Development of Molecular Assays for Detection of *Stenocarpella maydis* and *Stenocarpella macrospora* in Corn

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Abstract

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The causal agents of Diplodia ear rot are two species of the *Stenocarpella* genus, *S. macrospora* and *S. maydis*. In addition to ears, both pathogens can infect leaves and stalks, and both are present in most corn-production regions around the world. It is difficult to visually distinguish between the two pathogens based on plant symptoms and fungal signs. To facilitate accurate and rapid pathogen identification, polymerase chain reaction (PCR) assays were developed for identification of each species. Species-specific primers of 18 to 20 nucleotides in length were designed, targeting a portion of the internal transcribed spacer

(ITS) region of the fungal genome for conventional and real-time PCR assays. The conventional PCR method successfully amplified a single 1.7-kb and 800-bp fragment for each *S. maydis* and *S. macrospora* isolate, respectively. The real-time method was performed using SYBR green dye, and detection of each specific target pathogen was successfully obtained. In total, 82 *S. maydis* and 15 *S. macrospora* isolates were tested to evaluate the reproducibility of these primers. Both methods provide a rapid and specific tool for the detection of *Stenocarpella* spp.

Stenocarpella macrospora (Earle) B. Sutton and *S. maydis* (Berk.) B. Sutton, the only two species included in the *Stenocarpella* genus (9,12,47,48), have been identified as causal agents of Diplodia ear rot, Diplodia leaf streak, and Diplodia stalk rot (53). Both species are able to infect ears, stalks, and leaves (47,48) and, under specific conditions, these fungi can produce mycotoxins (10,45,55). *S. maydis* is recognized as one of the most persistent and important ear rot pathogens in the world (41). Typically, *S. macrospora* has been more significant in tropical and subtropical areas (15,20,24); however, the disease is commonly present in the United States, and increases in disease incidence have been observed across the Midwest in recent years (3,52).

Visually distinguishing between symptoms caused by these two species is challenging (6). *S. macrospora* produces long elliptical gray-green lesions with yellowish margins on leaves. Concentric zones are evident in the lesion around the point of infection origin. Pycnidia are typically observed in the older part of the lesion (19,24,53). Foliar lesions caused by *S. maydis* are oval and smaller than those caused by *S. macrospora* (19) and pycnidia production is less abundant across the lesion. Both diseases may be confused with other common corn foliar diseases, including northern corn leaf blight (caused by *Exserohilum turcicum*; 1) and Goss's bacterial leaf blight (*Clavibacter michiganensis* subsp. *nebraskensis*; 56). When ears are infected, both species can colonize the ear with white, cottony mycelia. Black pycnidia can be observed on the husk or on the kernel surface (6,13,24). Crop rotation and tillage are currently the only reliable methods for disease management. Hybrids with complete resistance to these diseases are not known, although some hybrids may be less susceptible than others.

In 2012, Diplodia ear rot, leaf streak, and stalk rot resulted in a combined loss of 0.97 million metric tons in the United States and Ontario, Canada (31). In addition to yield loss, in some subtropical and tropical corn production areas, cases of diplodiosis, a neuro-mycotoxicosis of livestock, have been reported and associated with *S. maydis* (17,23,28,35,37). Diplodiatoxin and diplonine have also

been associated with diplodiosis but these mycotoxins do not always cause the neurological signs associated with diplodiosis symptoms in livestock animals and, therefore, it is believed that the main causal agents responsible for this mycotoxicosis are still unknown (45,46,55). *S. macrospora* produces diplosporin and diplodiol, two mycotoxins that affect small animals (10,18). Field outbreaks were reported only in South Africa (16,28) until 1964, when Darvall reported a possible outbreak in Australia. Recently, Argentina (32) and Brazil (38) reported cases of animal poisoning by diplodiosis. Although there have been no reports of diplodiosis in the United States (22,51), corn grain is tested for the presence of these pathogens when exported to other countries. *S. maydis* is considered a regulated nonquarantine pest (RNQP) in Brazil, due to the negative impact it has on corn production (2,44).

Currently, *S. maydis* is considered a major pathogen throughout the corn-producing regions in the United States and, although *S. macrospora* is currently considered a minor pathogen in the United States, Latterell et al. (19) concluded that this pathogen could compromise corn production in humid areas of the United States. The distribution and prevalence of *S. macrospora* in the United States is not well known. Previous studies have focused on determining morphological differences between these two pathogens. There are clear differences in conidial dimensions and number of septa between the two species. *S. maydis* conidia are 15 to 34 by 5 to 8 μm , with 0 to 2 septae. *S. macrospora* conidia are almost triple the size of *S. maydis* conidia, being 55 to 106 by 6 to 14 μm and with 0 to 3 septae (12,24,47,48). There are also color variances between young and old cultures of each species when grown on potato dextrose agar (PDA) (8,25,36). Initially, both species have a white, cottony appearance on the media; however, after 2 weeks, *S. maydis* cultures turn grayish to dark brown. Cultures of *S. macrospora* maintain the original white appearance. Another distinguishable characteristic is that *S. maydis* reportedly produces higher numbers of pycnidia in culture (30). Pycnidia production of *S. macrospora* in culture increases when mycelium is removed from surface of culture. However, *S. macrospora* requires more time to develop pycnidia and, frequently, pycnidia are embedded in the media or at the edge of the plate (15,19,29), making it difficult to obtain conidia for identification (2).

Previous studies described the use of the polymerase chain reaction (PCR) method to identify *Stenocarpella* spp. (33,57). Primers designed for these assays were based on the internal transcribed spacer (ITS) region for *Stenocarpella* spp., and these assays were able to differentiate *Stenocarpella* spp. from other fungal

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species but were not specific enough to differentiate between the two *Stenocarpella* spp. (2).

According to Lievens et al. (21), the absence of an effective and accurate method to detect and identify pathogens is one of the major limitations of plant disease management. Although *Stenocarpella* spp. can be distinguished based on a combination of morphological and cultural factors, these techniques are time consuming and are not conducive to the accurate and rapid diagnosis required for regulatory agencies that monitor corn seed pathogens to prevent pathogen establishment in new areas and to monitor grain for the presence of pathogens that produce mycotoxins. *S. maydis* is considered an RNQP in Brazil, due to the negative impact it has on

corn production, and corn is routinely screened for presence of the pathogen (2,44). In fact, Casa et al. (6) reported that seed infected with *Stenocarpella* spp. was responsible for introducing the pathogen into areas in Brazil where it was not previously established. Currently, molecular assays for detecting and distinguishing *Stenocarpella* spp. are lacking. The objective of this study was to design and validate conventional and real-time PCR assays for *Stenocarpella* spp.

Materials and Methods

Fungal isolates and growth conditions. In total, 82 *S. maydis* isolates and 15 *S. macrospora* isolates collected in the United States

Table 1. Isolate code, year, and collection site of *Stenocarpella* spp. and other pathogenic species used in this study

Isolate code	Pathogen	Year	County, state
1-1-1	<i>Stenocarpella maydis</i>	2010	Tippecanoe, IN
1-1-3	<i>S. maydis</i>	2010	Tippecanoe, IN
1-2-4	<i>S. maydis</i>	2010	Tippecanoe, IN
1-3-3	<i>S. maydis</i>	2010	Tippecanoe, IN
1-6-2	<i>S. maydis</i>	2010	Tippecanoe, IN
1-11-2	<i>S. maydis</i>	2010	Tippecanoe, IN
1-11-4	<i>S. maydis</i>	2010	Tippecanoe, IN
1-13-2	<i>S. maydis</i>	2010	Tippecanoe, IN
1-14-4	<i>S. maydis</i>	2010	Tippecanoe, IN
1-18-1	<i>S. maydis</i>	2010	Tippecanoe, IN
1-19-1	<i>S. maydis</i>	2010	Tippecanoe, IN
1-20-4	<i>S. maydis</i>	2010	Tippecanoe, IN
2-2-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-3-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-4-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-6-2	<i>S. maydis</i>	2010	Tippecanoe, IN
2-7-4	<i>S. maydis</i>	2010	Tippecanoe, IN
2-8-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-13-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-14-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-16-1	<i>S. maydis</i>	2010	Tippecanoe, IN
2-20-1	<i>S. maydis</i>	2010	Tippecanoe, IN
3-1-1	<i>S. maydis</i>	2010	Tippecanoe, IN
3-2-2	<i>S. maydis</i>	2010	Tippecanoe, IN
3-3-1	<i>S. maydis</i>	2010	Tippecanoe, IN
3-3-2	<i>S. maydis</i>	2010	Tippecanoe, IN
3-4-4	<i>S. maydis</i>	2010	Tippecanoe, IN
3-5-2	<i>S. maydis</i>	2010	Tippecanoe, IN
3-6-4	<i>S. maydis</i>	2010	Tippecanoe, IN
3-8-2	<i>S. maydis</i>	2010	Tippecanoe, IN
3-9-1	<i>S. maydis</i>	2010	Tippecanoe, IN
3-9-3	<i>S. maydis</i>	2010	Tippecanoe, IN
3-9-4	<i>S. maydis</i>	2010	Tippecanoe, IN
3-10-1	<i>S. maydis</i>	2010	Tippecanoe, IN
3-10-2	<i>S. maydis</i>	2010	Tippecanoe, IN
3-11-1	<i>S. maydis</i>	2010	Tippecanoe, IN
3-11-2	<i>S. maydis</i>	2010	Tippecanoe, IN
3-12-3	<i>S. maydis</i>	2010	Tippecanoe, IN
3-12-4	<i>S. maydis</i>	2010	Tippecanoe, IN
1b.1	<i>S. maydis</i>	2011	Tippecanoe, IN
1b.2	<i>S. maydis</i>	2011	Tippecanoe, IN
2b.2	<i>S. maydis</i>	2011	Tippecanoe, IN
3a.1	<i>S. maydis</i>	2011	Tippecanoe, IN
4b.1	<i>S. maydis</i>	2011	Tippecanoe, IN
7a.1	<i>S. maydis</i>	2011	Tippecanoe, IN
8b.1	<i>S. maydis</i>	2011	Tippecanoe, IN
8b.2	<i>S. maydis</i>	2011	Tippecanoe, IN
9b.1	<i>S. maydis</i>	2011	Tippecanoe, IN
10a.1	<i>S. maydis</i>	2011	Tippecanoe, IN
10a.2	<i>S. maydis</i>	2011	Tippecanoe, IN
4a.1.1	<i>S. maydis</i>	2011	Indiana
4a.1.2	<i>S. maydis</i>	2011	Indiana
3b.1	<i>S. maydis</i>	2011	Indiana

(continued on next column)

Table 1. (continued from preceding column)

Isolate code	Pathogen	Year	County, state
1.2	<i>S. maydis</i>	2012	Tippecanoe, IN
1.4	<i>S. maydis</i>	2012	Tippecanoe, IN
2	<i>S. maydis</i>	2012	Tippecanoe, IN
2.1	<i>S. maydis</i>	2012	Tippecanoe, IN
3.1	<i>S. maydis</i>	2012	Tippecanoe, IN
3.2	<i>S. maydis</i>	2012	Tippecanoe, IN
3.3	<i>S. maydis</i>	2012	Tippecanoe, IN
3.4	<i>S. maydis</i>	2012	Tippecanoe, IN
4.1	<i>S. maydis</i>	2012	Tippecanoe, IN
5.1	<i>S. maydis</i>	2012	Tippecanoe, IN
5.2	<i>S. maydis</i>	2012	Tippecanoe, IN
6	<i>S. maydis</i>	2012	Tippecanoe, IN
7.1	<i>S. maydis</i>	2012	Tippecanoe, IN
8	<i>S. maydis</i>	2012	Tippecanoe, IN
9	<i>S. maydis</i>	2012	Tippecanoe, IN
5	<i>S. maydis</i>	2013	Tippecanoe, IN
8	<i>S. maydis</i>	2013	Tippecanoe, IN
10	<i>S. maydis</i>	2013	Knox, IN
13	<i>S. maydis</i>	2013	Knox, IN
15.1	<i>S. maydis</i>	2013	Howard, MO
15.2	<i>S. maydis</i>	2013	Howard, MO
44	<i>S. maydis</i>	2013	Cayugo, NY
45	<i>S. maydis</i>	2013	Tippecanoe, IN
1	<i>S. maydis</i>	2014	Gibson, TN
2	<i>S. maydis</i>	2014	Gibson, TN
3	<i>S. maydis</i>	2014	Gibson, TN
5	<i>S. maydis</i>	2014	Gibson, TN
6	<i>S. maydis</i>	2014	Gibson, TN
7	<i>S. maydis</i>	2014	Gibson, TN
1a.1.1	<i>S. macrospora</i>	2011	Indiana
1a.1.2	<i>S. macrospora</i>	2011	Indiana
P11-2.1a.1	<i>S. macrospora</i>	2011	Kosciusko, IN
P11-2.1a.2	<i>S. macrospora</i>	2011	Kosciusko, IN
4a.2	<i>S. macrospora</i>	2011	Indiana
4c.1.1	<i>S. macrospora</i>	2011	Indiana
10a.2	<i>S. macrospora</i>	2011	Indiana
10.1b	<i>S. macrospora</i>	2011	Indiana
1a	<i>S. macrospora</i>	2012	Tippecanoe, IN
1b	<i>S. macrospora</i>	2012	Tippecanoe, IN
4b	<i>S. macrospora</i>	2012	Tippecanoe, IN
1	<i>S. macrospora</i>	2013	Indiana
2.1a	<i>S. macrospora</i>	2013	Jennings, IN
2.2	<i>S. macrospora</i>	2013	Jennings, IN
4	<i>S. macrospora</i>	2014	Gibson, TN
ET	<i>Exserohilum turcicum</i>	2012	Tippecanoe, IN
Fv	<i>Fusarium verticillioides</i>	2012	Indiana
Fg	<i>Fusarium graminearum</i>	2012	Indiana
Af	<i>Aspergillus flavus</i>		Urbana, IL
Cmn	<i>Clavibacter michiganensis</i> subsp. <i>Nebraskensis</i>	2008	Pulaski, IN
Mp	<i>Macrophomina phaseolina</i>	2011	Grant, WI
Cg	<i>Colletotrichum graminicola</i>	2012	Illinois
Ps	<i>Phoma</i> spp.	2012	Hamilton, IN
Dp	<i>Diplodia pinea</i>	2012	Tippecanoe, IN

were included in this study (Table 1). Other pathogenic species that commonly infect corn or are closely related to the *Stenocarpella* genus were included to assess assay validity (Table 1). *Stenocarpella* spp. isolates were obtained from corn kernels and corn leaves collected between 2010 and 2014 from fields in Illinois, Indiana, Missouri, New York, Tennessee, and Wisconsin. Isolates were obtained from plant tissue by disinfecting leaves with 5% commercial bleach (a.i. 5.05% sodium hypochlorite) and Tween 20 (Bio-Rad Laboratories, Hercules, CA) for 10 s. Tissue was washed twice with water, dried for 4 h, and transferred to PDA. Cultures were stored at 28°C under 12-h light and dark cycles until pycnidia were visible (approximately 7 days). A single pycnidium was hand picked with a sterile needle and placed into a 1.5-ml Flex-Eppendorf tube (Eppendorf AG, Hamburg, Germany) with 150 µl of double distilled H₂O. After agitation, four drops of the suspension were transferred with a sterilized pipette to water agar, containing Bacto agar (Becton, Dickinson and Company, Sparks, MD) at 20 g/liter. Plates were incubated at room temperature for 24 h under dark conditions. Single germinated spores were picked and transferred to natural oatmeal agar (NOA) (40). Plates were stored at 28°C under 12-h light and dark cycles until the plate was fully colonized (approximately 7 days). Once a single-spore culture was obtained, a 5-mm plug was transferred to a new NOA plate already containing sterilized filter paper (8 by 8 cm). Plates were placed again at 28°C under 12-h light and dark cycles until the filter paper was fully colonized and pycnidia production was visible (approximately 15 days). Filter paper was removed from the plate with a sterile forceps and transferred to new, empty petri dish. The filter paper was completely dried, placed inside an envelope covered with foil paper, and stored at -20°C until further use.

DNA extraction. Pure cultures from storage were subcultured on fresh NOA plates. Plates were incubated at 28°C under 12-h light and dark cycles for 7 days, after which approximately 40 mg of fresh mycelium was collected by scraping from the colony surface. DNA was extracted from each isolate by a cetyltrimethyl ammonium bromide (CTAB) protocol derived from Saghai-Marooof et al. (42) and stored in nuclease-free sterile water at -20°C. DNA also was extracted from corn kernels and stalks infected by *S. maydis* in 2014 and from kernels and leaves infected by *S. macrospora* in 2014. DNA extraction from corn plant tissue also followed the CTAB protocol. DNA concentration was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). For conventional and real-time PCR assays, DNA concentrations were adjusted to 10 ng/µl.

ITS PCR and sequencing. The ITS regions of *Stenocarpella* spp. were amplified with ITS1F (CTTGGTCATTTACAGGAAGTAA; 14) and ITS4 (TCCTCCGCTTATTGATATGC; 54) primers. PCR amplifications were carried out in a 25-µl reaction volume containing 1 µl of template DNA (various DNA concentrations), 0.20 mM each deoxynucleoside triphosphate (Promega Corp., Madison WI), 4 U of Taq DNA polymerase (New England BioLabs Inc., Ipswich, MA), 1× PCR buffer (New England BioLabs Inc.), 0.60 µM each forward and reverse primer, and 16 µl of nuclease-free sterile water. PCR was conducted with a AB 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) with the following program: an initial heating cycle of 95°C for 2 min; 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min; and a 10-min final extension at 72°C. Each total product volume (10 µl) was loaded on a 1% (wt/vol) agarose gel in 150 1× Tris-acetate-EDTA buffer and stained with ethidium bromide. The gels were run at 50 V for 40 min and visualized using a UV-transilluminator FBTI-88 (Thermo Fisher Scientific Inc., Waltham, MA). PCR products were extracted from gels, purified, and sent to the Purdue Genomics Facility for sequencing. DNA sequences were BLAST analyzed to a database constructed from a partial genome assembly. The ITS region was located on a 7.8-kb contig (GenBank accession number KP164561). The sequence of this contig was used to identify two specific sets of primers for *S. maydis* for both conventional and real-time PCR assays. The sequence of the amplified fragments from *S. macrospora*

was aligned with the *S. maydis* sequence, and a single set of primers for this species was designed to use in both conventional and real-time PCR assays. Both species-specific sets of primers (Table 2) were synthesized by Integrated DNA Technologies (Coralville, IA).

Conventional PCR for the diagnosis of *Stenocarpella* spp. To determine the optimal annealing temperature of each primer set, temperature gradient PCR was carried out using a Mastercycler pro S (Eppendorf AG) with an annealing temperature range of 52 to 63°C for *S. macrospora* and 54 to 65°C for *S. maydis*. The optimal annealing temperatures for *S. macrospora* and *S. maydis* were 57 and 60°C, respectively. One negative control, containing nuclease-free sterile water instead of DNA, was included with each species assay.

For *S. maydis*, PCR were conducted in a 20-µl reaction volume containing template DNA at 10 ng/µl, 0.25 mM each deoxynucleoside triphosphate (Promega Corp.), Taq DNA polymerase (New England BioLabs Inc.) at 4 U/µl, 1× PCR buffer (New England BioLabs Inc.), 0.75 µM each forward and reverse primer (Table 2), and 11 µl of nuclease-free sterile water (Thermo Fisher Scientific Inc., Waltham, MA). Fragments were amplified using the Smay.F/Smay.R primers (Table 2) under the following program: an initial heating cycle of 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a 10-min final extension at 72°C.

For *S. macrospora*, PCR were conducted in a 20-µl reaction volume containing template DNA at 10 ng/µl, 0.25 mM each deoxynucleoside triphosphate (Promega Corp.), Taq DNA polymerase (New England BioLabs Inc.) at 4 U/µl, 1× PCR buffer (New England BioLabs Inc.), 0.25 µM forward primer (Table 2), 0.75 µM reverse primer (Table 2), and 12 µl of nuclease-free sterile water (Thermo Fisher Scientific Inc., Waltham, MA). Fragments were amplified using the Smac.F/Smac.R primers (Table 2) under the following program: an initial heating cycle of 95°C for 2 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min; and a 10-min final extension at 72°C.

Following PCR, amplified products were separated via gel electrophoresis and visualized as described above. Each reaction was performed three times. All amplified PCR products were resequenced for verification.

Real-time PCR for the diagnosis of *Stenocarpella* spp. PCR assays were performed using a LightCycler 480 Instrument (Roche Diagnostics Corporation, Hague Road, IN). For *S. maydis*, real-time PCR was conducted in a 20-µl volume reaction mix which included template DNA at 10 ng/µl, 1× iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.10 µM forward primer, 0.60 µM reverse primer, and 8.6 µl of nuclease-free sterile water (Thermo Fisher Scientific Inc., Waltham, MA). Isolates tested using the RT.Smay.F/RT.Smay.R primers were run as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The thermal profile for melting curve analysis consisted of a denaturation for 5 s at 95°C, lowered to 55°C for 1 min, then increased to 97°C with continuous fluorescence readings.

In total, 15 isolates of *S. macrospora* were amplified in a reaction volume of 20 µl which included template DNA at 10 ng/µl, 1× iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.25 µM forward primer, 0.75 µM reverse primer, and 7 µl of nuclease-free sterile water (Thermo Fisher Scientific Inc., Waltham, MA). These 15 isolates were tested with the same primers (RT.Smac.F/RT.Smac.R) and thermal cycling settings as those for the conventional PCR. The thermal profile for melting curve analysis was the same as for *S. maydis*.

Water control (nuclease-free sterile water) was included in each run for both assays. To evaluate specificity of each assay, the heterologous *Stenocarpella* spp., six fungal pathogens of corn, one bacterial pathogen of corn, and two pathogenic fungi not known to attack corn were also included in each run (Table 1). Each reaction was performed three times.

Detection limit for conventional and real-time PCR. The detection limit for each assay was determined by serial 10-fold dilution of

genomic DNA from pure fungal cultures and from corn seed. Dilutions ranged from 10 to 10×10^{-6} ng/ μ l for *S. maydis* and 10 to 10×10^{-3} ng/ μ l for *S. macrospora*. The PCR conditions for both assays described previously were used. Amplification was confirmed on a 1% (wt/vol) agarose gel and by melting curve profile for conventional and real-time PCR, respectively.

Results

Fragments of the ITS region were successfully isolated from the two *Stenocarpella* spp. using primers ITS1F and ITS4 (gel not shown). The primer set Smay.F/Smay.R, specific for *S. maydis*, generated a single and specific PCR product of 1.7 kb (Fig. 1A). This PCR product was observed in all 82 *S. maydis* isolates tested and in none of the *S. macrospora* isolated tested. No cross-amplification product was obtained when DNA from other fungal or bacterial pathogens was used (Fig. 1B). When DNA extracted directly from infected corn kernels and stalks (not pure culture) was tested using the conventional PCR set of primers, a specific PCR product of 1.7 kb was obtained (gel not shown).

The *S. macrospora*-specific primers Smac.F/Smac.R generated a single PCR product of 800 bp (Fig. 2A). This PCR product was observed only in the 15 *S. macrospora* isolates tested, and DNA extracted from other fungal or bacterial isolates did not generate a PCR product (Fig. 2B). When DNA extracted directly from infected corn kernels and leaves (not pure culture) was tested using the conventional PCR set of primers, a specific PCR product of

800 bp was observed on the corn kernels DNA; however, for the DNA extracted from corn leaves, a faint band was obtained (gel not shown).

Primers designed for the real-time assay were tested first in a conventional PCR assay. Two PCR products of 500 and 800 bp were generated by the primer sets RT.Smay.F/RT.Smay.R and RT.Smac.F/RT.Smac.R, respectively, for each target-species (gel not shown). No cross-amplification product was obtained when DNA from other fungal or bacterial isolates was tested.

The real-time PCR assay for *S. maydis* specifically amplified DNA from all *S. maydis* isolates tested (Fig. 3A). Positive *S. maydis* isolates had a range of melting peaks of 88.54 to 88.94°C (Fig. 3B and C) and cycle threshold (C_t) values of 23.18 to 26.16 (Fig. 3C). DNA extracted from corn kernels and stalks infected with *S. maydis* were tested with RT.Smay.F/RT.Smay.R primers, melting peaks were 88.74 and 88.94°C, respectively (Fig. 3B and C), and C_t values were 28.55 and 30.00, respectively (Fig. 3C). The *S. maydis*-specific real-time PCR assay did not amplify DNA of any of the *S. macrospora* isolates. Negative control samples showed a different range of melting peaks (64 to 78.01°C; Fig. 3B and C) compared with the *S. maydis* isolates. C_t values from most of the negative controls were ≥ 30 ; however, DNA of the nontarget species *Aspergillus flavus* and *E. turcicum*, reported C_t values of 26.08 and 27.21, respectively. The specific primers RT.Smac.F/RT.Smac.R produced an amplicon of the expected size for all *S. macrospora* isolates (Fig. 4A). *S. macrospora* isolates had a melting peak range of 89.39 to 89.80°C

Table 2. Primer sequence used to identify isolates of *Stenocarpella* spp.

Primer	Sequence 5'–3'	Annealing temperature (°C)	Product size (bp)	Product melt curve peak (°C)
Smay.F	CCTGCTATGCATAGGTCG	52.6	...	...
Smay.R	CAC CAG GCC GTT AAG CCT TA	57.4	1,700	...
RT.Smay.F	GTT TCC ATG ACC TGC TCA CG	56.3	...	...
RT.Smay.R	TGT TGC TCG GTT TCA GGC TTG	58.4	500	88
Smac.F	GGG CAA ATT TTC TCG GAG G	54	...	...
Smac.R	GCA GCT ATT CAG CGT TCA TC	54.1	800	...
RT.Smac.F	GGG CAA ATT TTC TCG GAG G	54	...	...
RT.Smac.R	GCA GCT ATT CAG CGT TCA TC	54.1	800	89

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products. **A**, Products of *Stenocarpella maydis* obtained with primers Smay.F/Smay.R. Lanes 1 to 5: isolates 3-10-1, 3a.1, 4a.1.2, 7.1, and 10a.2, respectively; lane 6 to 8: *S. macrospora* isolates 1a.1.1, 1a, and 4b, respectively; lane 9: control (nuclease-free sterile water); lane M: molecular size marker (Life Technologies, Grand Island, NY). **B**, Products of other fungal and bacteria species obtained with primers Smay.F/Smay.R. Lanes 1 to 9: *Phoma* spp., *Diplodia pinea*, *Fusarium graminearum*, *Aspergillus flavus*, *Clavibacter michiganensis* subsp. *nebraskensis*, *Exserohilum turcicum*, *Colletotrichum graminicola*, *Macrophomina phaseolina*, and *Fusarium verticillioides*, respectively; lane 10: control (nuclease-free sterile water); lane M: molecular size marker (Life Technologies).

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction products. **A**, Products of *Stenocarpella macrospora* obtained with primers Smac.F/Smac.R. Lanes 1 to 5: isolates 1b, 4a.2, 4b, 1a.1.1, and 1, respectively; lane 6 to 8: *S. maydis* isolates 2-2-1, 7.1, and 3a.1, respectively; lane 9: control (nuclease-free sterile water); lane M: molecular size marker (Life Technologies). **B**, Products of other fungal and bacteria species obtained with primers Smac.F/Smac.R. Lanes 1 to 9: *Phoma* spp., *Diplodia pinea*, *Fusarium graminearum*, *Aspergillus flavus*, *Clavibacter michiganensis* subsp. *nebraskensis*, *Exserohilum turcicum*, *Colletotrichum graminicola*, *Macrophomina phaseolina*, and *Fusarium verticillioides*, respectively; lane 10: control (nuclease-free sterile water); lane M: molecular size marker (Life Technologies).

Fig. 3. Melting curve analysis for *Stenocarpella maydis* using real-time polymerase chain reaction (PCR) assay. **A**, Amplification curves of *S. maydis* isolates and controls obtained with primers RT.Smay.F/RT.Smay.R. **B**, Melting curve analysis of the same samples shows the presence of the specific PCR product. **C**, Comparison chart with cycle threshold (C_t) values and melting temperatures. Superscript a: DNA extracted for other fungi isolated that are not the target of this assay and water are identified as negative controls. Superscript b: C_t values ± standard deviation. Superscript c: Cmn = *Clavibacter michiganensis* subsp. *nebraskensis*.

(Fig. 4B and C), and C_t values of 18.31 to 21.69 (Fig. 4C). DNA extracted from infected corn kernels and leaves with *S. macrospora* were tested with RT.Smac.F/RT.Smac.R primers, melting peaks were 89.18 and 88.25°C, respectively, and C_t values were 22.31 and 23.89, respectively (data not shown). The *S. macrospora*-specific assay did not amplify DNA from any *S. maydis* isolates or negative controls included in the assay (melting peak range of 76.74 to 83.28°C and C_t values = 25.00; Fig. 4B and C).

Detection limits of *S. maydis* in conventional and real-time PCR were 0.01 ng/μl with Smay.F/Smay.R primers (Fig. 5A) and 0.001 ng/μl with RT.Smay.F/RT.Smay.R primers (Fig. 5A). The detection limits of *S. macrospora* in conventional and real-time assays were 0.1 ng/μl with Smac.F/Smac.R primers (Fig. 5B), and 1 ng/μl with RT.Smac.F/RT.Smac primers (Fig. 5B). Detection limits of DNA extracted from infected corn seed were 10 ng/μl for *S. maydis* and *S. macrospora* in conventional PCR, and 1 ng/μl for *S. maydis* and *S. macrospora* in real-time PCR (data not shown).

Discussion

This is the first study to successfully develop multiple PCR assays with the specificity to distinguish *S. maydis* from *S. macrospora*. These pathogens are difficult to distinguish based on pathogen signs and disease symptoms, and previous diagnostic assays have been time consuming or nonspecific. Both conventional and real-time assays were able to distinguish between *S. maydis* and *S. macrospora* isolates collected from several states in the United States Corn Belt.

Real-time assays developed and validated here demonstrate that these techniques can be used to assess *S. macrospora* and *S. maydis* presence in both pure culture and in infected plant tissue. In this assay, two corn pathogens, *A. flavus* and *E. turcicum*, had slightly

higher C_t values (26.08 and 27.21, respectively) than the range of *S. maydis* isolates (23.18 to 26.16). In the *S. macrospora* assay, *Phoma* spp. had a C_t value of 19.95, which is in the C_t range of *S. macrospora* isolates (18.31 to 21.69). However, in both assays, the identity of each respective organism was easily differentiated from nontarget pathogens by examining the melting profile of the amplified products. When SYBR Green is used for detection, it is difficult to determine whether the C_t value obtained is due to nontarget amplicons or to nonspecific products. Because of this, identification of isolates using these protocols should be based only on the analysis of the melting curves. Melting curves and peak locations clarify target species as positive samples and nontarget species as negative samples. For instance, melting curve peaks for *A. flavus* and *E. turcicum* are 64.25 and 77.73°C, respectively, which are lower than the range of *S. maydis* isolates (88.54 to 89.94°C). *Phoma* spp. had a melting curve peak of 83.28°C, compared with the range of 89.39 to 89.80°C for *S. macrospora* isolates. Schroeder et al. (43) described the advantage that real-time PCR offers by observing the melting curves of each product. Melting curves are based on the GC amount, length, and sequence that each sample contains and, for that reason, melting curve peaks for the target *Stenocarpella* spp. may vary slightly when implemented in other laboratories (39).

SYBR Green was selected for use in the real-time assays due to the relative simplicity and low cost of the product (4,5,34). Buh Gašparič et al. (4) compared nine different real-time chemistries and concluded that SYBR Green is suitable for use in both qualitative and quantitative detection assays. However, SYBR Green binds with all double-stranded DNA, including nontarget amplicons as well as nonspecific products. This can result in fluorescence in samples that are free of the target pathogen (39). This could explain why fluorescence was

Fig. 4. Melting curve analysis for *Stenocarpella macrospora* using real-time polymerase chain reaction (PCR) assay. **A**, Amplification curves of *S. macrospora* isolates and controls obtained with primers RT.Smac.F/RT.Smac.R. **B**, Melting curve analysis of the same samples shows the presence of the specific PCR product. **C**, Comparison chart with cycle threshold (C_t) values and melting temperatures. Superscript a: DNA extracted for other fungi isolated that are not the target of this assay and water are identified as negative controls. Superscript b: C_t values ± standard deviation. Superscript c: Cmn = *Clavibacter michiganensis* subsp. *nebraskensis*.

observed in certain real-time PCR assays of nontarget organisms for which no amplicon was detected in conventional PCR assays. This potential disadvantage is remedied by examining the melting profile at the end of the real-time assay (39,43). Other assays using real-time PCR employing the fluorescent dye SYBR Green for the detection and diagnosis of plant pathogens have shown that the analysis of the melting profile allowed differentiation of the target with the nontarget species. Tessitori et al. (49) was able to confirm and differentiate two different citrus viroids, *Citrus exocortis viroid* and *Citrus viroid-IIb*, by the use of melting temperatures and their melting peaks. Torres et al. (50) also described the advantage of using the melting curve analysis to differentiate three different apple proliferation phytoplasmas ('*Candidatus Phytoplasma pyri*', '*Ca. P. prunorum*', and '*Ca. P. mali*') by their melting peaks. Therefore, we concluded that the use of SYBR Green and the analysis of the melting profile using real-time PCR is a reliable tool for the diagnosis of *Stenocarpella* spp.

S. maydis and *S. macrospora* were identified directly from corn kernels with both conventional and real-time PCR assays. Amplified bands were reliable enough to confirm the presence of both *Stenocarpella* spp. Positive confirmation in infected corn seed using the real-time assay was based on the melting peak observed in the melting curve analysis. Samples were concluded to be positive for *S. maydis* and *S. macrospora* if the melting peak was located at 88 and 89°C, respectively. The use of both assays on DNA samples extracted from infected corn seed simplifies the identification of this pathogen and increases the utility of these assays in seed-monitoring programs. However, weak bands were observed in the conventional PCR assay when DNA was extracted directly from corn leaves infected with *S. macrospora*. Because these

amplified bands were not as strong as those observed when *S. macrospora* DNA was amplified directly from corn kernels or from fungal cultures, we cannot reliably confirm *S. macrospora* directly from leaf tissue with the conventional PCR assay. However, both conventional and real-time PCR assays were able to reliably detect *S. macrospora* fungal presence at a detection limit of 0.1 and 1 ng/μl, respectively, from fungal culture, and 10 and 1 ng/μl, respectively, from infected corn seed. Detection of fungal presence in corn seed using DNA extracted from cultures or directly from infected tissue would be the primary use of these assays in regulatory agencies testing for seed quality, and incubation of infected tissue to increase the amount of DNA extracted from the pathogen may improve sensitivity in the assay (2). Further testing is needed to determine whether amplification of *S. macrospora* from infected leaves can be achieved with a conventional PCR assay.

Infected corn seed has been identified as an important source of inoculum for both *Stenocarpella* spp. (7,26,27), and can be regulated due to the potential for importing mycotoxin-affected grain into an area (11,16,28,32,38). Barrocas et al. (2) described the necessity of having a specific and accurate method for the identification of both *Stenocarpella* spp. for the Brazilian seed certification program, where *S. maydis* is listed as an RNQP and the Ministry of Agriculture has proposed that imported seed lots of certified seed may not contain more than 2% *S. maydis* (Administrative Regulation Number 47 of 19 February 2009). Inaccurate or unreliable diagnostic methods can negatively impact seed commercialization. The assays developed in this study can be useful tools in areas where *S. maydis* has been categorized as an RNQP.

Fig. 5. A, Dilution series for *Stenocarpella maydis* real-time (top image) and conventional (bottom/gel image) polymerase chain reaction (PCR) assays. Ten-fold dilutions were performed in each assay, with an initial concentration of 10 ng/μl to a final concentration of 0.00001 ng/μl. Gel image includes lane M: molecular size marker (Life Technologies, Grand Island, NY) and nuclease-free sterile water as control. B, *S. macrospora* real-time (top image) and conventional (bottom/gel image) PCR assays. Ten-fold dilutions were performed in each assay, with an initial concentration of 10 ng/μl to a final concentration of 0.01 ng/μl. Gel image includes lane M: molecular size marker (Life Technologies) and nuclease-free sterile water as control.

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