Cultural Techniques and Conditions Influencing Growth and Sporulation of *Cercospora zeae-maydis* and Lesion Development in Corn

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Journal Series Paper 8311 of the North Carolina Agricultural Research Service, Raleigh.

This research was supported in part from a grant from the Corn Growers' Association of North Carolina, Inc.

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Accepted for publication 23 August 1982.

ABSTRACT

Beckman, P. M., and Payne, G. A. 1983. Cultural techniques and conditions influencing growth and sporulation of *Cercospora zeae-maydis* and lesion development in corn. Phytopathology 73: 286-289.

Sporulating cultures of *Cercospora zeae-maydis* were obtained routinely by homogenizing freshly isolated or stored cultures in water, dispensing the homogenate on V-8 juice agar, and incubating the plates under a diurnal fluorescent light regime for 14 days. Sporulation was good on decoction media made from green or senescent corn leaves and on V-8 juice agar but was poor on potato-dextrose agar. Constant light inhibited conidial germination, mycelial growth, and sporulation. The optimum temperature range for germination and growth was 22–30 C. Cultures of the fungus could be stored successfully for at least 23 mo at 4 C on several media. Lesion development on corn plants was obtained by misting inoculated plants for 3 sec every 4 min between the hours 2000 and 1000 each day for 2 wk. Lesion development in the greenhouse was greater from June through August, when the greenhouse was kept at 22–28 C by wetpad cooling, than during the other months.

Additional key words: gray leaf spot, maize.

Gray leaf spot of corn (*Zea mays* L.), caused by *Cercospora zeae-maydis* Tehon and Daniels, was first reported in 1924 (8). The disease is most prevalent in the mountainous regions of Tennessee, Virginia, Kentucky, North Carolina, and South Carolina (2). The distribution of *C. zeae-maydis* and the severity of gray leaf spot have increased since 1970 with the increase in minimum tillage farming practices (2,6). Although several studies have described the characteristics of the disease in the field, little work has been reported on the biology of *C. zeae-maydis* and the development of lesions induced by the fungus under greenhouse conditions. Studies of this type have been limited, in part, by slow growth and poor sporulation of the fungus and failure to obtain lesions consistently on corn in the greenhouse. In our initial studies, routine techniques for obtaining sporulation in culture and lesion development in the greenhouse were unsuccessful. Others have reported similar difficulties (4,9). Also, substantial variability in growth and sporulation of *C. zeae-maydis* on artificial media has been reported (3). The objectives of this study were to determine conditions favorable for growth and sporulation of *C. zeae-maydis* and conditions conducive to lesion development in the greenhouse.

MATERIALS AND METHODS

Maintenance of isolates and inoculum production. Isolates of *C. zeae-maydis* grown on V-8 juice agar were stored on agar strips under sterile water in test tubes at 4 C. To prepare inoculum for spore production studies, 1 to 2-cm^2 agar strips from storage were placed in 500-ml Sorvall (Omnimixer, Sorvall, Inc., Norwalk, CT 06856) stainless steel cups containing 50 ml of sterile water and homogenized 30–60 sec at high speed. The homogenate was pipetted in 2 to 3-ml samples onto the appropriate agar medium in petri plates. After 5–15 min, the excess liquid was decanted.

To obtain spores for infection studies, plates were seeded with homogenate as described above and incubated in stacks of 10 at room temperature for 2 wk under either diurnal light (12 hr of fluorescent light, 12 hr of dark) or 11 days of continuous light followed by 3 days of dark. Conidia were harvested by flooding the plates with a 0.01% solution of Tween-20 surfactant and gently dislodging the spores with a camel's hair brush.

Effect of medium and light on spore production. Potato-dextrose agar (PDA), V-8 juice agar, green-corn-leaf decoction agar (GCLDA), and senescent-corn-leaf decoction agar (SCLDA) were examined for their effect on spore production. Difco PDA (American Scientific Products, Charlotte, NC 28224) was prepared according to instructions on the package. V-8 juice agar contained 19 g of Difco agar, 4 g of $CaCO_3$, and 300 ml of V-8 vegetable juice (Campbell Soup Co., Camden, NJ) per liter. GCLDA and SCLDA were prepared by simmering 120 g of green leaves and 35 g of dried-senescent leaves, respectively, for 20 min in 1.5 L of tap water. Decoctions were filtered through two layers of cheese cloth and combined with 19 g of Difco agar and 4 g of $CaCO_3$ per liter. Each medium was distributed into nine petri dishes. For each medium, three plates were seeded from each of three separate inoculum preparations. Plates seeded with homogenized cultures were enclosed in plastic bags in stacks of three with two unseeded plates on top for shading. The plates were incubated at 25 C under diurnal fluorescent light (12 hr of light and 12 hr of dark) for 14 days or under 11 days of constant fluorescent light followed by 3 days of dark. The effect of light regimes on spore production on V-8 juice agar was also measured by incubating three replicate stacks of three plates each, as described above, under constant light for 11 days followed by 3 days of darkness, or under diurnal light for 14 days, or in complete darkness for 14 days. The combined spores from the bottom two plates of a stack were harvested with 10 ml of water and counted at least twice with a hemacytometer. Incubation temperatures were recorded by a hygrothermograph.

Effect of temperature and light on spore germination and germ tube growth. Spores of uniform age were obtained for the experiments by seeding plates with a homogenized culture and incubating them under constant light for 9 days for conidiophore production followed by 2 days in the dark for spore production. Three drops of a spore suspension (5×10^4 conidia per milliliter) were placed on sterile glass slides and incubated in petri dishes containing two pieces of moistened filter paper. Three replications were used, with separate plates as replicates. A spore was

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considered germinated if its germ tubes were greater than half the length of the spore (>30 μm). A total of 100 spores was counted from four randomly located microscopic fields per replication. Average germ tube length was obtained from a total of 15 spores from three randomly chosen microscopic fields per replication.

The effect of temperature on percent spore germination was measured from 4 to 36 C at 4° intervals after 8, 12, 24, and 48 hr. The experiment was done three times for temperatures of 28 C and above. The effects of constant fluorescent light (27 lx) and of constant darkness on spore germination and length of germ tubes in sterile water and on V-8 juice agar also were determined after 20 hr at 25 C.

Longevity in vitro. In March 1979, cultures (first or second transfer after isolation) of *C. zeae-maydis* were grown on slants of PDA, water agar, SCLDA, SCLDA plus 10 g of dextrose per liter, and GCLDA plus 10 g of dextrose per liter at 20–25 C for a week and stored at 4 C. Also, strips of agar from cultures grown on PDA, SCLDA, or GCLDA for 1 wk were placed in sterile tap water in test tubes. The tubes were sealed with Parafilm "M" (American Can Co., Greenwich, CT) and stored at 4 C. In July 1980, five additional longevity in vitro (LIV) treatments were started from sporulating cultures on V-8 juice agar. In the first, cultures grown on V-8 juice agar slants were stored at 4 C. In the second, agar strips containing the fungus were placed in sterile tap water in test tubes at 4 C. In the third, agar strips containing the fungus were placed in 2-ml glass vials, quick-frozen in liquid nitrogen, lyophilized, sealed, and stored at 4 or −20 C. In the fourth, 2-ml samples containing 3×10^4 conidia per milliliter were pipetted slowly onto cooled sterilized silica gel beads according to the procedure of Perkins (5) and stored at 4 C. In a fifth LIV treatment, corn leaves with lesions were stored at −20 C. All LIV treatments were assayed in March 1981.

To assay for recovery of cultures stored on various media, a 1 to 2-cm^2 strip of agar containing the culture was placed in 50 ml of sterile water, hydrated for 15 min, homogenized as described earlier, and plated on V-8 juice agar. The silica gel beads were placed on V-8 juice agar, agitated briefly, and incubated in the dark.

Conditions for lesion development. Corn plants were inoculated 2–3 wk before anthesis with a conidial suspension of *C. zeae-maydis* containing 5×10^4 conidia per milliliter. The conidial suspension was either atomized or brushed with a camel's hair brush on both leaf surfaces.

Several methods for providing a suitable environment for infection and lesion development were examined. The two most successful methods involved a greenhouse misting system and a system constructed outside to simulate dew. In the greenhouse system, a fine mist was generated by misting heads (Catalogue No. 18-4700, A. H. Hummert Seed Co., St. Louis, MO 63103) placed 0.7 m apart over a greenhouse bench. The misting heads were mounted on pipes of interchangeable length to accommodate plants as tall as 2.5 m. The duration of the mist was regulated by 24-hr and 12-min time clocks. This system was constructed in a greenhouse maintained at 22–28 C during the summer with wet-pad cooling. The relative humidity in the greenhouse outside the misting area was 60–80% during the day and above 96% at night for at least 10 hr. Night mist, constant mist, and no-mist regimes were investigated for their relative effects on disease development. Plants were exposed to these mist regimes for 2 wk and evaluated for lesion development.

A new mist system devised to simulate leaf wetness by dew (7) was used outside. On nights without dew, this system provided regulated intervals of mist between 2100 and 0900 hours. The system was set to turn off when the leaf surface was wet but before runoff. Screening was placed around the corn plants to reduce evaporation by wind.

RESULTS

Effect of medium and light on spore production. Both medium and light influenced spore production by *C. zeae-maydis* (Table 1 and Fig. 1). An interaction was found between medium and light regime. Under diurnal light, sporulation of *C. zeae-maydis* was relatively low on PDA but abundant on all other media. When cultures were incubated under constant light followed by 3 days of dark, sporulation on V-8 juice agar and GCLDA + $CaCO_3$ was abundant, but all other media supported relatively little sporulation. The relatively small numbers of conidia produced on V-8 juice agar, SCLDA, and SCLDA + $CaCO_3$ in constant light followed by dark was the result of reduced colony numbers per plate in these treatments. Although spore production was greater on GCLDA + $CaCO_3$ than on V-8 juice under constant light followed by dark, colonies grown on GLCDA + $CaCO_3$ were loose, aerial, and consistently overrun with white sterile mycelium. This led to a greater number of mycelial fragments at spore harvest. When three light regimes were compared for spore production of *C. zeae-maydis* on V-8 juice agar, diurnal light supported greater spore production than did constant dark or constant light followed by dark (Fig. 1).

TABLE 1. Effect of culture medium and light on sporulation and colony morphology of *Cercospora zeae-maydis*

Medium[a]	Spores per plate ($\times 10^5$)[b] with		Colony characteristics
	Diurnal light[c]	Constant light[d]	
SCLDA	2.71 ± 0.16	0.15 ± 0.08	Compact, light gray
V-8 juice agar + $CaCO_3$	2.36 ± 0.16	1.31 ± 0.14	Very compact, gray to black
SCLDA + $CaCO_3$	1.83 ± 0.32	0.16 ± 0.02	Compact, mixed gray and black
GCLDA + $CaCO_3$	1.55 ± 0.34	2.79 ± 0.24	Very aerial, loose, white
PDA	0.02 ± 0.01	0.03 ± 0.01	Semicompact, black

[a] SCLDA = senescent-corn-leaf decoction agar, GCLDA = green-corn-leaf decoction agar, $CaCO_3$ = $CaCO_3$ at 4 g/L, PDA = potato-dextrose agar.
[b] Mean of three plates and standard error of the mean.
[c] Diurnal light = incubation for 14 days with 12 hr of fluorescent light (27 lx) and 12 hr of dark daily.
[d] Constant light = constant fluorescent light (27 lx) for 11 days followed by 3 days of darkness.

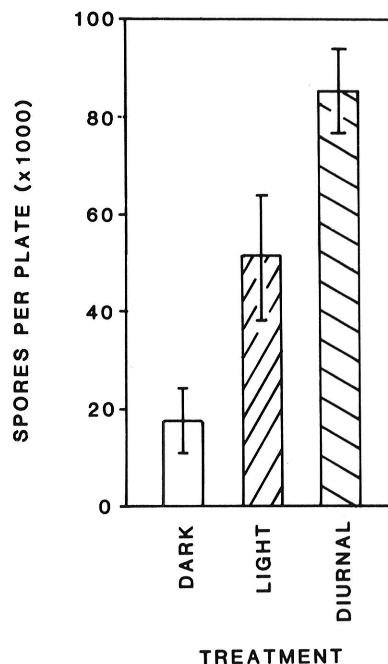

Fig. 1. Effect of light on spore production of *Cercospora zeae-maydis* on V-8 juice agar. Vertical bars represent standard error of mean. Dark = 13 days of dark; light = 10 days of fluorescent light followed by 3 days of dark; Diurnal = 13 days of 12 hr of fluorescent (27 lx) light daily.

Effect of temperature and light on spore germination and germ tube growth. The optimum temperature range for spore germination was between 22 and 30 C (Fig. 2). After 12 hr, more than 88% of the spores had germinated at temperatures between 17 and 30 C. No germination was observed at 36 C after 48 hr. The effect of temperature on germ tube growth paralleled its effect on spore germination (Fig. 2). Light inhibited both germination and germ tube growth of *C. zeae-maydis* (Fig. 3); the inhibition was more pronounced on V-8 juice agar than in water.

LIV. Sporulating cultures of *C. zeae-maydis* were recovered from all LIV treatments except from tissue with lesions stored at −20 C for 8 mo and spores stored on silica gel (Table 2). Few colonies were recovered from lyophilized material, and sporadic contamination associated with this procedure caused additional problems. Storage of isolates in sterile water on several media was convenient and resulted in good recovery. The recovered isolates had colony morphology similar to that of the original isolates and sporulated readily.

Conditions for lesion development. The optimum conditions for disease development were obtained with a greenhouse misting system. The system provided night misting (3 sec of mist every 4 min between the 2000 hours and 1000 hours the following day) for 2 wk. Under these conditions, sporulating lesions of *C. zeae-maydis* developed in 11–25 days at 22–28 C. These conditions allowed numerous lesions to develop and were conducive to coalescence of lesions, resulting in complete leaf necrosis. The lesions that developed were indistinguishable in appearance from lesions observed in the field. Increased misting tended to wash the inoculum into clumps or off the leaves. Additionally, the prolonged presence of free water reduced the amount of disease development compared with the amount on plants incubated under diurnal misting or no-mist treatments. Gray leaf spot also developed during the summer, but not during the winter, in greenhouses without misting. During the summer the relative humidity in the greenhouse was between 60 and 90% during the day and above 96% for at least 10 hr at night. Under these conditions, lesions readily developed but were delayed 3–6 days.

Gray leaf spot also developed readily outside the greenhouse under a simulated dew system. In general, the outdoor system was less effective and gave less consistent results than the greenhouse misting system for disease development.

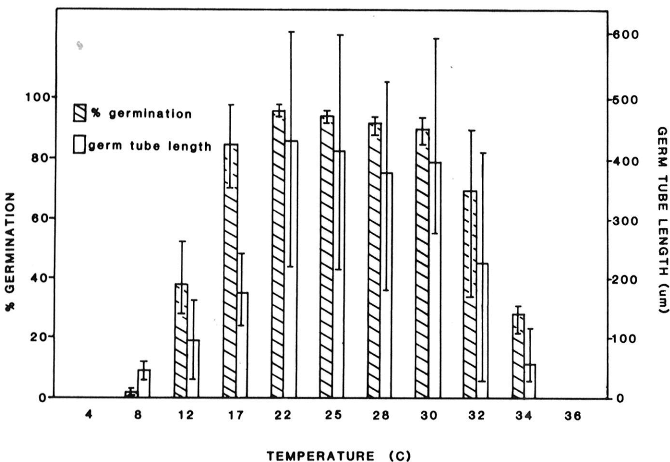

Fig. 2. Effect of temperature on spore germination after 12 hr and germ tube growth after 24 hr for *Cercospora zeae-maydis* in water. Vertical bars represent range.

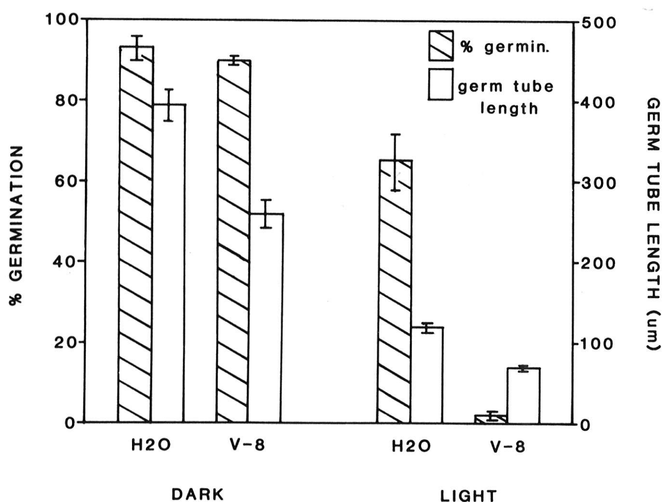

Fig. 3. Effect of fluorescent light (27 lx) on spore germination and germ tube growth of *Cercospora zeae-maydis* after 20 hr at 25 C on V-8 juice agar and in water. Vertical bars represent standard error of the mean.

DISCUSSION

Several factors influenced growth and sporulation of *C. zeae-maydis*. Serial transfer of single colonies of the fungus resulted in little growth, production of few conidia, and frequent production of white, sterile mycelium. Latterell and Rossi (4) reported that sporulating colonies could be maintained by transferring only conidia from sporulating colonies. Weaver and Elliott (9) reported that white, sterile mycelium could be induced to sporulate by homogenization in a blender before plating on media. We found the procedure of Weaver and Elliott to be effective in establishing sporulating cultures of the fungus. Several additional factors, however, influenced the relative growth and sporulation of *C. zeae-maydis*.

Culture medium influenced growth, colony morphology, and sporulation. The fungus grew well on most media, but spore production was the most consistent on V-8 juice agar. Fungal growth was good on PDA, but few or no conidia were produced. Latterell and Rossi (3) reported that various media supported different types or degrees of development of erect or submerged stromata and of subspherical bodies containing either macro- or microspermatia. In our studies, we observed an effect of medium on colony morphology, but no specialized structures were observed.

Constant light inhibited spore germination, germ tube growth,

TABLE 2. Survival of *Cercospora zeae-maydis* in storage

Treatment and medium used	Length of storage (mo)	Recovery[a]
Culture slants stored at 4 C		
V-8 juice agar	8	+
Potato-dextrose agar	23	+
Water agar	23	+
Green-corn-leaf decoction agar + dextrose[b]	23	+
Senescent-corn-leaf decoction agar	23	+
Senescent-corn-leaf decoction agar + dextrose[b]	23	+
Strips of agar containing fungus stored under water at 4 C		
Potato-dextrose agar	23	+
Green-corn-leaf decoction agar	23	+
Senescent-corn-leaf decoction agar	23	+
V-8 juice agar	8	+
Lyophilized strips of V-8 juice agar containing fungus		
Stored at 4 C	8	+[c]
Stored at −20 C	8	+[c]
Plant tissue with lesions, stored at −20 C	8	−
Spores of fungus stored on silica gel at 4 C	8	−

[a] Recovery determined by plating on V-8 juice agar and evaluating sporulation.
[b] 10 g of dextrose per liter.
[c] Only a few colonies were recovered.

and sporulation. Also, conidiophores formed under constant light, but conidial formation was inhibited, which agrees with the observations of Latterell and Rossi (3). Optimum sporulation of *C. zeae-maydis* occurred under diurnal light. If it is desirable, however, to have spores of approximately the same age, incubation of cultures under constant light for conidiophore production, followed by 3 days of darkness for conidia production, will lead to spores of approximately the same age. Because light has such a pronounced effect on this fungus in culture, light may play an important role in disease development.

The optimum temperature range for spore germination and growth was broad, and the fungus was able to grow well at 30 C. Growth at high temperatures was unexpected because this fungus is most commonly found in mountainous regions. However, recent studies have shown that the fungus can develop and cause disease in the piedmont of the southeastern United States if adequate periods of high humidity are present (P. M. Beckman and G. A. Payne, *unpublished*).

One of the limitations in the study of *C. zeae-maydis* and gray leaf spot has been the inability to readily obtain lesions in the greenhouse. Several techniques have been used by others to obtain lesions. Weaver and Elliott (9) induced infection by *C. zeae-maydis* on corn plants grown in 4-L flasks. Latterell and Rossi (4) obtained lesions by abrading leaves before inoculation. The procedure we have reported involves neither special containers for growing plants nor leaf abrasion. It does require, however, that specific moisture conditions be met. Intermittent misting at night followed by no misting in these day resulted in more lesions than did continuous misting. In fact, continuous misting resulted in fewer lesions than no misting. Although lesions were obtained throughout the year, the efficiency was much better in the summer when the greenhouse was cooled by wet-pad cooling. During the summer months, lesions could be obtained in the greenhouse with no misting. High humidity without free water or alternating periods of free water appear to be important in infection. This is consistent with the observation that continuous free water on the leaf surface reduces appressorial formation and penetration by *C. zeae-maydis* (1).

The conditions that are optimal for lesion development in the greenhouse are similar to conditions that occur in fields where gray leaf spot is present. The disease is more prevalent in mountain valleys where humidity is high for long periods due to fogs that persist until late morning.

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