Relationships of Infection by *Cercospora zebrina* to Seed Production and Seed Quality of Subterranean Clover

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ABSTRACT

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The potential for *Cercospora zebrina* to reduce seed production and affect seed quality of subterranean clover was evaluated in the field during four growing seasons. Cultivar Woogenellup was grown in plots from September to May of each year. Infection of foliage by *C. zebrina* was initiated by applying infested debris to plots in late winter. Control plots received autoclaved debris. Burrs (seed heads) were harvested each spring and counted. Seed were threshed, counted, weighed, measured volumetrically, and evaluated by a standard germination test. Over all years, plots infested with *C. zebrina* had 16–31% fewer burrs and 18–33% fewer seed than control plots (differences significant at $P < 0.01$) with no significant year × treatment interactions. Seed weight and size were reduced 6–24 and 5–21%, respectively, in infested plots as compared to controls, and year × treatment interactions were significant. Seed from control and infested plots did not differ significantly in frequencies of normal or abnormal germination, hard seed, embryo dormancy, or dead seed. Disease progress and flower production were compared during 2 yr. Rapid increase of foliar symptoms did not occur until 1–2 wk after peak flowering, and flowering was significantly reduced in infested plots only at those times. These results indicate that foliar infection by *C. zebrina* will significantly reduce seed production and seed quality of subterranean clover within or over years. It is unlikely, however, that the disease will eliminate seed production entirely during any one year for Woogenellup or earlier maturing cultivars when grown in climates similar to that of north Mississippi.

Additional keyword: Trifolium subterraneum.

Subterranean clover or subclover (*Trifolium subterraneum* L.) is an annual forage legume that is well adapted for use in grazing systems in the southeastern United States and in the Pacific Northwest (8). Subclover is grown in pastures to provide high-quality forage for grazing animals and to supply biologically fixed nitrogen for companion or subsequent grass crops. In the Southeast, subclover is sown or reseeds itself in late summer or fall, grows through the winter, and flowers and sets seed in the spring. New stands are reestablished in the fall by germination of seed produced on the same site. Production of adequate seed in the spring, therefore, is critical for successful reestablishment of subclover the following fall and in a continued annual cycle. Two unusual features of subclover that favor reseeding in grazed pastures are that stems grow procumbently over the ground, and that peduncles grow downward from stems after fertilization to bury developing seed heads in surface soil or thatch. This capacity for burial of seed accounts for the common name of the crop (8–10).

In 1979 and 1980, a new and severe Cercospora leaf and stem disease was discovered on subclover in Mississippi (11). The causal organism was identified as a new form of *Cercospora zebrina* Pass. that was highly virulent on subclover and largely specialized in pathogenicity to this host. Symptoms consisted of brown necrotic lesions on leaflets that often coalesced, girdling lesions on petioles that caused collapse of leaves, and elongated nongirdling lesions on stems. Of 15 other forage legume species and varieties tested, only rose clover (*Trifolium hirtum* L.) also developed severe symptoms after inoculation. Most cultivars of subclover were highly susceptible to the disease, but a few were resistant (11).

In 1980, a Cercospora disease was observed for the first time on subclover in western Australia (7). Although this pathogen also was identified as *C. zebrina*, isolates reportedly differed from those studied in Mississippi in temperatures optimal for pathogenesis and in their host range on other forage legumes (3). These observations suggest that different forms of the pathogen may be present in Australia and Mississippi.

Infection of subclover by *C. zebrina* in Mississippi is usually most evident with the advent of warm temperatures near the end of the spring growing season (11). Because flowering and seed development also occur at that time, it appeared that infection might reduce seed production or seed quality and thereby affect the ability of subclover to reestablish in the fall or to remain over years in a sward. Therefore, this study was undertaken to determine whether infection of foliage by *C. zebrina* affects the quantity or quality of seed produced by subclover in Mississippi. A preliminary report has been published (12).

MATERIALS AND METHODS

A similar experiment was established on a different site each year for 5 yr on land that had not supported subclover for at least 2 yr previously. Seed of cultivar Woogenellup were inoculated with compatible *Rhizobium* in a commercial peat mixture and planted in 1.49-m^2 plots (8 g seed per plot) on a prepared seedbed in September of each year. All plots were separated by 2.44-m alleys seeded to winter wheat (*Triticum aestivum* L.). Growth of the clover was initiated in September by rainfall or irrigation. In late February or early March of each year, plots were infested with dried debris collected from *Cercospora*-infected plants after their senescense in the field the previous spring. This debris originated from severely diseased plots of the experiment from the previous year and from naturally infected subclover stands. No consistent symptoms of other foliar diseases were observed in subclover from which debris was collected for inoculum. Debris from all sources was composited and mixed each year, air-dried for 3–4 mo in the greenhouse and laboratory, and coarsely ground in a Wiley mill to destroy burrs and remove seed. Approximately 300 g of debris was applied evenly to the top of foliage in each infested plot and brushed into the canopy by hand (11). Uninfested (control) plots received autoclaved debris. Infested and control treatments were applied to plots in a completely random design with five to 10

replicates each year.

Plants were observed weekly for disease symptoms and environmental damage throughout the winter and spring until senescense in May. Plots with significant damage caused by freezes or *Sclerotinia*, or control plots in which macroscopically visible foliar symptoms appeared before the onset of senescense (approximately mid-May) were deleted from the experiment each year. Plots were not mowed during the experiment to provide a thick canopy that would favor disease development.

After plants senesced completely in late May or early June, all debris was removed to ground level from a 0.25-m^2 sampling area in the center of each plot. Samples were air-dried for 1–2 wk on a greenhouse bench (25–38 C). Burrs were removed by hand from each sample of debris and counted. Seed were threshed by grinding burrs between a hand-held wood block and board lined with corrugated rubber. Seed from each sample were counted, weighed, and measured volumetrically.

Germination of seed was evaluated each year by a standard test (1) after storage for 3–4 mo at room temperature. Fifty seed were incubated between double-layer blotter paper in a 9-cm plate for 2 wk at 20 C with four replicate plates per sample (plot). After 4 days and 2 wk, frequencies of normal germination, abnormal germination, and dead seed were recorded (1). Seed swollen but not germinated at 2 wk were considered embryo dormant (10).

During 1986 and 1987, symptom development and flowering were monitored to compare their times of occurrence. Beginning in mid-March and continuing at biweekly intervals, a transect line was extended across the middle of each plot, and all flowers throughout the canopy within a zone 2.54 cm on each side of the line were counted. Simultaneously 10 fully expanded leaves were chosen randomly at mid-canopy height at approximately equidistant points along the line. Leaves were held in wet paper towels and examined for *Cercospora* lesions with a dissecting microscope at approximately 20× magnification. To verify the presence of the fungus in lesions, leaflets were incubated on water agar at 25–30 C for 24–48 hr under fluorescent light to induce sporulation.

Portions of seed collected in 1985 and 1987 were plated on agar to determine the incidence of *C. zebrina* in or on seeds. Seed were plated on Difco cornmeal agar, 20 per plate, four plates per source plot, after four treatments: no washing or surface disinfestation, 30-min wash in running tapwater, 1-min surface disinfestation in 1% NaOCl, and 5-min disinfestation in 1% NaOCl. Plates were incubated under 12-hr or continuous fluorescent light at 20–30 C for 7–11 days and examined periodically at 20 × for conidia or colonies typical of *C. zebrina*.

Data on the number of burrs and the number, weight, and size of seed were analyzed by the general linear models procedure of the SAS program (13). For each variable, a combined analysis was run with year, treatment, and replicate as experimental factors. Year× treatment interaction was used as an error term when it was significant. These data, and data on seed germination, also were compared separately for each year by analysis of variance.

RESULTS

In all plots that received infested debris, symptoms of foliar infection by *C. zebrina* became macroscopically visible and intensified from late April to early May of each year as previously observed (11). Some uninfested control plots developed symptoms during that period; these were deleted from experiments along with similar numbers of randomly chosen infested plots. Controls from which data were obtained remained free of symptoms until initial senescense as in the previous study (11) (Fig. 1). No symptoms suggestive of other diseases were observed more consistently in infested plots than in controls. Environmental conditions in late winter and spring appeared to be most favorable for growth of the clover and rapid development of disease during the 1982–83 and 1984–85 seasons. In 1985–86, disease development was less rapid because of a severe drought that occurred through late winter and spring, and in 1986–87 it was less rapid because of unusually cool temperatures that prevailed in early spring. In 1983–84, plots sustained unacceptable damage from a severe freeze in December, and the experiment was terminated.

Mean number of burrs and the mean number, size, and weight of seed from infested and control plots for 4 yr are given in Table 1 along with results of statistical analyses within years. Results of analyses over years are given in Table 2.

Infestation of plots with *C. zebrina* significantly reduced the numbers of burrs and seed in comparison to controls (uninfested plots) over all years without significant year × treatment interactions (Table 2). Decreases from control values were 16–31% for numbers of burrs and 18–33% for numbers of seeds in infested plots over all years.

Significant year × treatment interactions occurred for seed weight and size, and this necessitated using annual treatment means as replicate values for analysis over years. Under this condition, seed weight and size were reduced in infested plots over all years at $P = 0.057$ and 0.078, respectively (Table 2). Differences were significant at $P < 0.05$ within two and three individual years, respectively (Table 1). Decreases from control values were 5–21% for seed size and 6–24% for seed weight in infested plots over all years.

No significant differences related to seed germination were observed between control and infested plots for any of 3 yr. Ranges of mean values for both treatments over all years were as follows: normal germination at 4 days = 12–31%; total normal germination by 14 days = 24–36%; abnormal germination = 0.7–0.8%; and dead seed = 0.9–1.6%, embryo-dormant seed = 0.2–2.6%, and hard seed = 61–74%. The difference that most closely approached statistical significance occurred for hard seed in 1984–85, when the mean for control plots (70%) differed from the mean for infested plots (61%) at $P = 0.11$.

Observations of symptom development on leaves in relation to flowering of subclover during two seasons are given in Figure 1. In both years, flower production peaked in early April and declined and ceased several weeks before vegetative senescense. In 1986, flower production occurred during drought and reached its maximum near April 1 and declined sharply. In 1987, flower production was initiated during cool weather in early April and peaked later and more broadly. In both years, disease did not spread rapidly until after maximum flowering had occurred. Flowering in infested plots was not significantly reduced in comparison to controls until after significant increases in lesion counts were observed in infested plots.

Infestation of seed by *C. zebrina* occurred only at very low levels in each of 2 yr. From 3,200 seed from all plots from the 1984–85 season that were assayed by the four methods, sporulation by *Cercospora* was observed on only two seeds that had been plated without washing or surface disinfestation. From 3,200 seed from all infested plots from the 1986–87 season that were assayed, no growth or sporulation by *Cercospora* was observed.

DISCUSSION

Successful reseeding is an essential requirement for economical growth and utilization of subclover. Biological and physical factors that affect seed production need to be identified and evaluated to learn how to improve and manage the crop for the southeastern United States. Results of this study indicate that foliar infection by *C. zebrina* in the spring is one factor that significantly reduces seed production by subclover. Reductions in numbers of burrs and seed in plots infested with the pathogen were highly significant over all years despite strong variation in environmental conditions. This high overall significance, and the absence of significant year × treatment interactions, indicate that seed production will be reduced over years whenever the disease is present and infection is initiated by early spring. However, results also indicate that *C. zebrina* is unlikely to eliminate seed production entirely during any one season for Woogenellup or cultivars with similar flowering periods. The maximum reduction in seed production associated with the disease over 4 yr was 33%. The reason why greater reductions did not occur, as indicated by data from two seasons, is that a rapid increase in *Cercospora*-induced lesions does not commence until after peak flowering. This

relatively late disease development most likely results from a requirement by the pathogen for high temperatures for optimal growth and symptom development since host tissues of all ages are susceptible to infection (11). In different environments, with warmer winter and spring temperatures that would favor earlier disease development, or in cultivars of subclover that flower later than Woogenellup, rapid spread of infection might occur before rather than after peak flowering. In such situations, seed production might be drastically reduced or even eliminated by the disease.

Two possible approaches for maintaining control plots, with little or no Cercospora leaf spot, in the presence of infested plots in this study were to use foliar-applied fungicides to control the disease, or to use physical barriers to limit disease spread. Fungicides were not used to maintain controls because these might also have affected unrecognized, nontarget foliar or root pathogens or beneficial mycoflora. Seed yields obtained with fungicides then might not have typified yields that would occur in untreated stands in the absence of *C. zebrina*. Significant nontarget effects have been demonstrated with fungicides used to control a Cercospora disease of peanut (2). Alternatively, in this study as previously (11), wide borders of winter wheat were grown around all plots as physical barriers to limit movement of *C. zebrina* into controls. Although some symptoms did appear in controls by the end of the season (Fig. 1), this was mainly after most vegetative growth had ceased and natural senescensce of foliage had begun. It seems unlikely that this late disease development in control plots would have significantly affected their seed production. However, if the late occurrence of Cercospora leaf spot at low levels in controls was significant, results from infested plots would

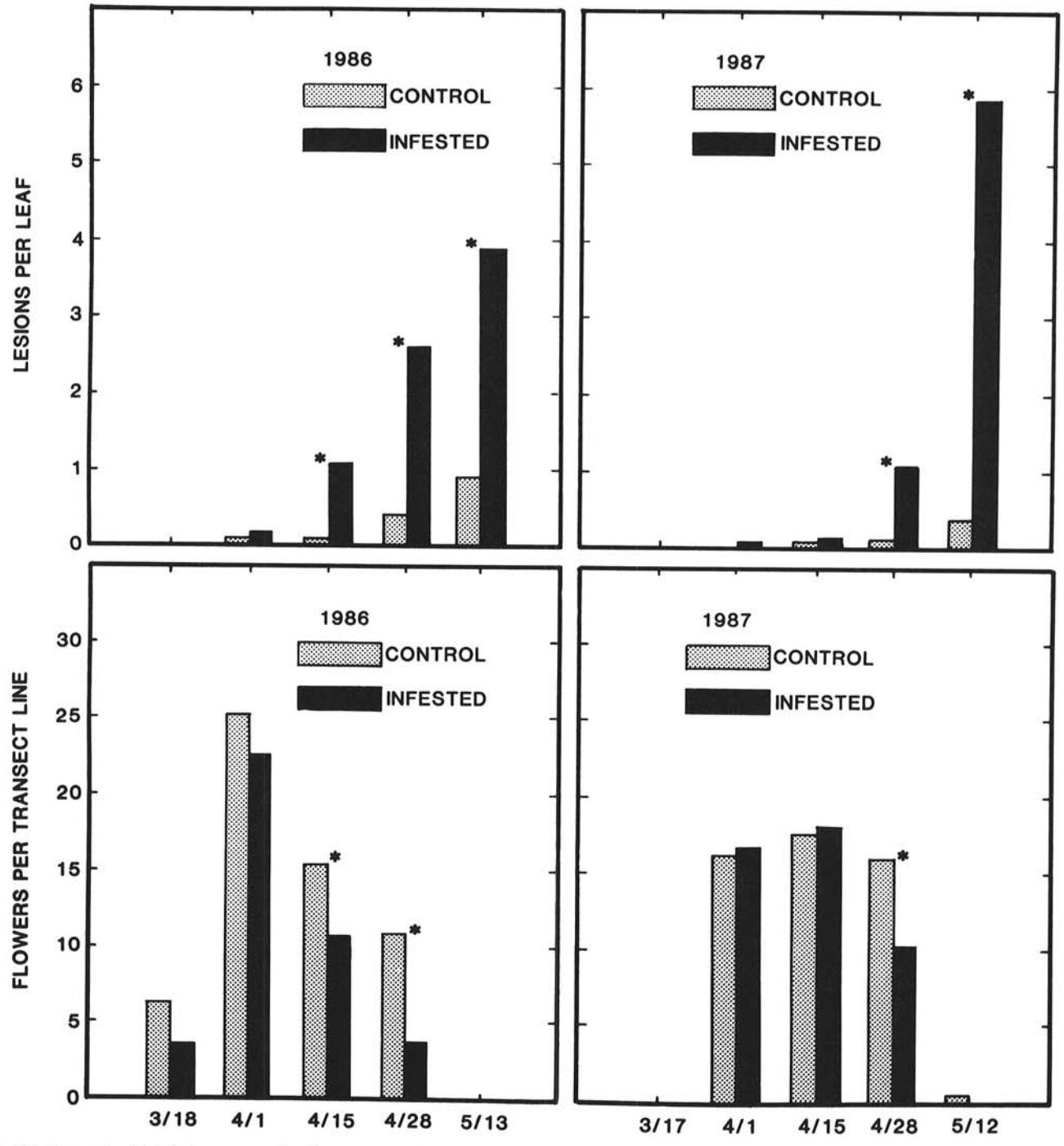

Fig. 1. Development of leaf lesions caused by *Cercospora zebrina* and flower production by subterranean clover grown in plots uninfested (control) or infested with the pathogen during two seasons at Mississippi State, MS. Each bar represents the mean value for samples collected or observed in 10 replicate plots. Asterisks indicate significant differences ($P < 0.05$) for sampling dates.

TABLE 1. Seed production and seed quality variables for subterranean clover grown in field plots uninfested (C) or infested (I) with *Cercospora zebrina* during four growing seasons

			Variable, treatment mean, and probability level (P) for difference between treatments							
			No. of burrs		No. of seed		Volume per 1,000 seed (cc)		Weight per 1,000 seed (g)	
Season	Treatment[a]	Replicates[a]	Mean	P	Mean	P	Mean	P	Mean	P
1982–83	C	9	1,225	0.024	3,392	0.177	10.91	0.050	9.05	<0.001
	I	9	1,005		2,795		8.56		6.85	
1984–85	C	5	1,658	0.137	4,614	0.065	10.06	0.070	8.75	<0.001
	I	5	1,356		3,510		9.27		7.71	
1985–86	C	10	914	0.001	2,397	0.002	11.10	0.273	9.09	0.170
	I	10	633		1,602		10.59		8.53	
1986–87	C	10	760	0.046	2,348	0.072	10.57	0.042	8.66	0.017
	I	10	603		1,893		9.59		7.77	

[a] Each replicate was one 1.49-m^2 plot of cultivar Woogenellup planted in September of each season and surrounded by 2.44 m of winter wheat. Treatments were applied in a completely random design as 300 g of autoclaved (C) or unautoclaved, infested (I) subclover debris per plot in February or March. Burrs with seed were harvested from a 0.25-m^2 sampling area in each plot in June.

TABLE 2. Sources of variation for analyses of variance of seed production and seed quality of subterranean clover grown in plots infested or not infested with *Cercospora zebrina*[a]

Variable	Replicate	Source of variation[b]	df[c]	Sum of squares	F	Probability $>$ F
No. burrs	Plot	Treatment	1	899,704	24.35	0.0001
		Year	3	5,656,431		
		T × Y	3	52,973	0.48	0.6989
		Error[e]	60	2,217,285		
No. seed	Plot	Treatment	1	8,528,649	18.52	0.0001
		Year	3	37,517,360		
		T × Y	3	794,992	0.58	0.6335
		Error	60	27,637,947		
Weight of seed	Plot	Treatment	1	21.58425		
		Year	3	7.55223		
		T × Y	3	7.09190	4.73	0.0050
		Error	60	29.96951		
	Annual[d] treatment mean	Treatment	1	21.58425	9.13	0.0567
		Error	3	7.09190		
Volume of seed	Plot	Treatment	1	20.83923		
		Year	3	15.16866		
		T × Y	3	8.98622	3.33	0.0253
		Error	60	50.90989		
	Annual[d] treatment mean	Treatment	1	20.83923	6.96	0.0778
		Error	3	8.98622		

[a] Data were obtained from 5–10 replicate plots of two treatments (infested with *C. zebrina* and uninfested) for each of 4 yr.
[b] T = treatments, Y = years.
[c] Error degree of freedom is the sum of $t(r-1)$ for each of 4 yr (Table 1).
[d] Annual treatment means were used as replicates when T × Y interactions were significant in analyses with plots as replicates. The T × Y mean square then was used as the error term.

represent underestimates of the impact of the disease on seed yield or quality.

It is not clear from these results whether repeated annual reductions in seed production, of the magnitudes observed here (Table 1), would eventually cause subclover to disappear from a site. This would depend on whether sufficient seed still remained each year to reproduce a stand of normal density by the time of flowering. Evaluation of stand regeneration of subclover over years in the presence of *C. zebrina* would require effective use of fungicides to maintain some plots as controls. For such studies, however, it would be desirable to also know how seed production, seed quality, and stand regeneration of subclover respond to fungicides in the absence of *C. zebrina*.

Counts of lesions on leaves (Fig. 1) accurately reflected times of disease spread and differences between treatments, but they did not always accurately reflect the total severity of disease symptoms. This was especially true for infested plots late in the season, when total leaf necrosis induced by *C. zebrina* was clearly higher than that suggested only by average numbers of lesions counted on sampled leaves. Although the pathogen appeared to cause little or no actual defoliation, lesions could be counted only on relatively young, intact leaves where little coalescense of necrosis occurred. Older leaves with large necrotic areas, or those with girdling lesions on petioles, became shriveled, tattered, or disintegrated and often could not be used to make lesion counts. Therefore, counts of lesions on leaves from infested plots late in each season (Fig. 1) should be viewed as underestimates of actual disease severity.

Results of this study on reduced seed production caused by *C. zebrina* are roughly comparable to a previous study reported from Australia (4). There, combined natural infection by *C. zebrina*, *Pseudopeziza trifolii* (Biv.-Bern.) Fuckel, and *Kabatiella caulivora* (Kirchn.) Karak. during one season correlated with reduced seed yields of up to 87% in several cultivars of subclover in comparison to fungicide-treated controls. Effects of the pathogens individually or interactions among them were suggested but not firmly established because none occurred independently. The fact that cultivar Woogenellup was among those least affected by *C. zebrina* in the Australian study strongly suggests that the strain of the pathogen was different than that found in Mississippi.

Although the potential importance of reduced seed production caused by *C. zebrina* in subclover is obvious, the importance of differences in seed quality induced by the pathogen is more difficult to ascertain. In this study, the size and weight of seed from infested plots were significantly less than from control plots in several individual years, and differences also were very close to the

accepted level of significance ($P = 0.05$) over all years when treatment means were used as replicates (Table 2). Previous studies with subclover have shown that large seed can be planted to greater depths in soil and will give plants that produce higher dry-matter yields than those from small seed (5). Differences in yields of plants from large and small seed may remain even throughout the life cycle of the crop, and initial differences in seed size may partly or entirely account for yield differences between some cultivars (6). These observations suggest that, when infection by *C. zebrina* causes production of smaller and lighter seed in a crop of subclover, as in at least several years of this study, subsequent stands produced from those seed might give reduced yields even if they are successfully regenerated. Further studies are needed to determine whether differences in seed quality induced by *C. zebrina* can be of sufficient magnitude to affect the productivity of subsequent stands of subclover.

LITERATURE CITED

1. Association of Official Seed Analysts. 1981. Rules for testing seeds. J. Seed Technol. 6:1-125.
2. Backman, P. A., Rodriguez-Kabana, R., and Williams, J. C. 1975. The effect of peanut leafspot fungicides on the nontarget pathogen, *Sclerotium rolfsii*. Phytopathology 65:773-776.
3. Barbetti, M. J. 1985. Infection studies with *Cercospora zebrina* on pasture legumes in Western Australia. Aust. J. Exp. Agric. Anim. Husb. 25:850-855.
4. Barbetti, M. J. 1987. Effects of three foliar diseases on biomass and seed yield for 11 cultivars of subterranean clover. Plant Dis. 71:350-353.
5. Black, J. N. 1956. The influence of seed size and depth of sowing on pre-emergence and early vegetative growth of subterranean clover. Aust. J. Agric. Res. 7:98-109.
6. Black, J. N. 1957. The early vegetative growth of three strains of subterranean clover (*Trifolium subterraneum* L.) in relation to size of seed. Aust. J. Agric. Res. 8:1-14.
7. Bokor, A. 1983. *Cercospora zebrina* Pass. found on *Trifolium subterraneum* L. in Western Australia. Australas. Plant Pathol. 12:24.
8. Knight, W. E., Hagedorn, C., Watson, V. H., and Friesner, D. L. 1982. Subterranean clover in the United States. Adv. Agron. 35:165-191.
9. McGuire, W. S. 1985. Subterranean clover. Pages 515-534 in: Clover Science and Technology. N. L. Taylor, ed. American Society of Agronomy, Inc., Madison, WI. 616 pp.
10. Morley, F. H. W. 1961. Subterranean clover. Adv. Agron. 13:57-123.
11. Pratt, R. G. 1984. A new Cercospora leaf and stem disease of subterranean clover. Phytopathology 74:1152-1156.
12. Pratt, R. G. 1987. Effects of a Cercospora leaf disease on seed production of subterranean clover. (Abstr.) Phytopathology 77:1707.
13. SAS Institute, Inc. 1982. SAS User's Guide: Statistics. SAS Institute, Inc., Cary, NC. 584 pp.