



## Research

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# Unpredicted impacts of insect endosymbionts on interactions between soil organisms, plants and aphids

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Ecologically significant symbiotic associations are frequently studied in isolation, but such studies of two-way interactions cannot always predict the responses of organisms in a community setting. To explore this issue, we adopt a community approach to examine the role of plant–microbial and insect–microbial symbioses in modulating a plant–herbivore interaction. Potato plants were grown under glass in controlled conditions and subjected to feeding from the potato aphid *Macrosiphum euphorbiae*. By comparing plant growth in sterile, uncultivated and cultivated soils and the performance of *M. euphorbiae* clones with and without the facultative endosymbiont *Hamiltonella defensa*, we provide evidence for complex indirect interactions between insect– and plant–microbial systems. Plant biomass responded positively to the live soil treatments, on average increasing by 15% relative to sterile soil, while aphid feeding produced shifts (increases in stem biomass and reductions in stolon biomass) in plant resource allocation irrespective of soil treatment. Aphid fecundity also responded to soil treatment with aphids on sterile soil exhibiting higher fecundities than those in the uncultivated treatment. The relative allocation of biomass to roots was reduced in the presence of aphids harbouring *H. defensa* compared with plants inoculated with *H. defensa*-free aphids and aphid-free control plants. This study provides evidence for the potential of plant and insect symbionts to shift the dynamics of plant–herbivore interactions.

## 1. Introduction

The development of intimate associations between microbial symbionts and their hosts has shaped the evolution of many organisms and allowed higher organisms to exploit otherwise inhospitable living environments [1,2]. However, although there is emerging evidence that microbial endosymbionts can shape the outcome of plant–herbivore interactions (reviewed in [3]), symbiotic associations are frequently studied in isolation from the community of organisms that interact with them. For example, a plant interacting with soil microbiota will be simultaneously engaging in interactions with a variety of other organisms, including insect herbivores. In addition, many plant sap-feeding insects host several types of microbial endosymbionts that supplement their nutritionally poor diet, modulate insect fitness and influence the outcome of trophic interactions (reviewed in [4,5]). For well-studied microbial symbionts of plants and insect herbivores, we can anticipate the influence of each individual host–symbiont pairing on plant–insect herbivore interactions [4,6]. However, such studies of two-way interactions (e.g. between a plant and its herbivore or an insect herbivore and its symbionts) cannot always predict the responses of organisms in a community [7–9]. Here, we adopt a community approach and simultaneously examine the role of both plant– and insect–microbial interactions in modulating the plant–herbivore interaction. To our knowledge, this is the first study to use a community approach to examine the interaction between microbial elements of the above- and below-ground components of a plant–herbivore system.

Soil management practices such as tillage, pesticide spraying and nutrient fertilization significantly alter the physical, chemical and biotic properties of the soil (reviewed in [10]) with potentially dramatic effects on the soil microbial community [11]. Compared with uncultivated sites, arable soils disturbed by tillage exhibit significantly reduced diversity, both in terms of species number and functional diversity of AM fungi, soil bacteria and other soil organisms [11]. Furthermore, some soil organisms that tolerate soil disturbance are often fast breeding generalists [11] that are less effective at capturing or recycling nutrients compared with species associated with uncultivated soils [12]. This raises the question of whether soil communities associated with cultivated and uncultivated soils differentially influence the nutritional quality of plants for insect herbivores.

Phloem-feeding aphids are important components of arable and natural food webs, causing direct and indirect damage to plants by removing resources and vectoring economically important plant diseases, as well as supporting a range of predator and parasitoid populations. A further interaction is with their community of bacterial endosymbionts. Most aphid species harbour the obligate bacterial endosymbiont *Buchnera aphidicola*, which provides essential nutrients to the aphid that supplement the nutritionally poor phloem sap diet [13,14]. In addition to *Buchnera*, many aphids harbour one or more types of facultative bacterial endosymbionts that influence aphid fitness and susceptibility to natural enemies [1,5]. For example, the facultative bacterial endosymbiont *Hamiltonella defensa* confers resistance to attack by parasitoid wasps in the pea aphid [8]. However, in the absence of parasitoids, the incidence of *H. defensa* declines in the aphid population [15,16], indicating fitness costs associated with harbouring this facultative endosymbiont that might relate to a low capacity for nutrient biosynthesis and dependence on *Buchnera*-derived nutrients [17]. This raises the intriguing possibility that endosymbiont infection could influence the aphid response to the host plant condition.

The aim of this study is to examine the potential interaction between soil community and insect endosymbiont status and the consequences of such an interaction for plant growth and insect performance. Our experimental system, conducted in controlled conditions under glass, comprised the potato aphid, *Macrosiphum euphorbiae*, feeding on potato, *Solanum tuberosum* var. Maris Piper and harbouring the facultative endosymbiont *H. defensa*. By comparing plant growth in sterile, cultivated and uncultivated soils and the performance of *M. euphorbiae* clones with and without *H. defensa*, we test the linked hypotheses that: (i) effects of soil cultivation will indirectly influence aphid performance and (ii) the range of aphid responses to these effects will vary with the presence of facultative symbionts. Our first prediction is that plant growth will be promoted by the more diverse soil microbial community associated with uncultivated, compared with cultivated soils, and that this will promote aphid development and fecundity. Our second prediction is that *H. defensa* infection will incur a fitness cost to aphids, and this will be exacerbated on plants grown in cultivated soils. To our knowledge, this is the first study to quantify the effects of *H. defensa* infection on fecundity of *M. euphorbiae* and furthermore, to examine the potential for indirect effects of aphid endosymbiont status on host plant growth.

## 2. Material and methods

### (a) Experimental material

Six clones of the potato aphid *M. euphorbiae* (AA09/03, AA09/04, AA09/06, AA09/12, AA09/13 and AA09/14) collected from a number of sites in Tayside in 2009 were cultured on excised leaflets of potato (cultivar Desiree) at 20°C with 16 L:8 D cycle. To confirm the presence or the absence of *H. defensa* in each aphid clone, DNA was extracted from fresh samples of approximately 10–15 adult aphids of each clone using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). The extraction protocol was modified for efficiency from the Qiagen Supplementary Protocol: 'Purification of total DNA from insects using the DNeasy Blood & Tissue Kit (DY14-Aug06)' to include an incubation of 70°C for 10 min followed by a centrifugation step (2 min, 6000g) after the addition of buffer AL. Diagnostic PCR was performed using forward primer PABSF and reverse primer 16SB1 to detect *H. defensa*, and to confirm the absence of other known facultative endosymbionts, according to the study of Douglas *et al.* [13] (specifically, the aphids were directly tested for the presence of *Regiella insecticola*). *Hamiltonella defensa* was confirmed to be present in three aphid clones (AA09/03, AA09/04 and AA09/06) and absent in three clones (AA09/12, AA09/13 and AA09/14), and subsequent screening (not shown) has confirmed the stability of the *H. defensa* infection in cultures of these aphid clones over periods of several months.

Soil was collected in June 2010 from two sites at the James Hutton Institute (JHI) in Dundee, Scotland, a field where cultivation has not occurred for over 6 years (uncultivated soil, high soil microbial diversity) and a field under long-term barley cultivation (cultivated soil, low soil microbial diversity). Soil was sieved to remove stones and large debris. A portion of the sieved soil was sterilized by autoclaving at 121°C for 3 h, allowed to cool for 24 h and then autoclaved at 121°C for a further 3 h.

### (b) Experimental design

The experiment comprised 189 plants in a randomized 2 × 7 × 3 factorial design featuring two levels of aphid herbivory (present or absent), seven levels of aphid herbivory (none, feeding by three different aphid clones lacking *H. defensa*, and feeding by three aphid clones hosting *H. defensa*) and three soil treatments (sterile, cultivated and uncultivated). The experiment was divided into three temporal blocks to allow the timing of aphid inoculation to be staggered, with a period of 24 h between each block inoculation. Each block contained three replicates of each treatment giving a total of 63 plants per block.

Pots were filled with a combination of background soil (sterile cultivated soil) and both soil types to control for potential nutritional or physical differences between the soils, so that any differences in plant or aphid traits could be confidently attributed to differences in the soil community. Sterile 10 l pots were filled as follows: 1 l of sterile cultivated soil, followed by 8 l of treatment soil (6 l of sterile cultivated soil mixed with 2 l of inoculum) and topped with 1 l of sterile cultivated soil. The inoculum comprised three soil treatments: sterile (1 l of sterile cultivated soil and 1 l sterile uncultivated soil), cultivated (1 l live cultivated soil and 1 l sterile uncultivated soil) and uncultivated (1 l sterile cultivated soil and 1 l of live uncultivated soil). Seed quality tubers of potato cultivar Maris Piper were supplied by JHI Dundee. Tubers of relatively uniform size were surface-sterilized by soaking for 15 min in 2% NaOCl solution followed by two 15 min rinses in deionized water. In each pot, a single sterilized tuber was planted heel-down below the top 1–1.5 l of soil. The date of tuber sowing was recorded as the start of week 0 of plant growth. Plants were grown under glass with supplementary lighting to achieve 16 L:8 D cycle and 18°C:14°C (day:night) temperature cycle. Plants were watered

daily taking care to prevent splashing that could transfer soil inocula between pots. Damage by Western flower thrips (*Frankliniella occidentalis*), which became apparent at week 7, was scored weekly using a five-point scale (1 = no damage; 5 = 75% leaves displaying damage over a large proportion of the leaf surface), and Thrip-Ex plus biocontrol (Koppert Biological, Veilengeweg) was introduced at week 9. At week 11, aphid-free control plants were covered with aerated bags when some plants became infested with non-experimental aphids (*M. euphorbiae* and *Myzus persicae*).

When plants were eight weeks old, two apterous adult *M. euphorbiae* females were confined in mesh-covered clip-on leaf cages of 2.5 cm internal diameter attached to the underside of terminal leaflets of leaves positioned approximately at mid-stem height on each plant assigned to an aphid treatment. Aphid cages were covered with small transparent, perforated bags to further secure the clip-on cages, and therefore minimize aphid escape from the inoculated leaflet. Leaflets of aphid-free control plants were caged in a similar manner. Adult aphids were removed once they had produced nymphs, and nymphs were then culled to a single individual per plant when 7 days old. Nymphs were monitored daily to record time (in days) to adulthood (TTA), adult morph (based upon the presence or the absence of wings) and time to the start of reproduction (or the pre-reproductive period  $T$ ). Reproductive output was recorded for a period of time equivalent to  $T$  (termed the post-reproductive period), after which the adult aphid was removed from the plant; nymphs were periodically removed from caged leaflets to prevent overcrowding. These parameters were used to calculate aphid  $R_m$  (the intrinsic capacity for population increase [18]).

Plants were harvested at 14 weeks after sowing. The number of stems was recorded, and plants were scored using a three-point scale to assess the extent of colonization by non-experimental aphids (1 = less than five aphids per plant; 2 = 5–50 aphids per plant; 3  $\geq$  50 aphids per plant). Plant stems were removed at the stem base and separated into leaves and stems. Roots and tubers were washed free of soil, and tubers were removed by severing the root material at the base of the stolon; where possible, the mother tuber was recovered. Tubers, stolons and mother tubers were oven-dried at 70°C for 3–4 days. Root material was oven-dried at 60°C for 7 days. Dry mass was recorded for all plant fractions.

### (c) Statistical analysis

ANOVA was applied to the data using the general linear models procedure of SAS (SAS 9.2, Cary, NC, USA) with leaf, stem, stolon, root, tuber, total plant mass and  $R_m$  alternately designated as the dependant variable. Block structure, soil type and aphid clone were input as independent factors. The main and interactive effects of soil type and aphid clonal line were analysed. There was no influence of any independent variable on non-experimental herbivores, and thus thrip and non-experimental aphid scores were included as covariates in addition to aphid morph and mother tuber dry mass. Three post-hoc orthogonal contrasts were included in the model. The first contrast (hereby referred to as *H. defensa*) tested for differences due to the presence of *H. defensa* within the main effect clonal line. We ran this contrast even when the main effect of clonal line was insignificant in the model, because the *H. defensa* contrast excluded aphid-free plants and tested a distinctly different question from the main effect. Running the *H. defensa* contrast within our main model reduced our Type II error by eliminating the need to run a second analysis on the data simply to answer the question of whether the presence of *H. defensa* itself altered any of the dependent variables. The second contrast (*Herbivory*), also run within the main effect clonal line, tested the *a priori* hypothesis that herbivory itself (regardless of clonal line or presence of *H. defensa*) altered plant-dependent variables. The

final contrast (*Tuber Mass*), run within the interaction between clonal line and soil type, examined the pattern of variation in tuber mass induced by different aphid clones between soil types. To satisfy the normality assumptions of the model, stolon and root dry mass values were log-transformed. The proportion of total plant mass contained in the roots (the root fraction) was calculated and analysed within the same model following arcsine square root transformation. To determine whether the non-parametric variable TTA was influenced by the independent variables block structure, soil type and aphid clone, we conducted a Cochran–Mantel–Haenszel test in the FREQ procedure of SAS, and to determine if TTA was influenced by *H. defensa* we conducted a Kruskal–Wallis test in the NPAR1WAY procedure in SAS. Results are reported at a significance level of 5% or smaller.

## 3. Results

Plant dry mass was altered by soil treatment (table 1). Dry mass of plants grown in sterile soil was on average 15% smaller than for plants grown in the cultivated and uncultivated soil treatments ( $F_{2,188} = 20.72$ ,  $p = 0.001$ ; figure 1a and table 1). Aphid fecundity responded to host plant soil treatment (table 1), with higher values of  $R_m$  achieved on plants growing in sterile soil compared with uncultivated soil ( $F_{4,107} = 3.73$ ,  $p = 0.0281$ ; figure 1b). None of the independent factors influenced TTA (Cochran–Mantel–Haenszel Statistic (d.f. = 1) = 0.8956,  $p = 0.3440$ ). Infection with *H. defensa* did not affect either aphid  $R_m$  (*H. defensa*-free  $0.105 \pm 0.005$ , *H. defensa*  $0.108 \pm 0.005$ ; table 2) or TTA (Kruskal–Wallis  $H_1 = 0.756$ ,  $p = 0.3847$ ); *H. defensa*-free  $8.018 \pm 0.154$ , *H. defensa*  $7.954 \pm 0.144$ ).

The *Herbivory* contrast (table 1) demonstrated that aphid feeding increased stem dry mass by approximately 20% ( $F_{1,188} = 11.85$ ,  $p = 0.0007$ ; figure 2a) and reduced stolon dry mass ( $F_{1,188} = 11.47$ ,  $p = 0.0009$ ; figure 2b) relative to control plants, irrespective of aphid clone and soil treatment.

The fraction of plant dry mass allocated to the roots (the root fraction) was smaller for plants exposed to aphids harbouring *H. defensa* compared with plants exposed to *H. defensa*-free aphids and to aphid-free control plants (table 1; *H. defensa* contrast,  $F_{1,188} = 5.39$ ,  $p = 0.0215$ ; figure 3). The root fraction was unaffected by soil treatment or aphid clonal line (table 1).

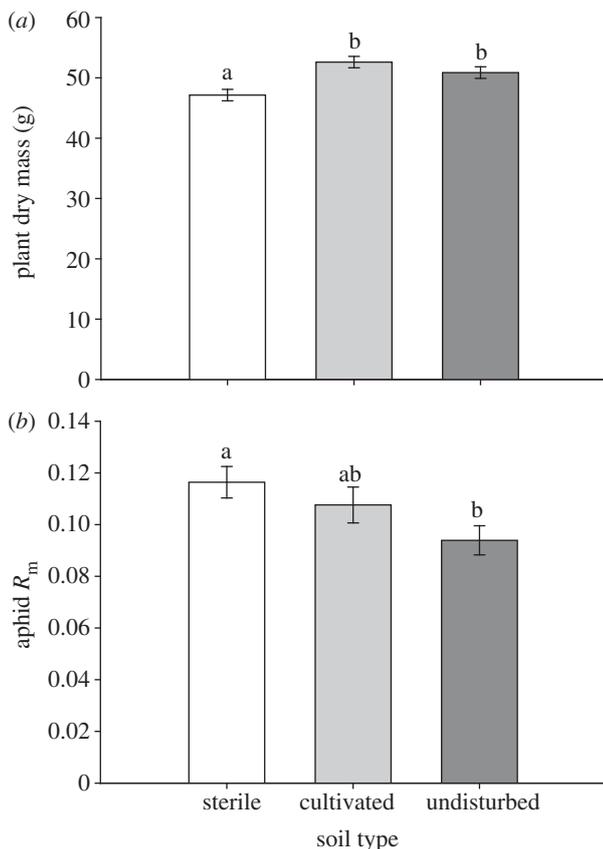
In contrast to the observed changes in stem and stolon mass, the effect of aphids on tuber dry mass depended on the aphid clone, irrespective of *H. defensa* infection status and soil treatment ( $F_{12,188} = 2.10$ ,  $p = 0.0196$ ; figure 4) principally due to a larger difference in tuber mass between sterile and cultivated soils in the presence of certain aphid clones (AA 09/06, AA 09/12 and AA 09/14 (*H. defensa* present in the former clone and absent in the latter two clones),  $F_{1,188} = 15.53$ ,  $p = 0.0001$ ; table 1, *Tuber Mass* contrast) and in aphid-free control plants.

## 4. Discussion

This is the first study to explore the potential for bottom-up and top-down interactions between soil community diversity and facultative bacterial endosymbionts of aphids mediated through the host plant. We observed clear effects of soil treatment on plant growth and aphid performance that indicate a significant impact of the soil community on the dynamics of plant–herbivore interactions. We also observed a surprising

**Table 1.** ANOVA of plant dry mass, showing variance ( $F$ ) and probability ( $p$ ) values. Contrasts are indented and italicized.

	total plant mass		stem mass		stolon mass		tuber mass		root fraction		
	d.f.	$F$	$p$ -value	$F$	$p$ -value	$F$	$p$ -value	$F$	$p$ -value	$F$	$p$ -value
block	2	1.17	0.3119	21.37	<0.0001	1.06	0.3496	7.08	0.0011	3.09	0.0481
clonal line	6	0.67	0.6711	2.79	0.0131	2.89	0.0105	0.74	0.6163	1.51	0.1782
<i>Herbivory</i>	1	0.74	0.3898	11.85	0.0007	11.47	0.0009	0.59	0.4433	2.40	0.1235
<i>H. defensa</i>	1	0.41	0.5214	0.57	0.4528	0.00	0.9910	1.19	0.2773	5.39	0.0215
soil type	2	20.72	<0.0001	18.63	<0.0001	8.15	0.0004	8.47	0.0003	0.16	0.8537
clonal line $\times$ soil type	12	1.59	0.0978	1.42	0.1607	1.19	0.2908	2.10	0.0196	0.99	0.4622
<i>Tuber Mass</i>	1	—	—	—	—	—	—	15.53	<0.0001	—	—
thrips	1	3.66	0.0576	3.22	0.0745	0.03	0.8567	2.73	0.1002	0.23	0.6303
wild aphids	1	0.78	0.3796	0.13	0.7235	0.00	0.9921	2.65	0.1057	2.72	0.1013
aphid morph	2	1.24	0.2933	0.11	0.8919	4.61	0.0113	1.63	0.1998	1.72	0.1825
mother tuber	1	2.96	0.0872	5.82	0.0170	1.66	0.1997	2.90	0.0907	11.41	0.0009
weight											
error	188										

**Figure 1.** (a) Total dry mass of *Solanum tuberosum* plants in response to three soil types. (b) Mean intrinsic rate of population increase ( $R_m$ ) of six aphid clonal lines feeding on *S. tuberosum* grown on three soil types. Values are least-squares means  $\pm$  s.e.m. and letters indicate significant differences.

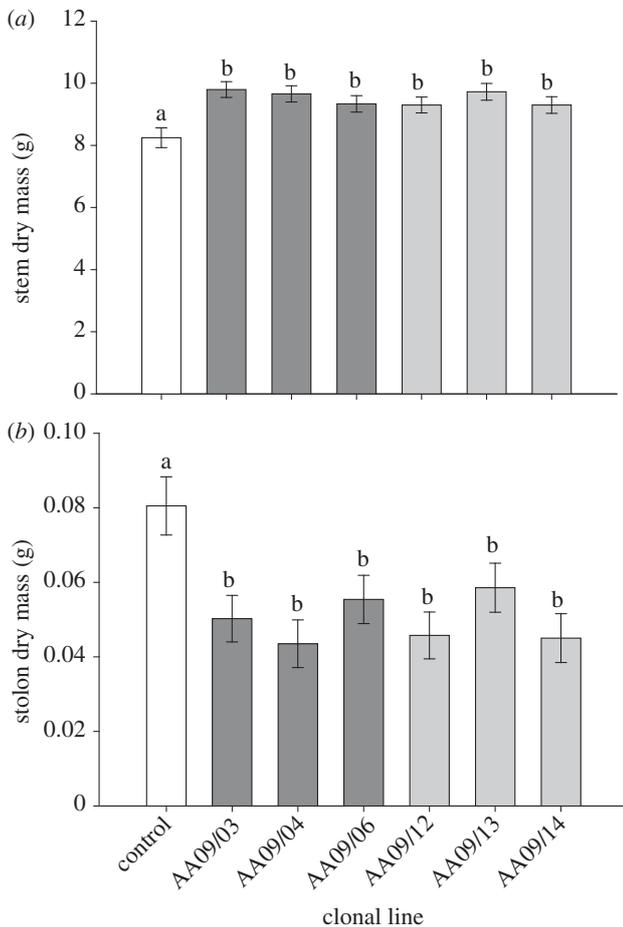
indirect effect of the aphid bacterial endosymbiont *H. defensa* on plant resource allocation.

As was initially hypothesized, aphid  $R_m$  varied in response to the soil in which its host plant was grown, providing evidence for indirect interactions between sap-feeding herbivores and the soil community. However, although we

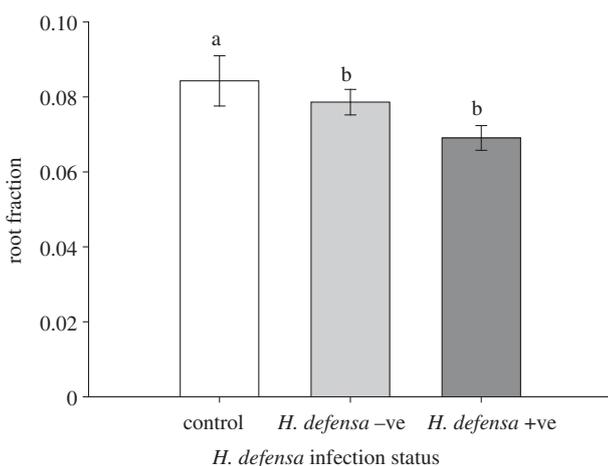
**Table 2.** ANOVA of aphid intrinsic rate of population increase ( $R_m$ ), showing variance ( $F$ ) and probability ( $p$ ) values. Contrasts are indented and italicized.

	d.f.	$R_m$	
		$F$	$p$ -value
block	2	4.36	0.0158
clonal line	2	2.98	0.0160
<i>H. defensa</i>	1	0.00	0.9707
soil type	4	3.73	0.0281
clonal line $\times$ soil type	2	1.46	0.1704
thrips	1	0.45	0.5062
wild aphids	1	1.53	0.2189
aphid morph	1	4.68	0.0333
mother tuber weight	1	0.49	0.4841
error	107		

initially postulated that  $R_m$  would be the greatest for aphid clones feeding on plants in the uncultivated soil treatment, aphid fecundity proved greatest in the sterile soil treatment, where plant biomass was significantly reduced relative to plants grown in the non-sterile soil treatments. Our hypothesis was based upon the assumption that plants grown on the high diversity uncultivated soil would be of greater benefit to herbivores than plants in the other two treatments, because associations with soil microbiota frequently augment plant acquisition of nutrients that are essential for herbivore survival, development and reproduction [19]. For example, plant tissue concentrations of phytosterols [20], sucrose [21] and phosphate [2] increase in the presence of soil microbiota. Although plant biomass was largest in the two live soil treatments, compared with the sterile soil-grown plants, it is possible that reduced aphid fecundity in these treatments was a product of tissue

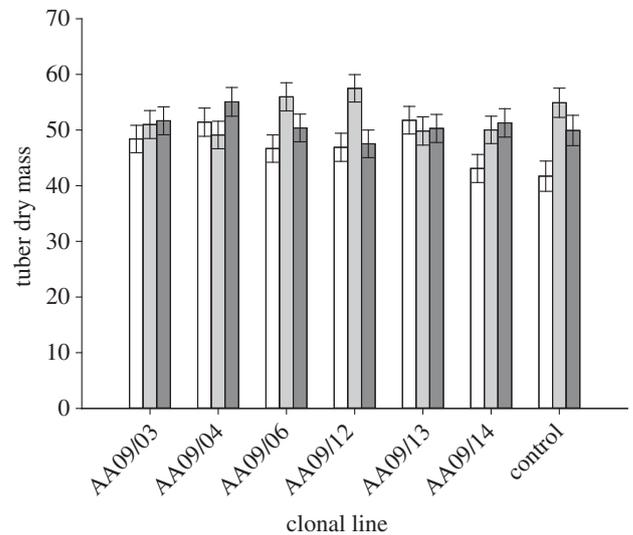


**Figure 2.** Dry mass of (a) stems and (b) stolons of *S. tuberosum* in response to feeding by six clonal lines of the potato aphid *M. euphorbiae*. Clones AA09/03, AA09/04 and AA09/06 (dark grey) harbour the facultative bacterial endosymbiont *H. defensa*, whereas clones AA09/12, AA09/13 and AA09/14 (light grey) were *H. defensa*-free. Control plants (white column) were not exposed to aphid herbivory. Values are least-squares means  $\pm$  s.e.m. and letters indicate significant differences.



**Figure 3.** Response of the root fraction (the proportion of total plant dry mass allocated to roots) to feeding by aphid clones either negative or positive for infection with the facultative symbiont *H. defensa*. Values are least-squares means  $\pm$  s.e.m. and letters indicate significant differences.

dilution of nutrients during rapid biomass accumulation [22] or increased plant resistance to herbivores mediated by soil microbes (reviewed in [23]). Alternatively, plants in the sterile



**Figure 4.** Response of tuber biomass to soil treatment and aphid clone. Clones AA09/03, AA09/04 and AA09/06 are positive for infection with the facultative symbiont *H. defensa*, and the remaining three aphid clones were free of *H. defensa* infection. Control plants were not exposed to aphid herbivory. Values are least-squares means  $\pm$  s.e.m. White bars denote sterile; light grey bars, cultivated; dark grey bars, undisturbed.

soil treatment may have been of increased quality to aphids compared with those on non-sterile soil due to compromised plant defences or more rapid onset of senescence, which can increase the flux of nutrients into the phloem to the benefit of phloem-feeding herbivores [24].

By contrast, no significant difference was observed in total plant biomass between the cultivated and uncultivated soil treatments, despite the fact that the disruptive effects of tillage [10,11] were likely to have reduced microbial diversity in the cultivated soil [25]. Although previous work [26] has demonstrated variability in total plant responses to soil community diversity, cultivated plants may be less likely to respond to variation in soil microbial communities [27].

Although aphids were present only in small numbers on each plant (while a single aphid was maintained upon each plant in the herbivory treatment any nymphs produced between counts would have been free to feed upon the plant within the confines of the clip cage. However, owing to the regular removal of nymphs, no nymph would have been able to feed for more than 48 h), aphid herbivory significantly altered plant resource allocation, increasing stem biomass and decreasing stolon biomass relative to control plants. Phloem-feeding insects such as aphids can disrupt source–sink relationships within their host plant by acting as a localized metabolic sink for photoassimilates [28]. This increase in resource demand has been linked to a compensatory increase in photosynthetic rate [28,29], which can alter the growth and physiology of the plant, potentially inducing an increase in the biomass of plants exposed to herbivory [30–32]. Such increases in growth could redirect resources away from sink tissues [32] such as the stolons and into structural tissues such as stems, although this effect did not extend to a decline in tuber mass. Response of tuber mass to aphid feeding was detected only when clone identity and soil treatment were taken into account, but there was no discernible pattern to this variation and any explanation for these effects, for example, owing to clonal differences in feeding rate and manipulation of resource allocation, are speculative.

The unanticipated decrease in relative allocation to the roots in the presence of aphids harbouring *H. defensa* could represent a new perspective from which to view the effects of this facultative symbiosis. Although a coincidental effect of aphid genotype cannot be ruled out, the lack of observed interaction with soil treatment or with aphid clone meet the criterion of a robust facultative symbiont effect (as defined by Douglas *et al.* [13]) on root allocation. The perturbation in root fraction poses significant questions, implying that *H. defensa* infection could influence aphid feeding or aphid manipulation of resource flux through the phloem. We initially proposed that *H. defensa* infection would impose a fitness cost on aphids and that this cost would be exacerbated in low diversity soil treatments, but aphid performance did not vary with *H. defensa* infection status. However, *H. defensa* is dependent upon its aphid host for the provision of eight essential amino acids [17] and therefore may compete with the aphid for the essential nutrients synthesized by *B. aphidicola* [1], which could conceivably lead to changes in the nutritional requirements and feeding behaviour of *H. defensa*-infected aphids. No other aspect of plant biomass examined in this study displayed a response to *H. defensa* infection but the feeding densities of aphid employed in this study were low, and it could be hypothesized that this observed decrease in root fraction could become more pronounced at higher feeding densities.

This study provides evidence for the potential of plant and insect symbiont systems to shift the dynamics of plant–herbivore interactions. Further research will be required to understand the mechanistic detail of the complex indirect interactions between plant–soil microbe mutualisms and aphid–bacterial mutualisms. Aphids are not the only agriculturally significant insects to carry facultative endosymbionts [1,5,33], and it has been argued that the ecology and physiology of symbiont-bearing insects cannot be fully understood outside the context of these symbioses [4]. The incorporation of insect symbionts into the design of soil–plant–insect interaction studies could provide further novel insight into the wider ecological significance of facultative symbioses.

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**Data accessibility.** Data are available via the Dryad Repository: <http://dx.doi.org/10.5061/dryad.7b79m>.

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