Development and Survival of *Cercospora zeae-maydis* Germlings in Different Relative-Humidity Environments

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ABSTRACT

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Conidia of *Cercospora zeae-maydis* were atomized onto polysulfone membrane discs and were incubated for 6 h at 100% relative humidity (RH). Discs were subjected to a range of relative humidities (39–100%) by suspending discs above glycerol solutions maintained at 25.0 ± 0.03 C. When germlings were maintained at 95 or 100% RH, germ tube elongation continued, and appressoria formed in 2–3 days. However, germ tube elongation did not occur when discs were subjected to relative-humidity environments below 95%. Germlings survived at 60% RH for 6 days, at 70% RH for 8 days, at 80% RH for 10 days, and at 90% RH for 15 days. When returned to 95% RH, viable germ tubes elongated and produced appressoria within 72 h. When discs were exposed for 6 days to daily 12-h periods of 95% RH and to 70–90% RH for the remaining 12 h, approximately 80% of the germlings produced at least one appressorium. Only 22–30% of the germlings produced at least one appressorium when the alternate 12-h period was at 60% RH or lower. When germinated conidia were placed in a 95% RH environment for periods of 6, 8, 10, or 12 h daily, with the balance of each day spent in 60 or 80% RH environments, germ tube development and appressorium formation proceeded at a rate that correlated with the accumulated hours spent at 95% RH.

Additional keywords: corn, gray leaf spot, maize.

Cercospora zeae-maydis Tehon & Daniels commonly causes gray leaf spot of maize (11) in the mountainous regions of Virginia, Tennessee, Kentucky, and North Carolina (7). In recent years, the disease has been increasing in prevalence and severity in the North-Central United States. The pathogen overwinters on crop debris (8); thus, conservation tillage practices favor the survival of the pathogen and primary inoculum until the next year. Overcast days during more than 50% of the the growing season, morning fogs, extended dew periods, and prolonged periods of high-relative humidity (RH) have been associated with disease development in Eastern states (7). Overcast days and morning fogs that result in extended dew periods are atypical for the North-Central region, yet significant disease development occurs. High-relative humidity is common in the North-Central region, however. During the summer months, the average relative humidity ranges from 60 to 85% (9), and higher relative humidities exist within the maize-crop canopy.

Beckman and Payne (2), using 12-h diurnal misting periods, successfully infected greenhouse-grown maize plants. During the summer, symptoms were also obtained on greenhouse-grown maize at Raleigh, NC, without misting, but the incubation period was 3–6 days longer than when misting periods were used. The relative humidity during the day ranged from 60 to 90% and was above 96% for at least 10 h daily (2). Based on these observations, we believe that relative humidity is an important factor during prepenetration processes.

Control and maintenance of constant relative humidities require precise temperature control, and when plants are added to the system, relative humidities can be affected greatly by transpiration. Therefore, prepenetration development of *C. zeae-maydis* was studied in the absence of the host plant, to minimize aberrations in relative humidity. The objective of the research was to determine the effects of relative humidity on germ tube elongation, appressorium formation, and germling survival of *C. zeae-maydis*.

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MATERIALS AND METHODS

Culture maintenance. A single isolate of *C. zeae-maydis* was used for all experiments. This isolate was obtained from J. Berry (Pioneer Hi-Bred International, Inc., Johnston, IA) and was maintained on V8-juice agar (2) amended per liter with 135 mg of streptomycin-sulfate. Mycelia from a sporulating culture were homogenized in sterile distilled water with a Waring blender. The homogenate was flooded over V8-juice agar in petri dishes, and the excess was decanted. Cultures were incubated at room temperature (22–25 C) with diurnal lighting (12 h of dark and 12 h of light from two 40-W cool-white fluorescent lamps placed 30–50 cm above cultures). For inoculum production, the cultures were placed under constant fluorescent light for 3–5 days to induce conidiophore production and afterward, were placed in continuous dark for 2–4 days to induce conidiation. This regime produced spores of nearly uniform age (2).

Inoculation and spore germination. Conidia from one 9-cm petri dish were harvested with 40 ml of sterile distilled water. A camel-hair brush was used to dislodge conidia. The suspension was filtered through four layers of sterile cheesecloth to remove most mycelial fragments. Membrane discs 4 mm in diameter were cut from 0.2-μm-porosity polysulfone membrane filters (Supor-200, Gelman Sciences, Ann Arbor, MI) and were placed on two sterile 9-cm filter papers moistened with 3 ml of sterile distilled water. Approximately 100 conidia were atomized onto each polysulfone disc with a Chromamist sprayer (Fisher Scientific Co., Pittsburgh, PA). The petri dishes were sealed with Parafilm and were incubated in the dark at room temperature (22–25 C) for 6 h, by which time about 98% of the spores had germinated. This resulted in germinated conidia (germlings), with germ tube lengths averaging 64.3 ± 9.1 μm, which were used in all experiments in this study. (In this paper, germling refers to the spore and all germ tubes arising from it.)

Transfer to relative-humidity regimes. After 6 h of incubation at 100% RH, polysulfone discs containing germlings were placed on a polyethylene mesh mat and were air-dried in a laminar-flow hood for about 30 min. The plastic mats supporting the discs were then transferred to humidity chambers (225-ml canning jars) containing glycerol solutions of known specific gravities, to achieve precisely controlled relative-humidity environments (5).

Specific gravity was measured with a hydrometer. A polyvinylchloride pipe fitting was placed in each jar to support the plastic mesh at a height about 1.3 cm above the glycerol solution. Lids were placed on the jars, and the jars were incubated (duration of incubation was an independent variable) in an enclosed, circulating water bath maintained at 25.0 ± 0.03 C.

Speed of moisture equilibration was determined by measuring the rate of moisture stabilization (by mass) of 25-mm polysulfone membrane filters moved between different relative-humidity environments. Filter discs equilibrated within 3 h, except for those moved from the 100% RH environment to the 95% RH, where as many as 12 h were required to reach moisture equilibrium.

Sampling and data collection. At various times, discs were removed, and the germlings were stained with either 0.1% acid fuchsin or 0.1% aniline blue in lactophenol (12) or nitro-blue tetrazolium, 0.5 mg/ml in 0.05 M sodium phosphate-sodium succinate buffer, pH 7.6 (3). The nitro-blue tetrazolium, a vital stain, was specific for succinic dehydrogenase activity, and active mitochondria were stained dark blue-black (3). The succinic dehydrogenase reaction was allowed to proceed for 12 h before viability was determined. Because the germlings were hyaline, 0.1% acid fuchsin in lactophenol was used to detect germlings that were no longer viable. A germling was considered viable if any cell in the germling was stained blue with nitro-blue tetrazolium.

Germ tube length, appressorium formation, and viability were determined microscopically (100×). Germ tube length was determined by measuring the apparently, longest germ tube of five germlings per disc (in some experiments more were specified). Selection of germlings was achieved by arbitrarily selecting five fields of view per disc and observing the germling nearest the center of each field of view. Appressorium formation was recorded as the percentage of germlings with at least one appressorium. A minimum of 25 germlings were observed per disc. (The distinctive, lobate appressorium of *C. zeae-maydis* begins as a hyphal swelling that develops convoluted, irregular, dichotomously branched lobes of varying shapes and dimensions [1]). Viability was the percentage of viable germlings per disc; a minimum of 25 germlings were observed per disc.

Continuous relative-humidity experiments. Germlings were maintained continuously at selected relative humidities, and discs were sampled periodically. In experiment 1, germlings were maintained in continuous 80, 90, and 95% RH for 120 h. Humidity chambers were arranged in a completely randomized design. Germ tube lengths were measured at 24-h intervals by measuring the longest germ tube on 15 random germlings per disc, two discs per replication, and four replications per treatment. Experiment 2 was similar to experiment 1, except it continued for 240 h, and the longest germ tube was measured on 10 germlings per disc, two discs per replication, and four replications per treatment.

In experiment 3, germlings were maintained in constant relative humidities of 39, 51, 60, 70, 80, 90, 95, and 100% and in free water, each for 15 days. Humidity chambers were arranged in a randomized complete-block design. Germ tube lengths and appressorium formation were recorded every 24 h, for 10 germlings per disc, two discs per replication, and two replications per treatment. After 15 days, discs in the 39–90% RH environments were transferred to a 95% RH environment, and after 72 h in this environment, germ tube growth and appressorium formation were recorded.

In experiment 4, germlings were maintained at 60, 70, 80, and 90% RH for 2, 4, 6, 8, and 10 days and then were transferred to 95% RH. Germ tube lengths were measured 0, 24, 48, and 72 h after transfer to 95% RH. Humidity chambers were arranged in a randomized complete-block design, with three replications, and five germling germ tubes per disc were measured.

Alternating relative-humidity experiments. In experiment 5, discs with germlings were placed in 60, 70, 80, and 90% RH environments for 12 h per day, alternating with a 95% RH environment for the remaining 12 h per day. Humidity chambers were arranged in a randomized complete-block design, with three replications. Two discs were sampled daily, the longest germ tube of each of five random spores per disc was measured, and appressorium formation was recorded. The alternating relative-humidity regimes were continued for 6 days. In a repeat experiment, environments of 39, 51, 60, 70, 80, and 90% RH were employed for 12 h per day.

In experiment 6, germlings were placed in a 95% RH environment for 6, 8, 10, and 12 h per day and were maintained in a 60 or 80% RH environment for the remainder of each day. Humidity chambers were arranged in a randomized complete-block design, with four replications. After 6 and 8 days, the percentage of germlings with at least one appressorium was recorded. Experiment 7 was similar to experiment 6, except determinations of the percentage of germlings with appressoria were made after 4, 6, 8, 10, and 12 days.

Experimental design and statistical analyses. Humidity chambers were arranged in a completely random or a randomized complete-block design, with two to four replicates (humidity chambers) per treatment (humidity regime). Samples were discs, and subsamples were individual germlings. In early experiments, two samples and 15 subsamples were taken. In later experiments, sample and subsample sizes were reduced because additional

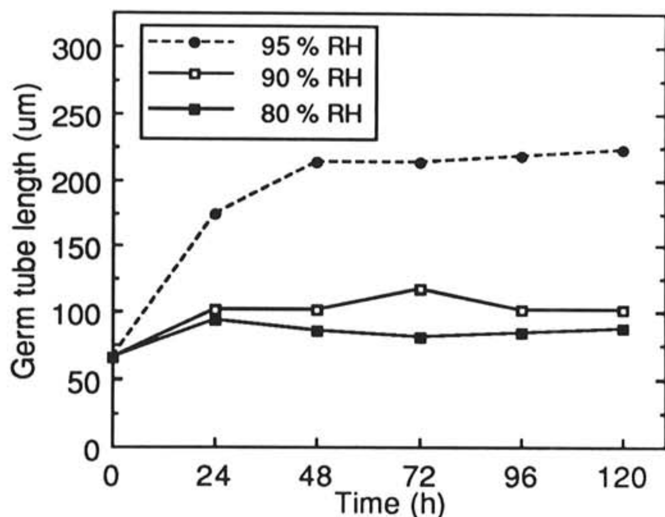

Fig. 1. Length of longest germ tube of *C. zeae-maydis* germlings, each germling maintained continuously at 95, 90, or 80% relative humidity for 120 h. Each value represents a mean of four replications, each with two samples and 15 subsamples per sample. LSD ($P = 0.05$) = 18.3 μm.

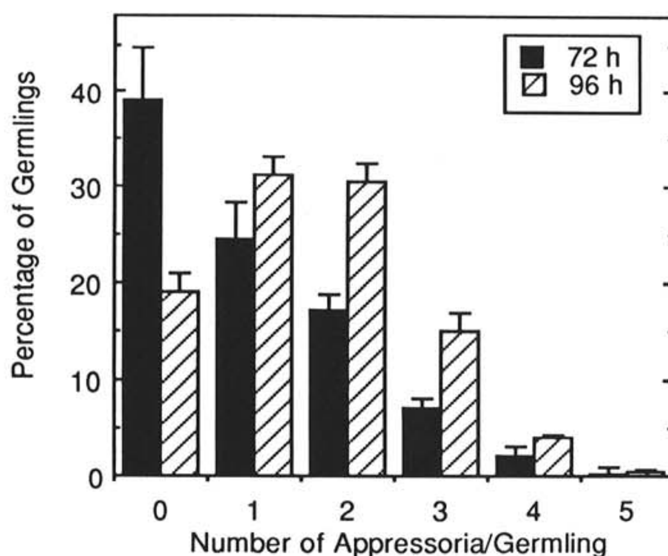

Fig. 2. Appressorium formation by *C. zeae-maydis* germlings after 72 and 96 h spent in 95% relative humidity. Each value represents a mean of four replications. The vertical bars represent mean standard errors.

sampling did little to improve precision, as determined by analysis of variance. Experiments were repeated at least once, and similar results were obtained. All statistical analyses utilized the general linear-models procedure of SAS (SAS Institute, Cary, NC [10]). In all experiments, a probability value of 0.05 was considered significant.

RESULTS

Continuous relative-humidity experiments. In experiment 1, germ tube lengths continued to increase very rapidly for 48 h at 95% RH, after which elongation either slowed or ceased (Fig. 1). Germ tube lengths found at 95% RH were significantly longer than those found at 80 and 90% RH, for all sampling periods. Appressorium formation occurred between 48 and 72 h spent at 95% RH, but no appressoria appeared in other relative-humidity treatments. After 72 h at 95% RH, about 80% of the spores had at least one appressorium.

In experiment 2, 94% of the germlings maintained at 80% RH and 89% of the germlings maintained at 90% RH were viable after 144 h. When germlings were maintained for 240 h at 80 or 90% RH, 78% and 42% of the germlings, respectively, were viable. Appressorium formation was observed only in the 95% RH environment. The number of appressoria per germling increased with time spent at continuous 95% RH. After 72 h at 95% RH, 39% of the germlings had no appressoria, but one germling had five (Fig. 2). After 96 h at 95% RH, 81% of the germlings had produced one or more appressoria.

In experiment 3, appressoria formed after 48–72 h in 95 and 100% RH environments but were never produced after 15 days in 39–90% RH environments. Fewer, but larger, appressoria were produced in 100% RH and in the presence of free water, compared with those in 95% RH. Germ tube lengths after 72 h were difficult to measure and were at least 400 μm in the 100% RH environment and averaged 275 μm in the 95% RH environment. In environments ranging from 39 to 90% RH, germ tube elongation did not proceed after equilibration periods. After being transferred to 95% RH environments, 94% of the germlings previously held at 90% RH resumed germ tube growth. After 72 h in 95% RH, about 30% of these germlings formed appressoria. Germlings previously in the 39–80% RH environments were nonviable.

In experiment 4, after 2 days spent at 60–90% RH, germ tube elongation resumed when the germling was transferred to a 95% RH environment (Fig. 3A). There was some lag in the resumption of germ tube elongation for germlings previously at 60 and 70% RH. After 72 h at 95% RH, the average germ tube length was essentially the same, regardless of prior relative-humidity treatments. Appressoria were observed on germlings in all treatments by 48 h, but more appressoria per germling were observed when germlings were previously held at 80 and 90% RH than when previously held at 60 and 70% RH. Nearly all germlings in all treatments had formed appressoria by 72 h. Appressorium formation of germlings incubated for 4 days at 60–90% RH before transfer to a 95% RH environment was essentially the same as that for germlings incubated for 2 days.

After 6 days of incubation at 60–90% RH, only germlings previously held at 80 and 90% RH rapidly continued germ tube elongation when transferred to a 95% RH environment (Fig. 3B). After 72 h in a 95% RH environment, germ tube lengths were much greater for germlings previously held in 80 or 90% RH environments, compared with germlings previously held in 60 or 70% RH environments. By 72 h, only germlings previously held at 80 and 90% RH formed appressoria. Germ tube elongation for germlings incubated for 8 days at 60–90% RH was essentially the same as that for germlings incubated for 6 days, shown in Figure 3B. However, a few germlings from the 70% RH environment continued germ tube elongation after 48 h.

Germ tubes on germlings held for 10 days at 60–90% RH continued to elongate after transfer to a 95% RH environment (Fig. 3C). Germ tube lengths were significantly greater on germlings from the 90% RH environment, compared with those from the 80% RH environment, but the rate of elongation was similar for both treatments (1.1–1.2 μm per hour). Appressoria were observed on germlings from both treatments after 72 h spent at 95% RH. None of the germlings previously maintained at 60–70% RH continued to grow when transferred to a 95% RH environment.

Alternating relative-humidity environments. In experiment 5, significant differences in the rate of germ tube elongation or germ tube length did not occur among germlings that were maintained

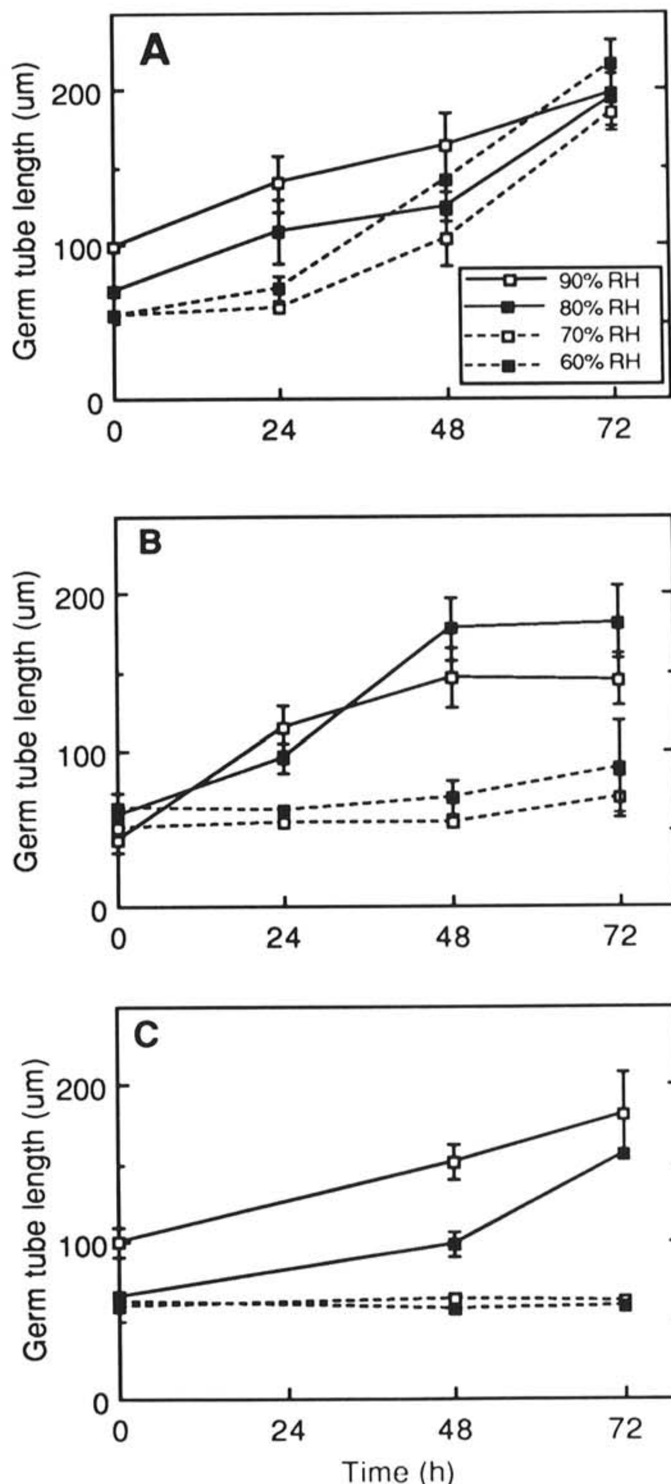

Fig. 3. Length of germ tubes of *C. zeae-maydis* germlings 0, 24, 48, and 72 h after transfer to 95% relative humidity (RH) from an initial period of **A**, 2 days, **B**, 6 days, or **C**, 10 days spent in 60, 70, 80, or 90% RH environments. Each value represents a mean of three replications, with five samples per replication. LSDs (P = 0.05) for 2-, 6-, and 10-day periods are 16.1 μm, 18.2 μm, and 12.0 μm, respectively. Vertical bars represent mean standard errors.

in 60, 70, and 80% RH environments for 12 h per day and in 95% RH for the remaining 12 h per day. The elongation rates were 21.9, 23.8, and 22.8 μm per day, respectively. The rate of elongation for germlings in the 90% RH for 12 h per day was 32.4 μm per day. Appressoria were observed in all humidity regimes by the end of the fourth day, however, a few appressoria were observed after the third day when germlings were maintained in 90% RH for 12 h per day. In the repeat experiment, there was no significant difference among the average germ tube lengths for the various alternating humidity regimes, and appressorium formation was observed in all humidity regimes by the end of the fourth day. When maintained in 39, 51, 60, 70, 80, and 90% RH for 12 h per day, 22, 30, 27, 75, 84, and 79% (LSD [$P = 0.05$] = 26%) of the germlings, respectively, developed appressoria.

In experiment 6, with 10 or 12 h per day spent at 95% RH, 85% or more of the germlings developed appressoria, regardless of the alternate relative-humidity regime (Table 1). Significantly fewer germlings produced appressoria when held at 60% RH for 16 h per day (8 h per day at 95% RH) than did those held at 80% RH for 16 h per day. Treatments involving 6 and 8 h spent at 95% RH daily were continued for 2 more days (Table 1).

In experiment 7, the number of germlings with at least one appressorium increased over time for all humidity regimes, with the exception of the day 8 sample, in which the number of appressoria decreased for germlings maintained in a 95% RH environment for 6 h per day (18 h per day at 80% RH) (Table 2). This discrepancy is explained by the abnormally high percentage of appressoria on day 6. The abnormality was evident from the results of repeated experiments. All humidity regimes had some germlings with appressoria after 4 days, including regimes in which the germlings were exposed to only 6 h of 95% RH daily and in which germlings were exposed to a total of 24 h at 95% RH (excluding the 6-h germination period at 100% RH). When the 6- and 12-h 95% RH treatments were compared, the number of germlings with appressoria after 12 days of 6 h per day spent at 95% RH were similar to the number of germlings with appressoria after 6 days of 12 h per day spent at 95% RH.

TABLE 1. Percentage of germlings with at least one appressorium after 6 and 8 days spent in a 95% relative-humidity environment for 6, 8, 10, or 12 h per day, with the rest of the day spent in a 60 or 80% RH environment

h/day in 95% RH	Alternate environment			
	60% RH		80% RH	
	Days		Days	
	6	8	6	8
6	22 (10.5)[a]	32 (5.0)	17 (6.0)	65 (3.5)
8	36 (2.5)	60 (0.2)	100 (0.0)	...[b]
10	94 (1.0)	...	94 (3.0)	...
12	85 (4.5)	...	93 (1.5)	...

[a]Standard errors given in parentheses.
[b]Data not collected because at least 85% of the germlings had appressoria by 6 days.

DISCUSSION

C. zeae-maydis germlings grew normally and formed typical appressoria (1) on polysulfone membranes, thereby allowing prepenetration phenomena to be studied in vitro. The number of appressoria per germling increased with increased time spent at 95% RH; one to two appressoria per germling were most common after 96 h. Beckman and Payne (1) reported that the formation of two to five appressoria per germling was most common in planta.

In our experiments, 95% RH was adequate for germ tube elongation and appressorium formation by *C. zeae-maydis* germlings, but ≤90% RH inhibited growth. Beckman and Payne (1) observed germ tube growth toward stomata and appressorium formation over stomata on plants subjected to intermittent misting in a greenhouse. However, in the presence of free water, appressorium formation and growth toward stomata were reduced. In the present study, longer germ tube lengths and fewer appressoria were observed in the presence of free water, compared to those observed at 95% RH. Prolonged periods of leaf wetness may cause extensive *C. zeae-maydis* growth on the leaf surface, with limited penetration. We have never obtained good inoculation efficiency in greenhouse experiments in which continuous periods of leaf wetness were maintained. If free water (after germination) is not a necessity for appressorium formation, one would assume free water is not needed for penetration and, thus, for disease development. In the field, however, a very slight temperature decrease is the difference between 95% RH and the dew point. Dew may be formed on plants when 90% RH is reached (4,6); radiation emitted from the leaf and evapotranspiration cool the leaf and allow dew deposition.

Our data show that germlings of *C. zeae-maydis* do not require continuously favorable moisture conditions for germ tube growth and appressorium formation. Germlings can develop during intermittent periods of favorable moisture and can survive extended periods of desiccation before penetration. Survival of germlings decreased with increases in time spent at relative humidities unfavorable for growth. Germlings survived continuous periods of 60% RH for 6 days, 70% RH for 8 days, 80% RH for 10 days, and 90% RH for at least 15 days. Survival during periods of desiccation was related directly to the relative humidity, except in experiment 2. In experiment 2, more germlings were viable after 240 h spent in continous 80% RH compared to germlings maintained in 90% RH. More germlings may have died in 90% RH because they had longer germ tubes and had expended more energy on germ tube growth than did germlings in 80% RH. It appears that *C. zeae-maydis* germlings can survive unfavorable humidities during the day and can resume prepenetration processes when favorable conditions recur. This information helps to explain why *C. zeae-maydis* can develop in the North-Central region of the United States.

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TABLE 2. Percentage of germlings with at least one appressorium after 4, 6, 8, 10, and 12 days spent in a 95% relative-humidity environment for 6, 8, 10, or 12 h per day, with the rest of the day spent in a 60 or 80% RH environment

h/day in 95% RH	Alternate environment									
	60% RH					80% RH				
	Days					Days				
	4	6	8	10	12	4	6	8	10	12
6	3 (3.0)[a]	7 (4.0)	30 (8.5)	45 (1.0)	41 (1.0)	17 (8.0)	89 (14.0)	65 (4.5)	98 (2.5)	100 (0.0)
8	3 (2.5)	19 (9.5)	64 (3.5)	...[b]	...	63 (16.0)	81 (16.0)	93 (7.5)	...	...
10	7 (2.0)	67 (13.0)	...	...	...	72 (1.5)	100 (0.0)	...	...	...
12	16 (10.0)	57 (14.0)	...	...	...	70 (5.0)	93 (2.0)	...	...	...

[a]Standard errors given in parentheses.
[b]Data not collected because further collection of data for a humidity regime was discontinued when more than 55% of the germlings in both alternate environments had appressoria.

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