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Context-dependent effects of induced resistance under co-infection in a plant–pathogen interaction

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Abstract

The ability of a parasite strain to establish and grow on its host may be drastically altered by simultaneous infection by other parasite strains, and dynamics under multiple infection have been suggested to be a major force driving pathogen evolution. Here, I studied whether hosts' induced defenses mediate dynamics of multiple infection of the fungal pathogen, *Podosphaera plantaginis*, infecting *Plantago lanceolata*. A laboratory study of sequential infections, where interaction between pathogen strains was prevented, showed that ability to establish remained unaffected, but prior infection elevates the host's resistance to the degree that subsequent infection development is significantly reduced. However, when inoculated plants and their healthy controls were planted back into their natural populations, hosts with prior infection became more heavily infected by the subsequent infections than the initially healthy plants. Hence, a controlled short-term laboratory study is a poor predictor of the host's ability to mediate multiple infection during the course of natural epidemics. These results have applied implications for priming where the plants' defenses are elicited to provide protection against further attack, highlighting the importance of testing priming under natural conditions for relevant time scales.

Introduction

Throughout the growing season, as epidemics proceed, the same host individual may be challenged by more than one pathogen strain of the same species. The resulting interaction may range along a continuum where at the one end we find superinfection with a single strain dominating the entire host, and at the other end of the continuum, we find multiple infection with several pathogen genotypes co-infecting the same host individual (de Roode et al. 2005; Bell et al. 2006; López-Villavicencio et al. 2007; Ben-Ami et al. 2008). If the sum of infections under co-infection is different from that under single infections, these within-host dynamics may have significant effects on key features of host–pathogen interactions such as symptom expression, transmission dynamics, and maintenance of pathogen variation (May and Nowak 1994; van Baalen and Sabelis 1995; Frank 1996; Read and Taylor 2001; Mideo et al. 2008; Mideo 2009). In general, co-infection is considered to select for increased within-host growth rates (May and Nowak 1994; Nowak and May 1994; van Baalen and Sabelis 1995; Frank 1996; Alizon et al. 2009), because the strains compete on the basis of their exploitation rates with the most competitive strain gaining a disproportionate share of the host (but see Buckling and Brockhurst 2008; Alizon et al. 2009 for exceptions). As empirical data are accumulating, the debate is no longer purely academic: Concomitant infections by several parasite genotypes and even species are frequently encountered (Lipsitch and Moxon 1997; Cox 2001; Read and Taylor 2001; López-Villavicencio et al. 2007).

The form of the interaction between different parasite strains within the same host may be classified as direct, exploitation or interference, competition among the pathogen strains or apparent competition mediated by the host's defense responses, and the two forms are not mutually exclusive (de Roode et al. 2005; Mideo 2009). Apparent competition, where abundance of one species

has effects on another species via a shared enemy, has been shown to be important for structuring species assemblages (Bonsall and Hassell 1997) and may similarly be very important for determining the community of parasites attacking a single host (de Roode et al. 2005; Alizon et al. 2009). However, to date, most models of within-host dynamics do not explicitly account for variation in mobilized host resistance properties for multiple infection (but see, e.g., Alizon and van Baalen 2008; Mideo and Day 2008; Choisy and de Roode 2010), and empirical data on the role of immune responses mediating mixed infections are limited. Theoretically, it has been shown that the mode of within-host competition can determine whether mixed infections select for higher or lower virulence (Choisy and de Roode 2010).

Few studies have looked at multiple infection in plants (Maltby and Mihail 1997; Meijer and Leuchtmann 1999; Wille et al. 1999; Hood 2003; Koskella et al. 2006; López-Villavicencio et al. 2007) where host-mediated competition may be particularly important. Plants lack the mobile defender cells and somatic adaptive immune system of vertebrates. In their defense against pathogens, plants rely on a two-step defense system where the first step recognizes and prevents infection by many classes of microbes, including nonpathogens (Jones and Dangl 2006). The second step is a signal transduction pathway that responds to both attempted and successful pathogen attack resulting in induced resistance against further attack (Ryals et al. 1996; Jones and Dangl 2006). This ability to coordinate the development of resistance when it is needed has been verified for numerous plant species (Heil and Baldwin 2002), which suggests that inducible resistance may play an important role in determining how dynamics of multiple infection are played out in plants. In the wild, we can expect plants to be continuously challenged by different pathogen strains even after becoming infected, yet very few studies have investigated the success of later arriving strains. Furthermore, it remains unclear whether inducible defenses are largely a laboratory phenomenon, and whether plants growing in the wild are permanently in the induced state (Heil and Baldwin 2002), or abandon it because it is too costly to maintain (Cipollini et al. 2003; van Hulten et al. 2006).

In recent years, the ability of plants to mobilize defense responses leading to enhanced resistance to both biotic and abiotic stresses has generated interest in the applicability of this trait (Conrath et al. 2002; Walters et al. 2005; Beckers and Conrath 2007; Walters and Daniell 2007; Jung et al. 2009). Today, there is a pressing need to find alternative means of battling plant disease as the everincreasing reliance on chemical pesticides is expensive, may impact negatively on the environment, and has often proved a short-lived solution in the face of rapidly adapting pathogen populations (McDonald and Linde 2002). Biotic agents, such as less virulent pathogens, necrotizing pathogens, or even compounds of the plants' own signaling pathway, may be used to boost the resistance of crops to subsequent pathogen attack in an environmentally friendly manner (Walters and Daniell 2007; Jung et al. 2009). Multiple genes are involved in the signal transduction pathway of induced resistance (Kazan and Schenk 2007), and hence, priming may prove more durable than use of R genes with major effects on cultivar resistance, which pathogen populations may quickly overcome (Lindhout 2002; Palloix et al. 2009).

Given that co-infection is a powerful driver of pathogen evolution and epidemiology, and that the applied implications of co-infections range from priming to other epidemiological interventions and virulence management (Ebert and Bull 2003), a precise understanding of the outcome and mechanisms of multiple infection is urgently needed. The aim of this study was to determine whether infection success of arriving strains – their establishment and subsequent growth – is affected by the hosts' responses to prior infection. The work was carried out with the *Plantago–Podosphaera* plant–fungal pathosystem that occurs naturally as a metapopulation in the Åland Islands SW of Finland (Laine and Hanski 2006). Multiple infections are considered common in this system because sexually produced spores, which result from two compatible mating types simultaneously infecting the same host, are frequently observed (Yarwood 1978; Laine and Hanski 2006). The role of hosts' responses in mediating the success of subsequent infection was studied both in the field and in the laboratory. In the laboratory, a pilot study suggested that prior infection may have significant impacts on the development of subsequent infections. A second laboratory experiment was carried out controlling for the effect of plant and pathogen genotypes that provided even stronger evidence of the host's induced defenses mediating subsequent infection. To test whether this result also holds under field conditions during the course of natural epidemics, experimentally infected and uninfected plants were planted back to the populations they were collected from as seeds, and their infection status was followed during the growing season.

Materials and methods

Host–pathogen system

The host plant *Plantago lanceolata* L. (Plantaginaceae) is a perennial plant that is considered an obligate outcrosser, a trait maintained both by protogyny and by an S-RNase-driven self-incompatibility system (Ross 1973). The seeds of *Pl. lanceolata* have no special dispersal mechanisms; as they ripen, they are simply dropped to the ground close to the mother plant (Bos 1992; van Damme 1992).

Clonally produced side rosettes from the axillary meristems are a common means of reproduction for *Pl. lanceolata* (Mook et al. 1992).

Podosphaera plantaginis (Castagne; U. Braun & S. Takamatsu) is an obligate powdery mildew fungus in the order Erysiphales within the Ascomycota (Yarwood 1978). In Finland, *Po. plantaginis* appears to be a specialist of *Pl. lanceolata* (A.-L. Laine, unpublished data). The fungus completes its entire life cycle on the surface of the host plant where it is visible as localized (nonsystemic) white powdery lesions. During the growing season, the pathogen is transmitted among hosts by clonally produced dispersal spores, conidia, that are passively carried by wind. At the end of the growing season in August, the sexually produced resting spores (cleistothecia) begin to appear, visible to the eye as black specks roughly 1 mm in diameter. During winter, as most host individuals die back to root stock, the local pathogen populations decline. Populations of *Po. plantaginis* consist of strains that vary in their growth and transmission on the same set of host genotypes (Laine 2004, 2005, 2008).

Resistance in *Plantago* against the fungal pathogen functions in the two-step manner typical of most plants (Jones and Dangl 2006). The first step is strain specific as the same host genotype expresses resistance against some strains (*i.e.*, recognition) of the pathogen while being susceptible to others (*i.e.,* nonrecognition) (Laine 2004). Once a strain has successfully established, its development is affected by both pathogen and host genotype (Laine 2007b). Infected hosts have reduced survival during drought periods compared with healthy hosts (Laine 2004), and drought is considered to be one of the main environmental stress factors affecting populations of *Pl. lanceolata* in Åland (Hanski 1999).

Plant and fungal material

Plants for the experiment were obtained as seeds from natural populations of *Pl. lanceolata* (population IDs for laboratory experiment 1: 273, 282, 463, 1006, and 1370; for laboratory experiment 2 and field experiment: 609, 877, 1915, 3484 and 3350) in the Åland Islands in September 2005 and 2006. Seeds of 15 haphazardly chosen individuals in each population were collected into paper envelopes and stored at room temperature. Seeds were germinated by placing them in 0.8-l pots in a 30% vermiculate – 70% potting soil mixture in greenhouse conditions of 16 h of light and at +22°C. Each plant genotype used in the laboratory experiment 2 and field experiment was cloned into four plants according to a method described by Laine (2004). Pathogen strains used in the experiments were collected from the Åland Islands in August–September of 2006 (population IDs 542, 877, 1915, 3484, and 3350) as infected leaves. Laboratory experiment 1 used bulked spore material, but genetically homogenous strains used in laboratory experiment 2 and the field experiment were obtained by repeating at least four single-colony inoculations (Nicot et al. 2002). The strains were confirmed to be different based on their differential ability to infect a test set of ten host genotypes known to differ in their resistance. The strains were maintained in Petri dishes on leaves of *Pl. lanceolata* in a growth chamber at 20 ± 2°C and a 16L/8D photoperiod, and they were transferred to fresh leaves approximately every 3 weeks. Repeated cycles of inoculations were performed prior to the experiments to obtain adequate stocks of sporulating fungal material. A summary of the differences between the three experiments is given in Table 1.

Table 1. A summary of the differences between the three experiments designed to test how prior infection affects subsequent infections via hosts' induced defenses.

	Laboratory expt. 1	Laboratory expt. 2	Field experiment
Aim	Test role of hosts' induced responses for co-infection, test methodology	Test role of hosts' induced responses for co-infection, test for differences among host genotypes	Test role of hosts' responses for co-infection in the field during natural epidemics
Host material	50 genotypes (10 genotypes from 5 populations)	19 host genotypes, each cloned into 4 plants	30 host genotypes, each cloned into 4 plants
Host population origin	273, 282, 463, 1006, 1370	609, 877, 1915, 3484, 3350	877, 3484, 3350
Pathogen material for 1st inoculation	Bulked spores from two source populations	Strain 1915.11	Strains 877.1, 3484.1, 3350.1
Pathogen material for 2nd inoculation	Bulked spores from two source populations (always from different populations than those used in 1st inoculation)	Strain 3350.5	Plants became naturally infected in the field
Pathogen population origin	542, 877, 1915, 3350	1915, 3350	877, 3484, 3350

Inoculation protocol and scoring of infection

For the three experiments described later, the protocol for the first inoculation was identical. Both in the field and in the laboratory experiments, the aim was to determine whether the hosts' response to the first infection affects subsequent infections by different pathogen genotypes. Hence, direct contact between the infections was prevented by sealing the first-inoculated leaf inside a pollination bag (PBS International). Pollination bags allow for a good spectrum of light inside the bags as well as flow of air and moisture, yet prevents movement of particles in the size range of the conidial spores (spores of powdery mildews contain the water required for germination, and hence, they are quite large, roughly within the range of 25–40 × 15–20 μm; Braun 1987). During the inoculation, the plant was protected from infection by placing it inside a transparent plastic bag with a single leaf exposed through a small hole. Spores from a sporulating colony of approximately 1 cm in diameter were evenly brushed onto this exposed leaf, and subsequently, a pollination bag was placed over the inoculated leaf and sealed at the base of the leaf for the duration of the experiment.

The purpose of sealing the leaf inside the pollination bag was to allow for infection to develop on the inoculated leaf but to prevent infection of other leaves by autoinfection because it would not have been possible to distinguish between the initial infection and subsequent infections. After the inoculations, the plastic bags covering the plants were carefully wiped with ethanol and removed the following day to ensure that no viable spores remained that could infect the rest of the plant. The experimental controls consisted of host plants that were not inoculated but received otherwise an identical treatment of being placed inside a transparent plastic bag for approximately 24 h and having one of their leaves sealed inside a pollination bag for the duration of the experiment. The plants were placed inside growth chambers at 20 ± 2°C with a 16L/8D photoperiod.

At the end of both laboratory experiments, all leaves on these plants were carefully checked for infection and only the leaves that were initially inoculated were confirmed to support infection demonstrating that the initial inoculation had infected only the target leaf as intended.

To confirm that mildew infection did not spread from the leaves contained in pollination bags under field conditions in the field experiment, 10 plants with one infected leaf sealed inside a pollination bag and ten plants with pollination bags sealing one noninfected leaf were placed outdoors in an exposed field at the University of Helsinki greenhouses on 1st of September 2009. *Podosphaera plantaginis* does not occur in this region, and hence, any infection on these plants would result from the infected leaves sealed in pollination bags. After 3 weeks, the plants were collected from the field and they were carefully checked for infection. Only the initially infected leaves sealed inside pollination bags showed signs of infection. Hence, infection does not spread from the pollination bags, and infection measured in the field experiment does not result from autoinfection.

Laboratory experiment 1

The aim of the experiment was to test the reliability of the method of sealing infections on the host plant and to determine whether hosts' response to the first infection affects the establishment and development of subsequent infections. The experiment was carried out in September 2006, and it consisted of 50 plant genotypes, each from a different maternal line. Ten genotypes represented five different populations (see Plant and fungal material), which were divided into 25 plants that received the first inoculation and 25 control plants. Inoculations were carried out with bulk fungal material collected from four different populations from different parts of the Åland Islands (population IDs 542, 877, 1915, and 3350) that had not been purified into single-genotype isolates. The inoculations were performed with spores bulked from two different populations, and the first and second inoculations were always performed with spores from different populations. To confirm that infection establishes and develops normally inside the sealed bags, a single leaf on ten plants was inoculated as in the experiment but left un-sealed and placed in a growth chamber for fourteen days.

Four days after the first inoculation, the plants were again sealed inside plastic bags with a single leaf exposed and inoculated by brushing spores from an infected leaf (approximately 1 cm in diameter of sporulating material) onto the target leaf. The time lag of 4 days should be sufficient for the activation of defense responses, such as the accumulation of pathogenesis-related (PR) proteins, that have been measured 3 days and onward in several host species inoculated with infective powdery mildew strains (Bryngelsson et al. 1994). The leaves for the second inoculations were taken from the same leaf spiral as the inoculated/control leaf so that they were of the same age. The plants were placed inside growth chambers, and after 10 days, the inoculated leaves were detached and infection was scored under a dissecting microscope. Infection was measured as a categorical variable ranging from mycelial growth to heavy spore production according to a key adapted from Bevan et al. (1993): 0 = no visible signs of infection, 1 = sparse mycelium but no conidia, 1.5 = mycelium producing very few conidia and colonies visible only under a dissecting microscope, 2.5 = colonies visible

with the naked eye but exhibiting sparse sporulation, 3 = profuse sporulation on colonies of moderate size (<5 mm diameter), and 4 = profuse sporulation on large colonies (>5 mm diameter).

Laboratory experiment 2

The aim of the experiment was to test whether the hosts' response to the first infection affected the establishment and development of the subsequent infection. Specifically, this experiment was designed to control for the effect of plant and fungal genotypes on the response by using paired cloned individuals in the infected and noninfected treatments and using the same purified pathogen strains on all host genotypes. The experiment was carried out in May 2007. Nineteen host plant genotypes were chosen for the experiment, each representing a different maternal line. Four clonal replicates, two inoculated and two controls, represented each genotype. All first inoculations were performed with the *Po. plantaginis* strain ID 1915.11 known to be infective on these genotypes, and all subsequent inoculations were performed using pathogen strain 3350.5, also infective on all 19 host genotypes.

Four days after the first inoculation, two leaves of the infected plants and a single leaf of the control plants were detached and placed on Petri dishes on moist filter paper. Detached leaves or leaf segments are generally considered to yield reliable estimates of host resistance that correspond to those of whole plants (Nicot et al. 2002). The extent and mechanical nature of detaching leaves is unlikely to trigger induced responses that affect pathogens to the extent that biotically generated damage does (Reymond et al. 2000; De Vos et al. 2006). The leaves were inoculated by brushing spores from an infected leaf evenly onto the leaves on the Petri dishes. The infection key described in laboratory experiment 1 was used for data analysis to identify which infections sporulated and which did not. The percentage of leaf area covered by fungal growth was also visually estimated.

Field experiment

The aim of the field transplant experiment carried out in the summer of 2007 was to determine whether prior infection by *Po. plantaginis* affects the infection success of later arriving strains on the same host plant under natural conditions. To obtain as realistic view as possible of subsequent infection probability and success on these infected and uninfected hosts, the plants were transplanted into natural populations in sympatry (*i.e.*, into populations they had been collected from). This was performed because previous studies have demonstrated that local mildew populations are often adapted to their local host populations' genetic structure (Laine 2005, 2007a, 2008).

In early July of 2007, three of the five *Pl. lanceolata* populations that had been sampled for seeds were determined to be infected by *Po. plantaginis* (IDs 877, 3484, and 3350). On 11–13 of July in 2007, when the cloned plants were 3 month old, two clones of each genotype were inoculated with *Po. plantaginis* in the laboratory. A leaf of each plant genotype was inoculated with a sympatric pathogen strain originating from the same population from which it had been collected in 2006 and maintained in the laboratory during winter. The strains had been determined to be infective on the host genotypes used in the experiment. The plants were inoculated as described previously. The experimental controls consisted of two clones of each genotype that were not inoculated but had one of their leaves sealed inside a pollination bag for the duration of the experiment. After the inoculations, the plants were placed inside growth chambers at +20°C with a 16L/8D photoperiod.

On July 15–17 2007, 4 days after the first inoculation, host plants were transplanted back to their populations of origin. In each population, there were 40 plants (four clones representing 10 genotypes), of which 20 had prior mildew infection and 20 were uninfected. Transplanting individuals into soil at these sites would have been impossible because *Pl. lanceolata* populations in Åland occur on rocky outcrops with extremely shallow soils. Hence, the plants were kept in their pots for the duration of the experiment. In each population, 40 plastic containers ($14 \times 10.5 \times 4.5$ cm) were placed on the soil in the proximity of naturally infected *Pl. lanceolata* individuals. In each population, the plants in their pots were then placed into these containers in a random order. Starting on July 24, the sites were visited every third day (1st day population 3350, 2nd day population 877, and 3rd day population 3484). At each visit, the number of healthy leaves and the number of infected leaves were counted on each plant (leaves that withered during the experiment, infected or healthy, were not counted). Handling the plants may have affected transmission of the fungus, but the effect was the same for controls and previously infected plants and hence should not bias the obtained results. To minimize the effect of spatial positioning (distance to infected individuals and position with respect to prevailing wind direction) on the infection probability and severity, the plants were randomized among the plastic containers at every visit. During the visits, the plants were watered if necessary. The plants were removed from the field on August 11–12 in 2007. After the plants had been removed from the field, all pollination bags covering the initially infected leaves were confirmed to be intact and sealed at the base. The plants were transported to the

laboratory where the bags were removed, and the leaves were checked to confirm that they were infected. Two of the plants in population 3484 were not infected and hence were removed from the statistical analyses.

Statistical analyses

Data on infection scores were analyzed with an ordinal regression as implemented in JMP 8.0.2. (2009 SAS Institute Inc.). All other analyses were carried out using SAS 9.1 (2008 SAS Institute Inc.) as generalized linear (GLM) or generalized linear mixed models (GLMM) with the GLIMMIX macro assuming binomially distributed errors with a logit link function for 0/1 data and Poisson distribution with a log link function for percentage data (Littell et al. 2006). For all analyses, I started out with full models and dropped nonsignificant interactions from the models in a stepwise manner.

In *laboratory experiment 1*, infection scores were compared between the bagged and nonbagged leaves with the bagging treatment as a class variable. Data measuring infection (0/1), sporulation (0/1), and infection scores of second infections on the previously infected and noninfected plants included treatment and plant population as fixed class variables.

In *laboratory experiment 2*, infection status was not analyzed as all leaves displayed some symptoms of infection. Infection severity was measured as the percentage of leaf area covered with fungal growth and whether the infection sporulated or not (0/1). The explanatory fixed variables were plant genotype and treatment. The plant individual and leaf, nested under plant genotype, were defined as random variables in the model.

In the *field experiment*, data were analyzed on whether the plants became infected or not (0/1) and severity of infection measured as the percentage of infected leaves of all leaves on each host individual. For both field and laboratory experiments, data on the percentage of infected leaves and the percentage of leaf area covered with fungal growth, respectively, were arcsin transformed. Following transformation, the data were confirmed to be Poisson distributed using statistical software package JMP 5.1/SAS (JMP 2002). Because the field experiment was performed in sympatry, i.e., plants were transplanted back to their population of origin, the effect of plant genotype could not be estimated across populations, and hence, host genotype was nested within population. Date of observation was included as a covariate in the model and treatment (prior infection or not) and population as class variables. The plant individual, hierarchically nested within genotype and population, that was repeatedly checked over time was defined as a random variable and identified as the subject of repeated measures in the model complying with a first-order autoregressive covariance structure.

For the field experiment, it was also tested whether the infected and control plants differed in the number leaves they produced with treatment, host population and genotype as explanatory fixed variables, and host plant individual as a random effect hierarchically nested within genotype and population.

Results

Laboratory experiment 1

Sealing the leaf inside a pollination bag was confirmed to be a reliable method. Infection class on inoculated leaves inside the pollination bags ($N = 50$) and on unsealed leaves ($N = 10$) did not differ (1.65 ± 0.15 SE vs. 1.55 ± 0.4 SE, respectively; df = 1, $\chi^2 = 0.068$, $P = 0.7947$), and at the end of the experiment, it was confirmed that infection was successfully sealed inside the pollinations bag, as no other leaves than the inoculated one was infected. At the end of the experiment, it was confirmed that on four plants, the first inoculation had not resulted in infection, and these plants were excluded from further analyses. Both infection success and sporulation were lower on plants with prior infection than on plants that were initially healthy, but these differences were not statistically significant (infection: 0.5 ± 0.12 SE vs 0.67 ± 0.1 SE; $F_{1,40} = 1.7$, $P = 0.2003$; sporulation: 0.41 ± 0.11 SE vs 0.5 ± 0.1 SE; $F_{1,40} = 0.73$, $P = 0.3964$). Infection score on plants with prior infection was lower than infection score on previously healthy plants, but this difference was not statistically significant (1.14 ± 0.16 SE vs 1.34 ± 0.2 SE; $\chi^2 = 1.2$, $P = 0.273$). Plant population did not have a significant effect on any of the response variables.

Laboratory experiment 2

In the laboratory experiment, all the leaves of the subsequent inoculations supported visible sign of the fungus, but there was variation in whether the infections sporulated or not, which ultimately determines the fitness of a given infection (Laine 2008). Sporulation was significantly higher in the control plants than in the previously inoculated plants (Fig. 1A; Table 2). Sporulation was not affected by host plant genotype (Table 2). The interaction term plant genotype × treatment was not statically significant ($P = 0.998$) and hence was not included in the final model. The percentage of leaf area covered by fungal growth (both mycelia and spores) was significantly lower in the inoculated plants than in the control plants. Plant genotypes also differed in the extent of their leaf area covered by infection (Fig. 1B; Table 2). The interaction term plant genotype × treatment was not statically significant

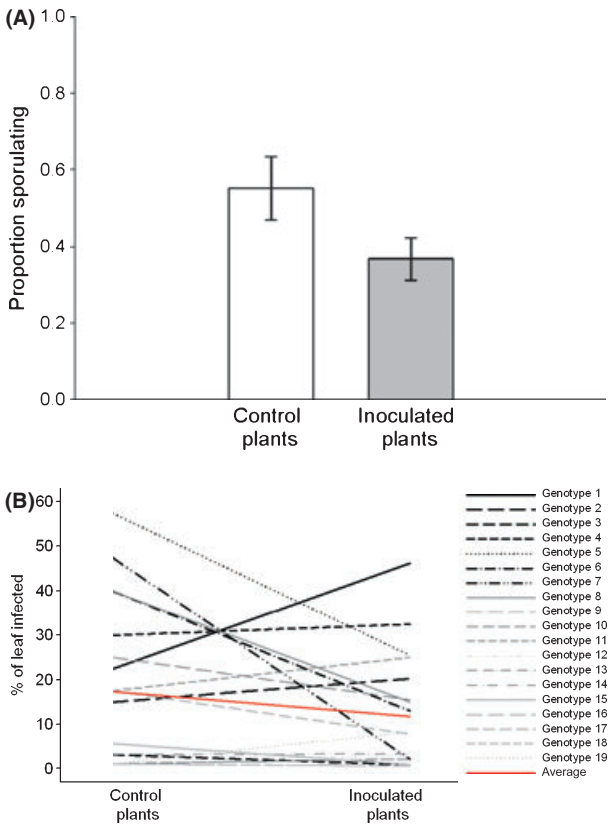

Figure 1 (A) Proportion of sporulating infections in the laboratory experiment on the leaves from initially healthy plants (controls) and on leaves from the inoculated plants. Error bars are based on standard errors of means. (B) Percentage of leaf area infected in the laboratory experiment on the leaves from initially healthy plants (controls) and on leaves from the inoculated plants on the 19 plant genotypes. Red line represents treatment averages.

Table 2. Results of a GLMM analyzing the sporulation (0/1) and percentage of leaf infected in the second laboratory experiment. Wald's *Z*-statistic is given for random effects, and the *F*-statistic is given for fixed effects.

Source	Estimate (±SE) for random effects	*Z*/*F*	*P*
Sporulation			
Host id (host genotype)	11.87 ± 4.47	2.66	0.004
Leaf (host id host genotype)	7.58 ± 11.01	0.69	0.2457
Residual	0.23 ± 0.04	6.01	<0.0001
Host genotype $_{18,37}$		0.64	0.8413
Treatment$_{1,37}$		13.82	0.0007
Percentage of leaf infected			
Host id (host genotype)	0.27 ± 0.13	2.14	0.0161
Leaf (host id host genotype)	0.26 ± 0.40	0.67	0.2518
Residual	0.06 ± 0.01	5.27	<0.0001
Host genotype $_{18,37}$		4.61	<0.0001
Treatment$_{1,37}$		12.90	0.001

($P = 0.1858$) and hence was not included in the final model. For the random effects in both the model of sporulation and of leaf area infected, the estimates for plant individuals were higher than the estimates of leaves (Table 2). This suggests that more of the variation in the data was explained by variation among the plants than by variation between leaves of the same plant.

Field experiment

In the field experiment, nearly all of the plants were infected by the end of the experiment (100% of the plants were infected in populations 877 and 3484, and 90% in population 3350), regardless of whether they had prior infection or not on the sealed leaf. The model variables – treatment, population, or host genotype (nested under population) – did not have a direct effect on the probability of becoming infected. However, the host genotypes within the three populations differed in the amount of time until they became infected (significant time × host genotype (population) interaction; Table 3). The interaction term plant genotype × treatment was not statically significant ($P = 0.9997$) and hence was not included in the final model. Infection severity, measured as the percentage of infected leaves within a plant, increased with time, although there were some fluctuations between

Table 3. Results of a GLMM analyzing the infection status and percentage of infected leaves in the field experiment. Wald's *Z*-statistic is given for random effects, and the *F*-statistic is given for fixed effects.

Source	Estimate (±SE) for random effects	*Z*/*F*	*P*
Infection			
Host id (genotype population)	0.2 ± 0.05	4.15	<0.0001
Residual	0.61 ± 0.04	15.3	<0.0001
Population $_{2,92}$		0.001	0.9999
Treatment$_{1,92}$		0.12	0.7293
Time$_{1,419}$		0.001	0.9685
Host genotype (population)$_{22,92}$		1.05	0.415
Time × host genotype (population)$_{24,419}$		1.93	0.0058
Percentage of infected leaves			
Host id (genotype population)	0.49 ± 0.03	14.35	<0.0001
Residual	0.08 ± 0.01	15.51	<0.0001
Population $_{2,88}$		147.58	<0.0001
Treatment$_{1,88}$		14.95	0.0002
Time$_{1,707}$		575.59	<0.0001
Host genotype (population)$_{26,88}$		5.27	<0.0001

dates because during the growing season, plants grow new leaves, and old leaves wither (Fig. 2; Table 3). The percentage of infected leaves was significantly higher in the inoculated plants than in the control plants (Fig. 2; Table 3). Percentage of infected leaves on the plants also varied significantly among the three populations (Fig. 2; Table 3), and within these populations, the plant genotypes differed significantly in their level of infection (Table 3). The interaction term plant genotype × treatment was not statically significant ($P = 0.7795$) and hence was not included in the final model. The previously healthy and infected plants did not differ in their overall number of leaves ($P = 0.14$), so their size does not explain the obtained results.

Discussion

These results show that the host's induced defenses are involved in mediating the dynamics of co-infection. In the laboratory, when differences between plant genotypes are controlled for, previous infection decreased the probability that a subsequent infection will reach sporulation and also decreased the spread of the pathogen across the leaf surface. These results are in line with earlier studies of multiple infection, showing that multiple infection is an important determinant of infection dynamics within a single host and that under conditions of multiple infection, the success of a strain cannot be predicted from its performance when alone (Nakamura et al. 1992; Thomas et al. 2003; Hodgson et al. 2004; de Roode et al. 2005). However, here I show that the outcome of multiple infection measured under controlled laboratory conditions is a poor predictor of dynamics of multiple infection under natural conditions. In the field experiment, where plants were most likely repeatedly challenged by different pathogen strains over a longer time period, hosts with prior infection supported a higher percentage of infected leaves than their initially healthy controls at the end of the growing season. Later, I discuss the possible reasons for the seeming discrepancy and what the evolutionary, epidemiological, and applied consequences may be.

Results of the laboratory studies show that prior inoculation alters host susceptibility as subsequent attack was lower on plants with prior infection than on initially healthy plants. This effect was more clearly demonstrated in the second laboratory experiment where the effects of plant and mildew genotypes were controlled for. Priming of the hosts defenses did not affect the second strains' ability to establish on the same host plant but there was a significant reduction both in sporulation rate and in pathogen growth (measured as percentage of leaf area infected) in the inoculated plants. Because the experimental design did not allow for direct interactions between the pathogen strains, the reduced infection may be attributed to induced resistance response of the host to the first attack. The ability to activate resistance as it is needed

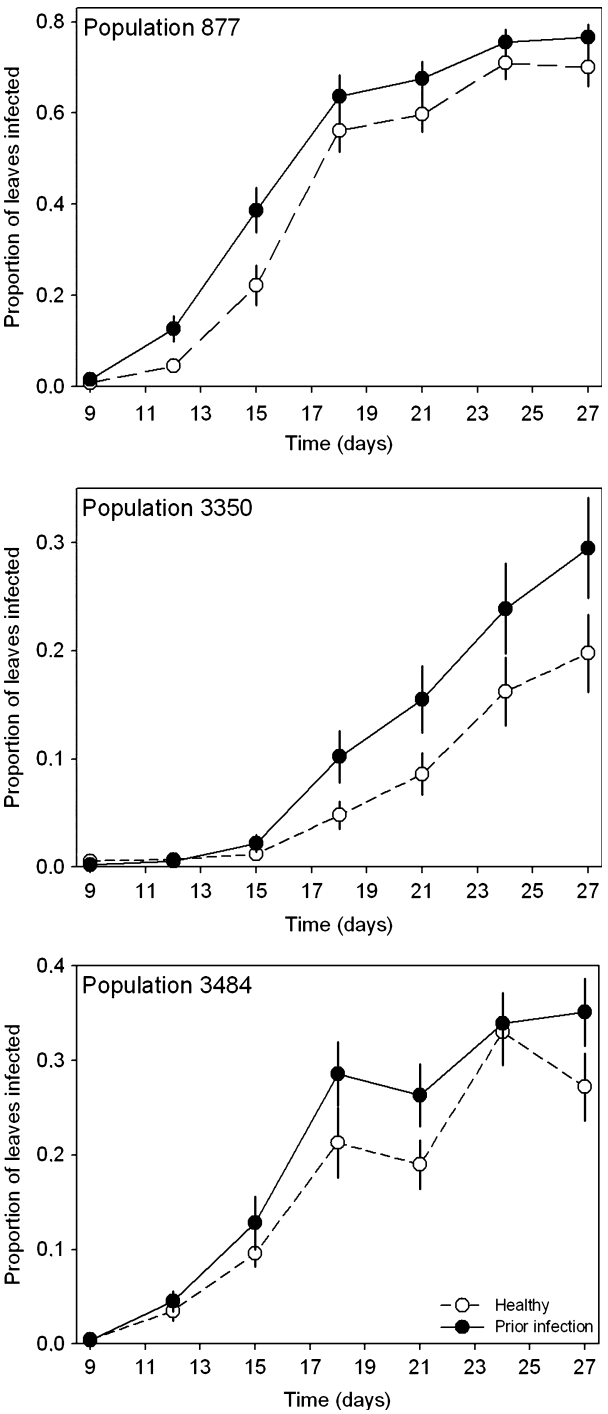

Figure 2 Proportion of infected leaves on experimentally infected and control plants in the three populations measured seven times during the 4-week field experiment. Error bars are based on standard errors of means.

under controlled conditions has been verified for numerous plant–pathogen interactions and it is considered to be a powerful mechanism providing protection against a wide range of parasites (see Bostock 2005 and references therein). Structural and biochemical changes in host plant cells, in particular the accumulation of PR proteins with antimicrobial properties, are considered responsible for the reduced success of subsequent infections (van Loon and van Strien 1999).

In the second laboratory experiment, plant genotype had a strong direct effect on the growth of the subsequent infection, as is evident in Fig. 1B. Some of the plant genotypes supported extremely low pathogen growth even in the control group, and hence, there was little possibility to respond with even lower growth in the plants that had been primed with prior infection. These strong genotype differences may explain why statistically significant differences were not detected in the first laboratory experiment where plant genotype was not controlled for. As visualized in Fig. 1B, in the plant genotypes where considerable pathogen growth was measured in the control treatment, there was variation in the magnitude, and even direction, of how the priming affected the subsequent infection, although the treatment × genotype interaction term was not statistically significant. The considerable variation among plant genotypes in inducible defenses can be understood as a 'cost-benefit' scenario where plants need to weigh the benefits of induced resistance against costs of reallocation of limited resources. Inducing resistance has been shown to be very costly for plants, tying up resources such as nitrogen (Heil et al. 2000) to the extent that lifetime seed production may be reduced (Smedergaard-Petersen and Stolen 1981; Heil et al. 2000; Redman et al. 2001). While elevated resistance may be a beneficial strategy for all genotypes of *Pl. lanceolata*, the costs associated with this trait may differ among the genotypes.

In the field experiment, the initially inoculated plants supported a higher percentage of infected leaves than their initially healthy controls throughout the course of the experiment. While this difference was more pronounced toward the end of the field trial, it is noteworthy that the initially inoculated plants supported more infection already after being in the field for 2 weeks (Fig. 2). Also, the three populations into which the plants had been transplanted differed significantly in the percentage of infected leaves. These population-level differences could be due to differences in local pathogen strains or population-level differences in the hosts' induced defenses. These differences could also reflect local differences affecting the epidemics, such as microclimatic conditions but it is currently not possible to tease apart what might have caused these differences. The field experiment differed in many fundamental ways from the laboratory experiments (i.e., using full plants with different aged leaves, plants were subject to multiple attack by presumably multiple pathogen strains, and the time period was longer). Also, in the field, it was not possible to distinguish between infection establishment and development. Hence, while results from the field should not be directly compared with the results from the laboratory experiment, it is noteworthy that both are sound tests of the role of induced defenses in mediating dynamics of co-infection, yet they yield qualitatively very different results. It seems unlikely that the differences could be attributed to differences among pathogen genotypes alone. The first laboratory experiment using bulk spore samples yielded qualitatively similar results, although not statistically significant, to the second laboratory results, suggesting that induced resistance does suppress subsequent infections regardless of pathogen genotype.

There are several, not necessarily mutually exclusive, possible explanations for the different results obtained in these experiments. First, it is plausible that the induced resistance observed in the laboratory trial does not hold during the course of the field experiment. We can expect the plants for the field study to also have responded with elevated resistance at the beginning, given that the inoculation treatment was identical for both the field and laboratory experiment. This initial response may have been so costly that during the 4-week field trial, the plants became more vulnerable to further attack than the control plants. While induced resistance is a well-studied phenomenon at the physiological as well as molecular levels (Ryals et al. 1996; Bostock 2005), very little is known about its durability over time and under repeated attack (Cipollini 2002; Walters et al. 2005). In the field, the effects of priming appear to be reversed at the time when the first field infections occurred, as the initially inoculated plants supported slightly higher levels of infection than their healthy controls already after 2 weeks of being placed in the field. Furthermore, it may be that the difference we see between the experimentally infected plants and their controls is accentuated as the control plants become 'primed' by their first pathogen encounter in the field. While these effects may also be short-lived, this later 'priming' could contribute to the difference observed between the treatments.

The second possibility is that the induced resistance of the inoculated host plant holds throughout the experiment, selectively filtering the arriving pathogens strains. If strains that are faster to develop and produce more spores were more likely to establish on the plants with induced resistance than on the previously healthy plants, then we could expect to see the initially inoculated plants supporting more infection by the end of the experiment. Such a

scenario would resemble apparent competition where the hosts' response to one parasite strain affects not only the abundance but also the genetic composition of the co-infecting of the parasite community (cf. Bonsall and Hassell 1997). This interpretation is supported by the theoretical model by Gandon and Michalakis (2000) where quantitative resistance was found to select for higher virulence than qualitative resistance. In the future, genotyping and/or obtaining infection profiles through inoculations of strains from the previously healthy and inoculated plants could confirm whether more virulent strains selectively establish on the primed plants.

The third possible explanation for the observed field result is that strains of *Po. plantaginis* respond conditionally to multiple infection. A conditional response through facilitation could explain the observed result if the first pathogen strain facilitates the development of subsequent infections (Cui et al. 2005). Like many pathogenic fungi, *P. plantaginis* is considered heterothallic meaning that the production of sexual spores through recombination takes place as two different mating types fuse (Agrios 2005). Hence, multiple infection is the prerequisite of sexual reproduction for this species, and facilitation of subsequent infections could be favored if they are a compatible mating type. The mating types of the strains used in the laboratory experiment were not characterized but if they were incompatible, and in the field, compatible strains caused some of the co-infections, their development may have been enhanced through facilitation.

Further studies are needed to identify which of the proposed mechanisms is operating behind the observed results. However, despite these unanswered questions, it is possible to draw some fundamentally novel conclusions concerning multiple infection dynamics: Under a given set of conditions (measured here in the laboratory experiment), multiple infection may result in decreased success of subsequent infection, while at the other extreme, prior infection results in overall higher levels of infection (as measured in the field). Much more experimental work is needed to understand whether the outcome is mediated by abiotic conditions or through a response to repeated attack, but these results demonstrate how variable and context dependent the dynamics of multiple infection may be. This variability may generate divergent evolutionary selection on parasite life history traits across space and time.

These results also have important consequences for applications aiming to predict and prevent parasite attack. From an epidemiological perspective, these results underline the challenges of modeling infection dynamics when under some conditions we may find less infection then would be predicted by parameters describing parasite transmission and host availability, while in other situations, we may find higher levels of infection than we would expect based on these parameters alone. Predictions generated by an epidemiological model on the within-population spread of *Po. plantaginis* have demonstrated such discrepancy with respect to real data (Ovaskainen and Laine 2006), and in part, this may be the result of spatially variable dynamics of multiple infection.

Further applied implications of these results relate to what is known as priming, where host's defenses are induced by a biotic or abiotic elicitor with the aim of increasing resistance against further attack (Conrath et al. 2002). It is widely documented that pathogens can induce resistance in plants to subsequent infections (Walters and Daniell 2007). Biotrophic fungal pathogens such as rusts and powdery mildews cause some of the most devastating diseases of crops worldwide, and both elicit resistance to subsequent infection (Cho and Smedegaard-Petersen 1986; Murray and Walters 1992). Using pathogens in disease control in the field may often be too risky as a control strategy (Walters and Daniell 2007), but pathogen elicitors have proven most valuable in identifying the signaling cascades involved in induced resistance (Pieterse and Van Loon 2007). In a breakthrough discovery, external spraying of azelaic acid, a component of the plant's own induced immunity involved in priming defenses, was shown to induce resistance against pathogen *Pseudomonas syringae* (Jung et al. 2009). The advantages of this as a means of crop protection cannot be overstated – priming using plant-based products is environmentally safe, and priming triggers pathways controlled by multiple genes (Kazan and Schenk 2007) making it potentially a more durable strategy than resistance governed by single major R genes. In this study, the first inoculations provided partial protection against subsequent infection in the laboratory, but in the field, these 'primed' plants became more heavily infected by the end of the 4-week trial. These results demonstrate how scenarios measured under controlled conditions need careful testing under field conditions for sufficiently long periods of time to make sure that the treatment has the desired effect on host resistance.

Data Archiving Statement: Data for this paper are available at: http://www.earthcape.com/. Access can be requested by contacting the author at email: anna-liisa.laine@helsinki.fi.

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