Seed Transmission of *Fusarium verticillioides* in Maize Plants Grown Under Three Different Temperature Regimes

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ABSTRACT

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Fusarium verticillioides can be seed transmitted and cause systemic infection of maize; however, the frequency of these phenomena has varied widely among and within individual studies. In order to better understand this variability, we evaluated the effect of temperature on the first step in the systemic infection process, the transmission of *F. verticillioides* from seed to seedling. Seed of a commercial maize hybrid were inoculated with a strain of *F. verticillioides* that had been transformed with a gene for green fluorescent protein (GFP). The seed were planted in a greenhouse potting mix and incubated in growth chambers. Plants were incubated at one of three temperature regimes designed to simulate average and extreme temperatures occurring in Iowa during the weeks following planting. Root, mesocotyl, and stem tissues were sampled at growth stages V2 and V6, surface disinfested, and cultured on a semiselective medium. At V2, >90% of root and mesocotyl tissues was infected by the GFP-expressing strain at all three temperature regimes. Also at V2, infection was detected in 68 to 75% of stems. At V6, infection of root and mesocotyl tissues persisted and was detected in 97 to 100% of plants at all three temperature regimes. Plants also had symptomless systemic infection of belowground and aboveground internodes at V6. Infection of the three basal aboveground internodes was 24, 6, and 3% for the low-temperature regime; 35, 9, and 0% for the average-temperature regime; and 46, 24, and 9% for the high-temperature regime. Seed transmission and systemic infection occurred at all temperatures and did not differ significantly among treatments. These results indicate that, if maize seed is infected with *F. verticillioides*, seed transmission is common and symptomless systemic infection can be initiated under a broad range of temperature conditions.

Additional keywords: endophytic infection, *Fusarium moniliforme*

Fusarium verticillioides (Sacc.) Nirenberg (synonym *F. moniliforme* J. Sheld.), *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun, & Marasas, and *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg are morphologically similar species and are among the most common *Fusarium* spp. infecting maize in most areas of the world. All three species cause identical symptoms in maize (*Zea mays* L.), including root rot, stalk rot, ear rot, and seedling blight. These species also produce toxic secondary metabolites (mycotoxins) that can be detected in symptomatic and symptomless plant tissues (20,21). The fumonisins, produced by *F. verticillioides* and *F. proliferatum*, are the most frequently occurring class of mycotoxins found in maize kernels. The most common fumonisins are fumonisin B₁, B₂,

and B₃ (20), but there are numerous others. Fumonisins have been proven to cause leukoencephalomalacia (LEM) in horses (29,37) and porcine pulmonary edema (PPE) in swine (29,37), and are associated with elevated rates of esophageal cancer in humans (20,35).

F. verticillioides, *F. subglutinans*, and *F. proliferatum* mycelia survive in maize residues (5,32) and in seeds (23). Maize plant infection results from inoculum spread by wind, rain (33), insects (41), and transmission from seeds (4,11,13,26,39). Seed-transmitted *F. verticillioides* can be found in symptomless tissues throughout the plant. In 1962, Foley found that *F. verticillioides* (reported as *F. moniliforme*) survives in the pedicel and abscission layers of the seed. Foley (11) and Sumner (42) determined that hyphae grow out of seeds and into the roots, mesocotyls, and stalks of the new plants. They used the term systemic infection to describe this symptomless colonization of the plants because they believed that the fungus moved from the roots to the remainder of the plant. Other authors have referred to this infection as endophytic (2). For many years, the uncertain significance of seed

transmission and systemic infection in relation to stalk rot and ear rot has been debated (14,23,26).

Recent studies have confirmed Foley and Sumner's conclusions that *F. verticillioides*-infected seeds are a source for root and stalk infection (2,13). In addition, other studies have demonstrated transmission of the fungus from the planted seed, through the plant, to the developing kernels (25,26). Kedera et al. (13) conducted an experiment in which seeds were inoculated in the laboratory with four different strains of *F. verticillioides* and planted in the field. The seed-inoculated strains (identified by vegetative compatibility) were recovered from 65% of the crowns, 34 to 54% of the nodes, 10% of the cobs, and 8% of the kernels. Results of recent studies by Desjardins et al. (6) and Desjardins and Plattner (7) support the occurrence of systemic infection and further suggest that this may contribute to mycotoxin contamination in grain. A strain of *F. verticillioides* that produced only fumonisin B₂ (FB₂) but not FB₁ or FB₃, was applied to seed at planting. At physiological maturity, fungi were isolated from the kernels and their fumonisin production was characterized. From the plants that were grown from seed inoculated with the FB₂-producing strain, 35 to 78% of the strains recovered from the kernels produced only FB₂. From these results, it was concluded that the FB₂-producing strain systemically infected the plants and produced FB₂ in the kernels.

The frequency of seed transmission and systemic infection have varied widely among and within individual field studies. In some experiments, seed transmission and systemic infection did not play a significant role in plant infection (10,14,26). In other experiments, seed transmission was detected in up to 80 to 90% of the belowground tissues of the plants, and symptomless systemic infection was detected in up to 50% of the plants at the seedling stage (4,26,46). Beyond the seedling stage, seed transmission was detected in up to 65% of the plant crowns, and symptomless systemic infection was detected in up to 34 to 54% of the stalk tissues and in 8% of the kernels (13). The various studies differed in fungal strains used, inoculation methods, maize genotypes, or environmental conditions. Results have varied even in studies conducted with

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the same fungal strain (25,26). These results suggest that environmental factors may affect the frequency of seed transmission and systemic infection by *F. verticillioides*.

In order to better understand the influence of environmental conditions on the frequency of systemic infection, we evaluated the effect of temperature on the first step in the systemic infection process, the transmission of *F. verticillioides* from seed to seedling.

MATERIALS AND METHODS

A *Fusarium* strain was isolated from Iowa maize kernels and identified as *F. verticillioides* based on morphological characteristics (30,31) and sexual fertility testing with *Gibberella fujikuroi* complex mating population tester strains (17) (generously provided by Dr. J. F. Leslie). The strain was deposited at the Pennsylvania State University Fusarium Research Center and designated M-8114.

Transformation. *F. verticillioides* strain M-8114 was transformed with genes for green fluorescent protein (GFP) and hygromycin B resistance using plasmid pIG-PAPA, generously provided by B. Gillian Turgeon (Cornell University, NY).

Plasmid pIGPAPA contains the gene *sGFP* attached to an isocitrate lyase promoter from *Neurospora crassa*. It also includes a hygromycin B phosphotransferase gene (*hygB*) for selection in fungi and an ampicillin resistance gene for selection in *Escherichia coli*. The plasmid was purified using QIAfilter Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) and was linearized with *Bam*HI.

Protocols for preparation of protoplasts were adopted from methods described for *Cochliobolus heterostrophus* by Turgeon et al. (43). Strain M-8114 was grown on complete medium with xylose (CMX) (44) for 7 to 10 days. A conidial suspension (9.5×10^6 conidia/ml) was prepared in liquid complete medium (CM) (16) and incubated at 30°C for 20 h on a rotary shaker at 150 rpm. The resulting mycelial mass was pelleted and then resuspended in 0.7 M NaCl solution. The mycelia then were incubated at 30°C for 1.5 h in enzyme osmoticum (chitinase at 0.1 mg/ml; Sigma-Aldrich, St. Louis), driselase at 7.5 mg/ml (Sigma-Aldrich), and Novozyme 234 at 7.5 mg/ml (InterSpex Products Inc., San Mateo, CA) (19) to digest the cell walls. Protoplasts were harvested by filtering the enzyme-osmoticum through two layers of cheesecloth and one layer of Nitex (pore size 20 μ m; Sefar America, Depew, NY). The filtrate was centrifuged for 15 min at $1,600 \times g$ and decanted. The pellet was washed by adding 10 ml of cold STC (1.2 M sorbitol, 10 mM Tris [pH 7.5], and 50 mM CaCl_2) and centrifuged for 15 min. The supernatant was decanted and the pellet was resuspended in 1 ml of STC.

Protocols for transformation were the same as described for *C. heterostrophus* by Turgeon et al. (43), except for the use of regeneration medium in place of molten acetamide medium. Linearized plasmid DNA (30 μ g) was added to a tube with approximately 3.5×10^6 protoplasts in 100 μ l of STC. Three additions of polyethylene glycol (PEG) solution (MW 3,500; 60% wt/vol; 10 mM Tris, pH 7.5; 50 mM CaCl_2) were made every 10 min in aliquots of 200, 200, and 800 μ l. During these incubation periods, the tube was rolled to mix its contents. STC (1 ml) was added to dilute the PEG solution. The contents of the tube were added to regeneration medium (0.1% yeast extract, 0.1% casein enzymatic hydrolysate, 1 M sucrose, and 2% agar) and incubated at 30°C for 24 h to allow protoplasts to regenerate walls and grow into colonies of mycelia. The plates were overlaid with 1% water agar with hygromycin B (Roche, Indianapolis, IN) at 100 μ g/ml. Colonies that were transformed were resistant to hygromycin B and, therefore, grew through the overlay in 4 to 5 days.

Plugs from the resulting colonies were transferred to petri dishes of CMX with hygromycin B at 100 μ g/ml. Colonies that were not inhibited by the antibiotic were checked for GFP expression with a fluorescence microscope. Thirty-five colonies were confirmed as transformants based on their resistance to hygromycin B and their ability to fluoresce. The transformants were placed on silica gel for storage at 4°C (46).

Transformant characterization. Three of the transformants were tested to confirm that the gene for GFP was mitotically stable. Two mitotic stability tests were conducted with transformants TXI-1, TXI-49, and TXI-79. The transformants were chosen based on their ability to fluoresce and a rapid radial growth on CMX similar to the wild type growth rate.

In the first test, sequential transfers were conducted to determine if gene expression was stable. The test was started by placing a single spore of the transformant on the outside edge of a petri dish containing CMX. Single spores were obtained by spreading a dilute spore suspension on water agar, inspecting the agar surface microscopically, and transferring single spores (and underlying agar) with a dissecting needle. For each transformant, three replicates were made. The petri dishes were incubated at room temperature under fluorescent lighting. When the colony covered the agar surface, a plug of mycelium was taken from the growing margin and transferred to the edge of a new plate of CMX. Four cycles of growth and transfer were done in this manner. Mycelium from the original petri dishes and the sequential transfers was checked for GFP expression with a Leitz fluovert inverted fluorescence microscope and the

results were recorded as positive or negative.

The stability of GFP expression through sporulation also was tested. Spore suspensions (100 spores/ml) of transformants TXI-1, TXI-49, and TXI-79 were prepared and 1 ml of the suspension was spread on each of 10 petri dishes of complete medium with sorbose (CMS) to restrict colony size (16). The dishes were incubated at 23 to 25°C under fluorescent lights for several days until the resulting colonies were approximately 1 to 3 mm in diameter. Each colony was checked for GFP expression using a fluorescence microscope. The number of colonies with GFP expression was recorded.

An experiment was conducted to determine whether transformation had affected the growth of the fungus in liquid culture. Spore suspensions (1.1×10^6 spores/ml) of TXI-79 (transformant) and M-8114 (wild type) were made. In 125-ml Erlenmeyer flasks, 1 ml of spore suspension was added to 29 ml of liquid CMX. For each strain, nine flasks of medium were inoculated. The flasks were placed on a rotary shaker at 100 to 125 rpm at 23 to 25°C. After 2 weeks, the contents of each flask were vacuum filtered onto preweighed filter papers. The filters were placed in petri dishes left partially open and placed in a 26°C incubator to dry for 1 week. The filter papers were then weighed and the dry weight of the mycelial mass was calculated.

A test also was conducted to determine whether transformation had affected the ability of TXI-79 to colonize maize stalk tissues. Thirty seeds of Cargill hybrid 1077 (Cargill Inc., Minneapolis, MN; currently Mycogen Seeds, Indianapolis, IN) were surface disinfested (2 min in 0.5% NaOCl and 0.5 min in sterile distilled water), planted in pots filled with pasteurized soil (1:2:1 peat:soil:perlite), and placed in the greenhouse. At tasseling (VT) (36), the plants were inoculated with either TXI-79 or strain M-8114 (wild type). Spore suspensions (1×10^6 spores/ml) of each strain were made from 7- to 14-day-old cultures grown on CMX medium 24 h before the inoculations and kept at 4°C. The spore suspension (0.5 ml) was injected into the corn plants by syringe just above the second aboveground leaf node. In all, 10 plants were inoculated with TXI-79 and 5 plants with strain M-8114. The stalk of each plant was harvested 46 days after inoculation. The stalks were cut into 30-cm pieces and disinfested for 3 min in 70% ethanol and 5 min in 0.5% NaOCl, and rinsed 2 min in sterile distilled water. Internodes were numbered and the center 2-cm portion of each internode (starting just above the second leaf node) was excised from the stalk, split longitudinally, and placed inner surface down on the medium. Tissues of plants that were inoculated with M-8114 were placed on Nash-

Snyder medium (28) without pentachloronitrobenzene (PCNB). The tissues of plants inoculated with TXI-79 were placed on Nash-Snyder medium without PCNB and with hygromycin B at 100 µl/liter. PCNB was excluded from the medium because incorporating hygromycin B made the medium semiselective for the transformants; however, preliminary experiments indicated that the combination of this antibiotic with PCNB was too inhibitory. The dishes were incubated at room temperature for 4 to 5 days in the dark to allow fungal mycelium to grow out of the tissues. Colonies were checked for the appropriate morphological characteristics or GFP expression.

Growth chamber experiments. Several experiments were conducted in growth chambers to assess the effect of temperature on seed transmission and systemic infection. In different experiments, plants were grown to either V2 or V6 (36). In each experiment, maize plants were grown from seed inoculated with strain TXI-79 and from seeds that were not inoculated (controls). Treatments consisted of three temperature regimes used for three repetitions of each growth stage. A spore suspension (1×10^6 spores/ml) was made from 7- to 14-day-old cultures of TXI-79 grown on CMX and kept at 4°C for 10 h or less until it was used as inoculum. Seeds of Cargill hybrid 1077 were surface disinfested (2 min in 0.5% NaOCl and 0.5 min in sterile distilled water), placed on sterile paper towels, and allowed to dry in a laminar flow hood. After the seeds were dry, they were placed in 125-ml Erlenmeyer flasks, 15 to 25 seeds per flask, with 25 to 50 ml of spore suspension or sterile distilled water. The flasks were placed on a rotary shaker at 100 to 125 rpm for 12 h. The inoculated and noninoculated seeds then were placed on sterile paper towels and allowed to dry in separate hoods.

Each time seeds were inoculated, a sample of 50 inoculated seeds was tested for infestation and infection by TXI-79. Twenty-five of these seeds were placed on Nash-Snyder medium without PCNB and with hygromycin B at 100 µl/liter of medium. The other 25 seeds were surface disinfested in 70% ethanol for 3 min and 0.5% NaOCl for 5 min, sterile distilled water for 2 min, and placed on the same medium. The plates then were incubated in the dark at 23 to 25°C for 4 to 5 days. Fungal mycelium was checked for GFP expression and the number of seeds yielding TXI-79 was recorded.

After the seeds had dried they were planted in pots (one seed per pot) filled with pasteurized soil (1:2:1 vermiculite:soil:peat by volume). For V2 experiments, 35 inoculated seeds and 15 noninoculated seeds were planted in 10-cm pots. For V6 experiments, 25 inoculated seeds and 10 noninoculated seeds were planted in 15-cm pots. Pots containing

inoculated and noninoculated seed then were arranged arbitrarily in the growth chamber.

Treatments consisted of three temperature regimes (low, average, and high) that were designed to simulate the range of temperature conditions that occur in Iowa during the weeks following planting. Results of a temperature study conducted over a 10-year period in Ames, IA (9) were used to determine the average day and night temperatures for each week during the growing season starting in early May, when the average night temperature was 10°C (adequate for maize germination and growth). This was designated the average temperature regime for our experiments. High and low temperature regimes were determined by calculating two standard deviations above or below the temperatures of the average regime. Each regime involved a weekly progression of day-and-night temperatures corresponding to the seasonal temperature progression (Fig. 1). The growth chambers were set for 14-h light periods (a combination of incandescent and fluorescent light), during which time the day temperatures were used, and 10-h dark periods, when the night temperatures were used. Light intensities in the growth chambers ranged across the chambers from 98 to 352 µmol m⁻² s⁻¹. Plants were watered as needed throughout the experiment and supplemental fertilizer was not used. Three growth chambers were used for the V2 experiments that were done in triplicate for each temperature

regime using a different growth chamber each time. Several growth chambers were used for the V6 experiments that also were done in triplicate for each temperature regime using a different growth chamber each time. Data loggers (StowAway and HOBO XT temperature loggers, Onset Computer Corporation, Pocasset, MA) were used to record the actual air and soil temperatures inside the chambers while the experiments were in progress.

At the appropriate sampling date (either V2 or V6), whole plants were harvested, rinsed thoroughly with tap water, surface disinfested (3 min in 70% ethanol, 5 min in 0.5% NaOCl, and 2 min in sterile distilled water), and dissected. For the V2 sampling date, the length of the mesocotyl and height of the stem (portion of the plant between the mesocotyl and the first leaf collar) were measured. The seed and primary root (approximately five 2-cm pieces) were placed on Nash-Snyder medium without PCNB and with hygromycin B. The mesocotyl and stem were cut longitudinally and the inner surfaces were directly placed on the medium. For the V6 sampling date, the stems were excised, surface disinfested, and measured from the soil surface to the sixth leaf collar. Then, the primary root and mesocotyl of the plants were sampled as described above. The internodes of the plants also were sampled by excising the center portions (1 to 2 cm) and cutting them longitudinally. Three belowground internodes were sampled and labeled 1 to 3 beginning with the

Fig. 1. Recorded air temperatures for growth-chamber experiments with maize plants grown from seed inoculated with *Fusarium verticillioides*. Three temperature regimes (low, average, and high) with a 14-h day and 10-h night cycle were implemented as treatments. Regimes were based on average weekly day and night temperatures from a 10-year weather study by Elford and Shaw (10). Low- and high-temperature regimes were determined by calculating two standard deviations below or two standard deviations above the temperatures of the average regime. Error bars are standard deviations, based on mean temperatures for three and six repetitions of experiments.

internode adjacent to the mesocotyl. The four basal aboveground internodes were sampled and labeled 4 to 7. The inner surfaces of the internodes were placed directly on the medium. Petri dishes were placed in clear, plastic containers and kept at 23 to 25°C in the dark. After 4 to 5 days, the tissues were checked for fungal growth that had GFP expression with a fluorescence microscope.

Data analysis. Data generated from the growth-chamber experiments sampled at V2 and V6 were analyzed to determine whether temperature affected the ability of TXI-79 to infect maize tissues (analysis of variance by PROC GLM of statistical program SAS, version 9.1; SAS Institute, Cary, NC). For the V2 experiments, a Latin Square analysis was used to determine the effects of chamber sequence (the order that each temperature regime was used in each chamber), growth chamber, and temperature treatment. Analysis for the V2 sampling dates was conducted separately for each tissue (root, mesocotyl, and stem). The V6 experiments could not be analyzed with a Latin Square analysis because more than three growth chambers were used. For these experiments, the analysis was conducted as a two-way analysis of variance for the effects of chamber and temperature. For each experimental unit at V6, below-ground and aboveground internodes were analyzed separately; the mean percentage of internodes infected with TXI-79 was calculated and used for the statistical analysis. A square-root transformation was used to equalize variances in the V6 internode analyses. Analysis of variance (SAS PROC GLM) also was conducted to deter-

mine whether chamber sequence, growth chamber, temperature regime, or inoculation affected plant height at V2 or V6.

RESULTS

Transformant characterization. The mitotic stability results indicated that GFP expression was stable through vegetative growth and sporulation in the transformed *F. verticillioides* strains. In the first experiment, for each transformant tested (TXI-1, TXI-49, and TXI-79), four of four consecutive transfers for all three replicates maintained GFP expression. In the second experiment, all 411, 340, and 273 colonies derived from conidia of TXI-1, TXI-49, and TXI-79, respectively, expressed GFP. From these results, we concluded that GFP would be an effective marker for *F. verticillioides* strain M-8114.

Transformation did not affect the growth of the strain in liquid culture. There was no significant difference in dry weight between TXI-79 and M-8114 ($P = 0.2564$), indicating that the strains grew at comparable rates.

Transformant TXI-79 and the wild-type M-8114 colonized maize stalk tissues at similar rates. A PROC GLM analysis of variance was conducted to compare the highest internode of each plant from which TXI-79 and M-8114 were recovered. There was no significant difference in the extent of maize stalk colonization by the two strains ($P = 0.1277$) (*data not shown*). From this, we concluded that transformation did not affect the ability of the fungus to colonize maize stalk tissues.

Growth chamber experiments. Our inoculation method provided us with high

levels of both seed infestation and infection. For all of the experiments, 100% of the seeds that were cultured without surface disinfection were infested with TXI-79, and 50 to 100% of the seeds that were surface disinfested and cultured were infected with TXI-79.

Air temperatures usually were maintained within 1°C of the desired temperature (Fig. 1). Soil temperatures were similar to air temperatures. The temperature regimes caused the plants to develop at different rates; therefore, the age of plants at V2 or V6 differed among the temperature treatments. Plants grown to growth stage V2 under the low-temperature regime were approximately 6 weeks old, whereas plants in the average- and high-temperature regimes were approximately 4 and 2 weeks old, respectively. At V6, the plants grown under the low-, average-, and high-temperature regime were approximately 12, 8, and 6 weeks old, respectively. Neither temperature treatment nor inoculation affected the number of plants that emerged or the final plant stand counts. Plants did not display symptoms of seedling disease.

The mean plant heights at V2 were 3.6, 5.3, and 5.0 cm for the low-, average-, and high-temperature regimes, respectively. Chamber sequence, growth chamber, and inoculation did not affect plant height ($P > 0.05$), but temperature regime did ($P = 0.0224$). For the V6 sampling date, the mean plant heights were 16.8, 22.4, and 26.0 cm for the low-, average-, and high-temperature regimes, respectively. For these experiments, an interaction existed between temperature regime and growth chamber ($P = 0.0071$). Therefore, conclusions could not be drawn on the effects of growth chamber and temperature on plant height.

At the V2 sampling date, TXI-79 was recovered from root, mesocotyl, and stem tissues of plants grown from inoculated seed under all three temperature regimes. The recovery of TXI-79 from these tissues was ≥ 72 , ≥ 68 , and $\geq 75\%$ at the low-, average-, and high-temperature regimes, respectively (Fig. 2). Recovery of TXI-79 from plant tissues grown from noninoculated seed was $\leq 2\%$. Chamber sequence and growth chamber did not significantly affect recovery of the fungus from plant tissues at V2 ($P > 0.05$). Similarly, recovery of the fungus did not differ among temperature treatments ($P > 0.05$; Table 1).

For the V6 sampling date, mean recovery of TXI-79 from roots and mesocotyl tissues was $\geq 97\%$ at all three temperature regimes; for all below-ground internodes, mean recovery ranged from 81.3% (low temperature) to 92.5% (high temperature) (Fig. 3). For specific belowground internodes, the range of means was ≥ 68 , ≥ 71 , and $\geq 87\%$ at the low-, average-, and high-temperature regimes, respectively, depending on the specific internode (Fig. 4). For

Fig. 2. Recovery of green fluorescent protein-expressing *Fusarium verticillioides* strain TXI-79 from tissues of maize plants at growth stage V2, grown from inoculated seed at three different temperature regimes. Each value represents the mean of three replicated experiments of 22 to 35 plants each. Error bars are standard deviations.

all aboveground tissues (internodes ≥ 4), mean recovery of TXI-79 ranged from 10.6% (low temperature) to 25.8% (high temperature) (Fig. 3). There was a steep decline in the recovery of TXI-79 from progressively higher internodes for all three treatments and the fungus was not recovered from tissues above the sixth internode (Fig. 4). Although there was a trend toward higher frequency of infected below- and aboveground internodes with increasing temperature, differences were not significant; nor did growth chamber significantly affect the mean number of infected below- or aboveground internodes (Table 1). TXI-79 was not recovered from below- or aboveground tissues of plants grown from noninoculated seed.

DISCUSSION

The influence of environmental factors on seed transmission of *F. verticillioides* has not been well characterized, and this may be the first report on temperature effects. Sachan et al. (38) compared seed transmission frequencies for *F. moniliforme* in rice at 20, 25, and 30°C, and reported that 30°C resulted in the highest level of seed transmission. The relevance of this report to *F. verticillioides* on maize is unclear, however, because the pathogen involved was likely *F. fujikuroi* (18), not *F. verticillioides*, despite both species once being recognized by the same species epithet (*moniliforme*). Our results indicate that temperature variation during the first few weeks after planting does not influence the frequency of seed transmission or variability in systemic infection frequency in the maize-*F. verticillioides* pathosystem. At growth stages V2 and V6, temperature did not significantly affect the transmission of TXI-79 from seed to seedling or the frequency of systemic infection in maize plants. Seed transmission occurred at a high frequency, which is consistent with some previous work (4,13,15,26,39). Our results also showed TXI-79 resided in the crown tissues of plants at the early growth stages, consistent with the results obtained by Lawrence et al. (15), who also found that the fungus does not colonize the entire plant until after tasseling. Oren et al. (34) grew maize seedlings in potting soil infested with a GFP-expressing *F. verticillioides* isolate and subsequently detected the isolate in aboveground tissues of 14-day-old maize seedlings. Infection could not be detected by microscopy of plant tissue, but only by culturing on a semiselective medium, similar to our study. The authors (34) did not report the percentage of plants in which infection could be detected, but suggested that there were only "trace" amounts of mycelium in the aboveground plant parts after 14 days. Maize seeds were inoculated in the same study, but the authors did not report detection of the GFP-expressing isolate in aboveground parts of plants grown from

inoculated seed. This result differs from ours, but might be explained by the use of fungicide-treated seed of a sweet maize cultivar in the study by Oren et al., who did not report on the success of their seed-inoculation method or present any quantitative results.

Seed-to-kernel transmission involves several stages, and seed-to-seedling transmission is only the first stage. Our results support the hypothesis that seed-to-seedling transmission is not the limiting stage for transmission of the fungus, as we have suggested previously (26). Appar-

ently, this stage of the process occurs at a high frequency under a broad range of temperature conditions. Seed-to-kernel transmission tends to occur at a lower frequency, indicating that limiting factors may operate at some later stage in the process.

The temperature treatments affected the growth of plants and most likely affected growth of the fungus as well. The optimal temperature for maize plant growth is 30°C (3) and the optimal temperature for *F. verticillioides* growth is 28°C (45) to 30°C (22). Minimum and maximum tem-

Table 1. Results of analysis of variance for the effects of experiment, growth chamber, and temperature on the recovery of green fluorescent protein-expressing *Fusarium verticillioides* strain TXI-79 from maize plant tissues sampled at growth stage V2 or V6 in growth chamber experiments

Growth stage, tissue	Effect	df	Mean square	F value	P value
V2					
Root	Experiment	2	63.4	5.98	0.1432
	Chamber	2	139.0	13.11	0.0709
	Temperature	2	45.4	4.28	0.1893
Mesocotyl	Experiment	2	12.4	0.17	0.8558
	Chamber	2	49.0	0.67	0.6003
	Temperature	2	27.4	0.37	0.7287
Stem	Experiment	2	117.8	1.30	0.4340
	Chamber	2	1,269.8	14.05	0.0664
	Temperature	2	74.2	0.82	0.5492
V6					
Root	Chamber	4	1.4	0.16	0.8537
	Temperature	2	1.4	0.16	0.8537
Mesocotyl	Chamber	4	1.8	1.00	0.4444
	Temperature	2	7.1	4.00	0.1111
Belowground internodes	Chamber	4	125.3	0.49	0.6433
	Temperature	2	94.9	0.37	0.7099
Aboveground internodes	Chamber	4	220.9	0.81	0.5049
	Temperature	2	183.0	0.67	0.5591

Fig. 3. Recovery of green fluorescent protein-expressing *Fusarium verticillioides* strain TXI-79 from tissues of maize plants at growth stage V6, grown from inoculated seed at three different temperature regimes. Root and mesocotyl values represent the mean of three replicated experiments of 21 to 25 plants each. Internode values represent the mean total percentages of infected belowground internodes (three per plant) and aboveground internodes (three per plant) for three replicated experiments of 21 to 25 plants each. Error bars are standard deviations.

temperatures for growth of this fungus are 4 and 36°C, respectively (24). None of the regimes included temperatures outside the range of growth for *F. verticilloides*; however, only the high-temperature regime reached the optimal temperature for host and pathogen growth. Therefore, the different temperature regimes caused the plants to develop at different rates, so that plants grown at the low-temperature regime were older and shorter than those grown at the average- and high-temperature regimes when they were sampled. It is possible that, because the low-temperature plants were shorter, the fungus did not need to grow as extensively to reach a given internode. It also is very likely that fungal growth was slowed in the low-temperature treatment; however, because these plants were older, the fungus had more time to colonize the tissues, resulting in a level of infection similar to the warmer temperature treatments. These factors illustrate that temperature effects on *F. verticilloides*–maize interactions are more complex than can be predicted by direct effects of temperature on fungal growth.

Maize seeds planted under cool conditions in the field often experience seedling disease (40); however, we did not observe symptoms in our study. It is likely that the *Pythium* spp. that primarily cause seedling disease at low temperatures (8) were not present in the pasteurized soil used in this study. The lack of seedling disease also can be attributed to the fact that soil moisture was not excessive in our study. Additionally, there is variability in aggressiveness among *Fusarium* strains (27), and

some strains have little capacity to cause seedling blight.

GFP was a suitable marker for *F. verticilloides* strain M-8114 (transformant TXI-79) and it allowed us to distinguish our strain from other naturally occurring *Fusarium* strains in the environment. GFP also proved to be more reliable and convenient than vegetative compatibility that has been used as a marker in previous studies (5,13,25,26). Unlike GFP, which was maintained during our experiments, vegetative compatibility can be lost if auxotrophic mutants become dysfunctional and cannot form heterokaryons or revert back to wild-type growth (5). Obtaining experimental results also is more efficient with GFP than with vegetative compatibility, which is more labor intensive.

Our results indicate that, if seed is infected with *F. verticilloides*, seed transmission is a common event and symptomless systemic infection can be initiated under a broad range of temperature conditions. The significance of these results depends on the frequency of *F. verticilloides* infection in commercial seed lots; infection frequency varies widely (1,12) but can be managed effectively through commercial seed-conditioning methods (12). Symptomless systemic infection also can be initiated by direct infection when seedlings contact inoculum in the spermosphere or rhizosphere (6,7,33), and some seedling infection in our study may have been due to externally (versus internally) seedborne inoculum. In fact, crop residue in the spermosphere or rhizosphere may be a common source of inoculum for systemic infection (5–7). The relative im-

portance of systemic infection as a pathway for kernel infection remains unclear; however, the primary significance of this pathway may be its contribution, if any, to mycotoxin contamination of grain. However, additional studies are needed to define this contribution.

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Fig. 4. Recovery of green fluorescent protein-expressing *Fusarium verticilloides* strain TXI-79 from individual internode tissues of maize plants at growth stage V6, grown from inoculated seed at three different temperature regimes. Internodes 1 to 3 were belowground; internodes 4 to 6 were aboveground. Each value represents the mean of three replicated experiments of 21 to 25 plants each.

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