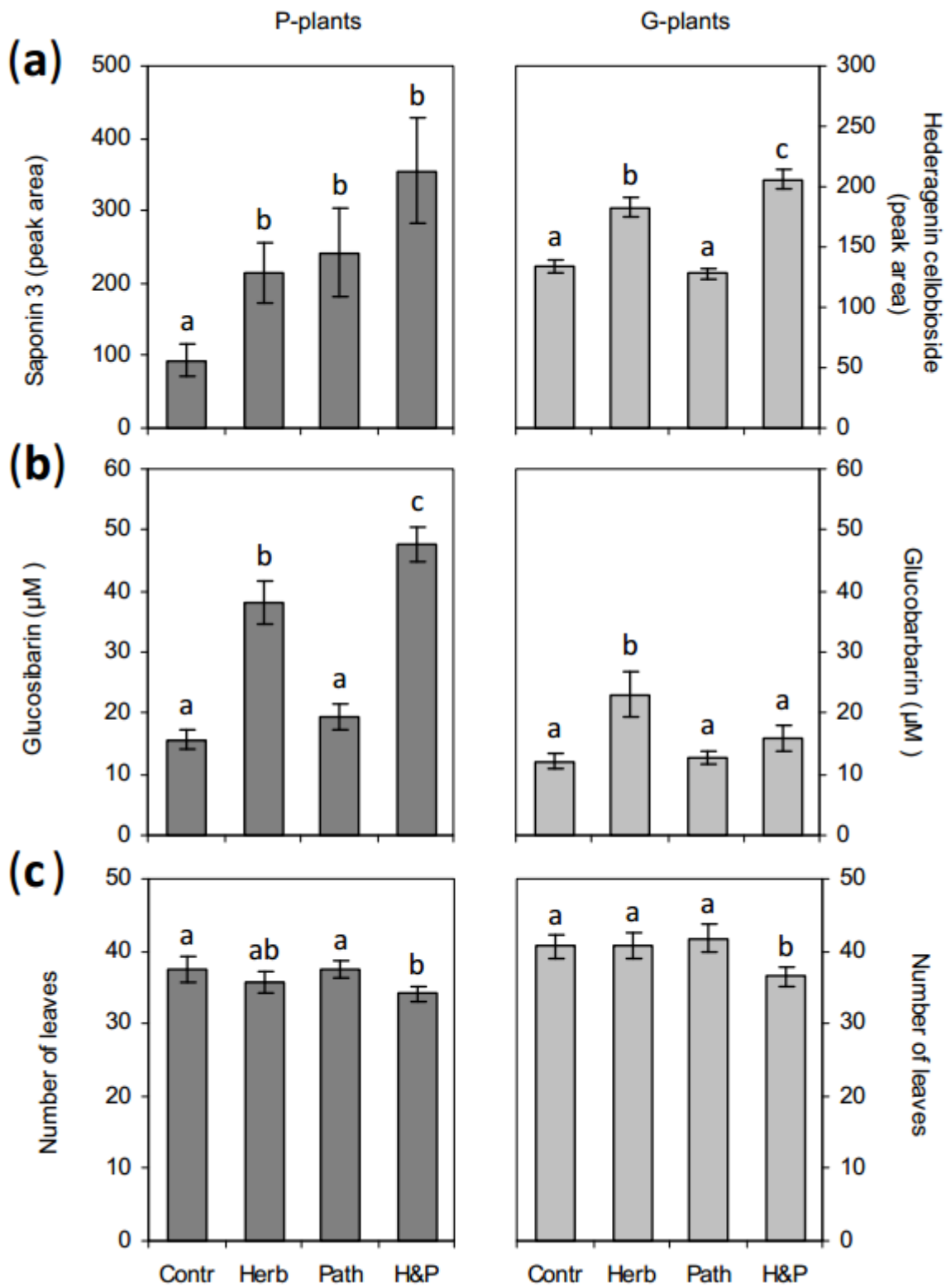


Figure 3



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 Traits	Error		Herbivore				Pathogen				Herbivore x pathogen			
	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				
<i>A. candida</i> 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			
	df*	MS [†]	df*	MS [†]	F/ X ² ‡	p	df*	MS [†]	F/ X ² ‡	p	df*	MS [†]	F/ X ² ‡	p
<i>A. candida</i> 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.