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## INBREEDING EFFECTS ON RESISTANCE AND TRANSMISSION-RELATED TRAITS IN THE *SILENE*–*MICROBOTRYUM* PATHOSYSTEM

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**Abstract.** Inbreeding in local host populations will be a common phenomenon in host–pathogen systems that are characterized by metapopulation dynamics, i.e., frequent extinction and recolonization of local host populations by small numbers of founding individuals. As an example of a pathosystem with metapopulation dynamics we investigated the impact of inbreeding in the host plant *Silene alba* on its interaction with the anther-smut fungus *Microbotryum violaceum*. Seeds from eight populations of *S. alba* were sampled, and five generations of sib mating resulted in 65 inbred lines, with inbreeding coefficients of  $f = 0, 0.25, 0.375, 0.5,$  and  $0.59$  per line. In a first experiment these lines were tested for active, biochemical resistance against fungal infection, by artificially inoculating individuals. The percentage of infected individuals differed significantly among populations, lines, and inbreeding levels, and both population-by-inbreeding level and line-by-inbreeding level interactions were significant. The most striking result was the strong variance in inbreeding effects among lines; inbreeding resulted in increased resistance in some lines and decreased resistance in others. In a second experiment for 12 inbred lines, originating from one population, active resistance and flower traits associated with passive resistance (avoidance) to this insect-vectored, florally transmitted disease were measured. Significant inbreeding depression was demonstrated for petal size and nectar volume. Thus inbreeding might enhance avoidance of spore transmission by insects. For both active resistance and all flower traits, significant line-by-inbreeding level interactions were found. The results indicate that the effect of inbreeding on the interaction between host and pathogen in this pathosystem is unpredictable at the local population level, because: (1) strong genotypic differences in inbreeding effect exist for both active and passive resistance, making the effect of inbreeding at the population level dependent on the genotypic composition of the (founder) population; (2) effects of inbreeding on active and passive resistance were not correlated, making the net effect of inbreeding on field resistance unpredictable; and (3) in several lines, evidence for epistatic effects was found, making the effect of inbreeding dependent on the actual inbreeding level of the genotype. The results underscore that most progress in the study of host–pathogen interactions may be expected from an integrated ecological and genetic approach.

**Key words:** biochemical resistance; host–pathogen dynamics; inbreeding; *Microbotryum violaceum*; *Silene alba*; transmission-related flower traits.

### INTRODUCTION

The numerical and evolutionary dynamics of the interaction between host plants and their pathogens in natural populations have been the subject of much research in the past decade (Burdon and Leather 1990, Fritz and Simms 1992, Clay and Kover 1996, Real and McElhany 1996). While both ecological and genetic questions have been investigated separately, it has been recognized that for a thorough understanding of host–pathogen interactions, an integration of ecology and genetics is indispensable (Alexander et al. 1996). For a number of natural host–pathogen systems, genetic

variation for both resistance in the host and virulence in the pathogen has been demonstrated (Parker 1985, Burdon 1987b, de Nooij and van Damme 1988, Jarosz and Burdon 1991, Alexander et al. 1993, Bevan et al. 1993, Davelos et al. 1996). The influence of the pathogen on the host population in these systems depends on the genotypic composition of both host and pathogen populations.

Inbreeding in local host populations will be a common phenomenon in host–pathogen systems that are characterized by metapopulation dynamics. As an example of a pathosystem with metapopulation dynamics, in this paper we investigated the impact of inbreeding in the host plant *Silene alba* (the white campion) on its interaction with the anther-smut fungus *Microbotryum violaceum*. Antonovics et al. (1994) argue that stable coexistence of host and pathogen in this system is probably only possible at the (regional) metapopu-

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lation level, while at the (local) population level frequent extinction and recolonization occur. When sites are recolonized, populations are founded by a very limited number of genotypes. If gene flow is limited, these populations will become increasingly inbred with time. Investigating inbreeding effects in this system, and in any other host–pathogen system that is characterized by metapopulation dynamics, is therefore important for understanding the dynamics of the interaction between host and pathogen.

In host populations, inbreeding or the mating among individuals related by descent, is a process that will change the genotype frequencies and may therefore affect host–pathogen interactions. The effect of inbreeding on disease levels in a host population can be brought about in two different ways. First, inbreeding may directly affect “active” or “biochemical” resistance, i.e., host responses to pathogen exposure that reduce the level of infection once the pathogen is encountered (Burdon 1987a). Second, inbreeding in the host may affect “passive” resistance, i.e., those host traits that reduce contact with the pathogen (avoidance) (Burdon 1987a). For instance, in systems where the pathogen is insect transmitted, any inbreeding effect on host traits that determines the attractiveness of an individual host plant to the insect vector, will change the transmission properties of the host–pathogen interaction.

Provided that there is genetic variation for the traits involved in active or passive resistance and there is some degree of genetic dominance for them, inbreeding will, in principle, result in inbreeding depression in these traits. However, in any system in which resistance is governed by both active and passive resistance mechanisms, the net effect of inbreeding in the host on resistance may be positive or negative, depending on whether the effects of inbreeding on separate components of active and passive resistance reinforce or oppose each other. Moreover, the observed pattern of inbreeding depression in a particular population may strongly depend on the genotypic composition of the population. Large genetic variation among genotypes in inbreeding depression, caused by genetic drift (Wright 1977) or by variation in the initial inbreeding level of the founding genotypes (Brewer et al. 1990, Dole and Ritland 1993), has been demonstrated in the red flour beetle *Tribolium castaneum* (Pray and Goodnight 1995, Stevens et al. 1997) and in annual populations of the plant *Mimulus guttatus* (Dudash et al. 1997). Finally, complex traits like (components of) resistance may be influenced by epistatically interacting genes. Epistatic interactions can either reinforce or inhibit inbreeding depression, resulting in a nonlinear relationship between the trait value and inbreeding level (Crow and Kimura 1970).

In plants, inbreeding depression has been demonstrated in many different life stages, ranging from seed production to adult reproduction and adult survival (for

review see Charlesworth and Charlesworth 1987). Studies of the effects of inbreeding on pathogen and herbivore resistance in plants have shown equivocal results. Significant inbreeding depression was found for resistance to two herbivores of maize (Ajala 1992), but in the seaside daisy *Erigeron glaucus*, effects of inbreeding on herbivore resistance depended on the resistance status of the parental plants used to generate the tested progeny families (Strauss and Karban 1994). In *Datura stramonium*, no effect of inbreeding could be detected on resistance to two herbivores (Nunez-faran et al. 1996). Significant inbreeding depression was found for rust resistance in slash pine (Matheson et al. 1995). However, documentation of inbreeding effects on host–pathogen interactions in natural populations is still scanty.

Here we report on investigations of inbreeding effects in a natural host–pathogen system of the short-lived perennial *Silene alba* (white campion) and the anther-smut fungus *Microbotryum violaceum*. The fungus is a host-sterilizing pathogen. Spores are produced in the anthers of its host plants and transmitted by insect pollinators. Field resistance of the host to the pathogen has an active and a passive component (Alexander et al. 1993, Thrall and Jarosz 1994, Biere and Antonovics 1996). Active or biochemical resistance is often quantified as the proportion of individuals that do not become diseased after (artificial) inoculation of hosts in the vegetative stage. Passive resistance to, or avoidance of, this florally transmitted pathogen is mediated by the production of a reduced number of flowers and late onset of flowering in reproductive hosts, both of which are interpreted as mechanisms reducing the probability of receiving fungal spores (Alexander et al. 1993, Thrall and Jarosz 1994, Biere and Antonovics 1996). Passive resistance may also be enhanced by decreased flower size and reduced nectar rewards (Shykoff and Bucheli 1995, Shykoff et al. 1997). Frequent extinction and subsequent recolonization seem to be normal features of the host dynamics in at least part of its range (Antonovics et al. 1994, McCauley et al. 1995, Thrall and Antonovics 1995).

Two experiments were conducted to investigate the effects of inbreeding on biochemical and passive resistance. In the first experiment we studied the effect of inbreeding level in the host on its biochemical resistance against artificial inoculation with the pathogen. This experiment was performed with progeny of host genotypes originating from eight host populations. In a second experiment we studied the effect of inbreeding level on flower size and nectar rewards, as putative components of transmission probability, in one of the eight populations.

## METHODS

### *Study organisms*

The white campion, *Silene alba* (Miller) Krause (= *Silene latifolia* Poiret, *Melandrium album* (Miller)

TABLE 1. Parental populations of the inbred lines in experiment 1, with the number of flowering individuals in 1992, the percentage of infected individuals in 1992 and 1993, and the number of inbred lines used in the experiment.

Population	Population size, 1992	Percentage diseased		No. lines in the experiment
		1992	1993	
MILLINGEN	1800	11.5	8.5	8
BYLAND	600	7.5	10.4	9
BEILEN	600	15.2	16.2	6
OOSTVOORNE	235	5.9	4.9	8
KWADEHOEK	150	10.0	6.8	7
HENGELO	125	28.3	15.0	12
BORGER	96	7.3	...	5
KRUININGEN	80	0	0.8	10

Notes: Population BORGER appeared to be extinct in 1993. In the *Results* section and figures, reference to these populations is made by the first three letters of their respective names.

Garcke, *Lychnis alba* Miller, *Silene pratensis* (Rafn) Godr. & Gren.), is a dioecious, short-lived perennial that grows (in the Netherlands) on disturbed roadsides and arable land. Individuals are frequently infected by the anther-smut fungus, *Microbotryum violaceum* (Pers: Pers) Deml & Oberw (= *Ustilago violacea* (Pers.) Roussel). The details of the interaction between the fungus and the host plant have been described in several papers (Baker 1947, Alexander and Antonovics 1988, 1995, Alexander 1989, 1990a, b, Alexander et al. 1993, Biere and Antonovics 1996). Diploid spores are produced in the anthers of infected hosts and are transmitted by insects such as bees, bumble bees, and hawk moths that serve both as pollinators of the host and as vectors of the disease. In both male and female individuals, infection leads to the production of spore-filled anthers, and the fungus effectively sterilizes the host.

Genetic variation for resistance has been demonstrated in the host (Alexander 1990b, Thrall and Jarosz 1994, Alexander and Antonovics 1995, Biere and Antonovics 1996). The genetics of host resistance is not known in detail, but the evidence available suggests that resistance may be a polygenic trait (Alexander 1990b). Variation in virulence of *M. violaceum* has been demonstrated within European populations of the host species *S. alba* (O. Kaltz and J. A. Shykoff, *personal communication*), but has not been reported for populations from the United States, where the pathogen was most likely introduced in the 19th century (Alexander et al. 1993).

#### Inbred lines

Seeds were collected in 1992 from eight populations in different parts of the Netherlands (Table 1). Three to five seed capsules were taken from 20 females per population, chosen in different parts of the population so as to minimize the possibility of collecting sibs. In

some populations seeds from fewer than 20 females were collected, either because the number of females in the fruiting stage was too small at the time of seed collection (BORGER, 10 females, and KRUININGEN, 12 females) or because effective seed set was low due to heavy seed herbivory by caterpillars of the noctuid *Hadena bicruris* (BEILEN, 10 females, and KWADEHOEK, 12 females).

Seeds were germinated in a growth cabinet (25°/15°C day/night temperature). Germination percentages were typically >80%. Twelve families per population (if possible), hereafter referred to as lines, were randomly chosen for further crossing.

In order to create a series of inbreeding lines in this dioecious species, a crossing scheme of four generations of full sib mating was applied. Each generation, each female was crossed with one randomly chosen brother. A single seed-descent approach was followed, i.e., one randomly chosen female was assigned as the parent of the next generation. To establish a base level of inbreeding ( $f = 0$ ) in the first generation, each female that was randomly chosen to initiate a line was crossed with a pollen mixture of five males from the same population, but from other lines. In this way five inbreeding levels per line and population were achieved, with inbreeding coefficients of (following the equation  $f_t = (1/4)(1 + 2f_{t-1} + f_{t-2})$  (Falconer 1981))  $f = 0, 0.25, 0.375, 0.50, \text{ and } 0.59$ , respectively.

For each generation eight randomly chosen seedlings per line were grown to flowering. Assuming a sex ratio of 0.5, this reduced the chance of finding only one sex in a particular line to <5%. Nevertheless, on several occasions, lines could not be pursued to the highest inbreeding level, because all eight, or all flowering, individuals were of the same sex. As germination percentages were generally high (>80%), no lines were "lost" because of insufficient germination. In this way 65 lines, unevenly distributed over the eight populations, were available for the experiments (Table 1).

#### Experiment 1

The effects of inbreeding on biochemical resistance were investigated in an experiment with eight populations (Table 1) and five inbreeding levels ( $f = 0, 0.25, 0.375, 0.50, \text{ and } 0.59$ ). One hundred seeds per inbreeding level per line per population were germinated in Petri dishes in a germination cabinet with a day/night temperature regime of 25°/15°C. Germination was first visible after 3 d, and nearly maximal after 6 d. Inoculations were performed according to a procedure described by Alexander and Maltby (1990). In each population, diploid fungal teliospores were collected from a single flower of two different host plants. Teliospore samples were subsequently germinated in the laboratory to isolate two haploid mating types from each sample. Inoculation medium was made by suspending the two mating types of each of the two strains that were collected from the same population; in this way

the inoculum consisted of four different fungal genotypes, representing the four different combinations of mating type. The haploid spores were suspended in water, and cultured while shaking overnight at 14°C. The following day, the frequency of conjugation was estimated under a microscope. Frequencies always exceeded  $10^6$  conjugated cells/mL. Seven days after the start of seed germination, 5 mL of the inoculation medium was added to each Petri dish with host seedlings. Petri dishes were then placed in a growth cabinet at 14°C for 3 d. For this experiment, eight types of inoculation medium were made, one for each population. Lines were inoculated with strains of their native population, except for the KRUIINGEN population, in which no disease was found in the field. These lines were inoculated with strains from the geographically nearest population (OOSTVOORNE).

This inoculation procedure generally leads to a high percentage of the individuals becoming infected (Alexander and Maltby 1990). Because inoculation medium is forced upon the seedlings, this experiment measures the biochemical component of resistance against infection and does not deal with traits influencing transmission.

After inoculation, between 20 and 25 seedlings per line per inbreeding level were planted in small peat pots ( $4 \times 4$  cm) with standard potting soil. Since the reliability of methods to observe successful establishment of the fungus in vegetative host stages is rather poor (Nilsson et al. 1994), plants were grown to the flowering stage, when successful infection can easily be assessed by observation of spore production in the anthers. Because the available space in the greenhouse was insufficient to accommodate the large number of inoculated host plants, plants were grown first in the greenhouse until they were large enough to be planted in the experimental garden of the Netherlands Institute of Ecology in Heteren, in early June 1994. Plants were planted in a grid of 77 rows of 76 individuals each, at interplant distances of 0.25 m. After every seventh row, a stretch of 0.75 m was left bare to serve as a path. Treatment combinations (population, line, and inbreeding level) were completely randomized across the grid, but for logistic reasons, individuals per treatment combination were planted in blocks of  $4 \times 5$  or  $3 \times 7$  individuals. A total of 5785 plants was planted for this experiment. After one week a heavy rainstorm washed away some of the plantlets that were not firmly rooted at that time. As a consequence, mortality in the experiment was relatively high (23%). However, since the fraction of individuals of a particular line that showed disease symptoms was not significantly correlated with the mortality rate of that line, there is no indication that estimates of resistance per line are biased due to differential mortality.

Individuals were monitored weekly for flowering. For each individual, week of anthesis, sex (male or female), and disease status (healthy or diseased) at first

flowering were recorded. Diseased females can be distinguished from diseased males by the presence of an aborted fruitbody in females. Diseased plants were immediately removed to prevent secondary spread of the disease, i.e., infections not due to inoculation. Since earlier observations (N. J. Ouborg and A. Biere, *unpublished data*) showed that occasionally the first flowers produced by an infected plant do not yet carry spores, disease status was recorded again 5 wk after anthesis, after which the individual was removed from the experiment. The restriction to a 5-wk period was chosen to minimize the chance of observing infections that were not the result of inoculation. Since the latent period of the fungus is on the order of several weeks (Alexander 1990a, Alexander et al. 1993, Biere and Antonovics 1996, Biere and Honders 1996), the recorded disease status of an individual 5 wk after the onset of flowering is expected to reflect only the artificial inoculation treatment, even if secondary spread of the disease were to occur in the experiment. Moreover, when plants are artificially inoculated in the vegetative stage they either become completely diseased or not diseased at all; in contrast, secondary (flower) inoculation would result in only a part of the flowers being diseased (Alexander and Maltby 1990). In this experiment no partly diseased plants were found, supporting the assumption that all disease phenomena resulted from artificial inoculation. Therefore the results were taken as a measure of biochemical (active) resistance.

The experiment was continued until the first periods of night frost at the end of November. Individuals that had not flowered at that time were classified as vegetative plants during the first season. A total of 3913 individuals survived until flowering and could be scored for disease status. Subsequent analyses of disease susceptibility were performed with these flowering individuals only.

### Experiment 2

In a greenhouse experiment, effects of inbreeding on traits putatively related to disease transmission and on biochemical resistance were investigated in detail for one of the populations (HENGEL0). Seeds of 33 progeny families, representing the treatment combinations of 12 lines and three inbreeding levels ( $f = 0, 0.375,$  and  $0.59$ ) for which enough seeds were available, were sown in Petri dishes on 13 January 1995. Overall germination was high (85.9%) and differed both between inbreeding levels ( $f = 0: 83.1\%, f = 0.375: 83.8\%,$  and  $f = 0.59: 91.4\%, \chi^2 = 42.64, df = 1, P < 0.001$ ) and lines (63.7–98.0%,  $\chi^2 = 64.6, df = 11, P < 0.001$ ; interaction line by inbreeding level  $\chi^2 = 41.0, df = 11, P < 0.001$ ). Approximately 30 uninoculated seedlings per family were planted on 6 February and used for measurements of nectar production and morphometric traits. Four weeks after planting, number of leaf pairs and length of the longest leaf were recorded. For

TABLE 2. Generalized linear model analyses of the effects of population, line (nested within population), and inbreeding level ( $f = 0, 0.25, 0.375, 0.50,$  and  $0.59$ ) on mortality rate, fraction of nonflowering individuals, susceptibility of host plants to fungal infection, and sex ratio in the host.

Source	Mortality			Nonflowering			Disease			Sex ratio		
	df	$\chi^2$	$P$	df	$\chi^2$	$P$	df	$\chi^2$	$P$	df	$\chi^2$	$P$
Population	7	48.8	0.0001	7	45.9	0.0001	7	33.7	0.0001	7	24.5	0.0009
Line(Pop.)	54	209.3	0.0001	42	111.5	0.0001	41	125.8	0.0001	41	36.0	0.6925
Inbreeding	1	39.9	0.0001	1	3.0	0.0821	1	6.7	0.0095	1	2.1	0.1527
Inbr. $\times$ Pop.	7	20.1	0.0054	7	40.5	0.0051	7	25.1	0.0007	7	8.3	0.3053
Inbr. $\times$ Line(Pop.)	54	229.2	0.0001	42	77.9	0.0006	41	142.4	0.0001	41	42.6	0.4007

$\approx 10$  flowering plants of each sex, line, and inbreeding level ( $n = 673$ ), the two primary flowers produced on the top branch-pair of the primary flower stalk were used for measurements of calyx length and width, petal blade length, and petal claw length. Nectar volume was measured one day after opening of a flower using 5- $\mu$ L microcapillary tubes. Total sugar concentration (gram sucrose equivalent per 100 g solution) was measured using a pocket refractometer (ATAGO Company, Tokyo, Japan) with a detection range of 0–90% (mass/mass).

Because we were interested in the correlation between inbreeding effects on passive and biochemical resistance within families, we assessed the inbreeding effects on biochemical resistance in the same families as in the first part of experiment 2. Eighteen seedlings per family were inoculated, in a similar way as described in experiment 1. The fungal strains used in this experiment were two HENGELO strains, but different from those used in experiment 1. Inoculated seedlings were planted in 11  $\times$  11 cm pots on 13 February and grown in a greenhouse under 21°/19°C day/night, and 16 h light. Disease status of 6–18 plants per family that survived and initiated flowering ( $n = 397$ ) was recorded weekly until 3 mo after anthesis. Plants producing flowers with spore-filled anthers were immediately removed from the experiment.

#### Data analysis

*Experiment 1.*—Effects of inbreeding, population, and line on disease susceptibility, sex ratio, mortality rate, and fraction of nonflowering plants were analyzed with a generalized linear model, with a binomial error distribution for the response variable, and a logit link function (SAS 6.12, procedure GENMOD; SAS Institute 1989). Population and line (nested within population) were treated as class variables. Since we were interested in the general direction and strength of inbreeding effects on susceptibility rather than in differences among any of the specific inbreeding levels, inbreeding was treated as a regression variable with five levels. Significance of differences was tested using type III likelihood ratio statistics. To improve convergence of the model, only lines for which at least four inbreeding levels with at least seven individuals were available were included in the analyses ( $n = 3218$ ).

Analyses were performed on pooled data for male and female individuals per line and inbreeding level combination, to avoid low expected cell frequencies. Average infection probability differed slightly among sexes, (males 20.1%,  $n = 1314$ , females 16.9%,  $n = 1904$ ;  $G = 5.2$ ,  $df = 1$ ,  $P < 0.05$ ), but since sex ratios were independent of inbreeding level and of interactions between inbreeding level and population or line (Table 2), estimation of inbreeding effects on susceptibility using pooled data for male and female individuals was preferred. Since different fungal strains were used for each population, “population effects” should be strictly referred to as the effect of specific combinations of populations and their native pathogens, but for simplicity we will use the phrase “population effect” throughout the paper.

*Experiment 2.*—Effects of inbreeding, line (treated as random effect), and sex on plant size and reproductive traits were analyzed using generalized linear models, with a normal error distribution for the response variable, and an identity link function (SAS 6.12, procedure GENMOD). Line and sex were treated as class variables. Quasi  $F$  tests based on type III analysis of Pearson’s chi-square values obtained from fitting the generalized linear model and constrained submodels, and an estimate of the dispersion parameter, were well in agreement with  $F$  tests obtained from conventional ANOVA (SAS 6.12, procedure GLM). Analogous to experiment 1, effects of inbreeding level and line on susceptibility of plants from population HENGELO were analyzed using generalized linear models with a binomial error distribution for the response variable, and a logit link function (SAS 6.12, procedure GENMOD). Data for males and females were pooled per line–inbreeding combination. Because different fungal strains were used in experiments 1 and 2 for population HENGELO, differences in percentage disease between the two experiments were not analyzed. For many of the traits, the response to inbreeding differed significantly among lines. To test whether the observed differences in response among lines followed a consistent pattern across all size and reproductive traits that were measured, we estimated standardized regression coefficients for each trait on inbreeding level, and analyzed differences among lines in their mean response for all traits using a one-way ANOVA.

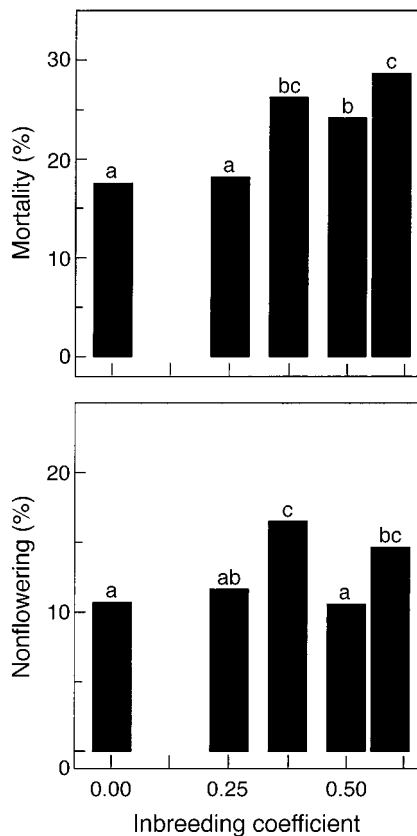


FIG. 1. Effect of inbreeding on mortality rate (top) and the fraction of surviving plants that did not flower in the first season (bottom). Data for all individuals in the experiment were pooled per inbreeding level. Bars within frames that do not share a common letter are significantly different from each other ( $P < 0.05$ ) in pairwise  $G$  tests.

## RESULTS

### Experiment 1

From the 5785 individuals in the experiment, 3228 (55.8%) were scored as healthy and 685 (11.4%) were scored as infected; thus 17.5% of the plants that could be scored with respect to disease status were infected at the end of the experiment. Of the plants that could not be scored, 530 did not flower and 1342 died before the end of the experiment, mostly during the rainstorm early in the experiment (9.2% and 23.2% of the total, respectively). There was a slight but significant increase in mortality with inbreeding level (Fig. 1 top, Table 2). By contrast, linear regressions of the fraction of nonflowering individuals on inbreeding coefficient were not significant (Fig. 1 bottom, Table 2). Neither mortality rate nor the fraction of nonflowering plants were correlated with infection rate (the proportion of plants for which disease status could be scored that became infected; correlation coefficients:  $-0.093$ ,  $P = 0.116$ , and  $+0.112$ ,  $P = 0.058$ , respectively,  $n = 287$  line-inbreeding level combinations). The absence of significant correlations indicates that patterns in the

observed infection rates are not merely a reflection of differential mortality or differences in the fraction of nonflowering individuals among line-inbreeding level combinations, but of differences in biochemical resistance.

Infection rates differed significantly between populations and between lines within populations (Table 2). Because native strains were used for each population, the population effect represents differences in infection rate among population-strain combinations. The effect of line indicates the existence of genetic variation for biochemical resistance within host populations. Infection rates were also affected by the level of inbreeding of the host (Table 2, Fig. 2). Surprisingly, the significant main effect of inbreeding level (Table 2) appeared to reflect an overall increase rather than a decrease in biochemical resistance after four generations of sib mating. Pooled data for all lines showed an overall 28% decrease in infection rate (increase in resistance) in the  $f = 0.59$  generation compared to the  $f = 0$  generation (Fig. 2: ALL). However, both populations and lines within populations strongly differed in their response to inbreeding (interaction effects, Table 2). For populations, the difference in overall infection rate between the lowest and highest inbreeding level ranged from a significant 71% decrease (Fig. 2: BEI) to a nonsignificant 32% increase (Fig. 2: BYL). The response of lines within populations differed significantly in both magnitude and direction, ranging from a change from complete resistance at  $f = 0$  to 57% infection at  $f = 0.59$  (BYL-1,  $G = 10.6$ ,  $df = 1$ ,  $P < 0.01$ ) to a change from 47% infection at  $f = 0$  to complete resistance at  $f = 0.59$  (HEN-11,  $G = 13.3$ ,  $df = 1$ ,  $P < 0.001$ ). Further inspection of the data showed that the relationship between inbreeding level and percentage disease was often nonlinear, both at the population and at the family levels. In many lines inbreeding led to an increase in infection rate up to the  $f = 0.375$  or  $f = 0.50$  level, followed by a decrease upon further inbreeding. Attempts to analyze quadratic regression components ( $f^2$ ) for the complete model presented in Table 2 failed because of nonconvergence of the complete generalized model. However, analysis of separate lines revealed that out of the 60 lines for which more than three inbreeding levels were available, 26 showed a significant (linear and/or quadratic) effect of inbreeding on infection rate, and in 17 of these cases a significant quadratic component was observed. We conclude, therefore, that the magnitude, direction, and shape of the response of resistance to inbreeding strongly varies among lines.

### Experiment 2

Inbreeding resulted in a decrease in plant size, as indicated by the reduced maximum leaf length of 4-wk-old plants (Table 3, Fig. 3). Inbreeding had a significant impact on flower size and floral nectar rewards (Table 3). After four generations of sib mating, petal blade length, and hence corolla diameter, was significantly

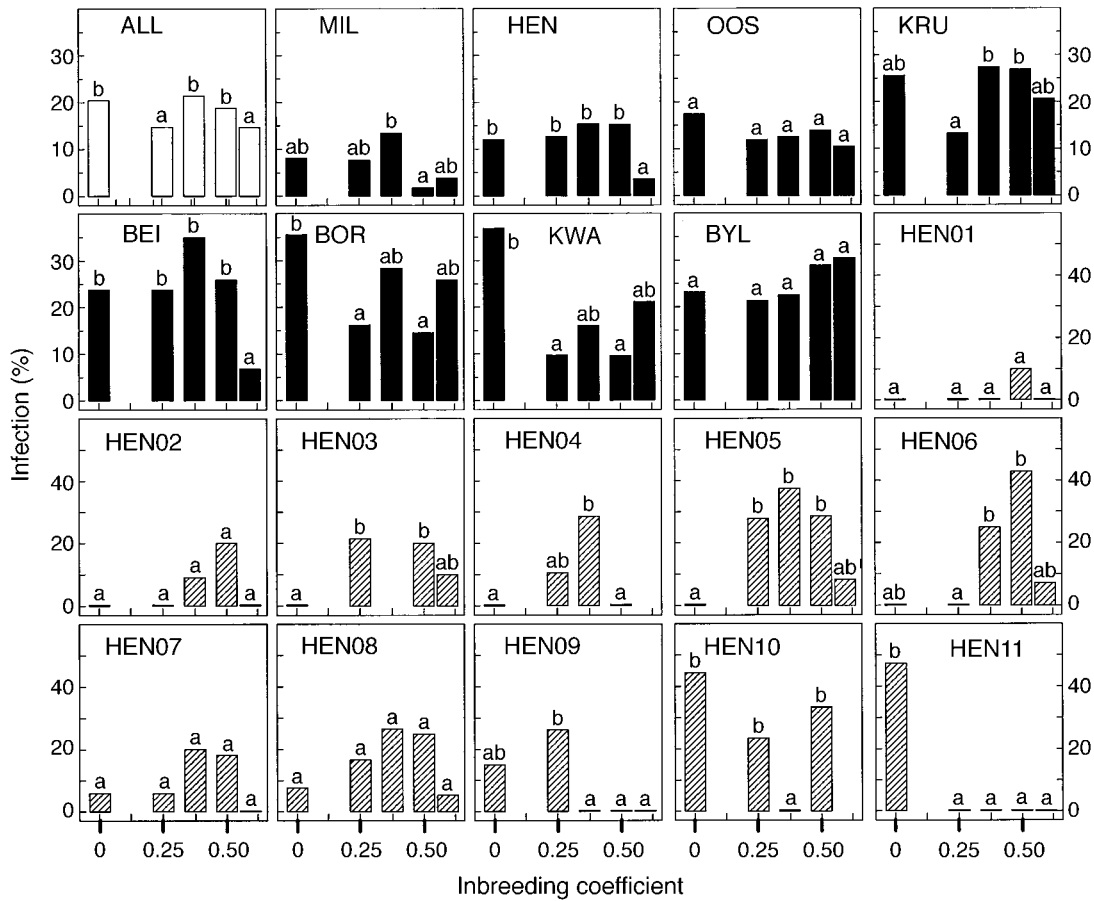


FIG. 2. Effect of inbreeding on susceptibility to the anther-smut fungus *M. violaceum* following experimental inoculation of *S. alba*. Open bars: data for all individuals included in the analysis (ALL,  $n = 3218$ ). Solid bars: data separated by population of origin (MIL–BYL). Hatched bars: data for one population (HEN) separated by line (HEN01–HEN11). Bars within frames that do not share a common letter are significantly different from each other ( $P < 0.05$ ) in pairwise *G* tests.

TABLE 3. Generalized linear model of the effects of inbreeding ( $f = 0, 0.375$ , and  $0.59$ ), line, and sex on leaf length, number of leaf pairs, and floral traits in *S. alba* (population HENGEL0).

Source	Leaf length ( $n = 668$ )			No. leaf pairs ( $n = 660$ )			Calyx length ( $n = 673$ )			Calyx width ( $n = 673$ )		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
Inbreeding	1, 11	29.45	0.000***	1, 11	0.92	0.358	1, 11	0.29	0.603	1, 11	1.00	0.340
Line	11, 620	4.12	0.000***	11, 612	5.45	0.000***	11, 625	4.80	0.000***	11, 625	2.73	0.002**
Sex	1, 11	2.04	0.181	1, 11	0.55	0.473	1, 11	14.00	0.003**	1, 11	511.13	0.000***
Inbr. × Line	11, 620	2.15	0.016*	11, 612	10.31	0.000***	11, 625	11.02	0.000***	11, 625	3.38	0.000***
Inbr. × Sex	1, 11	0.79	0.393	1, 11	0.00	0.957	1, 11	0.45	0.516	1, 11	1.62	0.229
Line × Sex	11, 620	1.72	0.065	11, 612	0.73	0.713	11, 625	1.83	0.046*	11, 625	1.34	0.199
Inbr. × Line × Sex	11, 620	1.68	0.074	11, 612	1.07	0.386	11, 625	1.08	0.376	11, 625	2.09	0.019*

Notes: Line was treated as a random factor. Quasi *F* values and *P* values are indicated.  
\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .



reduced in both male (reduced by 5.1%) and female hosts (10.4%) (Fig. 3). Nectar volume was strongly reduced (35.3%) by inbreeding in females, whereas no significant response was observed in males. As a result, nectar volume of females at the highest level of inbreeding reached the lower levels that were observed for males (Fig. 3). Calyx size, depth of the corolla (petal claw length), and nectar sugar content were not significantly affected by inbreeding in either of the host sexes.

Results of the inoculation study were similar to those of experiment 1. Inoculated plants of population HEN grown in the greenhouse showed a small, significantly positive main effect of inbreeding on resistance to the fungus ( $\chi^2 = 6.6$ ,  $df = 1$ ,  $P = 0.0103$ ); the proportion of infected plants after inoculation decreased from 25.9 ( $f = 0$ ) to 21.3 ( $f = 0.59$ ). However, as in the previous experiment, strong interactions between inbreeding and line were observed, indicating that lines responded differently to inbreeding with respect to biochemical resistance (Line:  $\chi^2 = 23.3$ ,  $df = 11$ ,  $P = 0.0158$ ; line by inbreeding interaction:  $\chi^2 = 39.6$ ,  $df = 11$ ,  $P = 0.0001$ ).

For all traits except leaf length and nectar sugar content, effects of inbreeding varied significantly among lines (Table 3). To illustrate these differences, we calculated standardized regression coefficients from linear regressions of trait values on inbreeding level for each line (Fig. 4). For most traits, lines with significantly positive and with significantly negative coefficients can be observed, indicating that not only the magnitude but also the direction of inbreeding effects differ among lines. Female hosts of lines that showed strong inbreeding depression in one of the eight size or reproductive traits tended to show strong inbreeding depression in the others as well. This is indicated by the significant difference in average values of the standardized regression coefficients among lines (Line effect for females:  $F = 4.19$ ,  $df = 11$ ,  $84$ ,  $P < 0.001$ )

for these eight traits (Fig. 4). By contrast, the response to inbreeding of males from specific lines varied strongly among traits, and no differences in average response of the different size and reproductive traits were observed among male host lines (Line effect for males:  $F = 0.49$ ,  $df = 11$ ,  $84$ ,  $P = 0.90$ ). There was no among-line correlation between inbreeding effects on biochemical resistance and inbreeding effects on any of the size or reproductive traits (females,  $r = -0.32$  to  $+0.23$ ; males  $r = -0.28$  to  $+0.40$ , all  $P > 0.19$ ).

#### DISCUSSION

Transient dynamics of the host–pathogen interaction at the local population level, driven by metapopulation dynamics at the regional level, have been put forward as one of the explanations for the often observed variation in resistance in local host populations (Parker 1992). Under a metapopulation scenario, host populations frequently go extinct, and new populations are established by recolonization of sites. Consequently, founder effects and inbreeding will shape the genetic structure of local host populations, influence the resistance levels and could be important regulating processes in host–pathogen dynamics. Therefore, investigating the response of resistance levels in the host to varying degrees of inbreeding is most relevant for our understanding of the dynamics of host–pathogen interactions.

We used the *Silene alba*–*Microbotryum violaceum* pathosystem as a model system to investigate the impact of inbreeding in the host on the interaction between host and pathogen. The system has been described as an example of metapopulation dynamics (Antonovics et al. 1994, McCauley et al. 1995, Thrall and Antonovics 1995). In North America, frequent extinction of local populations, followed by recolonization from neighboring populations, occurs (Antonovics et al. 1994, McCauley et al. 1995). McCauley et al. (1995) estimated the average effective number of col-

TABLE 3. Extended.

Petal blade length ( $n = 671$ )			Petal claw length ( $n = 672$ )			Nectar volume ( $n = 671$ )			Nectar sugar conc. ( $n = 594$ )		
df	$F$	$P$	df	$F$	$P$	df	$F$	$P$	df	$F$	$P$
1, 11	7.02	0.023*	1, 11	0.49	0.500	1, 11	5.12	0.045*	1, 11	0.29	0.603
11, 623	3.62	0.000***	11, 624	5.78	0.000***	11, 623	1.39	0.173	11, 546	1.33	0.200
1, 11	1.80	0.207	1, 11	142.51	0.000***	1, 11	27.50	0.000***	1, 11	4.77	0.052
11, 623	8.63	0.000***	11, 624	5.84	0.000***	11, 623	1.81	0.049*	11, 546	1.62	0.090
1, 11	2.63	0.133	1, 11	2.17	0.169	1, 11	3.20	0.101	1, 11	1.50	0.246
11, 623	1.59	0.097	11, 624	4.05	0.000***	11, 623	1.84	0.045*	11, 546	1.78	0.053
11, 623	0.98	0.464	11, 624	3.87	0.000***	11, 623	1.56	0.108	11, 546	1.15	0.318

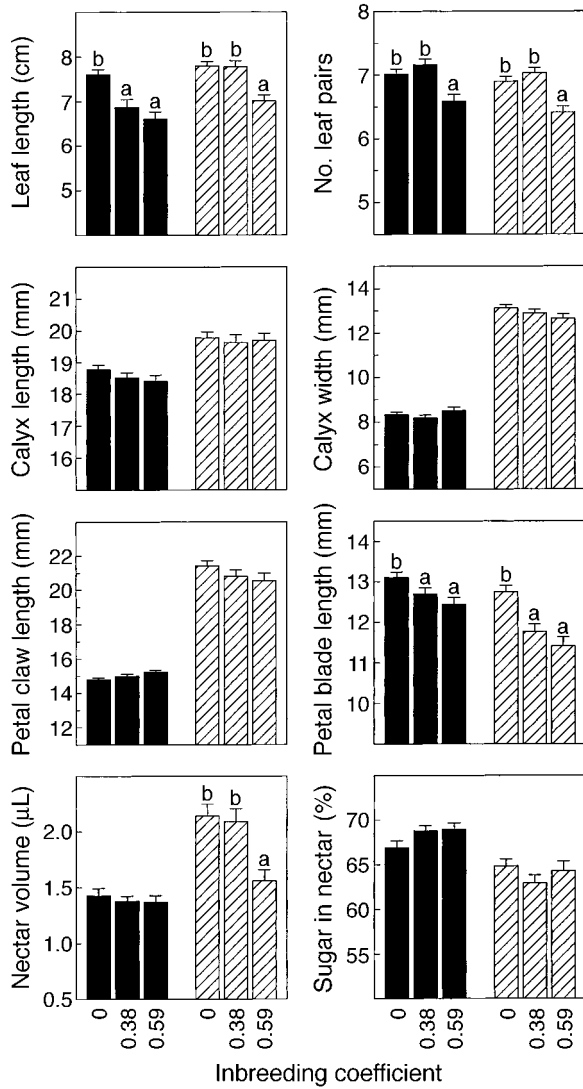


FIG. 3. Effect of inbreeding on floral traits in male (solid bars) and female (hatched bars) plants of *S. alba*. Mean values + 1 SE are indicated. Bars of each host sex within a frame that do not share a common letter are significantly different from each other (SNK test,  $P < 0.05$ ). The absence of letters for bars indicates the absence of significant differences.

onists in road verge populations of *S. alba* in the United States to be around four. Moreover, they demonstrated that in the founder pool there is only limited mixing of individuals from different populations. Thus, each host population is founded by a few genotypes, and inbreeding through mating among relatives will be a common process in local populations. McCauley et al. (1996) estimated  $F_{IS}$  for one population to be 0.017, with a 95% confidence limit of +0.108 to -0.074. However, the inbreeding level may vary considerably among populations, depending on their age and history, as well as among individuals within populations.

No detailed studies on metapopulation dynamics in this system in the Netherlands have been performed.

However, in a 3-yr survey of host populations in the Netherlands, extinctions and recolonizations were observed (N. J. Ouborg and C. L. Mudde, *unpublished manuscript*), suggesting that metapopulation dynamics could also be characteristic for Dutch populations.

Our results demonstrate that inbreeding in the host affects both active and passive resistance mechanisms. Active resistance, as measured by the results of forced inoculation, varied in a nonlinear way among inbreeding levels but on average was higher in the highest than in the lowest inbreeding level. However, significant variance in the response to inbreeding was found among populations (first experiment) and among lines within populations (both experiments), making statements about the direction of the inbreeding effect hard to generalize.

Our results indicate that inbreeding may also affect field resistance of plants through alteration of floral traits. Although significant variation in inbreeding response was found among lines for all flower traits, inbreeding consistently reduced flower size and, at least in females, nectar volume. For most other flower traits, lines with both significant inbreeding depression and significant inbreeding enhancement were found. Studies of deposition of *M. violaceum* spores in *Dianthus silvester* have shown that spore deposition significantly decreases with flower size, and tends to decrease with floral nectar rewards (Shykoff et al. 1997). Thus, inbreeding may increase passive resistance by enhancing avoidance of spore deposition. This effect was more prominent for females than for males.

Two types of results from our experiments are particularly important for our understanding of the interaction between host and pathogen. The first, most striking result is that inbreeding effects were strongly host genotype dependent. Significant interactions between inbreeding level and host genotype were found for all traits and in both experiments. The response of active resistance of lines differed even to the extent that inbreeding enhances resistance in some lines and reduces resistance in others.

Strauss and Karban (1994) also demonstrated that the effect of inbreeding in the host plant *Erigeron glaucus* on its resistance against the herbivorous thrips, *Apterothrips apteris*, was genotype dependent. Their experiment involved two inbreeding levels ( $f = 0$  and  $f = 0.5$ ), and genotypes were pooled into two groups (high resistance vs. low resistance). In experiments with a design similar to our own experiments, i.e., with a number of inbreeding levels and a series of genotypes/lines, differences in inbreeding effect among lines, comparable to the differences we found here, were discovered. Dudash et al. (1997) found significant variance among lines in inbreeding effects on lifetime relative fitness in the annual plant *Mimulus guttatus*. Pray and Goodnight (1995) report significant differences among lines in inbreeding effects on several life history parameters in the red flour beetle *Tribolium castaneum*. Significant

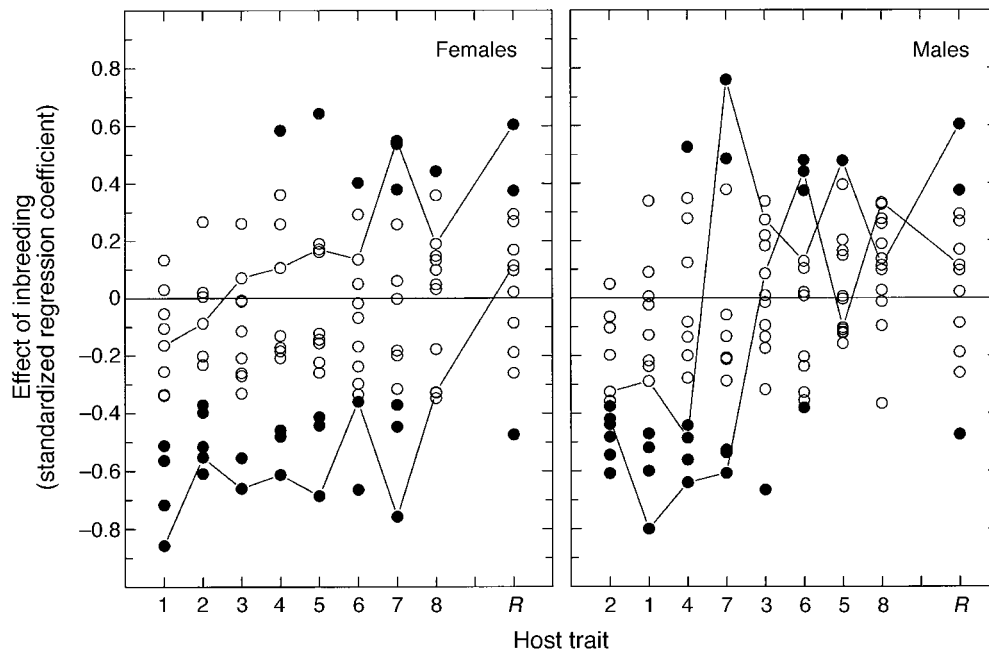


FIG. 4. Effect of inbreeding (standardized regression coefficients from linear regressions of traits on inbreeding coefficient) for nine different host traits in 12 host families of *S. alba*. Closed symbols indicate that effects of inbreeding are significant; open symbols indicate  $P > 0.05$ . Lines connect values for the two families with the highest and lowest overall responses. Traits: (1) Petal blade length; (2) Leaf length; (3) Nectar volume; (4) Number of leaf pairs; (5) Petal claw length; (6) Calyx length; (7) Calyx width; (8) Nectar sugar content; R, resistance (percentage of plants not infected after inoculation). Traits are ranked according to the mean overall response of families for each host sex.

among-line variation in inbreeding effects on susceptibility to parasitic infection has been found in *T. castaneum* (Stevens et al. 1997). Similar to our experiments, lines with inbreeding depression, lines with inbreeding enhancement, and lines not affected by inbreeding were found in each of these examples.

Inbreeding and inbreeding depression are normally treated as population phenomena. Variation in inbreeding effects among populations is explained by differences in inbreeding history (Brewer et al. 1990, van Treuren et al. 1993, Ouborg and van Treuren 1994) or by differences in inbreeding level among populations (Dole and Ritland 1993, Husband and Schemske 1996). However, we found differences in inbreeding effects among genotypes originating from the same population, indicating that inbreeding and inbreeding depression are genotype-level rather than population-level phenomena. The observed variation may be the result of genetic drift, founder events, and fixation of different alleles (Wright 1977), or of variation in the initial inbreeding coefficient  $f$ . Lacy (1992) used the term "historical accidents" to summarize these effects. In the *Silene alba* populations these accidents will be a consequence of the extinction/recolonization cycle of local host populations. The genotype(s) of the founder host(s) will determine the level of resistance against the fungus as a function of subsequent inbreeding. Given the large variation in response among genotypes, the response of resistance to inbreeding is unpredictable at the population level. Thus, a full

understanding and an accurate prediction of the outcome of the dynamic interaction between the host and the pathogen is only possible if the genotype composition and the within-population variance in inbreeding response is known.

A second striking result of our experiments is that there is no correlation between the effects of inbreeding on active and passive resistance at the genotype level. While the response of active resistance varies between "inbreeding enhancement" and "inbreeding depression", the responses of two important components of passive resistance (flower size and nectar volume) vary independently between "no effect" and "inbreeding depression". The implication of this result is that inbreeding may have reinforcing as well as opposing effects through active and passive resistance mechanisms. The net effect of inbreeding on field resistance is genotype dependent. This underlines the conclusion that the outcome of the dynamic host-pathogen interaction is unpredictable at the local population level.

Thus, prediction of host-parasite dynamics at the population level is frustrated by variation among genotypes in the magnitude and direction of their response to inbreeding. Predictions will be further complicated by variation among genotypes in the shape of their response to inbreeding. In 17 lines, a significant nonlinear component in the relationship between inbreeding level and active resistance was found. A significant curvilinear relationship between inbreeding

level and trait value is generally interpreted as evidence for epistatic interactions among genes involved in the expression of the particular trait (Crow and Kimura 1970) and has been found in other detailed inbreeding studies (Pray and Goodnight 1995, Dudash et al. 1997). Here, we interpret the nonlinearity as a reflection of the genetic complexity of the trait "active resistance." Although active resistance was operationalized simply as the fraction of host individuals that do not become infected after artificial inoculation, the processes involved in active resistance may be highly complex, and may be affected by many genes (Alexander 1990b).

Genome purging is often mentioned as an alternative explanation for nonlinear relationships between inbreeding level and inbreeding effect (Barrett and Charlesworth 1991). This could have affected the results, if during the process of creating the inbreeding levels, selection against deleterious recessive alleles decreases their frequency and purges the genome of its potential for inbreeding depression. However, in our experiments in each generation of sib mating, individuals were chosen at the seedling stage, when no knowledge of their potential level of active resistance was available. Therefore, no intended selection on active resistance was performed, and we are inclined to reject the purging hypothesis here.

The results have implications for the concept of increased risks of disease in small, inbred populations. In such populations the frequency of homozygotes will rise, as a consequence of loss of alleles by drift, and/or the effects of inbreeding (Soulé 1986). It has been argued that this may make these populations more susceptible to diseases (O'Brien and Evermann 1988). Evidence for this effect is reviewed by O'Brien and Evermann (1988), but others have questioned the validity of this evidence (Nunney and Campbell 1993). The genotype-dependent effects we found here demonstrate that for this pathosystem it is too much of a simplification to automatically link increased inbreeding to increased susceptibility to diseases. While we are dealing with a host plant, the same conclusion was reached for susceptibility of an insect host to parasite infection (Stevens et al. 1997). The evidence reviewed by O'Brien and Evermann (1988) mainly concerns vertebrate data. Taxonomic differences in physiological and genetic complexity of immune and resistance mechanisms prevent generalization of our results to other taxonomic groups. However, Stevens et al. (1997), following Caughley (1994), suggest that more rigorous experimentation might show that a link between homozygosity and disease susceptibility is less common than believed. Our data support this suggestion.

It has been argued that integration of ecology and genetics is indispensable for a thorough understanding of the dynamics of natural host–pathogen systems (Alexander et al. 1996). This argument was mainly based on the observed genetic differences in resistance among host genotypes. The results presented in this paper sup-

port this argument by showing that genetic variation exists for both active resistance and components of passive resistance in the host plant *Silene alba*. In addition, the results further extend the argument by showing that the effects of inbreeding are also different among host genotypes. Progress in our understanding of natural host–pathogen systems is therefore most likely to result from studies employing an integrated approach of ecology and genetics of the metapopulation dynamics of both host and pathogen.

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