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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Interactive impacts of a herbivore and a pathogen on two resistance types of *Barbarea vulgaris* (Brassicaceae)

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Abstract It is well known that pathogens and arthropod herbivores attacking the same host plant may affect each other. Little is known, however, about their combined impact on plant fitness, which may differ from simple additive expectations. In a 2-year common garden field experiment, we tested whether the pathogen *Albugo* sp. (white blister rust) and the herbivorous flea beetle *Phyllotreta nemorum* affected each other's performance on two resistance types (G-type and P-type) of the crucifer *Barbarea vulgaris* ssp. *arcuata*, and whether biomass, reproduction and survival of the plants were affected by interactive impacts of the antagonists. Most of the insect-resistant G-plants were severely affected by white rust, which reduced biomass and reproductive potential compared to the controls. However, when also exposed to flea beetles, biomass loss was mitigated in G-plants, even though apparent disease symptoms were not reduced. Most of the insect-susceptible P-plants were resistant to white rust; however, the number of flea beetle mines tended to increase in plants also exposed to *Albugo*, and biomass at the last harvest was slightly lower in the combined treatment. Thus, interactive impacts of the herbivore and pathogen differed between the two resistance types, with an antagonistic combined impact in G-plants, which lasted surprisingly long, and a slight synergistic impact in P-plants.

Keywords Plant performance · Synergism · Antagonism · Tolerance · Defence

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Introduction

In all ecosystems, plants are attacked by arthropod herbivores and pathogens. Infestation with these antagonists often results in decreased plant fitness (Marquis 1992), and in substantial yield loss in agro-ecosystems (Oerke and Dehne 2004). However, plants are not passive, defenceless objects; they have evolved a variety of mechanisms to resist their attackers, such as feeding-deterrent secondary metabolites and morphological structures. These give the plant an advantage upon attack by a pathogen or herbivore, but may be costly to produce (Baldwin et al. 1990; Agrawal 1999; Redman et al. 2001; Reudler et al. 2013).

Plant–antagonist interactions, however, are more than a dual relationship between a plant and an attacker. Plants are often exposed to a multitude of different enemies simultaneously, as different as generalist arthropod herbivores and specialist biotrophic pathogens. Complex interactions between the attackers, and between these and the plant, may ultimately affect performance of plants in ways that cannot be understood by considering each antagonist in isolation (Hatcher 1995; Paul et al. 2000; Stout et al. 2006; Hauser et al. 2013). Different antagonists on the same plant may thus facilitate or inhibit each other, either directly or mediated through plant responses (e.g., Röder et al. 2007; Turner et al. 2010). Whereas several studies

have investigated and discussed impacts of plant–antagonist interactions on antagonist performance (De Nooij et al. 1992; Stout et al. 2006; Thaler et al. 2010; Tack and Dicke 2013), comparatively little research has been done on the ultimate impacts of such interactions on plant performance. In a recent meta-analysis, Hauser et al. (2013) found no more than 35 studies that had measured the combined impacts of arthropod herbivores and pathogens on plant performance, covering only 29 plant species. This is surprising, given the large literature on plant–antagonist interactions. This suggests that we may not fully understand the functioning and evolution of plant–antagonist interactions, as cost and benefits of resistance against one antagonist, for instance, may be moderated by the presence of another. From an applied perspective, understanding interactions between multiple attackers and the magnitude of their impact on yield is crucial for efficient pest management.

Most experimental studies of plant–antagonist interactions have been based on short-term experiments, carried out under controlled conditions in climate chambers or greenhouses, and only a few long-term experiments of plants and their antagonists have been done under semi-natural or natural conditions (but see Dickson and Mitchell 2010; Cripps et al. 2011). Interactive effects, however, are often environment and time dependent (Burdon and Thrall 1999; Bostock et al. 2001; Rostás et al. 2003; Hauser et al. 2013), and additionally, resistance to antagonists may vary among populations (e.g., Fritz and Price 1988; Marquis 1990; Karban 1992). To estimate ultimate effects of antagonists on plant performance and yield, it is therefore necessary to observe long-term effects and to consider variation among populations, preferably under natural conditions.

Barbarea vulgaris ssp. *arcuata* (Opiz.) Simkovics (Brassicaceae) (henceforth named *Barbarea vulgaris*) is a short-lived outcrossed herbaceous plant that is attacked by several insect and pathogen species. Among them are the flea beetle *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae; henceforth named flea beetle) and the oomycete pathogen *Albugo* sp., causing white blister rust (henceforth named *Albugo*; the *Albugo* sp. infecting *B. vulgaris* may be an independent species, Choi et al. 2011; however, this is undecided). Some populations of *B. vulgaris* are resistant to flea beetles (Nielsen 1997), but are mostly susceptible to *Albugo* (ca. 80 % of the plants show symptoms upon infection), while other populations are susceptible to herbivores, but mostly resistant to white rust (less than 20 % of the plants show symptoms; van Mölken et al. 2014a, b). These two types of plant populations also differ in glucosinolates, morphology, phenology and other traits; they are genetically strongly divergent, reproductively somewhat incompatible (Toneatto et al. 2010, 2012), and occupy different geographical ranges in Eurasia (Hauser et al. 2012; Christensen et al. 2014). They have therefore been described as different 'plant types': the fleabeetle-resistant type as 'G-type' because it has glabrous rosette leaves, and the flea beetle-susceptible as 'P-type' because it has pubescent leaves (Nielsen 1997).

Herbivore resistance in the G-type is caused by production of the saponin compound hederagenin cellobioside, and probably others like oleanolic acid cellobioside (Shinoda et al. 2002; Agerbirk et al. 2003a; Kuzina et al. 2009; Nielsen et al. 2010; Augustin et al. 2012). The saponin(s) are lethal to the flea beetle larvae and larvae of other specialist herbivores, such as the diamondback moth *Plutella xylostella* (Shinoda et al. 2002) and *Pieris napi* (Renwick 2002), and feeding deterrent for adult flea beetles. The herbivore-susceptible and pathogen-resistant P-type produces other saponins, which have not been characterised yet (Kuzina et al. 2011). The mechanism of white rust resistance is unknown.

Based on the different resistances of the two plant types, we expect G-plants to be negatively affected by *Albugo* and P-plants by flea beetles. However, direct and indirect plant-mediated interactions between the herbivore and pathogen may modify each other's performance, and thereby, their combined impact on the plant. Interactions between different attackers may be synergistic or antagonistic (Hatcher 1995), and therefore, simultaneous infestation by flea beetles and white rust may either increase or mitigate the impact on performance of *B. vulgaris* compared to infestations by the herbivore or the pathogen alone. This may further depend on the resistances of the two plant types; even if plants of e.g., the G-type are resistant to flea beetles, they still seem to spend substantial resources on a defence that causes a lower biomass when exposed to flea beetles (van Mölken et al. 2014a).

An increased combined negative impact on the plants could arise by direct interactions between the arthropod herbivore and the pathogen, if e.g., the arthropods facilitate pathogen transmission and entry into plant tissues (Agrios 1980; Friedli and Bacher 2001; Kluth et al. 2002). Increased combined impacts could also result from interference between different plant defence signalling systems. Chewing or boring herbivores like flea beetles typically induce the jasmonic acid (JA) signalling pathway, while biotrophic pathogens such as *Albugo* induce the salicylic acid (SA) pathway. The two pathways are known to antagonise each other to some extent (Kunkel and Brooks 2002; Glazebrook 2005; Stout et al. 2006; Koornneef and Pieterse 2008; Koornneef et al. 2008), and hence, we may expect a synergistic interaction between flea beetles and white rust that may result in an increased damage and lower biomass of plants exposed to both. Additionally, resource loss due to attack of one antagonist may lower the plant's ability to compensate, and thus, above a certain damage level, may increase the negative impact of other antagonists, similar

to what Fournier et al. (2006) describe as 'compensation breakdown'. On the contrary, competition between different antagonists may reduce their combined negative impact on plant biomass (Karban et al. 1987; Hatcher et al. 1994; Fournier et al. 2006), and infestation by one attacker may lead to production of defence metabolites that are also active against others (Biere et al. 2004; Rayapuram and Baldwin 2008; Zhu-Salzman et al. 2008). Furthermore, plant compensatory growth and resource allocation in response to infestation by one antagonist may increase tolerance to other attackers.

Here, we studied the combined impact of white rust and flea beetles on *B. vulgaris* in a 2-year common garden field experiment, and asked whether interaction impacts were synergistically negative for the plant, antagonistically positive, or simply additive. Plants from three G-type and three P-type populations were exposed to the herbivore and pathogen alone and in combination, and the resulting damage by flea beetles and disease symptoms of *Albugo* were monitored. Biomass was determined at three harvests, and final survival at the end of the experiment was recorded. We could thus test whether the presence of white rust changes the damage and impact of flea beetles on *B. vulgaris* G and P-plants, and vice versa. Our results could further be compared to those from a similar experiment done under greenhouse conditions (van Mölken et al. 2014a).

Materials and methods

Plant material

Seeds for the experiment originated from a minimum of 20 plants from each of six populations on Zealand, Denmark; three G-type populations (Hedeland 55.626N 12.182E, Suserup 55.384N 11.548E, Svebølle 55.638N 11.337E) and three P-type (Brokøb 55.584N 11.423E, Halleby Ore 55.635N 11.344E, Trundholm 55.883N 11.567E). Seeds were stored at 4 °C until used for the experiment. Three hundred seeds from each population were sown in trays in a greenhouse in April 2011, and after 3 weeks, and 1 day before inoculation, 56 plantlets with four to five true leaves were randomly selected from each population, 336 in total. These were moved to a 15 °C climate chamber (16:8 h light dark) and covered with transparent plastic to keep a high level of humidity.

Inoculation

Albugo sporangia for the inoculum were obtained from infected *B. vulgaris* G-plants maintained in the greenhouse; this *Albugo* source originated from a spontaneous infection of *B. vulgaris* 2 years prior to the experiment. Inoculum was prepared as described in Dangl et al. (1992). Spores of *Albugo* were tapped on a glass slide, transferred to a glass vial, and 8 ml of deionized H_2O was added. A few drops of watery extract of G-type leaves containing saponins were added to improve suspension. The mixture was vortexed and incubated for approximately 30 min in a water bath at 15 °C until all spores were suspended. Concentration of the spore suspension was estimated by counting sporangia with a haemocytometer, and adjusted to a concentration of 9×10^4 sporangia per ml. The inoculum was then kept on ice.

Half of the selected plants were inoculated by pipetting 40 µl of the sporangia suspension on the four youngest leaves of each plant (10 µl in four droplets on each leaf). The other half of the plants were mock-inoculated with deionized water as a control. After inoculation, plants were covered with plastic and kept in the climate chamber (15 °C, 16:8 h light dark) for 4 days.

Field experimental design

Four days after inoculation (DAI), plants were transferred to an experimental field (20 × 30 m) at the university farm in Tåstrup, Denmark (55.672N 12.288E), in a randomized block design with four blocks of eight plots (2 × 2 m) each. Within each block, plots were randomly assigned to one of eight factorial combinations of plant types (G and P), *P. nemorum* exposure and *Albugo* infection, and controls. For each of these treatment combinations (plots), nine plants (three individuals per G-type and P-type population, respectively) were planted 30 cm apart. Thus, the experiment included 36 replicate plants per treatment, distributed over four blocks. To avoid edge effects, 16 plants derived from untreated surplus plants of the corresponding type were planted around the experimental plants.

Each plot was covered by a mesh tent (1.9 × 1.9 m) (MegaView Science, Taiwan) that had flaps extending below the sides; these were covered with soil to prevent insects from moving in and out of the tents. As precipitation was limited through the mesh tents, plots were evenly watered twice a week until week four after planting to avoid drought stress and facilitate establishment of plants. Mesh tents were removed from the plots in October 2011.

Flea beetle treatment

Flea beetles were reared in the lab from a susceptible line of *P. nemorum* as described in Nielsen (1997). Adult flea beetles used for the experiment were newly emerged and not sex determined. At 18 DAI, 17 flea beetles per tent were released into plots assigned to the herbivore treatment.

Herbivore and pathogen monitoring

Pathogen symptoms were monitored twice during the experiment: at 33 DAI, symptom development was low (<10 % of leaves with pustules) and only pathogen incidence (i.e., presence or absence of symptoms) was registered; at 60 DAI, disease symptoms were evaluated on a scale from 0 to 5, estimating the percentage of leaves covered with pustules (0: 0 %, 1: 0 to <10 %, 2: 10 to <25 %, 3: 25 to <50 %, 4: 50 to <75 %, 5: 75 to 100 %); all scorings were done by the same observer. Feeding damage of adult flea beetles and larvae was recorded by counting the number of holes on all leaves per plant at 33 DAI and 60 DAI and the number of leaf mines at 60 DAI. Pathogen symptoms and herbivore damage were largely absent on regrown plants after the first harvest (60 DAI).

Biomass

Aboveground individual plant parts were harvested three times during the experiment, with the first harvest in late June 2011 (60 DAI) and the second and third harvest of plants regrown after the first harvest in late August 2011 and late June 2012, respectively. Harvested material was dried in perforated cellophane bread bags at 60 °C for 48 h and weighed. Only vegetative plant parts were included in 2011, as *B. vulgaris* did not flower that year, as it requires vernalisation to flower. In 2012, all plants flowered, and therefore, plants including fruit-bearing shoots were harvested just before seed shattering. To use total aboveground biomass as a proxy for reproduction, we weighed fruit-bearing plant parts from a subset of 18 plants separately. Total plant biomass was highly correlated to the biomass of the fruit-bearing shoots ($N = 18$, $r^2 = 0.9854$, $p < 0.001$, data not shown), and thus, overall plant biomass was used as a measure for reproductive output.

Statistical analysis

Effects of treatments on pathogen symptoms and herbivore damage were tested with generalized linear mixed models (GLMM) in R 3.0.1 (R Core Team 2013), including the hierarchical random effects of blocks and plots and the crossed random effect of populations. The GLMM were calculated separately for the two types of *B. vulgaris* (G, P), so that the sample size per model was 144. In some cases, models could not be estimated with random population effects. Then population effects were included as fixed effects instead. Effects of treatments on pathogen symptoms were tested with binomial GLMM and logit link (function glmer of package lme4 1.0-5) on binary data (presence-absence of symptoms 33 DAI) or proportion data (average percentages of the symptom classes assessed at 60 DAI), respectively. Data on herbivore damage (number of holes and mines on the leaves) were over-dispersed compared to the Poisson distribution. Therefore, herbivore damage was modelled with negative binomial distribution using log link (package glmmADMB 0.7.7). The significance of fixed effects was assessed with Wald z tests.

We modelled effects of treatments on biomass of *B. vulgaris* using the whole data set including all three harvests, using a repeated-measures linear mixed-effects model (LMM) with normal distribution and identity link (package *nlme* 3.1-109 in R). The measurements of biomass were normalised using square-root transformation. Values were missing for 11 individuals, which were excluded from further analysis, giving an effective sample size of 831. The LMM included fixed effects of time, treatments and plant types and all possible interactions between them, as well as random effects of blocks, plots and plant individuals. Random effects of populations could not be included, because *nlme* does not allow for crossed random effects. Temporal autocorrelation of the repeated measures was accounted for by including a correlation structure with a lag of one time step (corAR1). As the variance increased markedly with time, we also included separate estimates of variance for each time step (varIdent). The significance of fixed effects was assessed using t and F tests.

Additionally, we calculated separate LMM for each plant type and time point (package glmmADMB_0.7.7 in R). These models included random effects of plots nested in blocks and the crossed random effect of populations next to the main effects of the treatments and their interaction. To test the interaction between the pathogen and herbivore on G-plant biomass in more detail, fixed effects (control, herbivore, pathogen, herbivore + pathogen) were hard-coded (coded as three dummy variables) and tested directly against the pathogen treatment.

As post hoc tests are not available for (G)LMM, we supplemented the statistical models described above with series of model variants that iteratively included each treatment category as the baseline, in order to test all pairwise comparisons of treatments.

Results

Herbivore damage

As expected, herbivore damage was higher in P-plants than in G-plants; the overall number of holes in plants treated with flea beetles was significantly higher in P-plants compared to G-plants (Fig. 1a–d; Table 1; additional GLMMs of plants treated with flea beetles, $p < 0.001$ for both 33 and 60 DAI). Also, in contrast to P-plants, hardly any leaf mines were found in G-plants in any of the treatments (Fig. 1e, f; Table 1).

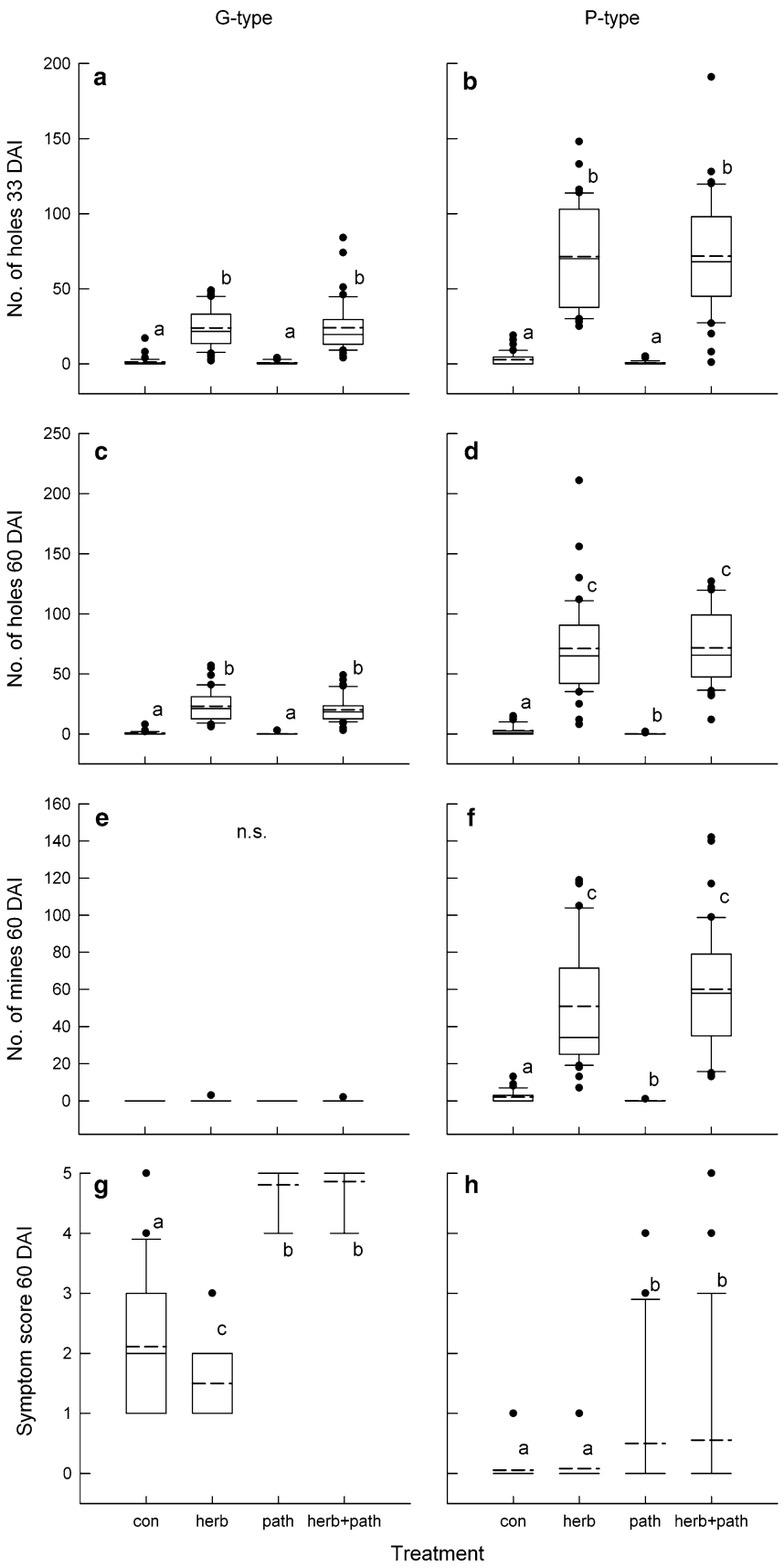

Fig. 1 Damage by the herbivore *Phyllotreta nemorum* and the pathogen *Albugo* in *Barbarea vulgaris* G-plants and P-plants infested with the herbivore (herb), the pathogen *Albugo* (path) and both (herb + path). G-plants are known from other studies to be resistant to the herbivore, and most P-plants to be resistant to the pathogen. Herbivore damage is shown as the average number of holes per plant produced by adult flea beetles at 33 days after inoculation (DAI) and 60 DAI for G-plants (**a**, **c**) and P-plants (**b**, **d**), and the average number of leaf mines per plant produced by flea beetle larvae at 60 DAI for G-plants (**e**) and P-plants (**f**). Pathogen damage is shown as symptom score on scale from 0 to 5 showing the average percentage of leaves with pustules per plant (0: 0 %, 1: >0 to <10 %, 2: 10 to <25 %, 3: 25 to <50 %, 4: 50 to <75 %, 5: 75 to 100 %) for G-plants (**g**) and P-plants (**h**). Box plots show medians (*solid line*), means (*dashed line*), 25th and 75th percentiles (*boxes*), 90th and 10th percentiles (*whiskers*) and outliers

Table 1 Mean values (±SE) of herbivore damage, pathogen symptoms, biomass and survival for G-plants and P-plants for control, herbivore, pathogen and combined treatments

Traits	G-type				P-type			
	Control	Herbivore	Pathogen	Herbivore + pathogen	Control	Herbivore	Pathogen	Herbivore + pathogen
Number of holes 33 DAI	0.53	23.89 ± 2.14	0.61 ± 0.21	23.97 ± 2.86	2.81 ± 0.80	71.39 ± 5.74	0.61 ± 0.23	71.69 ± 6.48
Number of holes 60 DAI	0.64 ± 0.32	23.19 ± 2.24	0.08 ± 0.08	20.08 ± 1.80	2.61 ± 0.71	71.06 ± 6.65	0.08 ± 0.06	71.72 ± 5.17
Number of leaf mines 60 DAI	0	0.08 ± 0.08	0	0.06 ± 0.06	2.03 ± 0.54	50.92 ± 5.47	0.03 ± 0.03	60.08 ± 5.49
% Plants with symptoms 33 DAI	0	0	88.9	72.2	0	0	8.3	16.7
% Plants with symptoms 60 DAI	100	100	100	100	5.6	8.3	19.4	16.7
Symptom score 60 DAI	2.11 ± 0.19	1.50 ± 0.11	4.81 ± 0.07	4.86 ± 0.06	0.06 ± 0.04	0.08 ± 0.05	0.50 ± 0.19	0.56 ± 0.23
Biomass first harvest (g)	16.96 ± 0.97	16.35 ± 0.76	12.42 ± 0.89	15.81 ± 1.09	16.48 ± 0.86	15.54 ± 0.97	17.40 ± 0.78	15.86 ± 1.00
Biomass second harvest (g)	10.07 ± 0.80	9.39 ± 0.43	6.03 ± 0.68	8.69 ± 0.93	12.53 ± 0.76	12.70 ± 0.72	11.23 ± 0.72	13.02 ± 1.02
Biomass third harvest (g)	47.66 ± 6.43	36.85 ± 4.42	13.41 ± 4.2	26.61 ± 5.58	51.52 ± 4.57	48.83 ± 5.36	56.67 ± 5.30	46.82 ± 6.07
% Survival at third harvest	86.1	100	69.4	69.4	100	100	100	91.7

Sample size for each treatment is $N = 36$, except for biomass second harvest: P-type pathogen treatment ($N = 27$), G-type pathogen treatment ($N = 25$) and G-type combined treatment ($N = 35$)

Table 2 Statistical significance (GLMM z tests) and effect sizes of the responses of herbivore and pathogen damage in *Barbarea vulgaris* to inoculations of *Albugo* (pathogen) and infestation with flea beetles (herbivore)

Source of variation	Herbivore damage holes 33 DAI	Herbivore damage holes 60 DAI	Herbivore damage mines 60 DAI	Pathogen incidence 33 DAI	Pathogen score 60 DAI
G-type					
Pathogen (P)	ns	ns	ns	ns	13.0***
Herbivore (H)	45.8***	121.5***	ns	ns	0.44**
P × H	ns	ns	ns	ns	14.2*
P-type					
Pathogen (P)	ns	0.05***	0.03**	ns	26.2***
Herbivore (H)	80.2***	47.6***	71.8***	ns	ns
P × H	ns	49.2**	82.3*	ns	ns

For interactions in the models, effect sizes were calculated as: $\exp(b_p + b_H + b_{P \times H})$, e.g., $\exp(-3.642 + 4.274 + 3.778)$ for interaction in the P-type model of mines and $\exp(2.5665 - 0.8266 + 0.9144)$ in the G-type model of pathogen score at 60 DAI

Asterisks indicate level of significance (*** <0.001, ** <0.01, * <0.05, *ns* not significant). Effect sizes are given as ratios between treatment and control for herbivore damage (holes and mines) and as odds ratios for pathogen incidence and score, i.e., in both cases, effect sizes were calculated as e^b (b = regression coefficient)

In G-plants, herbivore damage was not affected by the presence of the pathogen (Fig. 1a, c, e; Table 2). In P-plants, the combination of pathogen and herbivore slightly increased the number of mines at 60 DAI (Tables 1, 2); however, this was not statistically significant in the pairwise comparison of treatments (Fig. 1f).

Pathogen symptoms

Disease symptoms were much more frequent in G-plants than in P-plants, with on average a sixfold higher frequency of plants showing symptoms at 33 and 60 DAI and a ninefold higher score of symptoms at 60 DAI in inoculated plants (Fig. 1g, h; Table 1).

In G-plants, the percentage of plants with pathogen symptoms at 33 DAI was not significantly affected by the flea beetles (Table 2). At 60 DAI, non-inoculated plants also showed disease symptoms, probably due to spread of spores among tents or natural background infestation; however, the degree of symptoms was still significantly lower than in inoculated plants (Fig. 1g; Table 2). Interestingly, in these unintentionally infected plants, symptom score at 60 DAI was significantly lower in presence of the herbivore (Fig. 1g; Table 2).

In P-plants, neither the percentage of plants with pathogen symptoms nor the symptom score was affected by the flea beetles (Fig. 1h; Table 2).

Plant performance

At all three harvests, the biomass of G-plants was lower in the treatment with white rust only than in combination with flea beetles [however, this was only significant at first and second harvest; Fig. 2a, c, e; Table 3; Electronic Supplementary Material (ESM) 1]. Whereas the biomass of plants exposed to *Albugo* was 57 % lower than for control plants on average, it was only 31 % lower when plants were also exposed to flea beetles (Table 1). Overall, the biomass of G-plants was lower in the presence of white rust, with a stronger impact at the third harvest than at the first or second harvest (ESM 1).

Biomass of P-plants was somewhat lower in the combined treatment at third harvest than in the single treatments (Fig. 2f; Tables 1, 3). Plants exposed to flea beetles and *Albugo* had 4 % lower biomass compared to plants exposed to flea beetles alone and 17 % lower biomass compared to

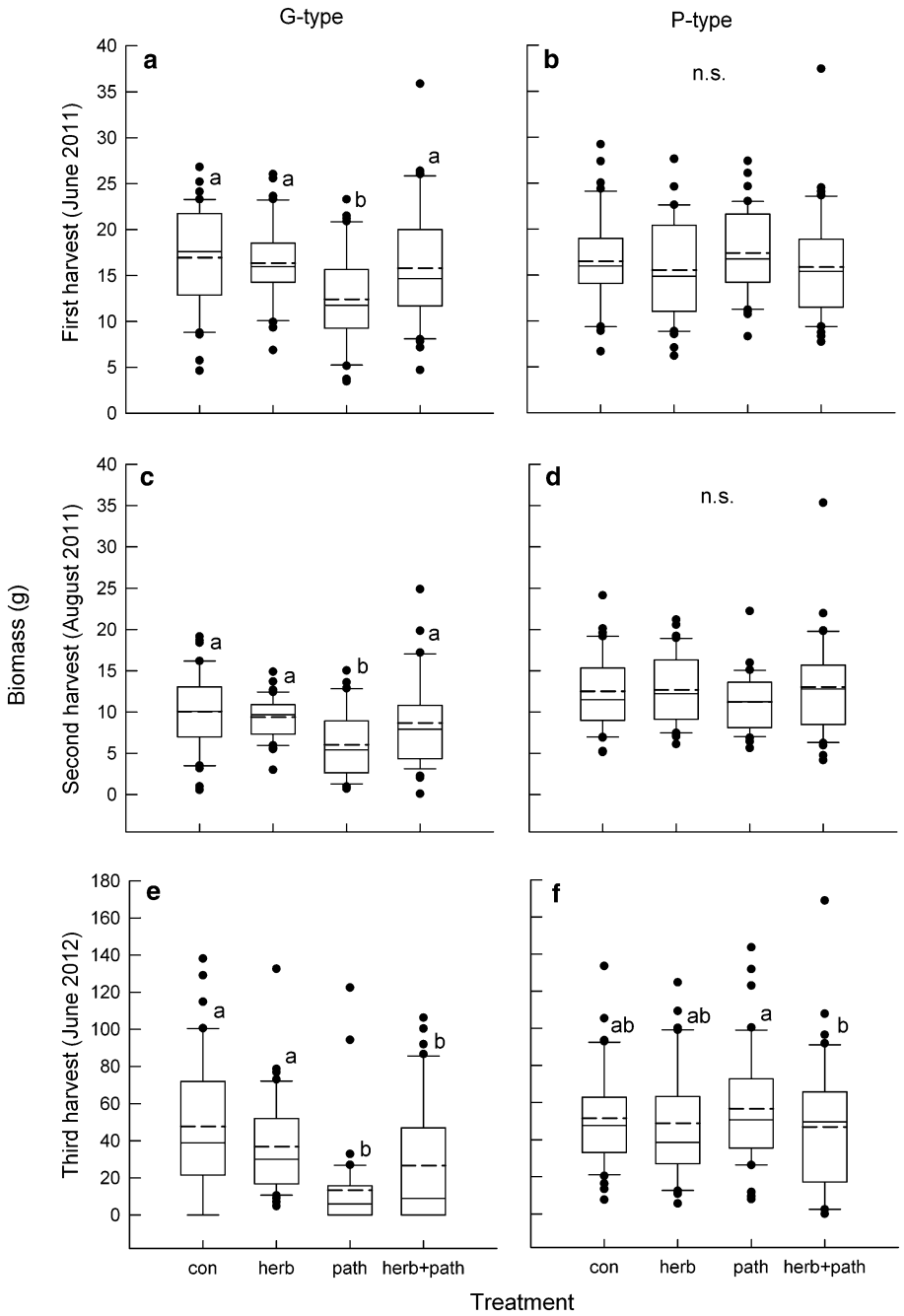

Fig. 2 Effects of *Phyllotreta nemorum* (herb) and *Albugo* (path) on aboveground biomass of *Barbarea vulgaris* G-plants and P-plants at first harvest (60 DAI) (**a**, **b**), second harvest (**c**, **d**) and third harvest (**e**, **f**). At the third harvest, almost all biomass was present in flowering stems, fruits and seeds. Box plots show medians (*solid line*), means (*dashed line*), 25th and 75th percentiles (*boxes*), 90th and 10th percentiles (*whiskers*) and outliers

Table 3 LMM of responses of *Barbarea vulgaris* G-plants and P-type biomass to single and combined treatments with *Albugo* (pathogen) and flea beetles (herbivore) for three harvests (time 1, time 2, time 3), regression coefficients are shown, indicating the effects on square-root transformed biomass values and significance levels from *t* tests

Source of variation	Biomass	
	Coefficient/*t* test	
	G-type	P-type
Time 1		
Control	0.61**	ns
Herbivore	0.57*	ns
Herbivore + pathogen	0.46*	ns
Time 2		
Control	0.75***	ns
Herbivore	0.73***	ns
Herbivore + pathogen	0.48**	ns
Time 3		
Control	3.48***	ns
Herbivore	3.17***	ns
Herbivore + pathogen	ns	−1.11*

Significance was tested against the pathogen treatment to illustrate effects of the pathogen alone compared to the control and the combined treatments. Asterisks indicate level of significance (*** <0.001, ** <0.01, * <0.05, *ns* not significant)

plants exposed to *Albugo* only. Neither the pathogen nor its interaction with flea beetles had any effect at the two other harvests (Fig. 2b, d; Table 3).

The herbivore alone had no significant effect on plant biomass, neither in G-plants nor in P-plants (Fig. 2; Tables 1, 3).

Survival

All plants survived the first year of the experiment, and at the third harvest, in 2012, 81.3 % of the G-plants and 98.0 % of the P-plants survived. In G-plants, survival tended to be lower in pathogen-inoculated plants, both in the treatment with white rust alone and in combination with the flea beetles (Table 1), and in P-plants, survival was lowest in the combined treatment with white rust and the flea beetles. However, these effects were not statistically significant.

Discussion

Our results show that biomass, reproductive potential and probably survival of *Barbarea vulgaris* can be affected by joint interactions with the pathogen *Albugo* sp. and the flea beetle *P. nemorum.* The interaction impact was most clear in the flea beetle-resistant and white rust-susceptible G-type of *B. vulgaris,* where addition of flea beetles clearly reduced the negative impact of *Albugo* on biomass. This was evident at all three harvests: directly after the controlled infestation with the herbivore and pathogen ended, after regrowth later that year and in the following summer, long after the experimental treatments had ended. Thus, the interaction between *Albugo* and flea beetles was antagonistic, with a net benefit for the plant. In contrast, we found a weak synergistic interaction between flea beetles and white rust in the flea beetle-susceptible and white rust-resistant P-type, where the number of larval mines tended to increase in the presence of the pathogen, plant biomass was slightly reduced at third harvest, and survival tended to be lower in the combined treatment.

The antagonistic interaction between *Albugo* and flea beetles in G-plants was most likely plant-mediated. Most G-plants readily develop white rust, probably because they do not recognise effectors from the pathogen or its specific strain (Göhre and Robatzek 2008), but its impact on the plants may be mitigated by defence reactions induced by exposure to flea beetles. Van Mölken et al. (2014a) showed that both saponins and glucosinolates are upregulated in G-plants by flea beetle attack and even more so when also exposed to *Albugo,* but not by *Albugo* alone. Likewise, genes involved in saponin biosynthesis are strongly upregulated upon attack by another specialist herbivore, *Plutella xylostella* (Wei et al. 2013). This may negatively affect performance of the pathogen, although the metabolites in themselves do not confer resistance (G-plants are mostly susceptible to *Albugo* at the non-induced concentrations at least; van Mölken et al. 2014a).

Disease symptoms were not visibly decreased by the presence of the flea beetles, except in control plants that developed white rust unintentionally during the experiment. Possibly, symptoms in inoculated plants were so severe that differences in degree of infection could not be detected by the visual scoring. However, it is difficult to draw conclusions based on these unintentionally infected plants, since time of infection is unknown and other factors, such as the position of plants, could be interfering. Contrary to our results, van Mölken et al. (2014a) found a significantly higher frequency of *Albugo* DNA in plants also exposed to flea beetles, and suggested that this could be caused by dispersal of spores among leaves by the beetles. This possibly contributed to a lower number of leaves produced by the dually infested plants (a synergistic effect) in that experiment.

Another possible explanation for the increased biomass of *Albugo*-infected G-plants when also exposed to flea beetles could be compensatory growth induced by herbivore feeding, which could balance the negative effect of the pathogen. Compensatory or even overcompensatory

growth upon herbivore attack is a well-known phenomenon (e.g., Strauss and Agrawal 1999; Agrawal 2000; Ruiz-R et al. 2008; Arab and Trigo 2011; Olejniczak 2011; Liu et al. 2012). If this was the case, we should also expect to see compensatory growth in treatments with the herbivore alone, but this did not occur. However, the impact of the herbivore alone may be too small to trigger a compensatory growth reaction, but enough to 'prime' plants and enable a quick response under severe pathogen attack.

In contrast to our results, van Mölken et al. (2014a) found no difference in biomass or reproduction between treatments, and suggested this to be due to the plants' ability to compensate for resource losses. We do not presently have a plausible explanation for the differences in direction and magnitude of interactive effects between our study and the corresponding greenhouse study of van Mölken et al. (2014a). However, the two experiments were conducted in different environments, time frames, and with different plant populations, which may account for differences to some degree. Plants in outdoor conditions, for instance, may be exposed to multiple stresses that may decrease overall plant vigour and the plants ability to compensate (Sciegienka et al. 2011).

Most P-plants were strongly resistant to *Albugo* and developed no symptoms at all, as was also found in previous experiments (van Mölken et al. 2014a, b). Resistance is probably based on recognition of highly specific pathogen effectors, as is common for biotrophic plant pathogens (Zhang et al. 2013). Exposure to *Albugo* also causes a strong induction of unknown saponins and the glucosinolate epiglucobarbarin (van Mölken et al. 2014a; alone or in combination with flea beetles). However, none of these seem to affect flea beetles (Agerbirk et al. 2001, 2003b; Kuzina et al. 2011). In our study, we even found a weak positive effect of *Albugo* on the number of flea beetle larval mines, as also found by van Mölken et al. (2014a), and a slight synergistic negative effect on biomass at the third harvest. In contrast, van Mölken et al. (2014a) found no interactive impacts on final biomass and reproduction. The small negative combined impact on plant biomass may to some extent be due to compensation. In a meta-analysis on plant–pathogen–arthropod interactions, Hauser et al. (2013) found that the combined impact of arthropods and pathogens was synergistic for size and number of plant parts, but additive for population growth and reproduction and antagonistic for whole plant biomass. This indicates that plants are able to compensate direct effects by resource allocation and by changes in photosynthesis and metabolism (Núñez-Farfán et al. 2007; Fornoni 2011). Thus, immediate interactions between plant antagonists, especially when they are not strong, may not always be reflected in plant performance (Fournier et al. 2006). However, only in the combined treatment with white rust and flea beetles, was survival of P-plants lower than 100 %, indicating that the combined impact of the herbivore and the pathogen may in some way still affect plant performance negatively.

Signal cross talk in plant defence has been shown to affect plant–antagonist interactions (Koornneef and Pieterse 2008; Thaler et al. 2010). However, our result on antagonism between *Albugo* and flea beetles on biomass of G-plants does not seem to involve such processes. Herbivory by tissue-damaging insects like flea beetles is known to induce the JA-signalling pathway, whereas biotrophic pathogens like *Albugo* mainly induce the SA-signalling pathway, and the two pathways are considered to antagonize each other (Kunkel and Brooks 2002; Glazebrook 2005; Stout et al. 2006). In that case, we should have expected a synergistic interaction between the herbivore and the pathogen. Yet the generality of cross talk in JA/SA-signalling is still discussed and may be more complex than previously thought (Thaler et al. 2012; Biere and Bennett 2013). In the P-type, however, negative cross talk may play a role. Here, we found a weak synergistic interaction for flea beetle larval performance (damage tended to be higher in pathogen-inoculated plants) and a slight reduction of plant biomass at third harvest, but no effect on pathogen performance. Generally, SA is thought to have a strong effect on JA suppression, but JA a milder effect on the SA pathway (Leon-Reyes et al. 2010), which could explain the asymmetric interactive effect between the herbivore and the pathogen on each other's performance. However, the increased herbivore damage in infected plants could be caused by other processes, e.g., changes in nutritional value of plants upon pathogen infection. Pathogen-infected tissues may accumulate carbohydrates, lipids and nitrogen compounds (Farrar and Lewis 1987), which may lead to preferential consumption of pathogen infected plants by herbivores (Ramsell and Paul 1990). Van Mölken et al. (2014a), however, did not find a higher concentration of nitrogen in pathogen-infected leaves of *B. vulgaris*.

Interestingly, the negative interactive impact of *Albugo* and flea beetles on the biomass of G-plants was still present at the third harvest, 1 year after the actual experimental treatment with the herbivore and the pathogen stopped. This indicates that priming and epigenetic effects may have contributed to this; recent results suggest that induction and priming may last much longer than previously believed (Conrath et al. 2002, 2006; Herrera and Bazaga 2011; Holeski et al. 2012; Verhoeven and van Gurp 2012; Worrall et al. 2012). Herbivory may induce resource allocation from the shoots to the roots (Trumble et al. 1993; Holland et al. 1996; Erb et al. 2009), and thus compensate for the negative effect of white rust on plant biomass. However, the decrease in loss of biomass in pathogen-inoculated plants when exposed to the flea beetles simultaneously is similar for all three harvests. If we expected herbivore-induced

resource allocation to account for a better performance of G-plants exposed to both *Albugo* and flea beetles, resources stored in the roots would probably be used for regrowth already after the first harvest, and thus it would be unlikely that we see the same effect after the second and third harvest. Consistently, van Mölken et al. (2014a) found no evidence for herbivore-induced root–shoot allocation of biomass under indoor conditions.

Albugo markedly reduced biomass at third harvest, which mainly consisted of fruiting stalks, indicating that the pathogen may decrease reproduction and propagation of G-plants, and that P-plants may have a potential selective advantage when exposed to *Albugo*. Overall, the impact of white rust on *B. vulgaris* was markedly stronger compared to flea beetles, suggesting that resistance to *Albugo* is a greater advantage for the plant than flea beetle resistance. Under greenhouse conditions, however, *Albugo* had only a small effect on plant performance, whereas the impact of flea beetles was comparatively strong. This suggests that interactions may vary depending on the environmental settings.

Implications

Impacts of antagonists on plant performance may depend on whether the plant is attacked by a single or by multiple antagonists. At the same time, direction and magnitude of these interactive effects may be different for different plant genotypes: the negative impact of *Albugo* on plant performance was substantially reduced by the presence of flea beetles, and this was only existent in the G-type of *B. vulgaris*.

A previous greenhouse study (van Mölken et al. 2014a) demonstrated that secondary metabolite levels differed between plants infested with a single antagonist and plants infested with the herbivore and the pathogen in combination. These biochemical changes may possibly explain the interactive effects we observed. However, detailed investigations of interactive effects on the plants' chemistry are required to disentangle the underlying mechanisms.

We found the same effects throughout several harvests, long after pathogen and herbivore infestation, indicating that antagonists and their interactions may have long-lasting effects on plants. In contrast to this study, which was conducted outdoors, the greenhouse study did not find any effect of *Albugo* or interaction between flea beetles and *Albugo* on plant biomass or reproductive output. This finding emphasizes the importance of considering environmental parameters when evaluating plant–antagonist interactions (Núñez-Farfán et al. 2007), and may be especially relevant for decision-making in integrated pest management, where impacts usually are to be evaluated under natural or semi-natural conditions.

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