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Combined effects of mutualistic rhizobacteria counteract virus-induced suppression of indirect plant defences in soya bean

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It is increasingly clear that microbial plant symbionts can influence interactions between their plant hosts and other organisms. However, such effects remain poorly understood, particularly under ecologically realistic conditions where plants simultaneously interact with diverse mutualists and antagonists. Here, we examine how the effects of a plant virus on indirect plant defences against its insect vector are influenced by co-occurrence of other microbial plant symbionts. Using a multi-factorial design, we manipulated colonization of soya bean using three different microbes: a pathogenic plant virus (bean pod mottle virus (BPMV)), a nodule-forming beneficial rhizobacterium (*Bradyrhizobium japonicum*) and a plant growth-promoting rhizobacterium (*Delftia acidovorans*). We then assessed recruitment of parasitoids (*Pediobious foveolatus* (Eulophidae)) and parasitism rates following feeding by the BPMV vector *Epilachna varivestis* (Coccinellidae). BPMV infection suppressed parasitoid recruitment, prolonged parasitoid foraging time and reduced parasitism rates in semi-natural foraging assays. However, simultaneous colonization of BPMV-infected hosts by both rhizobacteria restored parasitoid recruitment and rates of parasitism to levels similar to uninfected controls. Co-colonization by the two rhizobacteria also enhanced parasitoid recruitment in the absence of BPMV infection. These results illustrate the potential of plant-associated microbes to influence indirect plant defences, with implications for disease transmission and herbivory, but also highlight the potential complexity of such interactions.

1. Introduction

Plant odours are important sources of ecologically relevant information for other organisms, including insect herbivores and their natural enemies [1–3]. Recent work has made it clear that microbial plant symbionts, including both pathogens [4,5] and mutualists [6,7], can modify plant volatile emission patterns, along with other plant traits that influence plant–insect interactions. These microbial influences on volatile-mediated interactions may have potentially important ecological implications, including for the spread of plant diseases by herbivorous insect vectors [6,8–10]. However, much of the work that has explored such influences was conducted under controlled experimental conditions, with a narrow focus on the effects of individual microorganisms on a limited suite of herbivore or natural enemy behaviours [11]. Consequently, we have an incomplete understanding of volatile-mediated interactions in more realistic ecological contexts, where plants simultaneously associate with multiple microbial colonizers having diverse lifestyles and potentially conflicting interests [10].

Among plant-associated microbes, insect-vectored viruses are the best studied with respect to their effects on plant physiology and the modification

of plant-produced volatiles. In the case of vector-borne viruses, these effects often influence plant–vector interactions in ways that appear conducive to disease transmission [12]. For example, positive effects of virus infection on vector attraction to the odours of infected hosts have now been reported in many plant pathosystems [13,14], while counterexamples in which vectors use odour cues to discriminate against infected plants are rare or non-existent [15]. The presence of such patterns suggests that natural selection can favour viral genotypes whose effects on plant volatile emissions, and other host plant traits, influence the frequency and nature of plant–vector interactions in ways that are conducive to virus transmission [9,16]. To date, however, virus effects on host–vector interactions have typically been examined under highly controlled experimental conditions designed to isolate the effects of the virus on the host plant. Consequently, we know little about how virus effects on plant–vector interactions may be influenced by other ecological factors, including the presence of other microbial plant symbionts, which also have the potential to influence plant volatile emissions and other relevant traits, and whose interests may diverge from those of viral pathogens.

Common, non-viral plant symbionts with significant potential to modify host–plant chemistry include nitrogen-fixing rhizobia, which have coevolved with leguminous hosts [10,17,18], and plant growth-promoting rhizobacteria (PGPR) [19,20]. These belowground symbionts have previously been shown to influence plant defence pathways and plant phenotypes, with consequences for multi-trophic interactions [17,21,22]. For example, rhizobia colonization of lima beans reduced production of damage-induced volatiles via the octadecanoid, mevalonate and non-mevalonate pathways, but increased production of compounds produced by the shikimic acid pathway, causing changes in plant volatile emissions that reduced the attractiveness of lima bean plants to a specialist insect herbivore [17]. Studies on the PGPR *Pseudomonas fluorescens* show that these rhizobacteria can also influence herbivore-induced plant volatile emissions and the recruitment of parasitoid wasps, with positive or negative effects depending on the defence pathway induced by the attacking herbivore (jasmonic acid (JA) versus salicylic acid (SA)) [6,8]. The effects of individual rhizobacteria can also be influenced by the presence of other soil-borne microorganisms [23]. In such multiple-species scenarios, positive effects for the host plant may be more likely to arise from interactions between functionally distinct symbionts, which are less likely to be in competition with one another and may have complementary effects (e.g. on nutrient availability to the host plant) [23]. Such effects have been described for plant symbiosis with nitrogen-fixing rhizobacteria and arbuscular mycorrhizal fungi, which enhance nitrogen and phosphorous availability, respectively [23–25]. Soil-borne rhizobacteria also have the potential for both positive and negative interactions with biotrophic pathogens such as plant viruses [26,27]. The resulting effects of such interactions on host plant traits, including volatile emissions, may have implications for the behaviour of other organisms, including insect herbivores and their natural enemies, but these are currently not well understood.

To address the lack of information on plant-mediated interactions among viruses and other plant-symbiotic microorganisms [10] we documented effects of three different microbial colonizers, including the systemic plant virus bean pod mottle virus (BPMV), the nitrogen-fixing *Bradyrhizobium japonicum* and the PGPR *Delftia acidovorans*, on interactions among soya bean plants, a specialist beetle herbivore that serves as a vector of BPMV (*Epilachna varivestis* (Coccinellidae)), and a parasitoid natural enemy of the beetle (*Pediobious foveolatus* (Eulophidae)). We further documented the outcomes of co-colonization by these microbial players to gain insight into the relative strength of microbial effects on a shared plant resource. Because BPMV is transmitted to new plants only by the mobile adult stage of the beetle vector, we hypothesized that BPMV would induce changes in odour phenotypes that suppress the recruitment of the parasitoid, which attacks *E. varivestis* larvae. By contrast, because the success of rhizobacterial colonizers is enhanced when plants grow larger and produce greater root mass and higher levels of assimilated carbon, we hypothesized that both bacterial root colonizers would tend to enhance indirect plant defences. In the light of prior evidence of both additive and interactive beneficial effects on plant growth owing to co-colonization with PGPR and rhizobia [23,28], we further hypothesized that colonization by both bacterial species would have the strongest influence on induced plant defences and parasitoid recruitment.

2. Material and methods

(a) Bacteria, viruses and culture conditions

Our studies included all possible combinations of single, dual and triple colonization events (electronic supplementary material, table S1.1). Bacteria of each species were isolated from commercial inocula (BrettYoung) under sterile conditions, sub-cultured, and stored at −80°C as 30% glycerol stocks (see the electronic supplementary material, S1.1 for details). BPMV (*Comoviridae*) is an emerging viral pathogen of legumes, primarily soya beans and snap beans [29]. BPMV-infected leaf tissue was harvested and lyophilized, then stored at −20°C.

(b) Generation of plants for experiments and factorial design

Soya bean seeds (*Glycine max* cv. Williams 82) were sterilized for 5 min in a 10% sodium hypochlorite solution, washed with ultrapure water and germinated in a growth medium (Premier Pro-mix without mycorrhiza, Griffin Supplies) that had been autoclaved at 120°C for 40 min. Three-day-old seedlings were transplanted to individual 500 ml sterilized pots containing the same growth medium, then inoculated with rhizobacteria and infected with BPMV one week later, according to the factorial treatment design and inoculation methods described in the electronic supplementary material, S1.1 and table S1.1. Starting from the V1 stage, plants received 50 ml of a diluted, modified Hoagland's nutrient solution three times per week (see the electronic supplementary material, S1.2 for details). Plants inoculated with *B. japonicum* (alone or in combination with *D. acidovorans*) received the same nutrient solution but without nitrogen fertilizer, as nodule growth is strongly inhibited by the presence of nitrates in the soil [30]; this nutrient scheme thus introduces a potential confounding factor inherent to the study system (see the electronic supplementary material, S1.2 and the discussion section for information on the potential impacts of nutrient supplementation in the context of this study).

(c) Insects

Colonies of the parasitoid *P. foveolatus* and its beetle host (and BPMV vector) *E. varivestis* were established from insects initially provided by the New Jersey Department of Agriculture's Philip

Alampi Beneficial Insect Laboratory (Thomas Dorsey). After emerging from beetle mummies (electronic supplementary material, figures S2.1 and S2.2), adult *P. foveolatus* were kept in rearing cages in an incubator at 25°C with a L 16 : D 8 photoperiod and provisioned with honey and water (electronic supplementary material, figure S2.2 and video S3). *Epilachna varivestis* were maintained on uninfected *Phaseolus vulgaris* plants, under the same conditions as the parasitoids, but in separate incubators.

(d) Evaluation of microbial effects on odour-based foraging by *Pediobious foveolatus*

Pediobious foveolatus orientation preferences were evaluated via Y-tube olfactometer assays in a greenhouse at 23°C–25°C and 70% relative humidity. Plants and insects were moved to the greenhouse 24 h before each trial for acclimatization. For damage treatments, three fourth-instar beetle larvae were confined in clip cages and allowed to feed for 24 h prior to the bioassay. Larvae were removed prior to assays, and plants were placed inside glass domes with ports for air input and output. Charcoal-filtered, humidified air was pushed into the domes at a rate of 1.5 l min^{-1} and pulled into each arm of the Y-tube at 1.0 l min^{-1}. The Y-tube was oriented vertically inside an opaque box to obscure visual cues (electronic supplementary material, figure S2.3). Experiments were conducted between 11.00 and 17.00, corresponding to the peak of volatile release by damaged soya bean plants. Preliminary trials confirmed the attraction of wasps to plant odours versus empty control domes (electronic supplementary material, figure S2.4). We then tested the attraction of female wasps (n = 100 per treatment combination) to each of the following pairs of treatments: (i) *D. acidovorans* (Da) versus *B. japonicum* (Bj); (ii) control versus Da; (iii) control versus *B. japonicum* + *D. acidovorans* (Bj + Da); and (iv) control versus Bj. These bacterial treatment comparisons were conducted with uninfected (virus free) plants for the first round, then with BPMV-infected plants for the second round. A third set of comparisons, developed based on the results of the first two sets, compared Bj + Da uninfected versus Bj + Da infected and control uninfected versus control infected (see the electronic supplementary material, S1.3 for details).

(e) Evaluation of microbial effects on *Pediobious foveolatus* parasitism rates

Based on the results of the olfactometer bioassays, we examined *P. foveolatus* parasitism of larvae feeding on soya bean plants with select rhizobacteria and BPMV treatments, including: (i) Bj + Da versus control; (ii) Da versus control; (iii) Bj versus control; and (iv) Bj + Da versus Bj. These choice tests were conducted in two rounds (for uninfected and BPMV-infected plants) as described for the odour-based foraging experiment. Additionally, we tested the following mixed BPMV treatments: (v) Bj + Da uninfected versus Bj + Da infected and (vi) control uninfected versus control infected. These bioassays employed semi-natural set-up in a greenhouse under the same conditions as the Y-tube assays. Female wasps were released inside a fine-mesh tent (60 × 60 × 60 cm) containing two plants on which *E. varivestis* larvae were feeding (see the electronic supplementary material, S1.4 and figure S2.6 for details). A total of 120 larvae were tested for each of the treatment comparisons in a dual choice assay over a period of 10 days, using new wasps, larvae and plants for each test.

(f) Microbial effects on herbivore-induced volatile emissions of soya bean

Plant volatiles were collected in a growth chamber equipped with a push-pull volatile sampling system capable of simultaneous collection from 16 plants. This system enabled replication of each treatment two times within collection iterations (n = 5 total replications per treatment). However, pretests in this environment revealed that larvae reacted adversely to conditions inside the collection domes (reduced feeding), while adults behaved normally. Therefore, herbivore damage treatments for volatile collections were imposed using adults rather than larvae (implications of this difference are discussed below). Each plant was subjected to herbivory by three adult beetles over 24 h before collection and also during the collection period, using clip cages to control the leaf area removed. Stems of plants in vegetative stage 4 (three to four weeks old) [31] passed through an opening in a Teflon base supporting a 5 l glass chamber with ports for air input and output. Volatiles were collected for 7 h (11.00–18.00) through adsorption to traps containing 40 mg of SuperQ® (Alltech) (see the electronic supplementary material, S1.5 and figure S2.5 for details).

(g) Microbial effects on plant biomass, nodulation and bean pod mottle virus symptoms

Using two separate sets of undamaged plants, we assessed the effect of co-inoculation and BPMV infection on plant biomass and nodulation. Stage V4 plants in the first set were harvested to measure total shoot biomass (n = 20 plants per treatment). Roots from plants in the second set (n = 10–14 per treatment) were thoroughly washed, and nodules were harvested and placed separately in paper envelopes, then dried at 50°C for 48 h. The total dry biomass of the nodules was measured for each plant (nodules are only present in plants inoculated with *B. japonicum*, but control and Da-inoculated plants were checked for possible cross-contamination). BPMV symptom severity was also visually assessed for the second set of plants (n = 10–34 per treatment) using a 1–5 scale, where 1 = no symptoms and 5 = severe mottling, extensive stunting, strong leaf deformation and blistering.

(h) Statistical analyses

To analyse behavioural data, plant biomass, nodulation and BPMV symptoms, we used Bayesian generalized linear mixed models (GLMM) with Markov chain Monte Carlo (MCMC) estimation using the R package MCMCglmm [32] (see the electronic supplementary material, S1.6 and R code). We specified the multinomial family in both behavioural assays. Time to choose between the two arms was analysed using a Gaussian distribution in the GLMM. It was not possible to experimentally test all biologically relevant comparisons owing to logistical constraints. Therefore, we ensured that parasitoid attraction to plants with each microbial treatment was directly compared to the appropriate control treatment under the same conditions, then compared the proportions of wasps choosing plants with one microbial treatment (versus control) to the proportion choosing a second microbial treatment (versus control) to make inferences about the attractiveness of plants with microbial treatments relative to each other. We also used the proportion of parasitism in each treatment versus the same control to indirectly compare the parasitism rates across treatments. Plant biomass and nodule biomass were analysed using a Gaussian distribution, while BPMV symptoms were evaluated using the categorical family in the GLMM. From each model, we extracted the posterior mean (β), the 95% highest posterior density (HPD) intervals (credible intervals (CIs) are reported instead of confidence intervals), the p-value for the posterior distribution and the deviance information criteria value (DIC) for model comparison. Posterior means, which we used as our point estimates, were used to compare the treatment effect size across treatments.

To evaluate main effects and interactions among rhizobacteria and virus treatments with respect to the overall volatile blend we performed a permutational analysis of variance (PERMANOVA) using

the Euclidean dissimilarity matrix with 999 permutations in the R package *vegan* v. 2.5.1 [33]. As a follow-up, we used a random forest (RF) algorithm for variable selection to detect the most important compounds that account for significant differences among treatments in the PERMANOVA. We used out-of-bag (OOB) error rates as the importance score for variable selection implemented as backward elimination in the package *varSelRF* v. 0.7.5 [34] (ntree = 3000 bootstrap replicates, variable drop fraction = 0.2). Performance of the RF models was evaluated by the misclassification error rate. Additionally, we used empirical Bayes moderated *t* statistics in the R package limma [35] to identify differentially expressed compounds between the experimental treatments.

3. Results

(a) Microbial effects on odour-based foraging by *Pediobious foveolatus*

Preliminary tests in the Y-tube olfactometer (electronic supplementary material, figure S2.4 and table S1.2) confirmed that *P. foveolatus* prefer volatiles of herbivore-damaged plants compared to those from undamaged plants (pMCMC = 2.47×10^{-3}) or from empty chambers (pMCMC = 2.06×10^{-4}). In odour-based foraging assays employing only uninfected (BPMV-free) plants, parasitoid attraction to damaged plants was slightly enhanced when roots were colonized by Bj alone ($\beta = 0.993$, CI = [0.473, 1.55], pMCMC = 2.27×10^{-3}) and strongly enhanced by Bj + Da ($\beta = 2.19$, CI = [1.28, 3.22], pMCMC = 4.12×10^{-4}). Inoculation with Da did not have an effect on parasitoid attraction ($\beta = 2.20$, CI = [−0.27, 0.69], pMCMC = 0.38×10^{-4}) (figure 1*a*; electronic supplementary material, table S1.2). A GLMM comparing treatments across all trials, showed that the proportion of wasps choosing Bj + Da treatments over controls was much larger than the proportion choosing Da over controls ($\beta = 1.87$, CI = [1.05, 2.64], pMCMC = 2.06×10^{-4}) and slightly larger than the proportion choosing Bj over controls ($\beta = 1.1$, CI = [0.3, 1.93], pMCMC = 6.39×10^{-3}) (electronic supplementary material, table S1.2). When all plants in pairwise comparisons were infected with BPMV (figure 1*b*), Bj + Da colonization on roots again strongly enhanced parasitoid attraction ($\beta = 1.52$, CI = [0.05, 3.21], pMCMC = 2.97×10^{-2}), while Bj alone had only a slight positive effect on parasitoid attraction ($\beta = 0.68$, CI = [−0.01, 1.47], pMCMC = 5.3×10^{-2}). A GLMM showed that the proportion of wasps choosing Bj + Da treatments versus controls was slightly larger than the proportion choosing Da ($\beta = 1.09$, CI = [0.006, 2.26], pMCMC = 4.27×10^{-2}), but not significantly different from the proportion choosing Bj ($\beta = 0.53$, CI = [−0.70, 1.83], pMCMC = 0.373) (electronic supplementary material, table S1.2). In a direct comparison of plants with mixed infection status (figure 1*c*), uninfected controls were more attractive than BPMV-infected controls ($\beta = 0.631$, CI = [0.20, 1.08], pMCMC = 8.04×10^{-3}), while uninfected plants with Bj + Da colonization on roots were strongly preferred over BPMV-infected Bj + Da-colonized plants ($\beta = 1.34$, CI = [0.67, 2.04], pMCMC = 4.33×10^{-3}).

Using a mixed effect model to test the interaction of rhizobacteria-BPMV in the wasp responses during the foraging bioassays, we confirmed that dual inoculation (Bj + Da) had a stronger effect ($\beta = 2.15$, CI = [1.41, 2.91], pMCMC = 1.03×10^{-4}, electronic supplementary material, table S1.3) than single inoculation on the attraction of the parasitoid (Bj: $\beta = 1.01$, CI = [0.42, 1.64], pMCMC = 1.2×10^{-3}; Da: $\beta = 0.20$, CI = [−0.37, 0.76], pMCMC = 0.46; electronic supplementary material, table S1.3). Although there is a significant interaction effect between Bj + Da and BPMV infection ($\beta = 1.10$, CI = [0.50, 1.78], pMCMC = 4.12×10^{-4}, electronic supplementary material, table S1.3), we found that dual-inoculated BPMV-infected plants tend to be less attractive than Bj + Da plants without virus (Bj + Da − BPMV: $\beta = 1.103$; Bj + Da: $\beta = 2.15$, electronic supplementary material, table S1.3).

Figure 1*d* summarizes the mean time to choose for wasps in bioassays using only uninfected plants (figure 1*a*) and only BPMV-infected plants (figure 1*b*). Wasps took longer to respond to the odours of BPMV-infected plants than to those of uninfected plants ($\beta = 1.945$, CI = [1.83, 2.06], pMCMC = 1.03×10^{-4}, figure 1*d*; electronic supplementary material, table S1.4); however, this delay was reduced in the presence of both rhizobacteria (Bj + Da) ($\beta = -0.79$, CI = [−0.95, −0.63], pMCMC = 1.03×10^{-4}, figure 1*d*; electronic supplementary material, table S1.4). Furthermore, wasps also took less time to choose between plants colonized by both Bj and Da and bacteria-free controls even in the absence of the virus ($\beta = -0.51$, CI = [−0.62, −0.40], pMCMC = 1.03×10^{-4}, figure 1*d*; electronic supplementary material, table S1.4). By contrast, we did not find strong evidence that the presence of Da influenced the time of response for BPMV-infected plants ($\beta = -0.077$, CI = [−0.37, 0.21], pMCMC = 0.61, figure 1*d*; electronic supplementary material, table S1.4).

(b) Microbial effects on *Pediobious foveolatus* parasitism rates

In foraging assays allowing parasitoid contact with larval hosts feeding on uninfected (BPMV-free) plants (figure 2*a*), root colonization by Bj + Da strongly increased parasitism rates ($\beta = 3.11$, CI = [1.83, 4.53], pMCMC = 1.03×10^{-4}, electronic supplementary material, table S1.2) versus controls, while colonization by Bj alone had a smaller effect on parasitism rates ($\beta = 2.39$, CI = [0.88, 3.88], pMCMC = 8.25×10^{-4}, electronic supplementary material, table S1.2). Parasitism rates on uninfected Bj + Da colonized plants were greater than those on plants colonized by Bj alone ($\beta = 2.14$, CI = [0.86, 3.45], pMCMC = 1.65×10^{-3}, electronic supplementary material, table S1.2) (figure 2*a*), and further GLMM demonstrated that the proportion of larvae parasitized on Bj + Da plants over controls was higher than the proportion parasitized on Da plants over controls ($\beta = 2.19$, CI = [1.36, 3.1], pMCMC = 1.03×10^{-4}, electronic supplementary material, table S1.2). Plants colonized by Da alone did not have greater parasitism rates relative to controls ($\beta = -0.39$, CI = [−1.65, 0.825], pMCMC = 0.52, electronic supplementary material, table S1.2) (figure 2*a*) and had reduced parasitism rates relative to plants colonized by Bj alone ($\beta = 1.94$, CI = [1.05, 2.81], pMCMC = 1.03×10^{-4}, electronic supplementary material, table S1.2). In assays with BPMV-infected plants (figure 2*b*), we still observed strong positive effects of Bj + Da on parasitism rates over controls ($\beta = 2.52$, CI = [1.29, 3.76], pMCMC = 1.03×10^{-4}, electronic supplementary material, table S1.2), as well as over plants colonized by Bj alone ($\beta = 2.7$, CI = [1.42, 4.09], pMCMC = 2.06×10^{-4}, electronic supplementary material, table S1.2).

In assays comparing uninfected and BPMV-infected plants (figure 2*c*), parasitism rates were higher on uninfected plants regardless of the bacterial treatment (electronic supplementary

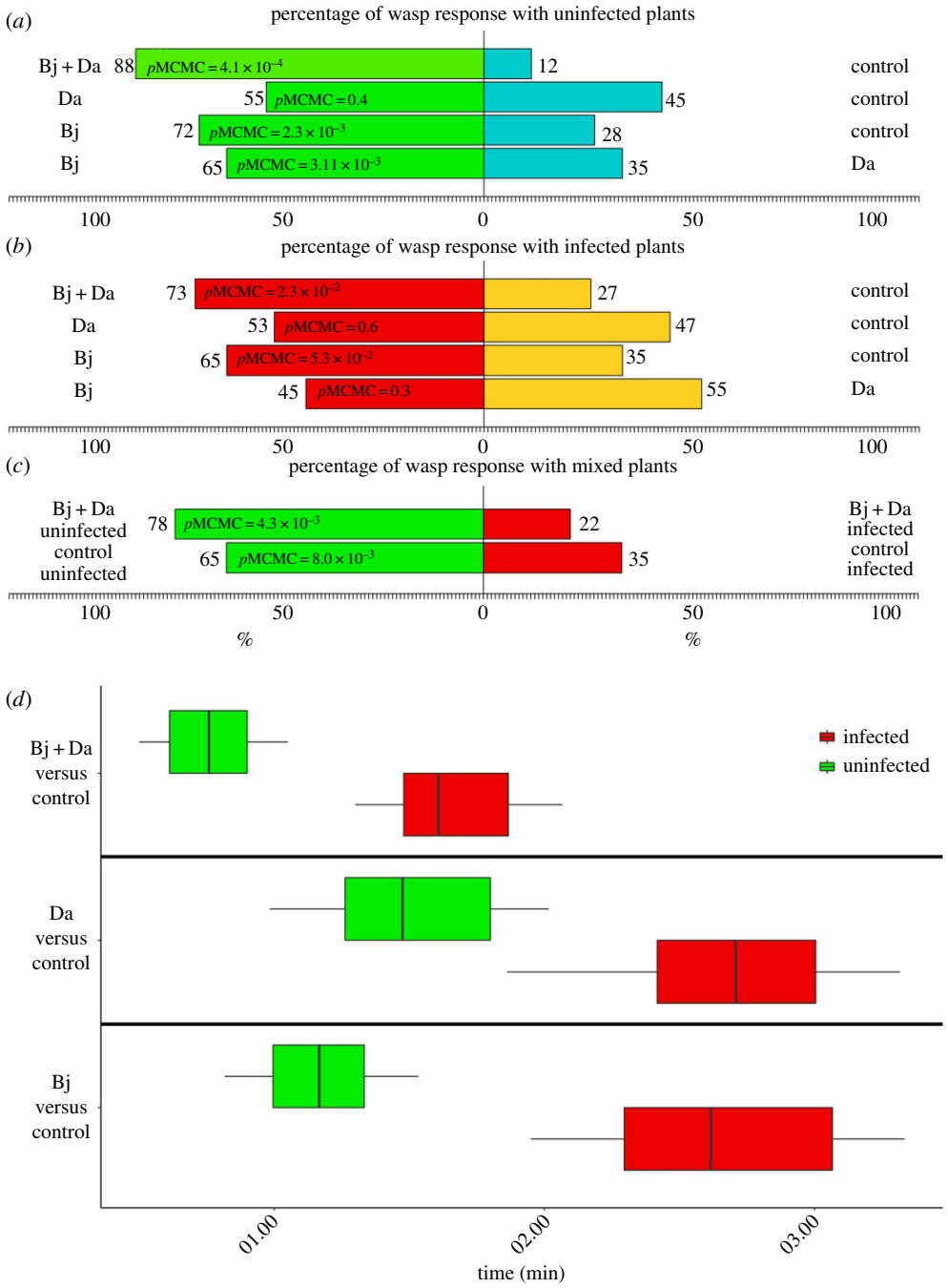

Figure 1. *Pediobious foveolatus* preferences for odours of *E. varivestis*-damaged soya beans under different rhizobacteria and virus treatments. Preferences of *P. foveolatus* were evaluated in a Y-tube olfactometer that presented two different odour sources simultaneously. (*a*) Percentages of individual wasps making a choice for each arm of the olfactometer for each treatment comparison in trials with uninfected (virus-free) plants. (*b*) Similar data for comparisons with BPMV-infected plants. (*c*) Similar data for comparisons between infected and uninfected plants. (*d*) Boxplots depicting the time to choose for wasps in each comparison. Wasps took longer to respond in BPMV-infected plants than in uninfected plants (pMCMC = 1.03×10^{-4}). Bj, *B. japonicum*; Da, *D. acidovorans*; Bj + Da, *B. japonicum* + *D. acidovorans*. (Online version in colour.)

material, table S1.2). A GLMM of the proportion of larvae parasitized *within* each direct comparison also suggests that BPMV infection reduces parasitism rates in most cases ($\beta = 0.81$, CI = [0.26, 1.34], pMCMC = 3.92×10^{-3}, electronic supplementary material, table S1.2). However, the proportion of larvae parasitized on uninfected Bj + Da plants versus controls did not differ from the proportion parasitized on BPMV-infected Bj + Da plants versus controls ($\beta = 0.181$, CI = [−0.68, 1.08], pMCMC = 0.69, electronic supplementary material, table S1.2). Additionally, a mixed effect model found that the interaction between BjDa and BPMV infection was not significant ($\beta = 0.63$, CI = [−0.56, 2.02], pMCMC = 0.337, electronic supplementary material, table S1.5). This indicates that benefits of Bj + Da colonization relative to bacteria-free controls were maintained even when BPMV infection was present.

(c) Microbial effects on herbivore-induced volatile emissions of soya bean

PERMANOVA, using the 19 emitted compounds as variables, revealed a significant main effect of virus infection on the volatile blend (pseudo-$F_{1,39} = 5.91$, $p = 0.008$; electronic supplementary material, table S1.6). A heatmap showing $\log^2$ fold changes in volatile emissions for all microbial treatments relative to the mean of uninfected controls reveals that BPMV

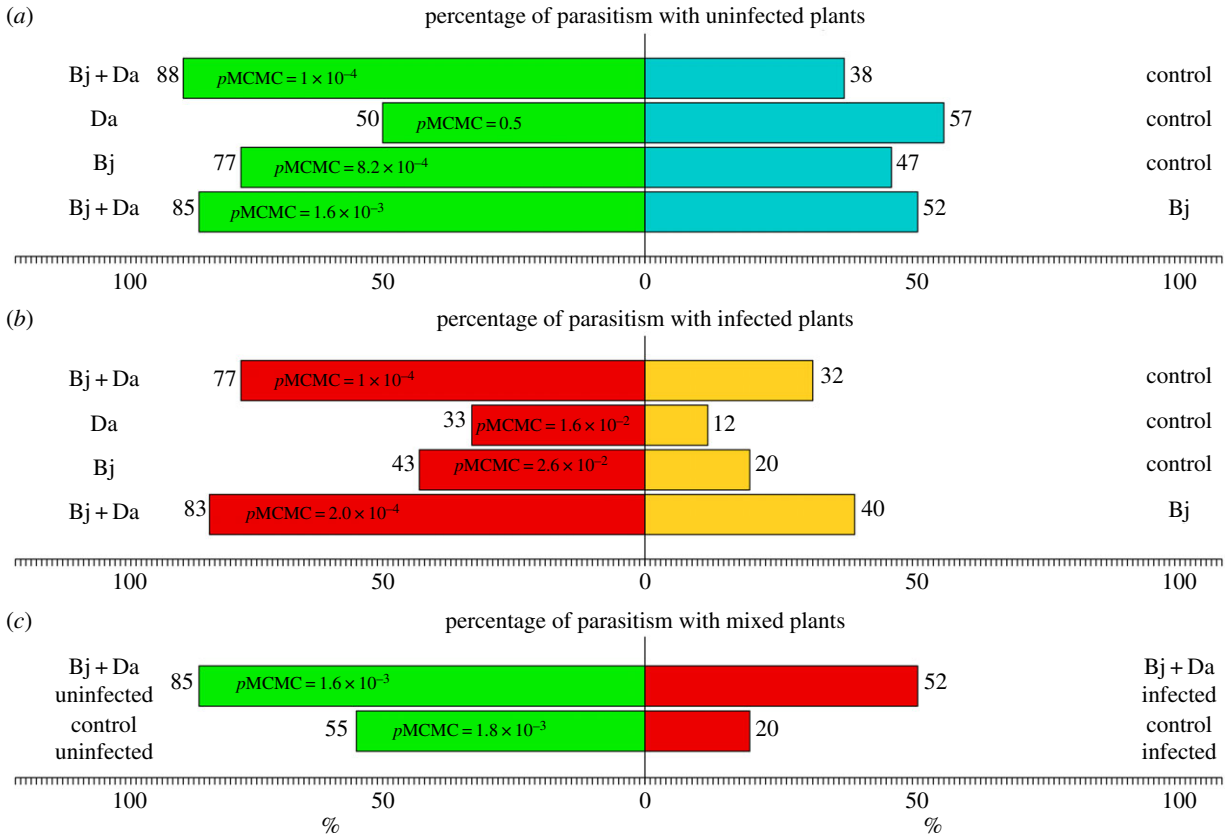

Figure 2. Parasitism rates on larval hosts residing on soya beans with different rhizobacteria and virus treatments. Dual choice comparisons of different rhizobacteria × virus treatments were selected based on observed preferences in odour-based assays and presented to wasps in a semi-natural foraging arena. Bars represent the percentage of parasitized larvae for each treatment across all tests performed for a given comparison (percentages for each comparison may not add to 100% because wasps could oviposit on larvae feeding on both plants) ($n = 120$ larvae per comparison). (*a*) Comparisons involving uninfected (virus-free) plants. (*b*) Comparisons involving BPMV-infected plants. (*c*) Comparisons between uninfected and BPMV-infected plants (with selected rhizobacteria treatments). Bj, *B. japonicum*; Da, *D. acidovorans*; Bj + Da, *B. japonicum* + *D. acidovorans*. (Online version in colour.)

infection suppresses the release of most compounds (figure 3). We found eight instances of a threefold or greater reduction in mean volatile production relative to control uninfected plants, with all of these instances (100%) being for BPMV-infected plant treatments (figure 3). RF analysis identified (*Z*)-3,7-dimethylocta-1,3,6-triene and (3*E*)-3,7-dimethylocta-1,3,6-triene as the best predictors of infection status (OOB error rate = 32.27%) (compounds G and F in figure 3). Both of these compounds were significantly reduced in BPMV-infected plants (*t*-test BPMV-infected versus uninfected: (*Z*)-3,7-dimethylocta-1,3,6-triene $t_{1,19} = -3.33$, $p = 9.0 \times 10^{-4}$; (3*E*)-3,7-dimethylocta-1,3,6-triene $t_{1,19} = -3.54$, $p = 4.0 \times 10^{-4}$; figure 3).

Single rhizobacterium inoculation did not significantly alter volatile blend (Bj: pseudo-$F_{1,39} = 0.18$, $p = 0.664$; Da: pseudo-$F_{1,39} = 2.09$, $p = 0.142$; electronic supplementary material, table S1.6). However, dual inoculation did have an effect in uninfected plants (pseudo-$F_{1,39} = 4.11$, $p = 0.035$; electronic supplementary material, table S1.6), and produced a similar, though marginally non-significant, trend in combination with BPMV infection (pseudo-$F_{1,39} = 3.13$, $p = 0.073$; electronic supplementary material, table S1.6). In the heatmap matrix of $\log^2$ fold changes in mean volatile emissions, there were 13 instances of a threefold or greater increase in volatile emissions relative to uninfected controls, with all of these instances (100%) being for Bj + Da uninfected treatments, while none were Bj + Da BPMV-infected treatments. RF analysis identified α-farnesene, (*Z*)-3-Hexen-1-yl acetate and an unidentified sesquiterpene (compounds N, D and Q in figure 3) as the best predictors of Bj + Da colonization (OOB error rate = 15.83%). Emission of (*Z*)-3-Hexen-1-yl acetate was significantly increased in Bj + Da uninfected plants compared to control, Bj, and Da uninfected plants (control: $t_{1,19} = 3.08$, $p = 2.0 \times 10^{-3}$; Bj: $t_{1,19} = 2.18$, $p = 3.0 \times 10^{-2}$; Da: $t_{1,19} = 3.28$, $p = 1.0 \times 10^{-3}$). Emission of α-farnesene was significantly increased in Bj + Da uninfected plants compared to Da uninfected plants ($t_{1,19} = 2.98$, $p = 3.0 \times 10^{-3}$).

(d) Microbial effects on plant biomass, nodulation and bean pod mottle virus symptoms

Co-inoculation of soya bean plants with *D. acidovorans* (Da) had no significant effect on nodulation by *B. japonicum* (Bj) (*p*MCMC = 0.62, electronic supplementary material, table S1.7). However, BPMV infection significantly reduced nodulation for both Bj and Bj + Da treatments even after adjusting for shoot biomass (*p*MCMC = 4.7×10^{-2}, electronic supplementary material, table S1.7; figure 4*a*). BPMV-infected plants had significantly less biomass than uninfected plants (*p*MCMC = 2.06×10^{-4}, electronic supplementary material, table S1.8; figure 4*b*). Across uninfected treatments, the effect of dual bacteria inoculation on shoot biomass, versus control, was stronger ($\beta = 2.01$, CI = [1.82, 2.19], *p*MCMC = 1.03×10^{-4}, electronic supplementary material, table S1.8) than single inoculations with Bj ($\beta = 0.58$, CI = [0.40, 0.77], *p*MCMC = 1.03×10^{-4}, electronic supplementary material, table S1.8) or Da ($\beta = 0.009$, CI = [−0.18, 0.19],

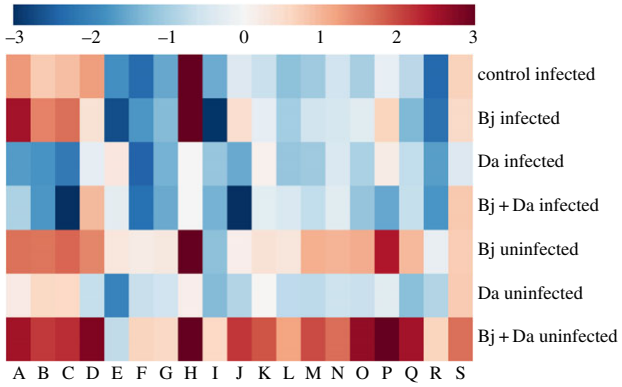

Figure 3. Heatmap depicting soya bean volatile signatures associated with rhizobacteria and virus treatments. Analysis of the entire blend by two-way PERMANOVA with 9999 permutations showed a significant effect of virus treatment (pseudo-$F_{1,39} = 5.91$, $p = 8.0 \times 10^{-3}$) and dual rhizobacteria colonization (pseudo-$F_{1,39} = 4.11$, $p = 3.5 \times 10^{-2}$) on volatile blend composition. The heatmap depicts $\log^2$ fold change in emissions of each compound from microbe-colonized plants relative to the mean value for uninfected microbe-free control plants. All plants received damage from *E. varivestis*. Letters indicate the following compounds: A. (E)-3-Hexen-1-ol; B. (Z)-2-methyl-butyl aldoxime; C. (E)-2-methyl butyl aldoxime; D. (Z)-3-Hexen-1-yl acetate; E. 2-ethyl-1-hexanol; F. (3E)-3,7-dimethylocta-1,3,6-triene; G. (Z)-3,7-dimethylocta-1,3,6-triene; H. 3-hexen-1-yl butyrate; I. Methyl salicylate; J. Indole; K. (E)-β-farnesene; L. Germacrene D; M. α-bergamotene; N. (E,E or E,Z)-α-farnesene; O. 3,7,11-trimethyldodeca-2,6,10-triene-1-ol; P. Unidentified sesquiterpene 1; Q. Unidentified sesquiterpene 2; R. (3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene; S. *Benzophenone*. Bj, *B. japonicum*; Da, *D. acidovorans*; Bj + Da, *B. japonicum* + *D. acidovorans*. (Online version in colour.)

*p*MCMC = 0.91, electronic supplementary material, table S1.8). BPMV infection reduced shoot biomass ($\beta = -0.42$, CI = [−0.61, −0.24], *p*MCMC = 2.06×10^{-4}, electronic supplementary material, table S1.8), but only the interaction with Bj + Da was significant ($\beta = -0.71$, CI = [−0.99, 0.47], *p*MCMC = 1.03×10^{-4}, electronic supplementary material, table S1.8). BPMV-infected plants inoculated with Bj + Da exhibited less pronounced viral symptoms than control, Bj or Da plants ($\beta = 21.31$, CI = [0.93, 47.9], *p*MCMC = 2.23×10^{-2}, electronic supplementary material, table S1.9; figure 4*c*).

4. Discussion

Consistent with our initial hypothesis, we found that BPMV disrupts the recruitment of parasitoid natural enemies of its vector, *E. varivestis*, to infected plants. *Pediobius foveolatus* wasps exhibited reduced attraction to BPMV-infected plants in volatile-based foraging assays (figure 1*c*) and were less efficient at locating and parasitizing *E. varivestis* larvae on BPMV-infected plants under semi-natural conditions (figure 2*c*). Wasps also took significantly longer to choose among rhizobacterial treatments when plants were infected with BPMV (figure 1*d*), and overall parasitism rates declined when the only hosts available were larvae feeding on BPMV-infected plants (figure 2*b*,*c*). Also consistent with our predictions, these viral effects were mitigated by the presence of plant mutualistic microbial symbionts, and particularly by the combined presence of *D. acidovorans* (Da) and *B. japonicum* (Bj), which elevated plant volatile emissions and restored parasitoid attraction and parasitism rates on BPMV-infected plants to levels similar to those seen in the absence of the virus (figures 1*a*, 2*a* and 3).

Together with positive effects of BPMV infection on soya bean palatability and quality for *E. varivestis*, which have also been documented elsewhere [36,37], the effects on indirect defences and parasitoid recruitment observed in the current study may fit with a broader strategy on the part of the virus to enhance feeding and virion uptake by the vector and thereby encourage the successful completion of larval development, which would produce mobile adult vectors capable of spreading the pathogen to new hosts [16]. It can be challenging to definitively distinguish virus adaptations for 'manipulating' plant chemistry to enhance vector transmission from merely fortuitous by-products of pathology; however, we have previously speculated that pathogen effects on host–vector interactions will tend to be broadly conducive to transmission because effects that disfavour transmission are likely to face strong negative selection [4,12]. In keeping with this expectation, similar effects on host phenotypes and vector survival are evident in other viral pathosystems [12,13].

Consistent with our hypothesis that functionally distinct rhizobacteria would elicit the strongest positive effects on indirect plant defences, we found that co-colonization of BPMV-infected plant roots with a combination of *B. japonicum* and *D. acidovorans* counteracted BPMV-induced suppression of indirect defence more strongly than either bacterial species alone. Indeed, dual colonization restored parasitoid attraction to, and parasitism rates on, BPMV-infected plants to levels near those observed for uninfected, dual colonized plants (figure 2*b*; electronic supplementary material, table S1.2). This recovery of the host plant's indirect defence phenotype is noteworthy, given that BPMV effectively suppresses volatiles and parasitoid recruitment when plants are single-colonized by either rhizobacterial species (figures 2*b* and 3). GLMM of parasitism proportions revealed that larvae-infested Bj + Da plants experienced higher parasitism rates than all other treatments under BPMV-infected *or* uninfected conditions (electronic supplementary material, table S1.2). Furthermore, using a factorial approach, we showed that these effects are enhanced when *D. acidovorans* is co-inoculated with *B. japonicum*, largely compensating for negative effects of BPMV on indirect defence (figures 1, 2 and 4), as well as on plant health (figure 4). Although root colonization by *B. japonicum* enhanced parasitoid attraction and parasitism on its own, its benefits for BPMV-infected plants were marginally significant in the absence of *D. acidovorans* (figures 1 and 2; electronic supplementary material, table S1.2), while single colonization by *D. acidovorans* had no significant effects on parasitoid foraging (figures 1 and 2; electronic supplementary material, tables S1.2 and S1.3).

The specific mechanisms underlying effects elicited by multiple microbial root colonizers are not well understood, in part owing to a lack of immune-pathway mutants for model legumes as well as logistical challenges associated with manipulating plant microbiomes [20,38–40]. There are several potential mechanisms by which such effects might be produced, including PGPR facilitation of additional colonization sites for rhizobia, PGPR production of plant hormones, direct effects of PGPR colonization on ethylene levels, and PGPR stimulation of flavonoid production by roots [39]. The failure to observe strong effects of *D. acidovorans* in isolation is consistent with the latter explanation, as in this scenario the benefits of *D. acidovorans* for the host occur indirectly via the induced

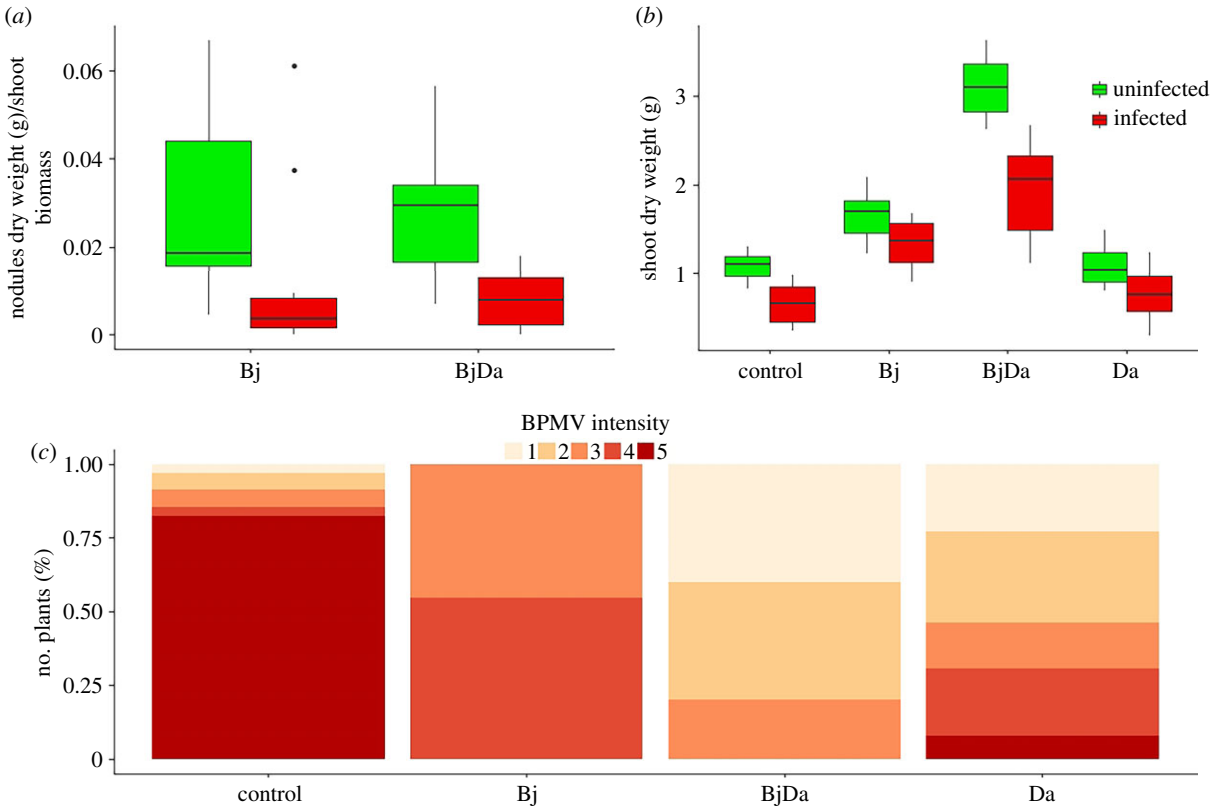

Figure 4. Microbial effects on plant biomass, nodulation and BPMV symptoms. (*a*) Effect of BPMV infection and rhizobacteria co-inoculation in nodule dry weights (g) (adjusted by shoot biomass) ($n = 10-14$ per treatment). BPMV significantly reduced nodulation (pMCMC $= 0.047$), but Da had no significant effect on nodulation (pMCMC $= 0.62$). (*b*) Effect of BPMV infection and rhizobacteria co-inoculation in shoot biomass (g) ($n = 20$ plants per treatment). BPMV significantly reduced plant biomass (pMCMC $= 2.06 \times 10^{-4}$). Single Bj and co-inoculation of both rhizobacteria species significantly increased plant biomass (pMCMC $= 1.03 \times 10^{-4}$). (*c*) BPMV symptomatology according to the rhizobacteria treatment. BPMV symptoms were scaled between 1 and 5 where 1 = no symptoms and 5 = severe symptoms across more than 50% of leaf surfaces ($n = 10-34$ per treatment). Plants that received both rhizobacteria treatments did not show severe BPMV symptoms (pMCMC $= 2.23 \times 10^{-2}$). Bj, *B. japonicum*; Da, *D. acidovorans*; Bj + Da, *B. japonicum* + *D. acidovorans*. (Online version in colour.)

release of flavonoids into the rhizosphere that enhance the recruitment of *B. japonicum*. In this case, *D. acidovorans* would function as a 'helper' rhizobacteria [39,41] to improve the performance of *B. japonicum*. A similar effect was reported in a recent study involving the application of *Delftia* in co-inoculation with *Sinorhizobium meliloti*, which found that *Medicago truncatula* roots produced significantly higher levels of several flavone signalling molecules (which enhance rhizobial expression of nodulation genes) under co-infection relative to microbe-free or single inoculation treatments [38]. The operation of a similar mechanism in our system might explain why we observed some positive effects of *B. japonicum* on indirect defence when inoculated singly (and even in the absence of nitrogen supplementation), but stronger positive effects (overriding the BPMV-induced phenotype) during co-inoculation with *D. acidovorans*.

It should also be noted that we provided supplemental nitrogen (in the form of potassium nitrate and ammonium nitrate) to plants without *B. japonicum* treatments but did not supplement those with *B. japonicum*, as the presence of nitrate inhibits root colonization by this microbe [30,42]. Therefore, we cannot exclude nitrogen supplementation as a potential driver of differences in parasitoid recruitment and volatile emissions between treatments receiving *B. japonicum* and those without this treatment, although we did verify that *B. japonicum* colonization compensated for the lack of nitrate in the soil and that plants with and without nitrate treatments had similar growth and nitrogen levels (figure 4*b*) [21]. A previous study in soya bean also found that plants given different nitrogen treatments had similar shoot biomass, emitted the same range of herbivore-induced volatile organic compounds, and elicited similar attraction of parasitoids [43]. It remains possible, however, that some of the observed effects of *B. japonicum* might be partially attributable to subtle differences in plant nitrogen sources. Such effects have so far received little attention in the context of indirect plant defence [21,44], although some previous work has raised the possibility that differences in the form of nitrogen supplied by rhizobia (versus fertilizer) might influence plant–herbivore interactions (e.g. [21]).

Our analyses of soya bean volatile emissions provide additional support for our initial hypotheses, as BPMV effects on volatile profiles are consistent with a suppression of indirect plant defences against the beetle vector (figures 1 and 2), while we also observed slightly enhanced positive effects of the two rhizobia species on the production of compounds known to attract natural enemies (figure 3) [1,45,46]. In the absence of the virus, dual colonization had strong effects on damage-induced volatile emissions; for example, three compounds, (*Z*)-3-Hexen-1-yl acetate, 3-hexen-1-yl butyrate and 3,7,11-trimethyldodeca-2,6,10-triene-1-ol (compounds D, H and O in figure 3), were emitted in higher amounts from uninfected, dual colonized plants relative to all other treatments (figure 3). In treatments with BPMV infected plants, dual rhizobacteria colonization produced a similar trend, though this effect was marginally non-significant (figure 3). Meanwhile, we did not

observe effects of inoculation with either individual rhizobia species on volatiles (figure 3). In particular, inoculation with Bj did not elicit differences in volatile emissions relative to bacteria-free controls that would explain the enhanced attraction of wasps to Bj treated plants in our previous assays (figures 1 and 2), although there was a positive fold-change in the amount of individual volatiles induced by *B. japonicum*. The more prevalent effects of dual colonization on volatiles may in part reflect additive or interactive effects of the two rhizobia species, similar to those observed in our behavioural and parasitism assays (figures 1–3). However, methodological differences between the behavioural and volatile-collection experiments may also have contributed to disparities between our volatile data and the parasitoid's behavioural responses. Plants in our behavioural assays were damaged with beetle larvae; however, as noted above, we experienced challenges in using larvae to induce damage treatments within the volatile sampling chambers and therefore used adult beetles instead. As simultaneous attack by both adult and larval stages occurs frequently in the field, we reasoned that damage by adults should be a reliable indicator of host presence for the wasps, and the resulting data are indeed broadly consistent with patterns observed in our behavioural experiments (e.g. with respect to the negative effects of virus infection and the positive effects of dual rhizobia colonization on indirect defences). However, differences in patterns of volatile induction by larval and adult feeding have been reported for at least one other coleopteran herbivore [47], and such differences might contribute to our failure to observe a statistically significant effect of single or dual rhizobia colonization in BPMV-infected plants that would explain the observed effects of these treatements on parasitoid behavioural preferences and parasitism rates (figures 1–3).

In overview, our results highlight the importance of developing and testing hypotheses regarding microbial effects on host phenotypes in complex systems that incorporate plant interactions with multiple organisms having different colonization strategies and lifestyles. They also suggest that understanding beneficial and antagonistic interactions among mutualistic and pathogenic plant symbionts may have important implications for predicting and managing disease transmission in natural and agricultural plant communities. In our system, co-colonization by *D. acidovorans* and *B. japonicum* produced the greatest beneficial effects on plant growth promotion and plant indirect defences against the chewing herbivore *E. varivestis* by promoting the attraction and parasitism of its natural enemy, *P. foveolatus*, even in the presence of virus infection. Together with our behavioural data, our analyses of plant volatile emissions demonstrate that microbes with different lifestyles and host associations can have significant effects on plant phenotypes that mediate indirect defences and thereby affect tri-trophic interactions among the host plant, insect herbivores and their natural enemies, with potentially important ecological implications, including for disease transmission by insect vectors. Future work in this and other model systems should focus on identifying the mechanisms underlying plant-mediation of positive and negative microbial interactions through transcriptomic and metabolomic approaches in the laboratory, with complementary field experiments to verify the robustness of observed effects under more complex scenarios.

Data accessibility. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.kh95v21 [48].

Authors' contributions. H.P., K.E.M., M.C.M. and C.D.M. designed the study; H.P. carried out the experiments and collected the data; H.P. and K.E.M. analysed the data. H.P. drafted the initial manuscript. All authors contributed significantly to revisions and gave final approval for publication.

Competing interests. We declare we have no competing interests.

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