A New Cercospora Leaf and Stem Disease of Subterranean Clover

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Cooperative investigation of USDA-ARS and the Mississippi Agricultural and Forestry Experiment Station (MAFES). Approved for publication as Journal Series Paper 5591 of MAFES.

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The author is grateful to K. E. Gourley for technical assistance and to K. W. Roy for advice on isolating and culturing *Cercospora*.

Accepted for publication 7 May 1984.

ABSTRACT

Pratt, R. G. 1984. A new Cercospora leaf and stem disease of subterranean clover. Phytopathology 74:1152-1156.

A severe disease caused by *Cercospora* was observed on foliage of subterranean clover at two locations in Mississippi. The pathogen was identified as a new, host-specific form of *Cercospora zebrina*. Symptoms developed on susceptible cultivars of subclover 8–10 days after leaves were inoculated with conidia under favorable conditions. Disease was most severe when plants were maintained at 25–31 C and incubated in a saturated atmosphere for ≥72 hr following inoculation. Symptoms developed more slowly at lower temperatures and were greatly reduced with shorter periods of saturation. Pathogenicity was evaluated on 11 species of *Trifolium* and *Medicago*; severe and consistent disease symptoms developed only on subterranean and rose clovers. Among 17 cultivars of subterranean clover, 14 were highly susceptible and three were moderately to highly resistant. Field plots were effectively inoculated by applying infested debris to foliage and by transplanting infected plants from the greenhouse; infested debris gave more uniform infection. Results suggest that the new Cercospora disease could potentially reduce forage production and reseeding by subterranean clover in the southeastern United States.

Additional key words: *Medicago sativa, Trifolium alexandrinum, Trifolium hirtum, Trifolium hybridum, Trifolium incarnatum, Trifolium nigrescens, Trifolium pratense, Trifolium repens, Trifolium resupinatum, Trifolium subterraneum, Trifolium vesiculosum.*

Subterranean clover (subclover) (*Trifolium subterraneum* L.) is an annual forage legume that is being grown increasingly as a winter pasture crop in the southeastern United States (9). It also has been utilized in pastures in California and Oregon since the 1930s (9,11), and in Australia since early in this century (12). Subclover is unique among annual clover species because it has a prostrate vegetative growth habit, and because the peduncles become geotropic and elongate after flowering which buries developing seed in soil and thatch (11). These morphological features enable subclover to grow, flower, and set seed even while receiving heavy and uninterrupted grazing pressure, and this accounts for much of its popularity with livestock producers (9,11,12).

Leaf and stem diseases caused by *Cercospora* on forage legumes are often referred to as "summer blackstem" diseases. Few taxonomic differences are apparent between isolates of *Cercospora* from different forage legumes (4,7,14). Horsfall (7) proposed that most isolates from *Trifolium, Medicago,* and *Melilotus* be considered as one species, *Cercospora zebrina* Pass., but Chupp (5) retained isolates from these genera in three different species. Cross-infection sometimes occurs following inoculations with isolates of *Cercospora* from different host genera and species, but isolates usually are most virulent on their hosts of origin (2,4,8,10).

Symptoms of diseases caused by *Cercospora* on forage legumes include gray to brown leafspots of various sizes and shapes, girdling lesions on petioles, and usually nongirdling lesions on stems (2,6,7). Symptoms develop most commonly on mature host tissue (8,18) and with warm temperatures and high humidity in late spring and summer (2–4,17).

The presence of *Cercospora* on subclover was recorded first in Brazil (13) and later in New Zealand (14), but no information on symptoms, disease severity, or losses was reported. Recently *C. zebrina* was reported as a pathogen of subclover in Australia, where it sometimes caused defoliation and reduced seed production (1). Apparent differences in susceptibility of subclover cultivars were noted, but it was not determined whether the disease was of major economic importance (1).

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No diseases caused by *Cercospora* have ever been reported to occur naturally on subclover in the United States. *Cercospora* isolates from alfalfa (*Medicago sativa* L.) and red clover (*T. pratense* L.) were only slightly pathogenic on subclover with artificial inoculations (4), and isolates from *Melilotus* were nonpathogenic on it (4,10).

In 1980 and in all subsequent years, a disease apparently caused by *Cercospora* was observed on subclover at Mississippi State, MS. It also was observed 350 km distant at Poplarville, MS, in 1981. Symptoms were sometimes very severe on subclover, but they did not occur on any of numerous other clover species or on alfalfa grown in adjacent field plots. This suggested that the disease on subclover was different from other diseases caused by *Cercospora* previously reported on red and white (*T. repens* L.) clovers and alfalfa in the United States (2,4,7).

The purposes of this investigation were to confirm the etiology of the new disease caused by *Cercospora* in subterranean clover, to determine the host range of the pathogen and conditions favoring pathogenesis, to evaluate reactions of host cultivars, and to compare methods for obtaining controlled infection in field plots.

MATERIALS AND METHODS

Isolation and identification of *Cercospora*. To isolate *Cercospora*, pieces of symptomatic leaf and stem tissue (≤1.0 cm in diameter) were dipped in ethanol, surface-disinfested in 1% sodium hypochlorite for 30–45 sec, rinsed in sterile distilled water, blotted on sterile filter paper, and plated on Difco cornmeal agar (CMA) (Difco Laboratories, Detroit, MI 48201). Alternatively, tissue was incubated in plates on saturated filter paper for 24–28 hr to induce sporulation by *Cercospora*. Areas of sporulation were touched with a sterile needle, and adhering spores were streaked on CMA. Transfers were made from dense, slow-growing colonies (3), and isolates identified as *Cercospora* following sporulation (see below) were stored on slants of CMA at 4 C. Taxonomic observations were made on conidia and conidiophores from naturally infected stems. Lengths and widths of 20 conidia and conidiophores from each of 10 stems were determined in water mounts at ×1,000.

Inoculum production, inoculation methods, and disease evaluation. Conidia were produced on senescent-soybean-pod agar (SSPA) (16). Hyphae from the edges of 7-day-old colonies on CMA were macerated with a loop in distilled water and fragments were streaked over SSPA. Plates were incubated at 25 C under continuous fluorescent light (1,630 lux). After 4 days, the plates were flooded with distilled water and scraped with a loop; suspensions of conidia, conidiophores, and hyphae were then restreaked over new SSPA. Transfers to SSPA were repeated, with 4-day incubation periods, until numerous conidia were present. The final crop of conidia was collected in distilled water and counted with a hemacytometer; concentrations were adjusted to $1–2 \times 10^4$/ml for use as inoculum in most experiments.

Germinated seed of subclover were planted in a mixture of loam, sand, and peat (1:1:1, v/v) in clay pots (375-cc capacity) (two plants per pot) and grown 6–8 wk at 23–28 C in the greenhouse or in growth chambers under conditions described previously (15). Compatible inoculum of *Rhizobium* was applied after 1 wk (15). Prior to inoculation, three to five leaves of similar size and age on each plant were marked with thin strips of masking tape around petioles. Inoculum was atomized onto foliage until runoff or streaked onto each marked leaf with a soft-bristled camel's hair brush dipped in swirled inoculum. Immediately after inoculation of plants, each pot was placed in a container (520-cc capacity), filled to the rim with water, within a transparent plastic bag that was sealed to create a saturated atmosphere. After 3–4 days, bags were removed and the plants were grown for 5–9 additional days before disease was evaluated. In each experiment, distilled water was atomized or brushed onto leaves of additional plants that were used as controls. These plants were incubated in saturated atmospheres and grown under the same conditions as inoculated plants.

Leaves marked with tape were harvested, and lesions caused by *Cercospora* were counted under a dissecting microscope. Each leaf was scored as follows: 0 = no lesions; 1 = 1–25 lesions (on all three leaflets together); 2 = 26–50 lesions; 3 = 51–100 lesions; and 4 = 100+ lesions. Treatments were compared by using mean scores of three to five leaves on each of 5–10 replicate plants. Differences were determined by analysis of variance and use of the Student-Newman-Keuls' test.

Establishment, inoculation, and evaluation of field plots. Subterranean clover cultivar Woogenellup was planted in 32 plots (each 1.2 × 1.2 m) in a randomized complete block design (four replicated blocks, eight plots per block) in September 1981. Each plot received 8 g of seed and was separated from all other plots by 2.4-m alleys that were planted to winter wheat. The eight treatments were: infested debris applied to plots in February; infested debris applied in March; noninfested debris applied in February; noninfested debris applied in March; infected plants transplanted into plots in February; infected plants transplanted in March; noninfected plants transplanted in February; and noninfected plants transplanted in March. One plot of each treatment was randomly located in each block. Infested leaf and stem debris was collected from severely diseased subterranean clover in the field after senescence the previous spring. Air-dried debris was stored in paper bags at room temperature for 9–10 mo prior to infestation of plots. Three hundred grams of debris was scattered evenly over each plot and brushed into the canopy by hand. Control plots received equal amounts of noninfested (autoclaved) debris. Infected plants were grown two per pot for 13 wk in the greenhouse, inoculated by spraying their foliage with suspensions of conidia, and transplanted to the field after 10 days when numerous lesions were evident on leaves. Plants from one pot were transplanted to the center of each plot. Control plots received noninfected transplants.

Samples of leaves were collected after disease symptoms became frequent. Lesions caused by *Cercospora* were counted on 10 leaves randomly selected out of a sample from the center, and 10 leaves out of a sample from the edges of the canopy of each plot. Means were compared by analysis of variance and with the Student-Newman-Keuls' test.

Fig. 1. Symptoms of infection by *Cercospora zebrina* on leaves of subterranean clover from the field: **A,** uninfected leaf; **B,** infected leaf with individual lesions of various sizes; and **C,** infected leaf with numerous lesions coalesced to cause large necrotic areas.

RESULTS

Symptoms of disease and isolation and identification of *Cercospora*. Lesions caused by *Cercospora* were observed on foliage of subclover in plots at Mississippi State each spring from 1980 through 1983, and in a stand grown for hay at Poplarville, MS, in 1981. Lesions on leaves were necrotic, rectangular to oblong, partly vein-delimited, ≤4 mm in diameter, with light tan centers and dark brown borders. They were generally similar to those illustrated for white clover and *M. lupulina* L. (7) (Fig. 1). Numerous lesions coalesced, which caused necrotic areas up to 1 cm in diameter. Centers sometimes disappeared from old lesions to give a shot-hole appearance. Lesions on petioles were dark brown, sunken, and elongated (up to 2 cm); they frequently girdled petioles and caused total leaf necrosis. Lesions on stems were more red-colored, up to 5 cm long, slightly sunken, and usually nongirdling. Symptoms of natural infection were usually first noticed in April and were common by May. Sometimes all foliage was killed in early May, several weeks before natural senescence was expected.

Fifteen isolates were obtained from leaves, petioles, and stems of plants from Mississippi State. Colonies on CMA were slow-growing (<1 cm/wk), gray to dark brown on top, and olivaceous to black underneath. All isolates produced numerous conidia on SSPA.

Conidiophores were observed as produced in the field on infested stems. They were straight or broadly curved, unbranched, multiseptate, multigeniculate, and pale to dark brown. They arose from single globose cells or up to three small cells beneath the epidermis, and they occurred individually or in small groups on lesions. Mean dimensions of 200 conidiophores from 10 stem lesions were 101.8 × 4.8 μm; ranges were 37–226 × 3.9–6.3 μm.

Conidia were produced when infested stems from the field were incubated in a moist chamber for 24–30 hr. They were needlelike, straight to broadly curved or bent, evenly tapered, and hyaline. Mean dimensions of 200 conidia from 10 stem lesions were 229 × 2.8 μm; ranges were 87–340 × 2–4 μm.

The morphology and dimensions of conidiophores were compatible with those described for *C. zebrina* by Chupp (5). Conidia were much larger than those described for *C. zebrina* from the field (5,7), but their sizes were well within ranges reported for conidia produced in the laboratory on clover and alfalfa tissue collected in the field (3). Therefore, the pathogen on subterranean clover was identified as *C. zebrina* sensu lato (sensu Horsfall [7]).

Conditions affecting pathogenesis. Disease severity increased with inoculum concentrations (Table 1). At high inoculum levels, initial symptoms (faint pepper-spots) first appeared after 3 days, and lesions were large and mature by 8 days. At low levels, symptoms did not appear until 7 days, and lesions did not appear mature until 12 days. Brush inoculations gave more numerous lesions on leaves than did spray inoculations (Table 1).

Numbers of lesions formed on leaves increased steadily as periods of incubation in saturated atmosphere bags increased from 24 to 96 hr. Mean disease scores after 0, 24, 48, 72, and 96 hr were 0.0, 0.40, 1.49, 2.26, and 3.28, respectively. Lesions appeared and enlarged most rapidly within 96 hr of saturation. No symptoms developed on three control plants used with each incubation period.

Effects of temperature on infection and disease development were evaluated with cultivar Woogenellup. Fifteen plants, each with four leaves brush-inoculated with 1.3×10^4 propagules per milliliter, were maintained in each of three growth chambers at ambient temperatures of 20, 25, and 30 C. Plastic bags were removed after 4 days, and lesions were scored after 11 days. Temperatures in the bags reached 5–6 C above ambient during light periods. Mean disease scores of plants in chambers with ambient temperatures of 20 and 25 C (3.6 and 3.2, respectively) did not differ significantly, but lesions enlarged and matured more rapidly on plants in the chamber at 25 C. No symptoms developed on three control plants at each of these temperatures. No symptoms occurred on plants in the chamber at 30 C, where temperatures approached 36 C during exposure to saturated atmospheres.

Reisolations of *C. zebrina* were attempted from leaves of 6-wk-old Woogenellup plants that were brush-inoculated with a mixture of spores from two isolates (1.2×10^4/ml) and maintained in the greenhouse for 10 days. Lesions produced on leaflets and petioles were identical to those observed in the field. Twenty pieces of tissue, each with a single lesion, were surface-sterilized and plated. Other infected leaves were maintained in a saturated atmosphere for 24–48 hr, and spores produced in 20 lesions were transferred to agar with a needle. *C. zebrina* was reisolated in pure culture from 17 lesions by plating of tissue and from 15 lesions by transfers of spores. In all instances, the morphology, growth rates, and pigmentation of reisolated cultures on CMA were identical to those of cultures used to inoculate plants. These results represent fulfillment of Koch's postulates for *C. zebrina* as a pathogen of subterranean clover.

Host range of *C. zebrina* from subclover. Eleven species of clovers and alfalfa were evaluated for reactions to *C. zebrina* from subclover. Results are in Table 2. Few or no lesions developed on leaves of arrowleaf, berseem, crimson, Persian, red, and white clovers, and alfalfa. Lesions were more numerous in ball clover, but some plants still developed no symptoms and none developed

TABLE 1. Severity of symptoms induced by *Cercospora zebrina* on leaves of subterranean clover inoculated by two methods and at five inoculum concentrations

Inoculum concentration[y]	Inoculation method and disease severity[z]	
	Spray	Brush
0	0.00 a	0.00 a
3,125	0.26 a	2.68 d
12,500	0.66 b	3.74 e
50,000	1.06 c	4.00 e
200,000	2.94 d	4.00 e

[y] Numbers of viable-appearing conidia (>90%), conidiophores, and hyphal fragments per milliliter as determined by hemacytometer counts. Inoculum was collected in distilled water from colonies sporulating on a soybean-pod agar medium (16).

[z] Five leaves on each of 10 subterranean clover plants (cultivar Woogenellup, 6 wk old) were inoculated by each method and at each concentration. Spraying consisted of atomizing inoculum onto leaves with a fine-mist hand sprayer until run-off. Brushing consisted of stroking the adaxial surface of each leaf seven times with a soft-bristled brush dipped in inoculum. Values are means of scores in which 0 = no lesions per leaf, 1 = 1–25 lesions, 2 = 26–50, 3 = 51–100, and 4 = 100+ lesions. Values not followed by the same letter differ significantly ($P = 0.05$) as determined by the Student-Newman-Keuls' test.

TABLE 2. Severity of symptoms induced by *Cercospora zebrina* on leaves of clover and alfalfa cultivars

Host	Common name	Cultivar or line	Disease severity[z]
Trifolium hybridum	Alsike clover	(unknown)	0.00 a
T. incarnatum	Crimson clover	Tibbee	0.00 a
		Chief	0.03 a
T. pratense	Red clover	Kenstar	0.00 a
		Kenland	0.09 a
T. vesiculosum	Arrowleaf clover	Yuchi	0.10 a
		Meechee	0.16 a
T. repens	White clover	Tillman	0.17 a
		Regal	0.19 a
T. alexandrinum	Berseem clover	(exptl. line)	0.23 a
T. resupinatum	Persian clover	Abon	0.36 ab
Medicago sativa	Alfalfa	Apollo	0.53 ab
		Vernal	0.56 ab
T. nigrescens	Ball clover	(unknown)	0.74 b
T. hirtum	Rose clover	(unknown)	3.13 c
T. subterraneum	Subterranean clover	Woogenellup	3.83 d
		Mt. Barker	3.94 d

[z] Three leaves on each of 10 plants of each entry (7 wk old) were brushed with inoculum (2.0×10^4 propagules per milliliter of distilled water). Values are means of scores in which 0 = no lesions per leaf, 1 = 1–25 lesions, 2 = 26–50, 3 = 51–100, and 4 = 100+ lesions. Values not followed by the same letter differ significantly ($P = 0.05$) as determined by the Student-Newman-Keuls' test.

severe symptoms. In contrast, most plants of rose clover and all plants of subclover were severely diseased. No symptoms of infection by *Cercospora* developed on three control plants of each species and cultivar.

Leaves of all species that developed lesions were incubated 22–40 hr in moist chambers and then examined; occasional to numerous conidiophores and conidia of *Cercospora* were observed in lesions on all species.

Reactions of subclover cultivars. Host reactions of 17 cultivars of subclover were evaluated in two experiments. Results of the first experiment, involving 16 cultivars, are in Table 3. Numbers and sizes of lesions were generally similar in 14 of the cultivars. In one other, Dwalganup, similar numbers of lesions developed, but these were very small and restricted in comparison to the other 14 cultivars. Cultivar Clare produced significantly ($P = 0.05$) fewer lesions than all other cultivars, and these lesions were restricted in size as in Dwalganup. No symptoms developed on two or three control plants of each cultivar.

In a second experiment, host reactions of four of the preceding cultivars and a fifth, Yarloop, were evaluated. Four leaves on each of 10 plants per cultivar were brush-inoculated (1.1×10^4 propagules per milliliter from two isolates of *Cercospora*). Plants were maintained at saturation for 4 days and symptoms were evaluated after 12 days. Numerous mature, enlarged lesions were present on Mt. Barker and Woogenellup. Significantly ($P = 0.05$) fewer lesions were present on Clare, Dwalganup, and Yarloop, and these were very small in size. Two plants of Yarloop had no lesions. Leaves on all plants of Yarloop also developed numerous small, red-purple spots that appeared to be hypersensitive reactions.

Disease development in field plots. Occasionally symptoms caused by *Cercospora* were first observed in inoculated field plots in late March 1982. By late April, symptoms were present in all inoculated plots and were first noticed in some control plots. Leaf samples were collected on 10 May, several weeks before senescence.

The disease was present in all control plots, but only at consistently low levels (Table 4). Infection was much greater in all inoculated plots. Infested debris gave more uniform infection than infected transplants. Infection was greater with inoculations in February than in March using transplants, but not using infested debris.

DISCUSSION

Results of this study demonstrate the occurrence of a potentially damaging leaf and stem disease caused by *Cercospora* on subterranean clover in Mississippi. The results also indicate that the organism on subclover differs in pathogenic behavior from other species or forms of *Cercospora* previously known on forage legumes in North America. Isolates from red and white clovers, alfalfa, and sweetclover were pathogenic on their hosts of origin and often on other hosts, but none of them were significantly pathogenic on subclover (3,4,8,10). In contrast, the organism from subclover in Mississippi is highly virulent on subterranean and rose clovers, but it is only weakly virulent or nonpathogenic on eight other species of clover and on alfalfa.

Taxonomic relationships between the *Cercospora* on subclover in Mississippi and congeneric pathogens known on other forage legumes in North America are not obvious or well defined. The pathogen is conservatively identified as *C. zebrina* because it occurs on a species of *Trifolium*, and because morphological characteristics and sizes of conidia, conidiophores, and basal cells are not incompatible with those described for *C. zebrina* by one or more authors (3,5,7). However, these same features also do not clearly separate the subclover pathogen from other species of *Cercospora* described on forage legumes (3–5,7). For this reason, this species identification is intended to represent the broad view as proposed by Horsfall (7,14).

Influences of environmental conditions on infection and disease development caused by *C. zebrina* on subclover are similar to those reported for diseases caused in other forage legumes by *Cercospora*. The length of time that subclover plants are maintained in a saturated atmosphere following inoculation is critical for infection and disease development; 72 hr was always required for severe disease to develop, and longer times resulted in more lesions. Nearly identical results were reported for the disease caused by *Cercospora* on red clover (4). The more rapid development of disease on subclover at 25–31 C than at 20–26 C also corresponds to effects of temperature on the disease caused by *Cercospora* on red clover. The high optimum temperatures for symptom development may be one reason for the appearance of severe symptoms only in late spring at Mississippi State. In Alabama, *C. medicaginis* also did not cause severe disease on annual burr clover (*M. arabica* All.) until April and May (18). In more northerly areas, diseases caused by *Cercospora* do not become frequent or important on forage legumes until summer (2,17).

Observations of host-pathogen interactions on 17 cultivars of subclover suggest that two forms or levels of resistance to *C. zebrina* may be present. Numbers and sizes of lesions were similar in 13 cultivars. Sizes of lesions were reduced in four other cultivars,

TABLE 3. Severity of symptoms induced by *Cercospora zebrina* on leaves of subterranean clover cultivars

Cultivar	Disease severity[z]
Clare	0.40 a
Dwalganup	1.26 b
Bacchus Marsh	1.26 b
Mt. Barker	1.54 bc
Dinninup	1.58 bc
Geraldton	1.60 bc
Cranmore	1.64 bc
Seaton Park	1.74 bc
Parkerville	1.78 bc
Woogenellup	1.88 bc
Nangeela	1.92 bc
Mississippi Ecotype	1.94 bc
Daliak	2.00 bc
Hepam	2.12 bc
Northam	2.48 c
Eden Valley	2.54 c

[z] Three leaves on each of five plants per cultivar (7 wk old) were brushed with inoculum (1.0×10^4 propagules per milliliter of distilled water). Values are means of scores in which 0 = no lesions per leaf, 1 = 1–25 lesions, 2 = 26–50, 3 = 51–100, and 4 = 100+ lesions. Values not followed by the same letter differ significantly ($P = 0.05$) as determined by the Student-Newman-Keuls' test.

TABLE 4. Severity of symptoms induced by *Cercospora zebrina* on leaves of subterranean clover in field plots inoculated by two methods and at two different times[x]

Control (C) or infested (I) inoculum	Inoculation method[y]	Inoculation time	Disease severity and areas of plots[z]	
			Centers	Edges
C	Transplant	February	0.23 a	0.08 a
		March	0.15 a	0.03 a
	Debris	February	0.03 a	0.03 a
		March	0.05 a	0.08 a
I	Transplant	February	2.73 c	0.85 bc
		March	1.83 b	0.45 ab
	Debris	February	1.68 b	1.55 d
		March	1.65 b	1.30 cd

[x] Cultivar Woogenellup was planted in 1.2 × 1.2-m plots (8 g of seed per plot, separated by 2.4-m alleys of winter wheat, in September 1981.

[y] Transplant = two plants from one pot, infected or not infected, transplanted to the center of each plot. Debris = 300 g of infested subclover leaf and stem debris, collected in the field the previous spring and autoclaved or untreated, brushed into the canopy of each plot.

[z] Data were obtained in May 1982, from random samples of leaves, and represent mean disease scores of four replicate plots in which 0 = no lesions per leaf, 1 = 1–25 lesions, 2 = 26–50, 3 = 51–100, 4 = 100+ lesions. Values within a column not followed by the same letter differ significantly ($P = 0.05$) as determined by the Student-Newman-Keuls' test.

and numbers of lesions were also reduced in one or two of them. These results suggest that it may be possible to develop resistant cultivars if a need is clearly demonstrated.

Results with inoculated field plots indicate that systematic evaluation and screening of subclover for resistance to *C. zebrina* could be accomplished effectively in the field. All plots inoculated with infested debris from the previous year's crop developed similar levels of infection. Furthermore, control plots of susceptible cultivars were kept largely free of disease by the use of wide borders of wheat. Although some infection did appear in all control plots by the end of the season, symptoms continued to be much more severe in inoculated plots as long as the plants remained alive.

The potential for this new disease caused by *Cercospora* to cause significant losses or to limit production of subclover in the southeastern United States is not known. The organism is highly virulent on susceptible cultivars, but it appears to require warm temperatures and prolonged high humidity for rapid and severe disease development. If disease does not become severe until near the end of the growing season in late spring, then losses in forage production may not be significant. However, since subterranean clover is grown as a reseeding annual species, it is still possible that the disease might cause significant losses in future stands if the quantity or quality of seed produced are reduced by infection.

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