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Dynamics of Multiple Infection and Within-Host Competition by the Anther-Smut Pathogen

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ABSTRACT: Infection of one host by multiple pathogen genotypes represents an important area of pathogen ecology and evolution that lacks a broad empirical foundation. Multiple infection of *Silene latifolia* by *Microbotryum violaceum* was studied under field and greenhouse conditions using the natural polymorphism for mating-type bias as a marker. Field transmission resulted in frequent multiple infection, and each stem of the host was infected independently. Within-host diversity of infections equaled that of nearby inoculum sources by the end of the growing season. The number of diseased stems per plant was positively correlated with multiple infection and with overwintering mortality. As a result, multiply infected plants were largely purged from the population, and there was lower within-host pathogen diversity in the second season. However, among plants with a given number of diseased stems, multiply infected plants had a lower risk of overwintering mortality. Following simultaneous and sequential inoculation, strong competitive exclusion was demonstrated, and the first infection had a significant advantage. Dynamics of multiple infection initially included components of coinfection models for virulence evolution and then components of superinfection models after systemic colonization. Furthermore, there was evidence for an advantage of genotypes with mating-type bias, which may contribute to maintenance of this polymorphism in natural populations.

Keywords: coinfection, superinfection, evolution of virulence, pathogen variation, *Microbotryum violaceum*, *Ustilago violacea*.

Infection of one host by multiple pathogen genotypes is an important force in disease ecology and evolution. Effects have been suggested for such broad-ranging traits as transmission dynamics, symptom expression, and selection on pathogen virulence and drug resistance (May and Nowak 1995; Ebert 1998; Hastings and Mackinnon 1998; Mosquera and Adler 1998; Read and Taylor 2001). However, empirical studies of multiple infection are difficult because the interactions cannot be observed directly and molecular or physiological markers are needed to distinguish pathogen genotypes. As a result, theoretical advances on the subject have greatly outpaced our knowledge of natural systems.

This is best represented by the large number of models for virulence evolution under multiple infection (i.e., May and Nowak 1994; van Baalen and Sabelis 1995; Frank 1996). In these models, pathogen genotypes compete on the basis of their rates of exploitation, which correlate positively with pathogen transmission but negatively with host survival. When a pathogen genotype infects the host alone, intermediate virulence can evolve (i.e., a moderate rate of exploitation) because it maximizes the pathogen's reproductive value by prolonging the host's lifespan. Under conditions of multiple infection, the most rapid exploitation rate is favored because death of the host stops reproduction of all resident genotypes and negates the advantages of lower virulence. A corresponding argument has been put forward for the evolution of prey exploitation strategies in metapopulations of predators (Pels et al. 2002). In fact, within-host dynamics have been viewed in much the same way as within-patch dynamics of a metapopulation, and mathematical models are frequently borrowed between these disciplines (May and Nowak 1995; van Baalen and Sabelis 1995; Mosquera and Adler 1998; Pels et al. 2002).

Emphasis has also been placed on the consequences of multiple infection for the maintenance of pathogen variation, which conceptually parallels the coexistence of competing species or genotypes in a patchy environment (Taylor 1990; Tilman 1994). Greater dispersal may allow genotypes to persist when they are inferior within-host competitors if there is a trade-off between dispersal and within-host competitiveness (Amarasekare and Nisbet 2001). This is possible because good dispersers more frequently colonize healthy hosts and initially proliferate in the absence of competition.

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Most models of multiple infection fall into two classes. Some allow coexistence within the host under exploitative competition (coinfection models; May and Nowak 1995). Alternatively, in a hierarchy of interference competition, the resident may be rapidly replaced by a superior genotype or defend against an inferior genotype (superinfection models; May and Nowak 1994). These particular types of interactions change the predicted population dynamics and the evolution of virulence (van Baalen and Sabelis 1995; Mosquera and Adler 1998; Chao et al. 2000), and this argues strongly for exploration of other reasonable forms of within-host competition. However, the extremely small number of well-understood empirical systems makes it difficult to judge which assumptions are most broadly applicable.

The details of multiple infection are best understood for only a few human, veterinary, and in vitro disease models (Ebert 1998; reviewed by Read and Taylor 2001). Reports of multiple infections in plants are even more limited (Van Alfen et al. 1975; Adachi and Tsuge 1994; Newton et al. 1997; Harrison and Robinson 1999; Meijer and Leuchtmann 1999; Christensen et al. 2000; Sanz et al. 2000). However, recent work by Wille et al. (2002) demonstrates the tractability of using natural genetic variation in plant-pathogen systems for multiple infection experiments. Some studies support within-host competition as an essential component to the evolution of increased pathogen virulence (Ebert and Mangin 1997; Ebert 1998; Turner and Chao 1998), but there is a lack of empirical evidence for multiple infections to facilitate the coexistence of competing pathogen genotypes at the population level.

In this study, I address multiple infection by *Microbotryum violaceum*, the systemic fungal pathogen that causes anther-smut disease of *Silene latifolia*. Even though this system is studied intensively and populations are known to be polymorphic for various markers (Kaltz and Shykoff 1997; Oudemans et al. 1998; Bucheli et al. 2000, 2001; Hood and Antonovics 2000; Hood et al. 2002), it remains an open question as to whether multiple infections occur and, if so, how frequently. Using artificial inoculation with a mixture of laboratory mutants, Day (1980) concluded that multiple genotypes of *Microbotryum* could simultaneously infect *S. latifolia*. However, Baird and Garber (1979) used some of the same mutants and similar methods but did not find evidence of multiple infection.

Two approaches were taken to understand the dynamics of multiple infections by *Microbotryum*. First, natural disease transmission was studied in experimental field populations. Pathogen genotypes were distinguished by whether they exhibited mating-type bias, a common polymorphism in natural populations that can be easily identified by its effects on colony morphology, as described in "Characterization of Fungal Samples." The occurrence of multiple infection was investigated by repeatedly sampling newly infected plants throughout a growing season and following the overwintering period. Second, multiple infection was studied under greenhouse conditions by direct inoculation onto plants. Plants were challenged with pathogen genotypes either simultaneously or sequentially with a brief time delay. The goals were to determine whether multiple infections are frequent under field conditions and whether multiple pathogen genotypes were present in the host simultaneously (i.e., as coinfections), to determine the distribution of the coinfecting genotypes within the host, and to determine whether the timing of infection influences the outcome of within-host interactions.

Material and Methods

Study System

Microbotryum violaceum (formerly *Ustilago violacea*) is a basidiomycete fungus that causes anther-smut disease of many perennials in the Caryophyllaceae (Fischer and Holton 1957). Nearly all infected plants that survive from year to year are systemically infected and express the disease in all flowering stems. When infected plants flower, *Microbotryum* forms diploid teliospores in the anthers, and insect pollinators then vector the spores to nearby plants (Jennersten 1983; Alexander and Maltby 1990). On germination, the fungus undergoes meiosis to produce haploid gametes (sporidia) that mate and produce infectious hyphae. Mating compatibility is determined by a single mating-type locus with two haploid self-incompatible alleles called *a*1 and *a*2.

The host, *Silene latifolia* (= *Silene alba*), is native to Europe and is naturalized in eastern North America. It produces branching erect stems from perennial rosettes, and it flowers during the entire growing season. The flowers are produced in dichasia, which are dichotomously branching inflorescences that contain a flower at the axis of each branch. *Silene latifolia* is dioecious, but the fungus sporulates in female hosts by inducing a malelike morphology with spore-bearing anthers. The sex of diseased plants can still be established by the presence of a rudimentary ovary in females.

Several studies have characterized variation within populations of *Microbotryum* (Kaltz and Shykoff 1997; Oudemans et al. 1998; Delmotte et al. 1999; Hood and Antonovics 2000). Particular attention has been given to the puzzle of mating-type bias, which is found at high frequencies in many populations even though biased genotypes carry alleles that are highly deleterious in the haploid stage (Hood and Antonovics 2000). It has been hypothesized that these biased genotypes may have a compensatory advantage during parasitism and that this may favor

their persistence at high frequencies (Antonovics et al. 1998).

Characterization of Fungal Samples

Mating-type bias in *Microbotryum* is caused by deleterious recessive elements linked completely to one mating type (Kaltz and Shykoff 1997; Oudemans et al. 1998; Hood and Antonovics 2000). Such biased genotypes produce a distinct colony morphology in vitro, which serves as a convenient, inexpensive, and rapid marker (fig. 1). When teliospores are incubated for 7–10 d on water agar at 15°C, biased genotypes produce large and small colonies in a 1 : 1 ratio, whereas nonbiased genotypes produce only large colonies (Hood and Antonovics 2000). The colony characteristics were determined using an inverted microscope with × 100 magnification. Samples were collected as single unopened flower buds (to avoid cross-contamination) and stored under desiccation at room temperature until being assayed. In nearly all cases, each flower bud contained a single genotype and could be characterized unambiguously as either biased or nonbiased. All sample assays were blind to the experimental treatments.

Experimental Populations

Seeds of *S. latifolia* were collected in bulk from a primary study population in Clover Hollow, Giles County, Virginia (location 6.1H in Oudemans et al. 1998). To establish the experimental populations, seeds were used to generate healthy target plants and to generate diseased source plants by inoculation. Source plants were prepared by germinating the seeds in petri dishes and then placing a drop of teliospore suspension on the shoot apex after expansion of the cotyledons (1,000 spores in 2 μL of water plus a surfactant; as described in Hood and Antonovics 2000). After 3 d in a growth chamber at 15°C, inoculated seedlings were planted into potting mix. Target plants were sown directly into potting mix. All plants were grown under greenhouse conditions for 2 mo before being transplanted into 20-cm pots at Mountain Lake Biological Station (University of Virginia, Giles County).

Teliospores for the initial inoculum were collected from naturally occurring *Microbotryum* in Clover Hollow and Goldbond, Giles County, Virginia (location 1.1F in Oudemans et al. 1998), which are separated by more than 15 km. These localities for initial inoculum were the basis of two replicate experimental populations that had source plants inoculated with fungal genotypes from either Clover Hollow or Goldbond. The Clover Hollow experimental population had an equal number of source plants inoculated with each of two biased and two nonbiased genotypes from that locality. Similarly, the Goldbond experimental population used two biased and two nonbiased genotypes from that locality. The experimental arrays each consisted of 64 source plants and 192 target plants arranged in 16 × 16 grids with 75-cm spacing between plants. The source plants were located centrally within the grids in an 8 × 8 square (fig. 2). Plants inoculated with biased or nonbiased genotypes were represented in equal numbers and arranged symmetrically with regard to distance from the target plants. An empty row in the horizontal and vertical centers of the arrays facilitated plant maintenance and plant disease sampling.

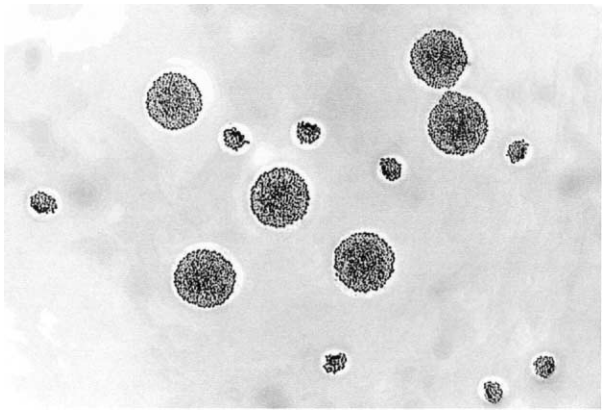

Figure 1: Cultural morphology for single-teliospore colonies of *Microbotryum* with mating-type bias. When grown under low nutrients and cool temperatures, large and small colonies are present in a 1 : 1 ratio. All of the colonies are of the large size for nonbiased genotypes under these growth conditions.

The experimental populations were established in mid-May 2000 and monitored for disease transmission every 3 d through August and then at least every other week until the first hard frost in October, which caused the stems to die back. Infected flowers buds were sampled from target plants as they became diseased. Each diseased stem was marked and sampled once on the first day if it was found to be diseased. Sampling of all source plants confirmed that they contained genotypes of *Microbotryum* with the correct biased or nonbiased colony morphology.

The experimental populations were left in the field to overwinter so that the persistence of coinfections could be studied. The following spring, diseased stems were sampled by the same method. To avoid effects of transmission during this second year, sampling was stopped 5 wk after flowering began (the minimum expected latent period based on transmission to healthy target plants in the previous season).

Inoculation Experiments

The same bulked collection of seeds from Clover Hollow and the same field-collected fungal genotypes from Clover

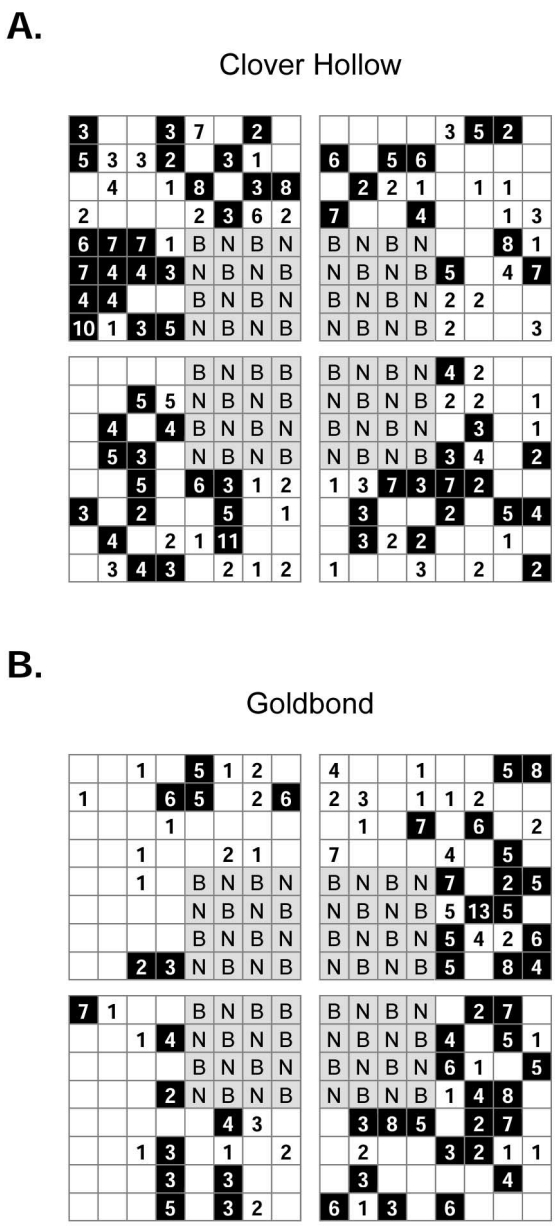

Figure 2: Two experimental populations of *Silene latifolia* identified by the natural populations (Clover Hollow and Goldbond) from which the original inoculum was obtained. The center of each array contains "source" plants infected with biased (*B*) or nonbiased (*N*) genotypes of *Microbotryum*, surrounded by healthy "target" plants. Data indicate the number of diseased stems sampled during the first season of growth. Black background indicates plants in which multiple infection was detected.

Hollow and Goldbond were used as in the field experiments. Genotypes from within a location were paired as biased with nonbiased at random and without replacement. Two pairs from Clover Hollow (pairs A and B) and a third pair from Goldbond (pair C) were used. Seedlings were inoculated as described in "Experimental Populations," and each plant had only a single shoot meristem at the time of inoculation. Within each experiment, the positions of plants were completely randomized.

In the simultaneous inoculation experiment, plants were either inoculated with a mixed pair of biased and nonbiased genotypes or were inoculated singly with only the respective biased or nonbiased genotype. When the inoculum was a mixed pair, the genotypes were equally represented in the same total inoculum concentration of 1,000 viable spores in 2 μL of water plus a surfactant. This avoided potential problems of dose effects on infection probabilities and allowed comparison of single and mixed inoculum treatments (Wille et al. 2002). Teliospore samples were assayed for viability the day before inoculation by plating a subset of spores on potato-dextrose agar and calculating germination frequency after 24 h. Each of the three treatments (two pure and one mixture) for each of the three biased + nonbiased pairs was replicated on 16 plants for a total of 144 plants. The 16 replicates were blocked over two starting dates that were 7 d apart because of the time involved in seedling and inoculum preparation.

In the sequential inoculation experiment, the same pairs of genotypes were used, and the interval between inoculations was 7 d. Plants received one of seven treatments: biased then nonbiased, nonbiased then biased, water then biased, water then nonbiased, biased then water, nonbiased then water, and water then water. Each of the treatments was replicated on 12 plants for a total of 252 plants.

Mature buds were sampled the day before they would have opened to avoid accidental disease spread within the experiment. Mature buds are readily recognizable by petals extending past the tips of the calyx. Buds were sampled daily for a period of 3 mo after inoculation, and samples were stored under desiccation until assayed for mating-type bias.

The following sampling design was applied to all plants in both the simultaneous and sequential inoculation experiments. All dichasia were sampled on the first stem to flower, and the apical dichasium was sampled from subsequent stems. A maximum of three flower buds were sampled per dichasium, including the first axial flower bud and the two immediately distal flower buds. Some flowers did not mature during the course of this study. Collected flower buds were labeled according to their positions within the plant, and all additional buds were removed and counted. As a control to test for the fidelity of mating-type bias expression, 63 flower buds (or 0.5%) from random plants inoculated with only one genotype of *Microbotryum* were assayed. All were infected by biased or nonbiased genotypes as expected.

An initial assessment revealed that about 2% of samples

Table 1: Summary of multiple infection in experimental populations

	Target plants	Diseased target plants	Plants with multiple diseased stems	Plants with multiple infection	Biased stems	Nonbiased stems	Average days of first to last sample	Average stems per diseased plant
Clover Hollow	192	111	93	63	172	212	18	3.5
Goldbond	192	86	66	50	196	120	19	3.6
Total	384	197	159	113	368	332	18	3.5

(n = 1,028) from only plants receiving the experimental mixed inoculations could not be classified because their ratios of colony sizes were between the expected 1 : 1 or 1 : 0 ratios of biased and nonbiased genotypes, respectively. These ratios were probably due to the presence of both biased and nonbiased genotypes within the same flower because this was used as the sampling unit. Therefore, to classify samples, the actual ratio of colony sizes was determined in random transects of the assay plates until at least 100 colonies had been counted. The status of samples was recorded as intermediate when the ratio of large to small colonies was not the 1 : 0 ratio of nonbiased genotypes but was more than two standard deviations from the mean 1 : 1 ratio of biased genotypes (n = 560; mean ratio plus two standard deviations is approximately a 3 : 2 ratio of large to small colonies).

Results

Experimental Field Populations

The two experimental populations did not differ for the following measures, and, therefore, the data were combined (table 1): the proportion of plants that were multiply infected (χ^2 = 1.21, df = 1, P = .27), the number of days from the first to the last sample per plant (Z-test = 0.31, P = .75), and the number of stems sampled per plant (log-transformed data; Z-test = 0.05, P = .96).

Multiple infection was detected in 71% of target plants with multiple diseased stems (n = 159), as indicated by the presence of both biased and nonbiased genotypes on separate stems of the same plant (fig. 2). Among multiple stems of a plant that were sampled on the same day, coinfection was detected in 74% of plants (n = 89). When a plant had more diseased stems, the likelihood that it would be multiply infected increased (fig. 3). Among diseased plants, the effect of sex on the likelihood of multiple infection was not significant, but there was a moderately significant interaction of sex by the number of diseased stems; females with two diseased stems had more multiple infections than expected, and females with larger numbers of diseased stems had fewer multiple infections than expected (LOGISTIC procedure in SAS; χ^2 for number of diseased stems = 8.06, df = 1, P = .005; χ^2 for sex = 2.57, df = 1, P = .11; χ^2 for the interaction = 4.59, df = 1, P = .032).

Separate stems within a plant were infected as independent units. To test for the independence of stems within a plant, it was assumed that each stem had an equal probability of being infected by a biased or nonbiased genotype. There was no difference between the observed proportions of multiply infected plants per number of diseased stems and the expected proportions if the stems were completely independent (combined binomial distribution probabilities as χ^2; Sokal and Rohlf 1969; χ^2 = 24.41, df = 20, P = .44). However, there is weak power in this statistic to suggest that the observed and expected proportions of multiply infected plants are actually the same and that there was no interdependence among the stems of a plant. This is due to the fact that the observed proportion of multiply infected plants was only 11% less than expected

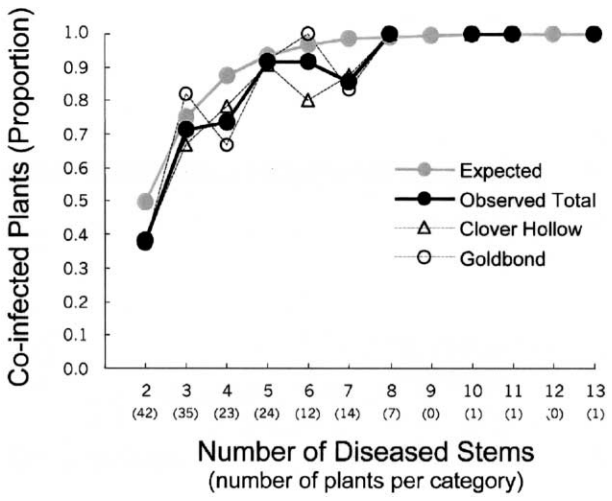

Figure 3: Multiple infection of *Silene latifolia* by *Microbotryum violaceum* in the first season for plants from the experimental populations (Clover Hollow and Goldbond) classified by the number of diseased stems. Expected values are the probabilities of obtaining multiple infection if one assumed all stems were completely independent with an equal chance of being infected with either of the two fungal genotypes. The observed total combines data from the two experimental populations.

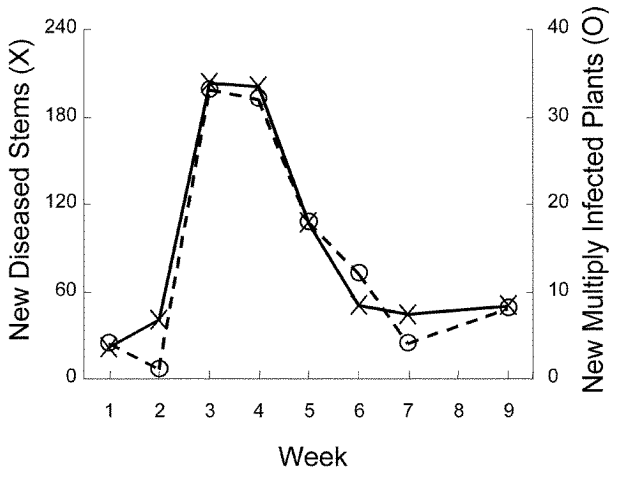

Figure 4: Dynamics of disease transmission and multiple infection over time. Data are the sum of observations per week, beginning from the first disease transmission at the end of July and continuing through the end of the season. The 6 : 1 ratio for the two vertical axes reflects the ratio of overall diseased stems to overall multiply infected plants in the first season.

(weighted average) and the level of sampling was limited (see fig. 3). Specifically, the confidence in avoiding a Type II error was low (measured as $1 - \beta = 0.22$; Rosner 1995). However, the level of sampling would have provided sufficient power to detect an interdependence among stems (i.e., to limit Type II error by $1 - \beta > 0.80$) if the actual proportion of multiple infected plants was more than 43% below the expected values (i.e., if about half of the time interdependence among stems prevented the occurrence of multiple infection, then this would have been detected).

The rate of disease transmission was highly variable over time and reflected a sharp peak in flowering of source plants in early July and a typical latent period of 5–6 wk (data not shown). However, there was no evidence for greater exclusion as the season progressed. The rate of multiple infection relative to disease transmission was constant over time (fig. 4). Also, there was no indication of an advantage of the first infection to exclude subsequent infections by the contrasting type of strain on separate stems; the biased status of the sample from the first diseased stem per plant predicted the biased status in the majority of stems for only 53% of multiply infected plants ($n = 113$; binomial distribution, $P = .29$). Finally, there was no correlation between the amount of time since the initial infection of a plant and whether subsequent samples were of the same biased status as was the first; proportions of samples matching the first in intervals from 1 to ≥5 wk: 0.54 ($n = 92$), 0.61 ($n = 140$), 0.50 ($n = 78$), 0.53 ($n = 38$), and 0.61 ($n = 41$).

Over the winter, a large number of plants changed status with regard to being diseased or healthy. Among the 134 surviving target plants, 27 were diseased in the second season, but only 10 of these had actually been diseased in the previous season; eight had been healthy, and nine had been vegetative. Mortality percentages were 83% for diseased plants ($n = 199$), 50% for healthy plants ($n = 139$), and 30% for vegetative plants ($n = 46$). The sex of diseased plants did not have a significant effect on mortality (LOGISTIC procedure in SAS; χ^2 for sex, df = 1, $P = .097$).

There was a highly significant positive correlation between the number of diseased stems in the previous season and the likelihood of overwintering mortality (fig. 5; CORR procedure in SAS; weighted correlation coefficient $r = 0.96$; $Z = 7.75$; Snedecor and Cochran 1989; $n = 9$; $P < .001$). Among plants classified by the number of diseased stems, those with multiple infection had a moderately significant lower mortality than did singly infected plants (LOGISTIC procedure in SAS; χ^2 for number of diseased stems = 8.24, df = 1, $P = .004$; χ^2 for multiple infection status = 4.43, df = 1, $P = .035$).

In the second season, separate stems within a plant were no longer independent with regard to multiple infection, meaning that a plant tended to have the same type of fungal strain on all of its diseased stems. More than one diseased stem was produced on 18 plants, for which the

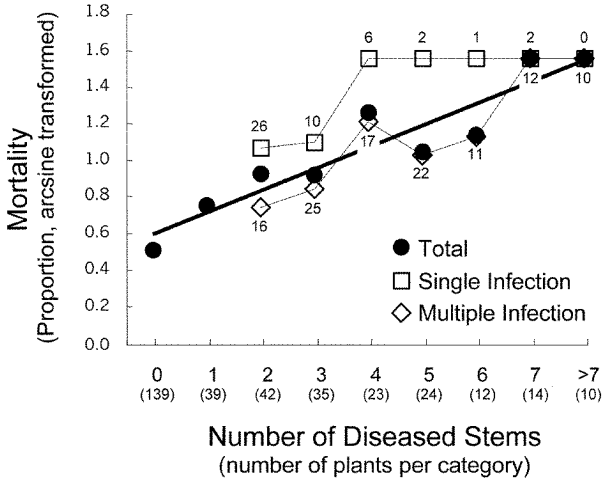

Figure 5: Overwintering mortality of *Silene latifolia* from the combined experimental populations, classified by the number of diseased stems in the first season. The class with 0 diseased stems includes plants that flowered healthy but excludes those that remained vegetative. Mortality for vegetative plants was 30% ($n = 46$; arcsine-transformed proportion = 0.30). The data are also separated according to whether plants with a given number of diseased stems were multiply or singly infected (numbers of plants per category are given next to their symbols).

average number of diseased stems was five. Only four of these 18 plants exhibited multiple infection, which was 61% (weighted average) less multiple infection than expected if the stems were still completely independent. This resulted in significantly fewer multiply infected plants than expected by chance (combined binomial distribution probabilities; $\chi^2 = 89.66$, df = 18, $P < .001$). Among the four multiply infected plants in the second season, three had been healthy or vegetative, and one had been multiply infected in the previous season.

Simultaneous Inoculation Experiment

In this experiment, 132 of the 144 plants flowered during the course of the study, and 123 of these were diseased (93% of flowering plants). There were no differences in the proportions of plants that flowered or that were diseased depending on the starting date, the pair or bias status of inoculum, plant sex, or whether the inoculum consisted of a mixture of genotypes or a single genotype (χ^2; smallest $P = .113$). The total number of flowers produced by diseased plants was significantly affected by plant sex (GLM procedure in SAS; $F = 19.38$; df = 1, 86; $P < .001$) and experimental starting date of the block ($F = 8.64$; df = 1, 86; $P = .004$), but there were no significant effects for other variables or interactions (next-smallest $P = .099$). On average, female plants produced 41 flowers, while males produced 59 flowers. Plants in the second block produced nine more flowers on average than did those in the earlier block.

Multiple infection was detected in a minority of plants given the mixed inoculum treatments (fig. 6). A single genotype was recorded in 31 of the 42 such diseased plants. Because sampling was not exhaustive, it remains possible that multiple infection was undetected in some plants. However, there was no difference in the level of sampling between plants in which multiple infection was detected and plants in which only a single genotype was detected (average number of samples for both was 10). Also, there was no effect of plant sex on the occurrence of multiple infection ($\chi^2 = 0.139$, df = 1, $P = .709$). Fungal samples of intermediate phenotype were detected only from plants receiving mixed inoculum treatments, and these samples were considered as a separate classification from biased or nonbiased (fig. 6). Some multiply infected plants contained significantly more samples of intermediate phenotype than expected by chance (χ^2 on the number of intermediate phenotype samples per plant across all multiply infected plants = 25.631, df = 10, $P = .004$).

There was no evidence for spatial segregation of genotypes within multiply infected plants, meaning that a single inflorescence (i.e., dichasium) was multiply infected as often as predicted by the ratio of genotypes within the stem as a whole (combined binomial distribution probabilities for all multiply infected plants; $\chi^2 = 28.05$, df = 20, $P = .108$).

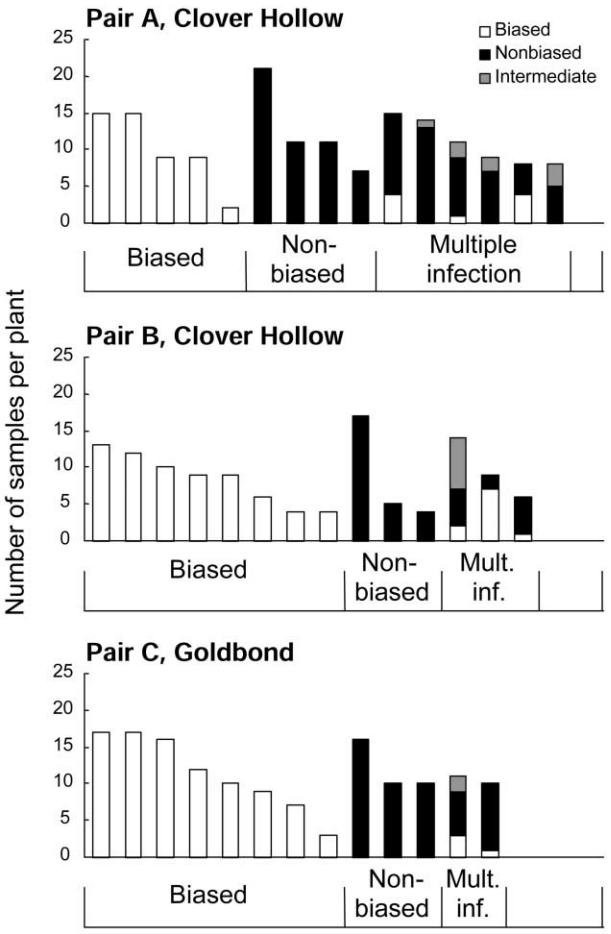

Figure 6: Characterization of pathogen samples from *Silene latifolia* following simultaneous inoculation with a mixture of biased and nonbiased genotypes of *Microbotryum violaceum*. Plants were classified as to whether only biased, only nonbiased, or a mixture of genotypes (multiple infection) were observed among the samples. Samples classified as intermediate fell between biased and nonbiased genotypes in the assays and may represent the multiple infection of single flowers.

Under simultaneous mixed inoculation, biased genotypes showed a slightly significant advantage over nonbiased genotypes. Twice as many plants given mixed inoculum had only biased genotypes ($n = 21$ vs. $n = 10$ for only nonbiased genotypes; fig. 6), and the trend was consistent across all pairs and replicates of the experiment (Fisher's exact test for heterogeneity among pairs and replicates; $n = 31$; $P = .454$; G statistic on pooled data for biased vs. nonbiased = 3.990, df = 1, $P = .046$).

Sequential Inoculation Experiment

In this experiment, 230 of the 252 plants flowered during the course of the study, and 199 of the inoculated plants were diseased (97% of flowering inoculated plants). All 26 of the water-treated plants that flowered were healthy. There were no differences in the proportions of inoculated plants that flowered or that were diseased depending on the pair or the biased status of the inoculum, the order of inoculum, or plant sex (χ^2; smallest $P = .352$). The total number of flowers produced by diseased plants was significantly affected by plant sex (GLM procedure in SAS; $F = 71.97$, df $= 1, 97$, $P < .001$), but there were no significant effects for other variables or interactions (next-smallest $P = .086$). On average, female plants produced 38 flowers, while males produced 73 flowers.

Samples from all plants given the single-genotype inoculum were consistent with their expected 1 : 1 or 1 : 0 ratios for colony sizes. Samples from the mixed inoculum treatments included a small number of intermediate phenotypes (fig. 7), and, again, some plants contained significantly more sample of intermediate phenotype than expected by chance (χ^2 on the number of intermediate phenotype samples per plant across all multiply infected plants $= 44.589$, df $= 14$, $P < .001$).

Generally, the genotype inoculated first had a highly significant advantage, although multiple infection was detected in a minority of sequentially inoculated plants (fig. 7). After sequential inoculation, a single genotype was recorded in 43 of the 58 diseased plants (excluding plants with fewer than two samples). For 37 of these plants, only the genotype inoculated first was present, and for six plants, only the genotype inoculated second was present (binomial distribution probability, $P < .001$). Furthermore, samples of the type inoculated first were more numerous than were samples of the type inoculated second in 13 of the 15 multiply infected plants (binomial distribution probability, $P = .004$). Again, there was no difference in the level of sampling between plants in which multiple infection was detected and plants in which only a single genotype was detected (averages were 12 and 10 sampled flowers, respectively; Z-test $= 1.26$; $P = .208$). Also, there was no effect of plant sex on the occurrence of multiple infection ($\chi^2 = 0.027$, df $= 1$, $P = .869$).

In contrast to the simultaneous inoculation experiment, there was evidence for spatial segregation of genotypes within multiply infected plants after sequential inoculation. An inflorescence was less often multiply infected than predicted by the ratio of genotypes within the stem as a whole (combined binomial distribution probabilities for all multiply infected plants; $\chi^2 = 40.19$, df $= 16$, $P < .001$).

Under sequential inoculation, no statistically significant

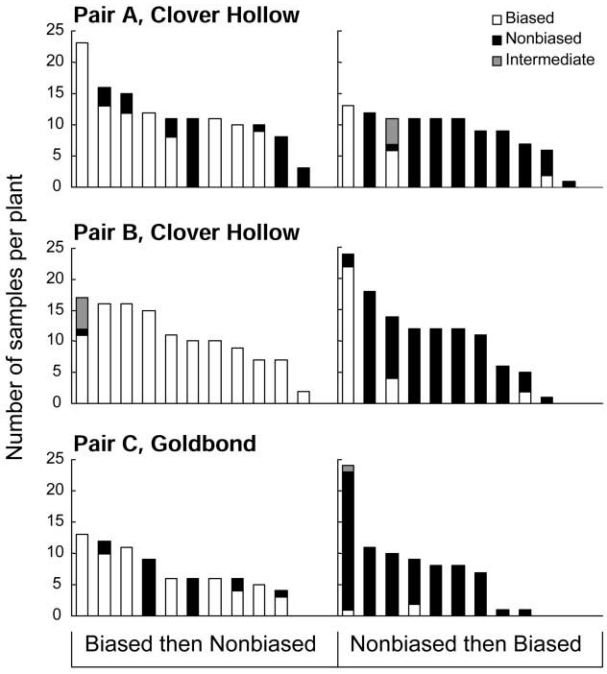

Figure 7: Characterization of pathogen samples from *Silene latifolia* following sequential inoculation with biased and nonbiased genotypes of *Microbotryum violaceum*. Sequential inoculations were separated by a 1-wk interval. Samples classified as intermediate fell between biased and nonbiased genotypes in the assays and may represent the multiple infection of single flowers.

effects of bias status were found. However, there was significant heterogeneity among the pairs with regard to whether the genotype inoculated first was the only genotype detected (Fisher's exact test; $n = 45$; $P = .017$). This was due largely to pair B, where no plants contained only the genotype inoculated second.

Discussion

Natural transmission of anther-smut disease in the field experiment frequently resulted in infection of *Silene latifolia* by multiple genotypes of *Microbotryum*. Because the disease is systemic, most previous studies assumed that the host was colonized only by a single fungal genotype, and all of its diseased flowers were assumed to represent collectively a fungal individual (i.e., Bucheli et al. 2000; Hood and Antonovics 2000). *Microbotryum* is instead able to infect separate stems of a plant as if they were completely independent. As the field season progressed, the appearance of disease on more and more stems was due much more to repeated infection than to systemic fungal colonization.

The high rates of mortality were unexpected and pre-

vented any rigorous investigation of how the genotypes interact during the overwintering period. For example, would there have been an advantage of the initial infection, and would coinfecting genotypes resolve themselves completely or segregate spatially within the host? However, there was an apparent influence of overwintering on the persistence of multiple infection in the field, and multiple infections were much more rare at the beginning of the second season than expected from the first season's data. Although this may seem to suggest a causal relationship between multiple infection and mortality, this is probably not the case. Pollinators are attracted to plants with greater numbers of flowers (Alexander and Antonovics 1995), and, thus, such plants are more likely to receive many doses of inoculum and become multiply infected. Meanwhile, it is reasonable to expect that greater resource allocation toward reproduction would also increase the risk of overwintering mortality. As a result of these interactions, the high rates of mortality effectively purged the experimental populations of large, multiply infected plants. This correlation among plant size, infection probability, and overwintering mortality might also have contributed to the previous report of increased mortality for plants that became diseased under field conditions (Alexander and Antonovics 1995).

Interestingly, plants with a given number of diseased stems were actually less likely to die when multiply infected than when singly infected. This apparent effect of within-host competition on virulence may be important to the evolutionary dynamics of this system, where overwintering survival of infected hosts is essential for disease persistence (Thrall et al. 1993). The mechanisms involved here and their long-term consequences need further study before they can be put into the appropriate theoretical context. However, multiple infection in other systems can have effects that are mediated through host resistance (Read and Taylor 2001) or by the direct spread of pathogen factors (e.g., hypovirulence dsRNA) that alter the expression of disease symptoms (Nuss and Koltin 1990).

It has previously been shown that latent infection of healthy or even vegetative plants can frequently persist through the winter and contribute substantially to the following year's diseased class (Alexander and Antonovics 1995). Furthermore, this study shows that such latently infected plants can also harbor persistent coinfections through the winter.

While the field study addressed multiple infection at the level of separate stems, there are finer subdivisions to the plant in which pathogen genotypes also interact. In the artificial inoculation studies, competitive exclusion can be evaluated at the level of plant meristems, which is the primary location where *Microbotryum* is thought to reside (Audran and Batcho 1982). Because seedlings were inoculated so early in development, all samples from a plant were necessarily derived from a single infected meristem. Two striking results were obtained. First, multiple genotypes can persist after simultaneously infecting a single meristem. This result is consistent with the previous report by Day (1980). However, by including a much larger number of replicates, this study shows that most often only one pathogen genotype dominates the host. Day (1980) found that three of four inoculated plants were multiply infected, but this may be partly due to the inoculation technique that used much older plants with 10–15 lateral meristems each. How genotypes sometimes share the extremely small space within a meristem is unknown.

The second important observation was that sequential inoculation had a major influence on the outcome of interactions between genotypes. The initial infection had a large advantage even though the interval between inoculations was very brief relative to the duration of infection. If not entirely excluded, the challenging genotype was often present only in a small part of the host and was spatially segregated at the level of separate reproductive meristems. These results indicate that *Microbotryum* genotypes exhibit some form of competitive exclusion once they have systemically infected plants, but the precise mechanism and its performance under field conditions remain to be determined. The additional question of whether a genotype-specific hierarchy exists with regard to this exclusion also requires further study, but this would be consistent with the significant effects of particular genotype combinations following simultaneous or sequential inoculations.

Beginning with healthy susceptible plants, the dynamics of multiple infection can be summarized as within-host diversity of infections that equals that of the nearby sources of inoculum by the end of the growing season. However, hosts that flower diseased in the following year are systemically infected, and if there is a high rate of mortality as in this study, they will have lower average within-host diversity. Such systemic infections then exhibit a degree of competitive exclusion that resists increasing the within-host diversity by subsequent challenges, although the strength and the mechanism of this exclusion needs further study. Therefore, components of both coinfection models initially and superinfection models after systemic colonization are expected. These dynamics make it difficult to fit *Microbotryum* to the existing theoretical framework, but this may be the case for other diseases in which systemic colonization is a prolonged process.

A polymorphism with two marker types was used in this study, and this limited the ability to determine the actual number of independent infections per plant. Natural populations of *Microbotryum* contain additional genetic variation (Kaltz and Shykoff 1997; Oudemans et al. 1998; Bucheli et al. 2000, 2001; Hood et al. 2002), and

multiple infection may result in a rich within-host diversity of pathogen genotypes. The degree to which this occurs, however, will depend largely on the spatial structure of pathogen variation in relation to movement between susceptible hosts. For example, one would expect the rates of multiple infection to be less than those observed in this study if source genotypes were distributed with a high degree of substructuring and a relatively short dispersal distance. It then follows that the forces responsible for such spatial structure are essential components to the biology of multiple infection. In *Microbotryum*, there is strong evidence for substructuring of genetic variation with regard to the mating-type bias polymorphism (Oudemans et al. 1998). Continued studies show that this reported structure has been maintained over a period of at least 9 yr despite almost a complete turnover of the plants in those patches (M. E. Hood and J. Antonovics, unpublished data). Therefore, accessing the extent and importance of multiple infection in natural populations would require an extensive and spatially explicit study using repeated sampling of diseased individuals and a wide range of genetic markers, such as microsatellites. Ongoing research that employs such techniques may be expected to confirm multiple infection in this system (A. Biere, personal communication). Current evidence comes from the work of Bucheli et al. (2000), where one sample from a natural population contained three microsatellite alleles. To date, however, there have been no studies that measure the degree of multiple infection in natural populations of *Microbotryum*, largely because this has not been seen as an important possibility. This study shows that multiple infection is likely to be the norm rather than the exception.

Mating-type bias of *Microbotryum* was used in this study not only because it provides a convenient and naturally polymorphic marker but also because of interest in the factors leading to the maintenance of this variation in the face of negative haploid selection (Antonovics et al. 1998). Biased genotypes produce viable haploids of only one mating type because the alternate mating type is linked to a highly deleterious allele that prevents haploid growth (Hood and Antonovics 2000). However, both mating types are required for conjugation and infection. It was previously suggested that very rapid mating among the immediate products of meiosis would minimize such deleterious haploid effects and that this mating would tend to be between the products of the same meiosis (i.e., intratetrad mating; Hood and Antonovics 2000). The fact that the haplo-deleterious allele was inherited so frequently in the field study, as seen by transmission of biased genotypes, supports the very rapid mating after meiosis either among products of one meiosis or among meiotic products of separate teliospores that have been deposited and germinated in close proximity. Because haploids carrying the deleterious marker do not survive, one would have otherwise expected a great deficiency of biased genotypes in the target plants.

As an alternative scenario, however, it was shown that biased genotypes could increase in frequency if an advantage in another stage of the life cycle was associated with the bias and, thus, compensated for negative haploid selection (Antonovics et al. 1998). Studies of haplo-deleterious alleles in other smut fungi have identified or suggested parasitic advantages of biased over nonbiased genotypes (Holton and Dietz 1960; Nielsen 1968). Such a compensatory advantage in *Microbotryum* was not seen in the measures of disease expression under greenhouse conditions. However, the results from the simultaneous inoculation experiment may indicate a within-host competitive advantage for these biased genotypes of *Microbotryum*; following mixed inoculation, they were the only genotype present in the plant significantly more often than nonbiased genotypes. This clearly warrants additional research to compare a broader range of biased and nonbiased genotypes.

If biased genotypes tend to have such an advantage, then there are important theoretical consequences for the maintenance of such genetic variation at the population level. Multiple infection is effective in this regard if a tradeoff exists between within-host competitive ability and dispersal (Amarasekare and Nisbet 2001). Maintenance of the mating-type bias polymorphism by this mechanism would imply a dispersal advantage of nonbiased genotypes of *Microbotryum*. Interestingly, nonbiased genotypes have a greater ability to proliferate during the haploid stage. Viable haploid sporidia can replicate in the nectar of healthy flowers and, thus, may be moved to new plants by insect pollinators, just like other nectar-inhabiting microorganisms (Lachance et al. 2001). Sporidia that carry a deleterious allele responsible for mating-type bias lack this ability. Nonbiased genotypes may thus have a dispersal advantage if sporidia contribute to new infections. However, this hypothesis should be treated cautiously until the actual role of sporidia has been conclusively determined.

Some key aspects of multiple infection that have been shown to be important in other systems remain unexplored in the anther-smut disease, such as the influence of variation for disease resistance in the host (Wille et al. 2002) and the level of relatedness among coinfecting genotypes (Frank 1992). However, this study establishes within-host dynamics as an important level of interactions. It also provides examples of the processes assumed in most theoretical disease models although in a sequential combination of coinfection and superinfection dynamics not often incorporated into existing work. Thus, there is the potential to broaden our understanding of disease ecology

and evolution by further exploring such systems in both empirical and theoretical contexts.

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