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Contrasting effects of necrotrophic and biotrophic plant pathogens on the aphid *Aphis fabae*

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Author Contributions: FAN and PEH conceived and designed the experiments, FAN performed the experiments, FAN and PEH analysed the data and wrote the manuscript.

Abstract

Phytophagous insects have to contend with a wide variation in food quality brought about by a variety of factors intrinsic and extrinsic to the plant. One of the most important factors is infection by plant pathogenic fungi. Necrotrophic and biotrophic plant pathogenic fungi may have contrasting effects on insect herbivores due to their different infection mechanisms and induction of different resistance pathways, although this has been little studied and there has been no study of their combined effect. We studied the effect of the biotrophic rust fungus *Uromyces viciae-fabae* (Pers.) Schroet (Basidiomycota: Uredinales: Pucciniaceae) and the necrotrophic fungus *Botrytis cinerea* Pers. (Ascomycota: Helotiales: Sclerotiniaceae) singly and together on the performance of the aphid *Aphis fabae* Scop. (Hemiptera: Aphididae) on *Vicia faba* (L.) (Fabaceae). Alone, botrytis had an inhibitory effect on individual *A. fabae* development, survival and fecundity, while rust infection consistently enhanced individual aphids' performance. These effects varied in linear relation to lesion or pustule density. However, whole-plant infection by either pathogen resulted in a smaller aphid population of smaller aphids than on uninfected plants, indicating a lowering of aphid carrying capacity with infection. When both fungi were applied simultaneously to a leaf they generally cancelled the effect of each other out, resulting in most performance parameters being similar to the controls, although fecundity was reduced. However, sequential plant infection (pathogens applied five days apart) led to a 70% decrease in fecundity and 50% reduction in intrinsic rate of increase. The application of rust before botrytis had a greater inhibitory effect on aphids than applying botrytis before rust. Rust infection increased leaf total nitrogen concentration by 30% while infection by botrytis with or without rust led to a 38% decrease. The aphids' responses to the two plant pathogens individually is consistent with the alteration in plant nutrient content by infection and also the induction of different plant defence

pathways and the possible cross-talk between them. This is the first demonstration of the complex effects of the dual infection of a plant by contrasting pathogens on insect herbivores.

Key words: *Vicia faba, Botrytis cinerea, Uromyces viciae-fabae,* tripartite interactions, induced resistance

Introduction

An insect herbivore not only has to contend with the direct effects of a variable and often poor-quality food source (Mattson, 1980), the vagaries of climate and a range of parasites and predators, but also indirect effects caused by other organisms exploiting this food resource (Hatcher, 1995; Rostás et al., 2003; Stout et al., 2006). One of the most important such groups of organisms are plant pathogenic fungi (Hawksworth, 1991). These can cause a range of effects to their host plants: increasing or decreasing nutrient levels in infected leaves or uninfected leaves on the same plant, altering the concentration of defensive chemicals, and up- or down-regulating an array of resistance pathways (Hatcher et al., 2004; Bostock, 2005; Stout et al., 2006). These changes to the plant can lead to insect herbivores having altered fitness when feeding on a plant infected by a pathogenic fungus (Hatcher, 1995).

Although there are now several well-studied model systems in which tripartite insect–plant–plant pathogen interactions have been elucidated (Hatcher, 1995; Rostás et al., 2003; Stout et al., 2006) most of these have only considered the interaction between one insect and one plant pathogen. However, in an attempt to bridge the gap between reductionist studies of pair-wise interactions and the holistic study of food webs and communities there is a need to study more complex consumer interactions on plants. It is difficult to predict the outcome of

single insect–plant–fungus interactions (Hatcher, 1995), and more complex systems will be harder still to predict without some guiding empirical studies of these more complex interactions.

Recent studies on the defences induced by necrotrophic and biotrophic plant pathogens and the cross-talk between these pathways and the induced defence pathways affecting insect herbivores suggest that these two types of fungal pathogen may affect insect herbivores in a contrasting fashion. For example, effective defence against biotrophic pathogens is largely due to programmed cell death (the hypersensitive response) and the associated activation of defence responses regulated by the salicylic acid (SA)-dependent pathway (Glazebrook, 2005; van Loon et al., 2006), whilst necrotrophic pathogens benefit from cell death, and thus activation of this pathway would only facilitate their infection (van Loon et al., 2006). Instead, necrotrophic pathogens may induce activation of jasmonic-acid (JA) and ethylene (ET)-dependent defence signalling (Glazebrook, 2005), with SA-induced pathways only becoming important later if the necrotroph starts to behave as a hemi-biotroph (van Loon et al., 2006). Similarly, insects with different modes of feeding may induce different pathways, with chewers often inducing the JA-dependent defence pathway, and sucking insects such as aphids the SA-dependent pathways (Walling, 2008; Thaler et al., 2010).

The SA and JA-dependent pathways do not exist in isolation; rather there is cross-talk between them, with activation of the SA-dependent pathway often leading to a down-regulation of the JA-dependent pathway and vice-versa (Bostock, 2005; van Loon et al., 2006; Robert-Seilaniantz et al., 2011), and thus we may expect the interaction between a necrotrophic pathogen and a sucking insect to be different than that between a biotrophic pathogen and a sucking insect on the same plant, but will lead to unexplored complications if the insect is exposed to several different pathogens simultaneously.

The broad bean, *Vicia faba* (L.) (Fabaceae) is attacked by a range of pests in Europe; among the most important are the aphid *Aphis fabae* Scop. (Hemiptera:Aphididae) (Banks & Macaulay, 1964), the necrotrophic pathogen *Botrytis cinerea* Pers. (Ascomycota: Helotiales: Sclerotiniaceae) (Davidson et al., 2004) and the biotrophic rust *Uromyces viciae-fabae* (Pers.) Schroet (Basidiomycota: Uredinales: Pucciniaceae) (Gaunt, 1983). Although *B. cinerea* often infects plants early in the season and *U. viciae-fabae* infects later in the year, both often occur together on the plant at a time when *A. fabae* is also present. Alone, both *U. viciae-fabae* and *B. cinerea* have been reported to increase aphid performance (Zebitz, 1988; Prüter & Zebitz, 1991; Zebitz & Kehlenbeck, 1991) most likely through increased mobilisation of carbohydrates and nitrogen through the plant and into infected leaves (Thrower & Thrower, 1966; Farrar & Lewis, 1987; van Kan, 2006; Choquer et al., 2007). However, we would predict from induced resistance studies that *B. cinerea* would enhance aphid performance by inducing the JA pathway and thus down-regulating the SA pathway (Bostock, 2005; Robert-Seilaniantz et al., 2011), while *U. viciae-fabae* would decrease aphid performance by co-stimulating the SA-dependent induced resistance pathway. This is assuming that, as has been found for many species of aphids, *A. fabae* in this system is sensitive to the resistance induced by the SA pathway (Walling, 2008; Thaler et al., 2010) The effect of dual infection on *A. fabae* is hard to predict, partly because of the lack of a predictive framework for such interactions (Thaler et al., 2010) and also due to the unknown effect of resource depletion caused by dual infection (e.g. Grueber & Dixon, 1988), but we would expect it to depend on the order of infection and thus the order of stimulation or inhibition of defence responses.

In this paper we first quantify the effect on *A. fabae* of individual infections of *V. faba* with *B. cinerea* and *U. viciae-fabae*, and then report the first experiments to determine the effect of concurrent or sequential infection of the two contrasting plant pathogens on the performance of the aphid.

Materials and methods

Experimental organisms

All experiments and plant rearing were carried out in a constant environment (CE) room at the University of Reading at 18–20°C and L16:D8 photoperiod.

Botrytis cinerea was obtained from a culture (originally isolated from the University of Reading Campus, Whiteknights, Reading, UK), maintained at the University of Reading, subcultured every 10 days on 4% malt extract agar and potato dextrose agar and incubated at 20±1ºC in constant darkness. Conidia were harvested from 16-day-old cultures by flooding the plate with sterile distilled water and dislodging the conidia with a sterile rod, and subsequent filtration through four layers of cheesecloth to remove mycelial debris. A 0.1 ml suspension of a $1x10^4$ conidia ml^{-1} inoculum was applied to the adaxial surface of each leaflet with a paint brush. After 30 min the plants were enclosed in polythene bags containing a saturated atmosphere at 20±1ºC for 48 hrs to allow spore germination before aphids were confined in a clip cage on the leaves. 0.1 ml sterilised distilled water was used as a control.

Uromyces viciae-fabae (originally isolated from the University of Reading Campus, Whiteknights, Reading, UK), was cultured permanently on *V. faba* at the University of Reading in a CE room at 20ºC and L16:D8 photoperiod. Uredospores were brushed from plants and stored at -20°C until use. Leaflets were evenly sprayed with a $1x10^5$ ml^{-1} suspension of spores in distilled water using a modeller's airbrush. The inoculated plants were incubated for 48 hrs in a saturated atmosphere in polythene bags in the CE room to allow germination and infection. In the CE room *U. viciae-fabae* usually showed first signs

of infection after 6 to 7 days and sporulated after 10 days. Plants were challenged with aphids 10 days after inoculation.

Black bean aphids, *Aphis fabae*, were obtained from a population maintained at the University of Reading (originating from aphids collected from the wild in Reading, UK). This population was reared on *V. faba* L. cv 'The Sutton' at 18–20°C and L16:D8 photoperiod in 51x69x69 cm perspex cages, with fan-assisted air circulation.

Effect of one fungus on individual aphids

Individual seeds of *V. faba* (cv 'The Sutton', Thompson and Morgan, Ipswich, UK – this cultivar was used in all experiments) were planted in 9-cm diameter pots of John Innes II compost. The plants were selected for treatment when five true leaves had appeared. For each fungus 60 plants were used, split equally between infected and uninfected plants. To measure local effects of infection, a leaflet on leaf 3 ('old') was infected with *B. cinerea* or *U. viciae-fabae* as above, and 2 or 10 days respectively after inoculation an aphid was placed in a clip cage on this infected leaflet. This time difference was selected so that the aphid encountered each pathogen at the same stage of development, i.e. sporulating pustules. Aphids were also caged on an adjacent uninfected old (leaf 3) leaflet and an uninfected new leaflet (leaf 5) on that plant to test if infection led to systemic effects in leaflets close to the source of infection, or newer, more distant leaflets. Aphids were also caged on an old (leaf 3) and young (leaf 5) leaflet on uninfected plants as a control.

Aphids were caged using 20 mm diameter perspex clip cages (Van Emden, 1972), supported so that leaflets maintained their normal position. First instar nymphs were transferred to the clip cages (one per cage) using a moistened paintbrush as soon as they were born. They were then kept on the leaf until they had matured and produced offspring for 10

days. The speed of aphid development was calculated as maturation time: the time in days between birth of an aphid and the birth of its first offspring. The mean relative growth rate (MRGR) of individual aphids in clip cages was determined after measuring the fresh weights of first-instar nymphs at the beginning and after 4 days of the experimental period on an electrical microbalance (Sartorius Supermicro S4), and MRGR (μg/μg/day) calculated as ($\text{Log } W_2 - \text{Log } W_1)/(t_2 - t_1$), where W_1 is weight at the first measurement (t_1) and W_2 weight at the second measurement (t_2). MRGR provides a measure of performance of the aphid which is likely to be directly related to the nutrition of the aphid, and independent of maternal effects (Van Emden, 1969; Wojciechowicz-Zytho & Van Emden, 1995), and is a measure of the growth of the aphid relative to its weight. The intrinsic rate of increase (r_m) (Wyatt & White, 1977) was calculated by recoding the number of nymphs produced by each individual during their first 10 days of reproduction (nymphs were removed from clip-cages as they were produced), using the formula $r_m = [c \ln(Md)] / D$, where Md is the number of nymphs produced by the adult in the first 10 days (D) of reproduction after the adult moult, the constant (c) has the value of 0.738 and is an approximation of the proportion of the total fecundity produced by a female in the first (D) days of reproduction (Wyatt & White, 1977).

Effect of two fungi on individual aphids

The first experiment demonstrated that the fungi, individually, had a greater local than systemic effect. In the second experiment this local effect was investigated in more detail, and the effect of the two pathogens combined, either applied at the same time or 5 days apart was studied. Thus, 24- day-old *V. faba* seedlings were inoculated with *B. cinerea* and *U. viciae-fabae* singly or together on two leaflets of leaf 3, as described above. When 29 days old, further seedlings (and some already infected, depending on treatment) were also infected

with the two fungi singly and together. This gave the range of treatments illustrated in Figure 2 (n = 20 for all treatments), encompassing single infection of both fungi at both times (24 and 29 days), sequential infection (with 5 days gap) of *B. cinerea* followed by *U. viciae-fabae* and vice-versa, and simultaneous infection by both fungi on both infection days. When the plants were 36 days old, one aphid was caged on an infected leaflet of leaf 3, as described above, and growth and fecundity parameters were recorded. The number of *B. cinerea* lesions was recorded per cm^2 10 days after inoculation and the number of *U. viciae-fabae* pustules 11 days post inoculation.

Effect of one fungus on aphid populations

Individual seeds of *V. faba* were planted in 13.5 cm diameter plastic pots filled with John Innes II potting compost, and raised in the CE room as above. To assess the effect of single-fungus infection on aphid population development 45 *V. faba* plants were inoculated 4 weeks after germination (when the fifth leaf had appeared) with *B. cinerea* on all leaves as described above and 45 control plants were sprayed with distilled water. Four days after inoculation four newly-moulted adult *A. fabae* from the stock population were confined in a clip cage on each plant for 24 hours and allowed to reproduce. Five of the resulting offspring were retained on each plant; the mothers and any other offspring were removed, along with the clip-cages. The plants were covered individually with perforated plastic bags (as used in Keary & Hatcher, 2004). A preliminary experiment (data not shown) demonstrated that the bags prevented aphids from escaping and did not inhibit plant or aphid development in our CE room conditions. Plants were placed in the CE room with treatments randomised, and at 3, 4, and 5 weeks after inoculation a subset of 15 inoculated and 15 control plants were randomly selected and destructively harvested, removing all aphids. The aphids were

counted, dried at 80°C to constant weight and weighed on an electrical microbalance (Sartorius Supermicro S4). The experiment was repeated using the same number of replicates with all leaves of infected plants inoculated with *U. viciae-fabae* spore suspension as above.

Nitrogen analysis

Vicia faba seedlings were raised as before in the growth room. At 4 weeks after germination (when the fifth leaf had emerged), a leaflet on leaf 3 was inoculated with *B. cinerea* and *U. viciae-fabae,* singly and in combination, as given in Figure 5. Two days after inoculation (for *B. cinerea*) and 10 days after inoculation (for *U. viciae-fabae*) infected leaflets were removed, oven-dried for 24 hours at 70°C, milled to a fine powder, and weighed (7–8 mg / sample). Percentage total nitrogen was determined by the Department of Soil Science at University of Reading, using the Roboprep /VG 622 system (Europa Scientific).

Statistical analyses

Three measurements, maturation time, fecundity and r_m required that the aphid survived until the end of the experiment. Unfortunately, a few aphid replicates died before the 10 day count of fecundity was over. Hence, analyses of variance for completely randomised designs were carried out using Genstat (version 8) (Lawes Agricultural Trust), since this was able to accommodate treatments having an unequal number of replicates. The effect of a single fungus on individual aphids was analysed by two-factor ANOVA with an added 'control': local infection (aphids reared on the leaflet from leaf 3 ('old') infected with fungus), with two systemic 'treatment' factors age of leaflet (old and young) x presence or absence of infection by *B. cinerea* or *U. viciae-fabae*. In this ANOVA design the factorial treatment structure age

x infection is nested within the factor control vs treatment (convstrt); the overall difference between control and treatment (i.e. between local infection and systemic or no infection) is calculated first and then the factorial effects of age and infection are calculated within this nested structure (Stern et al., 2001).

The effect of two fungi on individual aphids was analysed by two-factor ANOVA with factors presence of *B. cinerea* x presence of *U. viciae-fabae* . Each factor had three possible values: absence; presence at time 1 (24-day-old plant); or presence at time 2 (29-day-old plant), giving a 3 x 3 structure with 9 treatment groups. The number of lesions of *B. cinerea* and number of pustules of *U. viciae-fabae* were log transformed to meet the assumptions of ANOVA. Simple linear regression analysis was performed using Genstat (version 8.0) to determine the effect of the log number of lesions of *B. cinerea* and the log number of pustules of *U. viciae-fabae* on the mean relative growth rate (MRGR) of *A. fabae*.

Repeated-measurements ANOVA (Genstat version 8.0) was used to determine the effect of inoculation with *B. cinerea* and *U. viciae-fabae* on the population size and dry weight of *A. fabae* 3, 4, and 5 weeks after inoculations, compared to healthy plants.

Percentage nitrogen was analysed by a one-factor ANOVA (Genstat version 8.0).

Results

Aphis fabae feeding on *Botrytis cinerea*-infected *Vicia faba* leaflets had a slower growth rate (3 days longer maturation time and an almost 50% reduction in MRGR), a 65% reduction in fecundity and a halved r_m compared to aphids feeding on similar-aged leaflets on uninfected plants (Figure 1, Table 1). There was a lesser, but still significant systemic effect, with aphids reared on both young and old uninfected leaflets on infected plants having a 24%

reduction in MRGR, 35% reduction in fecundity and a 21% reduction in r_m compared to aphids reared on similar-aged leaflets on uninfected plants. There was less effect of leaf age on aphid performance, although aphids developed faster and had a higher r_m on younger rather than older leaflets (Figure 1).

Aphids feeding on *Uromyces viciae-fabae* infected leaflets, by contrast, had an improved performance compared to those reared on similar-aged leaflets on uninfected plants: maturation time was shortened by 2 days, MRGR was increased by 25%, fecundity by 39% and r_m was increased by 48% (Figure 1). No systemic effect of rust infection was observed, nor did leaflet age have an effect on aphid performance (Table 1).

The combined infection experiment (Figure 2) was concerned with local rather than systemic effects. The effects of *B. cinerea* or *U. viciae-fabae* infection alone mirrored that found in the first experiment, with *B. cinerea* reducing performance and *U. viciae-fabae* increasing it (Figure 2, Table 2), so that aphids feeding on rust-infected leaflets had a 3.5-fold greater fecundity and over 100% increase in r_m compared to those reared on *B. cinerea*-infected plants. When both fungi were applied simultaneously to a leaflet they generally cancelled the effect of each other out, resulting in most performance parameters similar to the controls, although combined infection reduced fecundity by a third (Figure 2). Sequential infection, (with infection by the second fungus five days after the first) led to a reduction in performance compared to the controls; increasing maturation time, a 70% decrease in fecundity and 50% reduction in r_m. The application of rust before botrytis had a greater inhibitory effect on aphids than applying botrytis before rust (Figure 2). The inhibitory effect of botrytis and the stimulatory effect of rust varied in linear relation to the lesion or pustule density in this experiment (Figure 3).

Both botrytis and rust infection inhibited the development of aphid populations on *V. faba* (Figure 4, Table 3), with infected plants having smaller populations of smaller aphids.

Botrytis reduced total nitrogen content in *V. faba* leaves by 48%, while rust increased nitrogen concentration by 30% (Figure 5). Combined infection reduced nitrogen by a similar amount to botrytis infection, and the order of infection did not affect nitrogen concentration.

Discussion

The results of single plant pathogen infections on individual aphids were consistent between the two experiments (Figures 1, 2), but were the opposite of that predicted in the introduction from likely interactions between induced resistance pathways. Rather than the predicted stimulation of *Aphis fabae* growth and development by *Botrytis* infection, the aphid had slower growth and lower fecundity when feeding on *Botrytis*-infected leaves. Likewise, rather than the expected decreased performance of the aphid on *Uromyces viciae-fabae* infected leaves, the aphid actually had increased performance when feeding on these leaves. These results are, however, consistent with the decreased nitrogen content found in the *Botrytis*-infected leaves and the increased nitrogen concentration found in the rust-infected leaves (Figure 5): *Aphis fabae* is sensitive to changes in nitrogen levels in plants (Van Emden, 1966; Jaenike, 1990).

Previous studies have found a varying response of aphids to *Botrytis* infection. Contrary to our results, Zebitz & Kehlenbeck (1991) found an increase in aphid performance on *B. cinerea*-infected *Vicia faba*, and suggested that this was due to the increased availability of amino acids resulting from the degradation of mesophyll cells in the phloem during pathogen-induced senescence, while Mouttet et al. (2011) found that *Rhodobium porosum* feeding on rose was negatively affected by *Botrytis* infection.

Few studies have investigated the effect of biotrophic pathogens on aphids (Hatcher, 1995; Rostás, 2003), although, similar to our experiment Prüter & Zebitz (1991) found an increase in *Aphis fabae* performance when reared on *Uromyces viciae-fabae*-infected *Vicia faba*, and this aphid also formed significantly larger colonies on *Cirsium arvense* plants infected with the rust *Puccinia punctiformis* (Kluth et al., 2002). By contrast, infection of barley by *Erysiphe graminis* reduced the weight and fecundity of two aphid species, *Sitobion avenae* and *Metopolophium dirhodium* (Pesel & Poehling, 1988).

The effect of local pathogen infection on aphid performance on *Vicia faba* is correlated with changes in nitrogen concentration in the leaves following infection (Figure 5). Whilst there is little consistency in whether a plant species will respond to a pathogen by increasing or decreasing leaf nitrogen content (Hatcher ,1995), such changes in leaf nitrogen concentration have been correlated with phytophagous insect performance in several studies (Hatcher, 1995). The amino acid composition of infected leaves can also be altered by fungal infection. *Botrytis fabae* altered the amino acid profile of *Vicia faba* (El Beih et al., 1988), and decreases in glutamic acid, aspartic acid and increases in asparagine, glutamine, serine and arginine concentrations were found in peach leaves infected by *Sphaerotheca pannosa* (Raggi, 1976) and bean plants infected by *Uromyces phaseoli* (Raggi, 1974). Given that aphids are sensitive to changes in composition of phloem amino acids (Douglas, 2006; Powell et al., 2006) it would be instructive to determine the effect of plant pathogens on these amino acids.

Apart from a change in nitrogen concentration, plant pathogens alter other chemical and physical aspects of leaves; some, such as carbohydrates and water are known to affect insects (Hatcher et al., 1995, 1997). The spore concentration of both pathogens applied to the plants in our experiments was selected to produce a medium-strength infection, but not to cover the whole leaf. The effect of the pathogen on the leaf tends to decrease as distance

from the pustule increases (Hatcher et al., 1995), and while it is unlikely that aphids, as plant suckers, would feed on the sporulating pustules they would have plenty of undamaged material on which to feed, and thus we suggest that gross morphological changes to the leaf are not responsible for the effects observed on aphid performance.

Although infection with plant pathogens can lead to an alteration in nutrient content in uninfected leaves on the plant, these systemic effects are usually weaker than the local effects (Hatcher et al., 1995; 1997) and are usually observed only when a significant leaf area has been infected (Hatcher, 1995). The infection of only one leaflet of *V. faba* with *B. cinerea*, however, is unlikely to lead to such changes in the physiology of the plant to account for the systemic effects observed on the aphids (Figure 1), and thus this is likely to be due to induced resistance. Botrytis kills host cells at very early stages of infection and causes extensive tissue death (Govrin & Levine, 2000; Mengiste, 2012). It is therefore unlikely to activate the hypersensitive response and associated activation of defence responses regulated by the SA-dependent pathway, as this would only facilitate infection - as has been demonstrated in Arabidopsis (Veronese et al., 2004). Rather, botrytis infection often induces the JA/ET signalling pathway in plants, which can be an effective defence against infection (Thomma et al., 1998; Ferrari et al., 2003; Glazebrook, 2005; Mengiste, 2012), and is insensitive to SA-signalled induced resistance in Arabidopsis and tobacco (van Loon et al., 2006).

Aphids appear to be able to induce both the SA and JA/ET pathways, can be affected by both (Cooper & Goggin, 2005; Tjallingii, 2006; Goggin, 2007; Thaler et al., 2010) and the induction of either pathway appears to have a variable effect upon them: aphids both being inhibited by necrotrophic fungus infection (Pratt et al., 1982; Moran, 1998) and stimulated (Prüter & Zebitz, 1991; Zebitz & Kehlenbeck, 1991; Johnson et al., 2003), depending on the species.

Biotrophic pathogens, such as the rust fungus *U. viciae-fabae,* induce the hypersensitive (HR) response by the SA-dependent pathway, leading to the activation of many defence activator genes (Glazebrook, 2005) and the development of necrotic lesions at the sites of pathogen entry. A few days after HR development systemic acquired resistance (SAR) can develop in uninfected parts of the plant, providing long-lasting resistance to a range of pathogens (Glazebrook, 2005; Vlot et al., 2009). This has been confirmed in *V. faba* where the development and exogenous applications of SA can induce systemic acquired resistance to *U. viciae-fabae* (Sillero et al., 2012). No systemic response of rust infection against *A. fabae* was observed in this experiment (Figure 1) and the increase in aphid performance on infected leaves is consistent with the increase in nitrogen observed in infected leaves (Figure 5), and has been reported also by Zebitz (1988) in this system. Thus, we can conclude that this aphid shows no evidence of being affected by products of aSA-dependent systemic pathway that could be induced by the rust fungus. Furthermore, as the SA and JA pathways are often mutually antagonistic, with the stimulation of one pathway leading to the inhibition of the other (Bostock, 2005; van Loon et al., 2006; Robert-Seilaniantz et al., 2011) this suggests that in this experimental system this aphid may induce, and is probably inhibited by, the defence products of the JA pathway (which is likely to be inhibited by rust infection and stimulated by botrytis infection) rather than the SA pathway. Although many aphids have been found to be affected by resistance induced by the SA pathway, some aphids can induce, and are affected by, the producets of the JA-mdiated induced resistance pathway (Cooper & Goggin 2005; Tjallingii, 2006; Goggin, 2007). However, the primary effect of both pathogens on the aphid seems to be via alteration in nitrogen concentration (Figure 5), supported by the significant positive correlation between pustule density and aphid performance (Figure 3) .

The first two experiments examined the effects of local (Figures 1, 2) and systemic (Figure 1) infection of one leaf per plant on individual aphids. The population experiment (Figure 4) examined the effect of whole-plant infection on the increase in aphid populations. The aphid population that developed on plants infected with either pathogen was significantly smaller (and composed of smaller individuals) than that which developed on the uninfected plants (Figure 4). Whilst this supports the negative effect of botrytis on the individual aphids it may be seen to contradict the positive effect of rust on the aphid observed in our individual-aphid experiments. However, both pathogens can significantly reduce the size and yield of *V. faba* (Lapwood et al., 1984; Rashid & Bernier, 1991; Sahile et al., 2010) and our results suggest that whole-plant infection reduces resource availability for the aphids, and thus lowers the plant's aphid carrying capacity. This was also observed by Prüter & Zebitz (1991). Furthermore, this experiment highlights the need to examine these interactions at the population level: interactions and mechanisms that may be important at the level of the individual may be unimportant at the population level.

As far as we are aware this is the first experiment in which the indirect effect of the infection of two plant pathogenic fungi together on an insect has been examined. Although the nitrogen concentration of dual-infected plants was as low as that in botrytis-only infected plants, in general the aphids performed better on dual-infected plants (when the two pathogens were applied simultaneously) than when fed on those infected with botrytis alone (Figure 2). Although aphid performance is overall is likely to be inhibited by the lack of nitrogen, if we postulate that these aphids are affected by JA-signalled rather than SA-signalled induced defences, then if the rust infection is able to inactive the JA-signalled defence, as suggested above, then the aphids will be able to perform better than on plants infected with botrytis alone. Further evidence for this hypothesis will be gained from experiments in which the effect of the aphid on the two pathogens and the effect of the

pathogens on each other are investigated. It is unfortunate that we were unable to measure concentrations of SA and JA in these experiments; this will be needed for confirmation of these effects.

The nature and extent of cross-talk between the signalling pathways depends on the timing and magnitude of their induction (van Loon et al., 2006), and this could be reflected in this experiment. Whether the pathogens were applied together or sequentially did not affect leaf nitrogen content, but it did affect aphid performance with greater inhibition of aphids when the pathogens were applied sequentially rather than at the same time (Figure 2). This suggests that concurrent infection may reduce the cross-talk between defence pathways and thus inhibition of defences effective against aphids.

Further experiments are needed to elucidate the mechanisms for the observed effects in this system, but the results suggest that aphids experience a complex and changing food source, with the possibility of their foodplants being infected by a variety of pathogens with their own widely different individual effects, and also effects from their combinations. The observation that these aphids are both significantly inhibited and also significantly enhanced by leaf pathogen infection raises issues for the control of these three pests of *Vicia faba*. There is the possibility that control measures against some of these pathogens could lead to a consequent increase or decrease in *Aphis fabae* performance and ultimately plant yield, and thus careful pest management will be essential. This will be investigated in subsequent experiments.

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Table 1

Statistical analysis of the effect of *Botrytis cinerea* (A) or *Uromyces viciae-fabae* (B) on the growth and fecundity of individual *Aphis fabae*. n=23. ***, P<0.001; **, P<0.01; *, P<0.05, ns, P>0.05. Two factor ANOVA (age of leaflet x presence/absence of systemic infection; together called 'treatment') with separate 'control' (aphids reared on an infected leaflet of leaf 3). Convstrt = overall difference between 'control' and 'treatment'. See text for further details of analysis.

A

		Maturation time (days)	MRGR	Fecundity	r_m
Source	df	MS	MS	MS	MS
Convstrt	1	46.37 ***	0.0508 ***	5279 ***	0.155 ***
Convstrt x Age of leaflet	1	8.183 ***	0.0001 ns	0.14 ns	0.003 **
Convstrt x Infection	1	62.81 ***	0.0351***	3769 ***	0.116 ***
Convstrt x age x infection	1	3.594 **	0.0001 ns	77.26 *	0.0001 ns
Residual	87	0.3537	0.0014	15.82	0.0003
Total	91				

B

		Maturation time (days)	MRGR	Fecundity	r_m
Source	df	MS	MS	MS	MS
Convstrt	1	143.3 ***	0.0245 ***	2358 ***	0.2658 ***
Convstrt x Age of leaflet	1	0.141 ns	0.0001 ns	41.3 ns	0.0001 ns
Convstrt x Infection	1	0.147 ns	0.002 ns	28.53 ns	0.0005 ns
Convstrt x Age x Infection	1	1.356 ns	0.008 ns	72.80 ns	0.0025 ns
Residual	112	1.100		38.89	0.0012
Total	116				

Table 2

Statistical analysis of the effect of *Botrytis cinerea* or *Uromyces viciae-fabae* infection, individually or combined on the growth and fecundity of individual *Aphis fabae* reared on infected leaves. Two-factor ANOVA (infection with *B. cinerea* x infection with *U. viciae-fabae*). ***, P<0.001.

		Maturation time	MRGR	Fecundity	Intrinsic rate of increase
Source	df	MS	MS	MS	MS
Botrytis	2	13.3 ***	0.01 ***	369 ***	0.03 ***
Uromyces	2	195 ***	0.08 ***	8718 ***	0.42 ***
Botrytis x Uromyces	4	28.6 ***	0.01 ***	671 ***	0.049 ***
Residual	144	0.89	0.002	14.9	0.001
Total	152				

Table 3

Statistical analysis of the effect of *Botrytis cinerea* (A) or *Uromyces viciae-fabae* (B) infection on the population growth of *Aphis fabae* on *Vicia faba.* Repeated measures ANOVA calculated. ***, P<0.001.

A

		Number of aphids	Dry weight of aphids
Source	df	MS	MS
Time after infection	2	8617 ***	0.278 ***
Infection	1	1193 ***	0.355 ***
Time x infection	2	4746 ***	0.198 ***
Residual	84	2713	0.002
Total	89		

B

		Number of aphids	Dry weight of aphids
Source	df	MS	MS
Time after infection	2	1394 ***	0.181 ***
Infection	1	2595 ***	0.413 ***
Time x infection	2	6631 ***	0.074 ***
Residual	84	2025	0.002
Total	89		

Figure legends

Figure 1 The local and systemic effect of *Botrytis cinerea* or *Uromyces viciae-fabae* infection of *Vicia faba* on the growth and fecundity of individual *Aphis fabae*. Means $\pm$ SE given. See Table 1 for statistical analysis. Solid bar, 'old' infected leaflet (on third leaf); hatched bars, 'old' uninfected leaflet (on third leaf); open bars, 'young' uninfected leaflet (on fifth leaf). Same letters signify no significant difference ($P>0.05$) between means, from LSD from ANOVA.

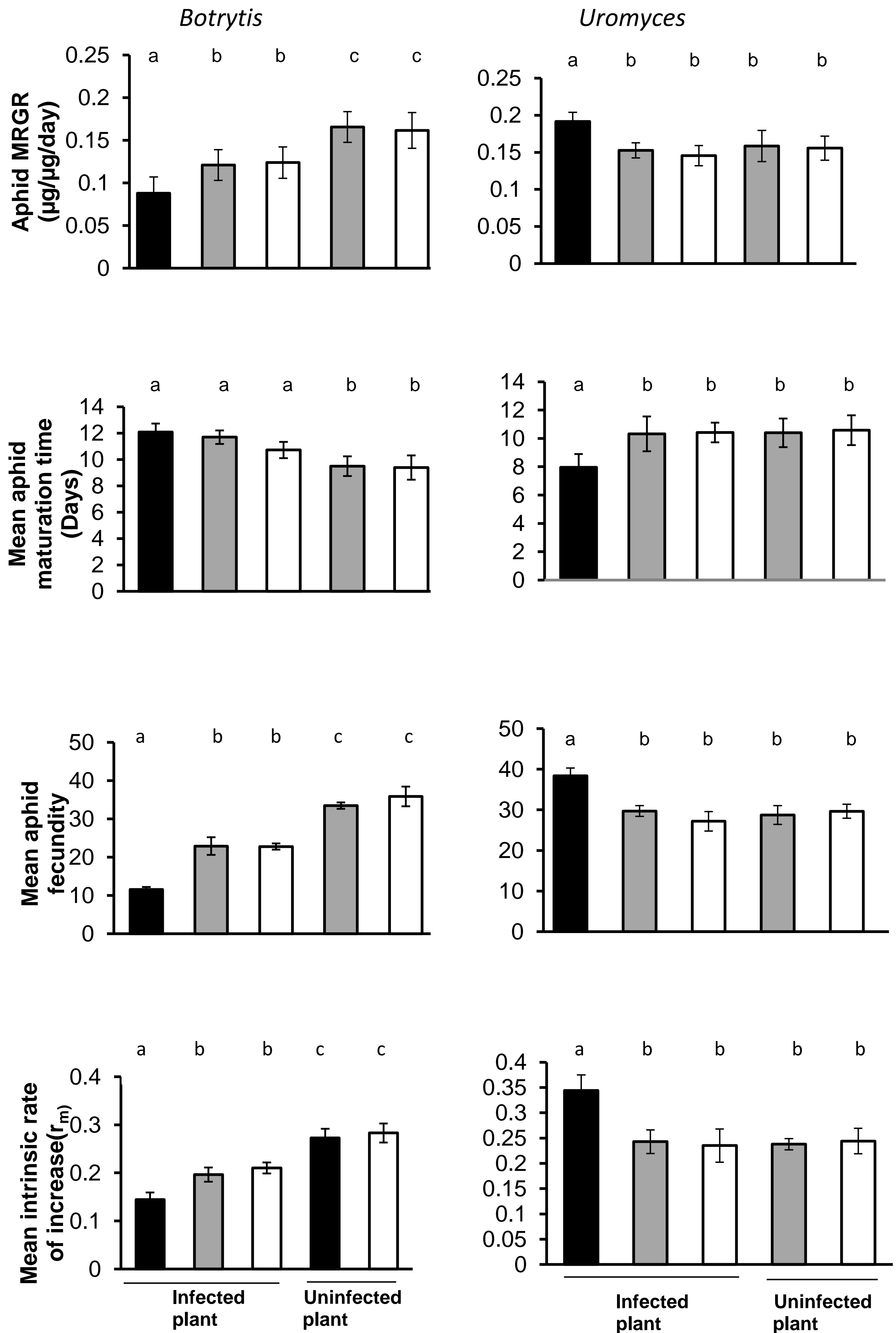
Figure 2 The effect of *Botrytis cinerea* or *Uromyces viciae-fabae* infection, individually or combined on the growth and fecundity of individual *Aphis fabae* reared on infected leaflets. Means $\pm$ SE given, n=20. X-axis legend key: Control, uninfected leaf; B, *Botrytis* infection; R, *Uromyces* infection; 24 or 29 days after plant germination; thus B24 indicates infected with *B. cinerea* 24 days after germination, and B24R29 indicates infected with *B. cinerea* 24 days after germination, and infected with *U. viciae-fabae* 5 days later (29 days after germination). Singly-infected leaflets are hatched, doubly-infected leaflets are cross-hatched. Summary results of two-factor ANOVA given (see Table 2 for statistical analysis): B, *Botrytis* infection; R, *Uromyces* infection; B x R, interaction term. ***, $P<0.001$. Same letters signify no significant difference ($P>0.05$) between means, from LSD from ANOVA.

Figure 3 Regression of lesion/pustule density of *Botrytis cinerea* (A) and *Uromyces viciae-fabae* (B) against aphid mean relative growth rate. A, $F_{1,102} = 268$, $P<0.001$, $r^2 = 0.722$. B, $F_{1,101} = 399$, $P<0.001$, $r^2 = 0.798$

Figure 4 The effect of *Botrytis cinerea* or *Uromyces viciae-fabae* infection on the population growth (number and dry weight) of *Aphis fabae* on *Vicia faba*. Mean $\pm$ SE given, =15. Solid line, uninfected plants; dashed line, infected plants. Summary results from ANOVA (see Table 3 for statistical analysis): I, infection; T, time; I x T, interaction term. ***, $P<0.001$.

Figure 5 Percentage dry weight nitrogen content of *Vicia faba* leaves infected with *Botrytis cinerea* (B) and/or *Uromyces viciae-fabae* (R), 24 or 29 days after plant germination (see Figure 2 for details). Mean $\pm$ SE given, n=5, except for control where n=2. Same letters signify no significant difference ($P>0.05$) between means, LSD from ANOVA ($F_{5,24} = 89.01$, $P<0.001$).

Fig. 1



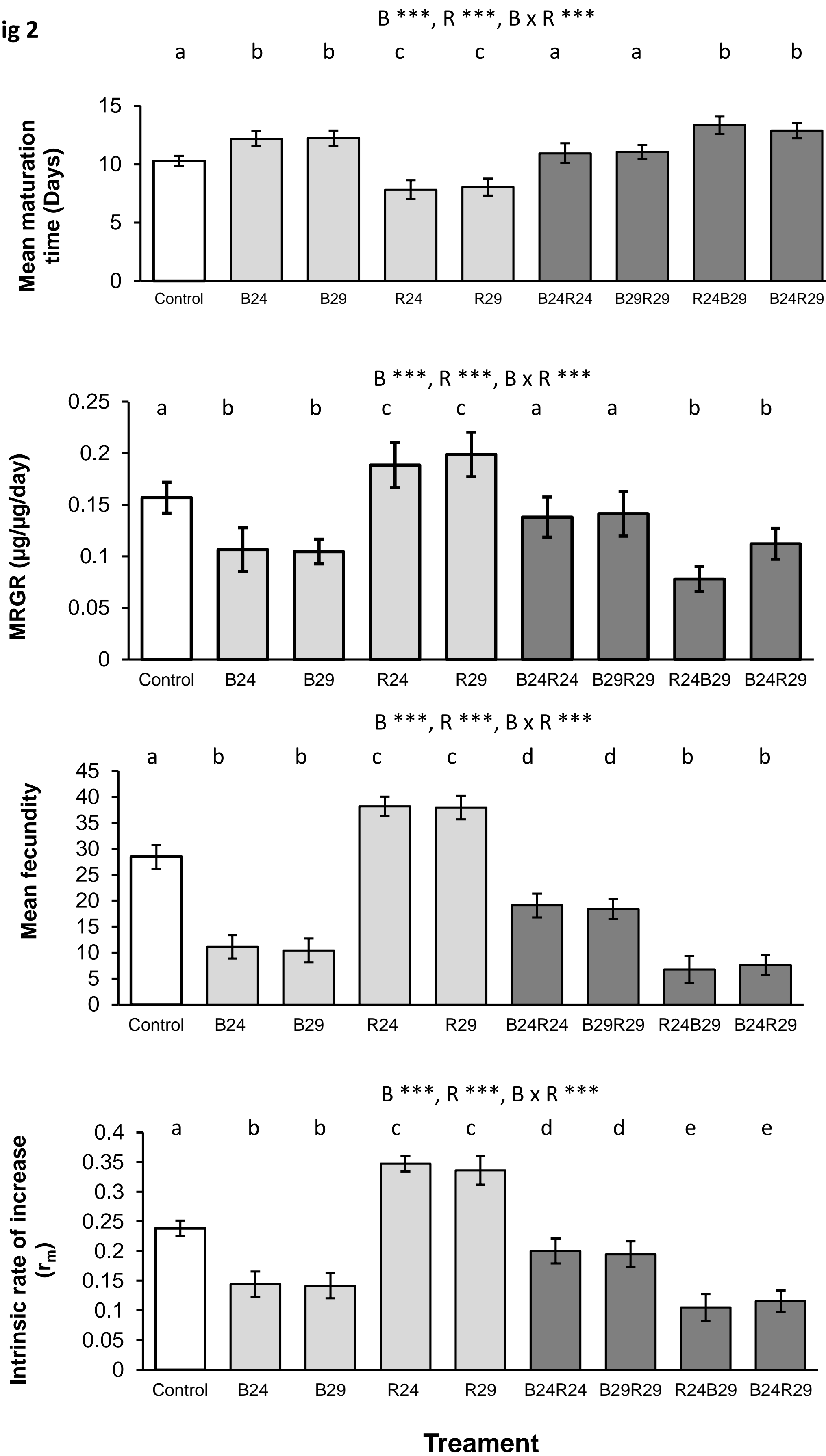
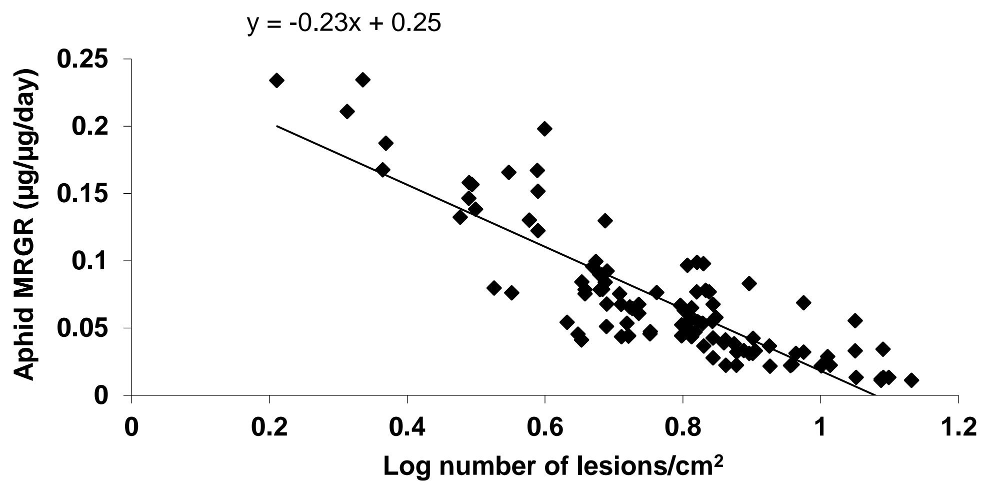


Fig 2
B ***, R ***, B x R ***
a b b c c a a b b
15
10
5
0
Mean maturation time (Days)
Control B24 B29 R24 R29 B24R24 B29R29 R24B29 B24R29
B ***, R ***, B x R ***
0.25
0.2
0.15
0.1
0.05
0
a b b c c a a b b
MRGR (µg/µg/day)
Control B24 B29 R24 R29 B24R24 B29R29 R24B29 B24R29
B ***, R ***, B x R ***
a b b c c d d b b
45
40
35
30
25
20
15
10
5
0
Mean fecundity
Control B24 B29 R24 R29 B24R24 B29R29 R24B29 B24R29
B ***, R ***, B x R ***
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a b b c c d d e e
Intrinsic rate of increase (r_m)
Control B24 B29 R24 R29 B24R24 B29R29 R24B29 B24R29
Treament

Fig 3

A



B

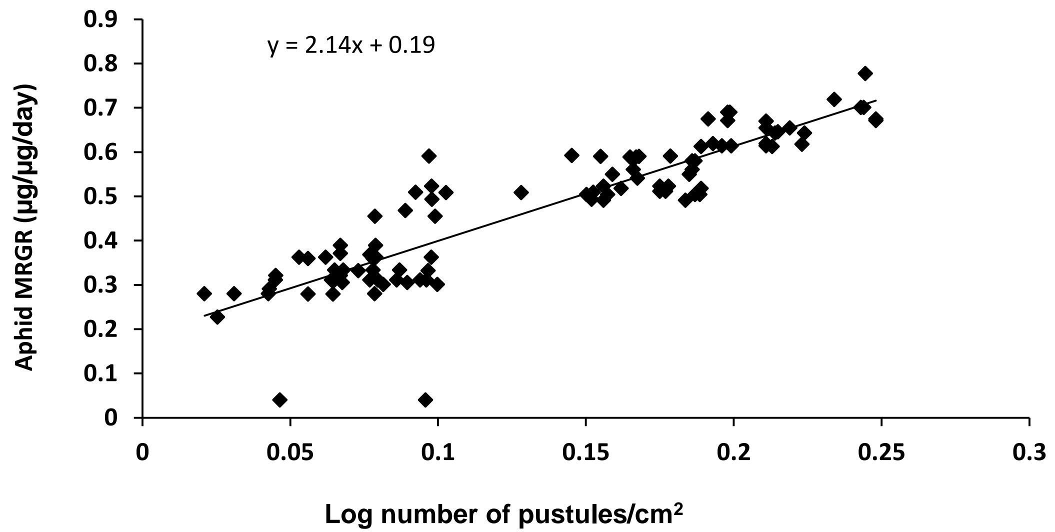
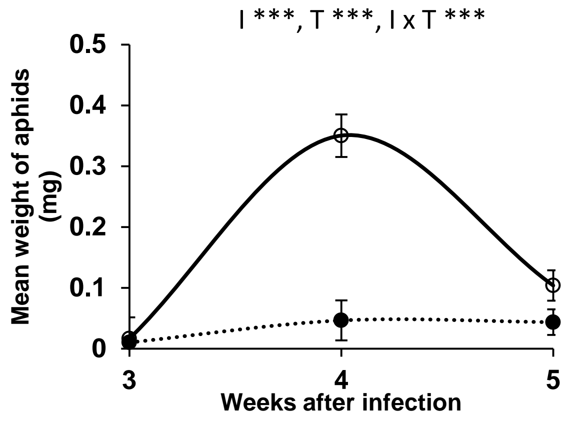
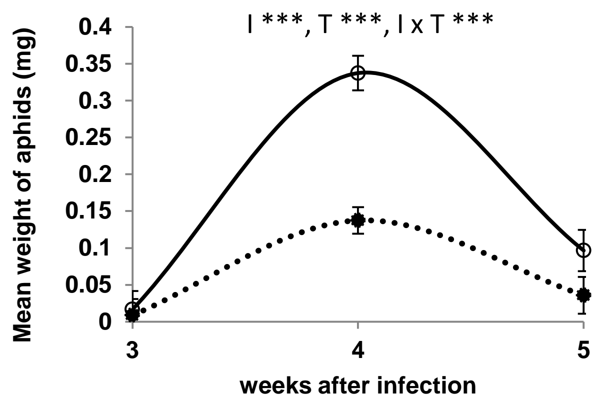


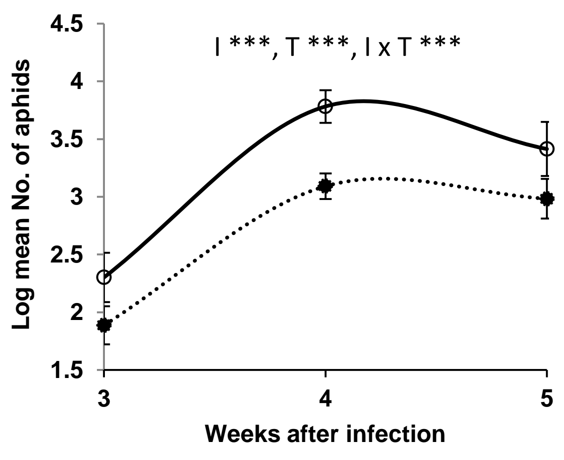
Fig 4

Botrytis



Uromyces





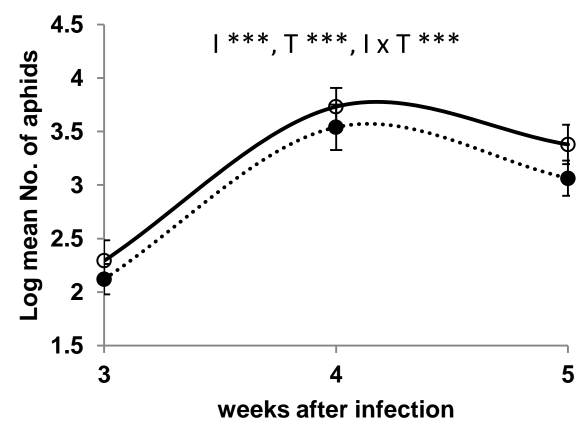


Fig 5