

Incidence and Patterns of Association of Pathogens in a Leaf Spot Disease Complex on White Clover in the Piedmont Region of North Carolina

Scot C. Nelson and C. Lee Campbell

Graduate research assistant and professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh. This research was supported by funds provided by the North Carolina Agricultural Research Service, Raleigh.

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ABSTRACT

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Incidence of pathogen species and their patterns of association in a leaf spot disease complex on white clover (*Trifolium repens*) were observed in plots and adjacent areas in a pasture during 1990-1991 and in a monthly (July-October) survey of commercial pastures during 1991. Leaf samples from seven pastures in five counties (Chatham, Franklin, Nash, Orange, and Wake) in the Piedmont region of North Carolina and one county in western North Carolina (Yancey) were examined. The presence or absence of pathogens was confirmed by diagnostic, visual symptoms and by observation of pathogen reproductive structures on leaves. The most prevalent diseases were black spot (*Pseudomonas andropogonis*), *Cercospora* leaf spot (*Cercospora zebrina*), and summer blight (*Rhizoctonia solani*). Certain diseases were conspicuous in certain sites or months, e.g., *Curvularia* leaf spot (*Curvularia trifolii*) in Wake County, *Cercospora*

leaf spot in Nash and Orange counties, and pepper spot (*Leptosphaerulina trifolii*) in October. Other diseases observed were *Stagonospora* leaf spot (*Stagonospora meliloti*), sooty blotch (*Polythrincium trifolii*), anthracnose (*Colletotrichum trifolii*), rust (*Uromyces* sp.), and *Pseudopeziza* leaf spot (*Pseudopeziza* sp.). The leaf spot disease complex comprised 10 pathogens at the regional level (Piedmont), five to eight pathogens within single pastures, two to five pathogens at the plant level, and generally one to two pathogens on a particular leaf. The results of a test for interspecific associations showed there was a significant net negative association among pathogen species at the leaf level. A test for pairwise pathogen associations indicated many negative associations and few positive associations at the leaf level.

The occurrence and associations of pathogen species are of central importance in the ecology of host-pathogen interactions in complex pathosystems, i.e., those with multiple pathogens on a single or multiple hosts. Within such pathosystems, biotic and abiotic factors influence the distribution and abundance of pathogen species. Subsequently, patterns of association result from interrelationships among organisms and from environmental factors. These patterns depend on whether or not organisms select or avoid the same habitat, have some mutual attraction or repulsion, or have no interaction.

Organisms that have similar patterns of resource usage have a high degree of "niche overlap" (16). Thus, pathogen species (e.g., foliar pathogens) in competition for a single resource (e.g., a leaf) tend to occupy the same niche. Such niche overlap generates affinity (or lack of affinity) for coexistence among species, known as interspecific association. Interspecific associations are of epidemiological interest, because they reflect spatial and temporal attributes of species diversity (24).

Species diversity and spatial scale of association may be important factors related to pathogen survival or occurrence and may help to understand factors important in the dynamics of epidemics. For example, pathogens that tend to co-occur at a regional or field level may not occur together at a plant or leaf level (due to exclusion or competition, for instance). Alternatively, pathogens with highly specialized niche requirements, i.e., a specific leaf wetness duration for infection that is satisfied primarily in a shaded, edge area of a pasture, may tend to be associated at the leaf or plant scale within specific areas. Thus, patterns of association and occurrence at different levels of spatial or temporal resolution may reflect important ecological and epidemiological information about pathogen species and diseases.

Statistical methods are available for testing whether an association between two species exists and for testing simultaneously whether species in a group are associated (16,24,25). One multiple-species technique, for example, compares the observed variance in total number of species (or individuals) in samples with the variance expected under the null hypothesis that density or occurrence of each species is independent of the others (25). This variance comparison technique for detecting overall associations has been applied to the ecology of arthropod and vertebrate species (16,25).

The ecology of the leaf spot pathosystem on white clover (*Trifolium repens* L.) within legume-grass pastures is extraordinarily diverse. Although white clover is an important perennial pasture species, it is subject to decline as a result of biological and climatic factors (11,13,14). The many leaf-spotting organisms that commonly attack white clover are potentially important components of white clover decline (8,9,15). Effective management of this complicated pathosystem resides in a better understanding of interrelationships among organisms and of species occurrence and diversity in the leaf spot complex.

There are few contemporary data on the occurrence of, and associations among, foliar pathogens of white clover in the United States. The concept of a community of pathogen species existing in a seasonal succession was developed in a synecological study of white clover pathogens in Alabama in 1953 (8,9). During a 12-mo period, species of *Alternaria*, *Cercospora*, *Colletotrichum*, *Curvularia*, *Sclerotinia*, *Stagonospora*, and *Stemphylium* were recovered from diseased leaves of white clover. Three groups of species were identified that reflect temperature-dependent, seasonal trends of pathogen occurrence, i.e., a high-temperature group (*Curvularia* and *Colletotrichum*), a low-temperature group (*Leptosphaerulina*, *Sclerotinia*, and *Stagonospora*), and an all-year group (*Colletotrichum* and *Stemphylium*). In North Carolina, a survey of pastures for diseases of forage legumes was

conducted in 1949; however, only species of *Curvularia*, *Leptosphaerulina*, and *Sclerotinia* were reported as pathogens of white clover (1).

In this paper we report on the prevalence, abundance, and species diversity of pathogens that have a role in leaf spot epidemics on white clover throughout the Piedmont region of North Carolina. Additional objectives of the research were to detect the presence or absence of overall associations among pathogen species, to identify associations among specific pairs of pathogen species, and to measure the relative strength of these associations.

MATERIALS AND METHODS

Establishment of experimental plots. Experiments were conducted in a 10-ha white clover/tall fescue (*Festuca arundinaceae* Schreb.) grass pasture (hereafter designated NCSU) grazed by dairy cattle in Wake County at the Unit 2 Forage Research Facility of North Carolina State University during 1990–1991. The pasture was established in 1967 on cleared forest land. Soil texture ranged from loamy sand to sandy loam.

Four plots were established in arbitrary locations representing dissimilar micro-environments within the pasture. Ten weeks before transplanting, plants within plots were sprayed with a broadleaf herbicide mixture (2,4-D, Dicamba, MCPP, approximately 5.2 ml a.i./L) until leaf runoff to eradicate existing weeds and clover from plot interiors and areas immediately surrounding plant lattices (Fig. 1). Plots consisted of two proximal, eight row by eight column lattices of 64 10-wk old transplants each of either the Southern Regional Virus Resistant germplasm, SRVR (10), or of the cultivar Regal (Fig. 1). Clover plant lattices were placed within the existing stand of tall fescue. Lattice dimension was 10 × 10 m, with plants on 1.25-m centers. Host genotype was assigned randomly to one of two lattice positions within each plot. Plots were enclosed by an electric fence (Gallagher Mini Strip Grazer, Gallagher Electronics Ltd., Hamilton, New Zealand) to prevent bovine interference. Plot orientation (long axis) was either north-south or east-west (two plots for each orientation).

Seeds of SRVR and Regal were inoculated with a compatible *Rhizobium* sp. and sown in 5-cm diameter (180 cm³ volume)

peat pots containing a pasteurized, 1:1 (vol:vol) mixture of washed river sand and Terra-Lite Metro Mix potting mixture (W. R. Grace and Co., Cambridge, MA). After being thinned to one per pot, plants were grown for 10–12 wk in a greenhouse with standard insect control.

One week before white clover was transplanted, fescue plot areas were harvested (to approximately 8-cm height) with a flail-chop harvester (Carter Manufacturing Co., Inc., Brookston, IN). Transplanting occurred on 4 May in 1990 and on 15 April in 1991. In 1990, holes (24-cm wide × 0.5-m deep) for transplants were drilled at lattice coordinates with a tractor-mounted auger. In 1991, shovels were used to prepare planting holes manually. Peat pots were not removed from plants for transplanting. The same planting positions were used in 1990 and 1991. Relative lattice positions for SRVR and Regal within plots were reversed between years. Transplants were allowed to grow for 4–5 wk, after which plot areas were harvested again. Subsequently, monitoring of the first growth period began 10–14 days after the harvest. Each growth period was 6 wk long. The first of the two consecutive 6-wk growth periods began on 25 June 1990 and 10 June 1991, respectively. Tall fescue and clover were harvested at the termination of growth period 1; harvested material was removed from plots and discarded. Monitoring of growth period 2 began 10–14 days after harvest at the end of growth period 1.

Because a severe epidemic of *Cercospora* leaf spot was observed in September 1989 on white clover grown for seed at a location near the experimental plots, the study in 1990 was designed with the assumption that *Cercospora* leaf spot would be the most prevalent, if not the only, disease in the pasture. Similarly, a prevalent leaf spot disease (with lesions similar in size, shape, and color to *Cercospora* leaf spot) was identified presumptively in the pasture in 1989 as *Cercospora* leaf spot. However, by early to mid-July 1990, this working assumption became inoperable as the disease in the pasture was identified correctly as black spot (*Pseudomonas andropogonis* (Smith) Stapp.) of white clover, the first report of this disease in North Carolina (20). Several other white clover leaf diseases also became prevalent in the pasture in 1990 (21). Therefore, beginning in late July 1990, incidence of foliar diseases caused by fungi and one bacterial

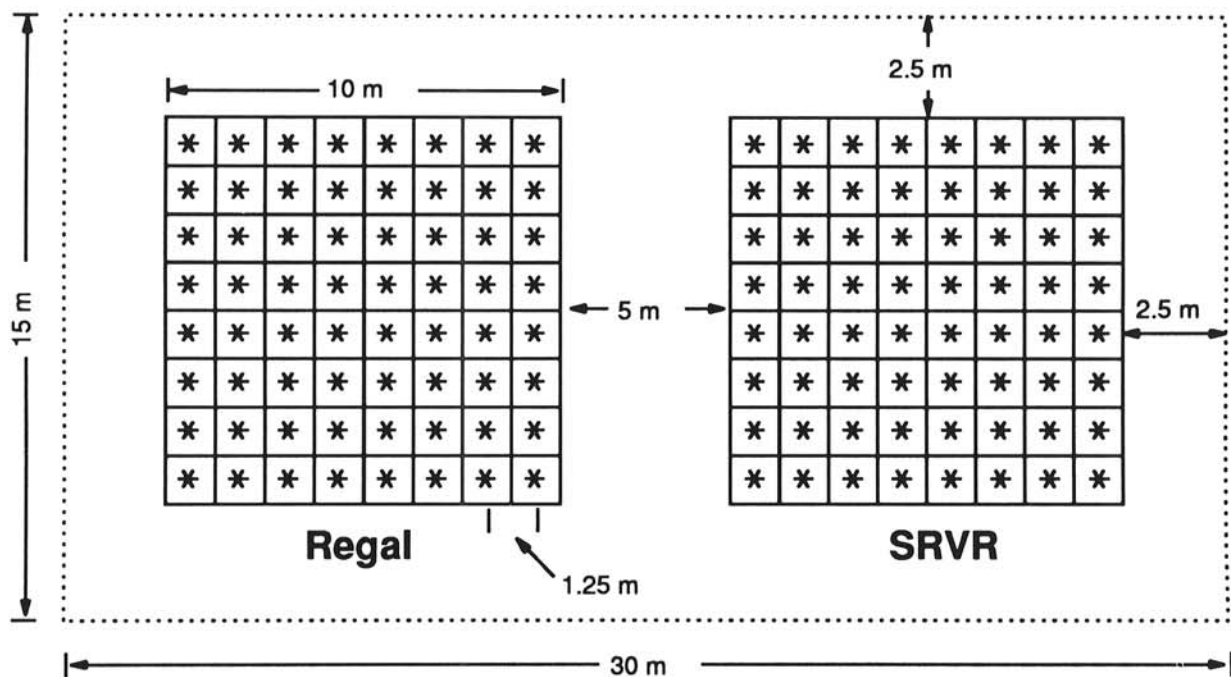


Fig. 1. Plot plan for experiments conducted in a 10-ha white clover/tall fescue grass pasture at the Unit 2 Forage Research Facility at the North Carolina State University near Raleigh in 1990 and 1991. Proximal 8 × 8 lattices contained 64 white clover plants of either Southern Regional Virus Resistant germplasm or cultivar Regal with tall fescue grass between plants. Lattice dimension was 10 × 10 m, with plants (denoted by asterisks) on 1.25-m centers. An unplanted area 2.5-m wide extended from lattice perimeter. Dotted line surrounding plot area represents electric fence used to prevent bovine interference.

pathogen was assessed biweekly on each of 512 plants (64 plants \times 8 lattices = 512). Virtually each leaf on every plant was examined in situ for pathogen incidence. The presence or absence of pathogen was confirmed via recognition of diagnostic symptoms or periodic, destructive sampling to allow microscopic inspection (40 \times) of leaves for pathogen reproductive structures. Pathogens were: *Cercospora zebrina* Pass. (*Cercospora* leaf spot), *Colletotrichum trifolii* Bain (anthracnose), *Rhizoctonia solani* Kühn (summer blight), *Stagonospora meliloti* (Lasch) Petr. (*Stagonospora* leaf spot), and *P. andropogonis* (black spot). Late in the 1990 growing season and during the 1991 season, additional pathogen species were identified and monitored: *Curvularia trifolii* (Kauffm.) Boedijn (*Curvularia* leaf spot), *Leptosphaerulina trifolii* (Rost.) Petr. (pepper spot), *Polythrincium trifolii* Kunze in J. C. Schmidt & Kunze (sooty blotch), and a *Uromyces* sp. (rust). During 1991, incidence of leaf-spotting organisms was assessed for each plant on a weekly basis.

On two dates in 1991 (23 July and 4 September) pathogen incidence and associations on a per leaf basis were assessed within three of the four plots and in pasture areas adjacent to the plots. One plot was omitted because of a lack of sufficient white clover plants outside the plot. Plants were sampled systematically for pathogen incidence. Within plots, five leaves were selected from each of the 512 plants by the following method. Plants were taken to approximate circles. Four points, equidistant from each other, were identified arbitrarily along a concentric, circular path approximately one-third the distance from the circumference (perimeter) towards the center of each plant. Four leaves were sampled by selecting the diseased leaf nearest to each of the four points. A fifth leaf per plant was selected as the diseased leaf nearest the center of the plant. In areas adjacent to plots, approximately 350 symptomatic leaves were collected from a total of 36 points equidistant along a zigzag path circumscribing the plot and extending approximately 20 m from plot perimeter. At each point, approximately 10 putatively diseased leaves were selected arbitrarily from a circular area about each point. Leaf specimens for each plot (350) were bulked in a plastic bag and mixed before pathogen incidence was assessed. Pathogen presence was confirmed by visual recognition of diagnostic signs and/or symptoms on the first 300 leaves removed arbitrarily from each bulked sample.

Pathogen survey. A survey of pastures for leaf spot pathogens of white clover was conducted in 1991. A total of eight white clover/tall fescue grass pastures were selected arbitrarily from five counties (Chatham, Franklin, Nash, Orange, and Wake) in the Piedmont region of North Carolina and one county (Yancey) in the mountainous region of western North Carolina; two pastures each were surveyed in Chatham and Orange counties. Samples from the Piedmont counties were collected 12–19 July, 8–13 August, 10–12 September, and 8–10 October. The pasture in Yancey County was sampled 19 July. Samples consisted of 300–400 putatively diseased leaves collected from circular areas surrounding sites (10–12 leaves per site) separated by 10–30 m along a W-shaped path through the pasture. Refrigerated leaf samples were bulked and mixed prior to examination within 48 h or for later inspection following pressing and drying of leaves at 45 C for 12–18 h. Pathogen presence was established by visual recognition of diagnostic signs and/or disease symptoms and microscopic examination of leaves for pathogen reproductive structures.

Data analysis. Interspecific associations were detected by analysis of pathogen presence/absence data with microcomputer software that performs tests (multiple-species case, two-species case) of interspecific associations and calculates several indices of relative strength of association (16). During the survey of Piedmont counties, presence/absence data for eight pathogens (*C. zebrina*, *Colletotrichum trifolii*, *Curvularia trifolii*, *L. trifolii*, *P. trifolii*, *P. andropogonis*, *R. solani*, and *S. meliloti*) for each date and pasture were analyzed first for overall multiple-species association. Pairwise tests of association for all species were conducted subsequently and supplemented by an index of relative strength of association (22). For the experimental plots, tests were

applied at the whole plant level and the leaf level. No attempt was made to identify individual plants during the survey of Piedmont pastures and tests for association were applied at the pasture level and at the leaf level.

Multiple-species associations. The test for interspecific association (multiple-species case) uses a variance ratio (*VR*) derived from a null association model to test simultaneously for significant associations (25). The null hypothesis is that there is no association among the species. This is true under two conditions: 1) the species are independent and 2) positive and negative associations between species cancel each other out (25). The alternative hypothesis is that there is a net positive or negative association among species.

The test for overall multiple-species association proceeds such that the total sample variance (σ^2) for the occurrence of *S* species in *N* sampling units is first calculated:

$$\sigma^2 = \sum_{i=1}^S p_i (1 - p_i) \quad (1)$$

where *S* = number of species (1...*S*), and $p_i = n_i/N$, wherein n_i = number of sampling units in which species *i* occurs ($i = 1$ to *S*) and *N* = number of sampling units, e.g., pastures, plants or leaves (1...*N*). Next, the variance in total species number (S^2) is calculated:

$$S^2 = N^{-1} \sum_{j=1}^N (T_j - t)^2 \quad (2)$$

where T_j = number of species found in each sampling unit ($j = 1$ to *N*), and t = mean number of species per sample. The variance ratio (*VR*)

$$VR = S^2/\sigma^2 \quad (3)$$

serves as an index of overall association. The expected value of *VR* under the null hypothesis is 1. A value of $VR > 1$ suggests net positive association among species. A value of $VR < 1$ suggests net negative association among species.

A statistic, *W*, which approximates a chi-square distribution, is computed that may be used to test whether deviations from 1 are significant.

$$\chi^2_{.05,N} < W < \chi^2_{.95,N} \quad (4)$$

where $W = (N)(VR)$. For example, if the species are not associated there is a 90% probability that *W* lies between the limits given by the chi-square distribution in equation 4.

Pairwise associations. As pointed out by Ludwig and Reynolds (16), the following test for pairwise interspecific associations is adequate for a single pair of species. When the number of species is greater than two ($S > 2$), the pairwise technique is inadequate, due to the fact that the pairwise comparisons would not be independent. Thus, one could not assign a probability to the distribution of the outcomes. However, the test for pairwise comparisons was applied to the pathogen species in the leaf spot assemblage for the purpose of providing insight into pathogen association and ecology, and as a vehicle for generating future hypotheses.

For each pair of pathogen species, *X* and *Y*, the following statistics were computed: *a* = the number of sampling units where both species occur; *b* = the number of sampling units where only species *X* occurs; *c* = the number of sampling units where only species *Y* occurs; *d* = the number of sampling units where neither *X* nor *Y* is found; *N* = the total number of sampling units ($N = a + b + c + d$). The null hypothesis is that the species are independent (i.e., there is no association). A chi-square statistic is used to test the null hypothesis of independence:

$$\chi^2_t = N(ad - bc)^2/mnr \quad (5)$$

where $m = a + b$, $n = c + d$, $r = a + c$ and $s = b + d$. The theoretical chi-square value for 1 df at the 5% probability level is 3.84. If $\chi^2_t > 3.84$, the null hypothesis that the co-occurrence

of species *X* and *Y* is independent is rejected; the alternative hypothesis is the species are associated. In this test, there are two possible types of associations. A positive association indicates that the pair of species occurred together more often than expected if independent [(the observed $a > E(a)$]. A negative association indicates the pair of species occurred together less often than expected if independent [(observed $a < E(a)$]. Ludwig and Reynolds present a complete discussion of this procedure, and of the use of Yate's correction formula when the chi-square test statistic is biased due to low frequency of species occurrence (16).

A third component of the study of species association was a measure of the degree or strength of the association. One effective index, the Ochiai index (OI) (22), measures the number of joint occurrences of the two species compared to the total occurrences of species *X* and *Y*, respectively. The Ochiai index is based on the geometric mean of a/m and a/r , and is equal to 0 at "no association" and 1 at "maximum association" (22):

$$OI = a / [(a + b)^{1/2} (a + c)^{1/2}] \quad (6)$$

RESULTS

Pathogen incidence in plots and adjacent pasture areas.

C. zebrina, *Colletotrichum trifolii*, *Curvularia trifolii*, *L. trifolii*, *P. andropogonis*, *P. trifolii*, *R. solani*, *S. meliloti*, and a *Uromyces* sp. were observed on plants during 1990–1991. Black spot (*P. andropogonis*) and summer blight (*R. solani*) were the most

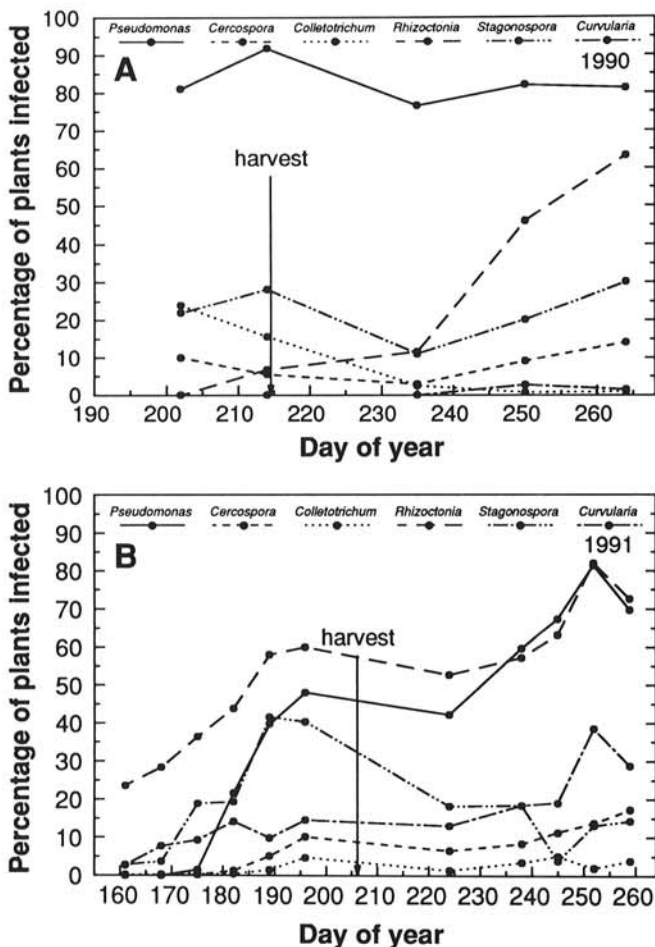


Fig. 2. Percentage of white clover plants infected with each of six pathogen species over time in A, 1990 and B, 1991 in experimental plots at the Unit 2 Forage Research Facility, North Carolina State University. Data points represent combined means of two treatments (germplasm) over four plots (512 plants). Harvest arrows indicate the end of the first of two 6-wk growth periods. Assessment based on visual recognition of diagnostic symptoms and microscopic examination for pathogen reproductive structures.

prevalent and destructive diseases (Fig. 2A,B). For the entire 1990 leaf spot epidemic and by the end of 1991, incidence of plants infected by *P. andropogonis* reached 80 percent. Warm weather and over 18 cm of rain in May 1990 favored the early occurrence and rapid spread of black spot in 1990.

The principal host for *R. solani* at NCSU was tall fescue. Symptoms of brown patch of tall fescue were evident throughout the pasture. White clover foliage in immediate proximity or in physical contact with *R. solani*-infected leaves of tall fescue was colonized rapidly by the fungus. Leaves of white clover had a water-soaked appearance during the early stages of infection. Later, white, cottony mycelia were conspicuous on the leaf surface. *R. solani* caused petiole collapse or complete leaf blight on white clover within 48 h of initial observation of symptoms.

S. meliloti infected a maximum of 30–40% of the 512 plants during 1990–1991. The disease was most important from June to July (day of year 152–213) and in September (day of year 244–274) (Fig. 2A,B). Cercospora leaf spot was a minor component of the disease complex during 1990 and 1991. Diseases of lesser incidence (although severe in some plots or on certain plants) that were observed in 1990 or 1991 included anthracnose, Curvularia leaf spot, sooty blotch, rust, and pepper spot. Curvularia leaf spot became established on a few plants in plot 2 in late August 1990. *Curvularia trifolii* was found throughout the pasture in 1991. However, Curvularia leaf spot was extremely severe on some plants in 1991, causing the defoliation of hundreds of leaves on certain plants.

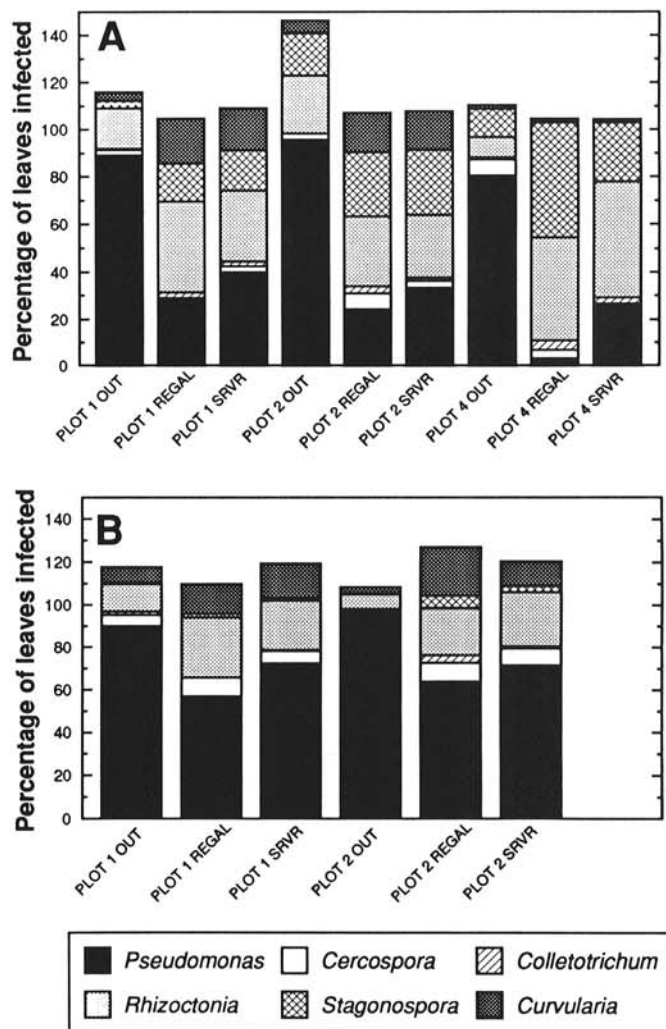


Fig. 3. Percentage of white clover leaves (selected from inside and outside plots at the Unit 2 Forage Research Facility, North Carolina State University in Wake Co.) infected by each of six pathogen species A, 23 July 1991, and B, 4 September 1991. Total percentage may exceed 100 because multiple pathogens were present on some leaves.

Examination of samples taken from within plots and in adjacent pasture areas on 23 July and 4 September 1991 indicated that, on a per leaf basis, the leaf spot complex comprised the same group of organisms both within and outside the plots (Fig. 3A,B). Incidence of bacterial leaf spot in the grazed regions surrounding the plots was significantly greater than within plots. Incidence of summer blight and *Stagonospora* were generally greater inside plots than in adjacent, grazed pasture areas.

Pathogen incidence in seven pastures. The nine pathogens observed in the experimental plots during 1990–1991 also were observed in samples from pastures in the Piedmont region. In addition, a *Pseudopeziza* sp. was detected on one leaf in the pasture

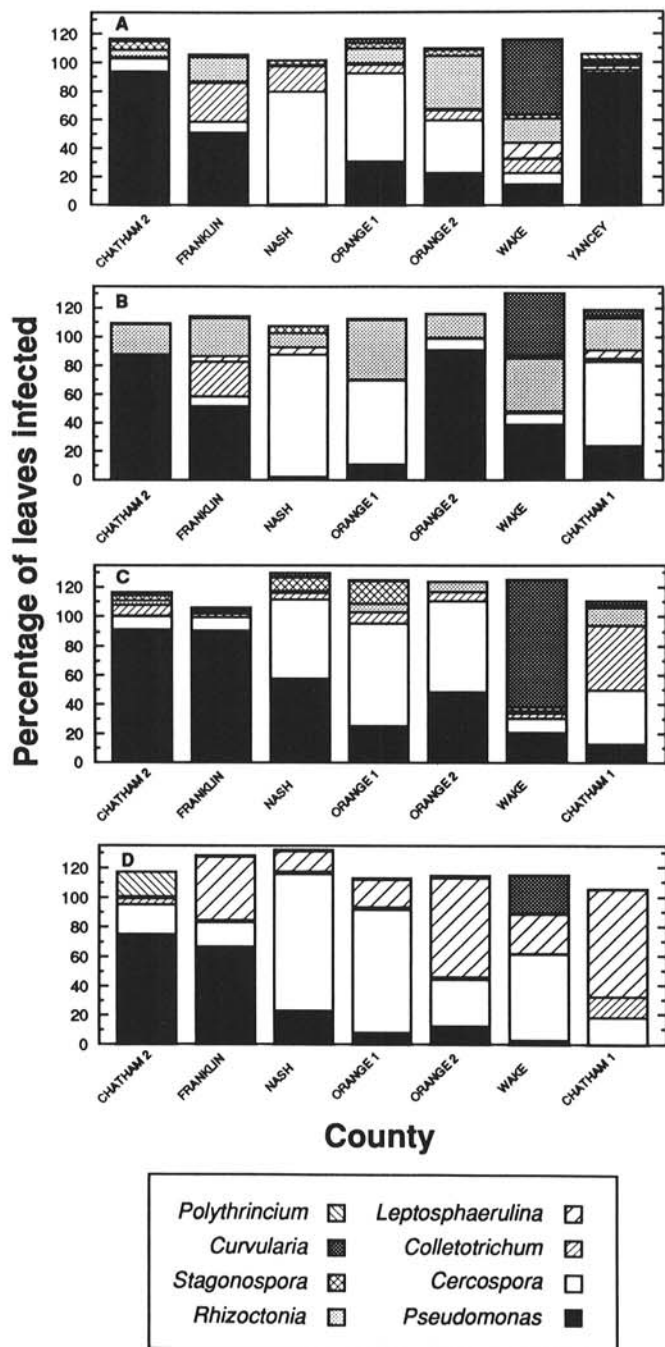


Fig. 4. Percentage of white clover leaves infected with each of eight pathogen genera in samples of six counties from the Piedmont region of North Carolina in 1991. A, 12–19 July; B, 8–13 August; C, 10–12 September; and, D, 8–10 October. Relative proportion of multiple infections (leaf infected by more than one pathogen) is reflected in the height of individual bars. Assessment based on visual recognition of diagnostic symptoms and microscopic examination for pathogen reproductive structures.

designated Orange 1 in July 1991. From five to eight pathogens were found in each pasture. All 10 pathogens were not found in any single pasture. Black spot (*P. andropogonis*), *Cercospora* leaf spot (*C. zebrina*), and summer blight (*R. solani*) were the most prevalent diseases (Fig. 4A–D). Black spot and *Cercospora* leaf spot were found in all pastures and months. Significant among-pasture variability was observed for incidence of specific pathogens and species diversity. In some pastures the disease complex was dominated by certain diseases (e.g., bacterial leaf spot in Chatham 2 and Yancey in July), whereas other pastures supported a more balanced population of pathogen genera (e.g., Wake in July).

Major shifts in species diversity and pathogen incidence were detected over time in most pastures (Fig. 4A–D). For example, in July 1991 in Orange 2 an approximate balance in disease incidence among black spot, *Cercospora*, and summer blight was detected, whereas in August 1991 in Orange 2 the complex was dominated by black spot. *Cercospora* leaf spot and black spot were also important diseases in Orange 2 in September, whereas pepper spot was the predominant disease in Orange 2 in October. In Wake County, incidence of *Curvularia* leaf spot was high throughout the season, and the disease was most abundant in early September. Incidence of pepper spot increased in all pastures from September to October.

Associations among pathogens in Piedmont counties. Relative proportions and numbers of multiple infections (infection of a single leaf by more than one pathogen) indicated temporal fluctuations and slight variability among pastures (Fig. 4A–D). The proportion of multiple infections relative to the total sample of 300 leaves for each pasture ranged from approximately 0.10 to 0.30. Most multiple infections (>99.0%) comprised only two pathogens, one of which was usually the predominant pathogen in the pasture. The greatest number of different pathogens observed on one leaf was four (e.g., *Colletotrichum*, *Pseudomonas*, *Rhizoctonia*, and *Stagonospora*).

Values of the test of multiple species interspecific association (conducted at the leaf level) were all significantly less than one, which indicated a net negative association among the pathogen genera in all pastures on each of the sampling dates (Fig. 5). Values for the variance ratio increased over time in several pastures (Franklin, Nash, Orange 1, and Wake). To indicate whether the net negative associations were due to either a balance of pairwise associations or primarily true negative associations among specific

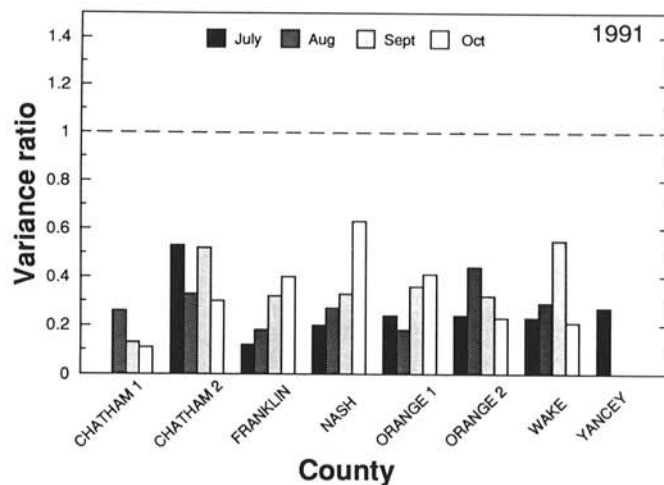


Fig. 5. Variance ratio (*VR*) from Schluter's test for overall interspecific association on pathogen incidence data from white clover leaves collected over four monthly intervals in 1991 in eight pastures from six counties in North Carolina. Yancey Co. is in western North Carolina. Other counties are located in a geographic cluster in the Piedmont region. Dashed line at $VR = 1$ indicates no association among the eight foliar pathogen species. All values are less ($P < 0.05$) than 1 based on a chi-square test statistic, which indicates an overall net negative association at the leaf level among pathogen species. Values significantly greater than 1 would indicate a net positive association among pathogens.

pairs, the chi-square test was extended to individual pairs of pathogens (16). A collection of significant unbiased associations (with each of seven species involved) and relatively few positive associations were detected (Fig. 6). A large proportion of the pairwise, negative associations were due to interactions involving *P. andropogonis*, *C. zebrina*, and *R. solani*.

Ochiai's index of strength of associations (which attains a value of 1 at maximum association) indicated that most negative, pairwise associations were relatively weak (Fig. 6). Strongest negative associations were between *Cercospora-Leptosphaerulina* (0.183), *Cercospora-Pseudomonas* (0.172), and *Curvularia-Pseudomonas* (0.154). The few significant and unbiased positive associations that were detected involved *Pseudomonas* or *Leptosphaerulina*, and were relatively stronger than the negative associations.

Associations among pathogens in plots and associated pasture.

The pasture used for experimental plots was similar to some Piedmont counties pastures with regard to species diversity and pathogen incidence (Figs. 3A,B and 4A-D). For leaves examined in July and September 1991 from outside plots, *P. andropogonis* was the predominant pathogen, occurring on over 85% of the leaves sampled (Fig. 3). The proportion of multiple infections (0.19-0.47) was slightly greater than in the Piedmont counties (0.10-0.30). An unusually large number of multiple infections occurred outside plot 2 in July, and the number of multiple infections in the grazed pasture (outside the plots) was found to be generally greater than in the ungrazed pasture (within plots). Within plots, more leaves were found to be infected with *Curvularia trifolii*, *R. solani*, and *S. meliloti*. The incidence of both *S. meliloti* and *R. solani* declined both within and outside the plots from July-September. Incidence of black spot increased within plots from July-September. Values for the variance ratio for NCSU, outside plots (0.45-0.49) and within plots (0.04-0.10) indicated a significant overall, net negative association among pathogen species at the leaf level. No significant, positive associations among pairs of pathogens were detected. Negative associations found between pathogens were similar to associations

detected in Piedmont pastures with regard to nature and strength of association (data not shown).

The test for overall association among pathogens was conducted at the plant level in experimental plots at NCSU in 1990 and 1991 (Fig. 7A-C). In 1990 (Fig. 7A), few values of *VR* were significant. There was a trend for increasing *VR* after harvest in several plots in 1991. During growth period 1 during 1991 (Fig. 7B), 12 significant, net negative associations were detected. During growth period 2 in 1991, seven significant positive associations among pathogens were observed (Fig. 7C), and only one positive association was found during growth period 1 in 1991. Values for variance ratio and pairwise associations indicate that cases of "no association" were the result of both offsetting, significant positive and negative associations, and truly nonassociated pathogens.

DISCUSSION

The leaf spot complex on white clover in North Carolina is not static with regard to time or spatial scale. A flexible concept of the leaf spot disease complex that is adaptable to different levels of spatial resolution (i.e., from the regional level to the leaf level) is needed. In other words, 10 pathogen genera comprised the leaf spot complex in the Piedmont region; however, all 10 diseases were not detected in any single pasture. At the regional scale, three pathogens dominated the white clover leaf spot complex (*C. zebrina*, *P. andropogonis*, and *R. solani*). A 'typical' summertime pasture in the Piedmont region of North Carolina usually hosts five to six leaf spot pathogens. Many of these pathogens are capable of being the dominant, leaf spot pathogen in the pasture. As determined in our experimental plots, a diseased, white clover plant within a pasture hosted, on the average, two to five leaf spot pathogens. A leaf was infected normally with one or two pathogens, a figure consistent with the average number of pathogens per leaflet (1.4-1.8) in the alfalfa leaf spot pathosystem (6,7).

Two primary groups of three pathogens each were observed in our plots and in adjacent areas, based on the progress curves for disease incidence (Fig. 2B). One group, *Pseudomonas*, *Rhizoctonia*, and *Stagonospora*, is represented by relatively high incidence in both 1990 and 1991 (compared to other pathogens) and curves that are, on average, roughly sigmoidal in shape for both growth periods in 1991. Another group (*Cercospora*, *Colletotrichum*, and *Curvularia*) maintained a relatively constant, low level of incidence. Dispersal of inoculum from disease foci to proximal plants within lattices is likely to have been more prevalent for the first group. Different temperature and wetness requirements among pathogens, or variability in host resistance may account for differences in multiplication rates among pathogens. Alternatively, notable declines in incidence of some pathogens at the end of growth period 2 1991 are probably the result of defoliation and conditions less favorable to pathogen reproduction and dispersal (e.g., reduced temperature and moisture) (S. C. Nelson and C. L. Campbell, unpublished data) (Fig. 2).

The concept that a leaf spot complex may comprise two or more groups of pathogens that respond similarly to environmental conditions has been addressed in studies of the alfalfa leaf spot system in North Carolina (6,7) and the white clover pathosystem in Alabama (8). Using a measure of strength of associations among pathogen species (6,7), Duthie and Campbell concluded that pathogens in the alfalfa leaf spot complex appeared to form two distinct groups, one group consisting of *L. trifolii* (Rost.) Petrak and *Stemphylium botryosum* Wallr. and a second consisting of *C. medicaginis* and *Colletotrichum* spp. Within each group, pathogens were associated strongly and positively, whereas between groups, pathogens were associated weakly or negatively. A similar concept was advanced in a study of temperature-dependent, seasonal trends of fungi in a white clover leaf spot complex in Alabama (8). Thus, the concept of the leaf spot disease complex on forage legumes is complicated by the potential existence of ecologically related groups of pathogens.

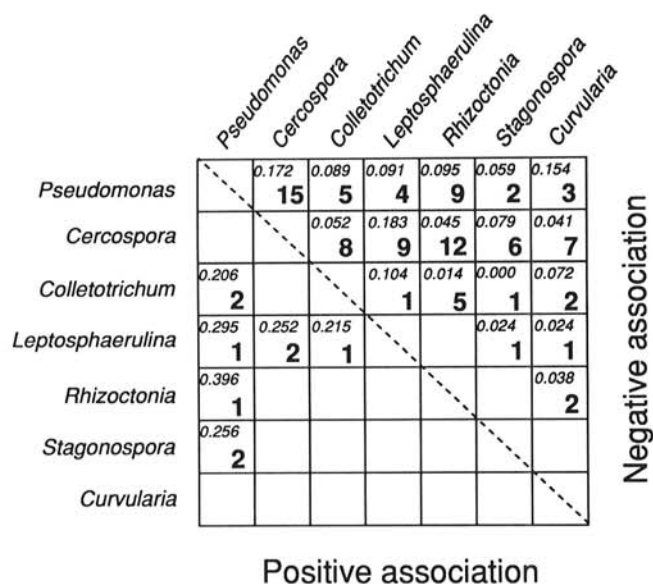


Fig. 6. Unbiased, significant pairwise positive and negative interspecific associations detected among seven genera of leaf spot pathogens observed on leaves of white clover collected over 4 mo in eight pastures from six counties in North Carolina in 1991. Large numbers in cells represent number of times (out of 28 possible samples) that a significant and unbiased association was detected. Italicized numbers in cells represent a mean value for Ochiai's index of relative strength of association (a value of one represents complete association) calculated from the significant associations. Empty cells indicate no (nil) association. Positive associations are arranged in the lower left of the matrix. Negative associations are located in the upper right portion of the matrix.

Interspecific associations among pathogens imply spatial relationships. Theoretically, if two pathogens each were distributed randomly throughout a plot, then there would be no association (no positive or negative association) between them. If the pathogens were associated positively, the spatial pattern of their occurrence should be similar. If the pathogens were associated negatively, their spatial occurrence should be different, if not unique. It was a simple matter to find evidence to support the above assumptions. A significant and unbiased positive association at the plant level was detected between *C. zebrina* and *Curvularia trifolii* in plot 2, SRVR, during 1991. Examination of the spatial pattern of diseased plants (Fig. 8A) reveals similarity between pathogens in cluster size, shape, and location of infected plants. In another plot at the same date, a positive association was significant between *P. andropogonis* and *R. solani*. Examination of the spatial pattern maps reveals a potentially significant

'edge effect' for both pathogens (Fig. 8). Similar clusters of diseased plants are evident in a comparison of the spatial pattern maps for black spot and summer blight (Fig. 8B). Clearly, ecological associations among species may have spatial analogs that reflect environmental and/or dispersal gradients.

The variability among pastures regarding species diversity and within/among dates regarding pathogen incidence in the Piedmont counties implies the potential existence of locally adapted pathogen biotypes or environmental conditions and circumstances particularly favorable to a specific pathogen. For example, the severe outbreak of *Curvularia* leaf spot in Wake Co. in July, August, and September is in stark contrast with the near absence of this disease in most other pastures. Although Allison (1) reported locally severe outbreaks of *Curvularia* leaf spot throughout North Carolina as early as 1949–1950, factors apparently exist that have limited the spread or adaptability of

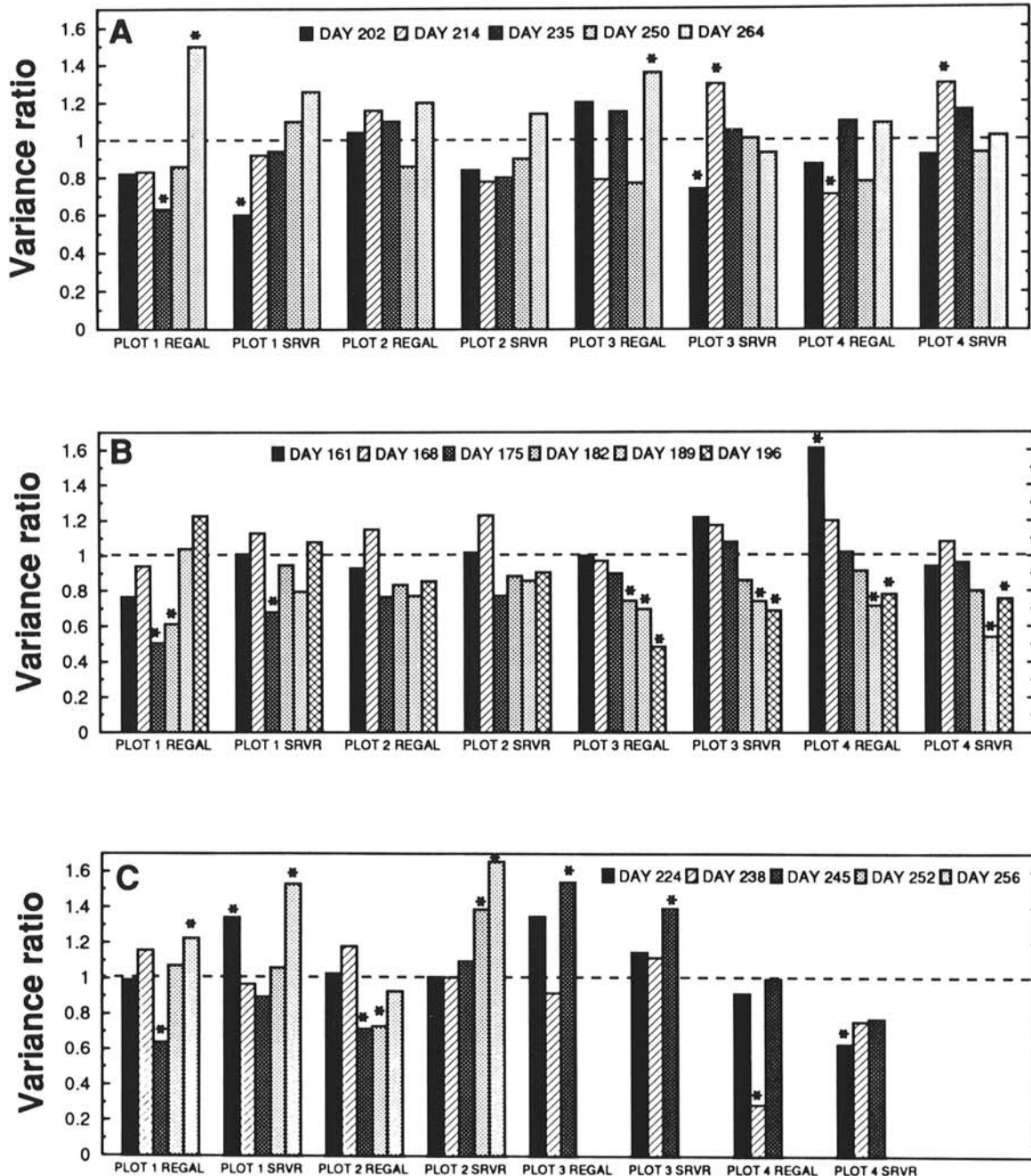


Fig. 7. Variance ratio (VR) from Schluter's test for overall interspecific association conducted on a per plant basis for incidence data on leaf spot pathogens on white clover in plots at the Unit 2 Forage Research Facility, North Carolina State University in Wake Co. Asterisks indicate values significantly ($P > 0.90$) greater or less than 1 (indicating overall positive and negative association, respectively) based on a chi-square test for association. Bars represent different assessment dates (day of year) for each plot. A, 1990. B, 1991, growth period 1. C, 1991, growth period 2.

Curvularia trifolii within North Carolina. Similarly, *Cercospora* leaf spot was severe, and *C. zebrina* presumably well-adapted to conditions at Nash and Orange 1. Variability among regional isolates of *C. zebrina* (19) and *L. trifolii* (23) from white clover in North Carolina with regard to virulence and cultural characteristics has been documented, lending plausibility to the existence of species biotypes in this region. Microclimates within pastures or regions may impact leaf spot species occurrence and diversity. Slope, aspect, shading, soil type, plant species, rainfall, and grazing effects should be examined experimentally to determine their effect(s) upon the local composition of the leaf spot assemblage.

Shifts in species occurrence and diversity over time may be attributed to the effects of bovine activity. Field notes from the pasture designated Orange 2 indicated that grazing activity increased significantly in this pasture between July and August. The rapid spread of *P. andropogonis* in this pasture between July and August (Fig. 4A,B) may have been influenced by the effect of grazing (wounding of plants, movement of cattle, and dispersal of bacteria). In the area adjacent to our experimental plots, black spot spread initially and rapidly through this grazed portion of the pasture. The marked differences between environments (grazed vs. ungrazed) regarding the incidence of black spot and other diseases (Fig. 3) lends credence to the hypothesis that grazing activity profoundly influences species diversity and pathogen associations in the leaf spot complex, especially when

wound-dependent pathogens (bacteria) or pathogens that thrive in dense canopies (e.g., *Rhizoctonia*) are present. A study of seasonal fluctuations in concentration of airborne conidia of *C. zebrina* and incidence of *Cercospora* leaf spot on subterranean clover (*Trifolium subterraneum* L.) in western Australia indicated that grazing interfered with disease development by removing much of the diseased material, thus preventing the development of severe epidemics (2).

Values from tests for interspecific association should be interpreted with caution and should be based on large sample sizes (16). The existence of a process may not be proved by the existence of a pattern. However, one advantage of using a measure of association based on presence/absence data is that once a mechanism of interaction is proposed or observed, one may assess or hypothesize an outcome of the interaction, without measuring the phenomenon (e.g., competition) directly. Thus, the primary utility of the variance ratio technique is to generate explanatory hypotheses.

The compelling indications of negative interspecific associations for leaf spot pathogens (overall and between specific pathogen pairs) at the leaf level may be used to generate a great number of explanatory hypotheses for the white clover leaf spot pathosystem. To explain why *L. trifolii* and *C. zebrina* are negatively associated (Fig. 6), we hypothesize that whereas the disease complex evolved as a whole, various component pathogen species evolved simultaneously so as to minimize co-occurrence (competition for space and resources) on a given leaf. This explanation is feasible for a host of rapid, indeterminate growth such as white clover and a pathosystem where available host tissue often exceeds the colonizing ability of the pathogen populations. Our observations in experimental plots during 1990–1991 indicate that *L. trifolii* is found most often on young and emerging leaves, whereas symptoms of *Cercospora* leaf spot usually appear on much older leaves. Thus, a likely explanation for negative associations between *L. trifolii* and *C. zebrina* centers about different niches associated with aging leaves of white clover. In the alfalfa-*Cercospora* system (4,5) and in the white sweet clover (*Melilotus alba* Desr.)-*Cercospora* system (12), older leaves are more susceptible to infection by *Cercospora* spp. than younger leaves. Younger alfalfa leaves are more prone to infection by *Leptosphaerulina* than are older leaves of alfalfa (15). Similarly, temperature optima for growth in vitro for *Cercospora* spp. (4,5,18) and *L. trifolii* (23) support the hypothesis that the negative associations observed between these two pathogens in our studies is the result of temperature-dependent shifts in pathogen populations. Pepper spot epidemics are favored by relatively cool, wet weather (14,17), whereas *Cercospora* leaf spot (also known as ‘summer blackstem’ on alfalfa) of forage legumes clover is favored by warmer temperature regimes (2,4,5).

R. solani was associated negatively with both *P. andropogonis* and *C. zebrina* (Fig. 6). Assuming that inoculum of both species is present simultaneously, a testable hypothesis would center about the mode of exclusion by *R. solani* that we observed in situ in our experimental plots during 1990–1991. A dramatically reduced incubation period and more rapid rate of leaf colonization for *R. solani* effectively exclude more slowly developing diseases. We observed the ability of summer blight to destroy a clover leaf within 48 h at NCSU. The incubation period for *C. zebrina* is approximately 4–5 days under optimum conditions (18,19). Therefore, *R. solani* can cause defoliation before symptoms of *Cercospora* leaf spot develop.

The white clover leaf spot pathosystem resembles a natural ecosystem in many ways. For example, we observed more than 40 plant species in the pasture where our experimental plots were located. Variability in the composition of the leaf spot complex likely reflects the great spatial and temporal variability among pastures with regard to host and pathogen populations, environmental conditions and cultural practices. Leaf spot is only a component of a more comprehensive biotic and abiotic disease-pest complex that includes root-infecting fungi, nematodes, numerous virus diseases and a number of important insects (3,11,14). This complex pathosystem presents unique and daunting

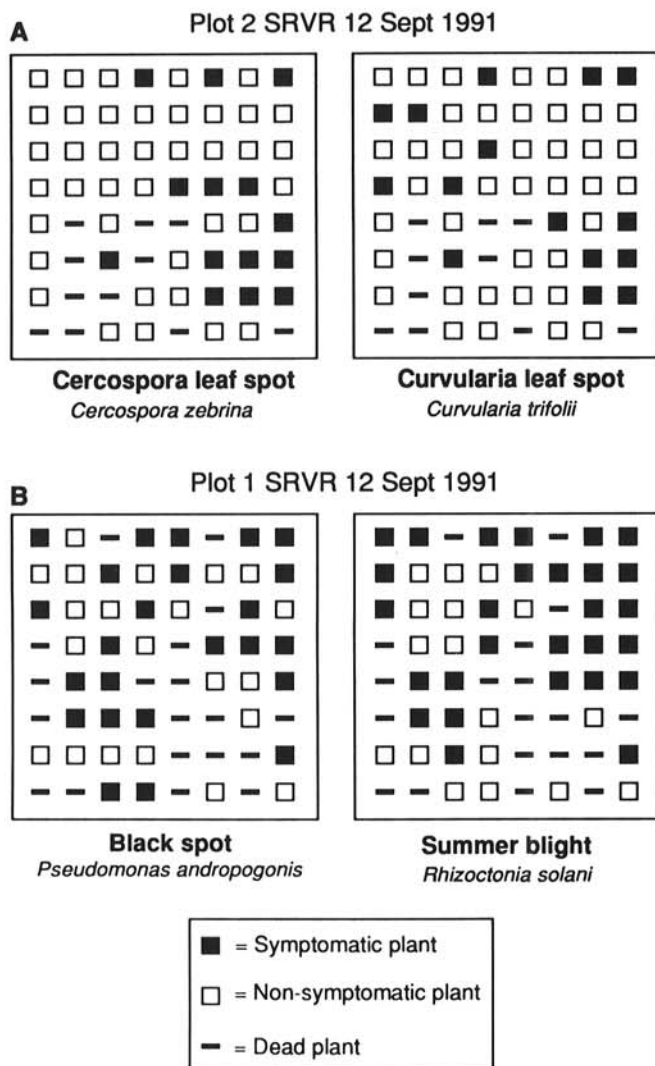


Fig. 8. Comparative spatial patterns of diseased white clover plants in eight row by eight column experimental plots in a pasture at North Carolina State University in Wake Co. A, *Cercospora* leaf spot and *Curvularia* leaf spot in Plot 2 (SRVR) on 12 September 1991. B, Black spot and summer blight in plot 1 (SRVR) on 12 September 1991.

challenges for the understanding, modeling, and management of the many diseases that occur on white clover within pastures.

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