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Mixed inoculation alters infection success of strains of the endophyte *Epichloë bromicola* on its grass host *Bromus erectus*

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Within-host competition in multiply infected hosts is considered an important component of host–parasite interactions, but experimental studies on the dynamics of multiple infections are still rare. We measured the infection frequencies of four strains of the fungal endophyte *Epichloë bromicola* on two genotypes of its host plant *Bromus erectus* after single- and double-strain inoculation. Double-strain inoculations resulted in fewer double, but more single, infections than expected on the basis of infection frequencies in single-strain inoculations. In most cases, only one of the two strains established an infection, and strains differed in their overall competitive ability. This pattern resembles the mutual exclusion scenarios in some theoretical models of parasite evolution. In addition, competitive ability varied with host genotype, which may represent a mechanism for the coexistence of strains in a population. Hence, considering the genetic variation in both host and parasite may be important for a better understanding of within-host dynamics and their role in epidemiology or (co)evolution.

Keywords: competitive exclusion; multiple infection; within-host competition

1. INTRODUCTION

Multiple infection—the presence of different parasite genotypes within the same host—may be an important determinant of host–parasite interactions (reviewed in Bull 1994; Levin & Bull 1994; Frank 1996; Read & Taylor 2000). Theory predicts that it influences within-host dynamics of infection (Hellriegel 1992; Levin & Bull 1994; Antia *et al.* 1996), transmission dynamics among hosts and parasite polymorphism at the population level (Levin & Pimentel 1981; Bonhoeffer & Nowak 1994; Nowak & May 1994), as well as evolutionary trajectories of parasite life history or virulence (Levin & Pimentel 1981; Bremermann & Pickering 1983; Frank 1992, 1994; Herre 1993; Bonhoeffer & Nowak 1994; Levin & Bull 1994; Nowak & May 1994; Van Baalen & Sabelis 1995; Gandon 1998; Gandon *et al.* 2001). A basic assumption in many mathematical models is that genetically unrelated parasite genotypes within the same host compete for limiting host resources, such as nutrients or space, or indirectly, via the host's immune system. Furthermore, because more competitive genotypes are assumed to replicate more rapidly within hosts or use up host resources more quickly, they will be more harmful to the host, generating a relationship between competitiveness and parasite virulence (Bremermann & Pickering 1983; Frank 1994; Levin & Bull 1994; Nowak & May 1994).

For mathematical convenience, models usually assume relatively simple, fixed patterns of within-host competition. In co-infection models, parasite genotypes coexist within the same host, but proliferate and transmit differentially according to their competitive ability (Bremermann & Pickering 1983; Frank 1992, 1994; Van Baalen & Sabelis 1995). In such models, a given parasite genotype always has the same (negative) effect on other strains (e.g. Frank 1994). Conversely, super-infection models assume rapid competitive exclusion, in which, upon encounter within the host, the less competitive parasite genotype is immediately eliminated and thus no longer transmitted to new hosts (Levin & Pimentel 1981; Nowak & May 1994; Gandon *et al.* 2001). Here, depending on whom they encounter, genotypes either win or lose in competition, so that they are ranked in a competitive hierarchy (Nowak & May 1994). The distinction between these two types of within-host competition is not trivial because they may lead to very different epidemiological and evolutionary dynamics, and produce different predictions for the mean virulence or the levels of polymorphism in the parasite (Van Baalen & Sabelis 1995; Mosquera & Adler 1998).

Experimental studies on within-host interactions are still rare (Read & Taylor 2000). Inoculation experiments demonstrated that within-host densities of parasite strains can be influenced by another co-infecting strain (see table 1 in Read & Taylor (2000)), suggesting direct or apparent competition between strains. One example exists of a slowly replicating parasite strain becoming outcompeted by faster growing ones over several generations of a serial passage experiment (Ni & Kemp 1992), but we know of no cases of immediate competitive exclusion, as envisaged by super-infection models. Nonetheless, experiments manipulating opportunities for multiple infection found evolutionary changes in the parasite-transmission route or virulence that can, at least partly, be explained by within-host competition (Bull *et al.* 1991; Ebert & Mangin 1997; Turner & Chao 1998).

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If empirical data on within-host competition are rare, even less is known about how competition depends on host genotype. Host variability may have a role in complex within-host interactions, because parasite genotypes often perform differently on different host genotypes (Thompson & Burdon 1992; Sorci *et al.* 1997; Ebert *et al.* 1998). Hence, parasite genotypes may be more competitive in some hosts, but less in others. To our knowledge, no studies have explicitly tested host-genotype effects on the outcome of within-host interactions. Furthermore, most work on multiple infection has focused on microparasites in animal hosts, and experimental studies on within-host interactions in plant host–parasite systems are quite rare (Van Alfen *et al.* 1975; Day 1980; Power 1996; Newton *et al.* 1997; Christensen *et al.* 2000).

We investigated the effects of host and parasite genotype on within-host competition in the fungal parasite *Epichloë bromicola* (Ascomycotina: Clavicipitaceae). This fungus grows endophytically within its host plant *Bromus erectus* (Poaceae). For sexual reproduction and horizontal transmission, fungal hyphae invade reproductive tillers and form fruiting structures (stromata) around developing inflorescences, thereby sterilizing the host (Kirby 1961; Schardl 1996; Saikkonen *et al.* 1998; Scott 2001). Sexual reproduction occurs on the stromata (mediated by insect vectors; Bultman *et al.* 1995). For transmission to new hosts, wind-dispersed fungal ascospores have to land on the inflorescence of a healthy plant and germinate on immature seeds (Chung & Schardl 1997), although stem or leaf tissues might provide an alternative route of entry (Brem & Leuchtmann 1999). Thus, multiple infection can occur if ascospores from different sources successfully infect the same plant. Endophytes typically grow in the nutrient-poor extracellular parts of their host, so that, in multiply infected hosts, fungal genotypes may compete over nutrients or space. Natural rates of multiple infection are unknown for this system, but reach up to 40% for *Epichloë sylvatica* on another grass species (Meijer & Leuchtmann 1999).

Previously, Wille *et al.* (1999) showed that double-strain inoculation can lead to mixed infection, with strains segregating into different tillers of the plant (see also Meijer & Leuchtmann 1999; Christensen *et al.* 2000). Here, we focus on within-host competition at the plant rather than tiller level. We compared infection success of four fungal strains on two host genotypes after single- and double-strain inoculation. This allowed us to test whether the probability of strains to infect a host plant was altered by the presence of another strain in the inoculum, and to what extent the outcome of interactions between strains were influenced by fungal or host genotype.

2. MATERIAL AND METHODS

(a) *Plant and fungal material*

Clonal replicates from two *B. erectus* Huds. genotypes, S1 and S9, were used in this experiment. Clones were derived from callus cultures, as described in Wille *et al.* (1999). The two seeds used to generate the callus cultures originated from two plants from a natural population near Nenzlingen, Switzerland, that differed in morphology, chitinase isoforms and random amplified polymorphic DNA (RAPD) banding profile (Wille 1999).

A single strain of *E. bromicola* Leuchtmann & Schardl was isolated from each of four plant individuals from natural populations in the Swiss Jura Mountains in 1994 (Groppe *et al.* 1995). Two of the four strains (A and N) originated from the population of Nenzlingen, the other two from the nearby populations of Movelier (M) and Vicques (V). The four strains represented different genotypes based on their RAPD–polymerase chain reaction (PCR) profiles (Groppe *et al.* 1995). Strains were grown on potato dextrose agar (PDA, Oxoid, Hampshire, UK, supplemented with the antibiotic tetracycline (50 mg l^{-1}) (Leuchtmann & Clay 1988)), and hyphal colonies were maintained by monthly transfer to fresh PDA medium.

(b) *Experimental set-up*

Plants of both genotypes (S1, S9) were inoculated either with single strains (single-strain inoculation treatment: A, N, M, V) or with their six pairwise combinations (double-strain inoculation treatment: A + N, A + M, A + V, M + N, M + V, N + V). We inoculated plantlets at the two- to three-leaf stage, as described in Leuchtmann & Clay (1988). For each strain, we prepared mycelial portions of *ca.* 0.5 mg. Using a fine syringe needle we inserted these mycelial portions into a stem slit near the meristem. For double-strain inoculations, two of these portions were mixed before insertion. Inoculations were carried out in six sessions during Autumn 1996. In each session, we inoculated plants of both genotypes with all the different single- and double-strain inocula. Some of the plants from single-strain inoculations had to be used for other experiments, and therefore sample sizes were smaller than in the double-strain treatment. Inoculated plants were incubated on sterile growth medium for two weeks and then planted in autoclaved sand (Wille *et al.* 1999). Seventy-four plants from single-strain (on average, 9.3 plants per strain and plant genotype; range: 6–13) and 173 plants from double-strain inoculation treatments (on average, 14.4 plants per strain combination and plant genotype; range: 10–20) survived until analysis. Tests for the presence or absence of fungal strains are described in detail in Wille *et al.* (1999). Briefly, plants were harvested six months after inoculation and DNA was extracted from the leaf blades. Successfully established strains were identified by diagnostic PCR using primers for size-variable microsatellite-containing loci of the fungus (for EMBL data access numbers see Wille *et al.* (1999)).

(c) *Data analysis*

First, we tested whether the proportion of infected plants differed among fungal strains or plant genotypes after single-strain inoculation. Second, to test whether strains interacted within hosts, we used infection frequencies after single-strain inoculation to calculate expected infection frequencies in the double-strain inoculation treatment. If strains do not interact, the expected frequency of double infections by a given fungal combination on a given plant genotype equals the product of the infection frequencies of each strain on this plant genotype after single-strain inoculation. Similarly, expected frequencies of single infections (e.g. after double inoculation with A + N) were calculated as the sum of two products: the probability of finding only A (infection frequency of A × (1 − infection frequency of N)) plus the probability of finding only N (infection frequency of N × (1 − infection frequency of A)).

Paired *t*-tests ($n = 6$ strain combinations × 2 plant genotypes = 12) were then used to compare expected and observed frequencies of (i) double infections; (ii) single infections; and (iii) double and single infections combined. We further tested for an overall correlation or difference between infection

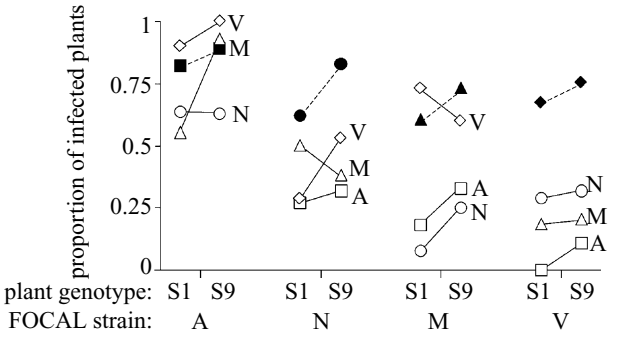

Figure 1. Infection frequencies of four *Epichloë bromicola* strains on *Bromus erectus* plants (genotypes S1 and S9) after single-strain inoculation (dashed lines, filled symbols), and after double-strain inoculation (solid lines, open symbols), together with each of three possible PARTNER strains (indicated by different symbols and letters next to symbols).

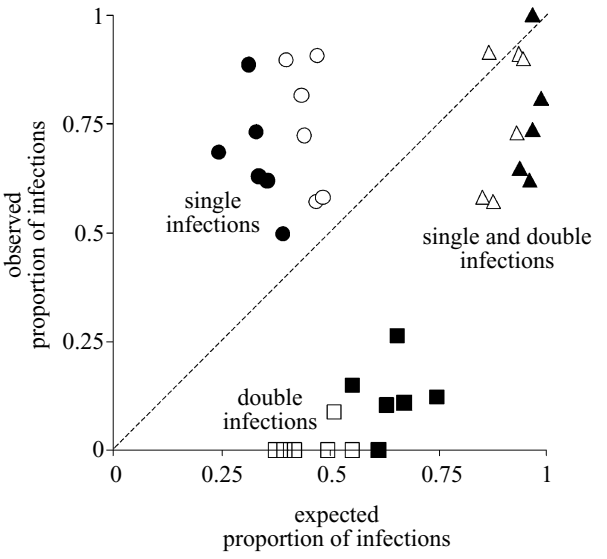

Figure 2. Relationships between observed infection frequencies after double-strain inoculation and expected proportions, calculated from infection frequencies of strains after single-strain inoculation. Observed versus expected values shown for double-strain infections, single-strain infections and single- plus double-strain infections combined, for each of the six pairwise strain combinations on the two plant genotypes. Dashed line, expected = observed. Open symbols, S1 plant genotype; filled symbols, S9 plant genotype.

frequencies of individual strains in single- versus double-strain inoculation treatments ($n = 4$ strains $\times$ 2 plant genotypes = 8). Third, to analyse variation in infection frequency after double-strain inoculations, we used the presence or absence of strains in the different pairwise fungal combinations as a response variable. A factorial model tested whether fungal strains differed in infection frequencies (= FOCAL strain effect), whether fungal strains differed in their effect on infection frequency of the other strain in the inoculum (= PARTNER strain effect), and whether the two plant genotypes differed in susceptibility. Inoculation date did not significantly affect infection probability (not shown) and was therefore removed from the initial fully factorial model.

Analyses of variation in infection frequency were carried out as logistic regressions (GLIM statistical package (Payne 1987), logit link function). Tests of Pearson correlation coefficients or *t*-tests were carried out with the JMP statistical package (SAS 1994) after arcsine transformation of infection frequencies.

3. RESULTS

(a) *Infection rates after single-strain inoculation*

Overall, 73% (54 out of 74) of the inoculated plants were found to be infected in the single-strain inoculation treatment. The frequencies of infection did not significantly differ among fungal strains ($\chi^2_3 = 2.38$, n.s.) or between plant genotypes ($\chi^2_3 = 1.45$, n.s.; strain $\times$ plant genotype interaction $\chi^2_3 = 0.22$, n.s.), although strain A tended to produce more infections than the three other strains, and plant genotype S9 was somewhat more susceptible to infection than genotype S1 (figure 1).

(b) *Expected versus observed infection frequencies in the double-strain inoculation treatment*

In the double-strain inoculation treatment, 135 out of the 173 plants (78%) became infected. Only 14 plants (8%) were doubly infected, far fewer than would have been expected (55%) from infection frequencies of the strains after single-strain inoculation (expected versus observed: $t_{11} = 13.1$, $p < 0.0001$; figure 2). Conversely, the proportion of plants carrying single infections (71%) was significantly higher than expected (38%; $t_{11} = -7.2$, $p < 0.0001$; figure 2). Overall, fungal strains produced significantly fewer single or double infections (79%) than expected (93%; $t_{11} = 2.72$, $p = 0.020$, figure 2). That is, more plants than expected remained healthy.

Infection frequencies of individual strains after double-strain inoculation were positively correlated with that after single-strain inoculation ($r = 0.72$, $p = 0.042$, $N = 8$). However, individual strains were considerably less successful in double- (42.6 ± 8.3 s.e.) than single-strain (73.7 ± 3.7 s.e.) inoculation treatments ($t_7 = -5.036$, $p = 0.002$). As shown in figure 1, infection success of strain A was only moderately reduced, or even increased, when inoculated together with strain V, whereas the three other strains experienced reductions in infection probability of up to 100% when inoculated with another strain (see also § 3c).

(c) *Variation in performance of individual strains in the double-strain inoculation treatment*

Differences among strains in infection frequencies were similar to those in single-strain inoculations, but considerably more pronounced (FOCAL strain effect: $\chi^2_3 = 49.1$, $p < 0.0001$): strain A was most successful, whereas the other strains produced intermediate (strains N and M) to low (strain V) infection frequencies (figures 1 and 3). These differences were mirrored in the effects of strains on the infection probability of the other strain in the inoculum (PARTNER strain effect: $\chi^2_3 = 19.1$, $p = 0.0003$). When inoculated together with strain A, strains were significantly less likely to establish an infection than when inoculated with strain V (figure 3). The relative impact of PARTNER strains on FOCAL strains varied across the different pairwise combinations (FOCAL $\times$ PARTNER strain interaction: $\chi^2_5 = 11.4$, $p = 0.0440$). For example, effects of the different PARTNER strains on

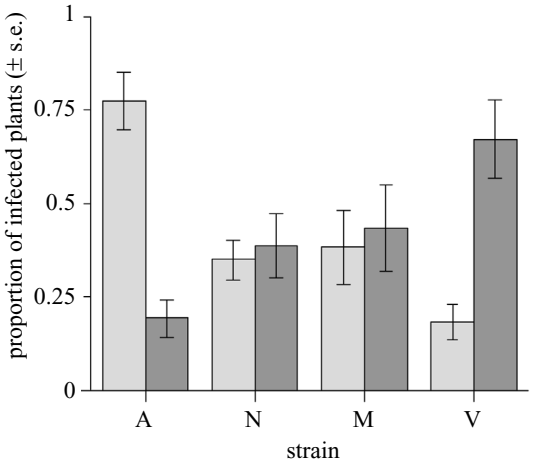

Figure 3. Infection frequencies of *Epichloë bromicola* strains after double-strain inoculation of *Bromus erectus* plants. Performance of each of four strains is shown as a FOCAL strain (mean infection frequency of this strain) or as a PARTNER strain (mean infection frequency of the other three strains when inoculated together with this strain). Light shading, FOCAL strain; dark shading, PARTNER strain.

infection success of FOCAL strain N were relatively similar, but infection success of FOCAL strains M or A varied considerably with the different PARTNER strains (figure 1). Moreover, interactions between strains from the same inoculum were also influenced by host-plant genotype (FOCAL × PARTNER strain × plant genotype interaction: $\chi^2_5 = 12.5$, $p = 0.0285$). These interactions are illustrated by non-parallel reaction norms (lines connecting performance on the two plant genotypes) of a given partner strain in combination with the different FOCAL strains (figure 1). For example, PARTNER strain M produced a reaction norm with positive slope together with FOCAL strain A, with negative slope together with strain N, and a flat reaction norm with strain V. As in the single-strain inoculations, the plant genotype S9 generally tended to be more susceptible than S1 ($\chi^2_5 = 3.2$, $p = 0.0736$).

4. DISCUSSION

This experiment indicates interactions between *E. bromicola* strains present on the same host plant. Individual strains were less likely to establish infections when inoculated together with another strain than when inoculated alone. Moreover, unlike in other experiments on within-host interactions (Read & Taylor 2000), usually only one strain established on a host plant, whereas the other one was lost (figure 2). This resembles the scenario of theoretical super-infection models, in which superior competitors eliminate inferior ones upon encounter within the host (Levin & Pimentel 1981; Bremermann & Pickering 1983; Nowak & May 1994).

Different mechanisms of competitive exclusion are possible. Infection by endophytes seems to occur only during early development of the plant (Latch & Christensen 1985). It may thus be crucial for the fungus to grow into not yet differentiated meristematic tissue to establish a systemic infection. At least *in vitro*, *E. bromicola* strains have been shown to differ in their hyphal growth rates (Wille 1999). Hence, faster growing strains may reach the meristem first and then monopolize space or nutrients. In plants, individual branches are often developmentally and physiologically independent units. Hence, such a 'first-come-first-served' mechanism would also explain exclusion at the tiller level in cases in which strains coexisted in the same plant (Wille *et al.* 1999; see also Christensen *et al.* 2000). Here, patterns of competitive exclusion at the whole-plant level seemed to be reflected at the tiller level, but the small number of doubly infected plants precluded conclusive statistical tests (not shown).

Fungal strains may also compete via the host's defence system. For example, faster growing strains may trigger host defences that suppress slower strains, similar to bacteriophages immunizing host cells against further infection (Model & Russell 1988). Furthermore, responses of host defence are often dose dependent (Ebert *et al.* 1998; Timms *et al.* 2001). Here, plants in the double-strain inoculation treatment received twice the total amount of fungal hyphae than in single-strain inoculations. Hence, threshold levels for activation of plant defence may have been reached that facilitated a specific response against individual strains, resulting in competitive exclusion. Higher fungal doses may also lead to elevated general defence levels. This could explain why plants remained uninfected more often than expected on the basis of single-strain inoculations (figure 2).

Alternatively, fungal strains may interact directly. Killer genotypes exist in other ascomycetes (Raju 1994), hypovirulence factors can spread horizontally among different genotypes of the pathogen *Endothia parasitica* (Taylor *et al.* 1998), and several endophyte species are known to produce toxic substances suppressing hyphal growth of other fungal pathogens (Siegel & Latch 1991; Stovall & Clay 1991). Whether this type of direct inhibition exists among *E. bromicola* genotypes remains to be tested. Clearly, to disentangle the mechanisms of competition, more information about infection dynamics is needed. This could be done, for example, by sequential inoculation (Christensen *et al.* 2000) to see if a head start in the infection process confers a competitive advantage.

Theory on the evolution of virulence often assumes that more competitive genotypes transmit more because of higher within-host growth. However, higher within-host growth also renders them more virulent, resulting in a trade-off between parasite transmission and survival of the infected host (Bull 1994; Frank 1996; Lipsitch & Moxon 1997). Obviously, the first part of these assumptions is met here: weak competitors will probably disappear after double-strain inoculation and thus will not transmit. Furthermore, there may be a direct positive relationship between within-host growth and transmission. Formation of the stromata necessary for production of transmission stages (ascospores) requires that fungal hyphae invade elongating reproductive tillers and take over the inflorescence before it becomes mature (Kirby 1961; Sun *et al.* 1990). Therefore, fast within-host growth may be a strongly selected trait. For example, in *Epichloë typhina*, stroma-forming strains grow faster than strains that remain asymptomatic (White *et al.* 1991).

However, the relationship between within-host growth and virulence is less obvious in our system. On the one hand, faster growing strains may be more harmful to their

host because they consume more resources or are more likely to sterilize host inflorescences. On the other hand, species of the genus *Epichloë* sometimes have positive effects on plant fitness, for example, by increasing drought resistance or competitive ability or by protecting the plant against herbivores (Clay *et al*. 1993; Schardl 1996; Saikkonen *et al*. 1998; Brem & Leuchtmann 2001). This may represent adaptations of a sterilizing, systemic parasite to ensure long-term transmission, but the plant may profit as well. If sterilization is incomplete, reduced host reproductive output may at least partly be compensated by a longer lifespan. Moreover, sterilized individuals of this clonal plant can still reproduce vegetatively, in particular in dense populations in which seed establishment is rare.

Our four fungal strains differed in their overall ability to out-compete their opponents and establish on a plant (figure 3). Clearly, if there is no price for being more competitive (e.g. through higher virulence), the most competitive genotype is expected to spread to fixation in a population. However, despite the overall differences, our results also indicate that within-host competition involved more complex interactions among different parasite and host genotypes. Fungal strains not only interacted differently with different partners, but also the outcome of these interactions varied with host genotype (figure 1). Hence, as long as less competitive strains are superior at least on some hosts, polymorphism may be maintained in the population (Regoes *et al*. 2000).

Whether this is a realistic scenario for our system depends on many factors, namely the frequency of parasite encounters on the same host, or the precise relationship between within-host competitive ability and transmission or virulence. Nonetheless, our data show that within-host competitive exclusion, a key assumption in certain theoretical models, may not be entirely unrealistic. Furthermore, traditional views of host–parasite coevolution are often based on fixed outcomes of interactions between single host and parasite genotypes. Our results suggest that these interactions can be modified if more than a single parasite genotype infects a host. This additional level of complexity may therefore be important for understanding the epidemiological and coevolutionary dynamics in host–parasite systems.

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