A GREENHOUSE SCREENING METHOD FOR RESISTANCE TO GRAY LEAF SPOT IN MAIZE

by

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ABSTRACT

Gray leaf spot (GLS) disease of maize (*Zea mays* L.), caused by the fungus *Cercospora zeae-maydis*, causes significant corn yield losses in Virginia and other mid-Atlantic states. A new greenhouse assay method with filter paper discs of *C. zeae-maydis* mycelia has been developed to evaluate corn germplasm for resistance to GLS. Mycelial inoculum obtained from cultures of mycelia in liquid malt media was pipetted at 100 *u*l samples onto each filter paper disc which was then adhered to the lower leaf surface by transparent tape. The inoculated corn seedlings were placed in a moist plastic chamber with high relative humidity provided by a humidifier. The first macroscopic symptoms induced by this inoculation method appeared 3 days after inoculation. This new inoculation method with mycelial discs was used on five corn genotypes (VA14, B68, PA875, B73, and MO17) to screen resistance to GLS disease. With this inoculation method, resistant and susceptible inbreds were easily differentiated based on lesion type. Resistant inbreds including VA14, B68, and PA875 were characterized by water-soaked appearance or small chlorotic flecks while susceptible inbreds like B73 and MO17 were characterized by more extensive

necrosis. Necrotic area under the mycelial disc was a good indicator for disease severity. However, the percent leaf area under discs affected by mycelia which reflected the total host responses was not appropriate to indicate disease severity. The effects of plant physiological factors on the expression of resistance to GLS was also investigated. Placing mycelial discs on lower leaf surfaces induced more responses than placing on upper leaf surfaces. Inoculation of lower older leaves induced more severe lesions than inoculation of upper leaves. The effect of cercosporin was investigated by inoculating corn seedlings with cercosporin-producing mycelia and with non-cercosporin containing mycelia. The former induced much more severe host response than the latter. Conidiation of *C. zeae-maydis* was examined with the mycelial inoculation method in the greenhouse. Conidiophores were found emerging from stomata as early as 15 days after inoculation in B73 and MO17 and limited only to necrotic tissue. No conidiation was observed in resistant genotypes VA14, B68 and PA875.

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INTRODUCTION AND OBJECTIVES

Gray leaf spot (GLS) disease of maize (*Zea mays* L.), caused by the fungus *Cercospora zeae-maydis* Tehon & Daniels (Tehon and Daniels, 1925), has increased in severity in recent years (Latterell and Rossi, 1983; Stromberg and Donahue, 1986). Although favorable weather is essential for the initiation of GLS disease, no-tillage practice of maize production has been reported to be associated with higher levels of GLS development (Roane, Harrison and Genter, 1974; Latterell and Rossi, 1983). It is estimated that between 10 and 25% of the corn yield potential can be lost annually in areas where GLS disease is a problem; when environmental conditions are optimum for disease development, losses can be much higher.

Genetic resistance appears to be the best means to control GLS disease, because conventional tillage and the application of fungicides may have adverse economic and environmental effects. Because most widely used commercial corn hybrids are susceptible to GLS, identification of GLS resistant inbreds would facilitate development of new resistant elite hybrids.

Several genetic studies have been conducted in the field to identify GLS resistant germplasm of corn (Roane, Harrison and Genter, 1974; Hilty, Hadden and Garden, 1979; Thompson, et al., 1987; Huff, Ayers and Hill, Jr., 1988; Elwinger, et al., 1990; Ulrich, Hawk and Carroll, 1990; Donahue, Stromberg and Myers, 1991). These studies identified several corn inbreds that were resistant to GLS disease and

concluded that GLS resistance was additive, quantitatively inherited, and "not very complex". Although field screening of large numbers of corn genotypes for GLS resistance is highly desirable, it is time-consuming and largely affected by weather conditions and locations which may result in variable disease levels. Thus, it would be an advantage to develop a reliable greenhouse screening method to evaluate GLS resistance of corn under controlled conditions.

Gray leaf spot disease favors high relative humidity. In previous studies conducted in the greenhouse, a moist chamber which provided high relative humidity for *C. zeae-maydis* infection of corn seedlings was necessary (Beckman and Payne, 1982 and 1983; Latterell and Rossi, 1983). Although the successful development of GLS symptoms was observed, genotype-dependent differences were never detected for susceptibility to *Cercospora zeae-maydis*, either in the greenhouse or laboratory (Beckman and Payne, 1982; Gwinn, Stelzig and Brooks, 1987).

The overall goal of this research is to develop a greenhouse screening technique to evaluate corn germplasm for resistance or susceptibility to the fungus *Cercospora zeae-maydis*. Specific objectives are: 1) to develop a successful inoculation method with mycelia of *C. zeae-maydis*, 2) to verify the reliability of the new method by testing five maize genotypes of defined GLS response in the field, 3) to examine the effect of corn leaf age on resistance to *C. zeae-maydis* by using the new method, 4) to investigate the effects of wild and mutant fungal mycelium on resistance to *C. zeae-maydis*, and 5) to observe conidiation of *C. zeae-maydis* after artificial inoculation.

LITERATURE REVIEW

Gray Leaf Spot and Its Distribution and Severity

Gray leaf spot of corn (*Zea mays* L.), caused by the fungus, *Cercospora zeae-maydis* Tehon & Daniels, was first found on corn leaves collected in Alexander County, Illinois, in 1925 (Tehon and Daniels, 1925). Roane (1950) collected and identified gray leaf spot in the mountain valleys of Virginia, near Blacksburg, in 1949 and 1950. The gray leaf spot (GLS) disease of corn remained a minor disease until the early 1970s. In 1974, Roane, Harrison, and Genter (1974) reported that early and intensive development of gray leaf spot disease was associated with the practice of no-tillage production of maize. In 1973, severe leaf damage caused by *Cercospora zeae-maydis* was also observed in fields in the mountains of North Carolina where GLS had previously been a minor disease (Leonard, 1974).

In recent years, gray leaf spot has increased greatly in distribution and severity throughout the southeastern, mid-Atlantic, and midwestern states including South Carolina, North Carolina, Virginia, Tennessee, Kentucky, West Virginia, Pennsylvania, Missouri, Iowa, and Maryland, (Hilty, Hadden and Garden, 1979; Latterell and Rossi, 1983; Ayers, Johnson, Jr. and Hill, Jr., 1984; Stromberg, 1986).

Although favorable weather is essential for the initiation of gray leaf spot disease, no-tillage practice has been reported to be associated with higher levels of gray leaf spot development (Roane, Harrison and Genter, 1974; Latterell and Rossi, 1983).

In Tennessee, the fields where minimum tillage had been practiced were found to be most severely infected by GLS (Hilty, Hadden and Garden, 1979). The use of no-till or reduced-tillage practices results in large amounts of debris from the previous year's crop remaining in fields. *Cercospora zeae-maydis* overwinters in corn debris, which then serves as a source of new inoculum.

Under conditions of severe infection, gray leaf spot may be expected to cause a reduction of corn grain yield. Grain yield losses as great as 20% have been reported in Tennessee (Hilty, Hadden and Garden, 1979). Losses caused by gray leaf spot are associated either with lodging or with the premature loss of photosynthetic area (Latterell and Rossi, 1983; Stromberg and Donahue, 1986; Donahue, Stromberg and Myers, 1991).

Biology of GLS Disease

Life cycle of the pathogen

The causal agent of GLS, *Cercospora zeae-maydis*, was first described by Tehon & Daniels (Tehon and Daniels, 1925). The fungus, which is a poor soil competitor, overwinters as mycelium in corn debris on the soil surface, which then may serve as a source of inoculum for the next year. Conidia are produced from mycelium in maize debris during wet weather and are wind-borne to new hosts. Conidia of *Cercospora zeae-maydis* are straight to slightly curved, hyaline, slender, and multiseptate. The size of mature conidia is about 70-180 *um* long and 5-6 *um* wide

with 6-12 septa. A species of *Mycosphaerella* is believed to be the perfect stage of *Cercospora zeae-maydis* (Latterell and Rossi, 1983; Shurtleff, 1980; McGee, 1988). In studies of overwintering and spore release of *Cercospora zeae-maydis* in corn debris in North Carolina, Payne and Waldron (1983) detected conidia in the air above corn debris as early as June 16. The concentration of airborne conidia was greatest during the week of Sep. 27 to Oct. 2, when the corn kernels were in dough to late dent stage of development (Payne and Waldron, 1983).

In culture, *Cercospora zeae-maydis* grows on V-8 and other agar media and morphological characters are often changed. Growth habit ranges from black, densely sporulating cushion-like colonies to white, cottony mycelial growth (Latterell and Rossi, 1983). In a study of variability in *Cercospora zeae-maydis*, significant variation in pathogenicity by testing disease efficiency and lesion length was found within 15 isolates obtained from different regions of the eastern United States, suggesting that genetic variability exists in populations of *Cercospora zeae-maydis* (Bair and Ayers, 1986).

Cercospora zeae-maydis, like other host-specific *Cercospora* species, produces the nonspecific toxin, cercosporin. Cercosporin was first isolated in 1957 from mycelia of *Cercospora kikuchii*, a soybean pathogen (Kuyame and Tamura, 1957). Cercosporin, a photosensitizing chemical, is activated by light and converted to an electronically excited state, which transfers this energy to oxygen to generate either superoxide ions or singlet oxygen. Both of these free radicals can kill plant cells by oxidizing proteins,

lipids, carbohydrates, and nucleic acids (Daub, 1982, 1987).

There is evidence that cercosporin may play an important role in disease development. In sugar beets, for example, high light intensity stimulates the development of disease symptoms, indicating the effect of cercosporin (Calpouzos and Stalknecht, 1967). Steinkamp and coworkers (1981) used cercosporin to inoculate sugar beet leaves and found that lesions induced by cercosporin resembled, in both size and appearance, the lesions induced by the fungus *Cercospora beticola*, suggesting that cercosporin may be involved in eliciting some disease symptoms. Furthermore, members of the genus *Cercospora* have an extremely broad host range, and cercosporin is produced by virtually all of them. The production of such a generalized and toxic compound as cercosporin may help explain the almost universal pathogenicity found in the genus (Daub, 1982).

Gwinn, Stelzig and Brooks (1987) treated corn leaf tissues with cercosporin and found that tissue from older corn was less sensitive to cercosporin than tissue from younger corn. They also tested the responses to cercosporin treatment in three corn genotypes with different resistance levels and did not observe varietal resistance. They concluded that neither age- nor cultivar-dependent resistance to *Cercospora zeae-maydis* could be explained on the basis of differential sensitivity to toxin. Carter (1992) also investigated germplasm sensitivity to cercosporin and indicated that sensitivity to the toxin was not correlated with varietal responses to *Cercospora zeae-maydis* in the field. However, in contrast to the results obtained by Gwinn, Stelzig and

Brooks, she found that 39-day old plants were significantly more sensitive to the toxin than 21-day old plants. Although conclusion is not consistent, these observations may lead to further investigation.

GLS infection process

Field observations of disease development show that characteristic GLS lesions are linear-rectangular and delimited in breadth by leaf veins. The lesions are tan initially and become gray when dense sporulation occurs under favorable conditions. Early symptoms of gray leaf spot infection are pinpoint lesions which are surrounded by a yellow halo if observed by transmitted light. These pinpoint lesions elongate and develop into the characteristic GLS lesions within 2-3 weeks, somewhat longer than most other foliar pathogens. Generally, mature lesions are 1-6 cm long and 2-4 mm wide (Latterell and Rossi, 1983). Under heavy disease pressure, lesions may coalesce and blight the entire leaf. In heavily infected fields, stalk lesions are common, resulting from spread of the pathogen through leaf sheaths. Stalk deterioration may result in severe lodging (Stromberg, 1986).

Disease progression of gray leaf spot of corn was also investigated in the greenhouse (Beckman and Payne, 1982). Swelling of germ tube terminals on the lower leaf surface was observed 2-3 days after spore inoculation. Abundant appressoria were observed over stomata after 4-5 days. Initial penetration through stomata by some appressoria occurred at 7-8 days after inoculation. Colonization of

corn leaves by fungal mycelium was confined to the air spaces and intercellular spaces within the parenchyma tissue of the mesophyll. As host tissue became necrotic, conidiophores bearing conidia were found emerging through the stomatal openings from stromata formed in substomatal chambers (Beckman and Payne, 1982; Latterell and Rossi, 1983). These conidiophores were observed to be limited only to necrotic tissue (Beckman and Payne, 1982).

Cercospora zeae-maydis, like some other members of the same genus, has an extended period between inoculation and lesion development, which is usually believed to be due to the ability of *Cercospora* to sustain its growth on the leaf surface for long periods before penetration occurs (Beckman and Payne, 1982).

Environmental effects on disease development

Environment has been found to have considerable influence on development of gray leaf spot of corn. In the early 1970's, gray leaf spot was found predominantly in the river valley fields and mountains of the Appalachian region of the United States in late season (Leonard, 1974; Roane, Harrison, and Genter,1974), suggesting that high relative humidity, moderate temperatures, abundant rainfall, and high light intensity may favor disease development. A study of the effect of environment on gray leaf spot in Kentucky compared weather conditions in locations where gray leaf spot was present with conditions in locations where the disease was absent (Rupe, Siegel and Hartman, 1982). They found that GLS was restricted to locations having

long daily periods of high relative humidity and leaf wetness; however, during the months of disease development (August and September) temperatures were similar between sites with and without the disease. They concluded high relative humidity was a more important factor than temperature in affecting GLS disease development.

Latterell and Rossi (1983) also concluded that relative humidity is probably more critical than any other single factor in promoting severe disease development. In spore inoculation studies in the greenhouse, they found that GLS lesions did not develop until a " dew " chamber was used to provide essential moisture (Latterell and Rossi, 1983). By investigating the relationship of GLS severity to temperature, relative humidity and rainfall Beckman and his coworkers (Beckman, Payne and Campbell, 1981) demonstrated that high temperature and low rainfall did not limit the range of GLS disease. They suggested that severe gray leaf spot may be related to the occurrence of high relative humidity.

Genetics of Host Resistance to Gray Leaf Spot

It is believed that the recent increase in prevalence of gray leaf spot may be associated with the adoption of no-till or reduced tillage agronomic practices, and continuous corn production (Roane, Harrison and Genter, 1974; Latterell and Rossi, 1983). Latterell and Rossi investigated gray leaf spot severity in several neighboring corn fields and concluded that there were far fewer lesions in the fields planted by conventional tillage than in the adjacent fields planted by no-till methods (Latterell

and Rossi, 1983). Stromberg (1986) concluded that control of gray leaf spot could be enhanced by using conventional tillage practices and crop rotation methods. However, in some areas where gray leaf spot is a major problem, no-till or reduced tillage practices are preferred because of severe soil erosion. Also, no-till methods cost less for growing corn. In addition, many farms that produce corn are dairy farms that traditionally use corn as a major source of feed. It is unlikely that these farms would use rotation methods (Huff, Ayers and Hill, Jr., 1988). Therefore, developing corn varieties resistant or less susceptible to gray leaf spot is practical and economical for control of this disease.

Sources of germplasm resistant to gray leaf spot may be available from corn varieties from other countries or from some inbreds which are not widely used for breeding commercial hybrids. A tropical variety from France, immune to gray leaf spot, has been tested in greenhouse (Latterell and Rossi, 1983). However, most inbred or tropical lines showing strong GLS resistance lack good agronomic characters desirable for use in breeding. Incorporating gray leaf spot resistance into commercial hybrids would be a better way to control this disease.

The inheritance of host resistance to the fungus *Cercospora zeae-maydis* has been studied by means of diallel analysis (Thompson, et al., 1987; Huff, Ayers and Hill, Jr., 1988; Elwinger, et al., 1990; Ulrich, Hawk and Carroll, 1990; Donahue, Stromberg and Myers, 1991). Based on general and specific combining abilities, these studies concluded that the inheritance of resistance to gray leaf spot is quantitatively additive

and controlled by few, rather than many, genes. The results indicated that while currently used commercial hybrids are largely susceptible to gray leaf spot, resistant inbreds do exist from which resistance genes could be transferred by conventional backcrossing and selection techniques.

Screening for Resistance to Gray Leaf Spot

Evaluation of resistance levels of hybrids and inbreds to gray leaf spot has been going on for many years. In 1973, Roane et al found very low resistance among 49 corn hybrids infected by *Cercospora zeae-maydis* naturally. The degree of resistance found was characterized by "chlorotic", as opposed to "necrotic", lesions on upper leaves (Roane, Harrison and Genter, 1974). In Tennessee, of 35 maize cultivars (including hybrids and inbreds) observed, only inbred T 222 had high resistance, indicating the resistance level was very low in the maize population tested (Hilty, Hadden and Garden, 1979). A diallel study of 14 elite inbreds was conducted to investigate the inheritance of reaction to gray leaf spot and the performance of these corn varieties (Donahue, Stromberg, and Myers, 1991). Data on GLS disease ratings, plant lodging, grain yield, and grain moisture were taken during the growing season. Disease severity was rated by this scale: 0 = no symptoms; 1 = trace of lesions below the ear; 2 = many lesions below the ear; 3 = large lesions below the ear and lesions on all leaves above the ear; 4 = all leaves with large lesions; 5 = all leaves dead. Based on the general combining ability (GCA) values for GLS rating, levels of resistance to GLS in the 14 inbreds were determined. Lines PA875 and

VA14 had the lowest GCA effects for GLS rating (-0.5 and -0.4, respectively), with B68, KB1250, NC250, VA17, and VA85 all being below zero. These lines were classified as resistant. Lines B73 and PA91 had the highest GCA effects of 0.6 and 0.5, with A632, H93, and VA22 all being strongly positive. These lines were classified as susceptible. Lines MO17 and VA35 were near zero in their GCA effects and were classed as intermediate. Similar results were also reported by Thompson, et al. (1987), Huff, et al. (1988), Elwinger, et al. (1990), and Ulrich, et al. (1990).

When evaluating resistance levels in the greenhouse and field, Latterell and Rossi (1983) observed several types of resistance to gray leaf spot, ranging from immunity (no lesions), high resistance (fleck-type lesions), to tolerance (slowly developing or relatively few lesions).

In field tests, environmental factors often influence the expression of disease. Thus, several researchers have attempted to develop resistance screening techniques in the greenhouse. The most important factor to be considered for screening resistance to gray leaf spot in the greenhouse is the presence of high relative humidity (Beckman and Payne, 1982 and 1983; Latterell and Rossi, 1983). Beckman and Payne (1982) also investigated the influence of plant genotype on disease expression by inoculating 10 corn cultivars with a spore suspension in the greenhouse, but they did not find genotype-dependent resistance expression among these cultivars. The symptoms they observed in the greenhouse were similar to the symptoms observed in fields.

The late-season appearance of gray leaf spot and the development of lesions beginning on the lower leaves suggests that susceptibility may be affected by plant or leaf age. To study the effect of plant maturity on susceptibility to gray leaf spot, Rupe and coworkers (1982) planted corn five times at 3-week intervals from May 9 to August 2 and found that on all five plantings initial disease symptoms didn't appear until the plants were near anthesis. The symptoms first appeared on the lower, then moved to the middle, and finally to the upper leaves.

In a study on genotype- and plant age-dependent resistance to gray leaf spot, 1-, 2-, and 3-month-old plant leaf disks from three cultivars with different degrees of resistance to *Cercospora zeae-maydis* were inoculated with a mycelial suspension of *Cercospora zeae-maydis* (Gwinn, Stelzig and Brooks, 1987). Significantly more stomata of older plant tissues were penetrated by the fungus, indicating that older leaf tissue is more susceptible to gray leaf spot. However, no varietal differences were detected, suggesting that the mechanism of age-dependent resistance to *Cercospora zeae-maydis* is different from the genotype-dependent resistance observed in the field.

MATERIALS AND METHODS

Plant Materials

The corn varieties used in this study consisted of five inbred lines: VA14, PA875, B68, B73, and MO17. These inbreds were selected based on previous field performances in tests for resistance to GLS (Donahue, Stromberg, and Myers, 1991; Elwinger, et al., 1990; Huff, Ayers, and Hill, Jr., 1988; Thompson, et al., 1987; Ulrich, Hawk, and Carroll, 1990) and represented a range of disease susceptibility and genetic background (Table 1). PA875 is the most resistant inbred in this group, VA14 and B68 are moderately resistant, while B73 and MO17 are relatively susceptible to GLS.

Seeds of these five inbreds were provided by Dr. E. L. Stromberg and Dr. M. A. Saghai-Maroof of Virginia Polytechnic Institute and State University. The seeds of these inbreds were planted in pots (one seed per pot), which contained mixed artificial potting soil made at the ratio of 1 part of vermiculite, 2 parts of weblite, one-half part of peat moss, 226.8 gram of osmocote 14-14-14, and 56.7 gram of lime. The greenhouse is equipped with artificial lights which are on 8 hrs a day in winter. The range of temperature in the greenhouse is between 22 and 30°C.

Fungal Cultures and Inoculum Production

The isolate of *Cercospora zeae-maydis*, collected by Dr. E. L. Stromberg in Mount

Table 1. Derivation, origin and level of disease resistance of maize inbreds used in greenhouse experiments on GLS responses.

Inbred line	Level of resistance	Derivation	Origin
B68	R*	(41.2504B x $B14^3$) Sel.**	Iowa***
B73	S	Iowa Stiff Stalk Syn. C_5 Sel.	Iowa
MO17	S	187-2 x C 103	Missouri
PA875	R	PA WF9 Syn.	Pennsylvania
VA14	R	(VA CBS Sel. x VA17) VA17	Virginia

* Levels of disease resistance were based on observations made in field previously (R = resistant, S = susceptible).

** Henderson (1984).

*** Lines having a state origin were released by that state's land grant institution.

Jackson of Virginia from corn leaves infected by gray leaf spot disease, was kindly provided by Dr. H. L. Warren (Virginia Polytechnic Institute and State University). This culture was derived from a single spore isolate. The culture was stored on V-8 juice agar media (300 ml of V-8 vegetable juice, 700 ml of distilled water, 3g of $CaCO_3$, and 15g of agar) in the refrigerator at 4 °C. To prepare mycelial culture for inoculation of corn in the greenhouse, mycelia were cultured on V-8 juice agar, potato dextrose agar (PDA), or Whites agar media (Table 2). Two or three weeks later, a piece of mycelial colony including some agar media was placed in a 50-ml aseptic plastic centrifuge-tube containing 20 ml of sterile water and homogenized 30-60 sec at high speed with a Tissuemizer homogenizer. The homogenate was pipetted in 1 ml samples into 100-ml flasks containing 25 ml Malt liquid medium. After culturing one week, 10 ml of mycelium suspension in Malt liquid medium was removed and homogenized 60 sec. The homogenate was pipetted in 3 ml samples into 250-ml flasks containing 100 ml Malt liquid medium. These mycelial suspensions were cultured one week more for preparation of inoculum. All petri dishes for solid culture and flasks for liquid culture were placed in an incubator at 25 °C and under diurnal light (12 hr of fluorescent light, 12 hr of dark). For liquid culture, the flasks containing mycelial suspension were placed on a shaker at 150 RPM.

GLS Screening Methods

Preparation of mycelial discs for inoculation. Mycelial suspension during log growth phase was collected after two rounds of liquid culture and homogenized 1 min at

Table 2. Culture media for *Cercospora zeae-maydis*.

Type of medium	Principle components (per liter medium)		Purpose
V-8 juice	V-8 vegetable juice $CaCO_3$	300 ml 3 g	Solid culture for mycelium growth
PDA	Difco potato dextrose broth	24 g	Solid culture for mycelium growth
Whites	Whites basal saltmix Sucrose	1 g 20 g	Solid culture for mycelium growth
Malt	Difco malt extract Peptone Glucose	15 g 3 g 30 g	Liquid culture for mycelium growth

high speed with a Tissuemizer homogenizer. The homogenate was centrifuged 3 min at high speed and the precipitate was washed with distilled water and centrifuged again. The new precipitate was washed with distilled water and centrifuged two more times. The fresh weight of centrifuged mycelium was measured. Finally, the mycelial suspension was made to a concentration of 450 mg fresh weight of mycelia per ml distilled water. 100 ul of the above mycelial suspension was pipetted onto each 6 mm-diameter filter paper disc (Whatman, grade 3) made by a paperhole punch, while the water vacuum was used to make filtering efficient. The same mycelial suspension was autoclaved for 20 min and pipetted onto filter paper discs as control.

Inoculation. Four plants for each of five maize varieties (VA14, PA875, B68, B73 and MO17) were inoculated at 4-6 week old with 8-10 leaves. Two leaves of each plant representing different leaf ages were inoculated by sticking three mycelium discs to the lower leaf surface with a piece of transparent packing tape on one side of the main rib at the middle position lengthwise of leaf. On the opposite side of the main rib, three filter paper discs containing autoclaved mycelium were stuck by transparent tape as control.

Environmental conditions. The inoculated plants were placed in a transparent plastic tent where a humidifier was used to provide approximately 90% relative humidity. The temperature in the greenhouse ranged from 22-30 °C.

Data collection. Observations were made at 3- or 4-day intervals. Types of host responses were recorded. Symptoms were rated by percent affected leaf area under

mycelial discs on a scale of 0-5 with 5 representing 100% of the leaf area affected under the mycelial discs. Disease severity was rated by percent leaf area covered by necrotic lesions on a scale of 0-5 with 5 representing 100% of the leaf area covered by necrosis.

Microscopy

Microscopy observations of conidiation were made on whole mount level. Inoculated leaf discs removed at 3- or 4-day intervals were fixed and cleared in the 1:1 acetic acid and alcohol solution for 24 hours. Cleared samples were stained with 0.5% aqueous trypan blue for 2-3 hrs, washed with distilled water for 2 min and observed under microscope.

RESULTS

Development of Inoculation System

In this study, mycelia of *Cercospora zeae-maydis* were used as inoculum to screen corn inbreds for resistance to gray leaf spot disease in the greenhouse. In preliminary experiments, we attempted to use *C. zeae-maydis* mycelial pads which were peeled from agar medium surface to inoculate corn leaves. While some lesions were induced by this method, the amount of response was small and lesion development was slow. Additionally, control experiments demonstrated that corn leaf tissues were sensitive to the fresh agar media. We also tried several other inoculation methods, including brushing corn leaf surfaces with conidial suspension, using microhumidity chambers, and spraying mycelial suspensions on plant leaves with an atomizer. However, no disease symptoms were observed after inoculation with these methods. Thus, a new technique based on applying filter paper discs containing cultured mycelia was developed.

The first step in making a mycelial inoculum was to investigate how to culture the fungal mycelia. Mycelia were initially cultured on agar media. Although mycelia grew well on V-8 juice, PDA and Whites agar media, Whites agar medium was used in this study to maintain mycelial growth because more toxin , visualized as reddish pigment, was produced by mycelia on this medium. Cultures were grown on Whites agar media for two weeks, and a piece of mycelial pad was taken from the surface of agar

medium and cultured in malt liquid media for 1 week after homogenization. Then two milliliters of this liquid culture was taken, homogenized in malt liquid media and then cultured on a shaker (Color plate 1A) for another week to increase the amount of mycelial suspensions.

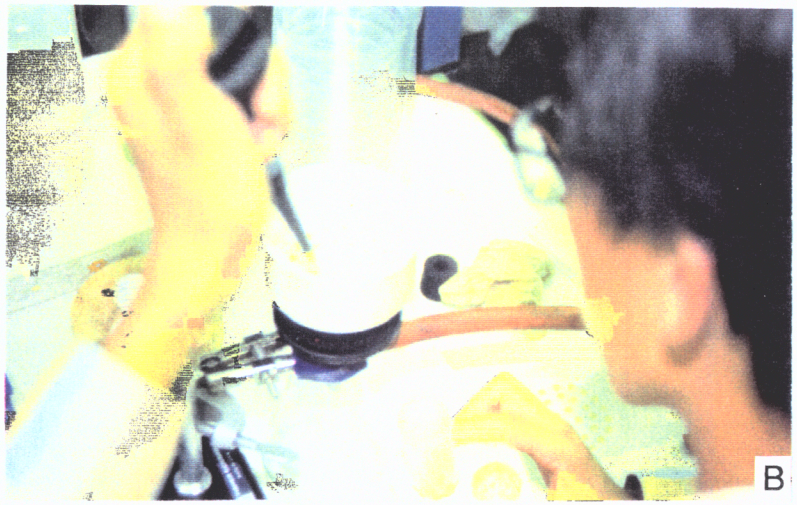
The second step was to prepare inoculum and pipet it onto filter paper discs (Color plate 1B). Whatman filter paper (grade 3) discs were used to carry mycelia because its thickness can hold adequate moisture and it was not toxic to plant tissues. Mycelial inoculum was pipetted to each filter paper disc at 100 ul of inoculum per disc so that each contained equal amounts of mycelia per unit area.

The third step was attaching filter paper discs of mycelia onto leaf surfaces (Color plate 1C, 1D) by using transparent tape. The tape also helped keep high moisture levels between corn leaves and tapes keeping filter paper discs from drying. Typically three identical discs were attached about 1 cm apart parallel to the leaf vein. Heat-killed mycelial discs were attached on the opposite side of the vein as control. All of the inoculated corn plants were placed in a plastic chamber where a humidifier provided a humid environment of 90% relative humidity for gray leaf spot disease to develop.

In order to determine the best part of the corn leaf to use for inoculation we performed experiments to inoculate fungal mycelia on upper vs. lower leaf surfaces. The results are shown in Table 3. More severe host responses were induced when mycelial discs were applied on the lower leaf surfaces. Application of mycelial discs

Color plate 1. Primary steps in the inoculation method with *C. zeae-maydis* mycelium impregnated filter paper discs included liquid cultures of mycelia in malt media (A), preparation of mycelium impregnated filter paper discs (B), and use of transparent tape to stick mycelium impregnated filter paper discs to lower leaf surfaces of corn (C, D).


A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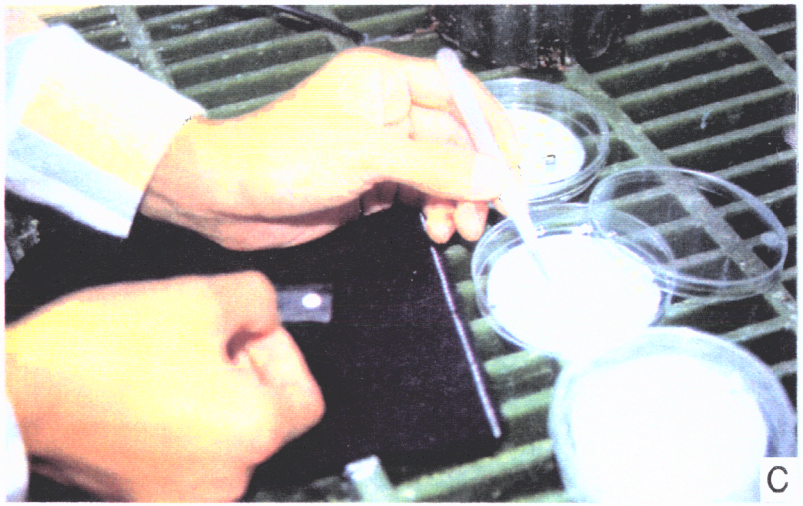

C

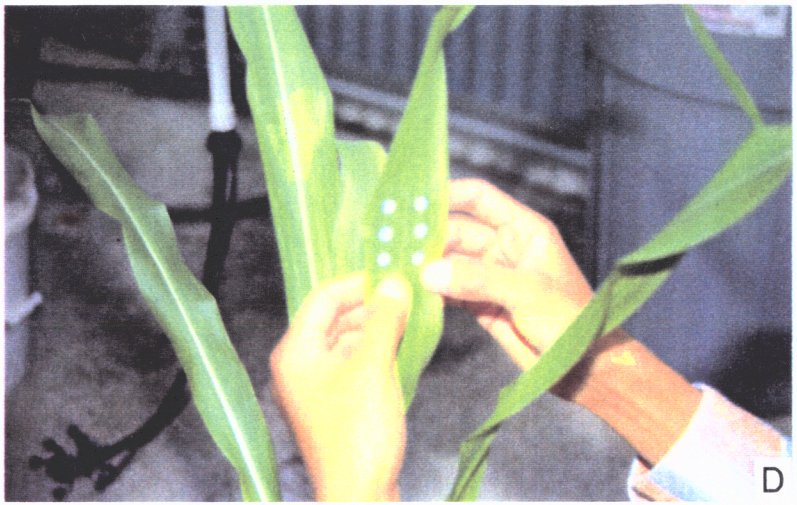

D

Table 3. Comparison of host responses from inoculating fungal mycelia on upper vs. lower leaf surfaces.*

Corn inbred	Applied on upper surface		Applied on lower surface	
	Total trials	No. causing responses	Total trials	No. causing responses
B73	12	0	12	8
VA14	24	5	20	20

* Plants were inoculated 6 weeks after planting with mycelium impregnated filter paper discs and data were collected 1 week after inoculation.

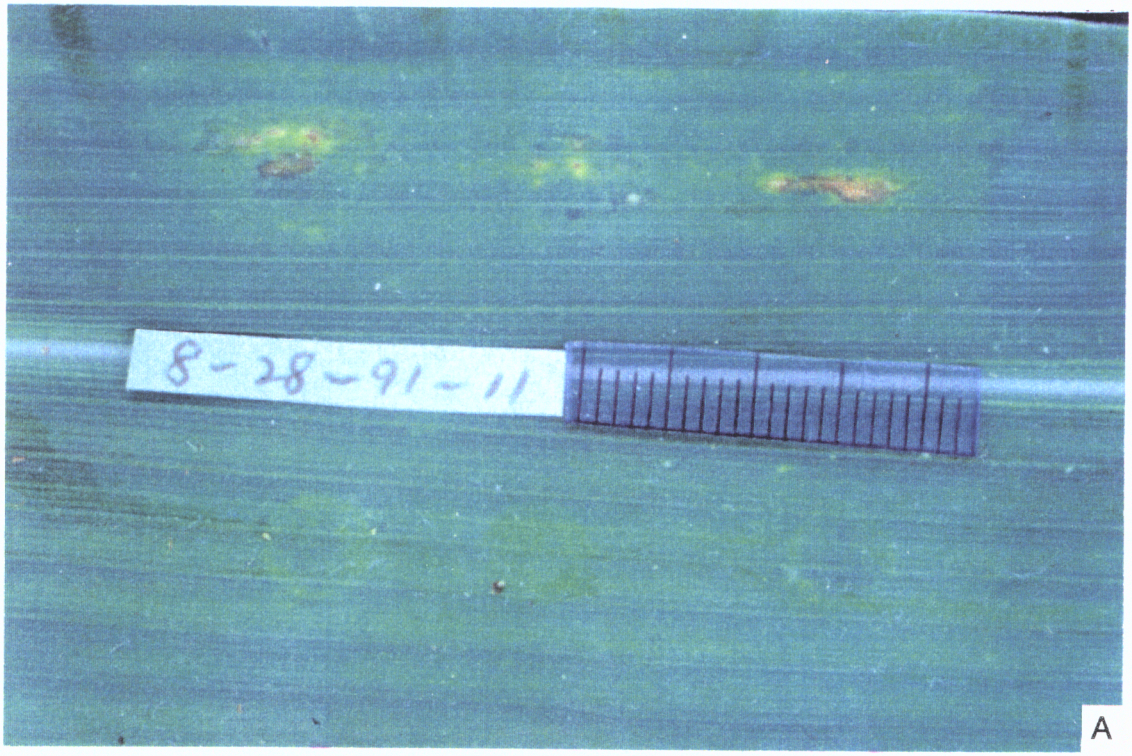
on the upper leaf surfaces caused reduced symptoms. Therefore, all subsequent experiments in this study were performed by placing mycelial filter paper discs on lower leaf surfaces.

Disease Responses of Host

We evaluated GLS resistance levels of five inbred lines (VA14, B68, PA875, B73, and MO17) by inoculating corn leaves with filter paper discs containing mycelia. The appearance of symptoms and the time course of development of host responses were different among these five lines. The first visible symptoms appeared 3-4 days after inoculation. Under favorable conditions, in B73 and MO17, more and more lesion spots appeared and enlarged with time (Color plate 2A, 2B). These necrotic lesion spots eventually coalesced and even expanded beyond the area of the original discs (Color plate 3).

Among the five genotypes tested in the greenhouse, there were three types of host responses observed. In B73 and MO17, the initial symptoms appeared as small dark chlorotic flecks 3-4 days after inoculation. Subsequently, these chlorotic flecks developed into yellow necrotic lesions (Color plate 4A). In inbred line PA875, symptoms were always water-soaked in appearance (Color plate 4B) and initially occurred 3 days after inoculation. These water-soaked symptoms were irregular in shape and had no sharp boundaries. They were usually bigger than other types of lesions in area and developed so fast that they coalesced and covered the entire leaf

Color plate 2. Development of susceptible lesions on MO17 induced by *Cercospora zeae-maydis* mycelial discs 8 days after inoculation (A) and 17 days after inoculation (B). The three mycelial discs were attached on the bottom leaf surface. Three heat-killed mycelial discs were placed on the opposite side of the leaf vein as controls.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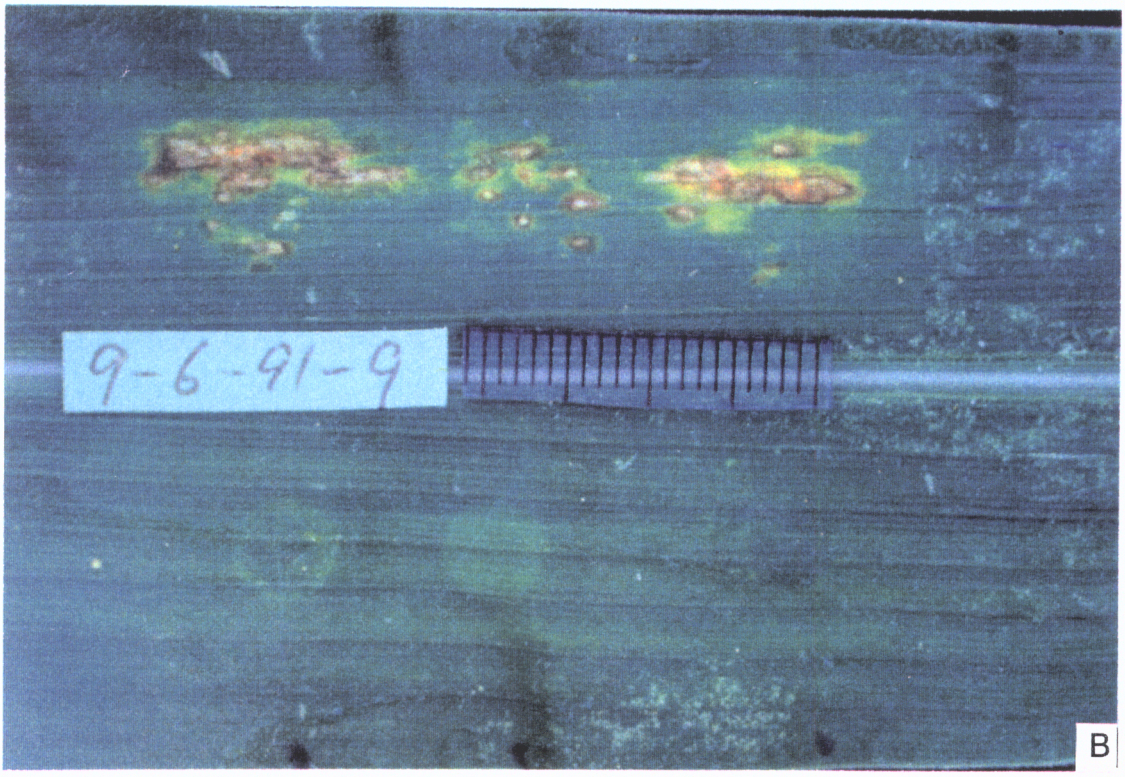
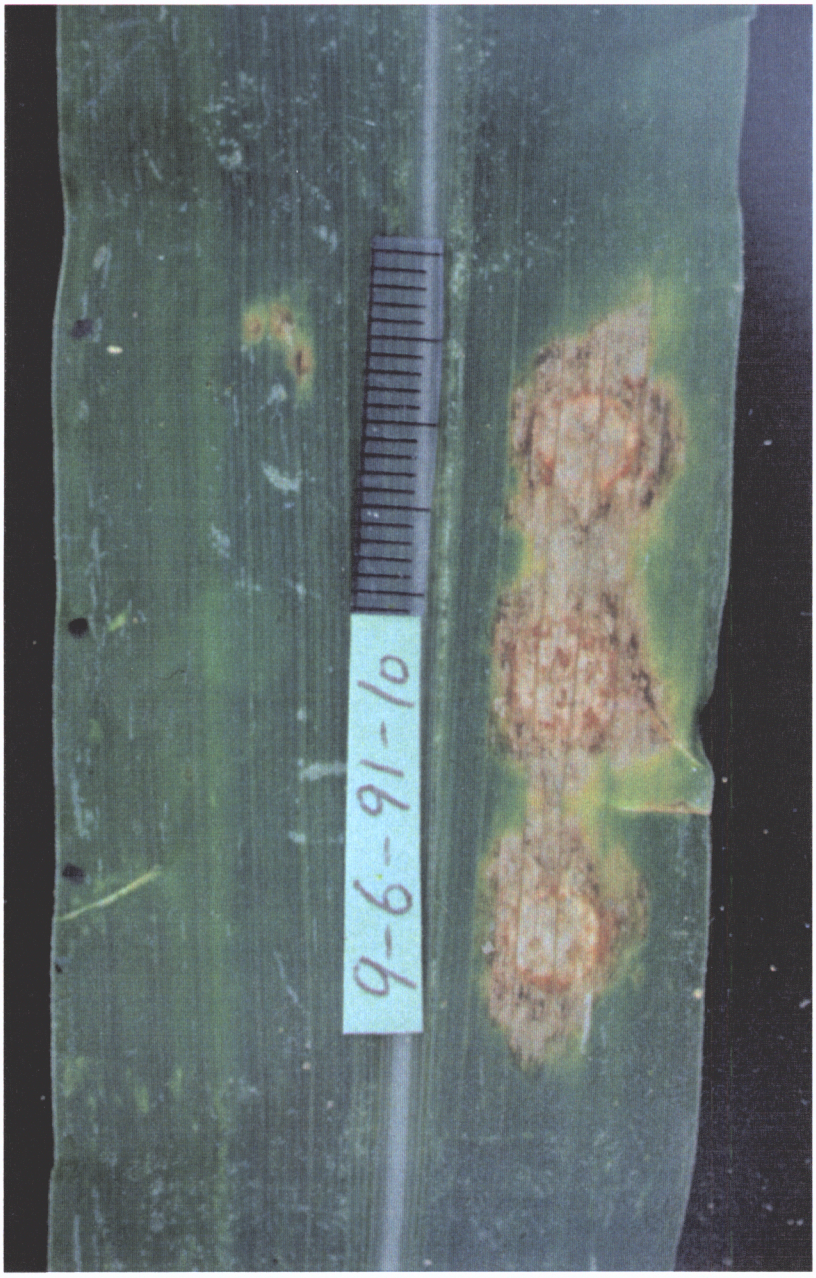
A


9-6-91-9
B

Color plate 3. Extensive necrotic lesions expanding beyond discs on MO17 induced by *Cercospora zeae-maydis* mycelial discs 17 days after inoculation. The three mycelial discs were attached on the bottom leaf surface. Three heat-killed mycelial disc controls were placed on the opposite side of the leaf vein.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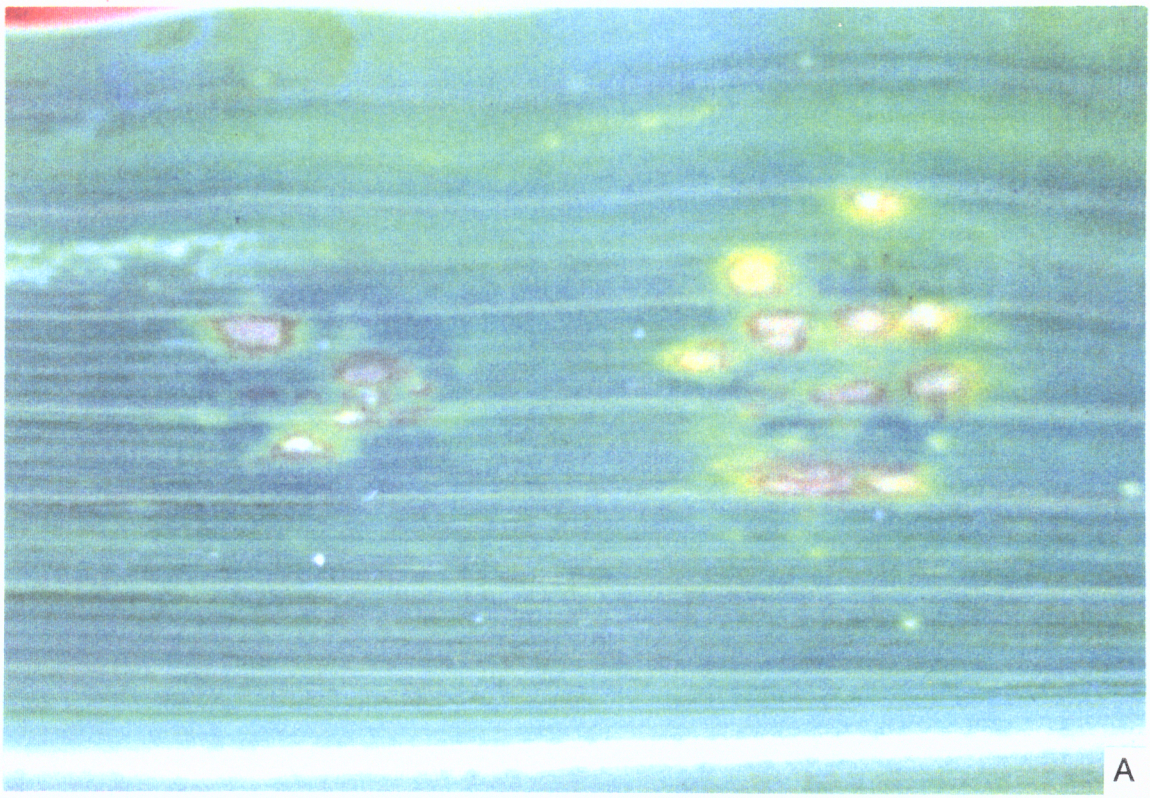
Color plate 4. Comparison of susceptible-type lesions on MO17 (A) with resistant-type responses on PA875 (B) 7 days after inoculation.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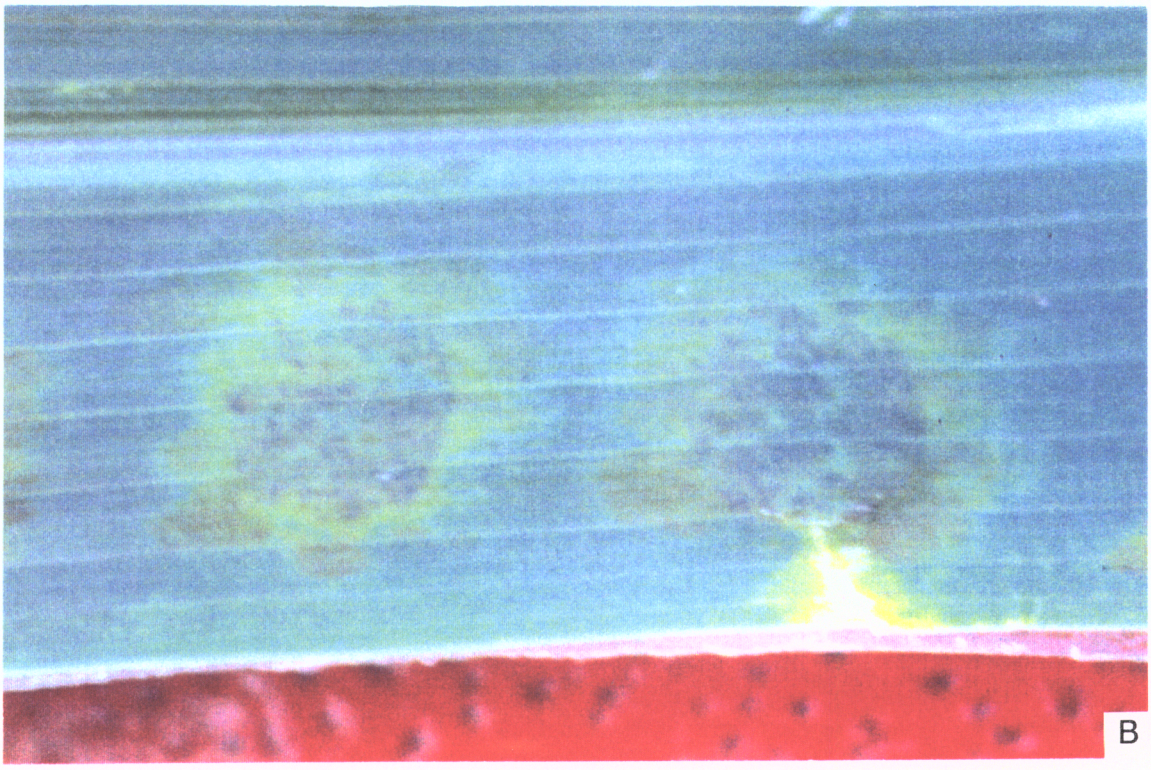
B

surface under the mycelial disc by 10-15 days after inoculation. These water-soaked leaf tissues were actually not dead. In VA14 and B68, the symptoms were either this water-soaked response or small chlorotic flecks, or a mixture of these. The chlorotic flecks were usually small and dark colored. The boundaries of these chlorotic flecks were very distinct. These chlorotic flecks enlarged very slowly and never became necrotic. An associated red pigment was occasionally visible in VA14 responses.

Quantitative measurements of host responses in the five inbreds were also investigated. First we assessed percent leaf area under each disc that was affected by exposure to mycelia. In Table 4, the symptoms observed in inbreds VA14 and B68 were small chlorotic flecks. They developed very slowly and resulted in relatively small affected leaf areas. The ratings were 0.8 in VA14 and 1.0 in B68 after 20 days. In B73, necrotic lesions enlarged very quickly and coalesced. Some lesions expanded beyond mycelial discs even after 8 days, when the rating for percent affected area was 4. At 20 days the rating for affected leaf area in B73 was 4.6. MO17 showed the same type of necrotic lesions as B73 but the rating was lower (2.6 at 20 days). However, this was much higher than the ratings for VA14 and B68. In PA875, the water-soaked responses resulted in high ratings of total affected area, with 4.7 at 20 days.

To evaluate disease severity of the five inbred lines exposed to mycelial discs in the greenhouse, we estimated the percent necrotic area observed under the mycelial discs. Table 5 shows the disease severity ratings measured by necrotic areas from the same experiment shown in Table 4. The ratings of B73 were highest, ranging from

1.3 at 4 days to 4.6 at 12 days. The range for MO17 was between 1.5 and 2.6 during the same period. The disease severity ratings remained at 0 in VA14, B68 and PA875 because they never showed necrotic lesions.

The Effect of Leaf Age on Host Responses to *C. zeae-maydis* Mycelia

In the experimental results shown in Table 6, the effect of leaf age was investigated by inoculating the 2nd through 5th upper leaves of 6-week-old plants in the greenhouse. There were four replicate plants of each genotype. Table 6 presents the differences in the degrees of host responses observed in different age leaves. For all of the five genotypes tested, older lower leaves were generally more sensitive to mycelial discs than younger upper leaves based on percent leaf area affected by mycelial discs. For example, in MO17, the leaf area affected was 0.7 on the second leaf from the top , and 4 on the fifth leaf from the top.

Differential Responses of Corn Inbreds to Wild and Mutant Fungus

The pathogen *Cercospora zeae-maydis* produces a toxin, cercosporin, which is a red pigment. The mycelial cultures used in these experiments produced very high levels of cercosporin. To examine the role of cercosporin in disease progression, we compared the host responses from wild mycelial cultures with those from a mutant isolate of *C. zeae-maydis* which does not produce cercosporin under conditions of these experiments. Filter paper discs containing either wild-type fungal mycelia or

Table 4. Percent leaf area affected by mycelial discs*

inbred line	Days after inoculation 4	6	8	12	20
VA14	0.2	0.3	0.7	0.8	0.8
B68	0.5	0.7	0.8	1.0	1.0
PA875	1.0	1.7	2.9	4.0	4.7
B73	1.3	2.8	4.0	4.6	4.6
MO17	1.5	1.6	1.7	2.1	2.6

* Plants were inoculated 5 weeks after planting and the 3rd top leaves were inoculated with three mycelial discs. There were four replicates of each corn genotype. Data, shown by mean value, were recorded on a 0-5 scale with 5 representing 100% affected area exposed to mycelial discs.

Table 5. Percent leaf area covered by necrotic lesions*

inbred line	Days after inoculation 4	6	8	12	20
VA14	0	0	0	0	0
B68	0	0	0	0	0
PA875	0	0	0	0	0
B73	0	2.8	4.0	4.6	4.6
MO17	0	1.6	1.7	2.1	2.6

* Plants were inoculated 5 weeks after planting and the 3rd leaf from the top of each plant was inoculated with three mycelial discs. There were four replicates of each corn genotype. Data, shown by mean value, were recorded on a 0-5 scale with 5 representing 100% necrotic area exposed to mycelial discs.

Table 6. The effect of leaf age on host responses*

Inbred line	Percent leaf area affected by mycelial discs			
	2nd top leaf	3rd top leaf	4th top leaf	5th top leaf
VA14	0.2	1.7	0.7	3.0
B68	0.2	2.8	2.5	4.8
PA875	2.0	3.3	5.0	5.0
B73	0.0	0.5	0.7	1.7
MO17	0.7	0.8	2.2	4.0

* Plants were inoculated 6 weeks after planting. Three mycelial discs were placed on each leaf. There were four replicates of each genotype. Data, showed by mean value, were recorded on a 0-5 scale with 5 representing 100% affected leaf area exposed to mycelial discs at 8 days after inoculation.

mutant-type fungal mycelia were attached to each side of a corn leaf rib. Table 7 presents the ratings of percent leaf area affected by wild versus mutant fungal mycelia 11 days after inoculation. Although some ratings were not as high as expected, i.e., the ratings for B73 and MO17 were somewhat low, it is clear that the host responses caused by wild-type fungus were much higher than those caused by mutant cultures. This shows that the wild-type fungus is more pathogenic than the mutant fungus which can not produce cercosporin. This supports a role for fungal toxin in the disease infection process.

Correlation of Disease Resistance with Conidiation

To investigate the role of sporulation of *C. zeae-maydis* during greenhouse exposure of corn lines to the fungus, we fixed inoculated leaf samples from four inbreds, VA14, PA875, B73 and MO17 for microscopic examination of conidiation. Leaf tissues under mycelial discs were collected at 3 - 4 day intervals from 8 days to 26 days. Conidiophores were not observed until 15 days after inoculation in B73 and MO17, the two susceptible cultivars. All of the conidiophores observed were limited to necrotic leaf tissue. Under the microscope, conidiophores bearing many conidia emerged in clusters from substomatal cavities (Color plate 5). This was particularly evident by their regular arrangement in rows on the leaf surfaces. During the period of observation from 8 days to 26 days, no conidiation was found in the two resistant cultivars, VA14 and PA875.

Table 7. The effect of pathogen type on host response*

Genotype	Percent leaf area affected by mycelial discs	
	wild type fungus	mutant type fungus
VA14	1.6	0.1
B68	0.4	0.0
PA875	3.0	0.7
B73	1.5	0.0
MO17	1.1	0.3

* Plants were inoculated 39 days after planting and the 3rd leaf from the top of each plant was inoculated with three wild type mycelial discs and three mutant type mycelial discs. There were four replicates of each genotype. Data, showed by mean value, were recorded on a 0-5 scale with 5 representing 100% affected leaf area exposed to mycelial discs at 11 days after inoculation.

Color plate 5. Conidiophores emerging from substomatal cavities on the leaf surface of MO17 19 days after inoculation with filter paper discs of *C. zeae-maydis* mycelium. 25x.

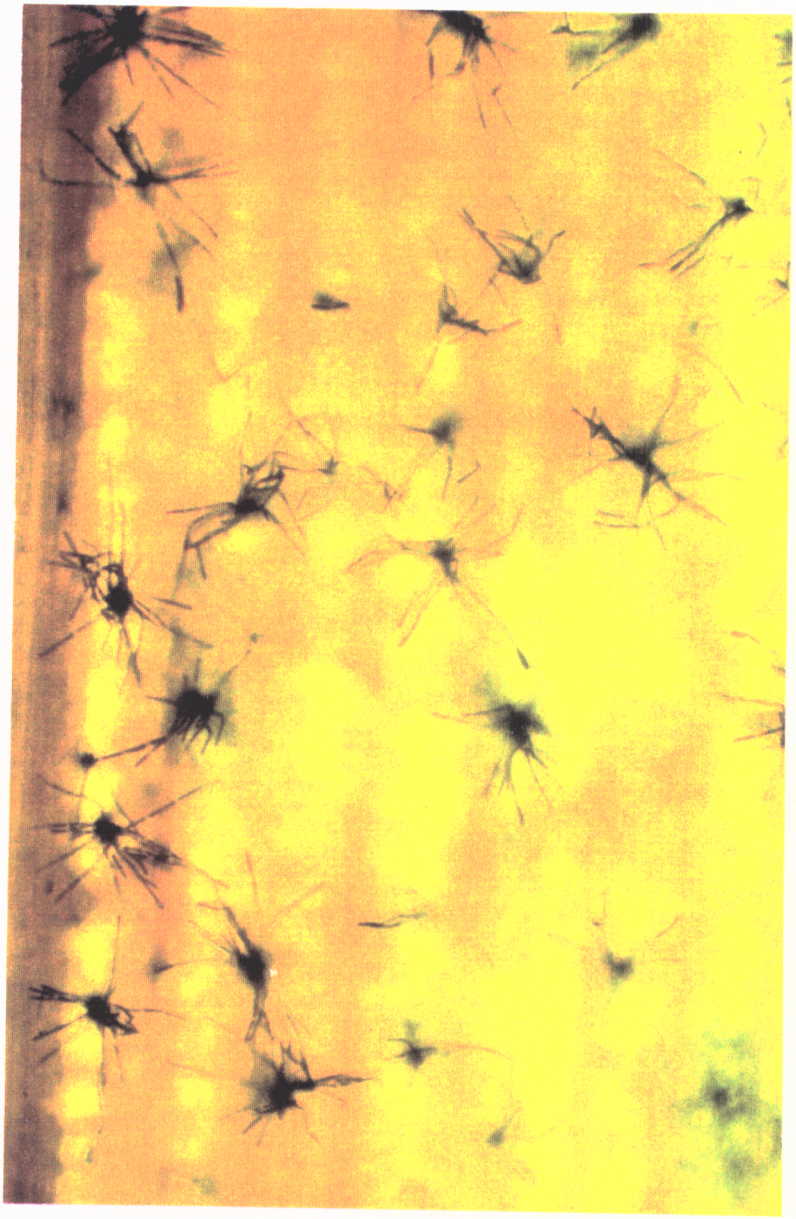

Table 8 shows conidiation responses examined 19 days after inoculation. In B73, on 9 of 13 leaf discs covered by extensive necrosis were conidiophores observed. No conidiophores observed on 11 leaf discs in VA14.

Table 8. The effect of host genotype on conidiation*

Genotype	No. of Leaf discs under mycelia examined	No. of Leaf discs having conidiophores
MO17	4	1
B73	13	9
VA14	11	0
PA875	5	0

* Plants were inoculated 5 weeks after planting. Conidiation was examined 19 days after inoculation.

DISCUSSION

Gray leaf spot of corn has become an increasingly important disease in corn producing areas in recent years. It is believed that development of GLS resistant maize germplasm has the potential to be the most effective and economical method of preventing yield losses due to GLS. Several field studies have identified GLS resistant germplasm, which may be used in breeding programs to develop high-yielding, GLS resistant elite inbreds and hybrids. However, field screening of large numbers of corn genotypes for GLS resistance is time-consuming and strongly affected by environmental conditions. Advanced lines must be field-tested for disease severity and yield loss, but initial screenings could be accelerated by the availability of greenhouse testing. In the greenhouse where environmental conditions are under control, disease reactions could be compared reliably.

Inoculation System in the Greenhouse

We believe that inoculation of corn seedlings with mycelial discs has advantages over previous methods of inoculation by spraying spore suspensions. Beckman and Payne (1982) investigated histologically the external growth, penetration, internal colonization, and sporulation after inoculation of corn seedlings with spore spray. They found that the disease development was established from spores through a long and complex process which included germ tube growth, appressorium formation, appressorium penetration, mycelium formation and growth, stroma formation, and

conidiation. Penetration of appressoria through stomata occurred 6-7 days after inoculation. After penetration, mycelia grew within parenchyma tissue of the mesophyll intercellularly from the substomatal cavity. The first visible disease symptom appeared as chlorotic dots only after the development of abundant fungal mycelia in the leaf tissue 9 days after inoculation. In contrast, our results using mycelial discs showed initial symptoms observed as early as 3 or 4 days after inoculation. Although the mechanism of infection starting with mycelia is not known yet, the fact that initial symptoms occur earlier than with spore application implies that mycelia can penetrate leaf tissue directly and independently of spore germination.

Inoculation of corn seedlings with filter paper discs containing mycelia also permitted quantitative measurements of host responses developing in a defined and limited leaf area. Each filter paper disc held defined amounts of mycelia which permitted us to accurately compare responses to *C. zeae-maydis* between hosts. In contrast, difficulty in spraying spore suspensions on leaves uniformly could easily result in inaccurate disease severity ratings.

The fungus *Cercospora zeae-maydis* favors high relative humidity during infection and colonization. Two plastic tent chambers provided with humidifiers were built in our greenhouse to provide high relative humidity up to 95%. These facilities were so important that without them no GLS lesions developed. This result is consistent with previous greenhouse and field studies (Beckman, Payne and Campbell, 1981;

Beckman and Payne, 1982; Latterell and Rossi, 1983), showing the importance of humidity and moisture for GLS development.The significance of high humidity and long periods of leaf wetness for GLS lesion development was also demonstrated by the usefulness of transparent tape for attaching inoculation discs. The transparent tape maintained a moist microenvironment between leaf surface and tape which provided the inoculum with the high relative humidity and moisture levels required for GLS disease development. The application of transparent tape also insured that the inoculum would stay on the leaf surface. We initially investigated methods of spraying or brushing liquid inoculum onto both leaf surfaces of corn as suggested by Beckman and Payne (1982). However, these inoculation methods didn't induce GLS lesions for us even in the presence of high relative humidity. These results indicated that the application of transparent tape could prevent the inoculum from being washed off leaf surfaces by water condensed on leaf surfaces, thus, enhancing the efficiency and reliability of GLS disease development.

The significant difference in host responses resulting from inoculating fungal mycelia on upper vs. lower leaf surfaces was surprising. It is so far unclear why mycelial discs on the upper leaf surfaces were less effective. It may be partially explained by the fact that the upper leaf epidermis has fewer stomata than the lower leaf epidermis through which mycelia could penetrate. It could also be that other differences in unknown chemical or physical composition of corn leaf surfaces could have produced this result.

Disease Response of Host in the Greenhouse

We evaluated five corn inbred lines including VA14, B68, PA875, MO17, and B73 which were inoculated with filter paper discs containing mycelia of *Cercospora zeae-maydis*. These lines covered susceptible and resistant response types in previous field trials. With our inoculation method, these five corn genotypes were easily classified into two groups according to host response type. One group, including MO17 and B73, was characterized by a necrotic lesion type. Another group, including VA14, B68 and PA875, was characterized by water-soaked or chlorotic-fleck response types. This grouping reflects GLS resistance evaluations in previous field tests of these varieties (Thompson, et al., 1987; Huff, Ayers and Hill, Jr., 1988; Elwinger, et al., 1990; Ulrich, Hawk and Carroll, 1990; Donahue, Stromberg and Myers, 1991). The necrotic lesion type was exhibited by field GLS susceptible lines in our tests. Water-soaked and chlorotic-fleck responses were observed in GLS resistant corn lines. These results support the conclusion that types of host responses to our tests are reliable in the evaluating corn germplasm for resistance to GLS.

Quantitative ratings of greenhouse tests were also helpful in evaluating host responses to the pathogen. Within all of the five tested varieties, the amount of necrosis covering leaf surface exposed to mycelia was positively correlated with susceptibility of these lines to GLS. In contrast, when evaluations were made on the basis of " total affected (Percent) leaf area " under discs the results were not reliable because they included chlorosis and water-soaked responses in addition to

necrosis. Chlorosis and water-soaked responses appeared to be associated with resistant phenotypes in some lines other than susceptibility to GLS. Thus, PA875, a field resistant line, showed extensive water-soaked responses to fungal mycelia in our tests but these lesions did not result in tissue death. We, therefore, conclude that quantitative rating based on necrosis (but not "total affected area") is a reliable indicator of GLS resistance in our greenhouse test.

Quantitative measurements of host responses to mycelial discs often showed variation between experiments. The variation levels among the five inbred lines were not the same. In PA875 and VA14, the variation was least between experiments. In B73 and B68, the variation between experiments was larger. The variations in MO17 were moderate.

Several previous studies of other plant diseases have reported similar results to those reported here. Nicholson and Warren (1975) evaluated 183 corn inbreds in the greenhouse for resistance to maize anthracnose. They found that neither lesion size nor extent of tissue coverage was an adequate measure of resistance to anthracnose under greenhouse conditions. However, they found that the type of lesion formed fit a pattern for susceptible, resistant and hypersensitively resistant host responses. In a study to investigate the components of resistance in peanut to *Cercospora arachidicola*, twenty genotypes of peanut were tested in the greenhouse (Ricker, Beute and Campbell, 1985). When five components of rate-reducing resistance were followed (number of lesions per leaf, lesion diameter, latent period, time until leaflet

defoliation, and sporulation), lesion diameter was always similar among genotypes and did not reflect disease resistance levels. As for lesion numbers, since rankings of genotypes by lesion number changed dramatically between trials, it was concluded that number of lesions was also an unreliable means to evaluate peanut genotypes for resistance to early leaf spot in the greenhouse. In another study conducted to examine effects of environmental conditions on expression of resistance to *Cercosporidium personarum* in peanut, temperature and duration of relative humidity were found to have a greater impact on lesion numbers than did genotype (Shew, Beute and Wynne, 1988).

It is likely that the variations in quantitative estimation of responses to GLS observed between our greenhouse experiments were strongly influenced by environmental factors. Although we placed a humidifier in the plastic chamber to provide continuous humidity and temperature was auto-controlled in the greenhouse, we did not achieve the absolute control of these conditions. In addition to humidity and temperature, light intensity also changed periodically in the greenhouse. Each of these environmental factors were probably involved in affecting the extent of responses to exposure to the pathogen on our experimental corn plants. These variations could be probably reduced by use of a growth chamber which would provide a more stable environment than the greenhouse.

The Effect of Leaf Age on Host Responses to *C. zeae-maydis*

In our experiments, it was clear that leaf age influenced the expression of resistance to gray leaf spot in corn genotypes tested in the greenhouse. Older lower leaves were more sensitive to *C. zeae-maydis* than the younger upper leaves. This result is analogous to how GLS disease develops in the field. Natural infection of corn plants by *Cercospora zeae-maydis* appears late in the growing season and usually begins with lower older leaves (Rupe, Siegel and Hartman, 1982; Latterell and Rossi, 1983). By investigating the relationship between photosynthesis levels and gray leaf spot disease severity, Donahue (1989) concluded that late season susceptibility to GLS in corn was related to factors associated with leaf aging. He suggested that young leaves remain resistant to GLS by maintaining a higher metabolic level, while old leaves are susceptible as they become senescent. However, there are alternative hypotheses that could explain the results of this study; e.g., that the newest leaves produce antibiotics or other disease inhibiting substances that are lost during the aging process.

The Effect of Cercosporin on the Expression of Resistance in Corn

Inoculation of corn seedlings with wild type mycelial discs induced more host responses than did inoculation with mutant mycelial discs which could not produce fungal toxin. This result suggests a role for the toxin in GLS disease development. This conclusion is supported by previous studies using purified cercosporin to treat host tissues. One such study was conducted to apply cercosporin to beet leaves (Steinkamp, et al., 1981). It was demonstrated that lesions induced by cercosporin

resembled, in both size and appearance, lesions induced by the fungus under greenhouse conditions, suggesting cercosporin may be involved in eliciting some of the disease symptoms. In the other two studies in corn (Gwinn, Stelzig and Brooks, 1987; Carter, 1992), while a sensitivity of host plant tissues to cercosporin was found, it was not correlated with varietal responses to *C. zeae-maydis* in the field. Thus, the mechanisms of cercosporin interaction with pathogen and host during the disease process is unknown.

The fungus *Cercospora zeae-maydis* has been reported to be variable in pathogenicity between different isolates (Bair and Ayers, 1986). Furthermore, Latterell and Rossi (1974) reported that cultures of *Cercospora zeae-maydis* exhibited striking variability with respect to growth habit, morphology, and metabolic by-products. They observed that some cultures produced brilliant red crystals on PDA; others produced none. Cultures that produced red pigment in yeast extract-dextrose liquid culture mutated to white cultures. Thus, since cercosporin has been demonstrated to be involved in the GLS disease development, cultures of *C. zeae-maydis* mycelium which produce extremely copious amounts of cercosporin may be more valuable for maximum pathogenicity in greenhouse screening methods.

Conidiation of *Cercospora zeae-maydis* in the Greenhouse

In our tests, conidiation occurred only in the two susceptible genotypes B73 and MO17. Conidiation was not observed in VA14, B68, and PA875. However, these

results don't preclude the possibility that conidiation could eventually occur in these genotypes because observations were ended 26 days after inoculation. It's interesting that conidiophores were limited only to necrotic tissue, which is consistent with previous observations (Beckman and Payne, 1982). Beckman and Payne observed the growth of mycelia toward the guard cell region of the substomatal cavity and the formation of stromata that filled the substomatal chamber as host tissue became necrotic. Subsequently conidiophores erupted through the stomatal openings from stromata. Our observations that conidiophores were arranged in rows on the leaf surfaces supports these previous results. The occurrence of conidiation in our experiments appeared to reflect the extent of fungal invasion of the leaf tissue.

SUMMARY AND CONCLUSIONS

In view of the recent development of gray leaf spot caused by *Cercospora zeae-maydis* into an important disease of maize, a new technique, artificial inoculation of corn seedlings in the greenhouse with filter paper discs containing mycelia of *C. zeae-maydis*, was developed. This technique is simple and reliable. One-week-old mycelial liquid cultures were homogenized, washed, centrifuged and finally suspended in distilled water to make mycelial inoculum, which was pipetted at 100 *u*l samples onto filter paper discs with a 6 mm diameter. These filter paper discs containing cercosporin-producing mycelia were placed on the lower leaf surfaces of corn seedlings and adhered by transparent tape. The inoculated plants were placed in a moist transparent plastic chamber with high relative humidity provided by a humidifier. Three or four days after inoculation, symptoms first appeared.

The new inoculation method with mycelial discs was used on five corn genotypes (VA14, B68, PA875, MO17 and B73) to test its reliability in screening for resistance to GLS in maize. Based on lesion type, these five genotypes of corn were classified into two groups. One group, including VA14, B68, and PA875, had resistant lesion types appearing as water-soaked appearance or small chlorotic flecks. Another group, including B73 and MO17, had susceptible lesion types appearing as necrotic lesions. Lesion type is a reliable criterion for evaluation of resistance or susceptibility in corn to *C. zeae-maydis*. Leaf area covered by necrotic lesions is also a good parameter for disease severity. However, the variation of lesion area in B73 between experiments

indicates that this disease severity rating could be seriously affected by environmental conditions like relative humidity, temperature, or light in the greenhouse. The percent leaf area under discs affected by mycelia reflects the total host responses but is not a good indicator of disease severity. With this inoculation method, disease symptoms appeared and developed within a shorter time period than with previous spore spraying methods. Our technique should, therefore, be helpful to plant breeders in rapid evaluation of corn germplasm in the greenhouse for resistance to GLS.

The effect of plant physiological factors on the expression of GLS resistance was also investigated. Mycelial discs placed on lower leaf surfaces induced greater responses than upper surfaces. Greater responses also occur on lower leaves of the plants than upper leaves, suggesting that leaf age is involved in disease expression.

Cercospora zeae-maydis produces cercosporin toxin which can kill plant cells in the presence of light. In all of the five corn genotypes tested (VA14, B68, PA875, B73, and MO17), inoculation with cercosporin-producing mycelia induced more severe host responses than with non-cercosporin producing mycelia, indicating that cercosporin plays an important role in the disease process.

Conidiation of *C. zeae-maydis* was also investigated with our mycelial inoculation technique. Conidiophores were found emerging from stomata as early as 15 days after inoculation in B73 and MO17 and were limited only to necrotic tissue. No conidiation was observed in resistant genotypes VA14, B68, and PA875 during this period suggesting that the conidiation process is delayed on resistant hosts.

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