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Ecological and genetic factors influencing a natural plant-pathogen interaction

Morrison, Janet Anne, Ph.D.

State University of New York at Stony Brook, 1994

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Ecological and Genetic Factors Influencing a Natural Plant-Pathogen Interaction

A Dissertation Presented

by

Janet Anne Morrison

to
The Graduate School
in Partial Fulfillment of the
Requirements
for the Degree of
Doctor of Philosophy

in

Ecology and Evolution

State University of New York
at Stony Brook

May 1994

State University of New York
at Stony Brook
The Graduate School

Janet Anne Morrison

We, the dissertation committee
for the above candidate for the
Doctor of Philosophy degree,
hereby recommend acceptance
of this dissertation.

Dr. Jessica Gurevitch, Associate Professor, Department of Ecology and Evolution, Dissertation Advisor

Dr. Charles Janson, Associate Professor, Department of Ecology and Evolution,
Chairperson of Defense

Dr. James Thomson, Professor, Department of Ecology and Evolution

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This dissertation is accepted
by the Graduate School

Abstract of the Dissertation
Ecological and Genetic Factors Influencing a Natural Plant-Pathogen Interaction

by

Janet Anne Morrison
Doctor of Philosophy
in

Ecology and Evolution

State University of New York
at Stony Brook

1994

Little is known about the ecological and genetic factors that influence plant-pathogen interactions in nature, although theories about the importance of pathogens assume that certain factors are paramount. The idea that pathogens regulate host population size assumes that pathogens respond strongly to host plant density. The idea that pathogens favor sexual reproduction in plants assumes that they respond strongly to host plant genotypes. As microscopic organisms, it is possible that plant pathogens are most affected by local environmental factors.

In this dissertation, the influences of some potentially important factors were examined in a three-year spatial study of a Long Island pine barrens population of the perennial rush *Juncus dichotomus* naturally infected by the smut fungus *Cintractia junci,* and in three multifactorial field experiments. For the spatial study, the relationships between disease and

density, nonhost plant density, soil water content, and host genetic variation were examined by comparing surface plots and spatial autocorrelations, and/or by calculating partial Mantel-test correlations. The experiments consisted of plantings and inoculations of propagated replicates of uninfected plants that were collected from the natural population and genotyped electrophoretically. One experiment tested the effects of host density, pathogen density, host genotype, and block heterogeneity; another tested the effects of heterospecific neighbors, host genotype, and block heterogeneity; the last tested the effect of soil water, host genotype, and block heterogeneity.

In the natural population, disease had stable spatial pattern, and disease and host density were positively correlated, with disease appearing to thin the population in a density-dependent manner. No associations were detected between disease and the other factors. Experiments showed that plants exposed to higher pathogen loads were more likely to be infected, that plants grown at high host density or in a dense stand of heterospecifics were smaller and less likely to become infected, that plant genotypes were variable for infection, that genotype X environment interactions were evident, and that environmental heterogeneity influenced infection.

To David, for his love, encouragement, faith, and help . . .

To Alex, for providing so much joy since February 10th, 1990 . . .

To Scott, for being the most important reason to finish this dissertation.

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is the one who is truly involved in my field of research. His comments on the dissertation helped me to better place my research into the field of plant-fungal interactions.

John MacDonald taught me how to do electrophoresis and started me off in SAS. Marie-Josee Fortin and Barbara Thomson generously gave time showing me how to do spatial analysis. Michael Axelrod, the director of the Stony Brook greenhouse, was very accommodating and helpful during the years that I grew plants there.

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1

Overview

Two ideas motivated this thesis research - that plant diseases are likely to have important impacts on plant population biology in nature but have been relatively ignored until recently, and that in order to understand the potential importance of plant diseases it is necessary to elucidate the ecological and genetic that factors influence plant-pathogen interactions. In this overview I will first generally discuss these two ideas and follow with a presentation of the approach I used to study one plant-pathogen interaction, some biological information on the studied organisms, an outline of the particular research in each chapter, and a summary and synthesis of the results.

On an individual-plant basis, disease can directly affect a host plant at a number of discrete stages in its life cycle (Agrios 1988). In the life cycle of a typical flowering plant there are four stages that plant disease directly affects if the plant is an annual and five if it is a perennial. Since disease can cause mortality it may directly affect seed survival, seedling survival, and for a perennial, the survival of perennating tissue. Disease can also influence plant growth, and if it is a disease that attacks the reproductive structures it

can influence flower production as well (Figure 1). As a class of organisms, plant pathogens clearly have the potential to be very important influences on host plant biology since they may impact so many different parts of a plant's life.

The importance of plant disease has long been recognized in agriculture, where it can have serious economic costs (Agrios 1988, pp. 17-30). The mere fact that so much time, money, and effort is put into fighting plant disease in crops is an indication of how influential pathogens can be in plant biology. In striking contrast is the lack of attention paid to plant disease in the study of natural plant populations (Burdon 1987a), which is only recently being remedied. Plant disease could be at least as important in natural populations as it is in agricultural populations. In many crops, plants are exposed to pathogens for only a part of their life cycle and, in addition, crop plants receive special care in the form of fertilizers, irrigation, and pesticides that may allow them to more readily resist disease. In nature, however, every plant life stage is a potential target of pathogen attack and the plant can rely only on its own resources for defense.

Since pathogens have the potential to directly influence both mortality and reproduction of individual plants, they also have the potential to impact the population biology of host plants in ways that can be important to both their ecology and evolution. Ecologically, the population

dynamics of a host plant population will be affected by a disease that increases mortality rates and/or decreases fecundity. The consequence may be a decrease in host population size and density (Janzen 1970, Anderson 1978, Burdon 1987a), which itself may lead to changes in the composition of the community to which the host plant belongs. Indeed, some of the more well-known plant disease epidemics in nature owe their fame to the harrowing changes they have wrought in their host plant's communities. For example, chestnut blight virtually eliminated one of the dominant canopy trees in temperate deciduous forests of North America, so that the composition of forest tree communities today is drastically different than what it was before the fungus was introduced (Day and Monk 1974). As a biotic disturbance that continuously opens gaps in space held by a host plant population, plant disease has been suggested as a critical factor promoting species diversity (Harper 1969, Janzen 1970, Connell 1971, Chilvers and Brittain 1972, Holt and Pickering 1985).

The greatest ecological effects in host plant populations (and their communities) theoretically occur when the pathogen responds to the plants in a density-dependent manner (Burdon and Chilvers 1982). The pathogen is expected to increase and have greater effect in a dense host population, where disease transmission is more efficient. It then can act to thin out the host population, leading to lower density and more room for other species

not attacked by that pathogen.

Disease influence on plant population biology may also have evolutionary consequences. Pathogens that cause mortality and/or reduce reproduction will do so differentially in plants that differ in resistance and thus the pathogen may act as an agent of natural selection. If plant resistance to a pathogen is genetically-based, heritable, and variable in the population, then selection by the pathogen may lead to the evolution of host plant characters that cause or are correlated with resistance (Alexander 1992).

As agents of selection that are alive, pathogens can themselves evolve in response to host plants. Theoretically, a pathogen population will most easily evolve virulence in a host plant population it encounters when the plant population has low genetic diversity. If one resistance allele is at high frequency in the plant population then the pathogen needs only to evolve virulence to overcome that one resistance allele in order for it to spread throughout the host population. In contrast, if the host population is very diverse genetically, with many resistance alleles at moderate frequency, then it should be more difficult for a pathogen population to evolve virulence. This reasoning has led many people to suggest that pathogens may therefore be at least partly responsible for the evolution and maintenance of sexual reproduction (Levin 1975, Jaenike 1978, Hamilton et al 1990). Sexually

produced offspring would be more likely to possess combinations of resistance alleles that differ from their parents and thus be more resistant to pathogen strains that were successful at infecting and reproducing in their parent's generation. This scenario depends on pathogens responding evolutionarily to host genotypes in a frequency-dependent manner.

Clearly, pathogens can have large effects on many aspects of an individual host plant life cycle, and because they may impact mortality and reproduction they may influence plant population biology as well. Thus, plant-pathogen interactions should be an important and promising area of research. The ultimate goal of such research would be to document and understand the actual role of plant diseases is in plant population biology. That role (or lack thereof) depends on whether pathogens primarily act on plant populations in a density dependent manner, respond to plant genotypes in a frequency dependent manner, or do neither, being influenced mainly by environmental conditions. This is where a problem arises in our understanding. We have very little information about exactly which factors plant pathogens respond to under natural conditions. Are pathogens primarily influenced by host plant density, so that disease builds up and causes the greatest effect where plants are at high density? Do pathogens primarily respond to host plant genotypes, so that disease increases where host plants are genetically susceptible and the population has low genetic diversity?

Perhaps pathogens are most affected by the physical environmental conditions for their survival and growth, so that disease develops where those conditions are favorable. Finally, it may be a combination of these processes that govern plant-pathogen interactions.

Although the number of empirical studies of natural plant-pathogen systems is mounting, there are very few that have attempted to look at multiple factors that may drive the interaction. For example, many studies have now documented that host plants in nature are genetically variable for resistance to disease (Burdon 1987a), but hardly any have shown that it is in fact the host genotype that actually determines whether infection occurs under natural field conditions. For example, even if a susceptible host genotype is present at high frequency in a population, disease may not develop appreciably in the population because the plants are at low density or because they grow in a site that is too dry for fungal spore germination. Without knowing what interacting ecological and genetic factors are actually important in a plant-pathogen system, it is not possible to conceptually understand the role that pathogens have in plant population biology. Thus a central research goal in the study of plant-pathogen interactions shouls be to identify what factors are important under natural field conditions. This is the subject of my thesis.

Identifying all the possible factors (and their interactions) that actually

influence plant-pathogen interactions in nature is obviously beyond the scope of a thesis. So, I first narrowed the problem by examining only one plant-pathogen interaction, in the hope that eventually enough different systems will be studied in this way so that we may be able to make some generalizations. My approach to the problem was two-fold. First, I studied the spatial and temporal patterns of naturally-occurring disease in one plant population over three years and compared its patterns with those of some hypothesized influential factors in the same site. A correspondence between disease pattern and a hypothesized factor's pattern suggests a possible causal relationship relevant to the actual natural population. Such a study is essentially descriptive and only correlative, but its power lies in the fact that it describes the patterns and relationships of factors in a real, ongoing plant-pathogen interaction.

I paired the natural population study with a series of three field experiments in which I manipulated some of the same putative factors that were studied descriptively in the natural population, using plant and pathogen material originating from the natural population and set up in sites very similar to the natural site. The ideal experiment would be a huge multifactorial design with all possible levels of all possible factors included and allowed to interact. Not only would such an experiment be physically impossible to carry out, it would probably yield uninterpretable results since

the human mind can perceive only so many dimensions at once. My alternative was to make the experiments multifactorial in order to look for possible interactions among the factors, but to limit each one to just a few factors, trying to control for the others experimentally. If only a series of single factor studies are done, then there can be no estimation of the prevalence of interactions in the system. At least if a series of experiments is done with several factors each, and if some of those factors are repeated in each experiment but in combination with different sets of other factors, then an attempt can be made to determine the general importance of interactions among the factors.

The power of this two-fold approach to studying the important factors in a plant-pathogen interaction is that the descriptive study and the experimental work are complementary. The relationships between disease and putative factors described from the natural population can be tested for causality in the experiments. In turn, the experimental results are relevant to an actual population and their results can be more fully understood in light of results from the natural population.

Seven factors and their relationship to disease were included in this research. Because of the theoretical importance of density-dependent interactions between plant and pathogens and frequency-dependent responses of pathogens to plant genotypes, I examined host plant density,

host plant genotype, and host plant genetic diversity as three of the factors. Because plant density and pathogen inoculum density are likely to be correlated where there is positive relationship between plant density and disease, in one experiment I also tested pathogen inoculum density as a factor separate from host plant density. I was also interested in how influential environmental factors are and how the environment might interact with host plant density and host genotype in determining the actual pattern of disease seen in nature. So, I also examined the effect of soil water content, the surrounding plant community, and general environmental heterogeneity. Soil water was chosen because many aspects of both plant and fungal biology are influenced by water, and in the natural population site the most noticeably variable physical feature was topography associated with a nearby bog, such that the site appeared variable for soil water content. The plant community of the host plant was chosen as a factor because it constitutes in large part the biotic environment of the host plant. The presence of heterospecifics in the vicinity of a host plant can affect transmission of the pathogen by acting as barriers to spore transmission, and can alter the physiological status of a host plant and thus its ability to defend itself against pathogen attack. Environmental heterogeneity was included as the block factor in the experiments as a way to account for the effect of all the other environmental variables that may contribute to a

pathogen's success at infecting a plant, but which were not explicitly measured or manipulated. The potential roles of each of these seven factors are discussed at greater length in the "Introduction" sections of the appropriate chapters.

I studied a population of the perennial rush *Juncus dichotomus* Ell. (Juncaceae) which was infected by the smut fungus *Cintractia junci* Schw. (Ustilaginales, Basidiomycetes). Details about the organisms and the site are in Chapter Two. Here I will just highlight a few important points. I began the study when the epidemic of smut in this population was firmly established and the frequency of disease was high enough to enable a study. The rush grows as a clump of shoots emerging from an overwintering rootstock. Each shoot has several leaves and bears a multi-flowered, wind pollinated inflorescence. The mating system is not known for this plant, but a high selfing rate is likely (see Chapter Two). Plants produce abundant seed, but I observed few young seedlings over three years of observations.

Fungi in the genus *Cintractia* only infect plants in the Juncaceae and Cyperaceae, and *C. junci* is known only from the host genus *Juncus* (Fischer 1953). In *J. dichotomus,* the smut may infect entire shoots or isolated fruits on otherwise healthy inflorescences. Infected shoots have zero seed production since the fungus systemically invades and deforms the entire inflorescence, using it for its own reproduction. In general, smuts

overwinter as teliospores or as mycelium in infected plants, and they have one generation per year, so spores arising from lesions produced in one year give rise to new infections that would produce spores the following year (Agrios 1988, p. 475).

Although the life cycle of this particular smut fungus has not been studied, it undoubtedly shares the following essential stages with other smuts (Agrios 1988 pp. 474-485). An infected lesion produces dikaryotic teliospores which are dispersed into the environment. The teliospore germinates and its two nuclei fuse and undergo meiosis. Haploid hyphae grow from the teliospore and may produce haploid basidiospores or sporidia. A haploid hypha or sporidium fuses with one of opposite mating type, produces an infective dikaryotic hypha that penetrates host tissue, and eventually gives rise to a new crop of teliospores. Germination and penetration may occur before winter, in which case the fungus overwinters as mycelium inside host tissue, or it may occur after the winter, in which case the teliospores overwinter. In the *J. dichotomus* - *C. junci* system the fungus appears perennial; once an infection is established in a plant, that plant will remain infected in subsequent years, and the proportion of shoots infected on the plant tends to increase.

Chapter Two presents the results of a spatial study spanning 1990 through 1992. In this study I measured disease frequency (percent of shoots

infected per square meter) each year in permanent square meter plots spaced on a regular grid throughout most of the population. In these same plots I also measured plant density (shoots per square meter) each year, soil water content in 1992, the percent cover of nonhost plants in 1990, and in a subset of plots I measured plant genetic diversity in 1992. My goal was to describe the progress of disease in the population through time and to determine if its spatial pattern matched that of any of the other variables, thus suggesting a causal relationship. I did this by creating surface plots of disease which I visually compared to surface plots of density and soil water, and by calculating correlations between disease and the various variables using partial Mantel tests, which allowed me to hold spatial position of the sampling points constant statistically. Standard correlation analysis was not appropriate since the data were spatially located and thus not independent from each other. I also compared the spatial autocorrelation structure of disease and plant density. These methods are discussed at length and referenced in Chapter Two.

Chapter Three presents the results of one experiment that tested for the influence on infection of heterospecific, nonhost neighbors/competitors, environmental heterogeneity, plant genotype, and their interactions. This experiment was set up twice - once in a xeric area and once in a mesic area, so it also allowed for comparison of results in two environmentally different

sites. Plant density, plant genetic structure, pathogen density, and pathogen genetic structure were kept constant experimentally throughout all treatment plots.

In Chapter Four an experiment is described that tested for the influence on infection of host plant density, pathogen density, host plant genotype, environmental heterogeneity, and their interactions. Host plant genetic structure, pathogen genetic structure, and the plant community were each kept constant experimentally throughout the plots in the experiment.

Finally, Chapter Five presents an experiment that tested the influence of soil water, host plant genotype, and environmental heterogeneity, and their interactions on infection by and recovery from the pathogen. Host plant density, host plant genetic structure, pathogen density, pathogen genetic structure, and the plant community were kept constant experimentally in all plots of the experiment.

All of the experiments were done in sites that had similar conditions to those from the natural populations - sandy soils, abundant light, and herbaceous vegetation. The plants used in the experiment were obtained healthy from the natural populations and successively propagated in the greenhouse for many months, yielding multiple copies of each individual. Starch gel electrophoresis was used to determine that the original individuals were genetically distinct. In all of the experiments the healthy plants were

artificially, individually inoculated from a teliospore suspension made from sporulating lesions collected from from many infected plants throughout the natural population.

The results from the spatial study and the three experiments are discussed in detail in the chapters. Here I will highlight and synthesize those various findings, starting with the relationship between disease and host density. In the natural population (Chapter Two) disease and density showed similar spatial pattern and autocorrelation structure, There was some evidence that the pathogen affected the host population in a density-dependent manner. In the first two years of the study, density and disease were positively correlated, but by the third year they were not. At the same time there was a decrease, on average, of plant density and the decrease was more pronounced in plots that started at high density. Plots that had no disease present in 1990 tended to increase in density between 1990 and 1991, while many plots that contained diseased plants decreased in density. The same pattern (but less pronounced) was seen for 1991-1992. So, it appears likely that over the three years of the study the pathogen is at least one important agent of density-dependent regulation of plant population size.

In contrast to those findings from the natural population, when plant density was tested experimentally in the field, I found that it was the low density plants that were more likely to be infected after artificial inoculation

(Chapter Four). An important difference between the natural population and the experiment was that in the natural population the relationship I saw between disease and host density was conceivably due to the effect of host plant density on every step of the disease cycle - from transmission between plants, to the effect of plant density on the microsite for spore germination, to intraspecific plant competition that could alter host resistance. In the experiment, however, I artificially inoculated each plant by hand and examined them for infection the following season, so transmission was not a factor. Instead, I looked only at the effect of host plant density on infection, skipping the inoculation/ transmission stages. I postulated that host plant density could influence infection through intraspecific competition stressing plants and making them less resistant, or that it could have the opposite effect; under higher intraspecific competition they could be too small and weak and therefore poor resources for the pathogen. There was also the possibility that the microclimate under a dense host canopy would differ from that under a low density host canopy, altering the fungal microsite. Either of these last two possibilities were supported by my result that plants at low density were more likely to be infected. I can not definitely determine which of the two mechanisms were important for this outcome, but there is some evidence for the poor pathogen resource hypothesis. Plants growing at low density were much larger than plants at high density and there was a

positive relationship between plant size and infection, regardless of the density treatment plants were in.

I found the same type of result in the experiment in which host plants were inoculated and grown with or without heterospecific neighbors in their plot (Chapter Three). Plants not growing under interspecific competition were larger and they were more likely to be infected. Again, regardless of the competition treatment, there was a positive relationship between plant size and likelihood of infection. This experiment spanned two years, however, so there was the possibility of disease transmission between plants so that the presence of heterospecifc nonhost plants could have acted as barriers to transmission. In the natural population, however, I could not detect any relationship between disease frequency and the abundance of nonhost plants, but the measures used for that study were very rough categories of nonhost cover (Chapter Two).

I also showed in the host plant density experiment that the effect of plant density was independent of the effect of pathogen inoculum density (Chapter Four), suggesting that it does have some importance aside from its influence on transmission. Plants that were inoculated with high pathogen density were more likely to be infected, but there was no interaction between plant density and pathogen density.

Under natural conditions, potential host plants grow in the presence

of both conspecifics and heterospecifics. My experiments showed that a high density of either is associated with smaller plant size and lower infection rates. I did not test whether disease was related to overall vegetation density (a combination of host and nonhost plants) in the natural population, but this is a promising direction for future work. It may be that intraspecific competition and interspecific competition have similar effects on plant and/or pathogen biology and influence infection rates in a similar way. The positive correlation I found between host plant density and disease in the natural population suggests that, at the time I observed the epidemic, the effect of host plant density on the interaction was to promote transmission of the pathogen and increase infections rates, rather than to decrease rates due to effects of competition. It would be interesting to follow an epidemic from its beginning in order to observe the possibly changing roles that host and nonhost density have at different times throughout disease development.

The natural population site appeared heterogeneous in soil water content, but the spatial study suggested that disease (i.e. shoot infection) and soil water were not associated (Chapter Two). This finding was largely corroborated by the results of the experiment in which I grew plants with and without supplemental water and watched inoculated plants for infection previously infected plants for recovery from the disease (Chapter Five). The

watering treatment had no detectable effect on shoot infection or recovery. In this experiment, I looked at fruit infection in addition to shoot infection and I did find that plants growing under wetter conditions were more likely to have elevated fruit infection. This may have been due to increased humidity in the plant canopy in watered plots, since fungal spore germination leading to fruit infection is likely to occur on flowers in the canopy.

Although soil water had little impact on disease, environmental heterogeneity, as measured by the block effect in the experiments, was consistently important to whether plants became infected. The only environmental factor I explicitly measured in the natural population was soil water content, but these experimental results suggest that there may well be other variable environmental factors in the natural population that help to explain the pattern of disease I observed. Other factors which may be of interest and which are likely to have varied in the blocks used in the experiments are soil nutrients and sunlight. The key point about environmental heterogeneity, regardless of what the factor or factors are which are important, is that infection can be influenced by the environment on the scale of meters. Plants in blocks that were just meters apart experienced different probabilities of infection. The relevance of this to natural populations is that disease may develop, or at least get started, in a population in an unpredictable fashion if the environment is very

heterogenous. Larger organisms that utilize plants, such as mobile insect or mammalian herbivores, may be able to average out small-scale environmental heterogeneity, and thus their interactions with plants is less likely to be influenced by small-scale environmental patchiness. In contrast, pathogens are microscopic, and so the environment they experience and are influenced by will be extremely local. It is not surprising then that environmental heterogeneity has such a consistent impact on infection rates. The significance of this for natural populations is that in a typically heterogeneous natural site it may be very difficult to predict the development of disease.

There is a perception that serious epidemics of disease in natural plant populations are less frequent than in agricultural situations. The usual explanation for this difference is that, in agriculture, plants are genetically more homogeneous and therefore subject to epidemics by pathogen strains that can easily evolve to overcome whatever few resistance factors are present in the genetically homogenous host (crop) population (Segal et al 1980). The striking influence of environmental heterogeneity in my infection experiments suggest a second possibility - that it is also the uniformity of crop field environments that promotes epidemics. Pathogens can evolve not just in response to host plant resistance factors, but also in response to their environment. Just as it is easier for a pathogen population to evolve

virulence on a genetically homogeneous plant population so it shouls be easier for it to evolve to survive, grow, and reproduce n a homogeneous physical environment. Modern agriculture is devoted to environmental uniformity so that machines can be used for every operation. Soil is plowed to uniform depth, fields may be evenly irrigated, and plants are of similar size and density. Nature is characterized by patchiness which may help prevent the development of epidemics.

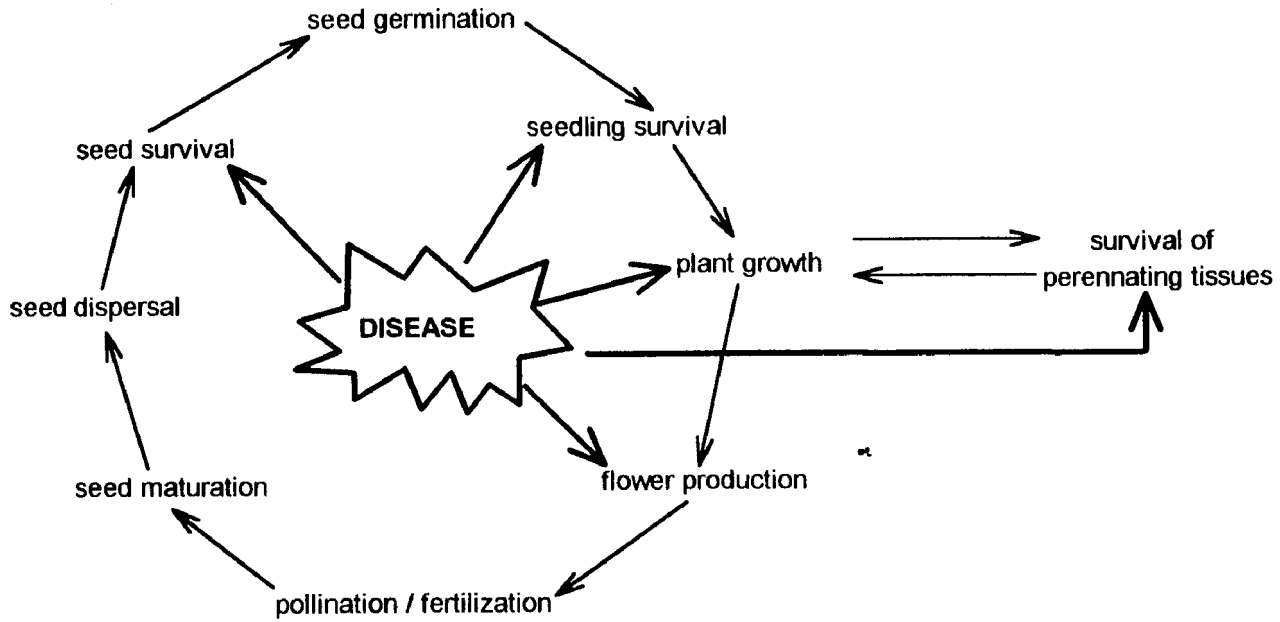
In all experiments I examined the effect of host plant genotype on infection (Chapters Three, Four, and Five), and in all experiments I detected variation for infection among the genotypes. Often the genotype factor interacted with other tested factors or blocks. In addition, in the experiment which was reproduced in a xeric and mesic site (Chapter Three) host genetic variation for infection could only be detected in the mesic site, even though the plant genotypes used in each were identical. The xeric site was more enviornmentally heterogeneous than the mesic site, suggesting that variation in infection due to environmental heterogeneity can overwhelm variation due to differential pathogen response to host plant genotypes.

My experimental results suggest that the host plant genotype has an effect on whether a plant becomes infected with the pathogen initially, although a genotype's phenotypic resistance may be contingent upon environmental factors. The plants used in the experiments were originally

taken from the natural population, so these results indicate that the population is variable for resistance to the pathogen in the natural site. Because of the theoretical importance of frequency-dependent selection on hosts by pathogens, I attempted to see if there was a relationship between host genetic diversity and disease in the natural population. I reasoned that if the plants were highly selfing and had limited dispersal then there could be appreciable spatial structure for genetic diversity even within one population, and that it could map onto spatial structure of disease. I used very few population subsamples for this test, however, and found no relationship. It is an interesting question to pursue further, since it is clear from the experiments that host plant genotype has some effect on the interaction.

Except for the weak influence of soil water, every factor I examined influenced the plant-pathogen interaction and some of these factors interacted. Clearly, it is not possible to state that, in this system at least, the plant-pathogen interaction depends solely on host plant density, or solely on the genetic interaction between host and pathogen. Small-scale environmental effects are also very influential. If this system operates similarly to other plant-pathogen systems in nature, then we still have a long road of research ahead in order to understand in any general sense how plants and pathogens interact. More multiple factor experiments done in conjunction with natural population studies are needed.

Figure 1. Disease influences on a typical flowering plant life cycle.


seed germination
seedling survival
seed survival
plant growth
survival of
perennating tissues
seed dispersal
DISEASE
seed maturation
flower production
pollination / fertilization

2

Smut fungus infection of a natural *Juncus dichotomus* population over three years.

INTRODUCTION

A first step in understanding the ecological and genetic factors that influence plant-pathogen interactions in nature is to examine the spatial patterns of the host plant, the disease, and potentially influential ecological and genetic variables over time in a natural population (Legendre and Fortin 1989). Few such studies have been carried out in natural populations (Alexander 1984, Burdon and Jarosz 1988, Burdon et al 1989, Frank 1992, Grosholz 1993). A descriptive study of this type can then be complemented by experimental work designed to test hypotheses generated by the descriptive data. This combined approach is necessary to understand the very complex interactions of physical and biotic variables which influence biotic interactions in nature.

Here I studied the infection of *Juncus dichotomus* by the smut fungus *Cintractia junci*. This plant disease has the potential to play an important role in the population biology of the plant, since it specifically attacks its host's reproductive structures, reducing or eliminating seed production, and

because infection is perennial in the host plant and can have long-lasting effects.The system was also useful for the approach outlined above. I could combine the spatial study of a heavily infected natural population with manipulative experiments using host and pathogen organisms from the same population, since plants were easily excavated, propagated, and inoculated with fungal spores collected from the field,

Here I report the results of a three-year spatial study of disease in one host population (see Study Site below). This study is complemented by a series of field and greenhouse experiments that control and manipulate factors to test hypotheses regarding the pattern of disease described here (see other chapters in this thesis). I had three main goals in this study. The first was to follow a sample of individually marked plants for several growing seasons to determine disease progress within plants over time. The second goal was to document the spatial pattern of the disease throughout the population over time. The third goal was to examine relationships between disease and a number of biotic and abiotic environmental variables in the site. To do this, I compared the spatial patterns of these variables and calculated correlations between them and disease frequency, thereby identifying which variables might be causal factors in the spatial pattern of the plant-pathogen interaction in the site.

In this study I examined the relationship of disease to host plant

density, soil moisture, the percent cover of nonhost plants, and host genetic diversity. Each of these variables has been postulated to play a critical role in determining disease frequency, and observations in the site suggested that the first three variables may have had patterns of spatial variability similar to that of the plant disease.

Host plant density typically is predicted to correlate positively with disease (Burdon and Chilvers 1982, Burdon 1987a). Higher host plant density may promote higher rates of disease transmission between plants; since the host plants are closer together, fungal spores have less distance to travel and would be more likely to intercept susceptible host tissue before they are deposited elsewhere. Increased stand density can also increase humidity in the canopy and facilitate the germination and growth of many plant pathogens. These effects are documented in both agricultural systems (Kranz 1978, Rotem 1978) and in some natural populations (Alexander 1984, Augspurger and Kelly 1984). In addition, once a systemic fungal pathogen has successfully been transmitted to and infected a host, it may manipulate host plant physiology so that the plant increases vegetative growth and tillering (Clay 1990a, Nus 1990). As a result, there could be a positive correlation between disease and host density measured as host tissue per unit area.

Host plant density could also be negatively correlated with disease

(see Chapter Four). At high density plants are expected to experience increased intraspecific competition, which itself can have negative consequences for the plants and could influence pathogen infection. The plants may be physiologically stressed and smaller, and both of these conditions can decrease the probability that a plant would become a host (Colhoun 1979, Auld et al 1990, Elmqvist et al 1993, Yahara and Oyama 1993). Systemic pathogens such as the smut fungus I studied here grow and sporulate within the tissues of the host plant (Fischer and Holton 1957) and thus they may require a certain level of host plant resources. Plants growing under strong competition may therefore be an inadequate resource for them (see Chapter Three). Also, small plants will be smaller targets for pathogen spores and so may be more likely to escape inoculation altogether. So the relationship between host plant density and disease could theoretically be either negative or positive.

The second environmental variable I measured, soil moisture within sample plots, is one of the most important physical environmental variables that can affect both fungal pathogen and host plant biology (Colhoun 1973, 1979, Harper 1977, Sussman and Dowthit 1982). As a smut fungus, *Cintractia junci* has teliospores that are released from the host plant and then must germinate outside of the plant, produce hyphal strands which fuse with those of an opposite mating type, and finally penetrate new host tissue

(Fischer and Holton 1957). Thus the fungal life cycle is dependent on physical parameters outside of the plant, and moisture conditions can strongly influence fungal pathogen biology (Agrios 1988). Similarly, soil moisture may influence host plant susceptibility to disease on both an individual and population level. Individually, host plant physiological resistance may be altered due to water stress (Colhoun 1979) while on the population level host plant density may be influenced and thus influence disease development as discussed above. So, it seemed likely that spatial variation of soil moisture in the site could well be correlated with that of disease due to its effect on both fungal and plant growth. Because fungal spores would experience soil moisture near the soil surface and plants would be more affected by water in soil near the roots, I measured soil moisture separately in both an upper and lower soil layer.

The third measured variable in this site was the percent cover of nonhost plants within sampled plots. This could be an important factor in plant-pathogen interactions in two major ways. First, it may be related to the degree of interspecific competition that potential host plants experience. Interspecific competition may act in the same manner as intraspecific competition, as discussed above, and so be negatively correlated with disease due to its adverse impact on host plant physiology and size. Nonhost plants may also act as barriers to incoming pathogen spores (Burdon and

Chilvers 1977a, Chin and Wolfe 1984b, Burdon 1987a) and thus potential host plants growing in close proximity to other species may be more likely to escape inoculation.

Finally, I measured the variation of host plant population genetic diversity within the population and correlated it with variation of disease frequency in this site. A large body of literature addresses this relationship (Leonard and Czochzar 1980, Bierzychudek 1988, Parker 1992) because it is central to theories concerning the evolution and maintenance of sexual reproduction (Hamilton et al 1990, Lively 1993, Roy 1993, Yahara and Oyama 1993) and to the understanding of plant disease epidemiology (Day 1978, Burdon 1987a, Marshall 1989). In general, greater host population genetic diversity is expected to impede the development of epidemics because it should be more difficult for pathogens to evolve virulence when faced with a wide range of host genotypes. Thus host population genetic diversity should be negatively correlated with disease frequency.

This prediction, however, would depend on how long the organisms had been interacting and their respective rates of evolution. A pathogen may well be able to initially invade and establish more easily in a genetically homogeneous and susceptible plant population. Over time, however, the frequency of resistant plant genotypes would be expected to increase while the susceptible ones decreased. Thus, host genetic diversity would increase

(Burdon, Groves, and Cullen 1981, but see Murphy et al 1982). At this point the correlation between disease frequency and plant population genetic diversity would appear positive rather than negative. After more time genetic diversity may decrease again as resistant plant genotypes came to predominate, making the correlation appear negative once again. These relative frequencies of resistant and susceptible genotypes, and thus population genetic diversity, would also be influenced by how quickly the pathogen evolved to overcome the defenses of resistant plants (Burdon 1987a). So, predicting whether the correlation between host population genetic diversity and disease frequency should be negative or positive is not straightforward. It depends on the history of the plant-pathogen interaction and their rates of evolution.

If there is a strong relationship between host population genetic diversity and disease frequency, and if host genetic diversity is spatially variable within the plant population, then it is possible to calculate a correlation between these two variables measured in plots within the population. There may be areas where the plants are genetically quite homogeneous and other areas where they are more heterogeneous. If so, it would be of great interest to know whether these different areas also differ in disease. A number of studies have now shown that natural plant populations, particularly those which are at least partial selfers, exhibit

spatial genetic structure for individual loci (Heywood 1991). Whether the overall genetic diversity of plant populations is typically spatially structured is a different question, and one for which no general answer is yet available.

The host plant *J. dichotomus* is most likely highly selfing (see Organisms below). The index of population genetic diversity used for predominately selfing species is D, known as gene diversity or average heterozygosity (Brown et al. 1990, Weir 1990). It defines high diversity as high heterozygosity within a plant sample averaged over the sampled loci, so it is a measure of overall diversity of the genes in a sample. However, D does not measure the diversity between whole-plant genomes. For example, in a clonal population of plants which are all heterozygous for a number of measured loci, there would be a very high D even though the individual plants are identical to each other genetically. In this case, whole-plant genetic diversity would be zero.

In this study I propose a second measure for whole-plant population genetic diversity which I call J (described in detail in the Methods). Depending on the history of the plant-pathogen interaction, such a whole-plant measure might be needed to understand the relationship between disease frequency and host genetic diversity. If the pathogen entered this population of plants when it was already established and if the plant genetic structure is relatively stable over time (due to long-lived individuals and a

high rate of selfing), then whole-plant diversity would be important. In areas of the population where the standing crop of plant genotypes is homogeneous, the pathogen population would more easily be able to evolve virulence because there would be fewer different host resistance alleles present.

In contrast, if the pathogen entered the host population when it was new and expanding and had more potential to evolve, then average heterozygosity would be important in the interaction. More heterozygous areas of the population would be able to evolve in response to the pathogen more readily than the more homozygous areas of the population.

Because I do not know the history of the plant-pathogen interaction in this site, I measured both D and J for samples of plants from plots located in low and high disease areas within the host population. The genetic data used to calculate the indices came from starch gel enzyme electrophoresis (see Methods).

All of the variables discussed above were spatially located, so I used statistical methods that take possible spatial structure into account (Legendre and Fortin 1989). Parametric correlations are inappropriate because spatially autocorrelated data violate the basic assumption of independence between the variates (Sokal and Rohlf 1981). Instead, I used Mantel tests to test for correlation between distance matrices of pairs of

variables. To test correlations without the variables' spatial structure as a covarying factor, I did partial Mantel correlations with a geographic distance matrix held constant statistically (see Methods below for details) (Smouse et al. 1986, Legendre and Fortin 1989, Sokal et al. 1992). To visually compare some variables I also generated surface plots with an interpolation method using kriging, which takes the spatial autocorrelation of the variable into account and uses as estimators only sampled points in the vicinity of the point to be estimated (Legendre and Fortin 1989). I also explicitly examined and compared the spatial autocorrelation structures of disease and host plant density. The spatial autocorrelation coefficient Moran's I is a measure of the independence of values of the variable within a distance class. If two variables are associated then they should exhibit similar patterns of spatial autocorrelation (Sokal and Thomson 1987).

ORGANISMS AND STUDY SITE

Juncus dichotomus Ell. (Juncaceae) is a perennial rush that typically grows in sunny habitats with poor soils. The underground parts of the plant overwinter and produce new leaves in the spring. By early summer the plants have also produced flowering shoots, which begin dispersing seeds in July. In late summer the aboveground tissue starts to gradually senesce until winter. In this population, typical plants range from about 20-30 cm in

height at full size and grow as a clump of, on average, 7.2 reproductive shoots each associated with several leaves (based on the mean of 115 plants measured in 1991). The only apparent vegetative spread is the gradual expansion of a clump's circumference, with no evidence of rhizomatous spread (personal observation based on excavating approximately 100 plants). Seeds are produced in abundance but I have seen very few new seedlings over four years of observation. Little is known about the mating system of *J. dichotomus*, although enzyme electrophoretic surveys of other populations suggest a high selfing rate (R. Brooks, personal communication). Plants from this population generally show low levels of electrophoretic variability (Morrison, unpublished data) and are able to set abundant seed when isolated from potential outcross pollen donors (Morrison, unpublished data).

The plant can be attacked by the parasitic smut fungus *Cintractia junci* Schw. (Ustilaginales, Basidiomycetes). This smut is found only on other members of the *Juncus* genus. Smuts are common fungal parasites of grasses, rushes and sedges and employ a variety of infection strategies, although the most common target is the plant's reproductive structure. In *J. dichotomus*, a systemically infected shoot produces a deformed inflorescence that produces no flowers or seeds. Instead, membrane-covered lesions that develop on the inflorescence break open to release black smut

teliospores. The dispersed teliospores germinate and produce hyphal strands that must fuse with a strand of opposite mating type in order to produce an infective mycelium (Fischer and Holton 1957). In experimental inoculations I have shown that plants can become infected when the base of the plant and clipped leaves are exposed to a teliospore mixture, with infection appearing in the growing season following the season of inoculation. Nothing else is known about the specific infection process of this plant-pathogen system.

Plants can be found with a wide range of infection. In the most severe cases all flowering shoots of the plant exhibit a systemic infection, in intermediate cases some shoots appear systemically infected while some shoots flower and produce seeds normally, and in the most benign cases no shoots are systemically infected, but isolated flowers on otherwise healthy inflorescences are infected (personal observation). It is possible that shoots that appear uninfected because no sporulation is observed may actually harbor latent infections of the fungal mycelium in plant tissue. I made an attempt to screen plants for mycelium with cytological staining but was not successful. More work in this area would be useful.

I studied these organisms in a sandy, open area within the Long Island pine barrens near the Swan Pond Biological Station of the State University of New York at Stony Brook in Manorville, New York. The study site is approximately 20m X 100m and is bordered on the eastern side by

wooded patches dominated by *Pinus rigida* - *Quercus spp.*, *Acer rubrum*, and a stand of *Vaccinium corymbosum*. The other sides are bordered by an old abandoned commercial cranberry bog. The main topographical feature is a slight increase in elevation as distance from the bog increases. In the central section of the site the *Acer rubrum* forest patch intrudes from the east so the available open habitat for *J. dichotomus* is restricted to a narrow strip about 4m across (Figure 1).

METHODS

INDIVIDUAL PLANT CENSUS

In the spring of 1991, before the development of inflorescences, I tagged a total of 115 *J. dichotomus* plants by tying brightly colored embroidery thread snugly around the base of the plant and taping labelled, colored plastic tape onto the thread. I did not know before tagging whether a plant was infected or healthy, since infection is only visible after the inflorescence starts to develop. I tagged the two plants which were closest to the center of $1m^2$ permanent plots which I established in 1990 on a regularly spaced grid (see below). In this way I sampled plants from throughout the entire population and was not biased by the infection status of the plant. If the plants in a plot grew too closely together for me to identify distinct individuals then I skipped that plot. Between 2-18

September 1991 I censused each plant and recorded the number of healthy and infected shoots it had. In 1992 I located 84 of the original 115 (see Results below) and censused them again in the same way between 8 August - 17 September.

I grouped these 84 into three disease stages for each year and calculated the transition probabilities of a plant moving from one stage in 1991 to another stage in 1992. The three stages were: 0% of shoots on a plant infected; greater than 0% but less than 100% infected; 100% infected. These categories are a good way to understand the effect that the pathogen has on host plant reproduction. If none of the shoots are infected then all can reproduce. If some of the shoots are infected then the pathogen prevents reproduction in those but the others still reproduce, while if all shoots are infected the pathogen has completely prevented host reproduction.

POPULATION AND COMMUNITY SAMPLING

In 1990 I set up a grid of plots throughout the main part of the host population, excluding some peripheral stands where the plants grew on heavily disturbed trails. I assigned spatial coordinates to permanently marked side-by-side pairs of $1m^2$ plots placed systematically every 6 meters in the X and Y directions on the grid (Figures 1 and 2). I also divided each plot into

four 0.25m^2 quadrants which were also assigned spatial coordinates. This systematic-clustered sampling design incorporates a variety of spatial scales and is useful for detecting spatial structure in the data (Fortin et al 1989). I also included 19 other permanent 1m^2 plots from four transects set up for a pilot study 1989. These transects are represented in Figure 1 as dotted lines.

In 1990, 1991, and 1992 I counted the number of infected and uninfected shoots in each plot, and calculated shoot density as the total number of shoots per quadrant and per plot, and disease severity as the percent infected shoots per quadrant and per plot. In 1990 there were 281 quadrants with the host plant present so in the subsequent years all of these quadrants were checked, except for those in a few plots along the transects where vandals removed plot markers in 1991.

In 1990 I also estimated the percent cover of nonhost plants in each quadrant using a cover scale ranging from 0% to <5% to 5-25% to 25-50% to 50-75% to 75-95% to >95%. In the analysis I assigned values to each quadrant to correspond to these categories: 0%, 5%, 15%, 37.5%, 62.5%, 85%, and 95%. For 1m^2 plots values I used the average from the four quadrants within a plot.

SOIL MOISTURE

In 1992 I sampled soil moisture throughout the site by taking soil

cores from each $1m^2$ plot on the grid 2 days, 5 days, and 10 days after saturating rain. Samples from all plots were taken within 2 hours of each other on the same day. Using a 2cm diameter soil corer I removed the top 15cm of soil from a fixed position relative to the center of each plot and divided the core into an upper 6cm soil layer and a lower 9cm soil layer. Immediately I placed each sample into a tightly sealed, labelled double plastic bag for transport back to the lab. Over the next 48 hours each sample was weighed to the nearest hundredth of a gram on an electronic digital balance and dried at 60° C to constant mass. The soil was weighed again after returning to room temperature. Percent soil water was calculated as 100(1-dry mass/wet mass).

Soil water potential was also estimated; it is a measure of the availability of water to the plant and takes into account the physical properties of the soil that affect water adhesion (Winter 1968, McCrane 1988). Soil samples from 15 locations throughout the site were sent to the Nutrient Analysis Laboratory at Cornell University for determination of curves of soil water potential versus percent soil water. From these curves I obtained values of soil water potential in each plot from the measured values of percent soil water. I assigned a curve to each plot based on the plot's proximity to one of the 15 sampled locations.

Although I collected data from two, five, and ten days after rain, I

only analyzed the disease-soil water correlation for the five day data because they represented the typical soil water heterogeneity that the plants and pathogen experienced (see Results below). After two days the soil was still quite saturated, with much less spatial heterogeneity of soil moisture than at the later dates. After ten days there was the most marked heterogeneity, but there were very few ten day stretches without rain during the growing season at this site (personal observation).

GENETIC DIVERSITY

For this part of the study I chose three plots where I knew disease levels were very high and three plots where I knew they were very low in order to maximize the chance of detecting a correlation between host population genetic diversity and disease frequency. I genotyped 15 plants from each plot using five variable enzymes: ADH, PGM, PGDH1, PGDH2, and IDH. To obtain a sample of 15 distinct individual plants I defined a plot by combining a pair of the side-by-side $1m^2$ plots into one. I counted the number of healthy and infected shoots in each of these six $2m^2$ plots and calculated shoot density and disease severity as above. I was unable to include more plots in this part of the study because of time constraints. This part of the study was done in the summer of 1993.

From the genetic information I calculated two measures of genetic

diversity for each of these 6 plots. One was D, gene diversity or average heterozygosity (Brown et al 1990, Weir 1990):

$$D = 1 - (\Sigma_l \Sigma_n p_{ln}^2) / m$$

where

p = frequency within the 15 plant sample of the nth allele at the lth locus

and

m = number of loci.

The index D is a measure of the average level of heterozygosity within the sample but it does not measure the amount of difference between whole-plant genomes. To measure that difference I propose a new index, J:

$$J = [\Sigma_{ij} (ap)] / \Sigma_{ij} a$$

$$= [\Sigma_{ij} (a (d/a))] / \Sigma_{ij} a$$

$$= (\Sigma_{ij} d) / \Sigma_{ij} a$$

where i,j are plant pairs for k plants,

a = the number of alleles,

p = proportion of alleles that differ between plant i and j, and

d = number of alleles that differ between plants i and j.

This index is the average proportion of alleles that are dissimilar between

pairs of plants weighted by the number of alleles sampled. The weighting is necessary because not all loci will always be determined for all samples; in this case in some of the plot samples only 4 of the 5 enzymes were resolvable. If all plants in a sample had identical genotypes at all alleles in all loci, then there would be zero whole-plant diversity and $J = 0$. If all plants in a sample had different alleles in all loci, then there would be maximum whole-plant diversity and $J = 1$.

SURFACE PLOTS

I generated three dimensional surface plots using the program SURFER (Golden Software, Inc.) for the variables disease severity in 1990, 1991, and 1992; shoot density in 1990, 1991, and 1992; and percent soil water by mass and soil water potential two, five, and ten days after a saturating rain in 1992. For all surfaces the data were from $1 m^2$ plots. SURFER allows the user several options for interpolating values between sample locations to estimate a continuous 3-D response surface. For all variables I chose kriging as the interpolation method and directed the program to search the 10 nearest sampled points to interpolate a value for every intersection on the X and Y gridlines on the plot. These plots have 10 gridlines in the X direction and 46 in the Y direction. The plots are displayed with a perspective view and 30° tilt.

SPATIAL AUTOCORRELATION

I used the Spatial Autocorrelation Analysis Program (SAAP) (Wartenberg 1985) to calculate spatial autocorrelation for the variables disease severity in 1990, 1991, and 1992 and shoot density in 1990, 1991, and 1992. For this analysis I used the variable values from the $0.25m^2$ quadrants in order to look at fine divisions of spatial scale. Because I was interested in comparing spatial autocorrelation of disease severity and density in plots where disease was present, I only included quadrants where at least one host plant was present so that there was a meaningful, nonmissing value for disease severity. The resulting sample sizes were 281 quadrants in 1990, 245 in 1991, and 183 in 1992.

I did two sets of analyses. First, I analyzed spatial autocorrelation of disease and density within 12 distance classes that ranged from the smallest to the largest possible distance (103 m) in the study site. I defined the distance classes to correspond to the particular distances between quadrants in my sampling design. For example, the class 0-2.1m included all quadrants that were within a pair of $1m^2$ plots, the next class, 2.1-9.1m, included all quadrants that were in adjacent pairs of plots, and the third class, 9.1-15.1m, included all quadrants that were two pairs of plots distant.

Second, I analyzed spatial autocorrelation of disease and density

within 9 narrower distance classes that ranged from 0m to 7m in order to examine the smaller distance classes at a finer spatial scale. These classes correspond to the various distances between quadrants within a $1m^2$ plot, between quadrants located in the two plots of a pair of $1m^2$ plots, and up to the distance between quadrants in different pairs of adjacent plot pairs.

The results are presented as correlograms that plot the value of the Moran's I autocorrelation coefficient for each distance class against the distance class midpoint. Values of Moran's I near zero are nonsignificant; that is, the values of the variable for samples within a nonsignificant distance class are independent of each other so a value can not be partly predicted by the other values falling within that distance class. If Moran's I is significant then the values from samples within the distance class are not independent and there is spatial structure present (Legendre and Fortin 1989).

MANTEL TESTS

For all variables except geographic distance, the matrices I used as input to the Mantel tests were dissimilarity matrices consisting of differences in the variable's values between all pairs of plots. Geographic distance matrices were assembled from pairwise geometric distances between all plots. The variables used in these correlations included disease severity in

1990, 1991 and 1992, shoot density in 1990, 1991, and 1992, percent cover of nonhost plants in 1990, and upper and lower layer percent soil water by mass 5 days after rain in 1992. I also tested genetic diversity indices D and J and disease severity in the 6 plots sampled in 1993. For all variables I used values from the $1m^2$ plots. Each test only included data from plots that had at least one host plant present for the disease year being tested in order to include only plots with nonmissing values for disease.

A plot of disease severity against shoot density in 1990 suggested density thresholds that might be important, but there was no association between the distance matrices of density measured continuously and disease severity. Therefore, I used categorical values to create the dissimilarity matrices for shoot density. If a plot had less than 51 shoots/m^2 it was assigned to the density category 0, if it had 51-300 shoots it was in density category 1, and if it had greater than 300 shoots it was in density category 2.

To test the correlation between two distance matrices **X** and **Y** I used Mantel tests (Mantel 1967) computed with the RPACKAGE program (Legendre 1985), which provides r_{XY}, a more easily interpreted normalized transformation of the Mantel statistic of association, Z_{XY} (Smouse et al 1986). Significance levels of these test statistics are calculated by comparing them to an empirical null distribution derived from Monte Carlo

sampling based on 249 permutations (Smouse et al 1986, Sokal et al 1992). In this procedure one of the matrices is held constant while the rows and columns of the other are randomly permuted, each time calculating the correlation statistic.

To test the partial correspondence between two variables X and Y with a third variable Z statistically "held constant", I employed the method of Smouse, Long, and Sokal (Smouse et al 1986), known as the partial Mantel test (Legendre and Fortin 1989). In this case the two matrices that are correlated are distance matrices of the residuals from a linear regression of variable X on variable Z, denoted **X'**, and variable Y on Z, denoted **Y'**. The same method can be used to correlate X and Y with more than one other variable held constant, in which case the distance matrices used are computed from the residuals from a multiple regression of X on the other variables and a multiple regression of Y on the other variables.

The Mantel test is a linear model like the more familiar Pearson's correlation coefficient, and so it only illustrates the linear component of the association between two distance matrices. If there is strong nonlinearity between two variables the Mantel test may fail to detect the relationship. Even though the effects of nonlinearity in the data have not been fully explored (Legendre and Fortin 1989), the method is being used in a variety of ecological and evolutionary studies (Sokal et al 1980, 1986, 1992,

Schnell et al 1985, Burgman 1987, Sokal and Thomson 1987) and is particularly useful for determining associations between spatially located variables (Legendre and Fortin 1989).

It would be useful to examine scatterplots of the relationship between the variables in order to look for possible nonlinearities. I did this with some variables and will do more in future analysis.

RESULTS

INDIVIDUAL PLANT CENSUS

Disease progress.

Plants with 0% infection in 1991 were most likely to remain disease free in 1992 (Table 1), but 16% did become infected. Plants with between 0% and 100% infection were most likely to remain at that stage of infection, but 16% recovered and became disease free while 14% worsened and became 100% infected. Only six plants had 100% infection in 1991; these were most likely to have had some disease-free shoots in 1992, but none of them recovered completely and 33% of them remained completely infected. This small sample, however, may not present an accurate representation of disease progress in this disease category. Overall, the percentage of infected plants in this 84-plant sample increased from 33% to 49% between 1991 and 1992.

The 31 plants that I lost track of between 1991 and 1992 could have been missing because their markers somehow were dislodged, because the vegetation grew too thick and obscured their markers from view, or because they died. The third possibility is the most likely since I marked the plants very securely and since I made very thorough searches for the marked plants in 1992. Assuming that missing plants were gone due to mortality, I did a G-test of heterogeneity (Sokal and Rohlf 1981) to see if 1991 plants in the three disease categories above were equally likely to be missing in 1992 (Table 2). Plants with 100% infected shoots were twice as likely to be missing in 1992 as were plants with 0% infection and about 3.5 times more likely to be missing than plants with intermediate infection ($G = 6.178$, $df = 2$, $P = 0.046$). In general smut infection is considered important for its effect on reproduction rather than for increasing mortality of host plants, but in this system it appears that the most severely infected plants may also be at higher risk for death, although since I am not certain missing plants died this conclusion should be viewed with caution.

Host plant size and amount of infection.

Host plant size (number of shoots per plant) and disease (proportion of shoots infected per plant) were not correlated with each other in this sample, using Kendall's coefficient of rank correlation with the normal

approximation (Sokal and Rohlf 1981) calculated with PROC FREQ in SAS 6.0 (SAS Institute 1990b) (two-tailed tests: 1991, $t_s = 0.94$, $P = 0.35$, $n = 84$; 1992, $t_s = 1.60$, $P = 0.11$, $n = 77$).

OVERALL YEARLY MEASURES - DISEASE AND DENSITY

From 1990 to 1992 disease severity on average in the host plant population increased slightly from 13% of shoots/m^2 infected to 16% infected (Table 3). In contrast, the host plant population density decreased from 132.6 to 59.8 shoots/m^2 from 1990 to 1992 (Table 3).

SPATIAL PATTERNS OF DISEASE, DENSITY, AND SOIL WATER

Surface plots.

The surface plots for disease severity, host shoot density, and various soil water measures illustrate the spatial heterogeneity of these factors at the study site over the three years of the study. Visual inspection and comparisons show a number of trends and patterns. In all surface plots there is a flat area which was forested and had no host plants present.

Disease severity (Figure 3) had clear, similar spatial patterns in 1990, 1991, and 1992, with distinct areas of high and low disease severity as measured by the percent of infected shoots in $1m^2$ plots. There were two areas of severe disease - one at the northern end of the site and one in the

middle and adjacent to the bog. Two areas with distinctly lower disease levels also appeared, with the lowest levels in the southern portion of the site and moderate levels in the narrow strip between bog and forest.

Shoot density, measured as the total shoots per $1 m^2$, also showed clear spatial structure (Figure 4). Areas with high and low shoot density were consistent from year to year and these areas appear to map onto the high and low areas in the disease plots, suggesting a positive correlation between host shoot density and disease severity. The plots also illustrate the reduction in shoot density from 1990 to 1992, as shown by the change in the shoot density mean (above), and they show that this reduction occurred throughout the entire site except in the vicinity of coordinates $X = 15$, $Y = 10$. There also appeared to be a reduction in overall spatial heterogeneity from 1990 to 1992.

Density-dependent reduction in shoot density is apparent; those areas with the greatest density in 1990 appear to have the greatest subsequent declines in shoot density (Figure 4). The scatterplots in Figure 5A and 5B illustrate that the more dense plots in both 1990 and 1991 had greater absolute decreases in shoot density the following year. This density-dependent decline could be due to the disease thinning out plants in the dense plots more than in less dense plots. Nearly all plots with 0% infection in 1990 showed an increase in density in 1991, while in plots with higher

rates of infection there tended to be a decline in shoot density (Figures 5C, 5D), and the plots with higher infection rates are also the plots with higher densities (Figures 5E, 5F, and see mantel correlation below). Further analysis is needed to evaluate density-dependent regulation of host density by the disease, but there is a strong suggestion that it may be operating in this system.

The surface plots for percent soil water by mass for the lower soil layer (Figure 6) show the presence of some spatial structure. The most marked feature was an area of higher water content in the narrow part of the site, which corresponds to a physical depression alongside a cranberry bog (personal observation). The spike near coordinates $X = 19$, $Y = 30$ corresponds to an edge of the host population which was near forest and the edge of another depression.

Soil water potential for the lower soil layer (Figure 7) generally exhibited the same spatial pattern as percent soil water, with the exception of 2 days after rain where the site looked more homogeneous. The scale used in the plots may have overemphasized the heterogeneity apparent on days 5 and 10 after rain, but it appears that there were areas in the site where water was much more readily available to plants. Comparison of lower soil layer water plots with disease plots (Figure 3) does not suggest any strong correlation between the two.

The surface plots for percent soil water by mass for the upper soil layer (Figure 8) show a very distinct difference between the narrow strip alongside the bog and elsewhere. Comparing this area with the same area on the maps of the lower soil layer, it appears that water retention was actually greater in the upper layer than it was in the lower layer. Elsewhere water retention was greater in the lower layer, as would be expected. The narrow region alongside the bog was a particularly compacted area due to human and probably deer traffic, possibly reducing water penetration to the lower layer.

Soil water potential surface plots for the upper layer (Figure 9) indicate that water continued to be easily available to plants in the narrow section of the site even after 10 days had passed since rain. Elsewhere, soil pressure began to decrease more rapidly in the upper layer than it did in the lower layer. Comparison of the surface plots for upper layer soil moisture to the disease surface plots again does not suggest a strong correlation.

Spatial autocorrelations.

In 1990, 1991, and 1992 disease was highly positively spatially autocorrelated at the smallest distance class, which ranged from 0-2.1 m (Figure 10). Positive spatial autocorrelation was still apparent at about 7 meters distance; at greater distances there was no clear pattern in 1990 or

1991, but in 1992 there was no further detectable spatial autocorrelation until the farthest distance class. Disease was clearly negatively spatially autocorrelated at the furthest distances in all three years, which is easily explained by reference to the disease surface plots - the most distant sampling points were in an area of low disease and an area of high disease. This result therefore probably reflects the chance arrangement of the plot rather than any real tendency for patchiness at this scale.

In order to examine the smaller distances in more detail, I also calculated spatial autocorrelation for disease severity (percent shoots infected) per 0.25 m^2) in 9 distance classes ranging from 0 to 7 m (Figure 11). Generally, spatial autocorrelation was most pronounced between 0 and 2 meters distant or, in other words, within pairs of 1m^2 plots on the sample grid.

I did the same analyses of spatial autocorrelation for shoots per 0.25m^2 . For the 0-103 m distance classes (Figure 12) the pattern was similar to that for disease in that up to about 7 meters distant shoot density was positively spatially autocorrelated. Increasingly, from 1990 to 1991 the intermediate distance classes, from about 10 meters to 50 meters, show negative spatial autocorrelation. The furthest distance classes were either not spatially autocorrelated or were positively so.

The very high positive spatial autocorrelation at the smallest distance

class in Figure 12 was clearly due to the high values of the two smallest distance classes shown in Figure 13. They correspond to the distances within one $1m^2$ plot.

ASSOCIATIONS BETWEEN DISEASE AND HOST DENSITY, NONHOST COVER, OR SOIL WATER

Host density and disease.

In 1990 and 1991, but not in 1992, disease severity (infected shoots per $1m^2$) was positively correlated with shoot density per m^2 (low, medium, or high) in the same year, even when geographic distance was held constant statistically (Table 4; DIS90,DENS90.space = 0.135, $P < 0.05$; DIS91,DENS91.space = 0.152, $P < 0.05$; DIS92,DENS92 = 0.06, ns). This indicates that their correlation was not simply due to some other unmeasured variable that followed the same spatial gradient as disease and density. Thus it appeared that there could be a true correlation between disease and density. However, the correlation between disease and density in 1991 became nonsignificant when 1990 disease severity was included in the partial correlation (Table 4,DIS91,DENS91.space,dis90 = 0.109, ns). This suggests that the positive correlation between disease and density may be due solely to their shared association with disease severity in the previous year. I could not test for this possibility for the 1990 disease-density

correlation since I did not have data for 1989 disease severity.

Disease severity in 1991 was highly positively correlated with shoot density of 1990, even with space held constant, but when disease severity in the previous year 1990 was included in the partial correlation, the association became nonsignificant (Table 4; DIS91,DENS90.space = 0.195, P = 0.004; DIS91,DENS90.space,dis90 = 0.111, ns). This again suggests that any positive relationship of host density in one year with disease severity in the next may be due to similar relationships both had with the previous year's disease level. There was no correlation between 1992 disease severity and 1991 shoot density (Table 4, DIS92,DENS91 = 0.103, ns).

No correlations were significant between disease severity and the uncategorized, continuous shoot density variable, so Table 4 only shows the correlations with the categorized version of shoot density.

Disease in adjacent years.

The strongest correlations by far were those between disease severity in 1990 and 1991 and between disease severity in 1991 and 1992. These were highly significant and had the highest values for the correlation statistic (Table 4; DIS90,DIS91 = 0.664,P = 0.004; DIS91,DIS92 = 0.596,P = 0.004). Also, the partial correlations stayed high and significant even with

geographic distance and shoot density from both years held constant (Table 4; DIS90,DIS91,space,dens90,dens91 = 0.653, P = 0.004; DIS91,DIS92,space,dens91,dens92 = 0.587, P = 0.004). This indicates that disease severity of the previous year was the main determinant of disease severity in the next year.

Other correlations.

Disease severity in 1990 was not significantly correlated with percent cover of nonhost plant species in 1990 (Table 4, DIS90,COMPCOV90 = -0.042, ns). This suggests that there was no effect on disease levels of either interspecific competition or a barrier effect. Since the Mantel correlation only tests for a linear relationship I also plotted disease against nonhost cover in a scattergram (Figure 14). It appears that plots with high nonhost cover are more likely to have 0% infected shoots than are plots with less nonhost cover. However, the highest disease levels are also found in plots with high nonhost cover. There may be a complex relationship, but more analysis than I have done here would be necessary to understand it.

As predicted by the surface plot comparisons (above), there was not a significant correlation between soil moisture and disease severity for either the lower or upper soil layer (Table 4; DIS92,UPWAT = -0.090, ns; DIS92,LOWAT = 0.015, ns).

Finally, there were no significant correlations between host population genetic diversity measured with either the D or J index and disease severity in 1993, the year I sampled plants electrophoretically for genetic diversity (Table 4; DIS93,JGEN93 = -0.083, ns; DIS93,DGEN93 = -0.007, ns; see Table 5 for index and density values). Because the sample size for this test was very small (6 plots) this result is in no way conclusive. Certainly it contains no hint of a relationship which is noteworthy particularly because the plots I chose for this test represent the ends of the disease continuum, with three having high disease levels and three having low levels. To test this further, I also did two-tailed Kendall rank correlations (Sokal and Rohlf 1981) for these variables in the six plots and they were also not significant (for J index: tau = -0.47; for D index: tau = -0.47).

DISCUSSION

This study had three goals - to monitor smut fungus infection of individual *Juncus dichotomus* host plants from the natural population over several years, to examine the spatial pattern of disease throughout the plant population over time, and to detect any important relationships between disease and various biotic and abiotic factors. This information could provide insight into the possible mechanisms that influence the development and persistence of the disease in this natural population.

At the individual plant level, the results show that infections were likely to remain in the plant from year to year. Both the host plant and the pathogen are perennial, so plants that are infected one year are usually infected in subsequent years and can be continuous sources of inoculum for new infections. Thus I would in fact expect to see similar spatial patterns of disease from year to year and perhaps an increase in the overall mean level of infection. There was a suggestion that disease may increase mortality of infected plants in a density-dependent fashion. If so, then over a sufficiently long period of observation cycling of disease may be expected.

The spatial analysis indicated clear areas of low and high infection that remained stable from 1990 through 1992. In addition, the mean level of disease in the sampled plots increased slightly over the three years while the density of the host plants declined. Apparently, once the *Cintractia junci* pathogen is established, it can persist for at least several years in the host population.

The perennial habit of *J. dichotomus* means that its evolutionary response to the pathogen threat will be slow, particularly compared to an annual host plant population. This is assuming that its generation time is longer than that of an annual. Even if sexual reproduction results in novel genotypes which are resistant to the disease, the older susceptible host genotypes will still be present for some time. I do not know what the

generation time or average life span of these plants is, but in the individual plant census I found that at least 73% of all plants censused in 1991 were still alive in 1992 (Table 2). Also, I have noticed very few seedlings recruited into the population over four years of study (personal observation). It would be necessary to follow disease in this population for several plant generations to determine the long-term stability of the pathogen population and the host plants' response to it.

Spatial autocorrelation of disease in this site suggests that plots within about 2-3 meters of each other had similar values of disease severity. Thus if there were a direct causal factor for disease severity, it should be spatially autocorrelated at the same scale. Spatial autocorrelation for one postulated factor, host density, looked similar to that of disease, in that plots within the closest distance classes were highly spatially autocorrelated. In addition, the surface plots of shoot density were very similar to those for disease severity. These results strongly suggest that shoot density and disease severity were positively related to one another.

A more complex picture emerges from the results of the Mantel test correlations. They show that indeed disease and density were significantly positively correlated in 1990 and 1991, with a slight trend in that direction in 1992 (perhaps the sample size was too small in 1992 to detect a significant correlation). The relationships held up when space was included in

a partial correlation, suggesting that they were not related simply due to some unmeasured third variable that followed a spatial gradient similar to disease and density. Similarly, the correlation between disease in 1991 and shoot density the year before was positive and significant and held up when space was held constant.

However, these positive correlations between disease and density within a year did not hold up when disease in the previous year was included in the partial correlation analyses. Meanwhile the correlations between disease severity in adjacent years were very high and highly significant no matter what other variables were included in their partial correlations. Because I was able to examine these correlations across years, I could determine that there was, at this stage in disease development within this natural population, a clear positive relationship between host density and disease that was confounded with the values of disease and density in adjacent years. This finding is consistent with the stable appearance of the disease pattern over the three years of the study evidenced in the surface plots of disease.

To know for certain how disease and density were related in this plant-pathogen system, it would have been necessary to follow disease development from the very beginning, before the disease was already established and recurring from year to year in a stable pattern. Twice I

attempted to start epidemics in uninfected natural populations of *Juncus dichotomus* by transplanting infected plants into the sites, but in both cases the infected plants did not survive the winter. This was unfortunate, because it may well be at the start of an epidemic that host density is important. Once a perennial pathogen is fully established in a perennial plant population, as it seems to be in the years I studied this system, then any relationship that might be there can be obscured by the strong year to year correlation of the disease pattern itself. Further work in this area with experimentally induced epidemics in host populations of different initial densities would be very fruitful.

Further analysis is needed, but inspection of the scatterplots in Figure 5, which plotted the change in density from one year to the next against disease level in the first year, suggests that the disease caused density-dependent thinning of the host population. This could explain why the positive correlation between disease and density disappeared by 1992. Earlier in the study, plots with the higher densities had higher disease levels, but by 1992 the higher density plots had decreased in density and thus were closer to the values of the lower density plots.

I also found no significant relationship between disease and the vegetation cover of nonhost plants. This indicates that the host plant's interspecific competitive regime was not an important factor determining

whether plants become infected even though it may be expected to be one influence on the vigor and physiological resistance of potential host plants. The lack of a relationship between disease and nonhost plant cover also suggests that there was no detectable barrier effect of nonhost plants on transmission in this natural population at the time of the study. The result should be viewed with caution, however, since the percent cover of nonhost plants is only a rough measure and also since the timing of the study could influence detection of a relationship. It is possible that early in the establishment of disease in this population the presence of nonhost plants may be very influential.

There was also no relationship between disease severity and soil moisture in either the upper or lower soil layers. Even though there is detectable spatial heterogeneity for soil water in this site, that variation does not map onto disease spatial variation in any simple way. So, the physical environmental factor of soil moisture as it may affect plant growth (the lower soil layer) or fungal growth (the upper soil layer) also appears unimportant in this interaction, at least during the time period of this study.

Finally, no evidence for a relationship between host population genetic diversity and disease was detected in this study. I sampled from the extremes of the disease range and still did not see a distinct difference in genetic diversity measured either as gene diversity, D, or with the measure

of whole-plant diversity, J. Since this may be due to low power I would suggest doing such a study in a different system where genotyping the plants is not so difficult and time-consuming.

The approach I have taken in this study proved to be useful in some ways but limited in others. Mapping the variables of interest and visually comparing their patterns and their spatial autocorrelation structures can be a partial test of theoretical hypotheses about the relationships between them and can lead to further, more quantitative tests. Here the comparisons supported the hypothesis that disease and host density are positively related. The comparisons also led me to test the hypotheses that there would be a positive correlation between disease in adjacent years, but not between disease and soil moisture. Mantel tests can then be used to analyze any hypothesized correlation between variables and, if they are correlated, further partial Mantel tests permit testing for correlation even when the variables values are spatially structured. The results of this type of analysis can then be used to refine further hypotheses that can be experimentally tested in other studies (see Chapters Three, Four, and Five).

So, this method can detect patterns and suggest hypotheses about relationships between the variables. Using this approach over multiple years seems to be essential for any understanding of the system. By measuring the change in density over several years and the relationship between disease

and density, I was able to observe density-dependent mortality of the plants possible caused by the pathogen. Also, it was only by comparing and correlating variables from year to year that I could discern that the apparent positive relationship between disease and host density within a year may be due solely to their positive relationships with disease from the previous year. This brings me to the limitation of this study. Although I followed this population for several years, I happened to study it in the established phase of the interaction. For a perennial population of host plants and for a perennial pathogen, I would probably need to watch the interaction from the start and follow it through several plant generations in order understand the relationships between the variables. The ecological and genetic processes which can potentially influence a plant-pathogen interaction are numerous and can interact. Only very strongly influential factors would be expected to correlate closely with disease and any correlations that were apparent would still need to be experimentally examined in order to determine causation.

Based on the findings from this study, I can state in summary that, for this perennial *Juncus dichotomus* population infected by the perennial *Cintractia junci* smut fungus the most important variable in the interaction presently is its previous history. Where there were high disease frequencies one year there will be high (perhaps higher) frequencies the next, relatively uninfluenced by other biotic or abiotic factors. The stability of the disease

combined with its effects of reducing or preventing host reproduction and possibly causing higher host mortality rates suggests that it will remain an important influence in the population biology of its host once it becomes established. There may be cycling of the spatial pattern of density and disease over a longer period of time, with dense areas of the population building up high disease levels which eventually thin the host population in those areas, while areas with no disease increase in density and presumably eventually succumb to disease and thinning themselves.

Studies of the spatial patterns and spread of disease and host population size over a much longer period of time than in this study will do much to advance our understanding of the effect of disease on host population dynamics. The key to understanding the pattern of disease spatial variation in perennial populations may be at the start of the interaction. Ideally, experimental epidemics should be started and followed over many generations in a series of natural and experimental host populations that differ in some of the theoretically important variables presented here.

Table 1. Disease stages of individually censused plants in 1991 and 1992. Percentages indicate the percent of shoots on a plant that were infected. No shoots means the plant had leaves, but no reproductive shoots. The transition probabilities of a plant moving from a stage in 1991 to a stage in 1992 are shown in parentheses for each 1991-1992 stage combination.

	0% INFECTED 1992	< 100 % INFECTED 1992	100 % INFECTED 1992	NO SHOOTS 1992	1991 TOTALS
0 % INFECTED 1991	41 (0.73)	9 (0.16)	0 (0.00)	6 (0.11)	56
< 100 % INFECTED 1991	2 (0.16)	16 (0.73)	3 (0.14)	1 (0.05)	22
100 % INFECTED 1991	0 (0.00)	4 (0.67)	2 (0.33)	0 (0.00)	6
1992 TOTALS	43	29	5	7	84

Table 2. Individually censused plants categorized by disease severity in 1991 and presence in the census in 1992. Percentages indicate the percent of shoots on a plant that were infected in 1991.

INFECTED 1991	FOUND 1992	MISSING 1992	1991 TOTAL
100%	6	7	13
> 0%, <100%	22	4	26
0%	56	20	76
1992 TOTAL	84	31	115

Table 3. Means, standard deviations and ranges for shoot density / m^2 and percent shoots infected / m^2 in 1990, 1991, and 1993.

Variable	N	MEAN	SD	MIN	MAX
Shoot density 1990	100	132.6	170.02	0	919
Shoot density 1991	97	102.8	122.50	0	630
Shoot density 1992	97	59.8	93.25	0	404
Infection 1990	80	0.13	0.17	0	1.00
Infection 1991	71	0.13	0.16	0	0.81
Infection 1992	54	0.161	0.18	0	0.88

Table 4. Matrix correlations between disease distances and host density, competitor cover, soil water, or host genetic diversity distances, and partial matrix correlations involving some of these distances, geographic distances, and host density distances from different years [a].

1990 Disease Correlations	N	r
1. (DIS90,DENS90)	80	0.155 * * *
2. (DIS90,DENS90).space	80	0.135 *
3. (DIS90,COMPCOV90)	78	-0.042
4. (DIS90,DIS91)	70	0.664 * * *
5. (DIS90,DIS91).space	70	0.660 * * *
6. (DIS90,DIS91).space,dens90	70	0.650 * * *
7. (DIS90,DIS91).space,dens90,dens91	70	0.653 * * *
1991 Disease Correlations		
(see 4-7 above)		
8. (DIS91,DENS90)	70	0.209 * * *
9. (DIS91,DENS90).space	70	0.195 * * *
10. (DIS91,DENS90).space,dis90	70	0.111
11. (DIS91,DENS90).space,dis90,dens91	70	0.045
12. (DIS91,DENS91)	70	0.160 *
13. (DIS91,DENS91).space	70	0.152 *
14. (DIS91,DENS91).space,dis90	70	0.109

15. (DIS91,DENS91).space,dis90,dens90	70	0.109
16. (DIS91,DIS92)	49	0.596 * * *
17. (DIS91,DIS92).space	49	0.592 * * *
18. (DIS91,DIS92).space,dens91	49	0.587 * * *
19. (DIS91,DIS92).space,dens91,dens92	49	0.587 * * *
1992 and 1993 Disease Correlations		
(see 16-19 above)		
20. (DIS92,DENS91)	49	0.103
21. (DIS92,DENS92)	49	0.055
22. (DIS92,UPWAT)	43	-0.090
23. (DIS92,LOWAT)	43	0.015
24. (DIS93,JGEN93)	6	-0.083
25. (DIS93,DGEN93)	6	-0.007

[a] All variables were measured in N $1m^2$ plots where host plants were present. DIS90, DIS91, DIS92, and DIS93 are distances (i.e. differences bewteen pairs of plots) in the percent of shoots in a plot infected in 1991, 1992, and 1993 respectively. DENS90, DENS91, and DENS92 are distances in shoot density per plot in 1990, 1991, and 1992 respectively. Shoot density was defined as low, medium, or high (see Methods). Space indicates geographic distances. COMPCOV90 is the distance in the percent of vegetation cover of nonhost plants in 1990. UPWAT and LOWAT are distances in the percent water in the soil by mass 5 days after rain in the upper and lower soil layers, respectively. JGEN93 and DGEN93 stand for distances of the J and D indices, respectively, of host genetic diversity in 1993. The matrix correlations, r, from Mantel and partial Mantel tests are followed by significance symbols based on 249 permutations of rows and columns of one of the two distance or residual distance matrices. Significances are indicated as: * $0.01 < P \leq 0.05$; * * $0.004 < P \leq 0.01$; * * * $P = 0.004$. The last probability is conservative, since it is the lowest that can be demonstrated with 249 permutations. The higher correlations marked with three asterisks are probably significant at $P << 0.004$.

Table 5. Population genetic diversity indices D and J and disease severity in six $2m^2$ plots.

X	Y	Total # shoots	% Infected shoots	D	J
10.5	93	588	46%	0.214	0.067
6.5	90	472	38%	0.271	0.127
0.5	84	129	28%	0.217	0.092
12.5	30	114	4%	0.232	0.103
18.5	18	123	0%	0.278	0.130
18.5	12	51	12%	0.309	0.187

Figure 1. Arrangement of plots on the sampling grid. Points indicate the centers of paired $1 m^2$ plots. Dotted lines represent 1989 transects along which are four or five additional plots each.

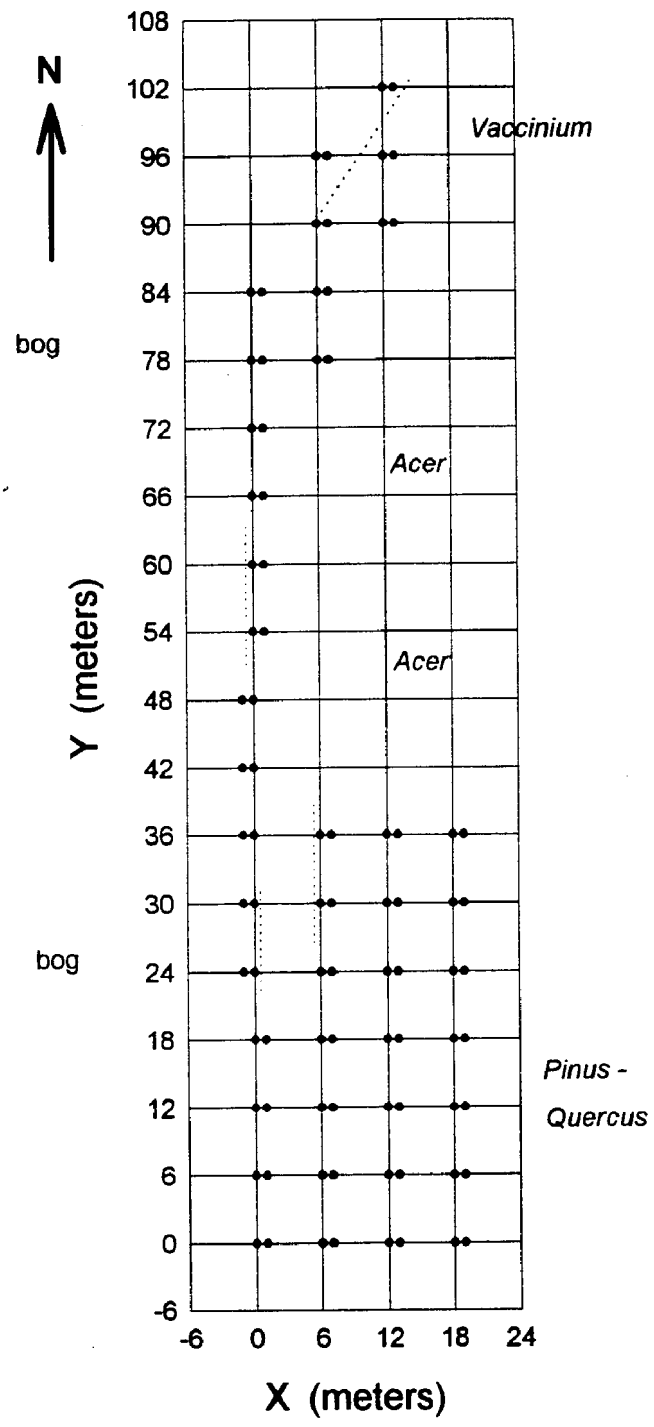

N
108
102
96
90
84
78
72
66
60
54
48
42
36
30
24
18
12
6
0
-6
Vaccinium
Acer
Acer
bog
bog
Pinus -
Quercus
Y (meters)
-6
0
6
12
18
24
X (meters)

Figure 2. Spacing of pairs of $1m^2$ plots on the sample grid (see Figure 1). Only four pairs are shown, but this arrangement is consistent throughout most of the site. In a few locations there are 7m in the horizontal direction between plot pairs.

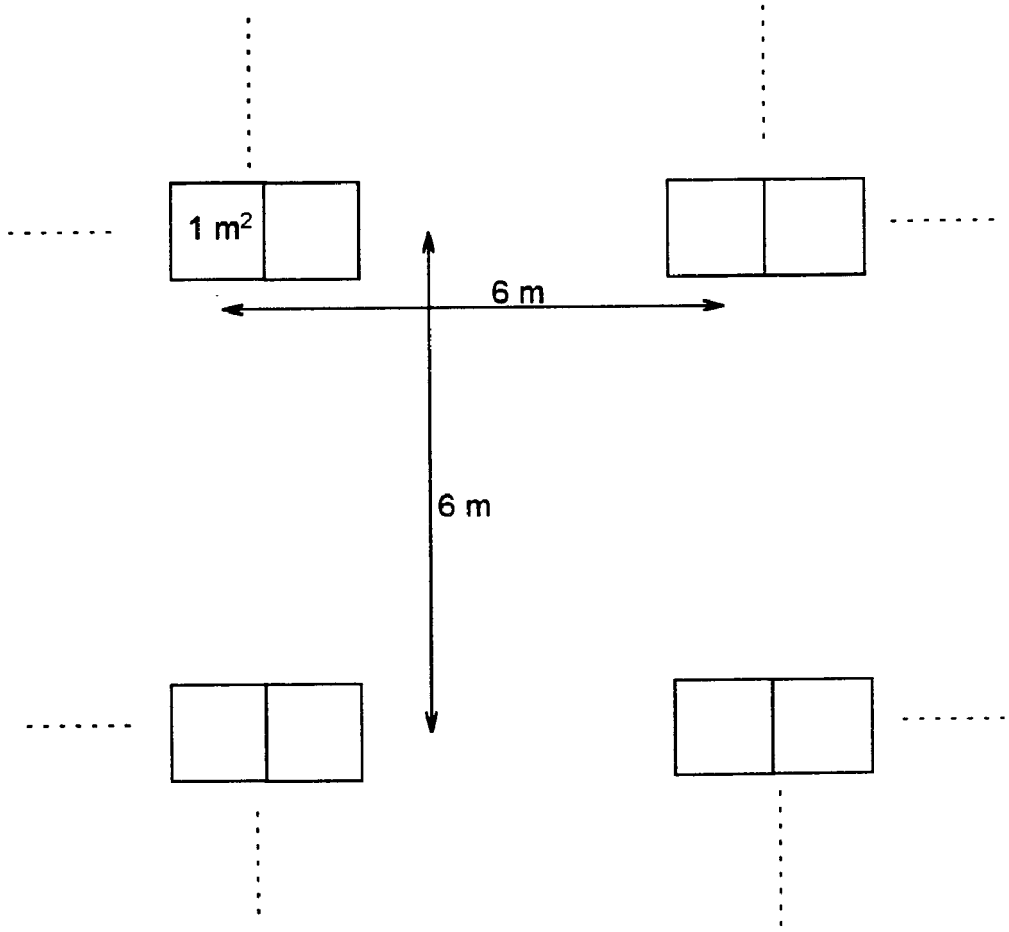

1 m²
6 m
6 m

Figure 3. The percent of shoots infected per $1 m^2$ in 1990, 1991, and 1992. The flat area was forested, with no host plants present (this applies to all surface plots).

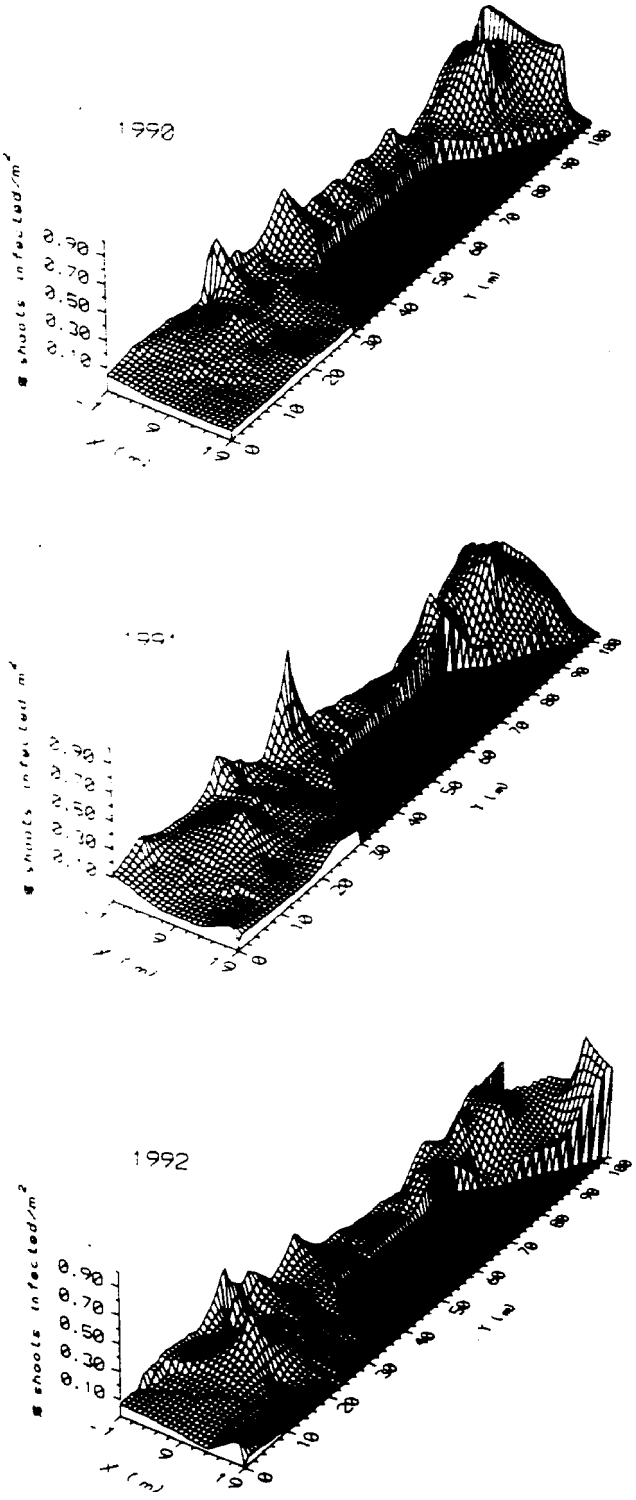

1990
1991
1992

Figure 4. The number of shoots per $1m^2$ in 1990, 1991, and 1992.

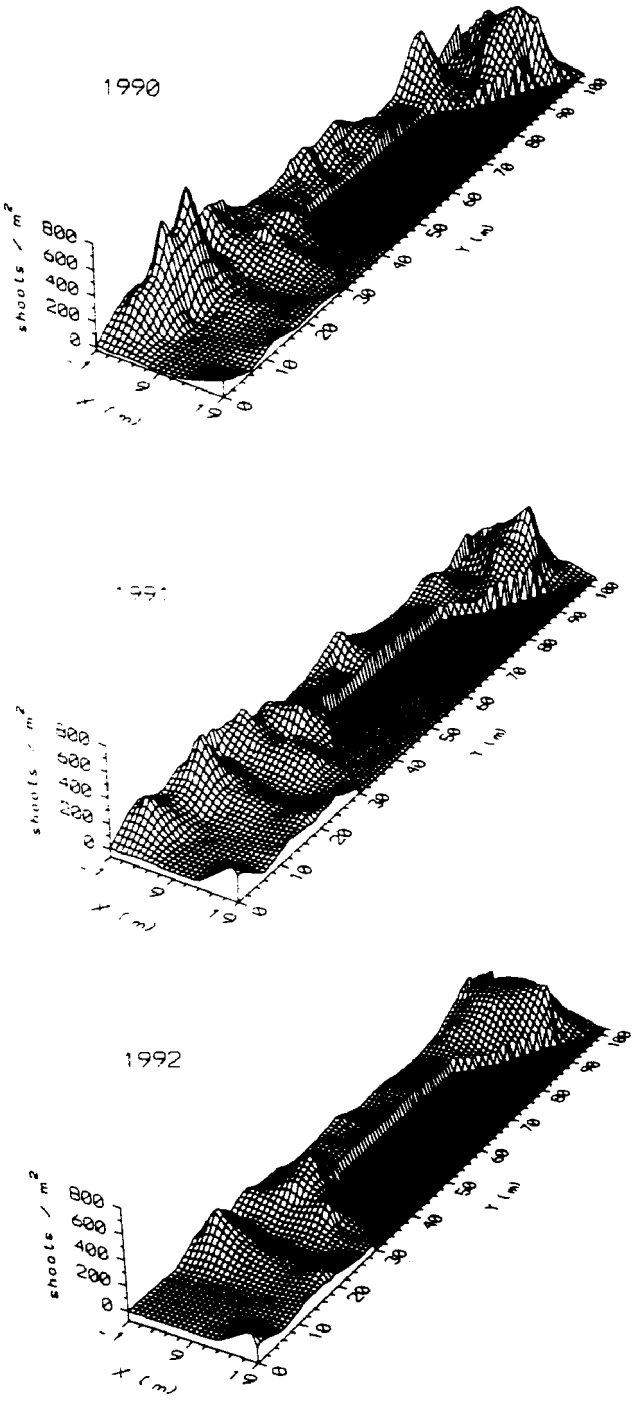

1990
1991
1992
shoals / m²
800
600
400
200
0
X (m)
Y (m)

Figure 5. Scatterplots of the relationships between (A) 1990 shoot density and the change in shoot density from 1990 to 1991; (B) 1991 shoot density and the change in shoot density from 1991 to 1992; (C) 1990 proportion of infected shoots and the change in shoot density from 1990 to 1991; (D) 1991 proportion of infected shoots and the change in shoot density from 1991 to 1992; (E) 1990 proportion of infected shoots and 1990 shoot density; (F) 1991 proportion of infected shoots and 1991 shoot density.

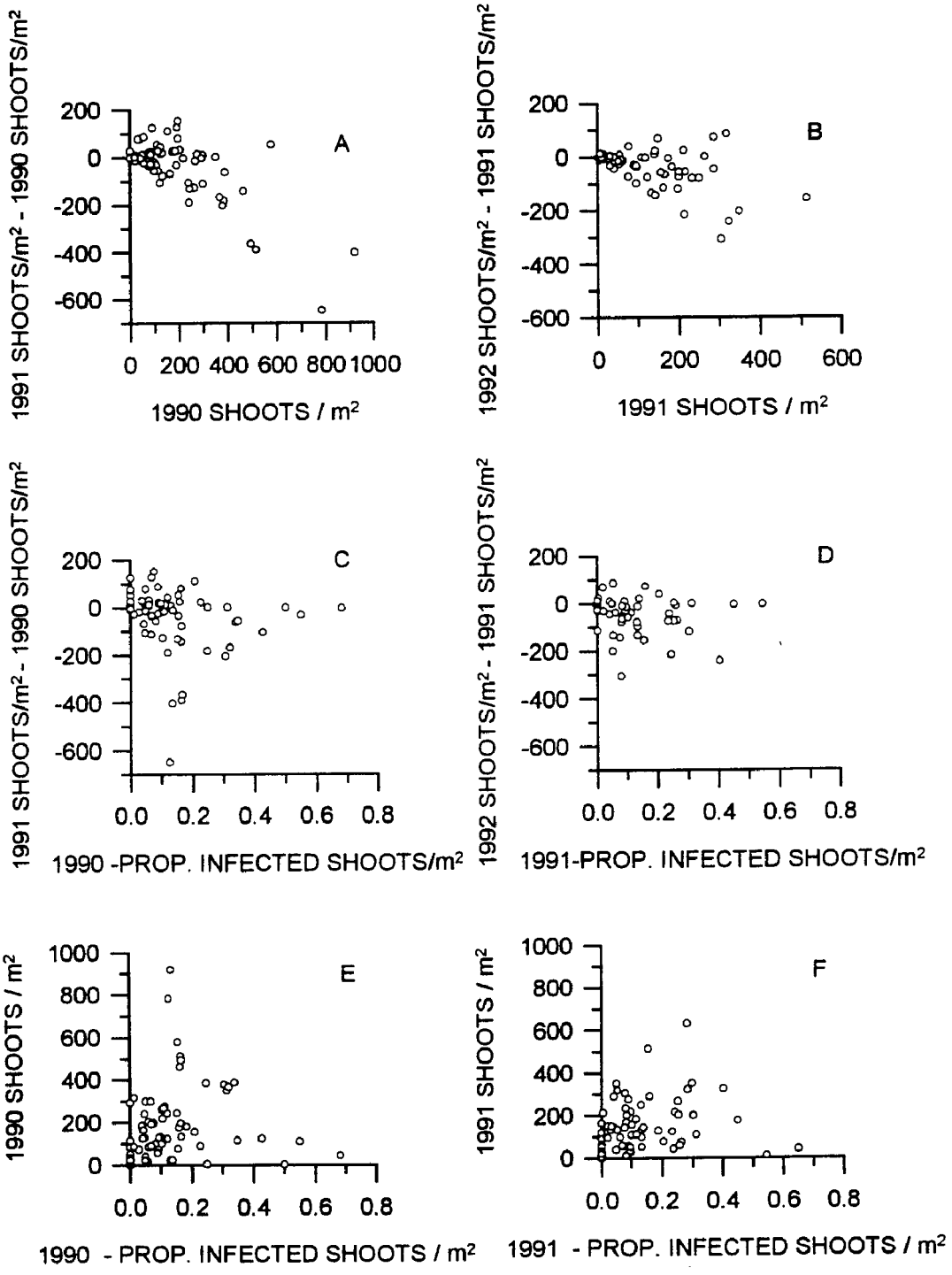
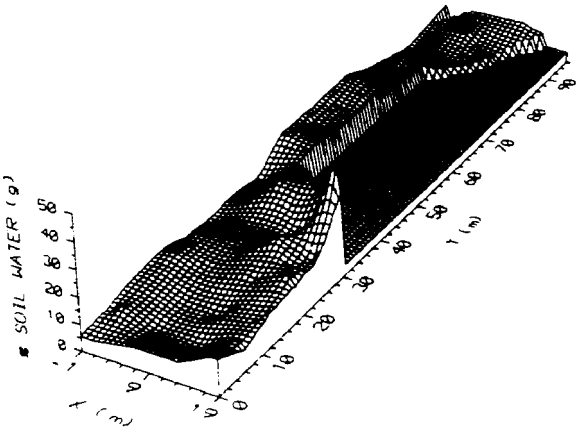
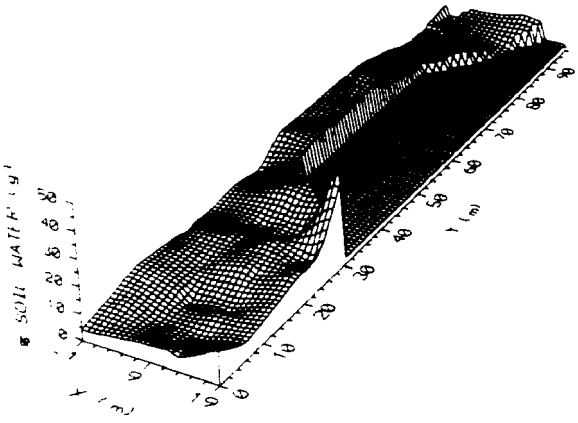

A
1991 SHOOTS/m² - 1990 SHOOTS/m²
200
0
-200
-400
-600
0 200 400 600 800 1000
1990 SHOOTS / m²
B
1992 SHOOTS/m² - 1991 SHOOTS/m²
200
0
-200
-400
-600
0 200 400 600
1991 SHOOTS / m²
C
1991 SHOOTS/m² - 1990 SHOOTS/m²
200
0
-200
-400
-600
0.0 0.2 0.4 0.6 0.8
1990 -PROP. INFECTED SHOOTS/m²
D
1992 SHOOTS/m² - 1991 SHOOTS/m²
200
0
-200
-400
-600
0.0 0.2 0.4 0.6 0.8
1991-PROP. INFECTED SHOOTS/m²
E
1990 SHOOTS / m²
1000
800
600
400
200
0
0.0 0.2 0.4 0.6 0.8
1990 - PROP. INFECTED SHOOTS / m²
F
1991 SHOOTS / m²
1000
800
600
400
200
0
0.0 0.2 0.4 0.6 0.8
1991 - PROP. INFECTED SHOOTS / m²

Figure 6. Percent soil water (by mass) per $1 m^2$ in the lower soil layer two, five, and ten days after rain.

2 DAYS AFTER RAIN - LOWER SOIL



8 DAYS AFTER RAIN - LOWER SOIL



18 DAYS AFTER RAIN - LOWER SOIL

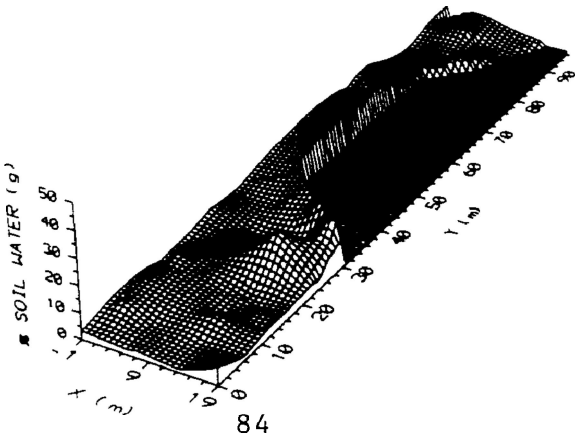
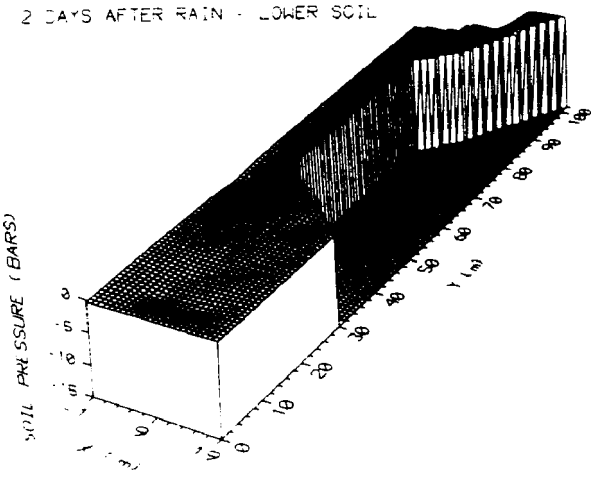
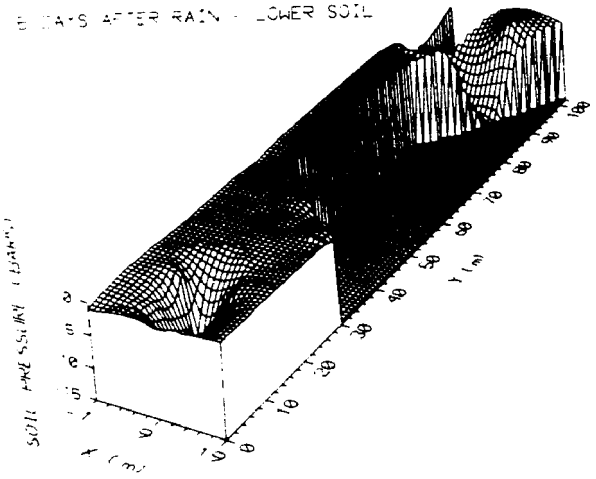


Figure 7. Soil water potential per $1 m^2$ in the lower soil layer two, five, and ten days after rain.


2 DAYS AFTER RAIN - LOWER SOIL
SOIL PRESSURE (BARS)
X (m)
Y (m)


LOWER SOIL
Y (m)

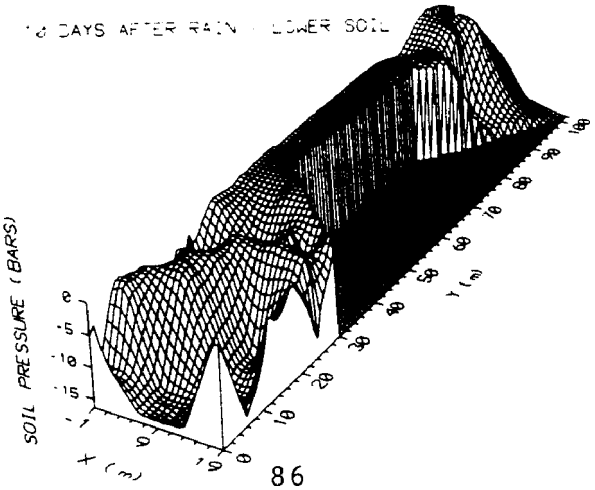
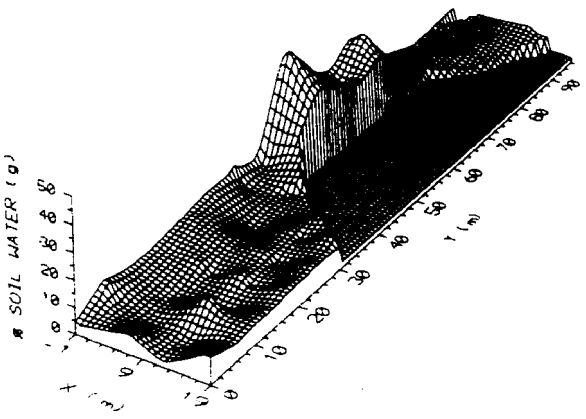
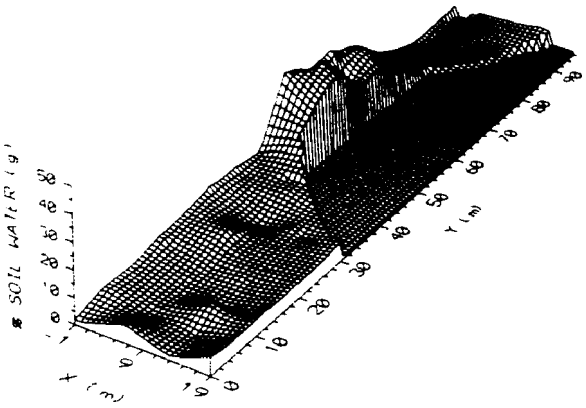

LOWER SOIL
SOIL PRESSURE (BARS)
X (m)
Y (m)

Figure 8. Percent soil water (by mass) per $1 m^2$ in the upper soil layer two, five, and ten days after rain.

2 DAYS AFTER RAIN - UPPER SOIL



5 DAYS AFTER RAIN - UPPER SOIL



12 DAYS AFTER RAIN - UPPER SOIL

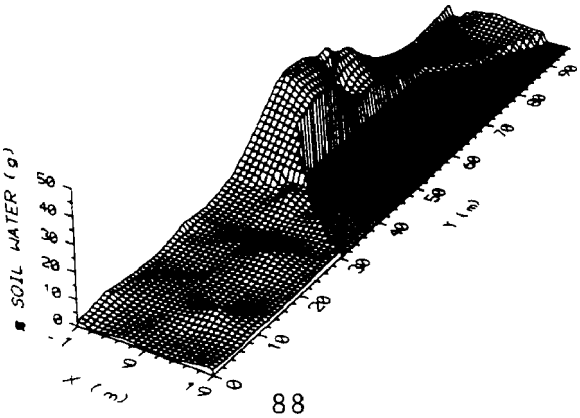
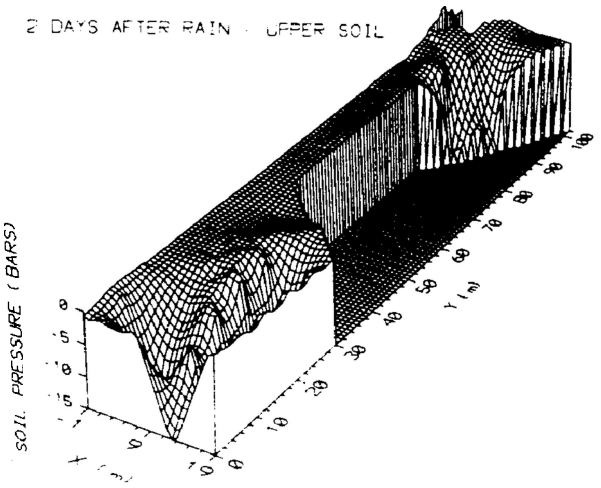
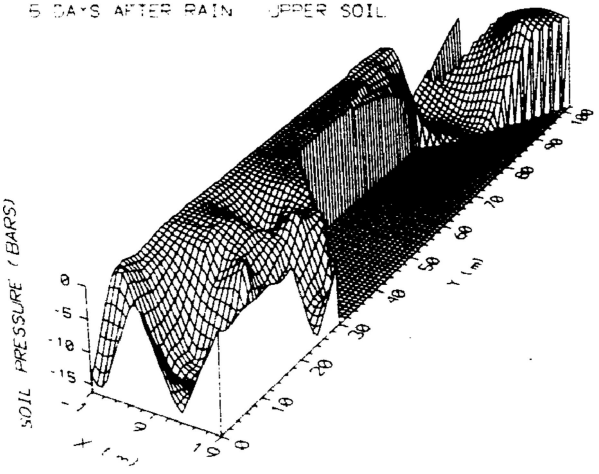


Figure 9. Soil water potential per $1 m^2$ in the upper soil layer two, five, and ten days after rain.


2 DAYS AFTER RAIN - UPPER SOIL
SOIL PRESSURE (BARS)
0
-5
-10
-15
X (m)
Y (m)


5 DAYS AFTER RAIN - UPPER SOIL
SOIL PRESSURE (BARS)
0
-5
-10
-15
X (m)
Y (m)

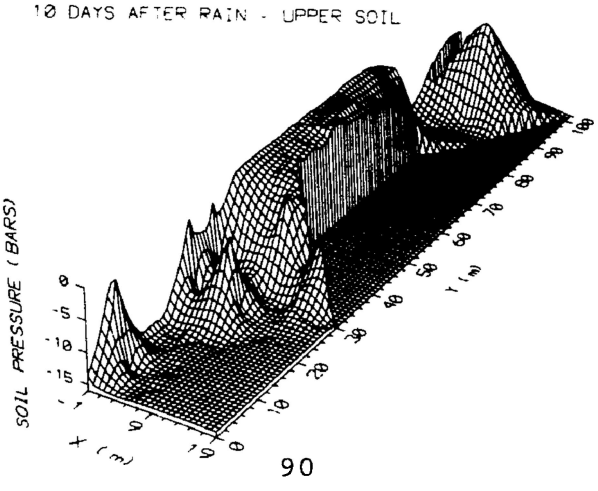

10 DAYS AFTER RAIN - UPPER SOIL
SOIL PRESSURE (BARS)
0
-5
-10
-15
X (m)
Y (m)

Figure 10. Spatial autocorrelation expressed as Moran's I of the percent of infected shoots per 0.25m^2 in distance classes ranging from 0m to 103m in 1990, 1991, and 1992. Points are plotted at the distance class midpoints. Points that were not significantly different than zero are indicated by open circles, significantly different from zero at $P<0.05$ by an open circle with a black center, at $P<0.01$ by a closed circle with a white bull's eye, and at $P<0.001$ by a closed circle.

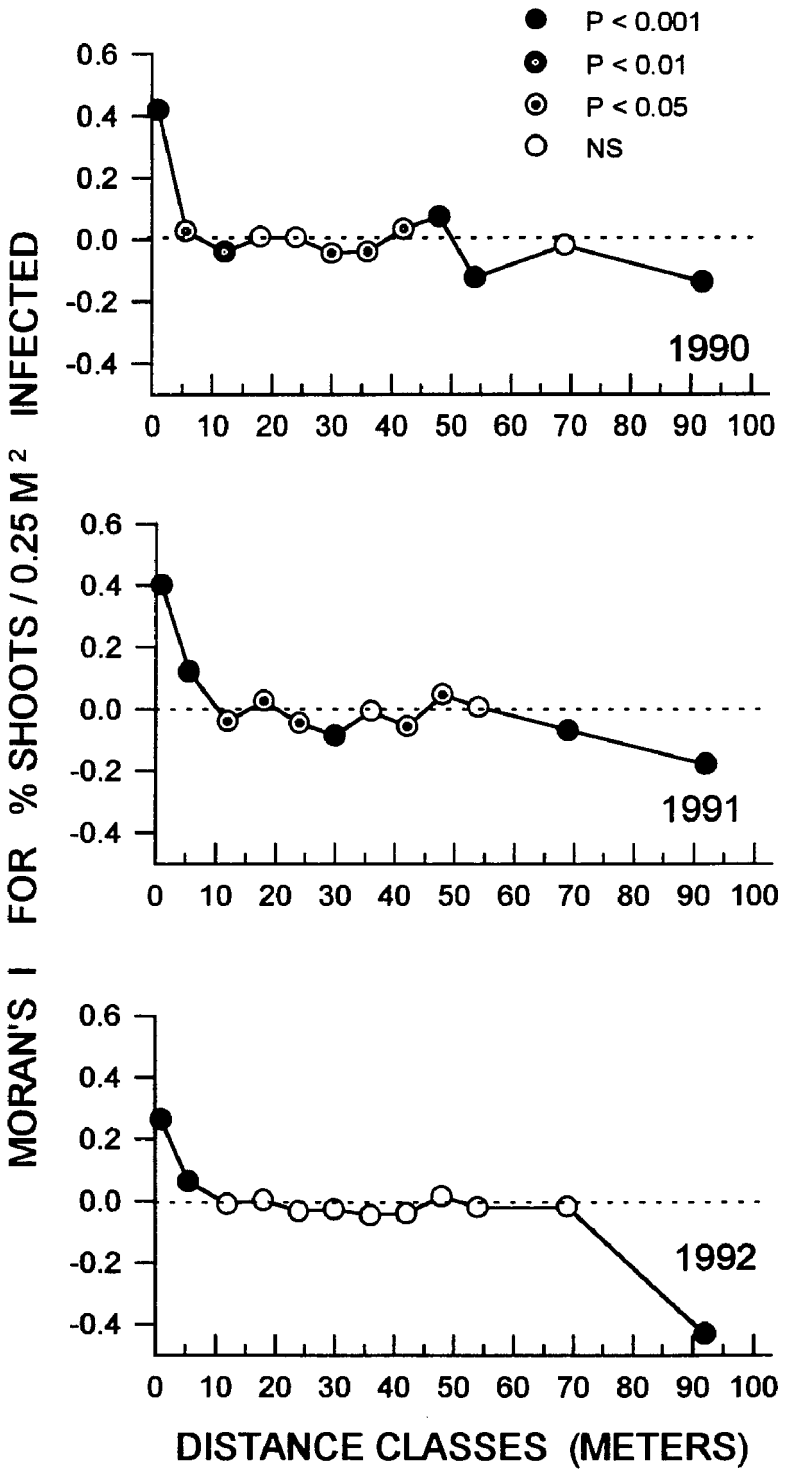

P < 0.001
P < 0.01
P < 0.05
NS
MORAN'S I FOR % SHOOTS / 0.25 M 2 INFECTED
0.6
0.4
0.2
0.0
-0.2
-0.4
1990
1991
1992
0 10 20 30 40 50 60 70 80 90 100
DISTANCE CLASSES (METERS)

Figure 11. Spatial autocorrelation expressed as Moran's I of the percent of infected shoots per 0.25m^2 in distance classes ranging from 0m to 7m in 1990, 1991, and 1992. Points are plotted at the distance class midpoints. For interpretation of symbols see Figure 9.

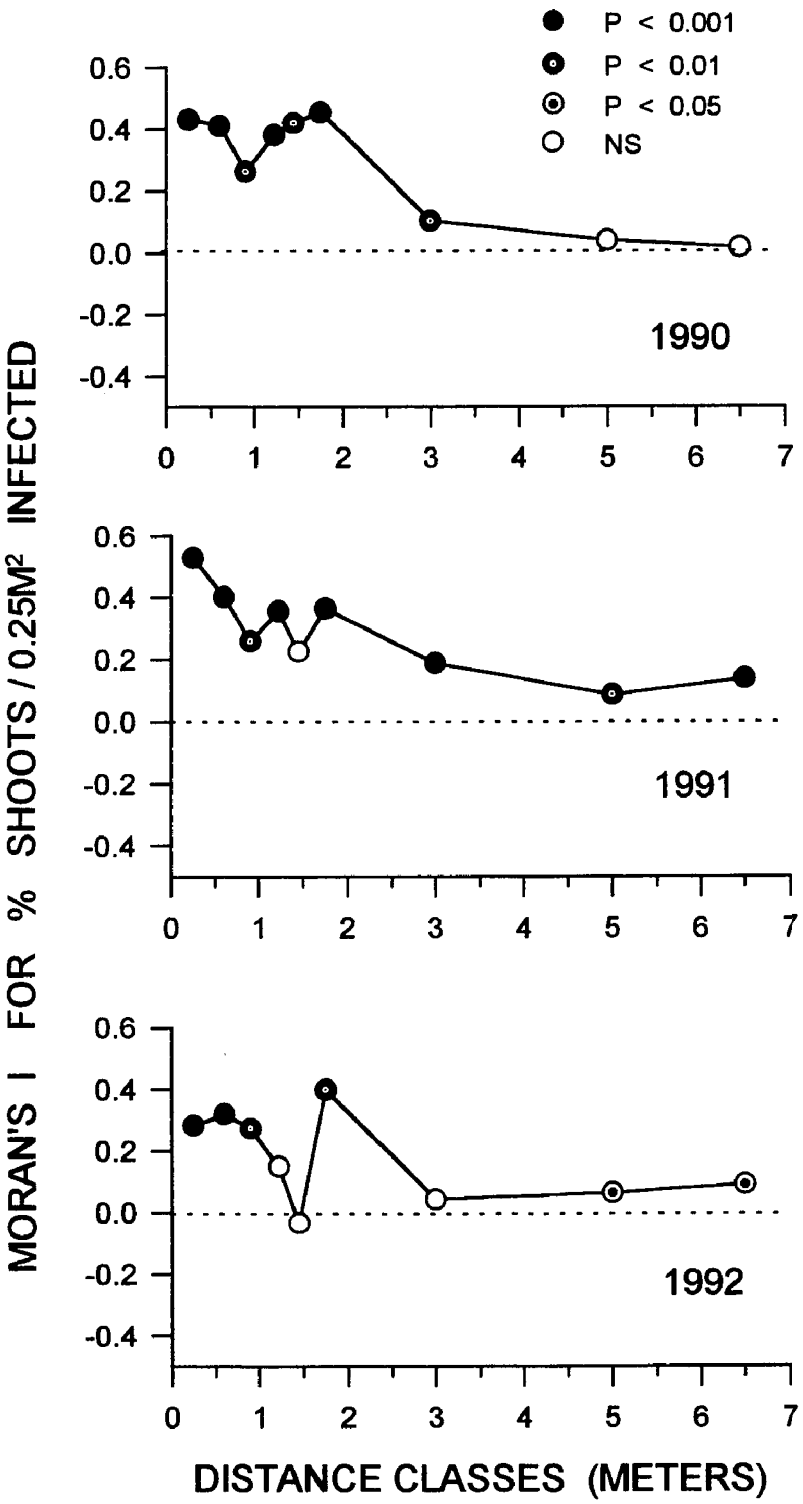

P < 0.001
P < 0.01
P < 0.05
NS
1990
1991
1992
MORAN'S I FOR % SHOOTS / 0.25M² INFECTED
DISTANCE CLASSES (METERS)

Figure 12. Spatial autocorrelation expressed as Moran's I of the number of shoots per 0.25m^2 in distance classes ranging from 0m to 103m in 1990, 1991, and 1992. Points are plotted at the distance class midpoints. Values near 0 are nonsignificant. For interpretation of symbols see Figure 9.

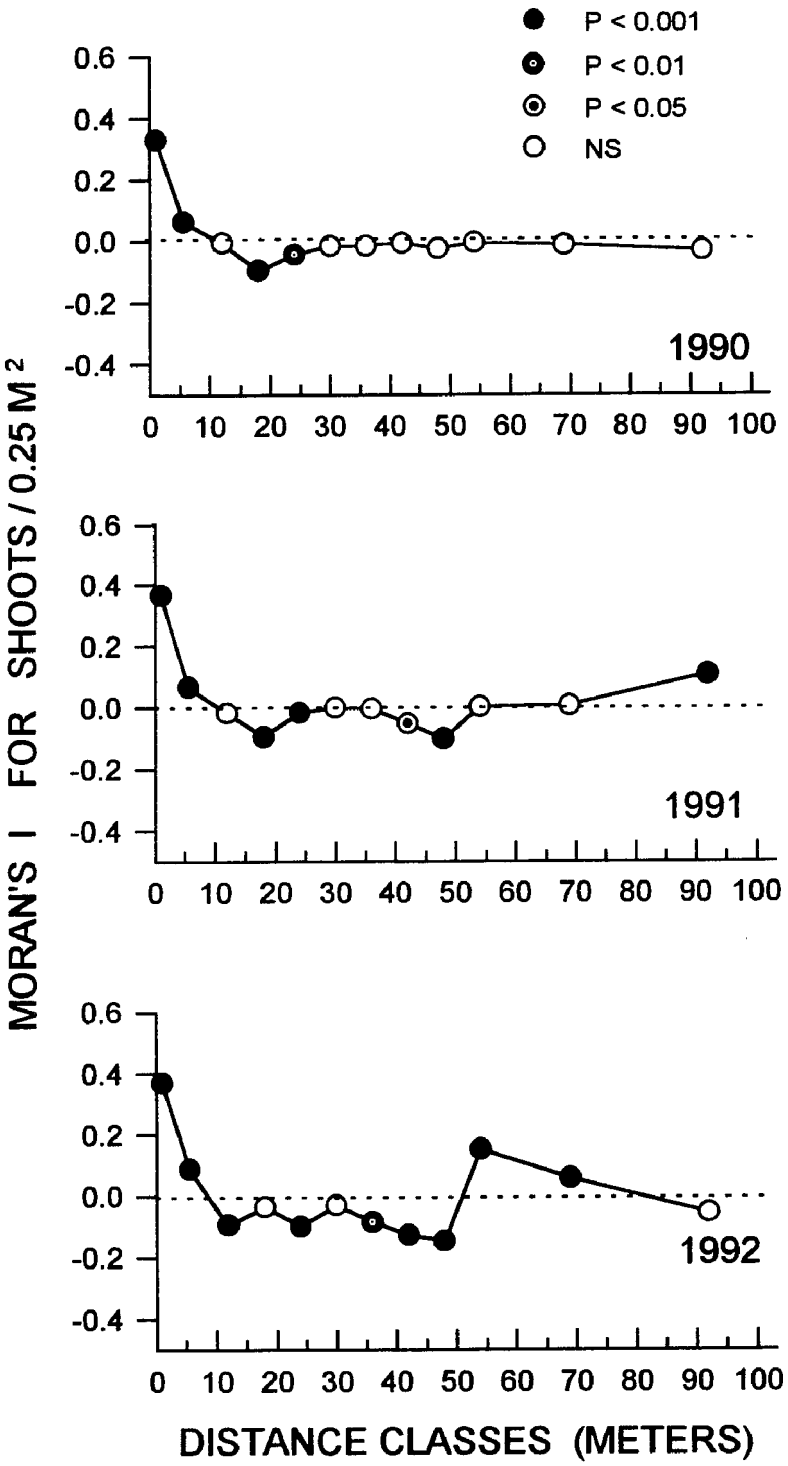

P < 0.001
P < 0.01
P < 0.05
NS
0.6
0.4
0.2
0.0
-0.2
-0.4
1990
1991
1992
0 10 20 30 40 50 60 70 80 90 100
MORAN'S I FOR SHOOTS / 0.25 M 2
DISTANCE CLASSES (METERS)

Figure 13. Spatial autocorrelation expressed as Moran's I of the number of shoots per 0.25m^2 in distance classes ranging from 0m to 7m in 1990, 1991, and 1992. Points are plotted at the distance class midpoints. Values near 0 are nonsignificant. For interpretation of symbols see Figure 9.

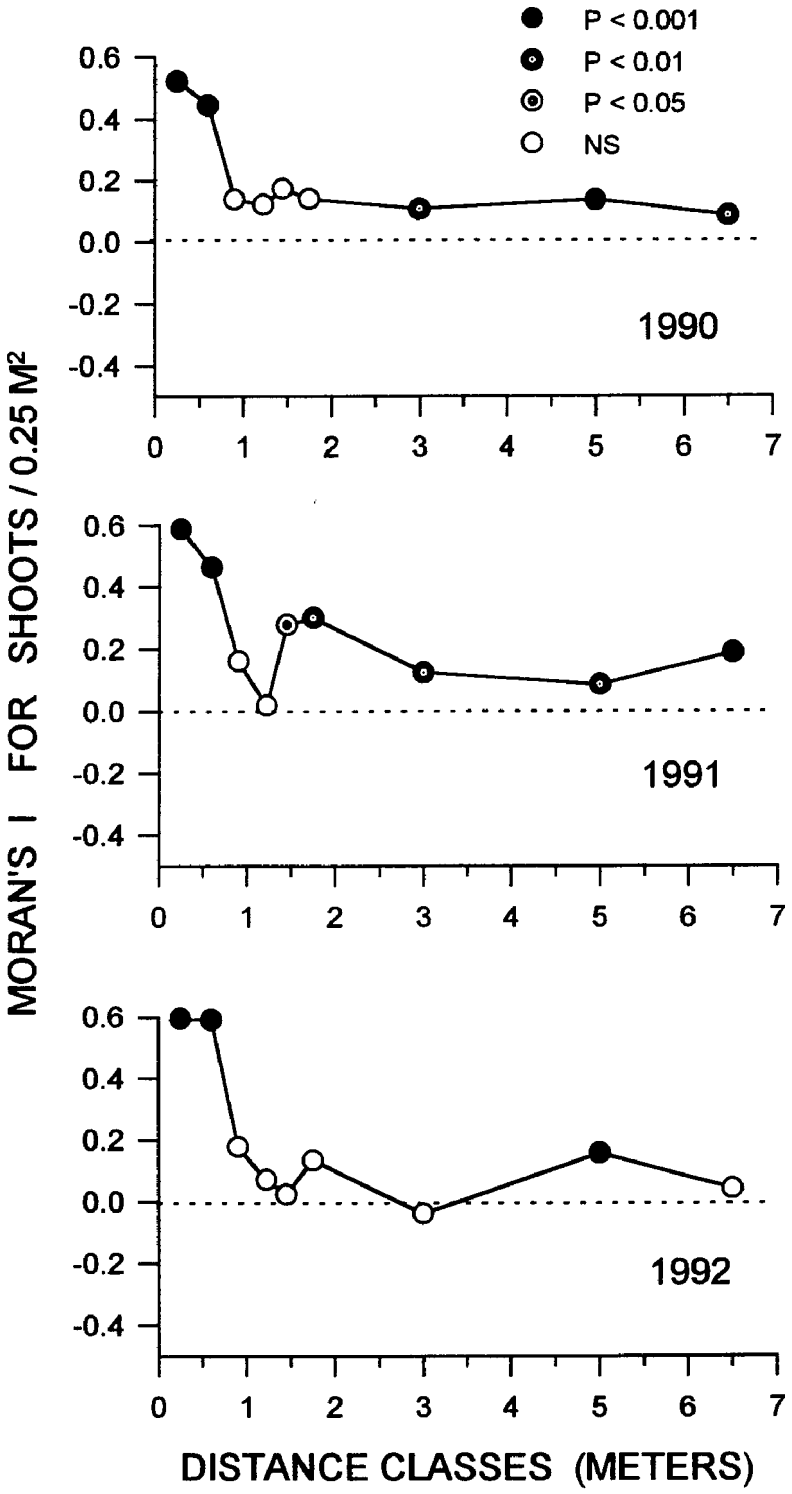
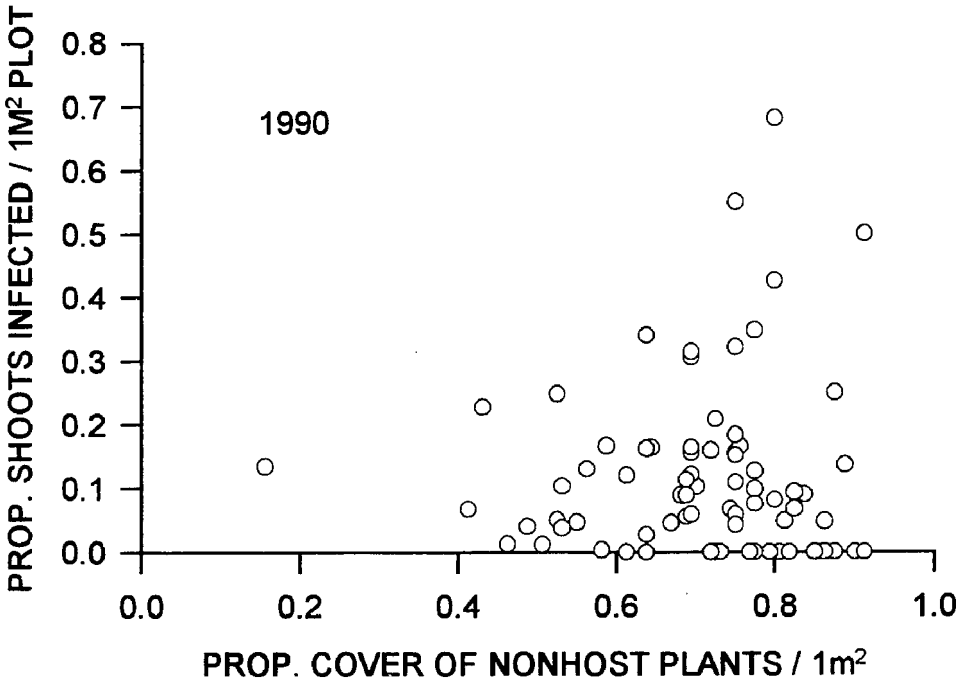

P < 0.001
P < 0.01
P < 0.05
NS
1990
1991
1992
MORAN'S I FOR SHOOTS / 0.25 M²
DISTANCE CLASSES (METERS)

Figure 14. Scatterplot of the relationship between disease (the proportion of host plant shoots infected in $1m^2$ plots) and the proportion of vegetation cover consisting of nonhost plants in $1m^2$ plots , in 1990.


PROP. SHOOTS INFECTED / 1M² PLOT
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1990
0.0
0.2
0.4
0.6
0.8
1.0
PROP. COVER OF NONHOST PLANTS / 1m²

3

Infection of *Juncus dichotomus* by the smut fungus *Cintractia junci*: an experimental field test of the effects of heterospecific neighbors, environment, and host plant genotype.

INTRODUCTION

Plant-pathogen interactions in natural populations are increasingly recognized as potentially powerful factors in plant population biology (Burdon 1987a, 1991, Bierzychudek 1988, Alexander 1992, Jarosz and Burdon 1992, Parker 1992, Ingvarsson and Lundberg 1993, Yahara and Oyama 1993) and in plant communities (Burdon and Shattock 1980, Burdon 1982, 1987a, 1991, Wills 1992, Brasier et al 1993, Crist and Friese 1993, Van der Putten et al 1993). However, little is known about the ecology of plant-pathogen interactions under natural conditions (Burdon 1987a) even though such knowledge is basic to our understanding of the conditions under which diseases become important in plant populations. The purpose of this study is to examine certain aspects of the ecology of one plant-pathogen system, the host plant *Juncus dichotomus* and the smut fungus *Cintractia junci*. In a multi-year field experiment I tested for an indirect effect of interspecific plant competition on this host plant-pathogen interaction, for

the influence of environmental heterogeneity, and for the response of different host plant genotypes to the pathogen in a variable environment. My results show that the ecological context of a plant-pathogen interaction can influence its outcome; under certain conditions host plants are much more likely to become infected than they are under other conditions. Similarly, the host plant genotype can influence infection, and the strength of this effect varies under different environments. Such results are to be expected by ecologists who understand that nature is complex and variable, yet few efforts have been made to experimentally demonstrate the influence of multiple ecological and genetic factors simultaneously in one plant-pathogen study. If we are to develop a predictive science in plant population biology, then we require this type of knowledge about the basic ecological conditions that affect important biotic interactions.

Competition is one of the most well-studied phenomena in plant ecology, yet most of the studies focus only on the direct effects of plant-plant interactions (Grace and Tilman 1990). I chose to examine the indirect effect that plant competition may play in the plant-pathogen interaction because, as an ecological phenomenon that can dramatically affect the physiology and vigor of a potential host plant, it could be very important in the plant's interaction with pathogens (Burdon 1987a). There has been some attention paid to the impact that pathogen infection has on plant

competition (Groves and Williams 1975, Burdon and Chilvers 1977b, Burdon et al 1984, Gates et al 1986, Paul and Ayres 1986, Paul 1989, Clay 1990c, Marks et al 1991) but little to the impact competition may have on infection (Boudreau and Mundt 1992, Finckh and Mundt 1992).

There are at least five ways in which competition could influence whether a host plant becomes infected. First, a host plant that is physiologically stressed due to resource depletion by competitors could also have inadequate resources for defense against parasites, predisposing it to disease (Burdon 1987a, Clay 1990c). Host physiological changes that predispose plants to infection have been shown in a variety of systems (Yarwood 1959, Colhoun 1979). Physiological stress factors tested include light, heat, mineral nutrition, moisture, and defoliation (Yarwood 1956, Colhoun 1979, Huber 1980, Auld et al 1990, Old et al 1990, Dudt and Shure 1993). In this study, I was able to examine whether stress from competition increases infection by measuring the effect of competition on host plant growth and comparing that to competition's influence on host plant infection.

Second, competition could also affect a plant-pathogen interaction through its effect on host plant size, in that larger plants are larger targets (Burdon 1987a). An important component of the disease cycle is inoculation (Baker 1978, Agrios 1988); infective pathogen propagules must somehow

come into contact with a host plant in order for infection to occur. For many types of pathogen dispersal, it is possible that host plant size could be very important in determining the frequency with which pathogen spores reach a particular plant. In insect-vectored pathogens, for example, a large, apparent plant may well be a more likely target for the pathogen. This effect is suggested by whitefly-vectored virus infection found more on larger *Eupatorium chinense* plants (Yahara and Oyama 1993), greater anther-smut infection of larger *Silene dioica* flowers (Elmqvist et al 1993) and larger floral displays of *Silene alba* males (Alexander 1989, 1990). If the disease is spread passively by wind currents or rain splash as it is in the *Juncus-Cintractia* system, large plants could also be bigger targets and intercept more spores. Such an effect is suggested by studies of bean rust infecting bean plants in competition with maize (Boudreau and Mundt 1992).

Third, there is another possible effect of competition through its influence on host plant size. Some infections by systemic fungal endophytes and other fungal pathogens can induce host plants to grow bigger (Watson and Wilson 1956, Clay 1984, 1986, 1990a, b, 1993, Antonovics et al 1987, Nus 1990) or to produce more flowers (Alexander and Maltby 1990)and could provide increased resources for fungal growth and reproduction. If the pathogen in fact requires a host plant of a certain size in order to grow and reproduce within its tissues, then by suppressing host

plant growth and size, competition may help prevent infection. In this experiment I investigated the relationships of competition, host plant size, and infection in order to determine whether larger plants were more or less likely to become infected and whether infected plants grew larger.

Fourth, the level of competition that a host plant experiences will also be linked to the local physical conditions for a pathogen propagule that lands on or near a plant. In a highly competitive situation, the plants are probably at high density. Such a biotic environment will alter a range of microsite conditions that can affect fungal pathogens such as temperature, soil moisture, and light (Agrios 1988). For example, such effects have been suggested in studies of anthracnose infecting dogwood grown in shaded forest or open field (Chellemi and Britton 1992, Dudt and Shure 1993) and bean rust infection on beans grown with and without maize (Boudreau and Mundt 1992). Pathogens may respond dramatically to differences in microsite, with some conditions excellent for spore germination and growth and other conditions inhibitory. Clearly, then, through its effect on the pathogen microsite, the biotic environmental context of a plant-pathogen interaction could be very important.

The possible effects of plant competition / biotic environment discussed above could occur due to either intraspecific or interspecific plant competition. Intraspecific competition is expected to increase as host plant

density increases (Clay and Shaw 1981). Because the frequency of infection is often positively correlated with host plant density (Antonovics and Levin 1980, Burdon and Chilvers 1982, Augspurger and Kelly 1984a, Burdon 1987a), separating the effects of plant competition from host density effects is best done by keeping host plant density constant and varying densities of interspecific plants. In the experiment reported here, I examine the effects of nonhost plants on host disease frequency by growing and inoculating constant densities of host plants in plots with or without other plant species present. In this case, a fifth effect due to interspecific plants is possible; nonhost plants can act as physical barriers to spore dispersal (Chin and Wolfe 1984b, Burdon 1987a). If host plants grow in a matrix of nonhost plants, they could benefit by protection from exposure to pathogen spores. This possibility would be supported in this experiment if the plants growing under higher competition were more likely to resist infection.

Even on a small spatial scale, the environment of a plant-pathogen interaction of course involves many other factors, besides the plant's competition regime, that may also influence the outcome of the interaction (Colhoun 1979, Agrios 1980, Burdon 1987a). So, in this study I took environmental heterogeneity into account by doing the same experiment in two different sites that differ significantly in soil moisture conditions and by using replicate blocks within each site. Environmental heterogeneity itself is

of considerable interest due to its possible interaction with host plant genetic variation for susceptibility (Simms and Rausher 1992). One of the most important goals of current studies in natural plant-pathogen interactions is to document whether or not host plants exhibit genetic variation for resistance to pathogens (Fritz and Simms 1992). This is a necessary condition if pathogens are going to act as agents of natural selection in host plant populations. Certainly host plant populations often do exhibit variation for genetically-based resistance mechanisms (Day, P.R. 1978, Miles and Lenne 1984, Burdon 1987a, Alexander 1989, 1992, Lawrence and Burdon 1989, Parker 1991, Penrose 1991). In the wild, however, variation due to host plant genotype may be overwhelmed by environmental heterogeneity that affects the plant-pathogen interaction (Jarosz and Levy 1988). Thus, a set of plant genotypes that exhibit significant variation in infection in a relatively homogeneous environment may be indistinguishable for infection under more heterogeneous conditions. I was able to examine this type of genotype X environment interaction in a limited way by comparing the response of identical sets of *Juncus dichotomus* genotypes to the *Cintractia junci* pathogen in the two sites.

STUDY ORGANISMS

Juncus dichotomus Ell. (Juncaceae) is a perennial rush that can be

found growing in sandy open areas of the Long Island pine barrens, where it can be attacked by the parasitic smut fungus *Cintractia junci* Schw. (Ustlaginales) (see Chapter Two for more details on these organisms). The plant flowers in early summer and begins dispersing seeds in July (personal observation). Infection can be detected when the smut fungus disrupts normal flowering. A systemically infected shoot produces an inflorescence structure, but it is deformed and produces no flowers or seeds. Instead, membrane-covered lesions which develop on the inflorescence break open, releasing black smut teliospores. The dispersed teliospores germinate and produce hyphal strands which must fuse with a strand of opposite mating type in order to produce an infective mycelium (Fischer, G.M. and Holton 1957).

Plants can be found with a wide range of infection. In the most severe cases all flowering shoots of the plant exhibit a systemic infection, in intermediate cases some shoots are systemically infected while some shoots flower and produce seeds normally, and in the most benign cases no shoots are systemically infected, but isolated flowers on otherwise healthy inflorescences are infected (personal observation). The fungus is perennial in the host plant and the proportion of shoots infected is likely to increase in the following year (Morrison, unpublished data). Shoot infection in one population I have studied (see Chapter Two) ranged from 0%-88%

shoots/m^2, so infection is spatially variable and can be locally severe. This experiment is part of a larger study to determine what ecological and genetic factors contribute to the spatial variation in infection that occurs in this population.

METHODS

THE EXPERIMENT.

In September of 1989 I collected 21 apparently healthy *Juncus dichotomus* individuals from the natural population described in Chapter Two, making sure that individuals were collected at least 0.5 meters apart and across the entire 20m X 100m population. I brought the plants to the greenhouse at SUNY Stony Brook where I washed their roots, planted them in standard Pro-Mix potting soil, and placed them in a common greenhouse bay. In January of 1990 and again in late March, late April, and early June 1990 I divided each plant at the rootstock into several ramets, repotted them, and placed them back in the bay. At the final propagation, I clipped off any flowering shoots and then grew the plants in two inch pots in the

greenhouse for another three weeks before transplanting to the field site. Throughout the propagation the plants were fertilized and watered as needed, with all plants receiving identical treatment. By growing the plants for nine months in a common greenhouse environment and successively propagating them four times, I minimized any possible variation among them due to environmental effects of their original collection locations. In the weeks just prior to the start of the experiment, the plants flowered in the greenhouse, which allowed me to check them again for infection. In the experiment I used only uninfected plants from the fourth propagation.

Since one goal of this experiment was to examine different plant genotypes for susceptibility, I needed to ensure that my 21 plant sample contained genetically distinct individuals. Dispersal distances are unknown for this plant; likewise, the breeding system of this species is not well studied, though selfing may be common (R. Brooks, personal communication and J. Morrison, unpublished data). I used starch gel electrophoresis to screen the 21 individuals for allozyme differences and found 7 polymorphic loci (ADH, PGM, IDH1, IDH2, 6PGDH, MDH1, MDH2) which let me determine that my set of 21 individuals contained at least eight distinct genotypes.

In late May of 1990 I set up 20 plots in a field location about 1 km from the natural population. This site is very similar to the home site, with

sandy soils and herbaceous vegetation. In fact, *J. dichotomus* grows at this site, although no naturally smutted individuals occur there. The experimental design consisted of 5 blocks in each of two sites. Each block had two adjacent 60X60 cm plots arranged as in Figure 1. I randomly assigned one plot of each pair to a vegetation removal treatment and removed all living vegetation before the experiment was begun. I did this by spraying the herbicide glyphosate (Roundup by Monsanto) into the plot, which I first surrounded by a tall cylinder of thin sheet metal. In this way no herbicide could drift into the other plot in the block, which was assigned to the no-removal treatment. After about 10 days I clipped off the dead vegetation at soil level. This technique kills the entire plant including the roots but leaves the soil undisturbed. In the other plot all vegetation was left intact, except that I removed with Roundup any *J. dichotomus* plants growing naturally in a plot. Several times in each year I maintained the initial removal treatments by gently uprooting any seedlings which had invaded the removal plots.

I set up the same experiment in two adjacent sites that differed in soil moisture in order to check for consistency of experimental results across environmentally heterogeneous space. The mesic site is alongside a cranberry bog and the soil is consistently wet, while the xeric site is slightly upland and the soil becomes very dry during the growing season (personal observation). On June 3, 1992, two days after a rainfall, I took soil cores

from the top 15 cm in each block in each site using a 2 cm diameter soil corer, weighed the samples before and after drying them in a 60° C drying oven, and calculated the percent soil water for each block. The xeric site was significantly drier than the mesic site (xeric mean percent soil water = 5.54%, n = 5; mesic mean = 10.61%, n = 5; t-test for the difference between two means assuming unequal variances, $t = -3.63$, $df = 6.4$, $P = 0.01$).

Planting began on June 26, 1990 and finished on June 28, 1990. I transplanted one copy of each of the 21 individuals into each plot in a hexagonal lattice design, with 5 cm between plants. This left a 20 cm unplanted outer border for each plot. Since each plot contained clones of the same 21 individuals, I have eliminated the possibility of plot-to-plot variation due to different genetic susceptibilities of the particular host plants in the plots. The 21 individuals were randomly located within the lattice. I planted each with its greenhouse soil plug intact in order to equalize the initial root environment of the transplants. I transplanted plants in no-removal plots directly into the vegetation matrix, and those in removal plots into the bare soil.

During planting I inoculated each plant with the pathogen by dipping two clipped leaves into 25 ml of a teliospore suspension, and then pouring those 25 ml into the soil plug at the base of the plant. I made the teliospore

suspension on June 26, 1990, the same day that the planting began, by crushing into deionized water fresh, sporulating lesions from over 100 plants from the natural, infected population. For each plant, I first shook the suspension before withdrawing the 25 ml for inoculation. Originally, I planned for each plant to receive an equal volume of inoculum from one common teliospore suspension, so that each would have equal probabilities of encountering both a certain number of spores and the same pool of pathogen genotypes. However, I needed to make a second suspension on June 28, 1990, and some blocks received inoculations from this second suspension. This second suspension was made in the same way from the same pathogen population, and I attempted by eye to make it the same concentration. In the analysis I test to see if there is an effect of inoculum suspension.

From July 31 through August 2, 1990 I examined the plants for any sign of infection and found none infected. In midsummer 1991 and 1992, I examined each plant again and counted the number of shoots with systemic infection and the number of healthy shoots. In early September 1992, I harvested the aboveground parts of each plant, dried them to a constant weight at 60° C in a drying oven, and measured dry weight per plant.

ANALYSIS

Host plant size and fitness.

Since I measured host plant size as shoots per plant on the same individuals in 1991 and 1992, I did repeated measures analyses with PROC GLM in SAS version 6.0. (Gurevitch and Chester Jr. 1986, SAS Institute Inc. 1989). The between-plant main effects were removal treatment and block and they were tested with repeated measures ANOVA; the repeated within-plants effect was year and it was tested with profile analysis (von Ende 1993). For the xeric site in this analysis I did not include data from the no-removal plot in block 2 since between 1991 and 1992 the plot was disturbed and I could not be sure of the individual plant identities in 1992. Similarly, any other plants which did not have positive identities in both years (usually due to lost plant markers) were deleted from the analysis. The SAS output for the year effects provides four different multivariate test statistics. Since they all provided equal F statistics, I only report the values for Pillai's trace in Table 1.

I also measured host plant size in just 1992 as aboveground dry mass and I measured host plant fitness as the number of uninfected reproductive shoots per plant in 1992. I analyzed both of these variables with a Model I two-way analysis of variance (Sokal and Rohlf 1981) with PROC GLM in SAS version 6.0, using Type III sums of squares (SAS Institute Inc. 1989,

Potvin 1993). Again, removal treatment and block were the main effects.

In field experiments blocks are usually treated as random effects but are not required to be (Potvin 1993). Since the blocks in this experiment were few, they may not be a random sample of the variation that occurs in the site, which is the criterion for treating them as a random effect. Consequently, I chose to consider blocks as fixed effects, so that all terms are tested over an error variance for univariate tests (Sokal and Rohlf 1981, Potvin 1993), or an error sums-of-squares-cross-products matrix for multivariate tests in the repeated measures analysis (Scheiner 1993).

I transformed both size measures and the fitness measure to $\log_{10}(x)$ so that the error terms were more normally distributed and to induce homoscedasticity (Sokal and Rohlf 1981). I tested the normality of the residuals from each analysis with PROC UNIVARIATE in SAS, which provides the Shapiro-Wilk statistic W, stem and leaf displays, boxplots, and normal probability plots. Either W or the graphic tests showed that the residuals approached normality or were normally distributed. I used the F-max test to see whether the variances of the 10 plots within each site were homoscedastic. Only the fitness measure in the xeric site was significantly heteroscedastic after transformation ($F_{max} = 5.62$, $df = 15, a = 10$), but moderate heteroscedasticity does not influence the overall tests of significance appreciably (Sokal and Rohlf 1981). In Figure 2 and Figure 8

the treatment means were backtransformed by taking the antilogs of the means and 95% confidence limits were calculated with transformed means and standard deviations and then backtransformed.

Host plant infection.

I measured host plant infection as the proportion of infected shoots per plant, and initially planned to analyze these data with factorial analysis of variance. However, the data turned out to be extremely skewed due to large numbers of plants no diseased shoots. No transformation could correct this. Thus I was unable to analyze the proportion data with ANOVA since the assumption of normally distributed error terms was severely violated. So, instead I categorized the plants with a binary response variable of shoot infection present/absent, which I could then analyze with a categorical analogue to ANOVA using the CATMOD procedure in SAS 6.0 (Freeman 1987, SAS Institute Inc. 1989). Biologically, binary categorization makes sense. It is likely that the event with long-term importance for the plant's fitness is, in fact, the initial infection. In the natural population I studied, plants which I censused for four years show that disease severity usually increased (unpublished data). The same holds true for the plants in this experiment (see Results below).

With CATMOD I used a three-factor, one-response model of

homogeneity (Freeman 1987) separately in the xeric and mesic sites to test the independent factors of block, removal treatment, year, and their interactions, with year as a repeated factor (SAS Institute Inc. 1989). In all analyses the three-way interactions of removal X block X year were not significant, so I then used a model that included only the main effects and the two-way interactions (Freeman 1987). As with the plant-size repeated-measures analysis, I excluded block 2 in the xeric site from this analysis. I used the same type of model to test the effect of site, removal, and year, pooling across the blocks within a site and combining the data from both sites.

I also analyzed infection differences among the eight electrophoretic genotypes within a site with categorical models of homogeneity. Again there were no significant three-way interactions, so only two-way interactions were included in the models. Because of low replication of each genotype within each plot, I needed to pool plots to do any analysis of host genotype differences. First I used genotype, site, and year as main effects, with year a repeated factor. I also tested each site separately, with just genotype and year as main effects. For the 1992 data, when the site effect was not significant (see Results below), I also pooled data from all 20 plots of the experiment (across both sites) and tested for a genotype X removal treatment interaction with a categorical model of homogeneity as above.

These categorical models test whether the distribution of the response variable (infection present/absent) is homogeneous across treatment groups. The analysis assumes that the data follow a binomial distribution, which can be used for hypothesis testing with the Neyman chi-square statistic, in which the observed counts of infection present/absent in the treatment groups are compared to expected counts calculated with a weighted least squares method (Freeman 1987). If the chi-square is significant, this means that the observed counts deviate from the expected by more than chance alone, which implies that the different treatment groups do not all follow the same binomial distribution and do not have equal probabilities of being diseased.

These data fit the assumptions of a binomial distribution (Freeman 1987): binary outcome (disease present/absent), fixed total sample size, independent probability of outcome, and independence of observing an outcome (all plants have the same probability that disease may be present). I used the most straightforward response function to model in a factor-response data structure with a binary outcome - the simple probability or proportion of healthy plants and diseased plants (Grizzle et al 1969, Freeman 1987). Sample size should be about 25x, where x is the number of response functions being modelled for each population or treatment combination (SAS Institute Inc. 1989). In this experiment I model one

response function, the probability of a plant being diseased, so for each treatment combination I should have 25 or more plants. In all of my analyses I approach or exceed this number.

I analyzed the relationship between infection in 1992 and host plant size in 1991 with logistic regression (Hosmer and Lemeshow 1989), which regressed the logit of the probability that a plant is infected on the number of shoots per plant in 1991. I used only plants that were uninfected in 1991. In order to avoid confounding the effect of 1991 plant size with the removal treatment, I did these logistic regressions separately for plants from the removal plots and the no-removal plots.

RESULTS

EFFECTIVENESS OF REMOVAL TREATMENT

First, I examined whether in fact the competitor-removal treatment had any impact on host plant vigor by analyzing host plant size. In both the xeric and mesic sites, plants which grew in the removal plots were significantly larger than those in no-removal plots. The repeated measures analysis shows that removal-plot plants had significantly more shoots per plant (Table 1, Figure 2) and in 1992 they also had greater mass (Table 2, Figure 2). Clearly, removing the surrounding vegetation from a host plant's plot had a relaxing effect on its competitive regime.

INFECTION OUTCOMES

Removal, year, block, and site effects

In the xeric site, host plants which grew in the removal plots were significantly more likely to be infected than were those growing in the no-removal plots (Table 4, Figure 3) but in the mesic site there was no significant difference (Table 5, Figure 3). In both sites, plants were more likely to be infected in 1992 than in 1991(Tables 4 and 5, Figure 3). There was a significant removal X year interaction in both sites also (Tables 4 and 5). As can be seen from Figure 3, this was because the removal treatment had different effects in the two years. In the xeric site, removal plots had more disease in both years, but categorical analyses done separately for the two years showed that in 1991 this was not a significant difference ($\chi^2 = 0.36$, $P = 0.549$, $df = 1$), while in 1992 it was ($\chi^2 = 9.14$, $P = 0.003$, $df = 1$). For the mesic site, Figure 3 shows that in 1991 removal plots had less infection than no-removal plots, while in 1992 they had more. However, neither of these differences were significant in separate categorical analyses by year (1991: $\chi^2 = 1.77$, $P = 0.184$, $df = 1$; 1992: $\chi^2 = 2.51$, $P = 0.13$, $df = 1$).

There was a highly significant block effect in the xeric site (Figure Table 4, Figure 4 (block 2 is included in this figure for illustration even though it was not in the analysis)), indicating that this site was very

heterogeneous for pathogen infection. It is possible that some of this heterogeneity was not due to physical differences among the blocks within the site, but could also have been due to using two different inoculum suspensions. When I set up the experiment, blocks 1, 2 and 3 received inoculum from the first suspension and blocks 4 and 5 received the second suspension. In the repeated measures categorical analysis I did pairwise contrasts for the blocks, excluding block 2 as discussed above (Table 4). These indicate that infection in block 1 is distinct from that in blocks 4 and 5, but that block 3 is not different from blocks 4 and 5 even though it received a different inoculum suspension. Thus it seems unlikely that the overall block heterogeneity was simply due to an inoculum effect. Instead, this site is inherently heterogeneous for the plant-pathogen interaction at the infection stage.

In the mesic site, the blocks only approach significant heterogeneity in infection ($P=0.08$, Table 5, Figure 4). In this site, blocks 3, 4, and 5 received inoculum from the first spore suspension and blocks 1 and 2 received the second suspension. The pairwise block contrasts for the mesic site (Table 5) indicate that this borderline heterogeneity is unlikely to be due to any inoculum difference.

In both sites there were highly significant interactions between the removal treatment and the blocks (Table 4 and 5, Figure 5). In some blocks

host plants in removal plots were more likely to be infected, but in other blocks they were less likely to be infected. This indicates that the combination of biotic and physical environment may be very important to the plant-pathogen interaction.

To make a more quantitative analysis of infection differences between the two sites, I pooled the blocks within each site and then used a categorical model of homogeneity with removal, site and year as the main effects (Table 6, Figure 3). The results are consistent with the separate site analyses above. The site difference is significant; plants in the xeric site were more likely to be infected than those in the mesic site. The removal treatment across the entire experiment was marginal ($P=0.07$), reflecting the combination of the xeric site where it was significant and the mesic site where it was not. Removal X site is marginally significant ($P=0.07$) which reinforces the finding that the removal treatment effect had different strengths in the two sites. The highly significant removal X year interaction across the whole experiment is consistent with the separate analyses, showing again that removal plots increased infection from 1991 to 1992 more than no-removal plots.

Host plant genotype, year, and site effects

There was no statistically detectable variation for infection among the eight host genotypes when I analyzed all 20 plots together with electrophoretic host genotype, site, and year as main effects (Table 8). However, when the sites were analyzed separately I found that, in the mesic site, host genotype did significantly affect whether or not plants were diseased, but in the xeric site it did not (Table 9, Table 10, Figure 6). There was also a significant genotype X year interaction in the mesic site (Table 10). Figure 6 indicates that the genotypes were more heterogeneous in the 1991 mesic site than in the 1992 mesic site. This is supported by separate RXC tests of independence (Sokal and Rohlf 1981), done with PROC FREQ in SAS 6.0), which test the interaction of genotype and disease in the two years. In 1991 the genotypes were heterogeneous for infection ($G = 17,85$, $df = 7$, $P = 0.01$) but in 1992 the heterogeneity was only marginal ($G = 12.14$, $df = 7$, $P = 0.10$).

A categorical model of homogeneity done for just 1992, with site and genotype as the main effects indicated that the two sites were not different in that year ($\chi^2 = 0.06$, $df = 1$, $P = 0.80$), so I pooled all 20 plots across sites in order to have large enough sample sizes to do a test of the genotype X removal treatment interaction. This analysis shows no significant interaction between genotypes and removal treatment (Table 11).

PLANT SIZE AND INFECTION

Since plants in the removal treatment had a higher probability of becoming infected and were also larger, I tested for a relationship between plant size and infection. First, however, to show that the number of shoots per plant is a reasonable measure of plant size, I did a Kendall rank correlation (Sokal and Rohlf 1981) of aboveground dry mass in 1992 with shoots per plant in 1992, since dry mass may be a better indication of overall plant size than shoots per plant. In both the xeric and the mesic sites, shoots per plant are highly correlated with dry mass (xeric site: $\tau = 0.664$, $n = 191$, $P < 0.001$; mesic site: $\tau = 0.595$, $n = 198$, $P < 0.001$; done with PROC FREQ in SAS 6.0).

The first plant size-infection relationship I tested was whether or not larger plants might have been bigger targets for the pathogen in one year and thus exhibited greater infection the next year. In both the removal and no-removal plots, the logistic regressions of infection in 1992 on size in 1991, done with plants uninfected in 1991 only, have significantly positive slopes, indicating that uninfected plants that were bigger in 1991 did have a higher probability of being infected in 1992 (Figure 7), regardless of the presence of neighbors. It is possible that the negative influence on infection caused by interspecific neighbors (Figure 3) was partly due to a nonhost barrier effect, but these results suggest that plant size alone is an important

factor.

Secondly, by holding constant statistically with analysis of covariance the size of the plants in 1991 and looking at the relationship of their size in 1992 with their 1992 infection status, I tested whether infection itself may have induced the plants to grow larger. Again, this analysis was done only on plants that were uninfected in 1991. For each site I pooled plants from all 10 plots and did an analysis of covariance (Sokal and Rohlf 1981), using the BIOM statistical package (Rohlf 1981), with size in 1991 as the covariate, with plants classified as infected or not infected in 1992 as the independent variable, and with size in 1992 as the dependent variable. Both 1991 size and 1992 were square-root transformed to improve normality. Residual analysis done with PROC UNIVARIATE in SAS 6.0 gave as a test of normality $W = 0.97$, $P = 0.30$ for the xeric site and $W = 0.99$, $P = 0.77$ for the mesic site.

In the xeric site there was no difference in 1992 size for plants that became infected between 1991 and 1992 and those that remained uninfected, with 1991-size as a covariate (Table 13). In the mesic site, however, plants that became infected between 1991 and 1992 were significantly larger by 1992, even with 1991-size held constant (Table 13). This indicates that infection did not induce increased size in the xeric site, but may have done so in the mesic site.

FITNESS AND DISEASE PROGRESS

Fitness. Removing interspecific neighbors can increase the infection probability for a plant, as it did significantly in the xeric site. At the same time, however, competitor removal consistently resulted in larger plants. So I tested whether or not plants in removal plots have lowered fitness as a result of infection.

As a correlate of fitness I measured the number of uninfected shoots per plant at the end of the experiment, in 1992. These healthy shoots were reproductive, produced normal inflorescences with flowers and seeds, while the infected shoots produced no flowers or seeds. For both the xeric and mesic sites, the analysis of variance on this fitness measure, with removal treatment and block as the main effects, indicated that competitor removal resulted in plants with more reproductive shoots per plant compared to plants in no-removal plots (Table 14, Figure 8). So, even though there was a tendency for plants in removal plots to be infected and thus have some nonreproductive shoots, after two years their fitness was still greater than plants in no-removal plots, due to their increased growth and greater healthy shoot production.

Disease progress within plants. Since the experiment ended after two years, I can not know for certain what the long-term fitness consequences would be for plants in removal versus no-removal plots. If

they are more likely to become infected in removal plots and if infection then spreads throughout the plant in subsequent years, it is possible that over a longer period of time than two years fitness of infected plants could decrease relative to uninfected plants.

To examine the spread of infection through host plants I compared disease progress from 1991 to 1992 for plants classified as with or without shoot infection in 1991. I measured disease progress by calculating the percentage of plants in which the proportion of infected shoots increased from 1991 to 1992.

In both sites, some 1991-infected plants recovered in 1992, some had decreased infection, and some remained at the same level. However, the majority of 1991-infected plants experienced increased infection, while the majority of 1991-uninfected plants continued to escape the disease in 1992 (Figure 9). G-tests of independence (Sokal and Rohlf 1981) done with PROC FREQ in SAS 6.0 show that (in both sites) 1991-infected plants were more likely to have increased infection than were 1991-healthy plants to become infected (xeric site, $G_{adj} = 9.05$, $df = 1$, $P < 0.005$; mesic site, $G_{adj} = 5.66$, $df = 1$, $P < 0.05$). So, a one-year-old infection tends to worsen in the plant by the second year and will do so more readily than will a new infection begin.

DISCUSSION

Competition, infection, and fitness.

In this system, plant-plant interspecific competition has a strong direct negative effect on host plant growth, but it also can have a strong indirect effect on the plant-pathogen interaction, being beneficial to the host plant by keeping infection at bay. A similar net benefit has been suggested for herbivory; herbivores may keep plant abundances below the level needed for a pathogen epidemic to develop (Bowers and Sacchi 1991). If such beneficial indirect effects on potential host plants were strong enough, there could be important implications for plant communities. In a site where a pathogen exerts strong selective pressure on its host plant, by being very abundant or very virulent, then there may be a long-term selective advantage to a plant that can survive and reproduce under strong interspecific competition, but which also escapes infection because of the presence of heterospecific neighbors. In turn, this could promote higher diversity in plant communities. It has been suggested previously that pathogens and other pests may in fact promote species diversity in a frequency-dependent manner, epidemics being more likely when susceptible plants are in monoculture (Chilvers and Brittain 1972, Burdon and Chilvers 1977a, Harper 1977, Chin. and Wolfe 1984a, Burdon 1987a, Clay 1990c). That argument emphasizes the barrier effect of nonhost plants, but I suggest

that the presence of nonhosts as competitors could have an additional effect on plant-pathogen interactions through their effect on plant size.

This adaptive scenario depends on interspecific competition being stronger than intraspecific competition. It is possible to imagine host plant characters that could evolve to promote a seed landing or growing preferentially under crowded conditions, such as short dispersal distance or germination in shade, but it is more difficult to imagine characters that could evolve to give a plant a higher probability of competing with a heterospecific rather than a conspecific. Perhaps a combination of characters would suffice. A dispersal distance long enough to remove a seed from its parental stand along with a shady germination requirement may work, for example.

It is also possible that the effect of interspecific competition on the plant-pathogen interaction seen in this experiment was due simply to the level of competition, regardless of whether it was intraspecific or interspecific. In another experiment in which host plants were grown and inoculated under low and high intraspecific competition (Chapter Four), plants were more likely to be infected under low host plant density, and they were larger on average. This result, combined with the result from the present experiment, suggests that both types of competition decrease the chances that a plant will become infected. In this case, there would be no particular consequences for the wider plant community, as there would be if

the effect were due to interspecific competition only.

If the effect is through plant size, as my data suggest may be the case, then we may expect some optimum host plant size where plants are big enough to survive and reproduce, but small enough to avoid either being found by a pathogen propagule or being a good resource for the pathogen. Thus we could see the evolution of characters (e.g. dispersal or germination mechanisms) within such a plant population that promote the survival and reproduction of potential host plants under competitive conditions.

For such a scenario to be possible, infection by the pathogen must result in a decrease in lifetime fitness. In the two years of this study I could not demonstrate any reduction in fitness in removal plots compared to no-removal plots due to infection, since the plants under removal still grew bigger and produced more reproductive shoots per plant. However, there may be long-term fitness consequences that I could not detect in just two years. Infection tends to worsen with time, with an increase in the proportion of shoots infected by the pathogen. In the natural population from which I took the experimental plants, I have seen many plants that are completely systemically infected. It seems likely that once a plant is infected it may eventually have its reproductive value reduced to zero. Thus, although there may be a short-term, transient benefit to a plant that is large and vigorous, there may well be an eventual fitness cost if that plants ends up as

a successful host for the pathogen. In future work it would be of great interest to follow inoculated plants longer in competitive and non-competitive situations and calculate their cumulative relative advantage and disadvantage.

Further experimentation would be necessary to understand more precisely how interspecific plant competition influences this plant-pathogen interaction (see methods of Boudreau and Mundt 1992), but the results of this experiment suggest some possibilities. Removing the surrounding vegetation from plots had a dramatic and consistent effect on plant size measured in a variety of ways from 1990 through 1992 in both the xeric and mesic sites. The removal treatment always resulted in bigger plants and this fact may be the key to why plants in these plots exhibited infection more frequently rather than less frequently.

There are two possible explanations for a host plant size effect. First, a healthy plant is a target for pathogen propagules. A larger target would come into contact with a larger number of infective propagules. If each propagule carried with it a probability of successfully infecting that plant, then the probability that the plant becomes infected is the sum of all the propagule's probabilities. In addition, with smut fungi generally, infectivity only results when two strains of opposite mating type fuse to produce an infective mycelium (Fischer, G.M. and Holton 1957, Thomas 1991), and

this mycelium needs to be very close to vulnerable host plant tissue for penetration (Snetselaar and Mims 1993), J. Groth, personal communication). Clearly, with more propagules on a plant, there is a greater chance that two of opposite mating type will be in close enough proximity to each other and to the plant to achieve fusion and penetration.

When the experiment was set up in 1990 I artificially inoculated each plant with equal volumes of teliospore suspension. I would therefore not expect a strong host target size effect as infection developed between 1990 and 1991. Indeed, there was not a consistent trend in the data in that year; the xeric site had slightly more infection in removal plots and the mesic site had slightly more in the no-removal plots (Figure 3).

Between 1991 and 1992, infection increased in both sites. There are two possible explanations for this. One is that some plants harbored latent infections from the 1990 inoculation that only appeared symptomatic in 1992. The other is that some plants became initially infected between 1991 and 1992 from spores that were released from plants already infected in 1991. I attempted to identify latent infections in plants with cytological staining of fungal mycelium, but was unsuccessful. Thus, I do not know which of these two alternatives are true.

If it was the case that the new infections seen in 1992 were due to new plant-to-plant inoculations in 1991. In this case, the target size of a

plant in 1991 might well make an important difference. Two lines of evidence support this idea. First, in 1991 plants in removal plots were on average bigger and in 1992 were more likely to be infected, with this a highly significant result in the xeric site. Second, the logistic regressions show that there was a positive relationship between 1991 size and infection in 1992 for plants newly infected between 1991 and 1992. A similar result was seen for bean rust infecting beans plants grown in competition with maize (Boudreau and Mundt 1992). So, the competitive regime that host plants experience may directly influence the interaction between the host and its pathogen by affecting target plant size.

Another component of the effect of competition on the plant-pathogen interaction could be how it influences the ability of infected plants to grow. Graminoids infected with systemic fungi often are induced to increase vegetative growth (Clay 1990b), perhaps so that the host plant produces enough resources for the pathogen's reproduction. If the *Cintractia junci* smut fungus relies on a high level of host plant growth to sporulate, and infection is only detectable when sporulation occurs, then it is possible that infection appeared greater in removal plots because it was only in them that host plants were able to grow big enough or fast enough to provide the necessary resources for the pathogen's reproduction. In this experiment, there is evidence that this might be the case. In the mesic site, plants which

became infected also became bigger, while in the xeric site the result was not significant but went in the same direction (Table 13). So, the competition (i.e. removal treatment) a host plant experiences may influence the plant-pathogen interaction in a second way; by suppressing host plant growth, it may deny the pathogen the resources it needs to reproduce itself within the host plant.

A different explanation for the increased infection present after competitor removal is that the nonhost plants present in no-removal plots may simply be acting as physical barriers to spore dispersal into the plots. Physical barriers can be very important to successful spore dispersal, as suggested by the fact that epidemics are more prevalent in single-species plantings (Harper 1977). This experiment presents evidence both for and against this hypothesis. Although there was an overall increase of infection under competitor removal, when I did separate analyses by year for the xeric site, the effect was only significant in 1992. Since I artificially inoculated plants in 1990, it was only between 1991 and 1992 that natural plant-to-plant spore dispersal took place. Thus, I would expect a barrier effect to appear only in the 1992 data, thus this result supports the barrier effect hypothesis. Against the hypothesis, however, is the result of the logistic regressions of new 1992 infection on 1991 plant size, which showed a positive relationship regardless of the presence of nonhost plants. If the

removal treatment was important only because of the barrier effect, then I would not expect the host plant size effect.

A final reason for the increased infection seen in removal plots may be that the physical microsite of the pathogen was altered, compared to the no-removal plots, in a way that promoted the pathogen. It is possible that the removal treatment enhanced the environment for the pathogen so that it had a better chance to disperse into those plots, germinated more easily there, or was better able to grow its infective mycelium. The overall density of vegetation was lower in removal plots (personal observation) and thus humidity, wind speed, and light could all be different compared to no-removal plots (Antonovics and Levin 1980).

Regardless of the exact mechanism, this experiment demonstrates that the interaction between host plants and pathogens can be altered by both direct and indirect effects from interspecific neighbors. Thus it is very important in field studies of plants and pathogens that the biotic environmental context of the interaction be addressed.

Environmental and genetic variation for resistance

Even though I used clones of the same 21 individuals in each plot of this experiment, I only detected variation among the eight electrophoretic genotypes when I analyzed the mesic site alone (Table 10). Among those

same genotypes in the xeric site I could not detect any genetic variation for resistance (Table 9), nor could I detect it when the data from both sites were analyzed together (Table 8). When I looked for genetic variation in the two years separately in the mesic site, I also could detect it only in 1991, not in 1992 (RXC tests and Figure 6). Even this result is based on rather slight differences in infection among the different genotypes. There were no genotypes that were 100% infected or 100% resistant. So it seems that there was some genetically based variation even in this small sample from the natural host population, but that it was rather subtle and difficult to detect.

Although the genotype X site interaction term (Table 8) was not significant when the sites were analyzed together, the differences in the genotypes' responses to the pathogen in the separate analyses of the xeric and mesic sites suggest some sort of genotype X environment interaction. Note that in the mesic site, where genetic variation was detectable, there was no significant block effect (Table 5). In contrast, in the xeric site where no genetic variation was detectable, even though the same plant genotype were present and they received inoculum from the same sources, there was highly significant block heterogeneity. The mesic site was more environmentally homogeneous for the plant-pathogen interaction than was the xeric site and it was in the mesic site that genetic variation could be

detected. There may be a hierarchy of effects operating in this system; if the physical environment that the pathogen experiences is very heterogeneous, it could overwhelm any heterogeneity the pathogen experiences due to host genotype, which may be a more subtle effect.

These results illustrate an important point about experiments attempting to detect plant genetic variation in response to a pathogen under field conditions. If this experiment had been set up only in the mesic site, I could have concluded that the host plants do show genetic variation for resistance. In contrast, if I had done the experiment only in the xeric site I would have concluded that there was no genetic variation detectable. These diametrically opposed results come from two sites that are mere meters apart, but which from the point of view of a pathogen are drastically different. This is not really surprising, considering that pathogens are microscopic organisms which experience the environment on a very small scale. Perhaps, in fact, we should expect host genetic variation only to be important in plant-pathogen interactions under relatively homogeneous environments.

Table 1. Repeated measures analysis on the number of shoots/plant. N DF = numerator degrees of freedom, D DF = denominator degrees of freeedom.

XERIC SITE

Within-plants effects	Pillai's Trace	F	N DF	D DF	P
Year	0.128	23.03	1	157	0.0001
Year X Removal	0.015	2.38	1	157	0.125
Year X Block	0.025	1.02	4	157	0.401
YearXRemovalXBlock	0.030	1.64	3	157	0.183

Between-plants effects	DF	MS	F	P
Removal	1	6.54	35.93	0.0001
Block	4	0.31	1.71	0.151
Removal X Block	3	0.06	0.33	0.801
Error	157	0.18		

Table 1 continued

MESIC SITE

Within-plants effects	Pillai's Trace	F	N DF	D DF	P
Year	0.359	101.0	1	180	0.0001
Year X Removal	0.019	3.55	1	180	0.061
Year X Block	0.077	3.77	4	180	0.006
YearXRemovalXBlock	0.078	3.81	4	180	0.005

Between-plants effects	DF	MS	F	P
Removal	1	6.88	56.34	0.0001
Block	4	0.14	1.12	0.351
RemovalXBlock	4	0.19	1.56	0.188
Error	180	0.12		

Table 2. Two-way ANOVAs on aboveground dry mass/plant (grams) in 1992.

XERIC SITE

Source	DF	MS	F	P
Removal	1	5.908	38.26	0.0001
Block	4	1.332	8.63	0.0001
Removal X Block	4	0.139	0.90	0.46
Error	189	0.154		

MESIC SITE

Source	DF	MS	F	P
Removal	1	3.593	39.46	0.0001
Block	4	0.042	0.46	0.76
Removal X Block	4	0.201	2.20	0.07
Error	197	0.091		

Table 3. Sample sizes for the plant size means in Figure 2.

Year	Site	Treatment	Variable	N
1991	Xeric	No Removal	# shoots	68
		Removal	# shoots	98
	Mesic	No Removal	# shoots	94
		Removal	# shoots	96
1992	Xeric	No Removal	# shoots	68
		Removal	# shoots	98
		No Removal	Dry mass	93
		Removal	Dry mass	97
	Mesic	No Removal	# shoots	94
		Removal	# shoots	96
		No Removal	Dry mass	101
		Removal	Dry mass	99

Table 4. Results of the categorical analysis of infection present/absent for the xeric site with pairwise contrasts for the block effect.

Source of variation	df	χ^2	P
Intercept * * *	1	325.6	< 0.001
Removal *	1	3.77	0.05
Year * *	1	9.63	0.002
Block * *	3	14.88	0.002
Removal X Year * *	1	6.81	0.009
Removal X Block * * *	3	18.49	<0.001
Year X Block	3	2.06	0.56
Residual	3	4.60	0.20
Block 1 vs. Block 3	1	3.36	0.07
Block 1 vs. Block 4 * * *	1	11.06	0.001
Block 1 vs. Block 5 * * *	1	11.41	0.001
Block 3 vs. Block 4	1	1.18	0.28
Block 3 vs. Block 5	1	1.82	0.18
Block 4 vs. Block 5	1	0.17	0.68

Table 5. Results of the categorical analysis of infection present/absent for the mesic site with pairwise contrasts for the block effect.

Source of variation	df	χ^2	P
Intercept * * *	1	631.3	< 0.001
Removal	1	0.03	0.85
Year * * *	1	27.41	<0.001
Block	4	8.33	0.08
Removal X Year * *	1	6.65	0.01
Removal X Block * * *	4	18.31	0.001
Year X Block	1	7.56	0.11
Residual	4	1.60	0.81
Block 1 vs. Block 2 *	1	5.52	0.02
Block 1 vs. Block 3 *	1	4.46	0.03
Block 1 vs. Block 4	1	0.01	0.92
Block 1 vs. Block 5 *	1	3.72	0.05
Block 2 vs. Block 3	1	0.10	0.76
Block 2 vs. Block 4 *	1	4.70	0.03
Block 2 vs. Block 5	1	0.15	0.70
Block 3 vs. Block 4 *	1	3.71	0.05
Block 3 vs. Block 5	1	0.01	0.93
Block 4 vs. Block 5	1	3.03	0.08

Table 6. Results of the categorical analysis of infection present/absent across both sites.

Source of variation	df	χ^2	P
Intercept * * *	1	744.4	< 0.001
Removal	1	3.18	0.07
Site *	1	4.47	0.03
Year * * *	1	28.31	<0.001
Removal X Site	1	3.18	0.07
Removal X Year * *	1	9.77	0.002
Site X Year	1	3.20	0.07
Residual	1	0.31	0.58

Table 7. Sample sizes for the categorical analysis.

Site	Block	Treatment	N
Xeric	1	No removal	19
		Removal	20
	2	No removal	20
		Removal	21
	3	No removal	17
		Removal	18
	4	No removal	20
		Removal	21
	5	No removal	12
		Removal	18
Mesic	1	No removal	18
		Removal	18
	2	No removal	20
		Removal	21
	3	No Removal	21
		Removal	18
	4	No removal	15
		Removal	19
	5	No removal	20
		Removal	20

Table 8. Results of the categorical analysis of infection present/absent for the eight electrophoretic host genotypes across both sites.

Source of variation	df	χ^2	P
Intercept * * *	1	619.1	< 0.001
Genotype	7	8.50	0.29
Site *	1	4.16	0.04
Year * * *	1	30.49	<0.001
Genotype X Site	7	9.96	0.19
Genotype X Year	7	12.68	0.08
Site X Year	1	2.35	0.12
Residual	7	10.39	0.17

Table 9. Results of the categorical analysis of infection present/absent for the eight electrophoretic host genotypes in the xeric site.

Source of variation	df	χ^2	P
Intercept * * *	1	220.0	< 0.001
Genotype	7	4.13	0.76
Year * * *	1	5.21	0.02
Genotype X Year	7	8.45	0.29

Table 10. Results of the categorical analysis of infection present/absent for the eight electrophoretic host genotypes in the mesic site.

Source of variation	df	χ^2	P
Intercept * * *	1	433.3	< 0.001
Genotype *	7	15.96	0.03
Year * * *	1	25.75	< 0.001
Genotype X Year *	7	14.63	0.04

Table 11. Results of the categorical analysis of infection present/absent for the eight electrophoretic host genotypes and the removal treatment in 1992.

Source of variation	df	χ^2	P
Intercept * * *	1	341.6	< 0.001
Genotype	7	9.03	0.25
Removal * *	1	6.03	0.01
Genotype X Removal	7	6.64	0.47

Table 12. Sample sizes of electrophoretic plant genotypes.

Site	Geno	N
Xeric	1	34
	2	24
	3	6
	4	16
	5	14
	6	24
	7	30
	8	18
Mesic	1	37
	2	25
	3	9
	4	20
	5	19
	6	26
	7	35
	8	19

Table 13. Analyses of covariance to detect whether plants that became infected between 1991 and 1992 were different in size (number of shoots/plant) in 1992 than plants which remained free of infection. Reported means and 95% CL are backtransformed.

XERIC SITE

Source of variation	df	MS	F
Adjusted means	1	0.591	1.417 ns
Error	94	0.417	

Group	Adjusted mean 1992 size	Lower 95% CL	Upper 95% CL
Plants infected between 1991-1992	5.6	4.50	6.73
Uninfected plants	4.8	4.10	5.51

Table 13
cont.

MESIC SITE

Source of variation	df	MS	F
Adjusted means	1	4.455	16.99***
Error	136	0.262	

Group	Adjusted mean 1992 size	Lower 95% CL	Upper 95% CL
Plants infected between 1991-1992	5.2	4.54	5.88
Uninfected plants	3.6	3.16	4.00

Table 14. Analysis of variance of number of uninfected, reproductive shoots in the xeric and mesic sites in 1992.

XERIC SITE

Source of variation	df	MS	F	P
Removal	1	1.323	9.73	0.002
Block	4	0.349	2.57	0.04
Competition X Block	4	0.158	1.16	0.33
Error	158	0.139		

MESIC SITE

Source of variation	df	MS	F	P
Removal	1	14.653	21.85	<.001
Block	4	0.627	0.94	0.44
Competition X Block	4	0.742	1.11	0.35
Error	190	0.671		

Figure 1. Layout of experimental plots.

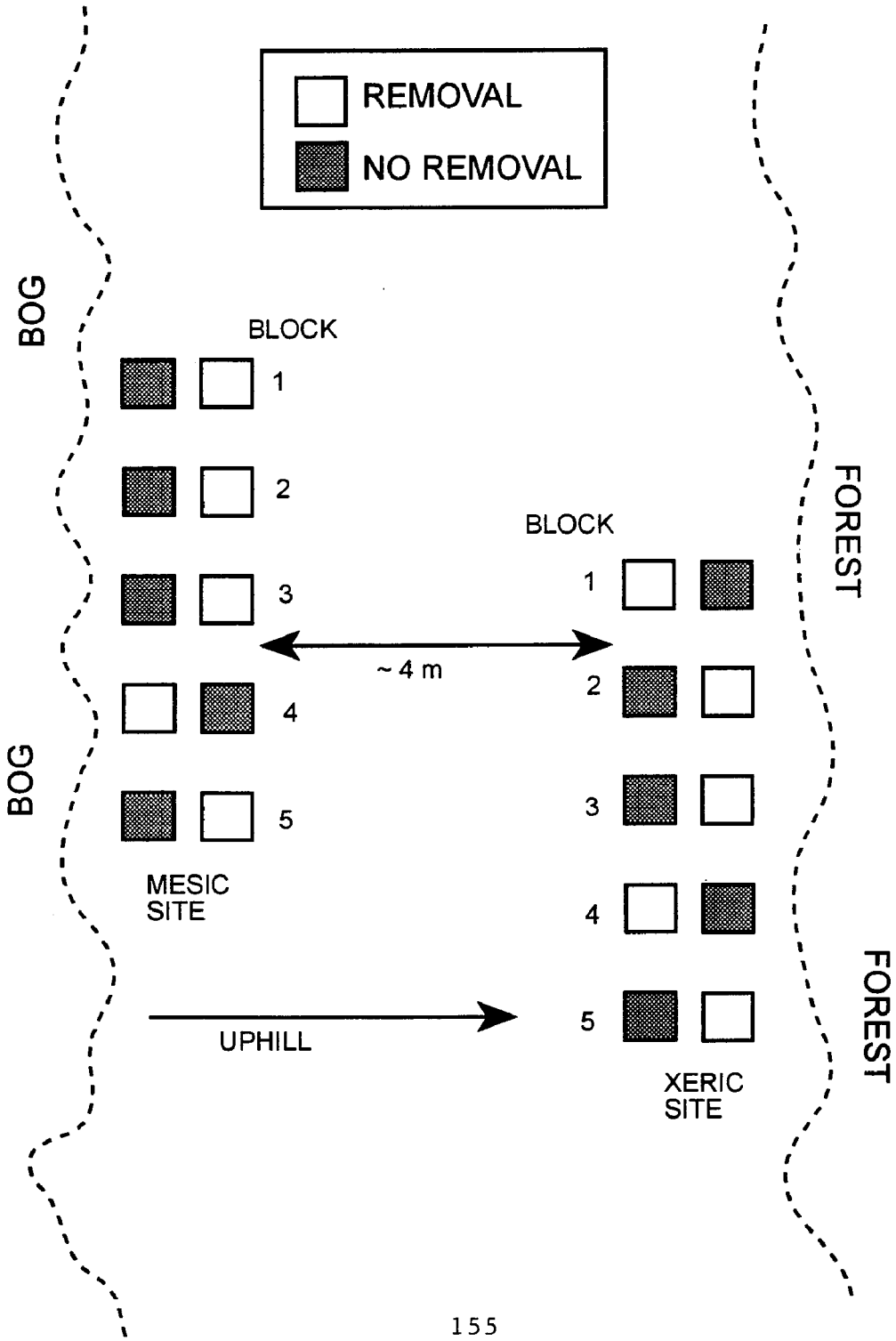

REMOVAL
NO REMOVAL
BOG
BOG
FOREST
FOREST
BLOCK
1
2
3
4
5
~4 m
BLOCK
1
2
3
4
5
MESIC SITE
XERIC SITE
UPHILL

Figure 2. Means and 95% confidence limits of host plant size in both sites in both years under the no-removal treatment and the removal treatment. Sample sizes are given in Table 3.

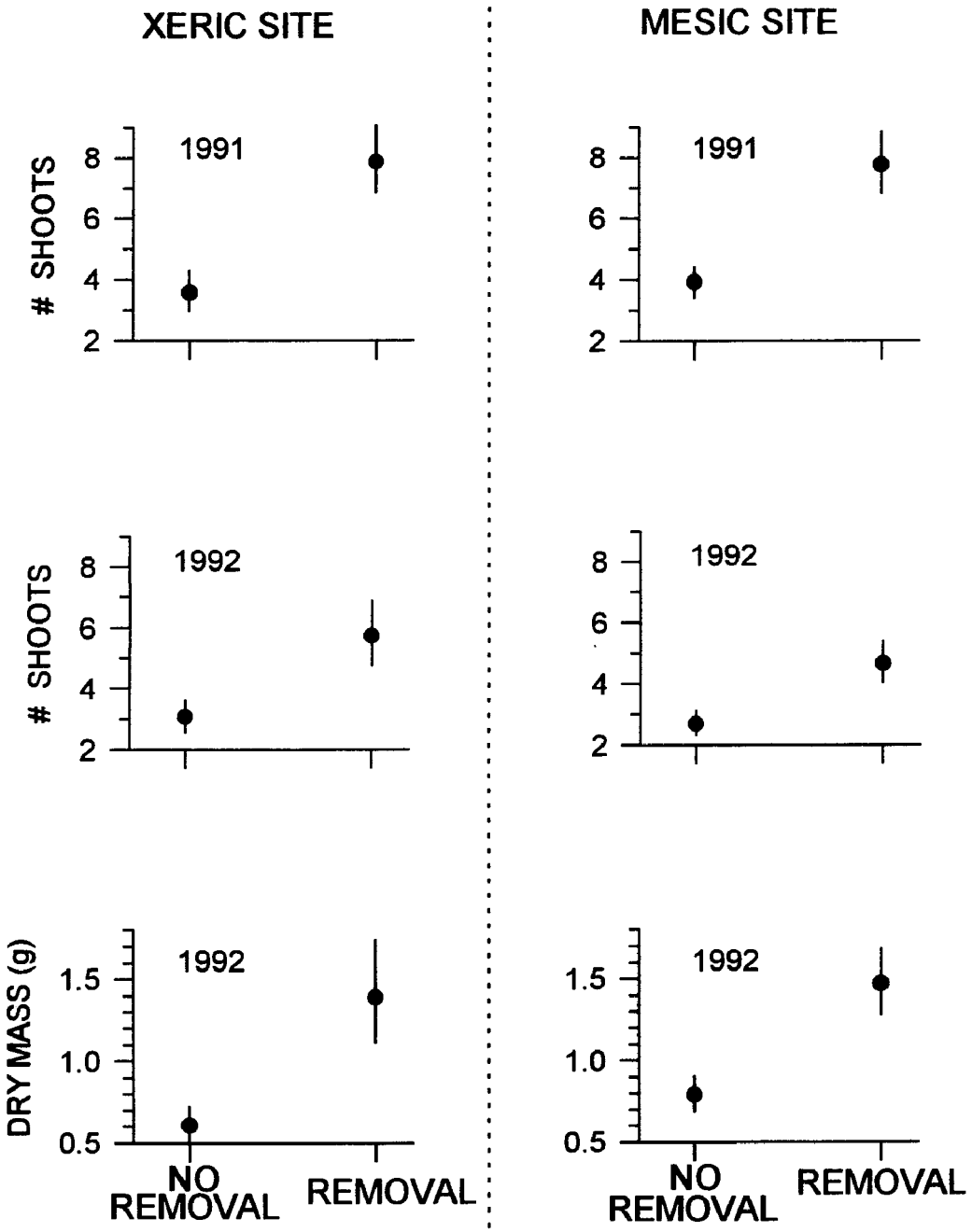

XERIC SITE
MESIC SITE
SHOOTS
1991
8
6
4
2
1992
DRY MASS (g)
1.5
1.0
0.5
NO REMOVAL
REMOVAL

Figure 3. The percentage of plants with shoot infection in 1991 and 1992 in the xeric and mesic sites under the no-removal treatment and the removal treatment. Sample sizes are given in Table 7.

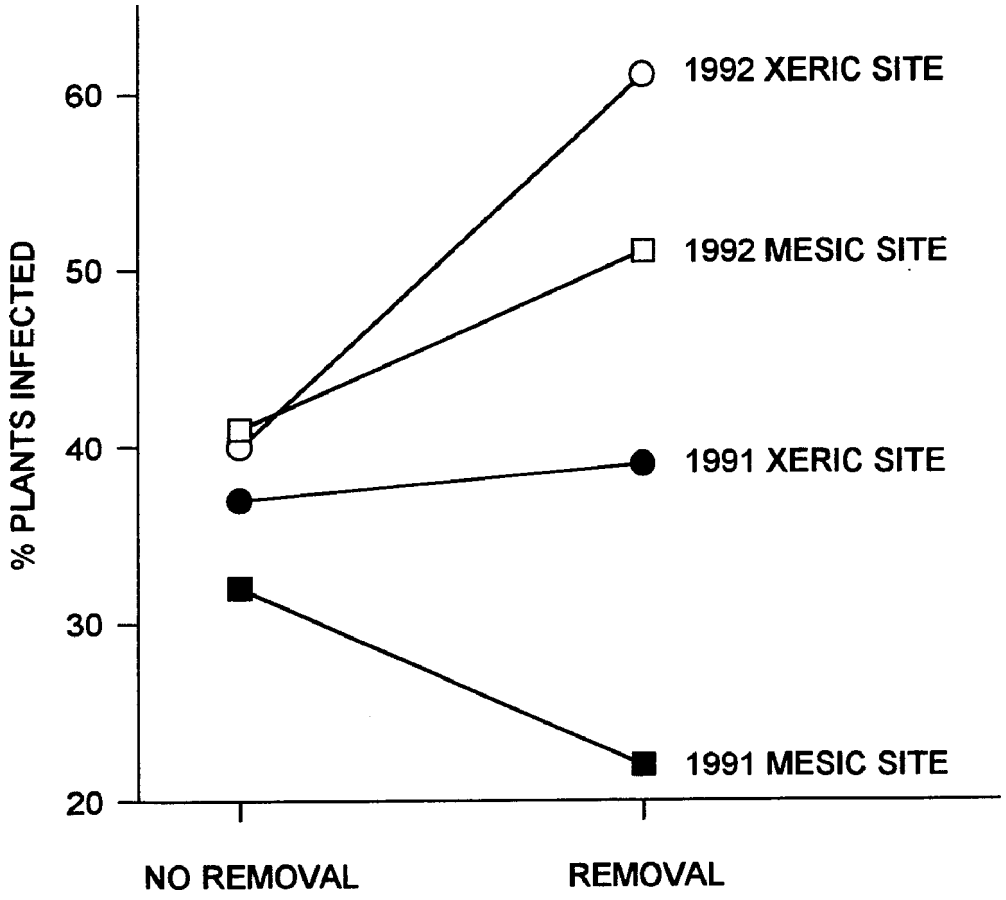

% PLANTS INFECTED
60
50
40
30
20
1992 XERIC SITE
1992 MESIC SITE
1991 XERIC SITE
1991 MESIC SITE
NO REMOVAL
REMOVAL

Figure 4. The percentage of plants with shoot infection in each of five blocks within the xeric and mesic sites in 1991 and 1992. Sample sizes are given in Table 7.

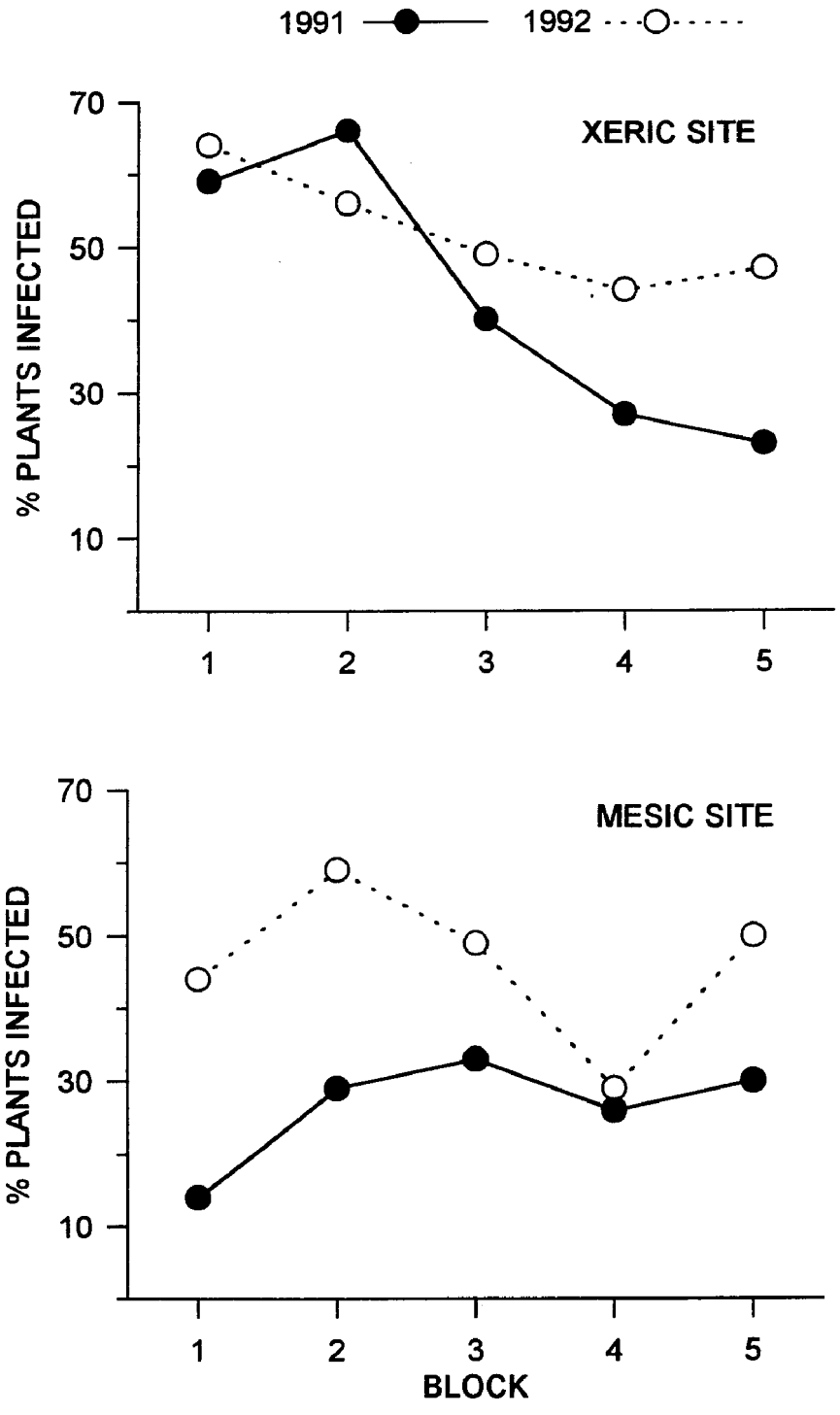

1991
1992
XERIC SITE
MESIC SITE
% PLANTS INFECTED
70
50
30
10
1
2
3
4
5
BLOCK

Figure 5. The percentage of plants with shoot infection in the removal and no-removal treatments in each of five blocks in the xeric and mesic sites in 1991 and 1992. Sample sizes are given in Table 7.

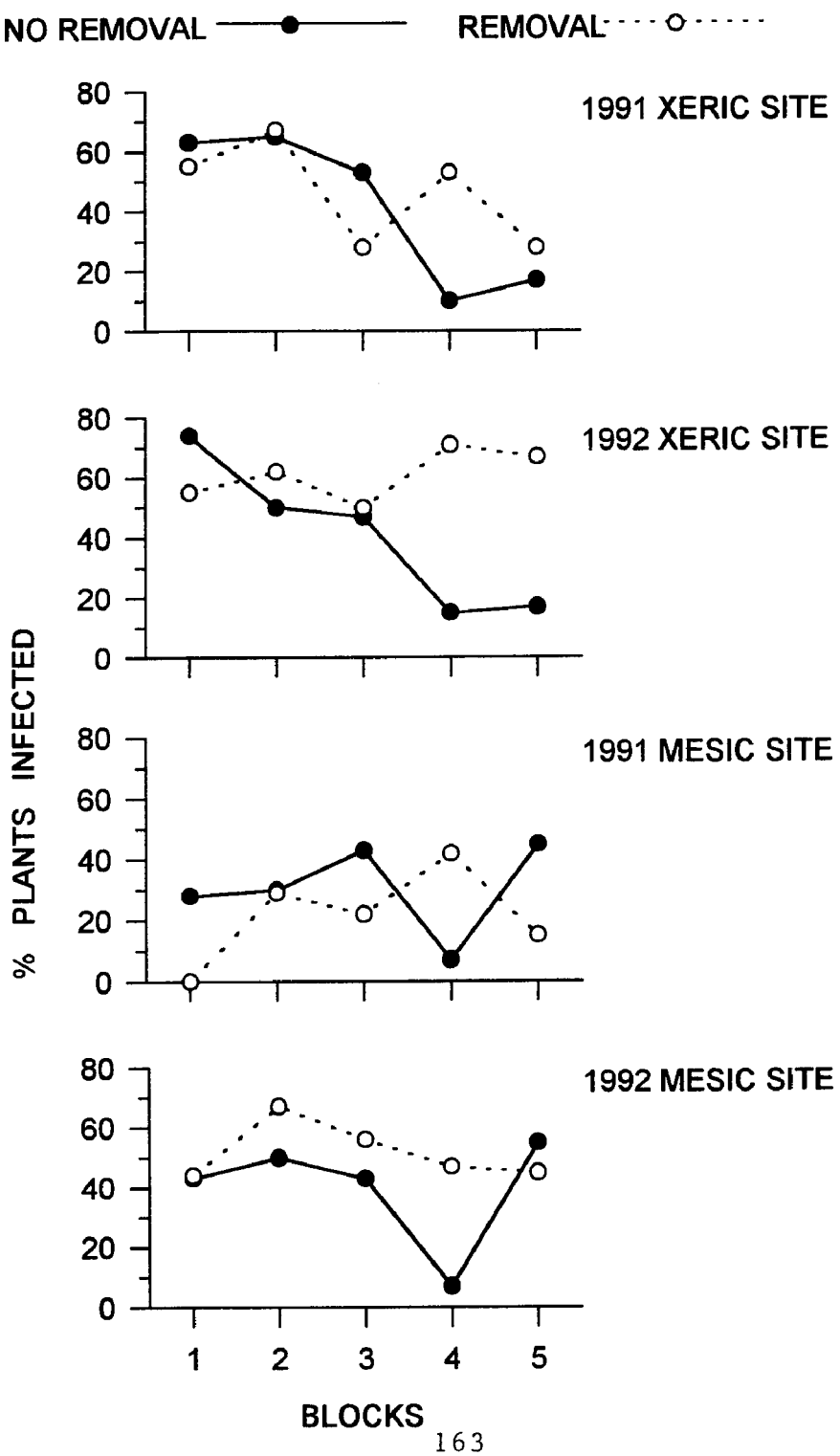

NO REMOVAL
REMOVAL
1991 XERIC SITE
1992 XERIC SITE
1991 MESIC SITE
1992 MESIC SITE
% PLANTS INFECTED
0
20
40
60
80
1
2
3
4
5
BLOCKS

Figure 6. The percentage of plants with shoot infection in eight electrophoretic genotype groups, in the xeric and mesic sites in 1991 and 1992. Sample sizes are given in Table 12.

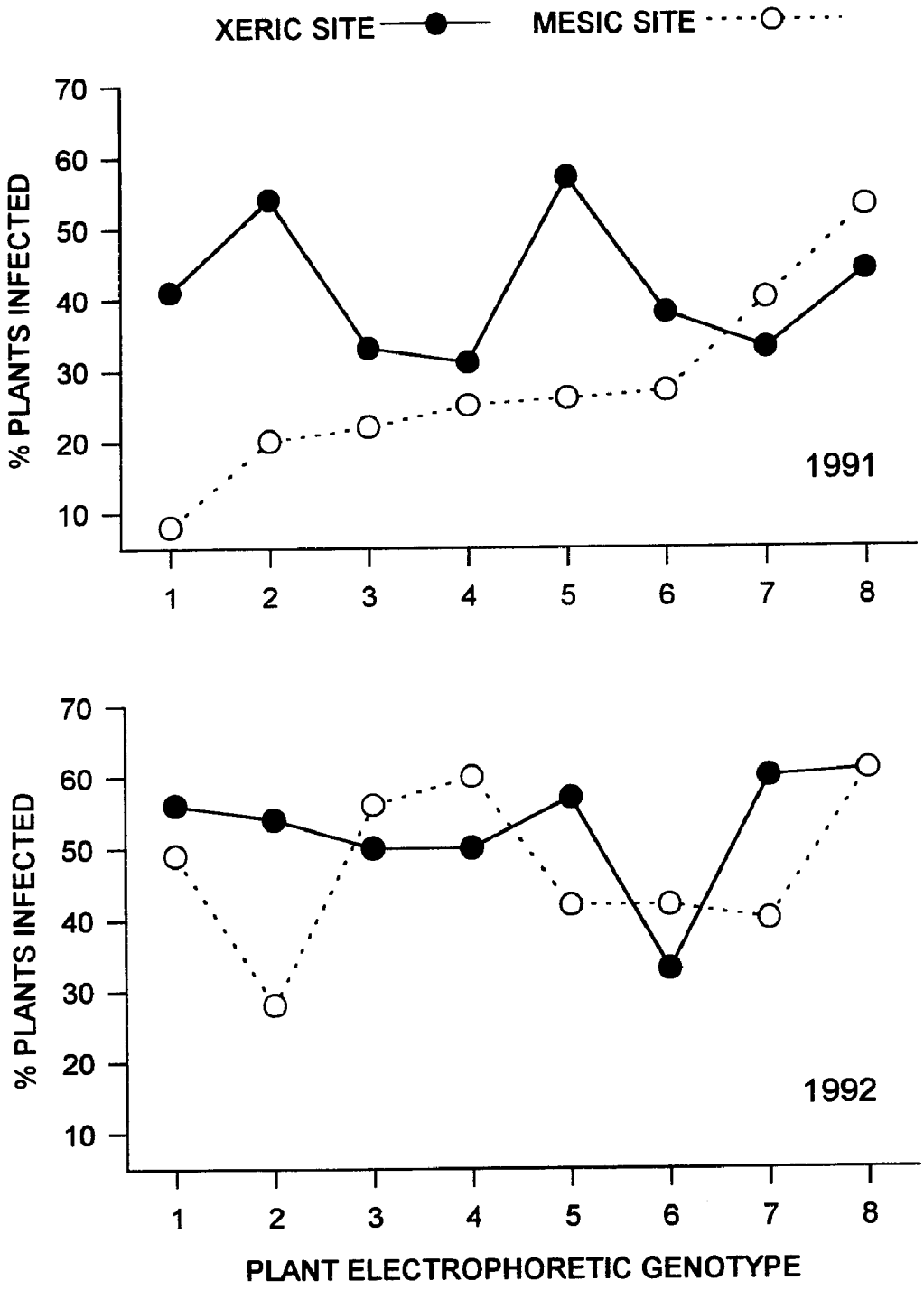
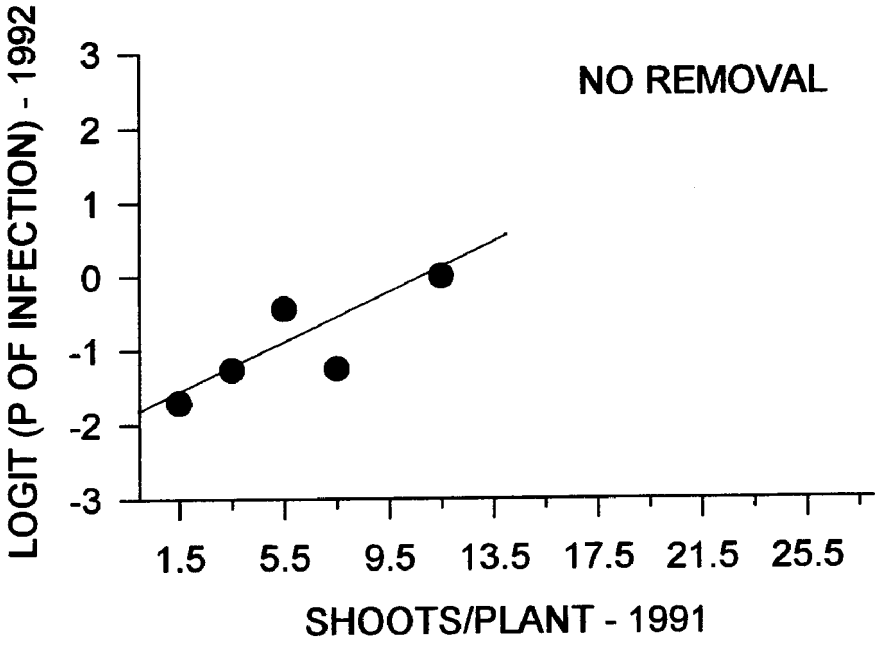

XERIC SITE
MESIC SITE
% PLANTS INFECTED
70
60
50
40
30
20
10
1991
1 2 3 4 5 6 7 8
% PLANTS INFECTED
70
60
50
40
30
20
10
1992
1 2 3 4 5 6 7 8
PLANT ELECTROPHORETIC GENOTYPE

Figure 7. Logistic regressions of shoot infection in 1992 on plant size in 1991 as measured by the number of shoots per plant, for both the xeric and the mesic sites. The regression lines are calculated from model parameters output from PROC LOGISTIC in SAS v. 6.0. The xeric site regression is significant at $P = 0.0454$ and the mesic site at $P = 0.0005$. The points are plotted to check that the relationship between the logit of infection probability and plant size is linear. The points are calculated by creating intervals of plant size, finding the proportion of plants in each size interval that had shoot infection, then taking the logit ($\ln(P/(1-P))$) of that proportion.


NO REMOVAL
LOGIT (P OF INFECTION) - 1992
3
2
1
0
-1
-2
-3
1.5
5.5
9.5
13.5
17.5
21.5
25.5
SHOOTS/PLANT - 1991

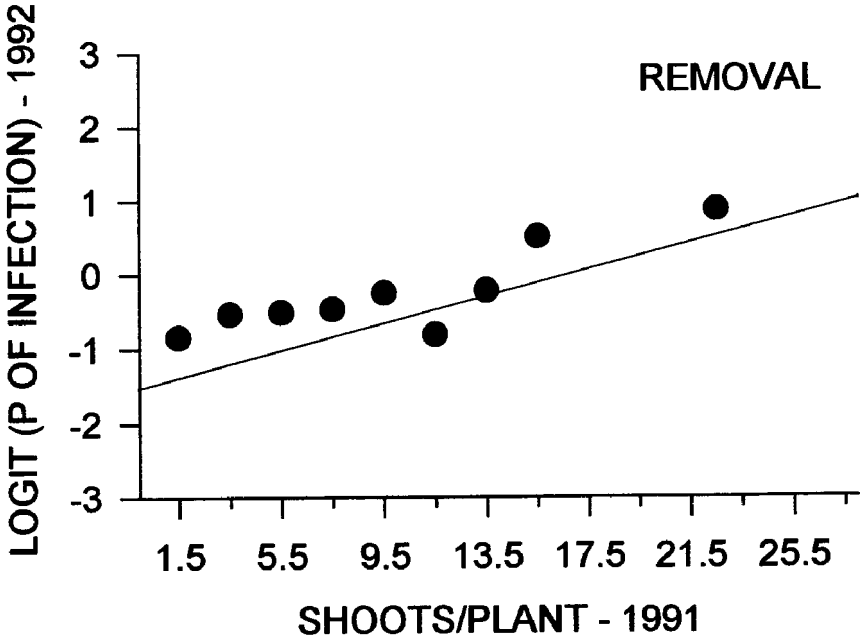

REMOVAL
LOGIT (P OF INFECTION) - 1992
3
2
1
0
-1
-2
-3
1.5
5.5
9.5
13.5
17.5
21.5
25.5
SHOOTS/PLANT - 1991

Figure 8. Means and 95% confidence limits of the number of uninfected reproductive shoots per plant in 1992 in the no-removal and removal plots in the xeric and mesic sites (xeric, no-removal, n = 81; xeric, removal, n = 87; mesic, no removal, n = 101; mesic, removal, n = 99).

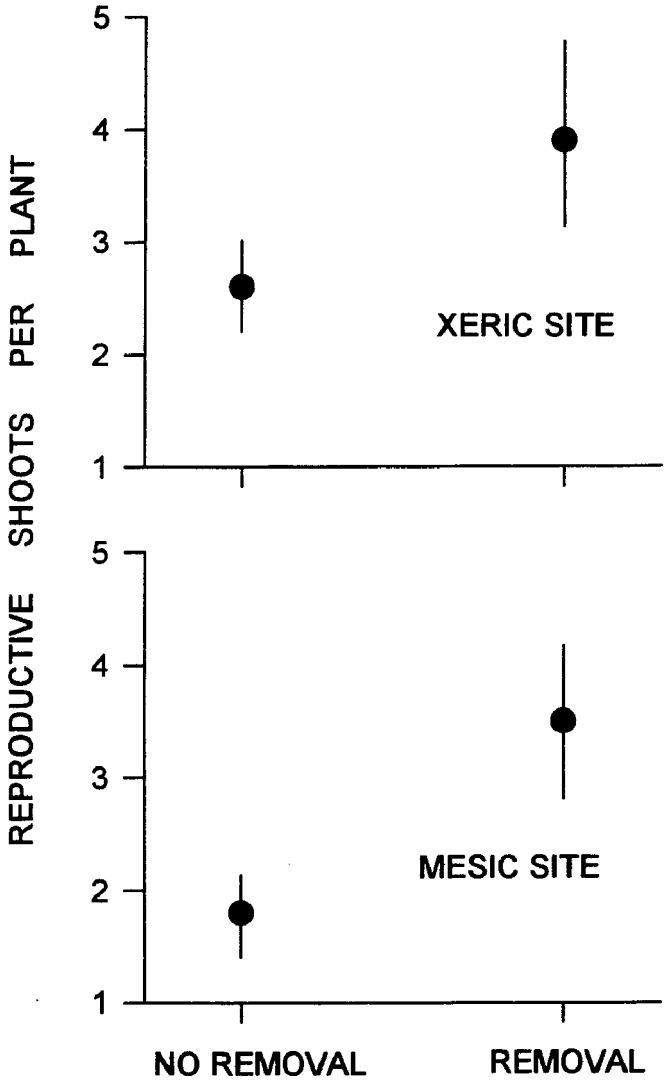
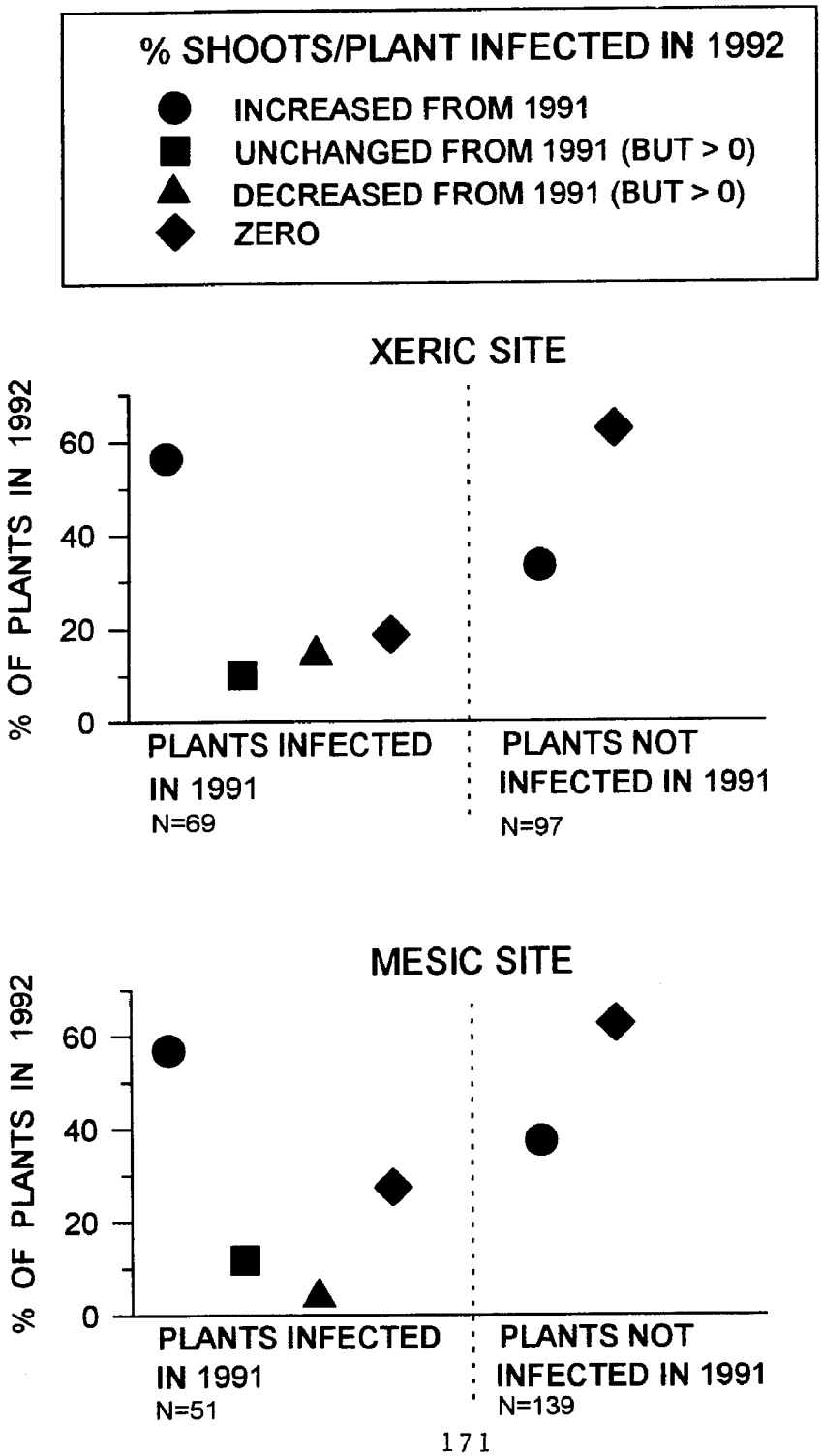

REPRODUCTIVE SHOOTS PER PLANT
5
4
3
2
1
XERIC SITE
MESIC SITE
NO REMOVAL
REMOVAL

Figure 9. A comparison of disease progress within plants classified as infected or not in 1991. Disease severity is measured as the percentage of shoots per plant with infection.


% SHOOTS/PLANT INFECTED IN 1992
INCREASED FROM 1991
UNCHANGED FROM 1991 (BUT > 0)
DECREASED FROM 1991 (BUT > 0)
ZERO
XERIC SITE
% OF PLANTS IN 1992
60
40
20
0
PLANTS INFECTED IN 1991
N=69
PLANTS NOT INFECTED IN 1991
N=97
MESIC SITE
% OF PLANTS IN 1992
60
40
20
0
PLANTS INFECTED IN 1991
N=51
PLANTS NOT INFECTED IN 1991
N=139

4

Infection of *Juncus dichotomus* by the smut fungus *Cintractia junci*: an experimental field test of the effects of host plant density, pathogen density, and host genotype in a heterogeneous environment.

INTRODUCTION

The interactions between plants and pathogens in natural populations are potentially subject to a wide range of environmental and genetic factors (Burdon 1987a). For example, a study of a natural population of the perennial host plant *Juncus dichotomus* and its smut fungus pathogen *Cintractia junci* indicated that spatial variation in disease frequency was similar to that for host plant density (Chapter Two). However, host plant density itself could be confounded in space with a number of other biotic and abiotic factors potentially important to the interaction (Antonovics and Levin 1980), such as pathogen density and genetic structure, plant community heterogeneity, soil moisture, or host genetic structure. With manipulative field experiments, factors hypothesized to be important can be studied while possibly confounding factors can be controlled. Such experimental work in this system has shown that when host plant genetic

structure, plant density, pathogen genetic structure, and pathogen density are kept constant, the infection of host plants can be influenced by the plants' heterospecific neighbors (Chapter Three), by the host plant genotype (Chapters Three and Five), by soil moisture to some degree (Chapter Three and Five), and by other environmental heterogeneity (Chapters Three and Five).

In this experiment I tested whether four theoretically important factors - host plant density, pathogen density, host genotype, and environmental heterogeneity (blocks) - influenced infection of *J. dichotomus* by *C. junci* when other factors were experimentally held constant. I created plots of plants that had identical, controlled genetic structure by using clonally propagated individuals, and I inoculated them from a common pool of pathogen spores, therefore controlling pathogen genetic structure as well. The plants were planted into plots that had all vegetation initially removed and subsequently weeded, so that the heterospecific biotic neighborhood of the host plants was similar among plots. I varied the density of host plants per plot and the density of pathogen spores per plot and replicated these treatments in blocks that corresponded to a topographical and vegetational gradient. Within plots I used replicates of 21 host individuals in order to study host variation for resistance. By using a factorial design I was able to examine not only the direct effects of these factors but also how they might

interact to promote or discourage pathogen infection. Even though the results of multifactorial experiments are more relevant to processes in natural environments than are the results of single factor studies (Gurevitch and Collins 1994), few plant-pathogen studies have examined more than one factor (but see (Burdon and Chilvers 1982, Augspurger and Kelly 1984b, Jarosz and Levy 1988) and (Strauss 1990) for a good example from plant-insect research.

All four factors examined here - host plant density, pathogen density, host plant genotype, and environmental heterogeneity - have been theorized to play important roles in mediating plant-pathogen interactions (Burdon 1987a). Theory and empirical studies show that host plant density can act in at least three ways to influence the establishment and spread of disease in plant populations. First, host density could influence the probability of infection through its effect on the host plant's physiological status. Crowded plants may be stressed due to intraspecific competition for light, water, or nutrients, and have fewer resources to allocate to defense, resulting in greater infection rates (Schoenweiss 1975, Colhoun 1979, Burdon 1987a, Clay 1990c). In contrast, for some pathogens physiological stress could render a potential host plant an inadequate resource for a pathogen that relies on host resources for growth and reproduction, leading to decreased pathogen success (Burdon 1987a, p. 57). Plants that have access to

abundant nitrogen, for example, produce succulent growth that may be more susceptible to diseases that normally attack such tissues than would be the older, slower-growing tissues of plants that lack nitrogen. For example, fertilized pear trees are more susceptible to fire blight, as is wheat to rust and powdery mildew (Agrios 1988, p.152).

Second, host density may influence disease development because it can alter the microclimate for fungal spore germination and / or hyphal growth (Rotem 1978, Agrios 1988, pp. 147-155). Consistent prediction of the direction of influence of host density has not been possible, although increased host density is usually thought of as promoting disease development due to increased humidity. In general, fungi require moist soil or atmospheric conditions for germination and growth and pathogenic fungi are no exception. However, host plant density also affects light levels and air currents within the canopy, which can have less predictable effects on fungal biology (Burdon and Chilvers 1982).

For purely physical reasons, dense stands of plants are also expected to increase the probability of transmission of pathogen propagules from one plant to another (Burdon 1987a, pp.41-48). In this experiment between-plant transmission influenced by plant density was not studied. Because I used artificial inoculation of each host plant I could focus on the post-transmission effects of host density on infection.

I chose pathogen density as the second factor in this experiment in order to separate its effect on the plant-pathogen interaction from that of host plant density. Pathogen density is a likely confounding factor in a correlation between host density and disease level detected after an epidemic has already been established (as it was in the natural population I studied). It is a chicken-or-egg problem. Once high infection rates are established in perennial populations (both this host plant and this pathogen are perennial), then dense host stands may be severely infected partly because of the host density effects described above, but also partly because there is high density of pathogen inoculum in those stands. In theory, there should be an increase of infection with an increase in the amount of inoculum potential, and one very important component of inoculum potential is the density of spores present (Baker 1978). Plants that are exposed to a higher number of spores per unit area have a higher risk of having at least one spore produce an infection (Alexander and Antonovics 1988), but see (Alexander 1990) .

The third factor examined in this experiment was host plant genotype. Plants can have genetically based resistance to pathogens, although the genetic basis of resistance in wild populations, including the *J. dichotomus* - *C. junci* system, is usually unknown (but see Burdon 1987b, Parker 1988, 1991). The most well-known model of resistance to pathogens

is the gene-for-gene model, which was developed for agricultural systems (Flor 1971, Burdon 1987a). It is an example of "race-specific resistance", where the resistance is to a particular pathogen genotype (i.e. "race"). It postulates a single gene of major effect for resistance in the plant matched by a gene for virulence in the pathogen, both inherited in simple Mendelian fashion. The resistance allele is dominant in the plant and the susceptibility allele is recessive, while in the pathogen avirulence is dominant and virulence recessive. Susceptible reactions occur when a pathogen homozygous for virulence encounters any host genotype (since it has virulence against even the host's resistance allele) or when a pathogen carrying the avirulence allele encounters a host homozygous for susceptibility. This and other forms of gene-for-gene interactions are known or postulated for over 25 agricultural plant-pathogen systems (listed in Burdon 1987, p. 68), including three plant-smut fungus interactions. However, the gene-for-gene interaction is not documented for many natural systems, which could be because it has not been looked for often or because the model is inappropriate (Barrett 1985). Major genes for resistance to pathogens have been found in natural populations of wild soybean (Burdon 1987b), groundsel (Harry and Clarke 1986, 1987), and hog peanut (Parker 1988).

In addition to race-specific resistance, plants can vary genetically for "race non-specific resistance," which responds similarly to a broad range of

pathogen genotypes (Burdon 1987a). The genetic basis of this type of resistance is not well documented, but is probably quantitative, the result of many genes with small additive effects. Variation may be found for every step of the interaction leading to infection, from the rate of host penetration, to survival and growth of fungal hyphae, to sporulation, and to length of the latent period (Burdon 1987a, pp. 53-57 and citations within). Race non-specific resistance is likely to be quite important in natural populations, although difficult to quantify (Barrett 1985, Burdon 1987a).

Plant characters leading to disease avoidance may also have a genetic basis in host plants that are otherwise susceptible (Burdon 1987a, pp. 60-61). For example, plant genotypes may vary phenologically such that the susceptible stage in some genotypes' life histories do not coincide with the infective stage of the pathogen (Agrios 1980). Host genotypes may also vary morphologically in a way that alters their exposure to disease, for example by having closed flowers that allow them to avoid a flower-infecting smut fungus (Russell 1978).

Any or all of the above genetically-based resistance mechanisms may be present in the *J. dichotomus-C. junci* plant pathosystem. If so, it is possible that the smut fungus is an agent of natural selection acting on the host plant, since infection diminishes or prevents reproduction in host plants and may also increase host plant mortality (Chapter Two). In general, it has

been suggested that pathogens might be very important selective agents in natural populations (Harlan 1976, Hamilton 1982, Dinoor and Eshed 1984, Burdon 1991). To know whether an evolutionary response to a plant pathogen might be occurring in a plant population it is necessary to document heritable genetic variation for resistance (Falconer 1981, Simms and Rausher 1992). Characterizing the resistance mechanism and its genetic basis and heritability was beyond the scope of this study, but as a first step I attempted to detect whether or not variation for resistance existed among the plant genotypes used.

The phenotypic expression of genetically-based resistance may be influenced by a wide array of environmental factors, illustrated by the concept of the disease triangle in plant pathology (Agrios 1988, p. 43), which states that disease is the result of interaction between host, pathogen, and environment. The strength and nature of an interaction between host genotype and environment will partly determine the potential evolutionary response of the host population to the pathogen. For quantitative resistance traits, which are probably quite significant in natural populations, the larger the environmental component of variance, the lower the heritable genetic variance (Simms and Rausher 1992). Environmental modification of phenotypic expression of major-gene resistance has also been documented (Burdon 1987a).

In this experiment, I tested for interactions between host plant genotype and two environmental factors - pathogen density (biotic) and spatial block (abiotic). Significant genotype-environment interactions in the experiment could have both ecological and evolutionary implications for the natural population from which the experimental plants were collected. Ecologically, the spatial pattern of disease in the natural population may be influenced by particular combinations across space of host genotypes and environmental factors. If so, the pattern of disease and its further spread could not be understood simply from the distributions of resistant and susceptible plant genotypes in a site. Evolutionarily, a host genotype - pathogen density interaction would suggest that the relative fitness of host genotypes would be different under different pathogen densities and, if pathogen and host density were correlated in a site, under different host densities as well. An interaction between host genotype and blocks would indicate that the genotype's relative fitnesses can be influenced by whatever environmental factors are variable among the blocks. Major factors that have been shown to influence the phenotypic expression of disease symptoms are soil moisture, soil nutrients, and light (Colhoun 1979). Thus, spatial heterogeneity in a site due to both biotic and abiotic factors could result in a mosaic environment upon which the relative fitness of particular plant resistance genotypes depends.

Abiotic environmental variables can also influence disease outright by affecting the biology of host plants and/or pathogens (Fischer, G.M. and Holton 1957, pp. 200-211; Agrios 1988, pp. 147-154). Although abiotic environmental variables were not explicitly measured in the blocks, testing block as the fourth main effect in the experiment can provide some idea of the direct influence that environmental heterogeneity has on infection rates in this system.

ORGANISMS AND STUDY SITE

Juncus dichotomus Ell. (Juncaceae) is a perennial rush that typically grows in open, sunny habitats on sandy soils, and it can be infected with the parasitic smut fungus *Cintractia junci*. Schw. (Ustilaginales, Basidiomycetes). A complete description of the plant and the pathogen may be found in Chapter Two, "Organisms and Study Site." The severity of smut fungus infection may range from an isolated infected fruit on otherwise reproductive, healthy shoots, to one or a few systemically infected shoots, to systemic infection of all shoots. In general, once an infection starts, it becomes worse in subsequent years (Chapter Two, Chapter Three). Shoot infection has the more important potential effect on host plant fitness, since infected shoots produce only deformed reproductive structures with no functioning flowers, but which the pathogen uses for its own spore

production. In this study I focused on shoot infection.

Both plant genotypes and pathogen inoculum were obtained from the natural population site described in detail in Chapter Two. The site for this experiment was approximately 1 km from the natural site and was in a similar open pine barrens habitat but had no naturally occurring healthy or diseased *Juncus dichotomus* plants. It was situated near the edge of a sunny field dominated by grasses and sedges growing in very sandy soil but with scattered small shrubs and trees. There was a gradual slope down from the center of the field toward the sparsely wooded field perimeter.

METHODS

In September of 1989 I collected 21 apparently healthy *Juncus dichotomus* individuals from the natural population, with collection sites at least 0.5 meters apart and situated across the entire population. I brought the plants to the greenhouse at SUNY Stony Brook where I washed their roots, planted them in standard Pro-Mix potting soil, and placed them in a common greenhouse bay. In January of 1990 and again in late March 1990, late April 1990, early June 1990, early June 1991, and early July 1991 I divided each plant at the rootstock into multiple ramets, repotted them, and placed them back in the bay. At the final propagation I attempted to make each plant of similar size, with three shoots per individual. At this point there

were at least 84 copies of each of the originally collected 21 plants. I will refer to a group of replicates from one original plant as a clone; thus there were 21 clones in the experiment. The plants then grew in flats of two inch pots (three flats for each clone) in the greenhouse for another three weeks before transplanting to the field site. During this time the positions of the flats in the bay were randomized to decrease variation among the clones due to the greenhouse environment. Throughout the propagation the plants were fertilized and watered as needed, with all plants receiving identical treatment. By growing the plants for 22 months in a common greenhouse environment and successively propagating them six times, I minimized any possible variation among them due to environmental effects of their original collection locations. In both summers the plants flowered in the greenhouse, which allowed me to check them for smut infection. I only used healthy plants in the experiment.

To document that the clones were genetically distinct I used starch gel electrophoresis to screen them for allozyme differences. Seven polymorphic loci (PGM, IDH2, PGDH-1, PGDH-2, MDH1, MDH2, AND MDH-3) allowed me to separate the clones into nine distinct electrophoretic genotypes. All clones grouped into one electrophoretic genotype had identical electrophoretic profiles. It is possible that clones assigned to one genotype may have other, undetected genetic differences and they may all

be unique, but I confined the genetic analysis below to the nine electrophoretic genotype groups.

During late July 1991 I prepared 12 experimental plots in the field described above by rototilling the native vegetation and then removing all dislodged plants by hand, in this way creating a uniform planting bed within each plot. The plots were arranged as in Figure 1, with three blocks of four plots each. The blocks followed a slight topographical gradient (uphill in block 1 to downhill in block 3) and a slight vegetational gradient (from more open field in block 1 to the field perimeter in block 3). Each plot in a block received one of four randomly assigned treatments: (1) high host plant density - high pathogen density (2) high host plant density - low pathogen density (3) low host plant density - high pathogen density (4) low host plant density - low pathogen density. Thus there were 3 replicates of each treatment combination.

In the high host plant density treatment, I randomly assigned 12 copies of each of the 21 clones to positions on a hexagonal lattice, with plants spaced 7 cm apart. In the low host plant density plots, 2 copies of each of the 21 clones were assigned at random on a hexagonal lattice 30 cm apart. A total of 1764 individual ramets of *Juncus* were planted out. The two spacings span the typical range of plant densities in the natural population (personal observation). As a result of the spacings and the

numbers of plants used, the high plant density plots were 0.95 m^2 and the low plant density plots were 2.34 m^2. The plants were planted over three days in the last week of July 1991 and were watered approximately every 2 days for the next week.

All plants were inoculated with a suspension of smut fungus teliospores in water which I made by crushing fresh sporulating lesions from the natural population into water the night before inoculation began. From this one suspension I made two batches of two different spore concentrations, one an order of magnitude more dilute than the other. In this way the batches contained the same genetic pool of pathogen spores and only differed in spore density. In the high pathogen density plots, I inoculated each plant with 10 ml of the concentrated suspension by dipping 3 clipped leaves into the liquid and then pouring it into the soil at the base of the plant. In the low pathogen density plots I did the same, using the diluted spore suspension. Most plants in block 1 were inoculated on 16 August 1991 and all remaining plants were inoculated on 17 August and 18 August 1991. In May 1992 each plot was weeded by hand and in September 1992 the number of healthy and infected shoots were counted on each plant.

Infection rates were quite low, so the percentage of infected shoots per plant had a highly skewed distribution that could not be transformed to approach normality. Therefore, I categorized each plant as having shoot

infection present or absent and analyzed the variation due to main effects and interactions with a categorical model of homogeneity (Freeman 1987) using PROC CATMOD in SAS 6.0. (Chapter Three, "Methods"). In one analysis I used host plant density, pathogen density, and block as main effects and included every plot. Replication of host genotypes was too low in the low plant density plots for a categorical analysis, so in a second analysis I used only the high plant density plots in order to examine host genotype, pathogen density, and block as main effects. There were no electrophoretic data for clone 62, so it could not be assigned to a genotype group. Thus, all ramets of clone 62 were excluded from this second analysis.

Logistic regression was used to examine the relationship between the logit of a plant's probability of being infected (ln(P/(1-P))) and its size (number of shoots per plant) and was done for plants from all plots, for plants in the low plant density plots only, and for plants in the high plant density plants only. Logistic regression is appropriate for a binary response variable (infection present/absent) and a continuous regressor (size) (Hosmer and Lemeshow 1989). It tests for a linear relationship between the logit of infection probability and plant size. A simple way to check that the relationship is linear is to create intervals of plant size and plot the midpoint of each interval against the proportion of plants in each interval that were

infected, transformed to their logits. This was done for all logistic regressions.

RESULTS

EFFECTS OF HOST PLANT DENSITY, PATHOGEN DENSITY, AND BLOCK ON INFECTION

Host plant density, pathogen density, and block all significantly affected the probability that plants had shoot infection (Table 1, Figure 2). Plants growing under high host density were less likely to be infected than plants growing at low host density. Plants that experienced a higher pathogen inoculum density were more likely to become infected than were those which were inoculated with the dilute pathogen density. Plants growing in block 1 were more likely to be infected than those in block 2 or block 3.

Pathogen density significantly interacted with blocks (Table 1). Although in all blocks the percentage of infected plants was higher under high pathogen density, this effect was much more pronounced in block 1(Figure 3). The three-way interaction between host density, pathogen density, and block was also significant. An examination of the values in Table 2 indicates, for example, that in blocks 1 and 2 the highest infection rate was in plots that had low host density and high pathogen density, but in

block 3 that combination had only an intermediate infection rate.

Through intraspecific competition, plant density can have dramatic effects on plant size, which in turn may be related to pathogen infection. From Figure 2 it is clear that, although plants in the low host plant density plots were more likely to be infected, they were also much larger (in number of shoots per plant) than those in the high host plant density plots. There is a suggestion of this pattern for the pathogen density and block effects also; greater disease was associated with slightly larger plants.

Logistic regression was used to test more directly for a relationship between the probability of infection and plant size. Using all plants pooled across all plots, the regression indicated that larger plants were more likely to be infected (Figure 4A). Plant size was not independent of the host plant density treatment, however, and host density had a significant effect on infection. So I also did separate logistic regressions for the low and high host density plots and both regressions had significant positive slopes (Figure 4B, 4C).

Comparing the plotted interval points to the logistic regression line in Figure 4 indicates that the positive relationship between plant size and disease was weaker for plants in the largest plant size interval (those with greater than 21 shoots). When plants with greater than 21 shoots were excluded from the regressions, the fit of the data to the model was better

and the regressions had greater positive slopes (Figure 4).

EFFECTS OF HOST PLANT GENOTYPE, PATHOGEN DENSITY, AND BLOCK ON INFECTION

Variation among plants according to electrophoretic genotype was an important influence on infection. The clones were grouped into their 9 electrophoretic genotypes for the following analyses, but even within these genotype groups there was a wide range of variability among the 20 clones (Figure 5). For example, no individuals of clone 41 became infected, while nearly 25% of clone 49 individuals were infected (Figure 5).

A categorical analysis of shoot infection presence / absence in the high host plant density plots indicated that the main effects of host genotype, pathogen density, and block all had highly significant influences on infection (Table 3). Mirroring the outcome in the categorical analysis for all plots (above), in this analysis the high pathogen density plots had more plants infected (10.6%, n=689) than the low pathogen density plots (3.51%, n=684). Similarly, block 1 had higher infection (12.82%, n=468) than block 2 (3.52%, n=454) or block 3 (4.66%, n=451). Infection among the nine electrophoretic plant genotypes ranged from 0% for genotype 11 to 19% for genotype 2 (Figure 6A). However, there did not appear to be any clear correspondence between infection rates and plant size among the

genotypes (Figure 6B) (Kendall's coefficient of rank correlation between genotype mean size and percent of plants infected, $\tau = 0.39$, $n = 9$, ns), due mostly to the unusually large size of genotype 9 plants. If genotype 9 is ignored, however, it again appears that larger plants were more likely to be infected ($\tau = 0.643$, $n = 8$, $P = 0.05$) (Sokal and Rohlf 1981).

Infection within plant genotypes was also differentially influenced by pathogen density, as indicated by the highly significant genotype X pathogen interaction (Table 3). Figure 7 illustrates that host genotypes differed in their responses to higher pathogen loads. Some experienced dramatically increased infection rates. For example, genotype 2 went from 7% of plants infected under low pathogen density to 31% infected under high pathogen density. Most other genotypes had less dramatic increases, while genotype 11 remained at 0% infection in both pathogen density treatments.

The genotypes also responded differently to the pathogen in the three blocks, as shown by the highly significant genotype X block interaction term in Table 3. Examination of Figure 8 indicates that this was due mainly to their differences in block 1, where plants were more likely to be infected. For example, clone 11 did not have increased infection in block 1 compared to the other blocks, clone 1 had decreased infection in block 1, and clone 14 showed a dramatically greater percentage of plants infected in block 1 than in the other blocks.

DISCUSSION

A *Juncus dichotomus* plant in a dangerous encounter with smut fungus disease has several routes of escape, based on the results of this experiment. It may have some genetically-based resistance mechanism that prevents it from becoming infected even if it is exposed to a very high dose of a wide range of pathogen genotypes and regardless of its environmental conditions. Alternatively, it may have weak genetic resistance, but may still escape infection by being located in an area that experiences a low inoculum potential. It may also escape because it is in an environment that is not very conducive to pathogen germination and growth or because the plant itself in that site is a poor resource for the pathogen, perhaps due to the stress of competition. All of these scenarios are supported by the results of this experiment and they illustrate an important point - that plant-pathogen interactions in nature are indeed the product of multiple factors interacting together in a sometimes unpredictable fashion.

Host plant density, pathogen exposure, and host genotype all influenced infection. Surprisingly, plant density had the opposite effect of what is usually predicted in plant-pathogen interactions; at high density plants were less likely to be diseased. This may be because disease transmission was not a factor in the experiment. It is likely that transmission would be facilitated when plants are at high density so that in subsequent

years, if the experiment had continued, disease might increase in high host plant density plots. The experiment did test host density as it influenced either the host plant as a resource for the pathogen or the pathogen's microclimate, although these two effects could not be separated. Host plants grown at high density were significantly smaller, so it is possible that they provided inadequate resources for the pathogen's survival, growth, or reproduction. I found a similar effect for host plants grown with and without interspecific competitors, where host plants were larger when released from competition but also were more likely to be infected (see Chapter Three).

The relationship between host plant size and infection was also suggested by the significant positive logistic regressions of infection presence/absence on plant size, even within the two different plant density treatments. Which variable was causal in this relationship is not clear. In some graminoids infection by a systemic fungus can induce plants to grow larger (Clay et al 1989, Clay 1990a) and I found a suggestion of such an effect for this pathosystem in another experiment (see Chapter Three). In some systems large host plants are more likely to become infected as they are larger targets for inoculum (Alexander 1990, Boudreau and Mundt 1992, Elmqvist et al 1993, Yahara and Oyama 1993), but that would not be the case in this experiment since I artificially inoculated the plants and they were similar in size at the start of the experiment. Plant size may also be an

indication of the host's overall vigor and richness as a pathogen resource. Assuming that, like most smut fungi, this one overwintered as a teliospore before germinating and infecting plant tissue (Fischer, G.M. and Holton 1957), then the infection was occurring and the fungus was surviving in the host tissue as the plant grew in the spring. Under high host plant density, where individual plants may have experienced greater intraspecific competition leading to smaller size, the fungus may have been unable to survive and/or sporulate.

It is also possible that the microclimate for spore germination or hyphal growth within the high host density plots was poor, although this would be surprising if true. Many fungal pathogens prefer a moist environment and although no explicit measurements of humidity or soil moisture were made in the plots, it was obvious from simple observation that the low host density plots were substantially drier than the high host density plots, at least near the soil surface. So, the evidence suggests that the poor pathogen resource hypothesis is most likely to be correct. It would be interesting to test this hypothesis further.

Pathogen density influenced infection as predicted - plants with heavier pathogen loads were much more likely to become infected, indicating that the range of pathogen densities used in the experiment were biologically realistic. Pathogen density could affect the probability that a plant becomes

infected in two ways. First, the chance that any one pathogen spore successfully infects a plant is probably very low, so exposure to many spores could make it more likely that one will be successful. Alternatively, it may be that pathogen spores have a synergistic effect on the plant such that infection is the result of many spores acting together (Baker 1978). Perhaps at some level of pathogen exposure, either at the site of penetration or during fungal growth inside the plant, host defenses become inadequate and infection can proceed. Until more is known about the infection biology in this system it is impossible to know exactly why higher pathogen loads resulted in greater infection rates.

One more possibility should be mentioned, however. The random assignment of plot treatments resulted in most of the high pathogen density plots being located in the eastern half of the experimental site instead of evenly distributed across the site. Thus high pathogen density could have been confounded with some other environmental variable that influenced infection and followed an east-west gradient. This is unlikely, however, given that plots in the eastern half of the site did not consistently have greater infection rates.

The effect of blocks was due to quite different responses of the plants to the pathogen in block 1 compared to blocks 2 and 3. The higher rate of infection of block 1 plants could be due to several factors. First,

these plots were inoculated first, so the spore suspension was fresher. When smut fungi teliospores are cultured in the laboratory on nutrient medium they germinate, grow hyphae, and if they come into contact with a hyphal strand of opposite mating type they can fuse and produce a rapidly growing, infective dikaryotic hypha called suchfaden (Fischer, G.M. and Holton 1957, p. 190). Once suchfaden is produced it must penetrate host tissue relatively soon (probably in the next 24 hours) or it becomes nonfunctional (J. Groth, personal communication). Assuming that the same phenomenon occurs in nature, then there may well be an inoculation time effect. After one or two days the proportion of viable suchfaden from spores that had germinated and fused in the suspension may have been lower.

There are three points arguing against a temporal effect, however. First, not all plants in block 1 experienced high infection rates, and some plots inoculated on the second or third days in blocks 2 and 3 had higher infection rates than some of the plots in block 1. Second, under laboratory conditions teliospores generally require an organic nutrient medium to germinate (Fischer, G.M. and Holton 1957, pp. 221-224). The suspension I made was plain water containing teliospores, so the only nutrients available were from whatever little organic material was attached to the spores themselves. Thus germination was likely to have occurred after the spores were in contact with the plant and/or soil, in which case the day of

inoculation would not have been very important. Third, smut fungi generally overwinter before germination occurs (Agrios 1988, p. 475), so it is likely that germination did not take place in this experiment until the spring following the summer inoculation.

The block effect could also have been due to an environmental difference that promoted infection in block 1 relative to the other blocks. Since block 1 was most distant from the wooded perimeter of the field, its plots received sun longer each day, and light conditions are known to influence fungal germination. Block 1 plots were also slightly uphill from the rest of the plots and so may also have been somewhat drier, although for most fungi this would result in less successful establishment. In another experiment in which I inoculated plants in a xeric and mesic site (see Chapter Three), infection was greater on average in the xeric site. Perhaps for *Cintractia junci* drier soil conditions do enhance fungal germination, penetration, growth, and/or sporulation, either through a direct effect on its biology or indirectly by influencing the host plant's physiological status. In other systems it has been shown that plants experiencing water stress are more likely to become infected (Colhoun 1979).

Finally, the host plant genotype was very important for the plant-pathogen interaction. Some genotypes were nearly or completely resistant even under high pathogen loads while others experienced 5-10% infection

rates under low inoculation and 15-30% rates under high inoculation. Certainly, further work to examine the genetic basis and heritability of resistance in this system would be worthwhile. If this resistance were sufficiently heritable and as variable throughout the natural population as it appears to be from this 20-plant sample, then it seems very possible that evolution driven by the pathogen could be occurring in the host population. Given that this smut fungus directly impedes host reproduction and that smuts typically have just one generation a year (Agrios 1988, p. 475), evolution in the host population due to this pathogen may be more rapid than in many other plant-pathogen systems where the pathogen's effect is more subtle or where it has multiple generations in one year, allowing it to co-evolve rapidly.

Host plant density, pathogen density, and the block factor all interacted to influence infection rates, as illustrated by the significant three way interaction term in Table 1 and the disease values in the 12 plots in Table 2. In addition, pathogen density interacted with the block factor, and host genotype interacted with both pathogen density and blocks. To the extent that the results of this experiment can be generalized for natural populations of host plants and their diseases, it is clear that predicting the outcome of a plant-pathogen interaction is no simple matter. Plants may have some genetically-based resistance mechanism to a pathogen, but its

phenotypic expression is likely to be influenced by some components of the environment. Thus it would be difficult to predict the evolutionary trajectory of resistance in a plant population in a typically heterogeneous environment. Also, disease development may be density-dependent, affected by both host and pathogen densities as it was in this experiment, yet strong spatial heterogeneity in the environment may influence the strength of that density-dependence.

The influence of only four factors on infection were tested in this experiment. Undoubtedly in nature a much greater number of interacting ecological and genetic factors have important effects on plant-pathogen encounters. Single-factor studies are absolutely unable to detect interactions and thus even their conclusions about the main effects they do test can be unrealistic under the heterogeneous conditions typical of nature. Hopefully, with more multiple-factor studies of a variety of natural pathosystems, we may begin to understand what the most consistently influential factors and interactions are and be able to eliminate others as unimportant.

Table 1. Results of the categorical analysis of shoot infection presence / absence.

Source of variation	DF	χ^2	P
Intercept	1	76.82	< 0.0001
Host plant density (HPD)	1	6.71	0.010
Pathogen density (PD)	1	9.79	0.002
Block	2	8.54	0.014
HPD X PD	1	0.00	0.999
HPD X Block	2	0.57	0.754
PD X Block	2	11.07	0.004
HPD X PD X Block	2	6.20	0.045
Block 1 vs. Blocks 2-3	1	8.43	0.004
Block 2 vs. Block 3	1	0.03	0.862

Table 2. The percentage of plants with shoot infection in each plot of the experiment.

PLOT	BLOCK	HOST DENSITY	PATHOGEN DENSITY	% INFECTED	N
1	1	Low	High	28.57	35
2	1	High	High	18.45	233
3	1	High	Low	7.23	235
4	1	Low	Low	5.56	36
5	2	High	High	5.73	227
6	2	Low	Low	8.11	37
7	2	High	Low	1.32	227
8	2	Low	High	15.38	39
9	3	Low	Low	14.29	42
10	3	High	Low	1.80	222
11	3	Low	High	5.26	38
12	3	High	High	7.42	229

Table 3. Results of the categorical analysis of shoot infection presence / absence in high host plant density plots, with host genotype, pathogen density, and block as main effects.

Source of variation	DF	χ^2	P
Intercept * * *	1	89.57	< 0.0001
Host genotype * * *	8	97.89	< 0.0001
Pathogen density * * *	1	20.28	< 0.0001
Block * * *	2	21.32	< 0.0001
Geno X Pathogen * * *	8	32.13	< 0.0001
Geno X Block * * *	16	27.70	0.0344
Pathogen X Block	2	0.01	0.9961
Residual	16	14.28	0.5775

Figure 1. Layout of experimental plots and treatments.

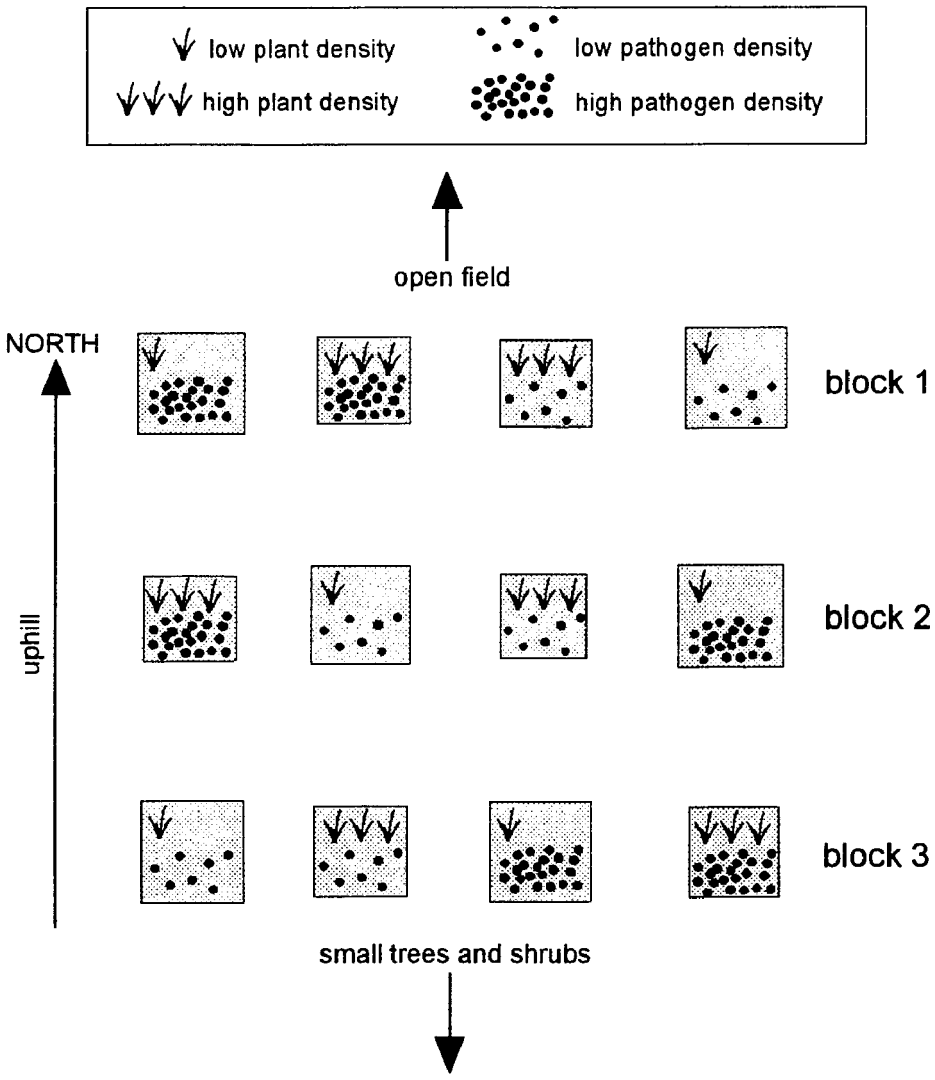

low plant density
high plant density
low pathogen density
high pathogen density
open field
NORTH
uphill
block 1
block 2
block 3
small trees and shrubs

Figure 2. The percentage of plants with shoot infection and plant size (number of shoots per plant) in (A-B) low (n = 227) vs. high (n = 1373) host density plots; (C-D) low (n = 799) vs. high (n = 801) pathogen density plots; and (E-F) blocks 1 (n = 539), 2 (n = 530), 3 (n = 531). The plant size graphs show means and 95% confidence limits.

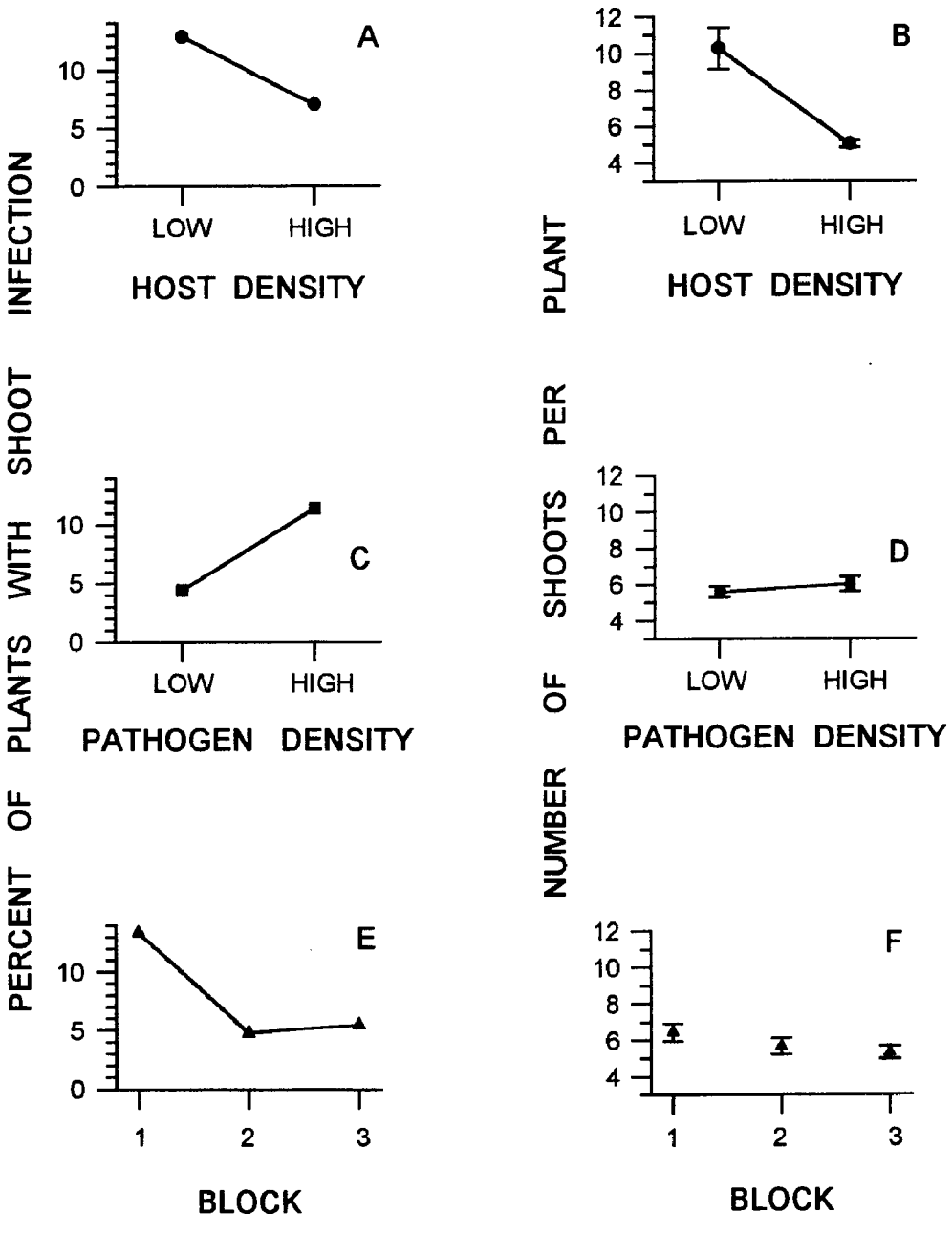

A
10
5
0
LOW
HIGH
HOST DENSITY
PERCENT OF PLANTS WITH SHOOT INFECTION
B
12
10
8
6
4
LOW
HIGH
HOST DENSITY
NUMBER OF SHOOTS PER PLANT
C
10
5
0
LOW
HIGH
PATHOGEN DENSITY
D
12
10
8
6
4
LOW
HIGH
PATHOGEN DENSITY
E
10
5
0
1
2
3
BLOCK
F
12
10
8
6
4
1
2
3
BLOCK

Figure 3. The percentage of plants with shoot infection in the low and high pathogen plots in blocks 1, 2, and 3. Number of plants in the low pathogen density plots in blocks 1, 2, and 3 respectively are 271, 264, and 264; for the high pathogen density plots they are 268, 266, and 267, respectively.

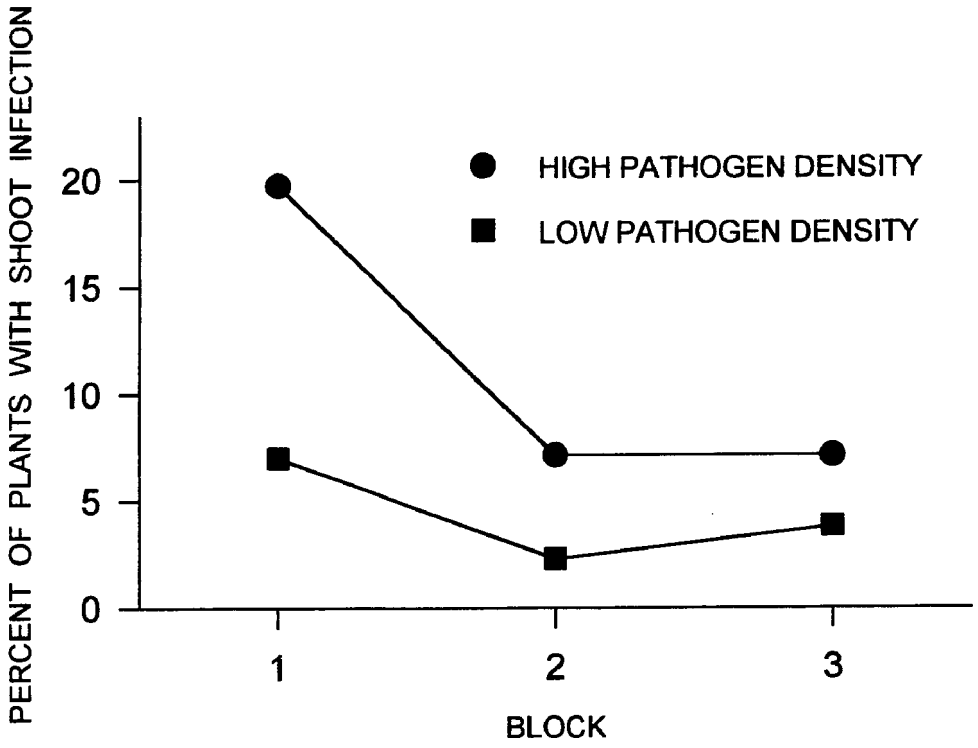

PERCENT OF PLANTS WITH SHOOT INFECTION
20
15
10
5
0
HIGH PATHOGEN DENSITY
LOW PATHOGEN DENSITY
1
2
3
BLOCK

Figure 4. Logistic regressions of presence / absence of shoot infection on plant size (number of shoots per plant). The dotted lines are the regression when all sized plants were included. The solid lines are the regressions for plants with less than 21 shoots. (A) Plants in all plots (dotted: $y = 0.11x - 3.21$, Wald chi-square = 60.94, $P = 0.0001$, $n = 1600$; solid: $y = 0.20x - 3.84$, Wald chi-square = 96.73, $P = 0.0001$, $n = 1568$); (B) plants in plots with low host density (dotted: $y = 0.05x - 2.51$, Wald chi-square = 6.82, $P = 0.0090$, $n = 227$; solid: $y = 0.14x - 3.35$, Wald chi-square = 11.02, $P = 0.0009$, $n = 203$); (C) plants in plots with high host density (dotted: $y = 0.17x - 3.68$, Wald chi-square = 65.06, $P = 0.0001$, $n = 1373$; solid: $y = 0.22x - 3.98$, Wald chi-square = 82.45, $P = 0.0001$, $n = 1365$). The plant size interval midpoints are plotted only to check that the relationship between the logit of infection probability and plant size is linear. For A and B the intervals ranged from 0-2, 3-4, 5-6, 7-8, 9-10, 11-15, 16-20, 21-55. The last interval for C was 21-47. The regression itself was calculated with all plant values, not the intervals.

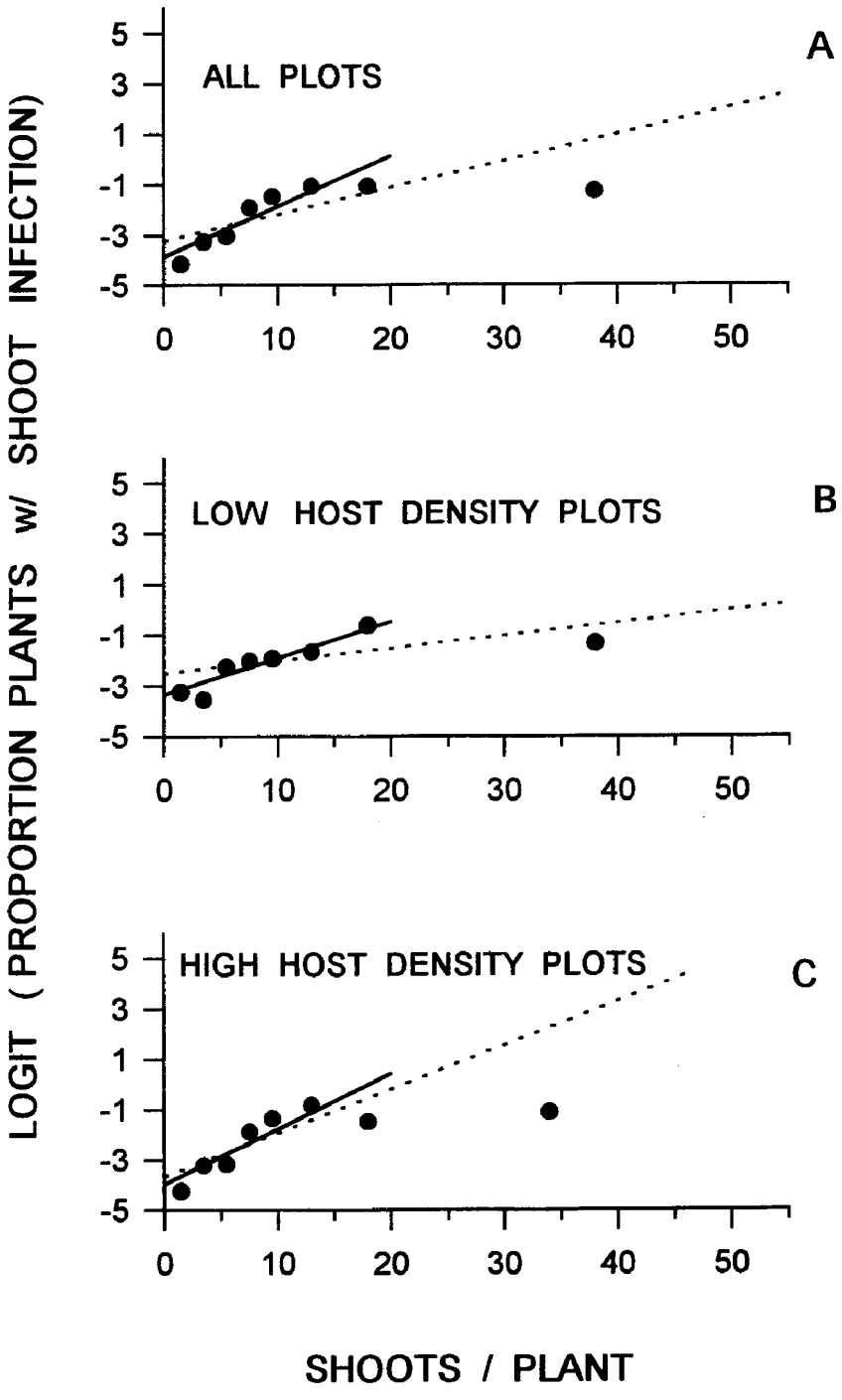

LOGIT (PROPORTION PLANTS w/ SHOOT INFECTION)
ALL PLOTS
A
LOW HOST DENSITY PLOTS
B
HIGH HOST DENSITY PLOTS
C
5
3
1
-1
-3
-5
0
10
20
30
40
50
SHOOTS / PLANT

Figure 5. The percentage of plants with shoot infection for each host plant clone in the experiment (with the exclusion of clone 62, for which there are no electorphoretic data and which was excluded in the genotype analyses). The clones are grouped according to their electrophoretic genotypes based on 7 polymorphic enzyme loci. Clones within one genotype had identical electrophoretic profiles. The following is a list of clone number and sample size: 41, n=63; 29, n=64; 45, n=68; 25, n=70; 26, n=66; 64, n=63; 53, n=58; 65, n=37; 9, n=68; 34, n=69; 61, n=67; 16, n=64; 22, n=61; 4, n=69; 15, n=71; 20, n=65; 52, n=69; 63, n=60; 23, n=68; 49, n=73.

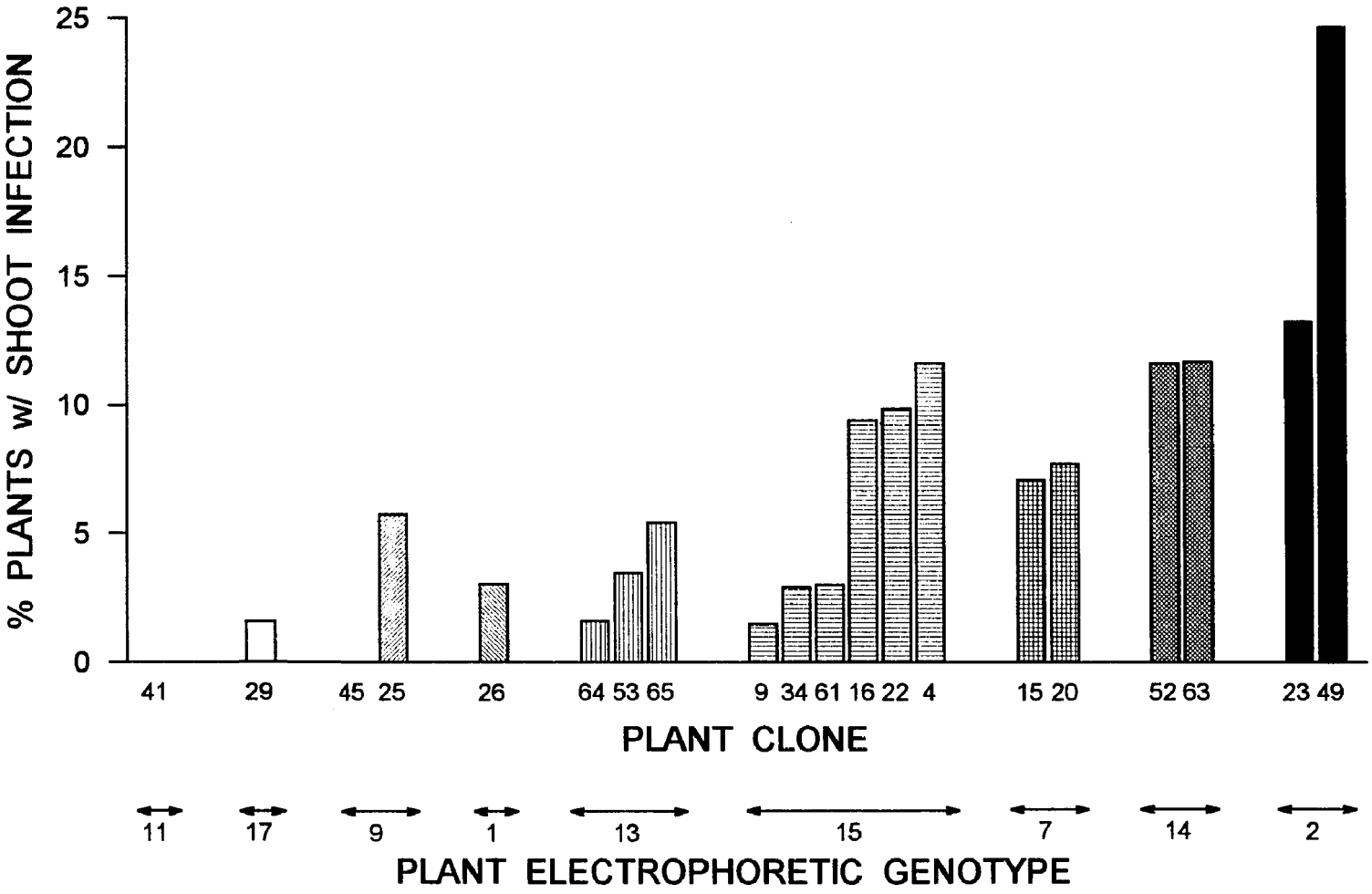

% PLANTS w/ SHOOT INFECTION
25
20
15
10
5
0
41
29
45 25
26
64 53 65
9 34 61 16 22 4
15 20
52 63
23 49
PLANT CLONE
11
17
9
1
13
15
7
14
2
PLANT ELECTROPHORETIC GENOTYPE

Figure 6. (A) The percentage of plants with shoot infection in each electrophoretic genotype group, pooled across all plots of the experiment. (B) Backtransformed means and 95% confidence limits of plant size (number of shoots per plant) for each electrophoretic genotype. For both graphs, sample sizes for the genotypes are: 11, n=63; 17, n=64; 9, n=138; 1, n=66; 13, n=158; 15, n=398; 7, n=136; 14, n=129; 2, n=141.

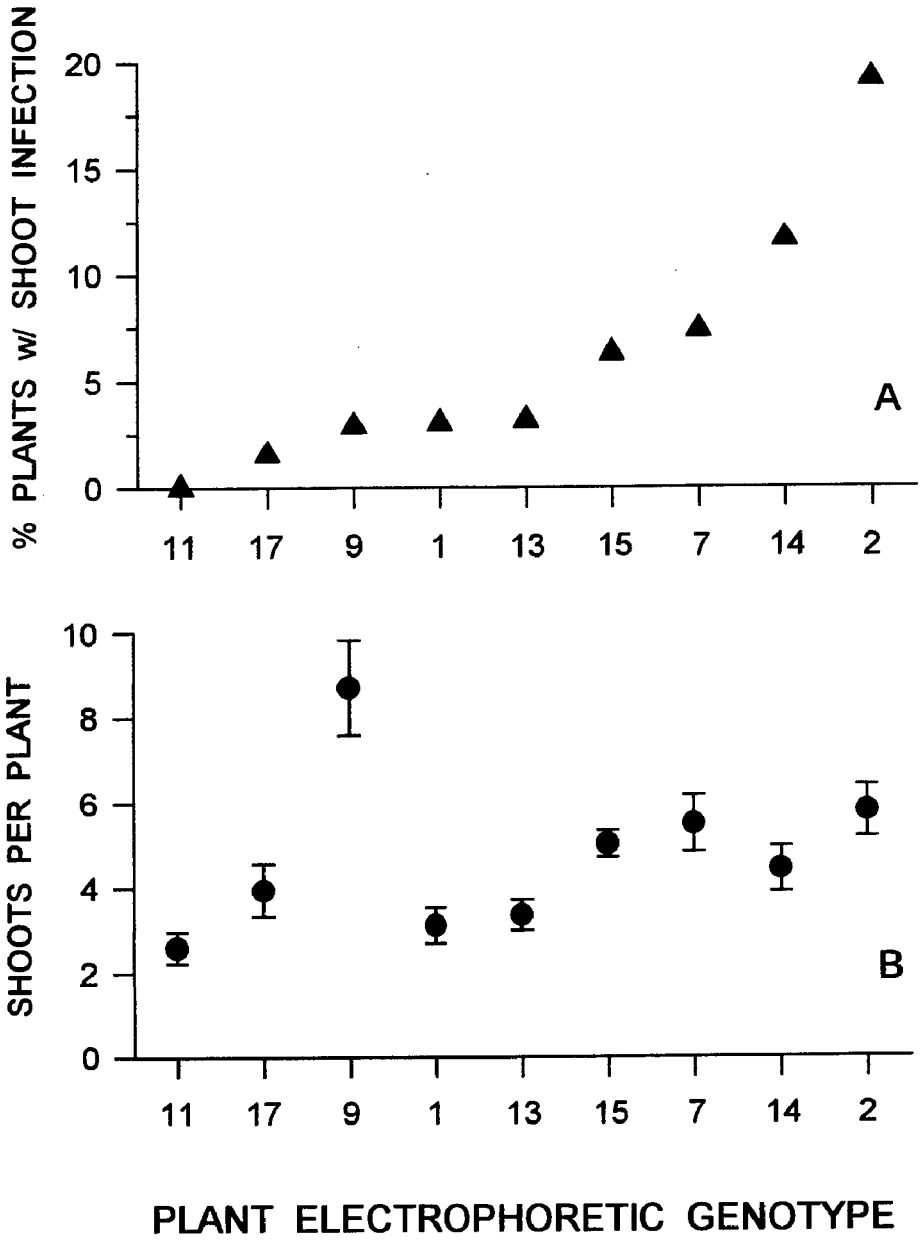

% PLANTS w/ SHOOT INFECTION
20
15
10
5
0
A
11
17
9
1
13
15
7
14
2
SHOOTS PER PLANT
10
8
6
4
2
0
B
11
17
9
1
13
15
7
14
2
PLANT ELECTROPHORETIC GENOTYPE

Figure 7. The percentage of plants with shoot infection in each electrophoretic genotype group in (A) the low pathogen density plots and (B) the high pathogen density plots. Sample sizes for the genotypes in (A) are: 11, n=33; 17, n=32; 9, n=67; 1, n=33; 13, n=78; 15, n=198; 7, n=68; 14, n=65; 2, n=71. In (B) they are: 11, n=30; 17, n=32; 9, n=71; 1, n=33; 13, n=80; 15, n=200; 7, n=68; 14, n=64; 2, n=70.

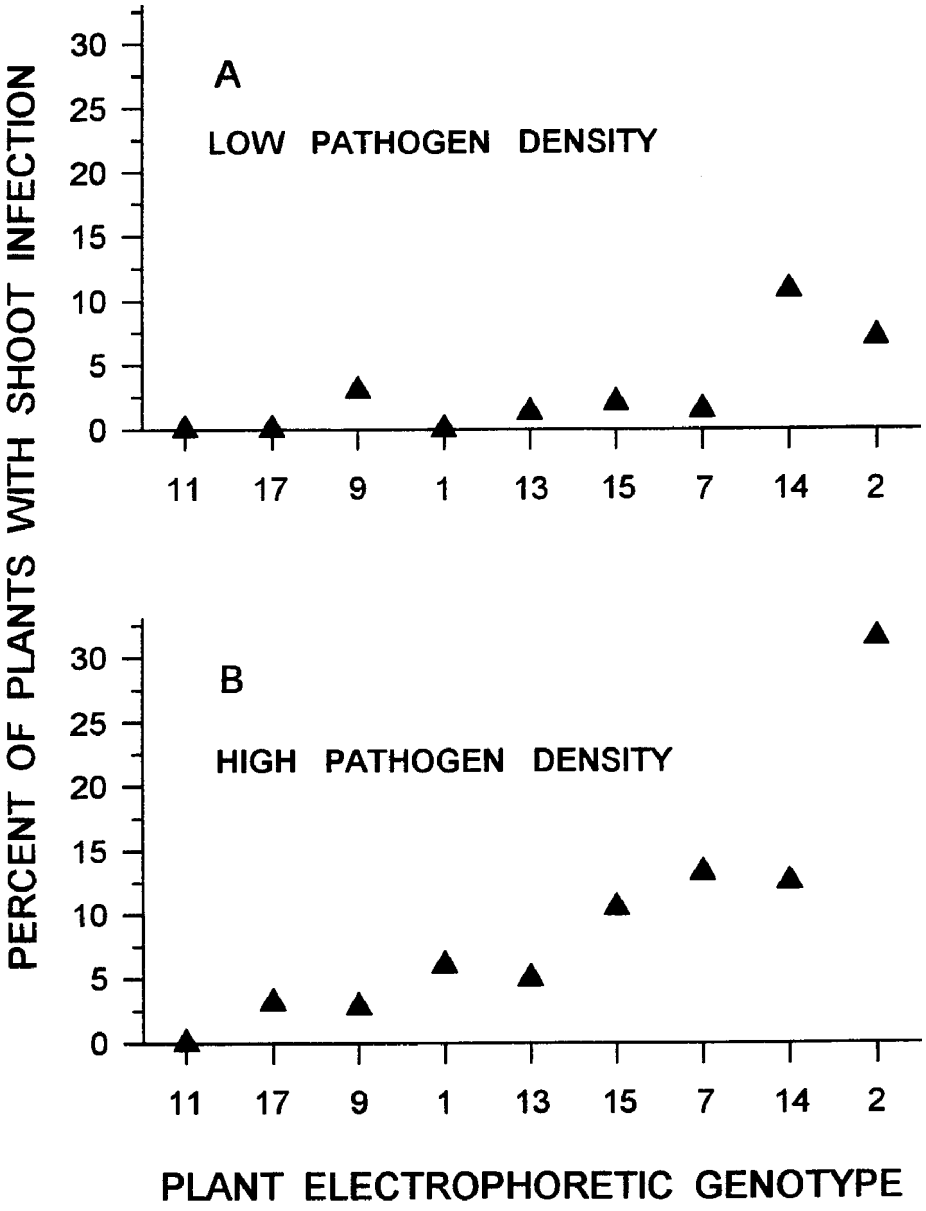
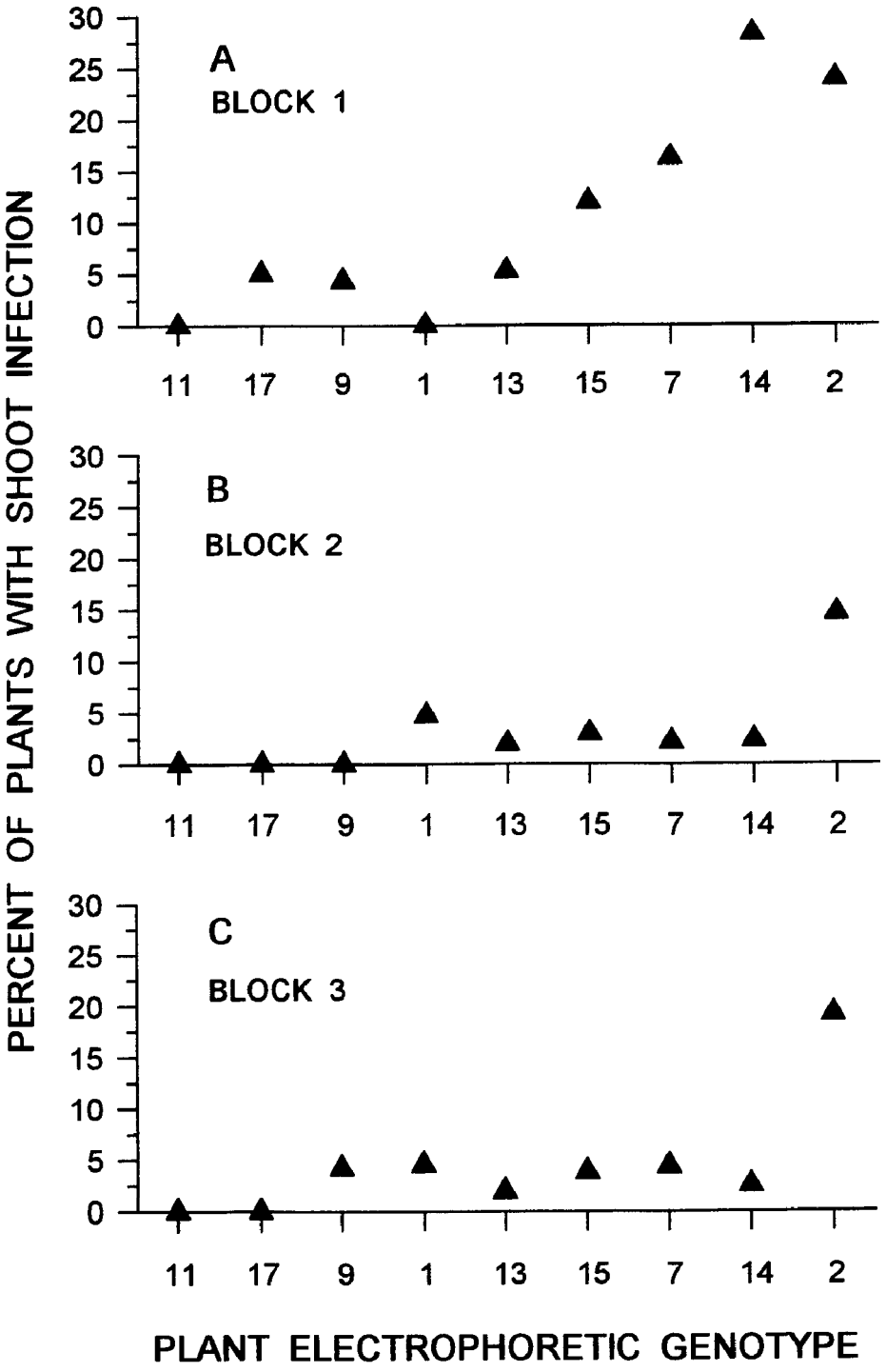

A
LOW PATHOGEN DENSITY
B
HIGH PATHOGEN DENSITY
PERCENT OF PLANTS WITH SHOOT INFECTION
0
5
10
15
20
25
30
11
17
9
1
13
15
7
14
2
PLANT ELECTROPHORETIC GENOTYPE

Figure 8. The percentage of plants with shoot infection in each electrophoretic genotype group in (A) block 1, (B) block 2, and (C) block 3. Sample sizes for the genotypes in (A) are: 11, n=23; 17, n=20; 9, n=46; 1, n=23; 13, n=57; 15, n=133; 7, n=43; 14, n=46; 2, n=46. In (B) they are: 11, n=20; 17, n=22; 9, n=44; 1, n=21; 13, n=51; 15, n=134; 7, n=47; 14, n=43; 2, n=48. In (C) they are: 11, n=20; 17, n=22; 9, n=48; 1, n=22; 13, n=50; 15, n=131; 7, n=46; 14, n=40; 2, n=47.


PERCENT OF PLANTS WITH SHOOT INFECTION
A
BLOCK 1
B
BLOCK 2
C
BLOCK 3
30
25
20
15
10
5
0
11 17 9 1 13 15 7 14 2
PLANT ELECTROPHORETIC GENOTYPE

5

Experimental infection and recovery in the *Juncus dichotomus* - *Cintractia junci* plant-pathogen interaction: effects of soil moisture, host plant genotype, and environmental heterogeneity.

INTRODUCTION

Many ecological and genetic factors may influence the interaction between host plants and their pathogens in natural populations (Burdon 1987a, Burdon and Jarosz 1988, Fritz and Simms 1992). These factors can be discovered through descriptive study of naturally occurring plant and pathogen populations and by experimentally manipulating these factors in controlled studies. In fact, both approaches are necessary and complementary; observations of the possible factors in the natural populations will suggest which factors need experimental study, and the experimental results will allow tests of hypotheses regarding the natural patterns.

In this manipulative experiment, I examined the effect of one such factor, soil water content, on the interaction between the rush *Juncus dichotomus* and a smut fungus pathogen, *Cintractia junci*. In addition, I

examined the effects of host plant genotype and environmental (block) heterogeneity. I experimentally controlled for host plant density and pathogen genetic structure, since both can influence the outcome of plant-pathogen interactions in a variety of ways (Burdon and Chilvers 1982, Parker 1992) (Chapter Two). I examined both the infection of healthy plants and the recovery of previously infected plants. Using uninfected and previously infected greenhouse-grown ramets set into field plots, I inoculated uninfected plants with the pathogen and observed them for signs of infection and watched the previously infected plants for signs of recovery. Both plants grew together in plots that were either watered or unwatered throughout the growing season.

I chose soil water as a factor for study because in initial field observations of these organisms in a natural population I noticed distinct patchiness of disease levels that appeared to be associated with soil water availability (Chapter Two). The population grows in a site with very sandy soils and is adjacent to a nearby wetland, so that in some areas of the site the conditions are extremely xeric while in other areas they are much wetter. It seemed likely that soil moisture might be an important ecological factor that could impact both pathogen and pathogen biology.

Cintractia junci spends a portion of its life cycle outside of host plant tissue. Teliospores are released from infected host tissue in the summer and

outside of the plant they germinate and produce hyphae which must fuse with an opposite mating type before penetrating host tissue (Fischer, G.M. and Holton 1957). Thus, during this time, the pathogen is vulnerable to the physical conditions in a site. Soil moisture and humidity are some of the most important physical factors that can impact the germination of spores and the growth of fungal hyphae, generally enhancing those processes (Rotem 1978, Sussman and Dowthit 1982, Davison and Tay 1987, Agrios 1988, Wilcox 1989). Consistent with this are the results of studies from several natural populations indicating that plants in shaded conditions are more likely to become infected, presumably because of increased moisture and humidity promoting pathogen success (Augspurger and Kelly 1984a, b, Jarosz and Burdon 1988, Jarosz and Levy 1988). In the agriculturally significant smut fungi that infect seedlings, soil moisture is very influential. The optimal soil moisture content, however, varies with the system studied; in some a wetter soil enhances infection while in others a drier soil does. In contrast, with those smut fungi that infect seeds, soil factors such as soil moisture have little influence on the outcome of infection. Instead, atmospheric humidity is important (Fischer and Holton 1957, pp. 199-204).

Soil water conditions may also impact the host plant in ways that can influence the plant-pathogen interaction. A lack of water that leads to plant stress may make a potential individual predisposed to initial infection by the

pathogen (Colhoun 1979). In contrast, it is also possible for a water-deficient plant to escape infection because it has become an unsuitable environment for the pathogen (Colhoun 1979, Ayres and Woolacott 1980, Auld et al 1990). The overall physiological condition of an infected plant should also influence its ability to recover from the disease. Thus plants which are water stressed and infected experience a double stress which in combination may be catastrophic (Ayres 1991).

Soil moisture is of course just one of many possible physical variables that might influence host and pathogen (Burdon 1987a). By including the soil water treatment with a general environmental block effect in this experiment, I can examine how important soil moisture is relative to other environmental heterogeneity. Although I did not measure specific environmental parameters in each block, differences among them that were independent of soil water could suggest further experiments designed to examine additional environmental variables.

The host plant genotype is an important factor to include in the study because genetically-based resistance to pathogens is common in many plant-pathogen systems but little is known about its importance in natural populations relative to environmental factors (Burdon 1987a). Also, knowing whether a host population is genetically variable for plant response to a pathogen is the first step toward understanding if the pathogen can act as a

selective agent in the host population (Alexander 1992) (for a more complete discussion of the role of host plant genotype in plant-pathogen interactions see the Introduction in Chapter Four). The results from this experiment, and from two other field experiments in which I also tested the effects of different host plant genotypes combined with other environmental factors (Chapters Three and Four), will indicate whether or not host plants in this system are genetically variable in their response to the pathogen and whether the effect of host genotype interacts with important environmental variables.

ORGANISMS AND STUDY SITE

Juncus dichotomus Ell. (Juncaceae) is a perennial rush that grows in sunny habitats with sandy soils (see Chapter Two for a complete description). When infected by the smut fungus *Cintractia junci* Schw. (Ustilaginales, Basidiomycetes), it can have shoot infections and/or isolated fruit infections. An infected shoot produces an inflorescence structure, but it is deformed and produces no flowers or seeds. Instead, membrane-covered lesions which develop on the inflorescence break open to release black smut teliospores. The dispersed teliospores germinate and produce hyphal strands which must fuse with a strand of opposite mating type in order to produce an infective mycelium. In experimental inoculations (this chapter, Chapter

Three, Chapter Four) plants became infected when the base of the plant and clipped leaves were exposed to a teliospore mixture, with shoot infection appearing in the growing season following the season of inoculation. The pathogen appears to be perennial, with the great majority of plants retaining shoot infection from year to year (Chapter Two, Chapter Three).

Although the details of fruit infection are unknown for this system, I hypothesize that it occurs after inoculation of individual flowers or flower buds earlier in the same season from spores that were in the soil. Flower infection happens with a number of other smut fungi (Fischer, G.M. and Holton 1957, Hassan and MacDonald 1971, Jennersten et al 1983, Snetselaar and Mims 1993), and is consistent with several field observations I have made of this system. First, different fruits on the same infructescence may be infected or not infected, suggesting that separate flowers rather than the entire inflorescence were exposed. This could only happen during the bud or flowering stages, when buds or flowers are distinctly separate structures. Second, within one fruit there may be both seeds and smut fungus spores. If the fungus had penetrated the plant previous to bud or flowering (say in meristematic tissue which later gave rise to reproductive structures), then I would expect it to have invaded and utilized every ovule within a flower. This is the case when a shoot is systemically infected - the entire inflorescence is deformed and all buds are invaded by the pathogen

and produce no seeds. Inoculation of buds or flowers could either be from spores that overwinter on the soil and are picked up and carried on the outside of the plant while it was growing to its reproductive stage, or from sporulating lesions that disperse teliospores at the same time as flowering (personal observation). However, the smut fungi are characterized as having only one generation per year (Agrios 1988, p. 475), which means that fruit infections that produce teliospores must have arisen from inoculations of spores produced in the previous season.

The experimental site had similar conditions to the plant's natural habitat with very sandy soils and abundant direct sunlight. Previously, it was an unused portion of a backyard with sparse weedy herbaceous vegetation and some planted grass. No *Juncus* plants or smut fungi were present on the site until they were introduced during the experiment. The site was surrounded on the southern and western side with a tall stockade fence. The western fence increasingly blocked direct sunlight across the site in the late afternoon (Figure 1).

METHODS

THE EXPERIMENT.

In September of 1989 I collected nine apparently healthy *Juncus dichotomus* individuals and four systemically infected individuals from the

same natural population where I had noticed spatial variation of disease and soil water conditions (these plants were a subset of a much larger collection). The plants were collected at least 0.5 meters apart and across the entire population. I brought the plants to the greenhouse at SUNY Stony Brook where I washed their roots, planted them in standard Pro-Mix potting soil, and placed them into two different greenhouse bays, one for the healthy plants and one for the infected plants.

In January of 1990 and again in late March, late April, and early June 1990 I divided each healthy plant at the rootstock into several ramets, repotted them, and placed them back in the bay. They then flowered and I checked each plant to be sure there were no signs of infection. I propagated and repotted them again in early winter of 1991 and one final time in June of 1991. These nine propagated healthy individuals are identified as clones 31, 46, 42, 44, 61, 43, 47, 36, and 66. Throughout the propagation the plants were fertilized and watered as needed, with all plants receiving identical treatment. By growing the plants for almost two years in a common greenhouse environment with successive propagations, I minimized any possible variation among them due to environmental differences in their original collection locations.

The infected plants received the same treatment except that they were propagated fewer times over the two years between collection and the

start of the experiment. In the summers of 1990 and 1991 the plants "flowered" and I checked them to make sure they remained infected. All plants showed 100% systemic shoot infections in both summers, i.e. they produced no healthy, reproductive shoots. I refer to these four propagated infected individuals as clones A, B, C, and D.

Since one goal of this experiment was to examine different plant genotypes for susceptibility, I needed to ensure that my nine plant sample contained genetically distinct individuals, even though I was careful to collect anatomically distinct individuals from separated locations in the field. I used starch gel electrophoresis to screen the individuals for allozyme differences and found 7 polymorphic loci (PGM, IDH2, PGDH-1, PGDH-2, MDH1, MDH2, MDH-3) which let me determine that my set of nine individuals were all genetically distinct, with the exception of clones 46 and 44, which had identical electrophoretic profiles. I did not do electrophoresis on the four infected clones, but the collection sites for these were widely spaced (from 10-80 m apart).

In early September 1991 I turned over the soil in the experimental site to make a uniform planting bed and in late September I put the plants outside at the site to let them acclimate for several days before planting into plots, which took place over several days. Plots within a block were 0.5 m apart and the distance between blocks was 1 m (Figure 1). Each plot

received three copies of each of the nine healthy plant clones, three copies of infected clones 10 and 11, five copies of infected clone 12, and two copies of infected clone 13. The plants were planted in rows in the plot according to the design in Figure 1. The positions of each clone were randomly assigned with the exceptions that infected plants were in the middle row and healthy plants were in the outer rows and that no two replicates of a clone could be adjacent. The placeholder plants (Figure 1) were included in order to make the biotic neighborhood for each experimental plant the same. No data were collected on the placeholders.

On October 2 I separately inoculated each healthy plant by pouring onto the base of the plant an identical volume of a common teliospore suspension made by crushing infected shoots from the natural population into water.

The supplemental watering treatment began in mid-April and was done almost daily. Within each block I randomly chose one plot to be watered throughout the growing season while the other plot only received natural rainfall (Figure 1). I watered with a sprinkler can, pouring the water around the base of the plants, avoiding exposing the canopy to supplemental water. Each watered plot received the same volume of water as all others. In order to reduce runoff from the watered plot to the unwatered plot in a block, I built a small ridge of soil between the two plots which prevented any

surface water going onto the unwatered plots. Within a block the watered and unwatered plots were 0.5 m apart.

In late August and September, after flowering, fruit set, and most seed dispersal, I examined each shoot of each plant for shoot and fruit infection. In mid September I harvested the aboveground plant parts, dried them to constant weight in a 60° C oven and measured dry mass per plant.

ANALYSIS

For the inoculated plants I analyzed plant size (number of shoots per plant) and fruit infection (the proportion of shoots on a plant with at least one infected fruit) as a split-plot analysis of variance with blocks (Steel and Torrie 1960) done in PROC GLM of SAS 6.0 using Type III sums of squares (SAS Institute 1990c, Potvin 1993). The whole-plot treatment factor was watering, since it had to be applied to the entire plot rather than to individual plants within a plot. The split-plot factor was host plant clone and it was replicated within each plot. I considered all factors as fixed, so all ANOVAs are Model I (Sokal and Rohlf 1981). As an experimental treatment, the watering factor is clearly a fixed effect. The block factor could be considered either as a fixed or random effect (Potvin 1993), but since this was a garden experiment I do not think that the six blocks necessarily represented a random selection of the heterogeneity that these plants would experience

in their natural environment. Also, I would not expect just six blocks to encompass the entire range of natural variation. The plant clone factor could also be considered either as fixed or random. Since nine clones is a small sample of the genetic variation that may be present in a natural population, I also did not expect them to be a very good random representation of the natural variation, so again I chose to analyze them as a fixed effect.

Plant size, measured as aboveground dry mass, was tested as a randomized block design without replication, with watering and block as the main effects tested over their interaction (Sokal and Rohlf 1981, pp. 348-352). Although dry mass was measured on many plants in each plot, plants could not be used as replicates in a split-plot design because their clonal identities were confused during harvesting. So, I used the mean mass in each plot for this analysis, pooling infected and uninfected plants within a plot. The data for one plot were missing and so were estimated as being equal to the marginal mean of he other plots in that treatment (Potvin 1993).

I tested the residuals from each ANOVA for normality with the Shapiro-Wilk statistic W and graphic tests generated by PROC UNIVARIATE in SAS 6.0 (SAS Institute 1990e). Homoscedasticity was tested with the F-max test (Sokal and Rohlf 1981). In the split-plot anovas the fruit infection data were arcsine-transformed and the plant size data were square

root-transformed.

The shoot infection data for the inoculated plants did not meet the assumptions of ANOVA due to extreme skewness, so plants were categorized as having shoot infection present/absent and analyzed with PROC CATMOD in SAS 6.0 (SAS Institute 1990a), using a categorical model of homogeneity (Freeman 1987) (see "Analysis" in Chapter Three for a discussion of this method).

In addition to analyzing fruit infection as a continuous variable with the ANOVA described above, I also analyzed fruit infection in the inoculated plants categorically by classifying plants as with/without severe fruit infection, defined as >66% shoots with fruit infection. I chose 66% as the breakpoint because 1) it is easy to conceptualize a categorical division of greater than or less than two-thirds infected and 2) there were consistently increasing numbers of plants with fruit infection greater than about 65% of shoots (Figure 5), indicating a natural grouping of high disease plants and lower disease plants. Essentially, this was one of many possible ways to roughly group plants into a higher and lower fruit infection category.

The relationship between plant size and fruit infection for the inoculated plants was analyzed with Kendall's coefficient of rank correlation with a normal approximation and a two-tailed test (Sokal and Rohlf 1981, pp. 602-606), using all plants pooled from all plots. The plant

size variable was the number of shoots per plant and the fruit infection variable was the percent of shoots per plant with at least one infected fruit. Tau was calculated from PROC FREQ in SAS 6.0 (SAS Institute 1990b). I also examined the relationship between plant size and the categorical measure of fruit infection with logistic regression (Hosmer and Lemeshow 1989) in which the logit (i.e. ln(P/(1-P)) of the probability that a plant had greater than 66% of shoots with fruit infection was regressed on the number of shoots per plant. It was done with PROC LOGISTIC in SAS 6.0 (SAS Institute 1990d). To check if the relationship was linear, I created intervals of plant size, calculated the logit of severe fruit infection for each interval and plotted the logits against the interval midpoints in Figure 7.

Recovery of shoots on infected plants was uncommon and those data also were inappropriate for analysis of variance. So, to analyze recovery in the previously-infected plants, I categorized them with a binary response variable of (1) at least one uninfected shoot or (2) no uninfected shoots. I analyzed the effect of watering treatment and block with a categorical model of homogeneity in PROC CATMOD, SAS 6.0 as above. Plant size (number of shoots per plant) for the infected plants was analyzed with split-plot ANOVA as described above for the inoculated plants, and plant size (dry mass) was analyzed by a randomized block design as also described above.

RESULTS

INOCULATED PLANTS

Shoot infection.

Out of the 306 initially uninfected but inoculated plants that survived the winter, 101 exhibited shoot infection in the summer of 1992. The plants that grew in the watered plots were no more likely to have infected shoots than those in the unwatered plots (Table 1). However, the block that plants grew in did influence infection rates; plants in block 3 were significantly less likely to become infected than those in the other blocks Table 1, Figure 2A). When block 3 was removed from the analysis, then the infection rates among the remaining blocks were homogeneous (Table 1). There was no evidence of any interaction between the watering treatment and the block effect (the residual chi-square in Table 1 was nonsignificant).

Replication of plant clones within each plot was low, preventing a categorical analysis as above, so since the watering and block factors were nonsignificant within blocks one, two, four, five, and six, I pooled across the plots in those blocks to test for variation among plant clones for infection. A G-test for heterogeneity (Sokal and Rohlf 1981, p. 745) between plant clone and presence/absence of shoot infection using PROC FREQ in SAS 6.0 (SAS Institute 1990b) showed no significant heterogeneity among the nine clones ($G^2 = 11.612$, $P = 0.169$).

Fruit infection

Like shoot infection, fruit infection (measured as the percent of shoots per plant with an infected fruit) was also unaffected by watering, but did vary among blocks (Table 2). In block 1 about 40% of shoots on the average plant had at least one infected fruit while in the other blocks closer to 80% had infected fruits (Figure 3A).

Plant clones were highly variable for fruit infection (Table 2), with plants of clone 44 averaging about 25% shoots with fruit infection, clone 42 averaging about 65%, and plants of most other clones averaging near 80% shoots with fruit infection. The effect of plant clone was similar in watered and unwatered plots and in the six blocks (nonsignificant interaction terms in Table 2). This split-plot analysis of variance had normally distributed residuals ($W = 0.985$, $P = 0.65$). The data from the 12 plots in the experiment were homoscedastic (F-max = 2.80, df = 24,25, a = 12, ns).

Most plants had fruit infection on most of their shoots but some plants had much less severe fruit infection (Figure 5). Plants which grew in watered plots were more likely to have severe fruit infection (defined as $>66\%$ shoots with fruit infection) than were plants in unwatered plots (Figure 6) and this difference became significant when block 1 was excluded from the analysis (Table 3). Plants in block 1 were significantly less likely to have severe fruit infection than plants in other blocks (Table 3, Figure 2B).

Plant size.

Plant clones were highly variable in number of shoots per plant (Table 4), with approximately a five-fold increase from the lowest mean in clone 66 to the highest in clone 42 (Figure 4B). Surprisingly, watering the plants had no effect on their size whatsoever, measured either as number of shoots (Table 4) or aboveground dry mass (Table 5). The plant's block also had no significant effect on plant size (Table 4, Table 5) and neither watering nor block interacted with the plant clone effect (Table 4). Analyses of residuals from these plant size ANOVAs indicated that they were normally distributed and the data were homoscedastic (for number of shoots split-plot, $W = 0.990$, $P = 0.93$, F-max $= 2.145$, $df = 25$, $a = 12$, ns; for dry mass randomized block, $W = 0.946$, $P = 0.535$, blocks F-max $= 11.57$, $df = 1$, $a = 6$, watering F-max $= 1{,}200$, $df = 5$, $a = 2$. ns).

Fruit infection and plant size.

Comparing fruit infection and plant size measures for plants in the six blocks (Figure 3) suggests a positive relationship, but the same comparison for the nine plant clones (Figure 4) suggests there may be a negative relationship. With all plants from all plots pooled, there was a slight but significant negative correlation between fruit infection and plant size (Kendall's coefficient of rank correlation, $\tau = -0.08$, $P = 0.037$).

Plant size and the categorical measure of fruit infection were also related negatively (logistic regression, slope = -0.044, Wald chi-square = 4.896, P = 0.027). This effect was clearly due to the influence of the largest plants, i.e. those with more than 21 shoots, which were much less likely to have severe fruit infection (with large plants excluded, logistic regression, slope = 0,008, Wald chi-square = 0.087, P = 0.768) (Figure 7).

RECOVERY PLANTS

Shoot infection

All infected plants at the beginning of the experiment still had shoot infections at the end, and most still had 100% of their shoots infected, but there was some evidence of recovery because some plants had some healthy shoots. The watering treatment did not influence which plants had healthy shoots, but the blocks plants grew in did (Table 6). No infected plants in block 3 had any healthy shoots at all, while in every other block at least some infected plants produced some healthy, reproductive shoots (Figure 8). When I excluded block three from the analysis, the block effect became nonsignificant (Table 6).

In order to analyze variation among the four plant clones for recovery, I pooled the data across the nonsignificant watering treatment within the homogeneous set of blocks one, two, four, five, and six and did a G-test for

heterogeneity. Heterogeneity among the clones only approached significance ($G^2 = 6.354$, df = 3, P = 0.096) (Figure 8B).

Plant size

In contrast to the inoculated plants, the previously infected plants growing with supplemental water were significantly larger than unwatered plants in both number of shoots (Table 7, Figure 9A) and dry mass (Table 8, Figure 9C). The block a plant grew in influenced its shoot number (Table 7, Figure 9B), but not its dry mass (Table 8, Figure 9D) and the four plant clones exhibited different sizes also (Table 7, Figure 8C). Analyses of residuals from these plant size ANOVAs on the infected plants indicated that they were normally distributed and the data were homoscedastic (for number of shoots split-plot, W = 0.980, P = 0.409, F-max = 6.051, df = 13,10, a = 12, ns; for dry mass randomized block, W = 0.951, P = 0.600, blocks F-max = 9.38, df = 1, a = 6, ns, watering F-max = 1,837, df = 5, a = 2, ns).

Comparison of the amount of recovery in the six blocks (Figure 8A) with plant size in the blocks (Figure 9B) indicates no clear association between recovery and plant size. Similarly, no relationship is apparent from a comparison between recovery in the four plant clones (Figure 8B) and plant size in the plant clones (Figure 8C).

DISCUSSION

This experiment demonstrated that the amount of soil moisture had little effect on the plant-pathogen interaction, but other aspects of environmental heterogeneity had a dramatic impact. Host plant variability also affected the interaction and its effect was not influenced by the environmental factors. To the extent that these experimental results can be extended to natural conditions, it is clear that an understanding of the interaction in nature between *Juncus dichotomus* and the smut fungus *Cintractia junci* requires consideration of the variability of plants in a population and the distribution of various environmental variables, although soil moisture is unlikely to be an important one.

Considering that soil moisture and humidity have known effects in many plant pathosystems (see Introduction), it is somewhat surprising that daily supplemental watering in this very xeric experimental site had so little effect on the plant-pathogen interaction. Out of four possible chances to detect an effect, watering influenced only one disease measure. It increased the likelihood that inoculated plants had severe fruit infection (>66% of shoots per plant with at least one infected fruit), but had no detectable influence on the more precise measure of fruit infection (percentage of shoots per plant with at least one infected fruit), or on the likelihood of shoot infection in the inoculated plants, or on shoot recovery in the previously

infected plants. Even the one significant effect was weak and mostly obscured by block heterogeneity.

There are several possible explanations for why soil moisture had such a weak influence in this interaction. First, soil water may in fact have little influence in this system. Watering was motivated by observations in the natural population of apparently similar patterns of disease levels and soil moisture. However, the experimental results agree with subsequent results from the natural population in which disease frequency and soil moisture were quantified; no correlation between disease and soil moisture was detected (Chapter Two). Since this particular plant-pathogen interaction has not been studied in the past, there were no previous findings to rely upon in hypothesizing about the effect of soil water. The contrast of my findings with other studies reporting an influence of water levels on plant-pathogen interactions illustrates the caution needed in generalizing, from a handful of studies, about the ecology of a diverse group of organisms.

Second, it is possible that soil moisture content can, in fact, strongly impact this plant-pathogen interaction, but that this experiment did not detect it. It is possible that the watered and unwatered plots were close enough that plants in the unwatered plots could get some of the supplemental water belowground. It is also possible that the watering treatment was insufficient and did not raise soil moisture content

significantly above the ambient level. This seems unlikely, since the treatment consisted of daily supplemental, saturating watering of naturally xeric plots. Also, the size of the previously-infected plants did respond to the watering treatment, suggesting that the plots did experience different water regimes with the watering technique I used. In a future experiment the watering technique could be changed so that plots are farther apart and irrigation is more constant than once a day, and/or it could be extended to earlier in the season or to the season before, when the plants were originally inoculated.

The results on plant size for the inoculated plants and the previously infected plants in the two watering treatments contradict each other, so they do not help clarify whether or not the treatment was strong enough to have consistent biological effects. Plant size of the inoculated plants was unaffected by supplemental watering, but watering the previously infected plants made them bigger. The watering treatment may have been more effective for the previously infected plants because of their position in the plots. They were planted in the central row (Figure 1A) and so they may have received a greater amount of water per plant, compared to the inoculated plants which were planted in the peripheral rows and so could have lost water to runoff. Even though watering did have a measurable effect on plant growth among the infected plants, it still had no effect on

their recovery from smut infection so, in terms of the plant-pathogen interaction, it was unimportant.

The one significant effect that watering had on the interaction was to increase the probability that inoculated plants developed severe fruit infection. Since watering did not affect the size of these plants, it is unlikely that the interaction was influenced through a host plant effect due to watering. Instead, watering probably improved the physical conditions for fruit infection by increasing humidity in the canopy and enhancing spore germination, mating fusion, or hyphal growth. If it is true that fruit infection resulted from penetration of individual ovules following inoculation of flowers, then it is very likely that the canopy was the site for pathogen germination, mating, growth, and penetration, since that is where the flowers were.

The largest plants in the experiment were less likely to have severe fruit infection (as indicated by the negative logistic regression), which is consistent with the above explanation for how watering may influence fruit infection. Although I did not measure plant height, my experience with these plants throughout my research suggests that those with more shoots are generally more vigorous and have taller shoots. If so, large plants may extend their inflorescences above the canopy where they would experience less humidity than within the plant canopy, resulting in less spore

germination and pathogen growth on the flowers, and thus less fruit infection.

In contrast to the mostly unimportant soil water factor, other environmental heterogeneity, as represented by the block factor, was consistently important for both infection and plant size. Although I measured no other environmental variables in this experiment, some hypotheses about which environmental factors were important can be made based on the patterns of infection and/or plant size in the different blocks.

Block one was different from the others for several variables. Among the previously infected plants, those in block one were smaller. Among the inoculated plants, those in block one had lower levels of fruit infection (measured both continuously and categorically), and they were slightly smaller in both shoots per plant and aboveground dry mass (but not significantly so). Even though this result for the inoculated plants suggests that fruit infection and plant size were positively correlated, it is unlikely that the similar effect of block one on both was simply due to a direct effect on plant size which in turn influenced infection; both the rank correlation and the logistic regression of fruit infection on plant size were slightly negative. Instead, it is likely that the environmental conditions of block one influenced plant size and fruit infection independently.

Block one was positioned such that it received direct sunlight for a

greater period of time than the other blocks, since it was located furthest from the north-south portion of the fence. With the very sandy, xeric soil in the experimental site, prolonged exposure to midsummer sun could cause increased water stress and thus decreased plant growth. Similarly, decreased humidity within the plant canopy might explain the reduced levels of fruit infection found in block one since fungal spore germination is often positively influenced by humidity. It would be interesting to experimentally manipulate both moisture and light together in order to separate the effects of these two environmental variables. It is also possible that soil nutrient levels differed among the blocks in a way that altered fruit infection and plant size, but I have no data to document this.

Block three also stood apart from the rest. Inoculated plants were less likely to have shoot infections if they grew in block three, and previously infected plants were less likely to have recovered shoots in block three. I have no obvious explanation for why block three is distinct in this way, but it illustrates further how important environmental heterogeneity on the scale of meters can be for a plant-pathogen interaction.

There was also heterogeneity among plant clones that was independent of environmental effects due to watering or blocks. Since the genetic basis of resistance in this plant is unknown, I can only hypothesize about the reasons that the plant clones show variation. The patterns of clone

variation for the inoculated plants for both fruit infection and plant size are close to mirror images (Figure 4), which suggests that the basis of the clones' difference in fruit infection might be their size differences. Those clones with lower proportions of fruit-infected shoots appeared to be those that had more shoots per plant. The negative fruit infection - plant size rank correlation and the negative logistic regression of categorized fruit infection on plant size also support this idea.

There are several reasons why the largest clones may have been better able to avoid fruit infection by the smut fungus. As discussed above, the inflorescences of larger plants may escape high humidity levels within the canopy. In addition, large plants may also have more resources at their disposal for defensive purposes, so that they could resist infection at the site of penetration, or by inhibiting hyphal growth into the ovules.

In contrast to the results for the inoculated plants, the previously infected clones showed no correspondence between plant size and recovery. Clones that were larger and presumably more vigorous did not appear any more or less likely to produce healthy, reproductive shoots than smaller clones. Since the clones were only marginally different in recovery, this lack of relationship with plant size is tentative, but perhaps no clear relationship should be expected. Once this perennial, systemic infection takes hold in a host plant, as it had for all of the previously infected plants used here, the

amount of resources that a plant can garner may have little impact on its ability to recover from the infection. Thus an infected plant clone that is better able to utilize resources in a site and so grows larger than another infected clone may simply be providing more resources for its already-established parasite. The outcome would be that both the smaller clone and the larger clone remain completely infected, but the larger clone produces more smutted shoots.

Although the variation in recovery among infected plant clones was only marginal, it is worth some further discussion. An important point is that variation among them is difficult to interpret. First, I did not do electrophoresis on these clones, so I am not certain they are genetically distinct, although they were collected from distant locations in the original natural population. Second, they were collected from the field already infected, so the pathogen strain(s) inhabiting them may very well be genetically variable themselves. Thus any difference in recovery among the clones might be due to differences among the plants or the pathogens or both.

Documenting that genetically distinct host individuals vary in response to a pathogen is the first step in determining whether a disease may have an evolutionary impact on a plant population. The second step is identifying which traits are responsible for their different responses. For

example, here I hypothesize that plant size is an important trait for fruit infection. The third step is to demonstrate that those traits have a genetic basis and to estimate their heritability. In this plant-pathogen system, further work would be needed to determine the genetic basis and heritability of plant size. It may be that the clones' variation in fruit infection is a phenotypic difference only, either related to size or to some other phenotypically variable trait. It is also possible that other genetically controlled traits are important for avoiding fruit infection.

In summary, soil moisture had little effect, but other aspects of the environment which varied on a scale of meters had a clear influence on this plant-pathogen interaction. Host plant variability, either genetically-based or phenotypic, was also important in some aspects of the interaction. These results support the concept that host - parasite interactions in nature are very complex. Only a series of more and more refined experiments aimed at characterizing the important ecological and genetic factors could lead to a good understanding of a natural plant-pathogen interaction. This experiment was useful as a step toward eliminating one and promoting other of the many possible variables that may be important in this *Juncus-Cintractia* system.

Table 1. Categorical analysis of the effect of watering treatment and block on presence / absence of shoot infection of inoculated plants.

WITH ALL BLOCKS

Source of variation	DF	χ^2	P
Intercept * * *	1	154.67	<0.0001
Watering	1	1.44	0.230
Block *	5	11.10	0.050
Residual	5	7.39	0.193
Block 3 vs. others * *	1	7.84	0.0051

WITH BLOCKS 1,2,4,5,6

Intercept * * *	1	143.27	<0.0001
Watering	1	0.38	0.535
Block	4	3.39	0.495
Residual	4	6.56	0.161

Table 2. Split-plot analysis of variance for inoculated plants of the percent of shoots on a plant with at least one infected fruit Whole-plot factors were tested over error (a) and sub-plot factors over error (b).

Source of variation	DF	MS	F	P
WHOLE-PLOT				
Blocks *	5	1.314	8.63	0.017
Watering	1	0.095	0.62	0.469
Error (a)	5	0.152		
SPLIT-PLOT				
Plant clone * * *	8	1.542	14.10	0.0001
Clone X Watering	8	1.119	1.09	0.371
Clone X Block	40	0.125	1.14	0.270
C X W X B	40	0.125	1.14	0.276
Error (b)	198	0.109		

Table 3. Categorical analysis of the effect of watering treatment and block on fruit infection severity (> 66% or ≤ 66% shoots with fruit infection) for the inoculated plants.

WITH ALL BLOCKS

Source of variation	DF	χ^2	P
Intercept * * *	1	722.31	< 0.0001
Watering	1	3.19	0.074
Block * * *	5	47.66	< 0.0001
Residual	5	3.64	0.602
Block 1 vs. others	1	42.97	< 0.0001

WITH BLOCKS 2-6

Intercept * * *	1	757.53	< 0.0001
Watering *	1	4.43	0.035
Block	4	3.41	0.492
Residual	4	2.24	0.691

Table 4. Split-plot analysis of variance (for inoculated plants) of the total number of shoots per plant. Whole-plot factors were tested over error (a) and sub-plot factors over error (b).

Source of variation	DF	MS	F	P
WHOLE-PLOT				
Blocks	5	3.256	2.42	0.178
Watering	1	0.003	0.00	0.963
Error (a)	5	1.340		
SPLIT-PLOT				
Plant clone * * *	8	13.720	28.01	0.0001
Clone X Watering	8	0.361	0.74	0.659
Clone X Block	40	0.635	1.30	0.127
C X W X B	40	0.698	1.42	0.061
Error (b)	200	0.490		

Table 5. Randomized block analysis of variance for the inoculated plants of aboveground dry mass per plant in grams. The analysis was done on a per plot basis using the mean mass within each plot since individual plant IDs could not be matched to individual mass values (see Methods).

Source of variation	DF	MS	F	P
Watering	1	0.321	1.82	0.235
Block	5	0.159	3.67	0.090
Error (W X B)	5	0.087		

Table 6. Categorical analysis of the effect of watering treatment and block on presence / absence of uninfected shoots on infected plants.

WITH ALL BLOCKS

Source of variation	DF	χ^2	P
Intercept * * *	1	27.41	<0.0001
Watering	1	0.00	0.999
Block * * *	5	27.67	< 0.0001
Residual	5	1.64	0.897
Block 3 vs. others * * *	1	27.41	<0.0001

WITH BLOCKS 1,2,4,5,6

Intercept * * *	1	27.26	<0.0001
Watering	1	5.93	0.926
Block	4	0.01	0.204
Residual	4	1.63	0.804

Table 7. Split-plot analysis of variance for infected plants of the total number of shoots per plant. Whole-plot factors were tested over error (a) and sub-plot factors over error (b).

Source of variation	DF	MS	F	P
WHOLE-PLOT				
Blocks *	5	3.651	6.41	0.031
Watering *	1	5.403	9.48	0.028
Error (a)	5	0.570		
SUB-PLOT				
Plant clone *	3	4.720	3.13	0.029
Clone X Watering	3	0.464	0.31	0.820
Clone X Block	15	2.335	1.55	0.104
C X W X B * *	14	3.488	2.31	0.009
Error (b)	94	1.509		

Table 8. Randomized block analysis of variance for the infected plants of aboveground dry mass per plant in grams. The analysis was done on a per plot basis using the mean mass within each plot since individual plant IDs could not be matched to individual mass values (see Methods).

Source of variation	DF	MS	F	P
Watering *	1	1.670	7.94	0.037
Block	5	0.290	1.38	0.366
Error (W X B)	5	0.210		

Figure 1. (A) The arrangement of inoculated and infected plants in each plot. Plants were spaced 7 cm apart. (B) The arrangement of the 12 plots in the experimental site. Plants near the fence experienced increased shading.

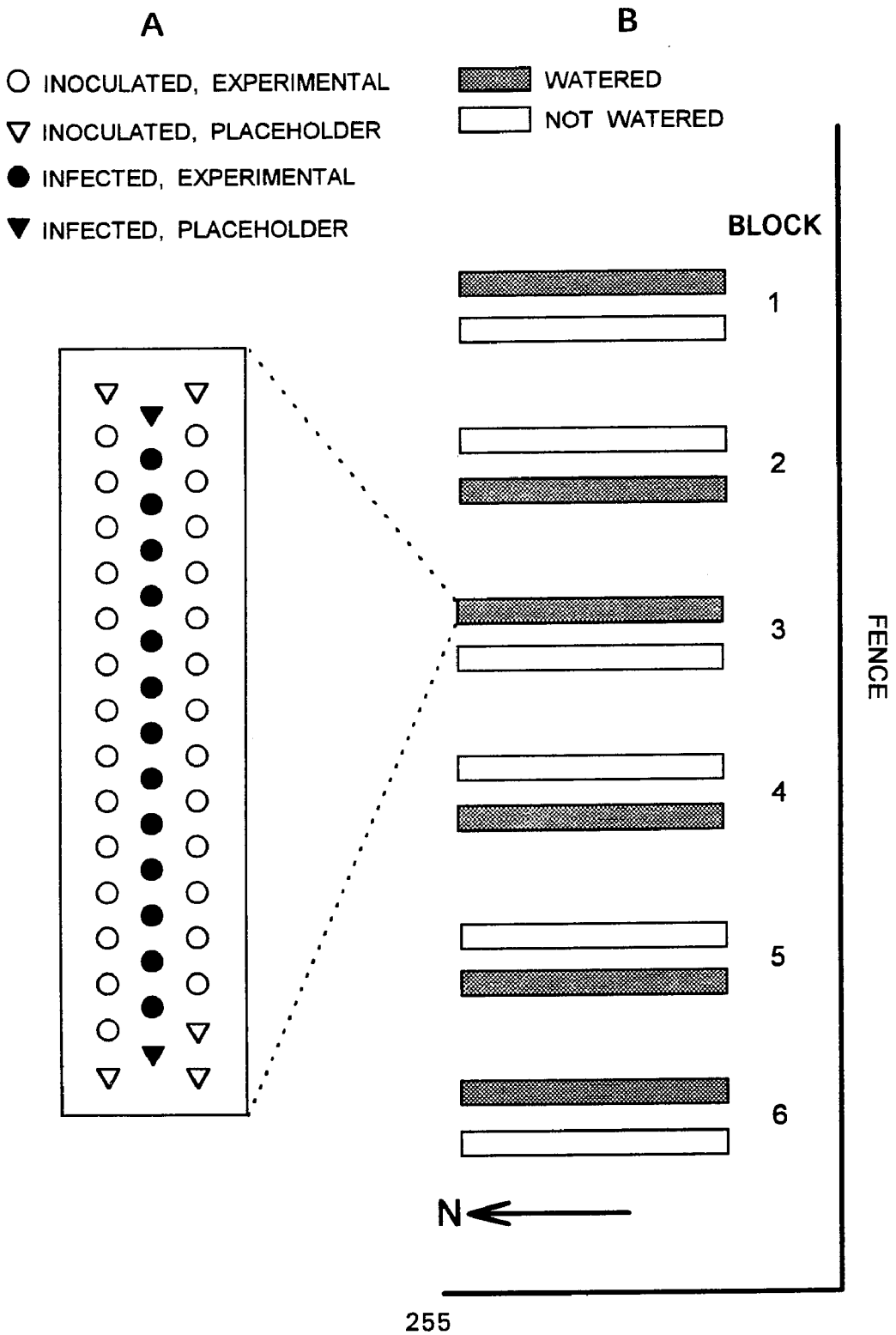
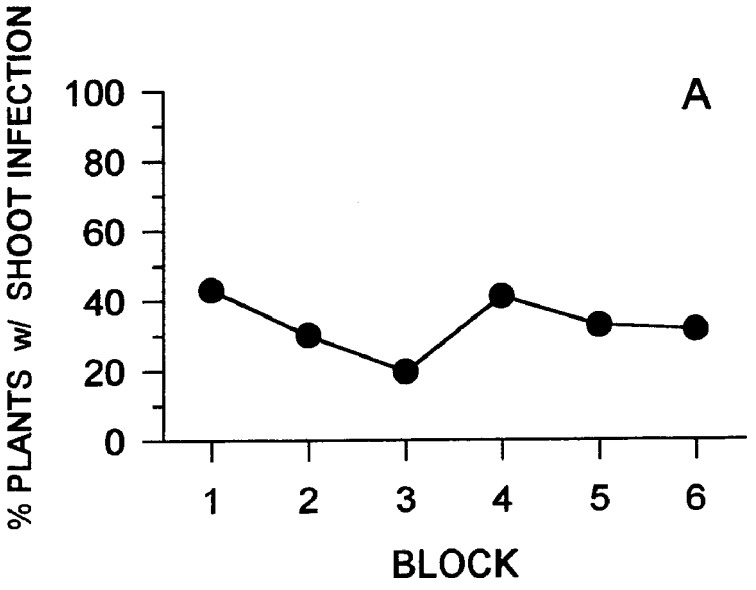

A
B
INOCULATED, EXPERIMENTAL
INOCULATED, PLACEHOLDER
INFECTED, EXPERIMENTAL
INFECTED, PLACEHOLDER
WATERED
NOT WATERED
BLOCK
1
2
3
4
5
6
FENCE
N

Figure 2. The percentage of inoculated plants in blocks 1-6 which exhibit (A) shoot infection and (B) heavy fruit infection (> 66% of shoots with at least one infected fruit). Block sample sizes for both graphs are shown above the block axis in B.


% PLANTS w/ SHOOT INFECTION
A
100
80
60
40
20
0
1
2
3
4
5
6
BLOCK

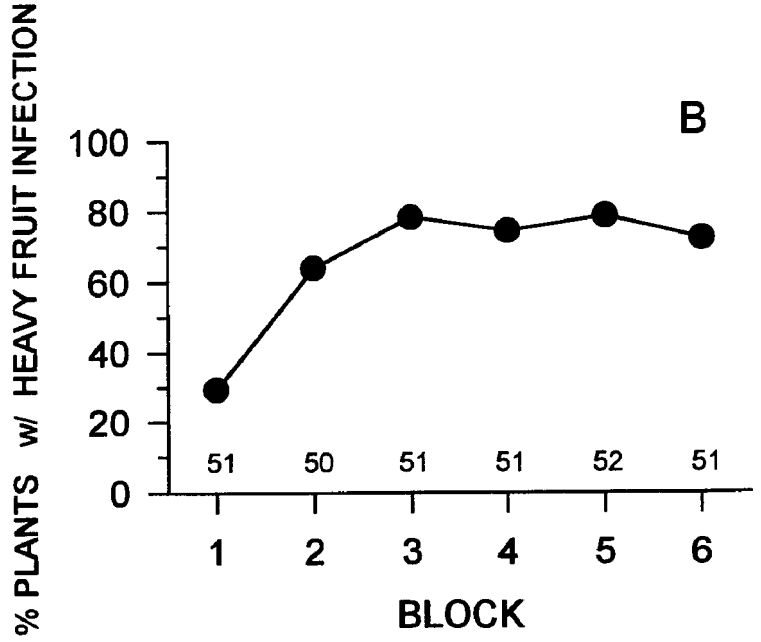
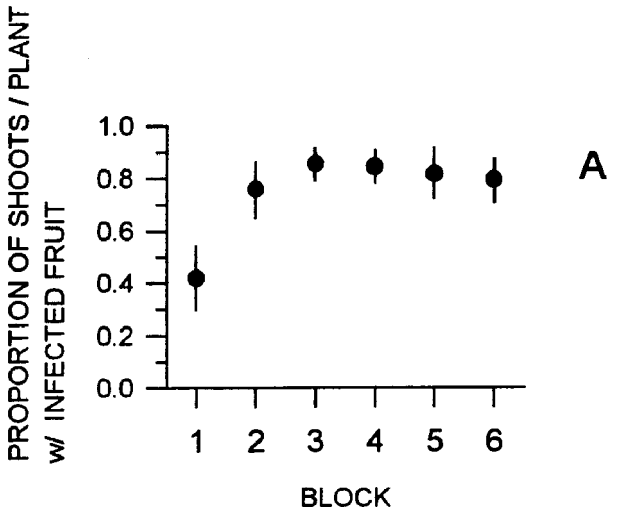
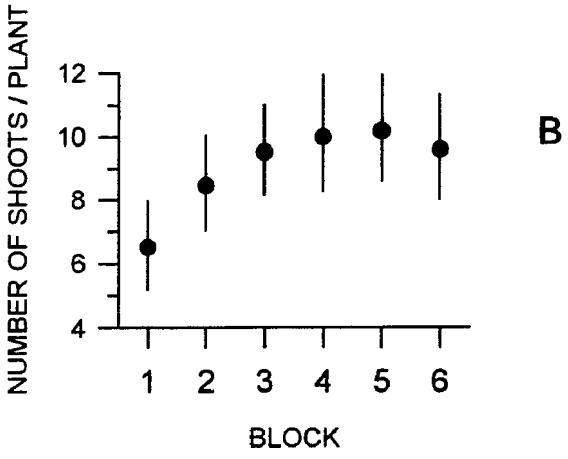

% PLANTS w/ HEAVY FRUIT INFECTION
B
100
80
60
40
20
0
51
50
51
51
52
51
1
2
3
4
5
6
BLOCK

Figure 3. Back-transformed means and 95% confidence limits in the six blocks for the inoculated plants of (A) the proportion of shoots per plant with fruit infection (B) the total number of shoots per plant. Sample sizes for each block are shown in Figure 2B. (C) Means and standard deviations of aboveground dry mass per plant in grams in the six blocks. Sample sizes in blocks 1-6 are 52, 50, 25, 51, 52, 50, respectively.


PROPORTION OF SHOOTS / PLANT
w/ INFECTED FRUIT
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
BLOCK
A


NUMBER OF SHOOTS / PLANT
12
10
8
6
4
1
2
3
4
5
6
BLOCK
B

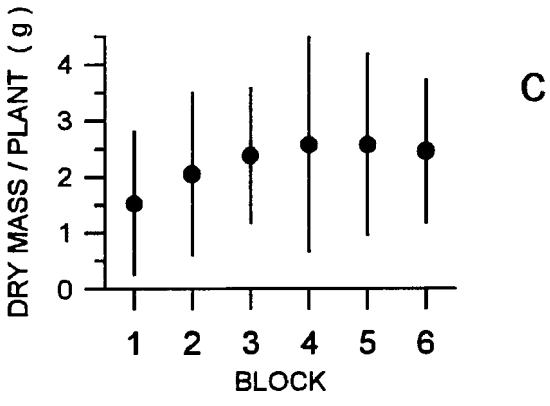
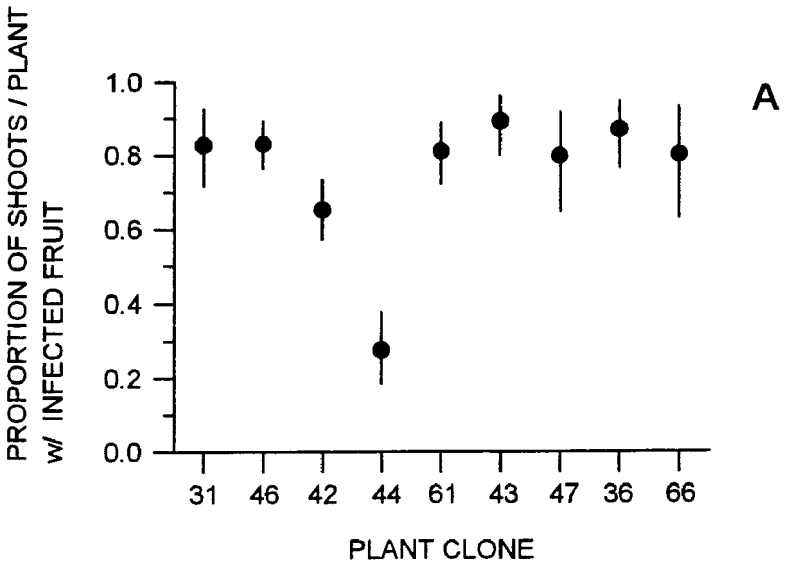

DRY MASS / PLANT (g)
4
3
2
1
0
1
2
3
4
5
6
BLOCK
C

Figure 4. For the nine inoculated plant clones, (A) backtransformed means and 95% confidence limits of the proportion of shoots per plant with fruit infection (sample sizes for clones 1-9 are 34, 36, 35, 35, 35, 31, 34, 35, 31, respectively) and (B) means and 95% confidence limits of the number of shoots per plant (sample sizes for clones placed from left to right on the graph are 34, 36, 35, 35, 35, 32, 34, 35, 32, respectively).


PROPORTION OF SHOOTS / PLANT
w/ INFECTED FRUIT
1.0
0.8
0.6
0.4
0.2
0.0
31
46
42
44
61
43
47
36
66
PLANT CLONE
A

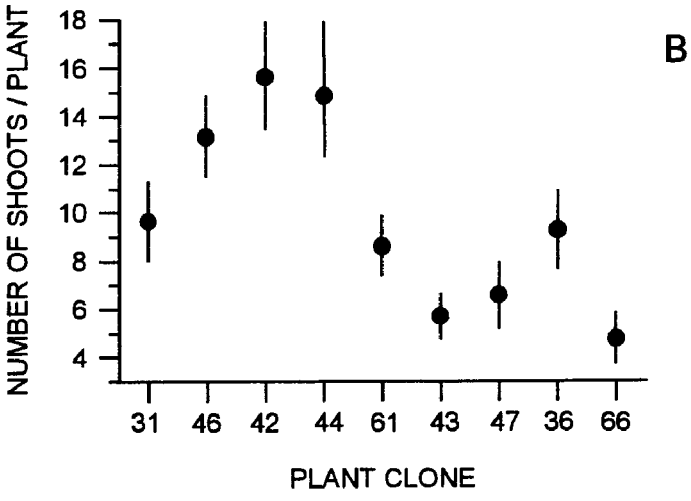

NUMBER OF SHOOTS / PLANT
18
16
14
12
10
8
6
4
31
46
42
44
61
43
47
36
66
PLANT CLONE
B

Figure 5. Frequency distribution for the inoculated plants of the percentage of shoots per plant with at least one infected fruit.

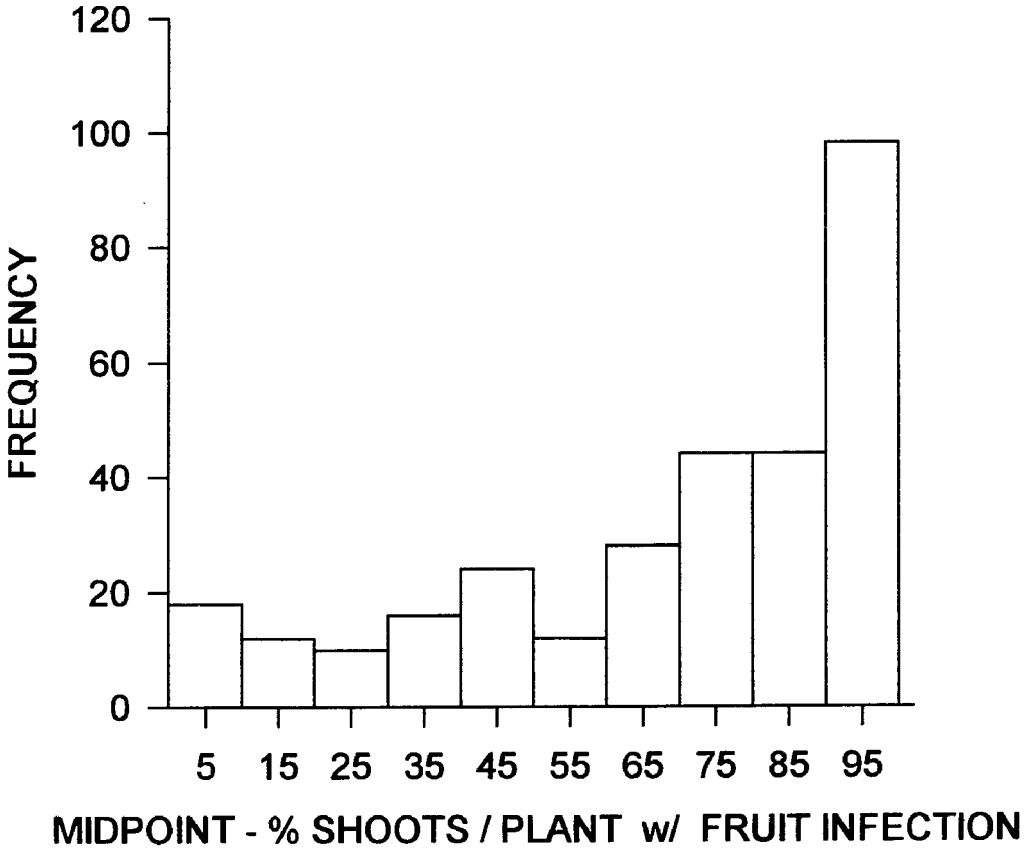

120
100
80
60
40
20
0
FREQUENCY
5
15
25
35
45
55
65
75
85
95
MIDPOINT - % SHOOTS / PLANT w/ FRUIT INFECTION

Figure 6. For the inoculated plants, the percentage of plants in the watering treatments with heavy fruit infection (> 66% of shoots with at least one infected fruit).

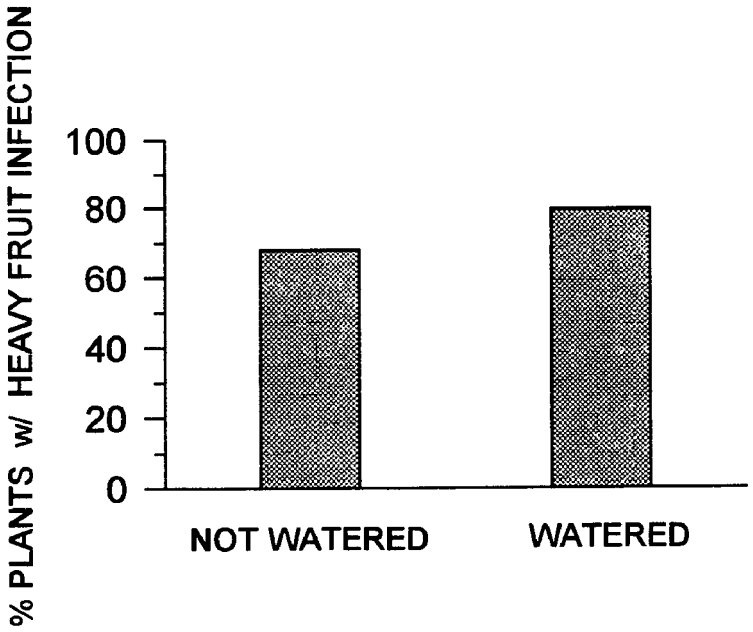

% PLANTS w/ HEAVY FRUIT INFECTION
100
80
60
40
20
0
NOT WATERED
WATERED

Figure 7. For inoculated plants, logistic regression of the logit of the probability that >66% of a plant's shoots had fruit infection regressed on the number of shoots per plant. The solid line shows the significant negative regression for all plants. The dotted line shows the nonsignificant regression when plants with greater than 21 shoots were excluded. The points represent midpoints of eight plant size intervals and are plotted to check the linearity of the relationship . Sample size for the regression with all plants was 306 and for the regression without the largest plants sample size was 289.

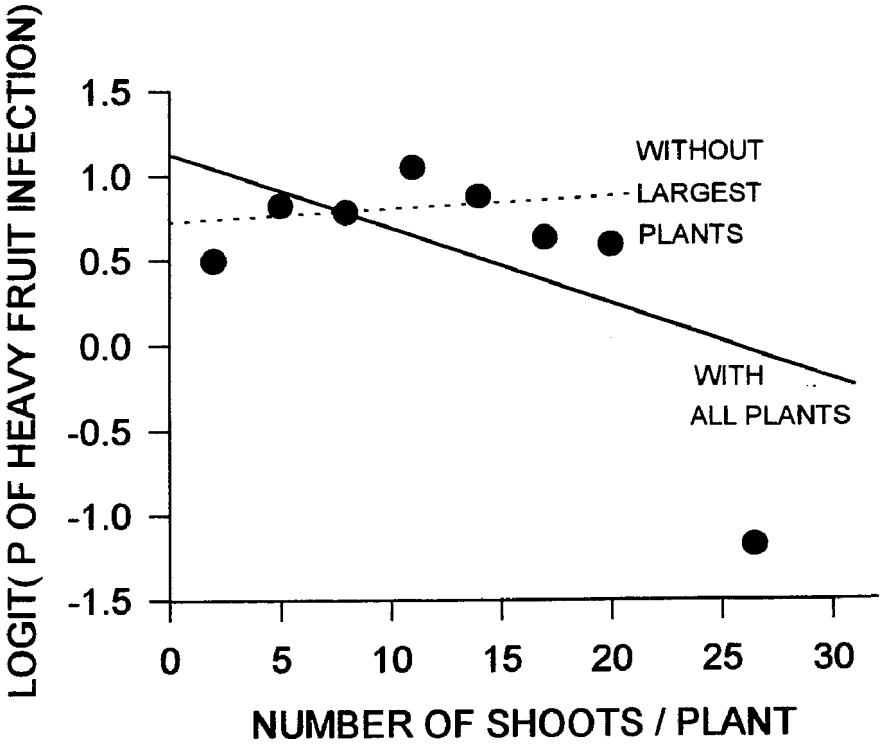

LOGIT(P OF HEAVY FRUIT INFECTION)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
0
5
10
15
20
25
30
NUMBER OF SHOOTS / PLANT
WITHOUT
LARGEST
PLANTS
WITH
ALL PLANTS

Figure 8. For the infected plants the percentage of plants with at least one uninfected shoot among (A) the six blocks (n = 20, 24, 23, 24, 23, and 24, for blocks 1-6) and (B) the four plant clones (n = 26, 31, 58, and 23 for clones A-D). (C) Backtransformed means and 95% confidence limits of the number of shoots per plant (n = 27, 32, 59, and 23 for clones A-D).

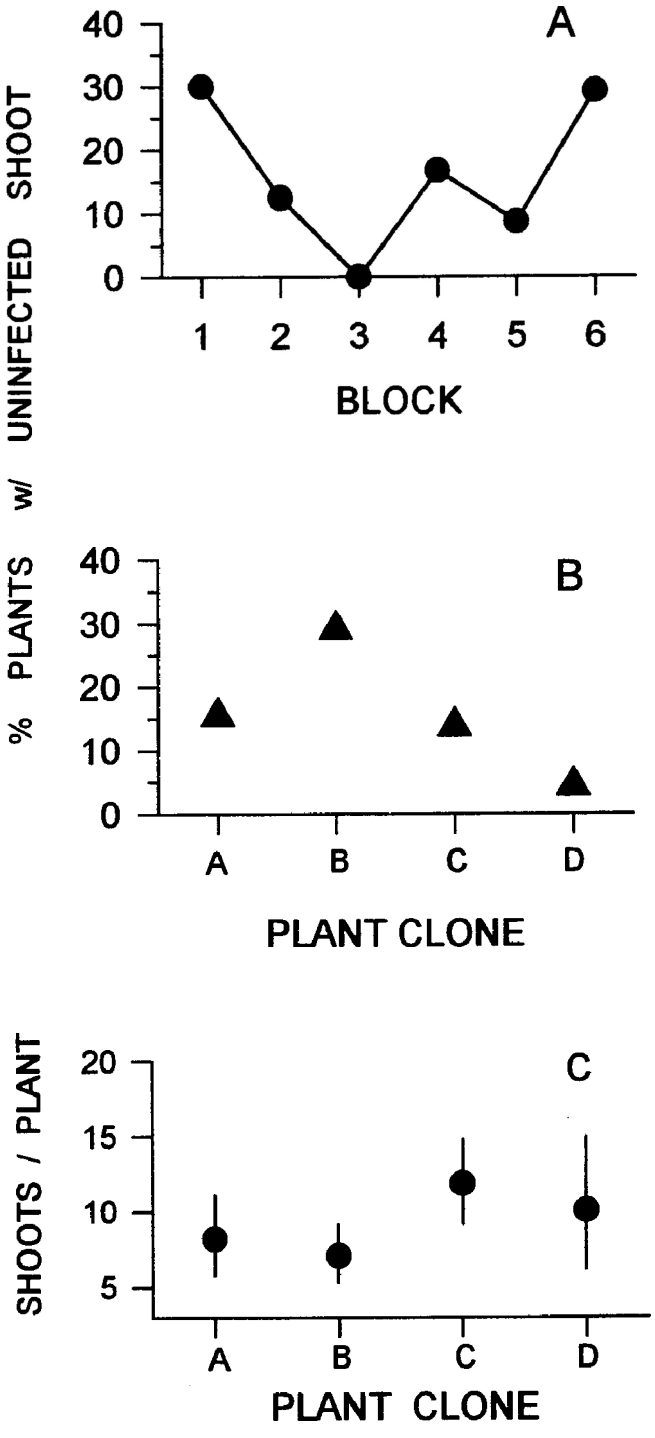
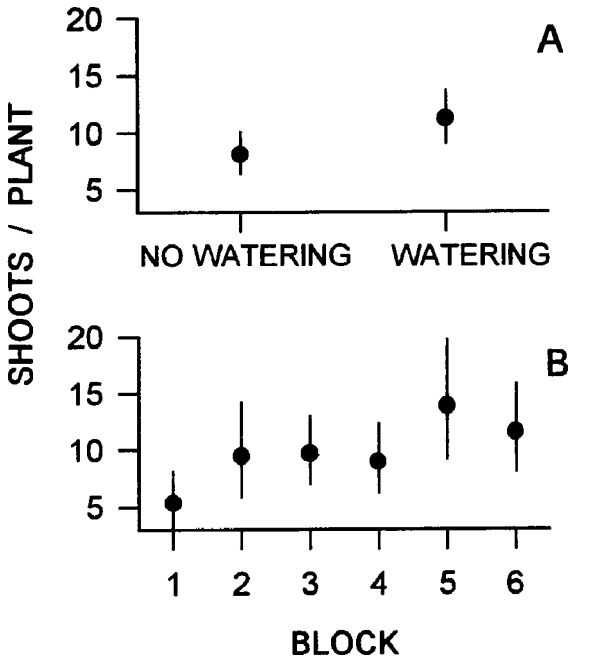
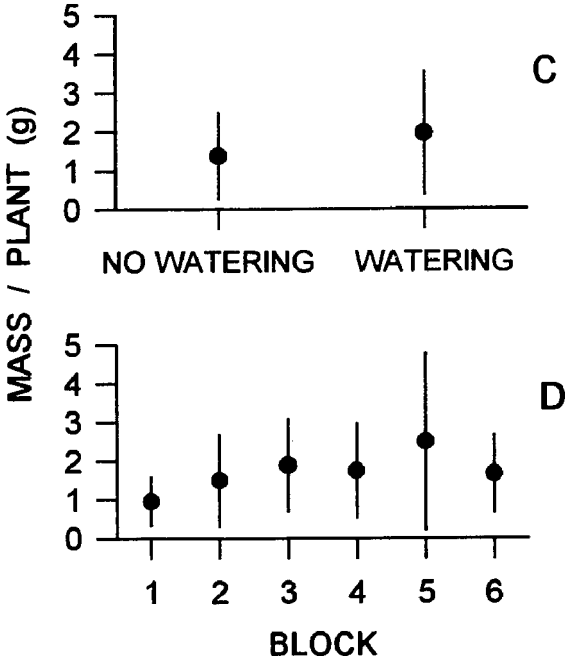

A
40
30
20
10
0
1
2
3
4
5
6
BLOCK
% PLANTS w/ UNINFECTED SHOOT
B
40
30
20
10
0
A
B
C
D
PLANT CLONE
SHOOTS / PLANT
C
20
15
10
5
A
B
C
D
PLANT CLONE

Figure 9. For the infected plants, transformed means and 95% confidence limits of (A) the number of shoots per plant in the watered and unwatered plots (n = 68 and 73, respectively); (B) the number of shoots per plant in blocks 1-6 (n = 22, 24, 23, 24, 23, and 25, respectively); Means and standard deviations for aboveground dry mass per plant in the (C) watered and unwatered plots (n = 58 and 76, respectively); (D) aboveground dry mass per plant in blocks 1-6 (n = 22, 24, 12, 24, 25, and 27, respectively).


A
B
SHOOTS / PLANT
20
15
10
5
NO WATERING
WATERING
1
2
3
4
5
6
BLOCK


C
D
MASS / PLANT (g)
5
4
3
2
1
0
NO WATERING
WATERING
1
2
3
4
5
6
BLOCK

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