Pathogen Relatedness Affects the Prevalence of Within-Host Competition

B. Koskella,[1,*] T. Giraud,[2,†] and M. E. Hood[3,‡]

1. Department of Biology, Indiana University, Bloomington, Indiana 47405;
2. Ecologie, Systématique et Evolution, Université Paris-Sud, Orsay Cedex, France;
3. Department of Biology, University of Virginia, Charlottesville, Virginia 22903

Submitted January 23, 2006; Accepted May 9, 2006; Electronically published June 5, 2006

abstract: Although the evolutionary consequences of within-host competition among pathogens have been examined extensively, there exists a critical gap in our understanding of factors determining the prevalence of multiple infections. Here we examine the effects of relatedness among strains of the anther-smut pathogen *Microbotryum violaceum* on the probability of multiple infection in its host, *Silene latifolia*, after sequential inoculations. We found a significantly higher probability of multiple infection when interacting strains were more closely related, suggesting mechanisms of competitive exclusion that are conditional on genotypic characteristics of the strains involved. Pathogen relatedness therefore determines the prevalence of multiple infection in addition to its outcome, with important consequences for our understanding of virulence evolution and pathogen population structure and diversity.

Keywords: coinfection, virulence, kin selection, *Ustilago violacea*.

As disease spreads through a population, some individual hosts will be exposed to more than one pathogen strain. The resulting interaction between strains can be either multiple infection, where pathogen strains compete directly for resources within the host, or exclusion, where only a single pathogen strain persists. The ecological and evolutionary consequences of multiple infection have been examined extensively using theoretical models (van Baalen and Sabelis 1995; Mosquera and Adler 1998), but empirical evidence remains scant (Brown et al. 2002; Massey et al. 2004; Gower and Webster 2005). When multiple infection occurs, the performance of pathogens competing against one another is often not predicted by their performance as single infections (Nakamura et al. 1992; Weeds et al. 2000; Cushion et al. 2001; Thomas et al. 2003; Hodgson et al. 2004; de Roode et al. 2005). In particular, theoretical and experimental studies show that the relatedness among pathogen strains can greatly influence the outcome of within-host competition (Frank 1992; Chao et al. 2000; Puustinen et al. 2004; Jäger and Schjørring 2006). This is principally because high relatedness decreases the evolutionary conflict associated with sharing of host resources. In addition, relatedness may provide a metric on which the pathogen can base conditional responses that regulate interactions with competitors. For instance, if interference mechanisms are used to optimize inclusive fitness of the strains involved, a greater genetic distance between the strains could elicit a higher level of antagonistic response. Although examples of conditional interference abound in the ecological literature, it has only rarely been examined with regard to pathogen interactions, and even then, the studies only explored the relative performance of strains during multiple infection (Cushion et al. 2001; Massey et al. 2004). Thus, there is a critical lack of investigation concerning interference that may occur when a novel strain attempts colonization of a previously infected host, despite its importance to the prevalence of multiple infection and the resulting effect on disease severity and long-term dynamics (van Baalen and Sabelis 1995; Mosquera and Adler 1998; Brown et al. 2002; Massey et al. 2004).

Microbotryum violaceum, the fungal anther-smut pathogen of plants in the Caryophyllaceae, has proved to be a useful model for investigating competition among pathogen strains for host resources (Day 1980; Hood 2003; Van Putten et al. 2003). Previous work revealed that multiple infections can be a common phenomenon under field conditions, that there are significant effects on host survival, and that there exist mechanisms of competitive exclusion between different pathogen strains (Hood 2003). The goal of this study was to determine whether the pathogen's ability to exclude a subsequent infection is dependent on

* E-mail: bkoskell@indiana.edu.

† E-mail: tatiana.giraud@ese.u-psud.fr.

‡ Corresponding author. Address for correspondence: Department of Biology, Amherst College, Amherst, Massachusetts 01002; e-mail: mhood@amherst.edu.

Am. Nat. 2006. Vol. 168, pp. 121–126.

the relatedness between competing strains. Based on known mechanisms of conditional interference (Deleu et al. 1993; Glass and Kaneko 2003; Kauserud 2004), our results agree with the prediction that multiple infections would occur more often with interactions involving the most closely related strains.

Methods

Microbotryum violaceum is a sexually transmitted fungal pathogen of plants in the Caryophyllaceae and resides in meristematic tissues of its host, *Silene latifolia* (Audran and Batcho 1982). The pathogen produces spores (i.e., teliospores) within the host's anthers that are spread to healthy plants by insect pollinators. The pathogen on *S. latifolia* is native to Europe and was introduced to North America. Materials were obtained from the Mountain Lake metapopulation in Giles County, Virginia, which has been the subject of a long-term census (Antonovics 2004): the pathogen was sampled from populations 1, 6, and 7 (1.1F, 6.1H, 7.2D, respectively, in Oudemans et al. 1998), and the host was sampled as a mixture of seeds from several plants in the 6.1H population. These populations are separated by 10–15 km. Each pathogen population is polymorphic for mating-type bias, a heritable marker causing variation in the morphology of cultures and thus allowing for identification of multiple strains within a host, as shown previously (Hood 2003). One pathogen strain with mating-type bias ("biased") and one without ("nonbiased") were isolated from each population.

In order to assess the genetic distance between strains, microsatellite and amplified fragment length polymorphism (AFLP) analyses were used. *Microbotryum violaceum* DNA was extracted from spores of strains as previously described (Lopez-Villavicencio et al. 2005). Fungal strains were genotyped using all available microsatellite markers that could be amplified: GR2, GR3, GR4, GR11, GR15, GR18, GR19, GR22 (Giraud et al. 2002), GR26 (Lopez-Villavicencio et al. 2005), L6, L11, L14, L17, and L18 (Bucheli et al. 1998). However, no microsatellite variation was found. The AFLP protocol and data collection described in an article by Lopez-Villavicencio et al. (2005) were used with the following combinations of selective primers: P20/M12 (13 polymorphic bands), P12/M25 (19 polymorphic bands), P12/M12 (14 polymorphic bands), P20/M25 (four polymorphic bands), and P21/M13 (three polymorphic bands). Polymorphic AFLP fragments of strong intensity were scored as binary characters for each isolate. Genetic distances were calculated for AFLP data using the following methods: Euclidean, Jaccard (1908), Nei and Li (1979), and Sokal and Michener (1958). Mega (ver. 2.1) software was used for construction of neighbor-joining dendrograms and bootstrap replication.

We infected host plants, all from the same source populations, with a single resident strain of *M. violaceum* from either a biased or nonbiased strain from one of the three populations. Plants were germinated in petri dishes and then inoculated with a teliospore suspension at the single-meristem stage, according to Hood (2003). Plants were grown under greenhouse conditions until the phenotype of infecting strains could be confirmed. In order to determine strain morphology, we collected the first mature flower bud produced by each plant, and if it was diseased, the teliospores were plated on water agar dishes and examined using an inverted microscope with × 100 magnification after an incubation period of 7–10 days at 15°C. Samples were collected before the flowers opened in order to minimize cross-contamination of teliospores. Plants that either did not reach maturity or did not become diseased from this initial inoculation were removed from the experiment. Diseased plants were then challenged with a second dose of inoculum that was given as a teliospore suspension poured over the plants on each of three consecutive days. These challenge inoculations consisted of pathogen strains that contrasted with the resident strain (biased vs. nonbiased; Hood 2003) and were from the same or different populations, thus being, respectively, more or less genetically related, according to analysis of AFLP variation (fig. 1). Plants were then cut back to allow for new flowering stems. After the expected latent period of 4 weeks, the first mature flower bud from each stem produced over the following 2 months was collected, and the fungal spores were assayed for mating-type bias phenotype.

Two runs of this experiment were conducted sequentially, using the same pathogen strains and collection of plant seeds. Due to space constraints, run 1 included only half of the cross-inoculation matrix of resident strains ver-

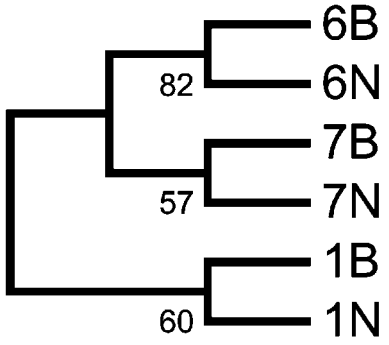

Figure 1: Genetic distance of *Microbotryum violaceum* strains used as inoculum. Neighbor-joining dendrogram is based on analysis of 53 polymorphic AFLP bands. Pathogen strains are identified by their population of origin (1, 6, or 7) and phenotype as biased (B) or nonbiased (N). Bootstrap values shown = 100 replications based on Euclidian distances.

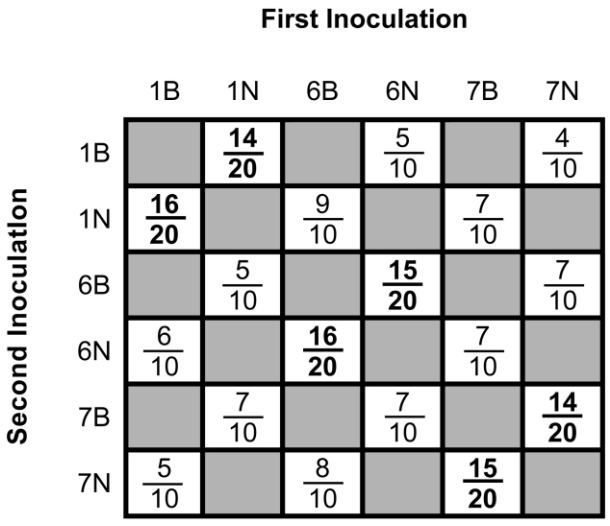

Figure 2: Experimental design of sequential inoculation by *Microbotryum violaceum*. Sequential inoculation design for run 2 of the experiment is shown by pathogen strains used at the first or second inoculation. Pathogen strains are identified by their population of origin (1, 6, or 7) and phenotype as biased (B) or nonbiased (N). Within-population challenges are in bold. Numbers within boxes indicate how many plants were given the initial inoculation (denominator) and how many were successfully diseased by the initial pathogen strain and were thus given a second challenge inoculation (numerator).

sus challenge strains, and for each population, 50 plants were inoculated initially, with 10 receiving within-population challenges and 40 receiving between-population challenges. In order to confirm the results of run 1 and have equal numbers of within- and between-population challenges, a second run was conducted using the complete crossing matrix. In run 2, for each population, 40 plants received within-population challenges, and 40 plants received between-population challenges (fig. 2). A multiple infection was considered successful when the challenging strain was recovered from one or more flowers produced by the already infected plant over the 2-month collection period, as determined by the mating-type bias phenotypes observed when culturing the strains. Statistical differences among the number of successful coinfections across all within- and between-population treatments were calculated using Fisher's exact test. In addition, overall infection success of biased versus nonbiased strains was compared using χ^2 tests of independence after initial inoculation and challenge inoculation.

Results

All plants that became diseased after the initial inoculation were confirmed to have the correct strain phenotype according to the treatment they received. In both runs of the experiment, the number of successful multiple infections was significantly greater for within-population challenges than for between-population challenges after sequential inoculations. Because there was no difference between biased and nonbiased strains with regard to initial infection frequency ($\chi^2[1, N = 240] = 2.382$, $P = .12$) or coinfection success ($\chi^2[1, N = 164] = 2.906$, $P = .09$), results were combined according to whether challenging strains were within or between populations. Among plants that became diseased in run 1, only four of 59 (6.8%) between-population challenges were successful at invasion, while five of 18 (27.8%) within-population challenges were successful ($P = .028$ for success of combined within- vs. combined between-population challenges using a one-tailed Fisher's exact test). In run 2, only four of 77 (5.2%) between-population challenges were successful, while 16 of 87 (18.4%) within-population challenges were successful ($P = .008$; fig. 3).

Multiple infection occurred across resident strains from all populations as well as across resident strains of both biased and nonbiased phenotypes. The intensity of sampling did not differ between the treatments, with one flower being sampled from an average of five separate stems produced per plant in both the within- and between-population treatments. The sampling method used did not include analysis of all flowers that could be produced by

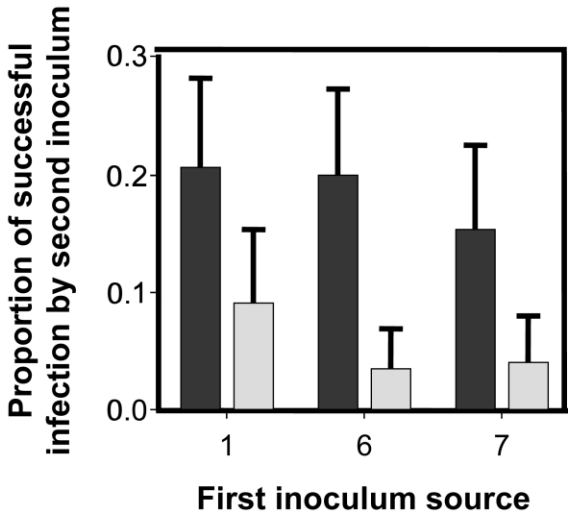

Figure 3: Proportion of successful multiple infection for within- and between-population challenges. Shown are the proportions of successful multiple infections across different inoculum source populations (1, 6, or 7) for run 2 of the experiment. Black bars represent within-population challenges, and gray bars represent between-population challenges. Results are means for both biased and nonbiased samples combined from each population ± SEM.

infected plants, thus allowing for the possibility that a subset of multiple infections remained undetected. However, both the initial infection rate and rate of coinfection were similar to those in a prior study (Hood 2003) in which sampling was more thorough. Also, there was no evidence that strains of *M. violaceum* from the same population as the host seeds were more likely to cause disease overall than strains of the fungus from other populations ($P = .429$, Fisher's exact test). From the first inoculation onto the bulk seeds from host population 6.1H, the majority of plants became diseased with each of the different sources of inoculum (62.5%–82.5%; fig. 2).

Discussion

This study demonstrates the importance of pathogen relatedness in predicting competition for host colonization and thus the prevalence of multiple infection. The results suggest exclusionary mechanisms occur between more distantly related strains and the absence of such mechanisms allowing more closely related strains to both colonize the host. Although all strains were genetically distinct, the lack of variation at microsatellite loci suggests low neutral genetic diversity. Even the level of polymorphism observed with AFLP data appears low compared with similar studies conducted in the pathogen's native range (Lopez-Villavicencio et al. 2005). The apparent lack of genetic diversity in our study may be due to a bottleneck event during introduction of the pathogen to North America. Despite the low neutral diversity, however, important differences regarding the outcome of infection were found that significantly affect the competitive interactions among pathogen strains. This discrepancy between neutral variation and adaptive variation has been seen in animal systems as well (Edmands and Harrison 2003) and may prove to be a widespread phenomenon.

The results show that the ability of *Microbotryum violaceum* to colonize its hosts is dependent on the relatedness of competing strains. One potential mechanism of resident exclusion is a vegetative incompatibility system for nonself recognition, as occurs in a wide range of fungi (Glass and Kaneko 2003). In fungi where vegetative incompatibility is well established, it is manifested through direct contacts between fungal hyphae (for a review, see Glass et al. 2000), which may occur during colonization of plant meristems by *M. violaceum*. The genetics of vegetative incompatibility often involve multiple loci with many alleles, and the number of differences between strains determines the strength of their antagonism. Because vegetative incompatibility systems are hypervariable and subject to balancing selection, differences at such loci may persist even as variation in other molecular markers is purged (Kauserud 2004). Moreover, such incompatibilities may arise by very simple, even single amino-acid changes in the gene products (Deleu et al. 1993). Therefore, this proposed incompatibility mechanism is consistent with the observed disconnect between the levels of neutral and adaptive variation in our study. However, alternative interference mechanisms must also be considered (Cushion et al. 2001; Wille et al. 2002). For example, various organisms, including fungi (Stovall and Clay 1991; Poulsen and Boomsma 2005), parasitic plants (Puustinen et al. 2004), and bacteria (Massey et al. 2004), may secure nutrient resources by using toxins or incompatibility compounds to which the producing strain is insensitive. In some systems, disease resistance mechanisms or pathogen-specific immunity may play an important role in the ability of resident infection to exclude challenging strains (de Roode et al. 2005) and has recently been implicated in the dynamics of multiple infection among closely related pathogen genotypes (Jäger and Schjørring 2006). Future studies establishing the mechanisms of interference by *M. violaceum* will be important for predicting the associated costs of competition and the effects on disease dynamics that are predicted by multiple infection models (Chao et al. 2000).

This study has major implications for two main areas of theoretical work where within-host competition is considered important. First is the idea that multiple infections may affect a pathogen's diversity and population structure (Mosquera and Adler 1998). When, as these results indicate, competitive exclusion occurs between less related strains, frequent migration should result in a higher probability of challenging strains being incompatible with previously established infections. This dynamic would lead to a negative relationship between migration frequency and the proportion of genetic variance that is found at the within-host level. The invasion success of a migrant pathogen strain may also be negatively affected by conditional interference since the migrant is restricted to colonizing only healthy hosts. This limitation on gene flow may be quite severe when disease frequencies within populations reach high levels, as in the Mountain Lake metapopulation of *M. violaceum* and *Silene latifolia* (Antonovics 2004). Once established within a host, however, the migrant could better secure the resource by excluding the majority of challenging infections. With regard to metapopulation dynamics, such complex interactions of pathogen relatedness and exclusionary mechanisms remain to be explored from both theoretical and empirical perspectives.

The second major implication of this study regards the long-term coevolutionary dynamics of the disease system, which are also thought to be strongly influenced by the prevalence of multiple infection. Theoretical studies suggest that the type of within-host competition can drastically affect pathogen life-history evolution (Brown et al. 2002). Specifically, theory predicts that the direct com-

petition for host resources, a consequence of multiple infection, leads to selection for increased rates of exploitation and thus greater virulence (van Baalen and Sabelis 1995; Mosquera and Adler 1998; but see Chao et al. 2000; Brown et al. 2002; Massey et al. 2004). The demonstration that strain relatedness regulates the prevalence of multiple infection supports the idea that conditional interference between strains may act as a key factor in limiting virulence evolution and disease dynamics of natural populations, in accordance with kin selection theory (Frank 1996; Chao et al. 2000). If multiple infections are common only among closely related strains, then naturally occurring infections would actually generate less of the evolutionary conflict that is expected to select for raised virulence. It is clear from our results that in order for researchers to more accurately address the large body of theoretical work on this subject, a better understanding is needed of both the frequency and the genetic heterogeneity of naturally occurring multiple infections.

Acknowledgments

We would like to thank J. Antonovics, F. Bashey-Visser, J. Cianciolo, C. Lively, J. Partain, A. Pedersen, F. Ransler, D. Repasky, and J. A. Shykoff for helpful comments on earlier drafts and inspiring conversation. We especially thank J. Enjalbert and O. Jonot for help with molecular markers. This work was supported by National Science Foundation grant DEB-0346832.

Literature Cited

Antonovics, J. 2004. Long-term study of a plant-pathogen metapopulation. Pages 471–488 *in* I. Hanski and O. Gaggiotti, eds. Ecology, genetics, and evolution of metapopulations. Elsevier Academic, Burlington, MA.

Audran, J. C., and M. Batcho. 1982. Comportement d'*Ustilago violacea* (Pers) Rouss au sein des tissus vegetatifs et reproducteurs du *Silene dioica* (L). Revue de Cytologie et Biologie Végétale–Le Botaniste 5:59–63.

Brown, S. P., M. E. Hochberg, and B. T. Grenfell. 2002. Does multiple infection select for raised virulence? Trends in Microbiology 10: 401–405.

Bucheli, E., B. Gautschi, and J. A. Shykoff. 1998. Isolation and characterization of microsatellite loci in the anther-smut fungus *Microbotryum violaceum*. Molecular Ecology 7:665–666.

Chao, L., K. A. Hanley, C. L. Burch, C. Dahlberg, and P. E. Turner. 2000. Kin selection and parasite evolution: higher and lower virulence with hard and soft selection. Quarterly Review of Biology 75:261–275.

Cushion, M. T., S. Orr, and S. P. Keely. 2001. Time between inoculations and karyotype forms of *Pneumocystis carinii* f. sp. *carinii* influence outcome of experimental coinfections in rats. Infection and Immunity 69:97–107.

Day, A. W. 1980. Competition and distribution studies of genetically marked strains of *Ustilago violacea* in the same host plant. Botanical Gazette 141:313–320.

Deleu, C., C. Clave, and J. Begueret. 1993. A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserine*. Genetics 135:45–52.

de Roode, J. C., M. E. H. Helinski, M. A. Anway, and A. Read. 2005. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. American Naturalist 166: 531–542.

Edmands, S., and J. S. Harrison. 2003. Molecular and quantitative trait variation within and among populations of the intertidal copepod *Tigriopus californicus*. Evolution 57:2277–2285.

Frank, S. A. 1992. A kin selection model for the evolution of virulence. Proceedings of the Royal Society of London B 250:195–197.

———. 1996. Models of parasite virulence. Quarterly Review of Biology 71:37–78.

Giraud, T., E. Fournier, D. Vautrin, M. Solignac, and J. A. Shykoff. 2002. Isolation of 44 polymorphic microsatellite loci in three host races of the phytopathogenic fungus *Microbotryum violaceum*. Molecular Ecology Notes 2:142–146.

Glass, N. L., and I. Kaneko. 2003. Fatal attraction: nonself recognition and heterokaryon incompatibility in filamentous fungi. Eukaryotic Cell 2:1–8.

Glass, N. L., D. J. Jacobson, and P. T. K. Shiu. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. Annual Review of Genetics 34:165–186

Gower, C. M., and J. P. Webster. 2005. Intraspecific competition and the evolution of virulence in a parasitic trematode. Evolution 59: 544–553.

Hodgson, D. J., R. B. Hitchman, A. J. Vanbergen, R. S. Hails, R. D. Possee, and J. S. Cory. 2004. Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. Journal of Evolutionary Biology 17:1018–1025.

Hood, M. E. 2003. Dynamics of multiple infection and within-host competition by the anther-smut pathogen. American Naturalist 162:122–133.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Société Vaudoise des Sciences Naturelles Bulletin 44:223–270.

Jäger, I., and S. Schjørring. 2006. Multiple infections: relatedness and time between infections affect the establishment and growth of the cestode *Schistocephalus solidus* in its stickleback host. Evolution 60:616–622.

Kauserud, H. 2004. Widespread vegetative compatibility groups in the dry-rot fungus *Serpula lacrymans*. Mycologia 96:232–239.

Lopez-Villavicencio, M., E. Enjalbert, M. E. Hood, J. A. Shykoff, C. Raquin, and T. Giraud. 2005. The anther smut disease on *Gypsophila repens*: a case of parasite sub-optimal performance following a recent host shift? Journal of Evolutionary Biology 18:1293–1303.

Massey, R. C., A. Buckling, and R. ffrench-Constant. 2004. Interference competition and parasite virulence. Proceedings of the Royal Society of London B 271:785–788.

Mosquera, J., and F. R. Adler. 1998. Evolution of virulence: a unified framework for co-infection and superinfection. Journal of Theoretical Biology 195:293–313.

Nakamura, T., T. Konishi, H. Kawaguchi, and J. Imose. 1992. Estimation of relative fecundity in *Eimeria tenella* strains by a mixed infection method. Parasitology 104:11–17.

Nei, M., and W. H. Li. 1979. Mathematical modelling for studying genetic variation in terms of restriction endonuclease. Proceedings of the National Academy of Sciences of the USA 74:5269–5273.

Oudemans, P. V., J. Antonovics, S. M. Altizer, P. H. Thrall, L. Rose,

and H. M. Alexander. 1998. The distribution of mating-type bias in natural populations of the anther-smut *Ustilago violacea* on *Silene alba* in Virginia. Mycologia 90:372–381.

Poulsen, M., and J. J. Boomsma. 2005. Mutualistic fungi control crop diversity in fungus-growing ants. Science 307:741–744.

Puustinen, S., T. Koskela, and P. Mutikainen. 2004. Relatedness affects competitive performance of a parasitic plant (*Cuscuta europaea*) in multiple infections. Journal of Evolutionary Biology 17:897–903.

Sokal, R. R., and C. D. Michener. 1958. A statistical method for evaluating systematic relationships. University of Kansas Science Bulletin 38:1409–1438.

Stovall, M. E., and K. Clay. 1991. Fungitoxic effects of *Balansia cyperi*. Mycologia 83:228–295.

Thomas, M. B., E. L. Watson, and P. Valverde-Garcia. 2003. Mixed infections and insect-pathogen interactions. Ecology Letters 6:183–188.

van Baalen, M., and M. W. Sabelis. 1995. The dynamics of multiple infection and the evolution of virulence. American Naturalist 146: 881–910.

Van Putten, W. F., A. Biere, and J. M. M. Van Damme. 2003. Intraspecific competition and mating between fungal strains of the anther smut *Microbotryum violaceum* from the host plants *Silene latifolia* and *S. dioica*. Evolution 57:766–776.

Weeds, P. L., R. E. Beever, and P. G. Long. 2000. Competition between aggressive and non-aggressive strains of *Botrytis cinerea* (*Botryotinia fuckeliana*) on French bean leaves. Australasian Plant Pathology 29:200–204.

Wille, P., T. Boller, and O. Kaltz. 2002. Mixed inoculation alters infection success of strains of the endophyte *Epichloe bromicola* on its grass host *Bromus erectus*. Proceedings of the Royal Society of London B 269:397–402.

Associate Editor: Judith L. Bronstein
Editor: Michael C. Whitlock