Molecular Diagnostic Assay for Detection of the Butternut Canker Pathogen *Sirococcus clavigignenti-juglandacearum*

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ABSTRACT

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Butternut canker, caused by the fungal pathogen *Sirococcus clavigignenti-juglandacearum*, is present throughout the range of butternut (*Juglans cinerea*) and is the primary cause for its decline. A quick and reliable method for identification of *S. clavigignenti-juglandacearum* would provide a valuable tool for the detection of the pathogen on propagative material to avoid spread, as well as assist studies targeted at the epidemiology of this pathogen, in particular the dissemination of the pathogen by seeds of the butternut. The objective of this study was to develop a diagnostic assay to detect *S. clavigignenti-juglandacearum* in butternut plant tissue. The primers were developed using an alignment of internal transcribed spacer (ITS) sequences from isolates of *S. clavigignenti-juglandacearum* and several closely related species. These primers were tested on *J. cinerea*, 48 isolates of *S. clavigignenti-juglandacearum* recovered from diseased trees, and 26 species of other fungi recovered from butternut tissue. The primers amplified a product from the DNA of all isolates of *S. clavigignenti-juglandacearum*, detected its DNA at a concentration as low as 1 pg/μl, and detected the pathogen at a concentration of 1×10^3 spore/ml. The primers developed in this study will be a valuable tool for the detection of *S. clavigignenti-juglandacearum* present on butternut seeds, and as a rapid diagnostic tool for early detection of the pathogen on butternut trees.

Butternut (*Juglans cinerea* L.) is a medium-sized hardwood tree that is native to the eastern North American forests. In recent years, this tree has been decimated by the fungal pathogen *Sirococcus clavigignenti-juglandacearum* N.B. Nair, Kostichka & J.E. Kuntz, which causes butternut canker. Butternut canker was first reported from southwestern Wisconsin in 1967 (24), and the pathogen causing the disease was described for the first time in 1979 (16). It is unclear how long the canker disease has existed in North America, but research reported by Furnier et al. (3) suggests that *S. clavigignenti-juglandacearum* was recently introduced into North America. Evidence supporting the hypothesis of a recent introduction includes the following: numerous recent first reports of the occurrence of the disease and its documented rapid expansion in North America, lack of genetic variation in the pathogen population (3), symptoms that are an obvious diagnostic

feature and therefore not likely overlooked in the past (16), and the fact that disease-free trees are rarely found within affected populations of native butternut (20).

S. clavigignenti-juglandacearum grows under the bark layer after the spores have found an entry point. The fungus then forms thin, black stromatic tissue under the bark, and eventually forms a number of stromatal columns composed of interwoven mycelia, which lift the dying bark layers of the tree until they rupture (33). The pathogen is primarily spread via rain splash and wind dispersal (33). Long-distance movement of the pathogen likely takes place by insect transmission (5,11) and within infected seed of butternut and black walnut (*Juglans nigra* L.) (9). In addition, the fungus can continue to produce spores on dead trees for up to 2 years (33). *S. clavigignenti-juglandacearum* may also be able to infect and colonize other hardwood species. Ostry and Moore (18) found that in greenhouse inoculations, the pathogen was able to colonize several genera of hardwoods, including *Juglans*, *Carya*, *Quercus*, *Castanea*, and *Prunus*, and several commercially important Persian walnut (*J. regia* L.) cultivars were moderately to highly susceptible.

Since its initial report in North America in 1967, butternut canker was subsequently

reported in Canada in Quebec in 1990, in Ontario in 1991 (1), and in New Brunswick in 1997 (7), where it was thought to have been present for at least 7 years. The rapid spread of the pathogen into Canada, combined with the devastating effect of this disease, led to the butternut being designated as an Endangered Species in Canada in November 2003 (17). A recovery plan is being developed to achieve viable, self-sustaining, and broadly distributed populations within, and possibly north of, the current range of butternut in Canada (17). This will require the identification of source material to be increased and planted across the range of butternut, and the collection of seeds from various locations to conserve the native germplasm and conserve potentially resistant material. The most critical steps in the recovery program will be to ensure seed is free of the pathogen before it is distributed to recovery plan collaborators and participants for planting.

To assist in the recovery project, a rapid and reliable diagnostic method to test for the presence of *S. clavigignenti-juglandacearum* would help determine if seeds and seedlings were free of disease before planting. A molecular identification assay would be ideal. The use of a polymerase chain reaction (PCR) assay is one approach that allows for the detection of small amounts of DNA. The PCR method is a specific and sensitive assay for identification of pathogens, and is well documented in its utility (8,14,35). The PCR is also reproducible for amplification of diagnostic molecular markers and could be used for identification and detection if species-specific primers were available. PCR-based diagnostic assays have been developed for several tree-infecting fungal species (6,22,23,38), as well as seed-infecting fungi (2,12,21,27,30). The focus of this research was to develop a process that will allow for the extraction of DNA and identification of the pathogen from plant material, including leaves, petioles, stems, trunk tissue, and seed pericarp and cotyledons. Therefore, the objective of this study was to develop a rapid, sensitive, and species-specific PCR-based diagnostic assay for the presence of *S. clavigignenti-juglandacearum* in infected butternut tissues.

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MATERIALS AND METHODS

Fungal isolates. Three isolates of *S. clavignenti-juglandacearum*, including the type-culture described by Nair et al. (16) and two isolates recovered from infected butternut in Ontario, were used for the development of the species-specific oligonucleotide primers. An additional 45 isolates of *S. clavignenti-juglandacearum* from 12 locations (Table 1), including isolates recovered from trunk cankers, stem cankers, terminal buds, leaves, and nuts, were used for validation of the primers. Other fungi recovered from *J. cinerea* (Table 2), including those from the nut wash experiment and those from trunk, stem, leaf, and bud tissues, were included. Several species of fungi from our culture collection were also used to evaluate the specificity of the primer pair. Prior to DNA extraction, isolates were stored on potato dextrose agar (PDA) slants.

DNA sequencing and species-specific primer design. DNA was extracted using two methods. For the first method, isolates were grown in potato dextrose broth for 14 days, mycelia were collected and ground in liquid nitrogen, and DNA was extracted using the DNeasy Plant Mini Kit by Qiagen (Qiagen Inc., Valencia, CA). The second method was a modified boiling mini-prep extraction. For this method, isolates were grown on PDA for 5 to 14 days, and a sterile toothpick was used to transfer approximately 5 µg of mycelia to a 1.5-ml tube containing 500 µl of 4× TBE buffer. Mycelia were heated at 95°C for 10 min. For PCR, 2 to 5 µl of supernatant was added directly to the reaction. The universal primers ITS1 and ITS4 (37) were used to amplify a portion of the 18S region, all of the ITS1, the 5.8S region, ITS2, and a portion of the 28S region of the ribosomal DNA. A 50-µl reaction consisting of 10 µl of 5× Green GoTaq reaction buffer (Promega Corp., Madison, WI), 5 µl of 25 mM MgCl₂, 1 µl containing 10 mM each dNTP, 0.25 µl of GoTaq Taq polymerase, 5 µl each of 5-µM concentration of primers ITS1 and ITS4, 2 µl of DNA at a concentration of 10 ng/µl, and 21.75 µl of sterile deionized water was completed in a 0.2-ml thin-walled microcentrifuge tube. PCR parameters were 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min; and completed with 72°C for 5 min followed by 4°C. PCR products were purified using Qiaquick spin columns (Qiagen). For sequencing, 2 µl of either ITS1 or ITS4 at 5 pmoles/µl was added to 2 µl of purified DNA (20 ng/µl) product. Sequencing was completed at the University of Guelph Genomics Facility. Sequences were aligned and edited using BioEdit. For fungi recovered from *J. cinerea*, sequence data were compared with known sequences deposited in the National Center for Biotechnology Information (NCBI) nonredundant database to assist with identification.

To design primers that were specific to *S. clavignenti-juglandacearum*, sequence data were used to search the NCBI database to identify species with the most similar sequence data. The eight species with the most similar internal transcribed spacer (ITS) sequence profiles were included in an alignment using ClustalW. After the alignment, a variable region within the intergenic spacer region 1 (ITS1) was identified. Using the Primer3 Plus software (34), primers were designed that spanned this variable region. This primer was named SCJF3 (5'-GTGGAGTGAGGAGCAGAC-3') and was used as the forward primer, and the universal primer ITS4 (37) was used as the reverse primer.

Primer specificity and sensitivity. To evaluate the sensitivity of the primers, a

DNA serial dilution ranging from 10 ng to 10 fg was used. DNA from all three isolates used in the alignment was evaluated at all DNA concentrations. The primers were tested for specificity on 48 isolates of *S. clavignenti-juglandacearum* (Table 1) and 32 other fungal isolates (Table 2). For PCR using the SCJF3/ITS4 primer pair, a 50-µl reaction consisting of 10 µl of 5× Green GoTaq reaction buffer (Promega), 5 µl of 25 mM MgCl₂, 1 µl containing 10 mM each dNTP, 0.25 µl of GoTaq Taq polymerase, 5 µl each of 5 pmol/µl concentration of primers SCJF3 and ITS4, 2 µl of DNA at a concentration of 10 ng/µl, and 21.75 µl of sterile deionized water was carried out in a 0.2-ml thin-walled microcentrifuge tube. PCR parameters were 95°C for 5 min; followed by 35 cycles of

Table 1. Isolate designation, tissue sample, collection location, phenotype on potato dextrose agar (PDA) and date collected for *Sirococcus clavignenti-juglandacearum* isolates used to evaluate the specificity of the SCJF3/ITS4 primer pair

Isolate	Tissue	Location	Phenotype ^a	Date collected
SCJ1	Stem	Arlington, WI, USA	Dk, D	1978
SCJ2	Stem	York, ON	Dk, R, Sp	01/2008
SCJ3	Stem	York, ON	Sec, R, Sp	01/2008
P-005	twig	Simco Lake, ON	Dk, R, Sp	7/18/2008
WB22	Stem	Cambridge, ON	L, Sp, R	3/25/2009
GA1-1	Stem	Guelph, ON	Dk, R, Sp	4/14/2009
GA5-1	Stem	Guelph, ON	Dk, R, Sp	4/14/2009
Bud 2-3	Terminal bud	Guelph, ON	L, R, Sp	4/14/2009
Bud 2-2	Terminal bud	Guelph, ON	Dk, R, Sp	4/14/2009
WB2-4	Stem	Cambridge, ON	L, Sp, R	3/25/2009
Bud 2-1	Terminal bud	Guelph, ON	Dk, R, Sp	4/14/2009
GA 2-3	Stem	Guelph, ON	Dk, D	4/14/2009
259L-LB11	Lateral bud	Guelph, ON	Dk, D	4/25/2009
B6L-LB23	Lateral bud	Guelph, ON	Dk, D	4/25/2009
B6L-TB11	Terminal bud	Guelph, ON	Sec, R, Sp	4/25/2009
B6L-TB12	Terminal bud	Guelph, ON	Dk, D	4/25/2009
WB1-2	Terminal bud	Cambridge, ON	Dk, D	5/5/2009
WB1-28	Lateral bud	Cambridge, ON	Dk, R, Sp	5/5/2009
WB1-8	Lateral bud	Cambridge, ON	L, Sp, R	5/5/2009
WB4-16	Bud scar	Cambridge, ON	L, Sp, R	5/5/2009
WB4-8	Lateral bud	Cambridge, ON	Sec, R, Sp	5/5/2009
WB-32	Outer pericarp	Cambridge, ON	Dk, D	7/8/2009
WB-34	Seed cotyledon	Cambridge, ON	Sec, R, Sp	7/8/2009
WB-36	Outer pericarp	Cambridge, ON	Dk, R, Sp	7/8/2009
WB-37	Outer pericarp	Cambridge, ON	L, Sp, R	7/8/2009
70-13	New stem	Arkell, ON	Dk, R, Sp	6/15/2009
70-16	New stem	Arkell, ON	Dk, D	6/15/2009
70-17	Stem canker	Arkell, ON	Dk, D	6/15/2009
70-18	Stem canker	Arkell, ON	Dk, R, Sp	6/15/2009
70-19	Leaf lesion	Arkell, ON	Dk, D	6/15/2009
70-20	Leaf lesion	Arkell, ON	Dk, R, Sp	6/15/2009
70-21	Leaf lesion	Arkell, ON	Dk, D	6/15/2009
70-BW1	Black walnut	Arkell, ON	Dk, D	6/15/2009
P12-2-1	Terminal bud	Big Rideau Lake, ON	Dk, D	6/28/2009
P12-2-5	Leaf lesion	Big Rideau Lake, ON	Dk, R, Sp	6/29/2009
P12-3-5	Terminal bud	Big Rideau Lake, ON	Dk, D	6/30/2009
P-003	Twig	Simco Lake, ON	Dk, R, Sp	7/17/2008
P-008	Twig	Simco Lake, ON	Dk, D	7/30/2008
P-010	Twig	Simco Lake, ON	Dk, D	8/3/2008
P-013	Twig	Guelph Lake, ON	Dk, D	8/8/2008
P-017	Twig	Conestoga Lake, ON	Dk, D	8/14/2008
P-019	Twig	Hockley Valley PNR, ON	Dk, D	8/22/2008
P-029	Twig	Simco Lake, ON	L, Sp, R	8/14/2008
P-034	Twig	Bradford, ON	Dk, D	8/26/2008
P-037	Twig	Brockville, ON	Dk, D	8/28/2008
P-043	Twig	Charleston Lake, ON	Dk, D	9/11/2008
P-045	Twig	Big Rideau Lake, ON	Dk, D	9/16/2008
P-046	Twig	Big Rideau Lake, ON	Dk, D	9/17/2008

^a Abbreviations for phenotypes refer to appearance of mycelia on PDA and are as follows: Dk = dark, L = light, Sec = sectorial, D = diffuse, R = rings, Sp = sporulating.

95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and completed with 72°C for 5 min followed by 4°C. To rule out a possible inhibition of the PCR reaction or lack of template DNA, universal primers ITS1 and ITS4, which amplify the intergenic spacer region, were used to amplify the fungal DNA of the respective fungi recovered from butternut tissue and culture collections.

Infected plant tissues were also used to evaluate the primers. Bud, leaf, petiole, stem, and trunk tissues with visible symptoms of infection (Fig. 1) were sampled

from butternut trees. To confirm the presence of *S. clavignenti-juglandacearum*, a portion of the affected area was surface sterilized and cultured on acidified PDA. DNA was extracted from these tissues using the MoBio Power Soil DNA extraction kit (Mo Bio Laboratories Inc., Carlsbad, CA). To improve the efficiency of DNA recovery after plant tissue was added to the first bead tube of the MoBio kit, tubes were incubated at 75°C for 15 min prior to the vortexing step. The PCR reaction of DNA extracted from infected plant tissue was the same as mentioned previ-

ously except 5 µl of DNA and 18.25 µl of sterile water were added to the reaction for a final volume of 50 µl.

DNA extraction from conidia and nut-wash water. To evaluate the potential of the *S. clavignenti-juglandacearum* specific primer pair as a diagnostic tool during the dehusking process, a laboratory simulation was designed to determine if the primers could detect the presence of *S. clavignenti-juglandacearum* in the nut-wash water. DNA was extracted from spores suspended in dH₂O, butternut nut-wash water, and from butternut nut-wash water amended with spores of *S. clavignenti-juglandacearum*. Spore suspensions of 10⁶, 10⁵, 10⁴, 10³, 10², or 10¹ conidia in 100 ml of dH₂O were used for these experiments. For the nut-wash experiment, individual nuts that were previously dehusked were submerged in 40 ml of sterile H₂O and shaken for 30 min. Ten nuts were included, with six receiving 10⁶, 10⁵, 10⁴, 10³, 10², or 10¹ conidia of *S. clavignenti-juglandacearum*, and four were untreated. Nuts were removed and rinse water was centrifuged at 10 × g for 90 min. Excess water was removed, leaving the resulting pellet in approximately 1 ml of water. The tube was vortexed to disrupt the pellet, and the contents were transferred to a 1.5-ml tube. The contents were centrifuged at 10 × g for 15 min, and the supernatant was removed leaving approximately 100 µl. The pellet was resuspended and used for DNA extraction. The samples were then subjected to three freeze-thaw cycles (2 min in liquid nitrogen, 2 min at 75°C, repeated three times with the last thaw extended to 30 min). The contents of the tube were then transferred to a bead tube, and DNA was extracted using the MoBio PowerSoil DNA extraction kit.

RESULTS

Fungal isolation. Morphology differed among the 48 isolates of *S. clavignenti-juglandacearum* tested in this study (Table

Table 2. List of fungal isolates^a used to evaluate the specificity of the SCJF3/ITS4 primer pair

Isolate	Genus	Species	Source
BE11	<i>Alternaria</i>	<i>alternata</i>	<i>J. cinerea</i> endocarp
N60C2	<i>Alternaria</i>	<i>alternata</i>	<i>J. cinerea</i> nut surface
BC21	<i>Aspergillus</i>	<i>tubigenis</i>	<i>J. cinerea</i> cotyledon
N30A3	<i>Aureobasidium</i>	<i>pullulans</i>	<i>J. cinerea</i> nut surface
W15B4	<i>Dothioraceae</i>	sp.	<i>J. cinerea</i> nut surface
P12-1-7	<i>Epicoccum</i>	<i>nigrum</i>	<i>J. cinerea</i> branch
WB-35	<i>Epicoccum</i>	sp.	<i>J. cinerea</i> branch
BE33	<i>Fusarium</i>	<i>oxysporum</i>	<i>J. cinerea</i> branch
WB-36	<i>Fusarium</i>	<i>graminearum</i>	<i>J. cinerea</i> branch
WB26	<i>Fusarium</i>	<i>lateritium</i>	<i>J. cinerea</i> branch
BE21	<i>Fusarium</i>	<i>equiseti</i>	<i>J. cinerea</i> endocarp
N30B1	<i>Fusarium</i>	<i>proliferatum</i>	<i>J. cinerea</i> nut surface
N60B4	<i>Fusarium</i>	<i>graminearum</i>	<i>J. cinerea</i> nut surface
W30C1	<i>Fusarium</i>	<i>solani</i>	<i>J. cinerea</i> nut surface
W60C3	<i>Fusarium</i>	<i>proliferatum</i>	<i>J. cinerea</i> nut surface
W30B3	<i>Geotricum</i>	sp.	<i>J. cinerea</i> nut surface
P12-3-2	<i>Gnomonia</i>	<i>leptostyla</i>	<i>J. cinerea</i> branch
W1	<i>Melanconis</i>	sp.	<i>J. cinerea</i> branch
70-12	<i>Melanconis</i>	<i>juglandis</i>	<i>J. cinerea</i> branch
W11	<i>Nectria</i>	sp.	<i>J. cinerea</i> branch
N30B4	<i>Penicillium</i>	<i>glandicola</i>	<i>J. cinerea</i> nut surface
W30B2	<i>Penicillium</i>	spp.	<i>J. cinerea</i> nut surface
W14	<i>Phoma</i>	<i>glomerata</i>	<i>J. cinerea</i> branch
W16	<i>Phoma</i>	sp.	<i>J. cinerea</i> branch
P12-1-1	<i>Phoma</i>	sp.	<i>J. cinerea</i> branch
N60B3	<i>Plectosphaerella</i>	<i>cucumeria</i>	<i>J. cinerea</i> nut surface
F1M3	<i>Botryosphaeria</i>	<i>obtusa</i>	Culture collection
115-2	<i>Cryphonectria</i>	<i>parasitica</i>	Culture collection
	<i>Fusarium</i>	<i>graminearum</i>	Culture collection
	<i>Fusarium</i>	<i>verticillioides</i>	Culture collection
Win80	<i>Ophiostoma</i>	<i>ulmi</i>	Culture collection
SS357	<i>Sclerotinia</i>	<i>sclerotiorum</i>	Culture collection

^a Isolates were identified to genus or species level using both morphological traits and sequence data from the ITS1, 5.8S, and ITS2 regions of the ribosomal DNA.

Fig. 1. Symptoms of *Sirococcus clavignenti-juglandacearum* infection on butternut (*Juglans cinerea*). **A**, Terminal bud cross-section; **B**, bud dieback; **C**, leaf; **D**, leaf with production of conidia from lesions in culture; **E**, trunk canker; **F**, liquid discharge from cankers; and **G**, stem.

1). Colony morphology differed on PDA. Four distinct phenotypes were observed: cultures were (i) dark, with diffuse mycelia, and without rings; (ii) cultures were dark, with diffuse mycelia, rings, and sporulating; (iii) cultures had light-colored mycelia, rings, and sporulating; or (iv) cultures were sectorial (both dark and light mycelia), with rings, and sporulating (Fig. 2). There was no difference in the ITS sequence among any of the four phenotypes observed.

DNA sequencing and species-specific primer design. Oligonucleotide primers were developed based on the ITS1 and ITS2 regions of isolates SCJ1 (ATCC 3662), which is the type culture, and SCJ2 and SCJ3, which were recovered from *J. cinerea* in Ontario. The sequence data for SCJ1, SCJ2, and SCJ3 were deposited in the GenBank database under accession numbers GU183863, GU183864, and GU183865, respectively. The ITS region was identical for all three isolates sequenced. The species found to have the most similar sequence data were from the genus *Gnomonia*, and were recently reclassified to the genus *Ophiognomonia* (28). There was little sequence similarity with other species of *Sirococcus*. A variable region within ITS1 was identified as an optimal position for designing a forward primer specific to *S. clavignenti-juglandacearum*. The universal primer ITS4 (37) was used as the reverse primer.

Primer specificity and sensitivity. The SCJF3/ITS4 primer pair amplified a putative 467-bp amplicon in each of the 48 isolates, which included the four different phenotypes, tested in this study (Fig. 3). The primers did not amplify DNA from the other 32 fungal isolates tested, including 26 isolates recovered from various infected butternut tissues (Table 2). In addition, the boiling mini-prep extraction method was successful at extracting DNA from mycelia and spores of *S. clavignenti-juglandacearum* that could be used in PCR reactions using the specific primers (Fig. 3B). DNA extracted from isolates of *S. clavignenti-juglandacearum* using the Qiagen DNeasy kit had a concentration ranging from 20 to 50 ng/μl. DNA concentrations were measured using a Nano-drop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). It was this DNA that was used in the serial dilutions to test the sensitivity of the SCJF3/ITS4 primer pair. The primers were able to amplify *S. clavignenti-juglandacearum* DNA at concentrations as low as 10 pg, and a faint band was often seen with as little as 1 pg of starting DNA.

Detection of *S. clavignenti-juglandacearum* in butternut tissue and nut-wash fluid. The primers designed in this study were also used to amplify DNA of *S. clavignenti-juglandacearum* directly from infected tissue from butternut trees. In all, 33 pieces of plant tissue were sampled

Fig. 2. The four phenotypes of *Sirococcus clavignenti-juglandacearum* observed on potato dextrose agar. Phenotypes included cultures with **A**, light mycelia with rings and sporulation (L, R, Sp), **B**, sectorial cultures with rings and sporulation (Sec, R, Sp), **C**, dark mycelia with rings and sporulation and dark pigmentation of the agar (Dk, R, Sp), and **D**, dark and diffuse mycelia with no rings and dark pigmentation of the agar (Dk, D).

Fig. 3. Polymerase chain reaction (PCR) product amplified from the internal transcribed spacer region of rDNA, using the SCJF3/ITS4 primer pair, from 48 isolates of *Sirococcus clavignenti-juglandacearum* collected in Ontario. DNA was extracted using two methods: **A**, the DNeasy Plant Mini Kit by Qiagen or **B**, a modified boiling mini-prep extraction method, for which a sterile toothpick was used to transfer approximately 5 μg of mycelia to a 1.5-ml tube containing 500 μl of 4× TBE buffer, and was denatured at 95°C for 10 min. For PCR, 2 to 5 μl of the supernatant was added directly to the reaction. A negative control and 100-bp ladder are shown.

from three locations in southern Ontario (Table 3). Amplification of pathogen DNA was successfully detected in 22 of the 33 pieces of plant tissue including tissue from black walnut (*J. nigra*) and hickory (*J. ailantifolia* Carr. var. *cordiformis*). The presence of *S. clavigignenti-juglandacearum* was confirmed from infected buds, stem tissue lesions, petiole lesions, leaf lesion, seed outer pericarp, seed cotyledons, trunk cankers, and exudate from trunk cankers using the SCJF3/ITS4 primer pair (Table 3, Fig. 4). The primers amplified DNA from asymptomatic terminal buds and seed sampled from butternut, but did not amplify DNA from asymptomatic stems and leaves (Table 3). *S. clavigignenti-juglandacearum* was also successfully cultured onto acidified PDA from infected buds, stems, petioles, leaves, seeds, and trunk cankers. However, as the season progressed into July and August, it became increasingly difficult to culture the fungus from lesions attributed to *S. clavigignenti-juglandacearum* infection, but the PCR diagnostic assay continued to detect the presence of the pathogen in infected tissue. Attempts to isolate the pathogen from trunk canker exudates were inhibited by the presence of large amounts of bacteria. However, the molecular diagnostic was able to detect the pathogen in the presence of the bacteria in the exudate.

In addition to testing the primers for their ability to detect *S. clavigignenti-juglandacearum* DNA in infected plant tissue, the primers were also evaluated for their efficacy in detecting the pathogen during the dehusking process. The pathogen was not detected from seeds that had

already been dehusked and used in the nut-wash simulations. However, the presence of *S. clavigignenti-juglandacearum* was detected in the laboratory simulation of the dehusking process via spiking nut-wash water and subsequently extracting DNA from these samples. The detection of *S. clavigignenti-juglandacearum* conidia by the SCJF3/ITS4 primer pair was similar when DNA was extracted from conidia suspended in water or when conidia were added to the nut-wash process, indicating that a sufficient number of spores were not lost in the centrifugation steps to decrease total DNA yield. The *S. clavigignenti-juglandacearum* specific primers were able to detect the presence of the pathogen at a concentration of 1×10^3 spores in laboratory nut-wash simulations. This result was similar when DNA was extracted from spores suspended in H_2O .

DISCUSSION

In the present study, a PCR-based diagnostic assay was developed to detect *S. clavigignenti-juglandacearum* in infected plant tissue and from spores during a nut-wash process, using pathogen-specific primers derived from the ITS region of the rDNA of *S. clavigignenti-juglandacearum*. Nuclear ribosomal DNA continues to be an attractive source for designing species-specific primers for the detection of the pathogen of interest. This is the result of several factors, the first being that a large and growing database of ITS sequences from a variety of fungi is readily available from GenBank. Secondly, the ITS region is highly conserved within individual species,

yet variable among species (4,10,13). This study found that DNA sequences of the eight isolates of *S. clavigignenti-juglandacearum* were identical over a 595-bp region spanning from the 3' end of the 18S rRNA gene to the 5' end of the 28S rRNA gene. The lack of variation in the ITS region of the eight isolates is consistent with the view that the ITS region is conserved within species (36) and that there is little or no genotypic variation among isolates of *S. clavigignenti-juglandacearum* in North America (3).

Symptoms of infection by *S. clavigignenti-juglandacearum* on butternut trees include discrete, elliptical to fusiform cankers involving the bark and wood of twigs, branches, stems, and even exposed buttress roots (16). Young cankers commonly originate at leaf scars, buds, lenticels, and bark wounds, including insect wounds and natural bark cracks (16). These symptoms plus trunk cankers and developing cankers on younger branches are the basis for visual disease assessment. Therefore, in sampling for PCR-based detection, plant tissue from all areas of the host were sampled to ensure that DNA extraction and PCR amplification for the detection of the pathogen would be successful for all tissues. PCR amplification of fungal DNA isolated from several types of plant tissue allowed for the amplification of presumably small amounts of biomass of *S. clavigignenti-juglandacearum*. The effects of inhibitors on the PCR reaction commonly found in the DNA extracts from plant materials were overcome by using the MoBio PowerSoil DNA extraction kit, which is designed for isolation of high-quality DNA from environmental samples. There was even an observable difference in band intensity among the different plant tissue samples (Fig. 4). This was likely due to the variable amounts of fungal biomass in the individual samples.

For a diagnostic assay to be successfully employed, it requires a high level of specificity and sensitivity. The specificity of the SCJF3/ITS4 primers have proven to be reliable, as DNA from several fungi commonly found on butternut were not successfully amplified, including the closely related fungi *Ophiognomonia leptostyla* (Fr.) Sogonov and *Melanconis juglandis* (Ellis & Everh.) A.H. Graves. In addition, the primer pair successfully amplified the target region of 48 isolates of *S. clavigignenti-juglandacearum* from 11 locations in Ontario. In regards to sensitivity, the assay was able to detect as little as 100 fg of DNA, and the assay was able to detect as few as 1,000 spores of *S. clavigignenti-juglandacearum* in a simulated nut-wash process. The assay was also able to consistently detect the pathogen in a variety of infected tissues, including leaves, buds, petioles, stems, seed pericarp and cotyledon, trunk tissue, exudate from trunk cankers of butternut, as well as

Fig. 4. Polymerase chain reaction of DNA extracted from symptomatic lesions on 1, terminal bud; 2, branch; 3, petiole; 4, leaf; and 5, petiole tissue; and from 6, asymptomatic petiole tissue using the SCJF3/ITS4 primer pair. Lanes 7 and 8 are the positive (*Sirococcus clavigignenti-juglandacearum* DNA) and negative (no DNA) controls, respectively. Numbers in the plant tissue images correspond with lane numbers in the gel image. The gel is 1.5% agarose with a 100-bp ladder shown.

leaves of *J. nigra*, and stems and buds of *J. ailantifolia* var. *cordiformis*. Therefore, the diagnostic assay developed in this study using the SCJF3/TTS4 primer pair satisfies both the specificity and sensitivity requirements for diagnostic applications.

The diagnostic assay developed in this study could prove to be valuable in the early detection of the pathogen on seed, nursery stock, and scionwood. However, each of these has its limitations. Because butternut seed is generally in small supply, destructive testing to access the cotyledon is generally not an option. Therefore, sampling the pericarp prior to dehusking, and the rinse water during the dehusking process may be viable alternatives. In addition, seeds that fail to germinate and symptomatic seedlings can be tested to determine if the pathogen was responsible for the failed germination or the visible symptoms, respectively. Where the diagnostic assay may prove to be the most valuable is in the testing of nursery stock and scionwood before it is transferred to a new location to ensure that it is disease free, or to

ensure that diseased individuals are removed from production to prevent further spread of the pathogen.

Although there is much interest in conserving butternut and managing butternut canker, most of the research has been focused on finding host resistance (15,19,20,25,26). However, there are still many unanswered questions regarding the epidemiology of this disease, primarily the long-distance spread and potential for seed transmission of the pathogen. Some type of vector is suspected for this pathogen, primarily because of the geographically scattered nature of butternut and the rapid spread of the pathogen throughout North America. The most likely sources of long-distance spread are insect vectors (5,11,29), seed transmission (9), and/or wind-blown spores (31,32). The development of a rapid diagnostic assay for the presence of *S. clavignenti-juglandacearum* in this study will enable future epidemiological studies to test various sources of pathogen spread, such as seeds, insects, alternative hosts, or wind-blown spores.

Table 3. Tissue samples, collection location, date collected, and symptoms present on different plant tissues used to evaluate the *Sirococcus clavignenti-juglandacearum* molecular diagnostic assay^a

Plant tissue	Location	Date	Symptoms	Presence of isolate ^b
<i>J. cinerea</i> current year stem	Guelph, ON	6/12/2009	Dieback	+
<i>J. cinerea</i> current year stem	Guelph, ON	6/12/2009	Reddish lesion	+
<i>J. cinerea</i> current year stem	Guelph, ON	6/12/2009	No	—
<i>J. cinerea</i> stem canker	Guelph, ON	6/12/2009	Canker	+
<i>J. cinerea</i> catkin	Guelph, ON	6/12/2009	Frost damage	—
<i>J. cinerea</i> trunk canker	Guelph, ON	6/12/2009	Canker	+
<i>J. cinerea</i> leaf	Guelph, ON	6/12/2009	Fungal lesions	—
<i>J. cinerea</i> seed pericarp	Cambridge, ON	7/9/2009	No	+
<i>J. cinerea</i> seed pericarp	Cambridge, ON	7/9/2009	No	—
<i>J. cinerea</i> seed cotyledon	Cambridge, ON	7/9/2009	No	+
<i>J. cinerea</i> terminal bud	Cambridge, ON	7/9/2009	Dieback	+
<i>J. cinerea</i> terminal bud	Cambridge, ON	7/9/2009	No	+
<i>J. cinerea</i> current year stem	Cambridge, ON	7/9/2009	Dieback	+
<i>J. nigra</i> seed pericarp	Cambridge, ON	7/9/2009	No	—
<i>J. nigra</i> petiole tissue	Cambridge, ON	7/9/2009	Fungal lesion	+
<i>J. nigra</i> petiole tissue	Cambridge, ON	7/9/2009	Fungal lesion	—
<i>J. cinerea</i> trunk canker exudate	Guelph, ON	7/16/2009	Canker ooze	+
<i>J. cinerea</i> trunk canker exudate	Guelph, ON	7/16/2009	Canker ooze	+
<i>J. cinerea</i> trunk canker	Guelph, ON	7/16/2009	Canker	+
<i>J. cinerea</i> trunk canker	Guelph, ON	7/16/2009	Canker	+
<i>J. ailantifolia</i> var. <i>cordiformis</i> terminal bud	Guelph, ON	7/16/2009	Dieback	+
<i>J. ailantifolia</i> var. <i>cordiformis</i> terminal bud	Guelph, ON	7/16/2009	No	+
<i>J. ailantifolia</i> var. <i>cordiformis</i> stem	Guelph, ON	7/16/2009	Dieback	—
<i>J. nigra</i> leaf	Aylmer, ON	8/11/2009	Fungal lesion	—
<i>J. nigra</i> leaf	Aylmer, ON	8/11/2009	Fungal lesion	—
<i>J. nigra</i> petiole	Aylmer, ON	8/11/2009	Fungal lesion	+
<i>J. nigra</i> petiole	Aylmer, ON	8/11/2009	Fungal lesion	+
<i>J. cinerea</i> leaf	Guelph, ON	8/27/2009	Fungal lesion	+
<i>J. cinerea</i> leaf	Guelph, ON	8/27/2009	Fungal lesion	+
<i>J. cinerea</i> petiole	Guelph, ON	8/27/2009	No	—
<i>J. cinerea</i> petiole	Guelph, ON	8/27/2009	Fungal lesion	+
<i>J. cinerea</i> terminal bud	Guelph, ON	8/27/2009	No	—
<i>J. cinerea</i> terminal bud	Guelph, ON	8/27/2009	Dieback	+

^a Symptomatic and asymptomatic tissues were collected from butternut (*Juglans cinerea*), black walnut (*J. nigra*), and heartnut (*J. ailantifolia* var. *cordiformis*).

^b The (+) and (–) symbols represent the positive amplification of *S. clavignenti-juglandacearum* DNA using the SCJF3/TTS4 primer pair and the failure to amplify *S. clavignenti-juglandacearum* DNA, respectively.

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