An In Planta Method for Assessing the Role of Basidiospores in *Rhizoctonia* Foliar Disease of Tomato

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ABSTRACT

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A tomato (*Solanum lycopersicum*) foliar blight disease of unknown etiology was observed in North Carolina (NC) during 2005 to 2006. Symptoms included necrotic lesions and blighted leaves, with signs of white mycelial growth on abaxial leaf surfaces. The morphology of isolates from symptomatic leaves was consistent with that of *Rhizoctonia solani*. Because the pattern of symptom expression suggested that basidiospores were the primary inoculum source, Koch's postulates were fulfilled using a method to generate basidiospores in planta. Isolates were characterized by morphology, DNA sequence analysis, hyphal anastomosis, and somatic hyphal interactions. Phylogenetic analyses and hyphal anastomosis criteria support placement of the isolates in *R. solani* anastomosis group 3 (AG-3). Tomato foliar blight isolates from NC form a single phylogenetic group with tomato isolates of *R. solani* AG-3 from Japan and are more closely related to *R. solani* AG-3 isolates from potato than tobacco. Isolates exhibited both compatible and incompatible hyphal interactions when paired in vitro. To our knowledge, this is the first detailed report of tomato foliar blight caused by *R. solani* AG-3 in North America. A comprehensive description of the technique employed for producing basidiospores is presented with potential utility for understanding foliar disease etiology in other *Rhizoctonia* pathosystems.

A foliar blight disease of tomato (*Solanum lycopersicum* L.) of unknown etiology was observed during 2005 in a commercial field located in Rowan County, North Carolina (NC). This disease was subsequently found in four fields in two additional NC counties (Henderson and Polk) in 2006. The symptoms appeared as brown necrotic lesions and blighted leaves in the mid- to upper canopy (Fig. 1A), and signs of white mycelial growth were observed on the underside of infected leaves (Fig. 1B). Isolates obtained from symptomatic leaf tissue displayed morphological characteristics consistent with that of *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* (A.B. Frank) Donk) (4,31).

R. solani is a species complex composed of a diverse assemblage of soil fungi that vary with respect to host specificity, life history, and morphology. Historically,

fungi in this species complex have been placed into anastomosis groups (AGs) based on their somatic hyphal interactions with tester strains. At least 13 AGs of *R. solani* have been recognized (2,5,6,23). More recent analyses of ribosomal RNA and β -tubulin genes support the traditional AG classification and suggest that the AGs of *R. solani* share a common evolutionary history (16,17). Although fungi in the *R. solani* species complex can cause diseases of more than 500 genera of plants, host range is generally more limited within an AG. For example, *R. solani* AG-3 is predominantly pathogenic on members of the family Solanaceae that include eggplant,

pepper, potato, tobacco, and tomato (5,14,29).

The symptoms of *R. solani* infection on tomato are typically expressed in or near the soil, and include seedling damping-off, root necrosis, basal stem cankers, and fruit rot (20). This pattern of symptom expression results from the colonization of plant tissues by fungal hyphae or sclerotia present in the soil, and often results from splash dispersal of these propagules onto leaves and stems by rain (12). However, numerous foliar diseases of *R. solani* have been reported on other cultivated (e.g., bean, lettuce, soybean, sugar beet, and tobacco) and noncultivated hosts (12,26,27). For these pathosystems, disease results primarily from infection by sexual spores (i.e., basidiospores) which are produced under warm ($\geq 20^{\circ}\text{C}$), humid ($\geq 90\%$ relative humidity) conditions and often are dispersed in the dark (26,27). There are several examples of foliar diseases caused by *R. solani* AG-3 within the family Solanaceae. Target spot disease of tobacco is attributed to infection by basidiospores of the teleomorph stage of *R. solani* AG-3 (19). Sexual spores of this fungus on potato have been reported to cause leaf lesions, although the role of basidiospores in disease epidemiology is not well understood (27). There is also one published report of tomato foliar blight in Japan caused by *R. solani* AG-3 (13).

The objectives of this study were to (i) verify that *R. solani* was the causal agent of the tomato foliar blight observed in NC from 2005 to 2006 by demonstrating

Fig. 1. A, Blight symptoms on a tomato leaf infected by *Rhizoctonia solani* anastomosis group 3 (AG-3). **B,** Necrotic lesions and mycelial growth on abaxial side of *R. solani* AG-3-infected tomato leaves.

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Koch's postulates using a recently developed in planta-based method to produce basidiospores as inoculum and (ii) characterize tomato foliar blight isolates based on phylogenetic analysis of ribosomal DNA (rDNA) sequences and somatic hyphal interaction criteria. A preliminary report of this research has been published (18).

MATERIALS AND METHODS

Examination of diseased samples and isolation. Leaves from more than 25 plants exhibiting blight symptoms and signs of fungal infection were collected from three tomato cultivars (Mountain Fresh, Crista, and FL 47) from five fields in Rowan, Henderson, and Polk Counties of NC in 2005 to 2006. Disease incidence in each field ranged from 5 to 15% of the entire planting. Slide mounts of mycelium scraped from the underside of infected leaves were initially examined with a compound microscope to observe morphological characteristics.

Isolations were conducted by aseptically excising 3-mm² sections of leaf from the leading edge of symptomatic tissue. Leaf sections were rinsed in deionized H₂O, embedded in acidified 2% water agar (pH

6.2), and incubated at 25°C in the dark. Isolates were transferred to potato dextrose agar (PDA) (Difco Laboratories, Detroit) and incubated at 25°C to establish pure cultures. A preliminary identification of each isolate was made based on both macroscopic and microscopic characteristics of fungal structures and mycelium. Twenty isolates with hyphal and nuclear characteristics of *R. solani* were isolated and stored for further examination in this study (Table 1). Plugs of mycelia, 5 mm in diameter, were taken from 1 cm behind the leading edge of the colony and placed in 2.0-ml cryogenic vials (Corning, Corning, NY) containing 500 µl of potato dextrose broth (PDB; Difco Laboratories) and incubated for 48 h at 25°C. A 500-µl aliquot of sterile 50% glycerol (Sigma-Aldrich, St. Louis) was added to each cryogenic vial before storing at -80°C.

Isolate characterization by DNA sequencing and phylogenetic analyses. Sequence analysis of the internal transcribed spacer (ITS) regions of the rDNA repeat as well as anastomosis criteria were used to determine the AG identity of the isolates of *R. solani* (5,16,30). The ITS rDNA sequences were obtained for six

isolates: Tom7b, Tom19a, Tom21f, Tom22a, Tom23a, and Tom23e. For each isolate, three plugs (5 mm in diameter) of actively growing mycelium on PDA were transferred to a 10-cm-diameter petri dish containing 15 ml of PDB and incubated at 25°C in the dark for 5 to 7 days, until colonies had reached the edge of the dish. The mycelia were separated from the broth by vacuum filtration onto Whatman no. 1 filter paper (Whatman International, Ltd., England) and frozen at -80°C. The frozen mycelium of each isolate was ground in liquid nitrogen (Airgas National Welders, Morrisville, NC) with a mortar and pestle.

DNA was extracted from the ground, frozen mycelium using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD). The following modifications were made to the manufacturer's protocol for extraction from fungal tissue: 100 µl of diamond beads were added to each tube along with buffer AP1 and RNase to facilitate cell lysis, and elution was carried out using 50 µl of sterile deionized water. DNA was stored at -20°C until amplified by the polymerase chain reaction (PCR). Reactions for PCR amplification were prepared in a total volume of 25 µl comprising 1 µl

Table 1. Source, anastomosis group (AG), and accession numbers of sequences deposited for isolates of *Rhizoctonia solani* used in this study

Isolate ^a	Host	Location	AG, subgroup	GenBank accession number ^b
Tom7a	Tomato cv. Mountain Fresh	Rowan Co, NC	AG-3	N/A
Tom7b	Tomato cv. Mountain Fresh	Rowan Co., NC	AG-3	GQ885147
Tom7c	Tomato cv. Mountain Fresh	Rowan Co., NC	AG-3	N/A
Tom19a	Tomato cv. Crista	Polk Co., NC	AG-3	*
Tom19b	Tomato cv. Crista	Polk Co., NC	AG-3	N/A
Tom21a	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	N/A
Tom21b	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	N/A
Tom21c	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	N/A
Tom21d	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	N/A
Tom21e	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	N/A
Tom21f	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	*
Tom21g	Tomato cv. Mountain Fresh	Henderson Co., NC	AG-3	N/A
Tom22a	Tomato cv. Crista	Polk Co., NC	AG-3	N/A
Tom22b	Tomato cv. Crista	Polk Co., NC	AG-3	N/A
Tom22c	Tomato cv. Crista	Polk Co., NC	AG-3	N/A
Tom23a	Tomato cv. FL 47	Henderson Co., NC	AG-3	*
Tom23b	Tomato cv. FL 47	Henderson Co., NC	AG-3	N/A
Tom23c	Tomato cv. FL 47	Henderson Co., NC	AG-3	N/A
Tom23d	Tomato cv. FL 47	Henderson Co., NC	AG-3	N/A
Tom23e	Tomato cv. FL 47	Henderson Co., NC	AG-3	*
OKA-6	Tomato	Japan	AG-3	AB000023
OKA-9	Tomato	Japan	AG-3	AB000024
1	Tobacco	United States	AG-3	AB000001
30	Tobacco	United States	AG-3	AB000002
1600	Tobacco	United States	AG-3	AB000004
1614	Tobacco	United States	AG-3	AB000005
4Rs (ATCC 14006)	Potato	United States	AG-3	AF354064
5Rs (ATCC 44660)	Potato	United States	AG-3	AF354107
6Rs (ATCC 14701)	Potato	United States	AG-3	AF354106
ST3-1	Potato	Japan	AG-3	AB000041
ST4-1	Potato	Japan	AG-3	AB000042
ST6-3	Potato	Japan	AG-3	AB000043
Rhs1AP (ATCC MYA-4579)	Potato	Maine	AG-3	N/A
Rs161	Potato	Hyde Co., NC	AG-3	N/A
AH-1 (ATCC 76126)	Peanut	Japan	AG-4	N/A
Rh-165 (ATCC 76127)	Sugar beet	Japan	AG-4	N/A
2Rs (ATCC 66158)	Rice	United States	AG-1-IA	AF354097

^a Isolate names with the prefix "Tom" represent those isolated in this study. "Tom" isolates with the same number originated from the same plant but different leaves (a, b, c, and so on).

^b GenBank accession number with the prefix "GQ" represents isolate sequenced in this study, N/A = not available, and * = sequence determined in this study to be identical to that of GenBank accession number GQ885147.

of extracted DNA, 1 µl each of 10 µM solutions of primers ITS5 and ITS4b (Integrated DNA Technologies, Coralville, IA), 12.5 µl of 2× PCR master mix (Fermentas, Glen Burnie, MD), and 9.5 µl of DNase-free water (Fermentas) (15,36). Amplifications were performed with a Mastercycler eppgradient S model thermal cycler (Eppendorf, Westbury, NY). The cycle parameters were an initial denaturation at 96°C for 5 min followed by 30 cycles of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension period at 72°C was held for 7 min. A UV transilluminator was used to visualize PCR products. The reaction products were purified with the QIAquick PCR purification kit according to the manufacturer's spin protocol.

PCR-purified product (2 µl for each isolate) was inserted into a pCR 2.1 vector using the Invitrogen TOPO TA cloning kit (Invitrogen, Carlsbad, CA) with the following modification to manufacturer's specifications: 0.5 µl of TOPO vector was added to 5.5 µl of total volume of each cloning reaction mixture. Vectors were transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen). Three transformant colonies were picked per isolate and grown in Luria-Bertani broth at 37°C for 24 h. Pellets of bacterial cells were formed by centrifuging the overnight *E. coli* cultures at 3,000 rpm for 20 min at 4°C. The supernatant was discarded and the pellets were stored at -20°C. The vector was extracted from the *E. coli* cells using the Qiaprep Spin Miniprep Kit (Qiagen). The cloned ITS regions were amplified from the vectors using PCR with dye terminator chemistry. Reaction mixtures contained 7 µl of a 1:15 dilution of the vector, 0.5 µl of BigDye v.3.1 cycle sequencing terminator (Applied Biosystems, Foster City, CA), 1.5 µl of BigDye sequencing buffer (Applied Biosystems), and 1 µl of 10 µM M13 primer solution (Integrated DNA Technologies). Vector map and primer sequences are available through Invitrogen. The sequencing PCR parameters were an initial denaturation at 96°C for 2 min followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. The sequences were determined using an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems). Accuracy of ITS sequences obtained in this study was confirmed by comparison with those deposited in GenBank, and one representative sequence was deposited in GenBank (Table 1).

Sequences were compared with those published by Gonzalez et al. (16) for *R. solani* AG-3 isolates from potato, tobacco, and tomato, with an isolate of *R. solani* AG1-1A from rice included as an outgroup taxon. Sequences were aligned using the ClustalW2 software program (European Bioinformatics Institute, Cambridge, UK)

and manually edited using MacClade software to exclude ambiguous regions (Sinauer Associates, Inc. Publishers, Sunderland, MA) (21). The maximum likelihood criterion in Phylogenetic Analysis Using Parsimony (PAUP) was used for the phylogenetic analysis, which included a heuristic search using random stepwise sequence addition with 1,000 replicates (32). The outgroup taxon was defined before calculating bootstrap values from 1,000 replicates.

Isolate characterization by hyphal anastomosis. The putative identity of tomato foliar blight isolates Tom7b, Tom7c, Tom19a, Tom21f, Tom22a, Tom23a, and Tom23b was confirmed by pairing with representative AG-3 tester isolates (Rh51AP [ATCC MYA-4579] and Rs161). Tomato foliar blight isolates Tom7b and Tom7c were also paired with representative AG-4 tester isolates (AH-1 [ATCC 76126] and Rh-165 [ATCC 76127]). The anastomosis interaction was also observed between 12 of the tomato foliar blight isolates paired with each other. Microscopic examination of hyphal interactions followed the technique described by Carling (5) and nuclear staining was performed with phenosafranin (Sigma-Aldrich) (30). For each paired isolate, one mycelial plug (5 mm in diameter) was taken from 1 cm behind the leading edge of an actively growing colony on PDA and was placed on the upper surface of a glass slide coated with 2% water agar. The plugs of paired isolates were placed 3 cm apart on each slide. Three replicate slides were prepared for each pairing, and each isolate was paired with itself as a control. A plastic box containing the slides and a moist paper towel was covered and incubated in the dark at 25°C for 24 to 48 h. When the mycelia of the paired isolates grew together, the hyphae were stained to enable visualization of the contact zone with one drop each of aqueous solutions of 0.1% phenosafranin and 3% potassium hydroxide (Sigma-Aldrich). Each slide was observed using a light microscope at ×100 and ×400 magnification. Anastomosis reactions were scored based on the categories defined by Carling et al. (5,7).

Isolate characterization by macroscopic somatic hyphal interactions. Tomato foliar blight isolates were paired on petri plates containing 30 ml of PDA with 1% activated charcoal (3) and examined for their resulting somatic hyphal interactions as described by MacNish et al. (22). Self-pairings were used as controls and three replicate plates were prepared per pairing. Plates were incubated at 25°C in the dark for 5 to 7 days and somatic hyphal interactions were scored as compatible or incompatible as described by Charlton and Cubeta (11).

Demonstration of pathogenicity. Tomato plants (cv. Mountain Fresh) were grown in a 22°C growth chamber in pots

(10 cm in diameter and 7 cm deep) containing 2:2:1 potting mix of pine bark:peat moss:vermiculite supplemented with lime. The chamber was illuminated by 40-W cool, white fluorescent bulbs set to a photoperiod of 12 h of light. Plants were watered as needed with deionized H₂O. Inoculum was produced for pathogenicity experiments on tomato using the following procedures. Isolates Tom7b, Tom19a, Tom21f, Tom22a, Tom23a, Tom23c, and Tom23e were retrieved from cryogenic preservation and grown on PDA at 25°C in the dark for 4 to 5 days. For each isolate, four 5-mm-diameter mycelial plugs were added to a 100-ml glass jar containing 20 g of sterile oat grains. The oat grains had been moistened by 20 ml of deionized H₂O before autoclaving. The oat grain inocula were incubated at 25°C in the dark for at least 10 days, until the grains were fully colonized. Ten colonized oat grains were placed approximately 1 cm deep in the soil around the base of each 3- to 4-week-old tomato plant. Both single isolates and 17 representative pairs of isolates were added to the soil of a single tomato plant for each treatment. When isolates were paired, soil was amended with five colonized oat grains of each isolate. Each plant was placed in a clear plastic box and sprayed with deionized H₂O as needed to maintain high humidity. When mycelial growth was observed on stem tissue, tape mounts were stained with lactophenol blue as described by Millar (24) and observed microscopically for basidia at ×400 magnification.

When basidia or basidial initials on infected tomato stem tissue were observed, this tissue was used to inoculate healthy 4- to 5-week-old tomato plants using the following procedure. Stem sections 4 to 8 cm long were excised aseptically from the leading edge of symptomatic stem tissue with a scalpel and taped to the inner lid of a plastic box above a healthy tomato plant. The stem tissue remained taped to the lid for the duration of the experiment, allowing the basidiospores to forcibly eject onto the leaf tissue. Each plant was then sprayed with deionized H₂O after inoculation and incubated in the dark for 25 h before resuming the 12-h photoperiod. Reisolation from symptomatic leaf tissue, which occurred from 4 to 10 days after inoculation, was carried out as described above for the original isolations from field samples. Reisolated cultures were checked for somatic compatibility with the isolates used to generate inoculum on PDA with 1% activated charcoal as described above. This experiment was conducted twice.

RESULTS

Examination of diseased samples and isolation. Tape mounts of symptomatic leaf tissue contained multinucleate, hyaline-to-brown hyphae 5 to 13 µm wide, without clamp connections, typically with slight constrictions at branch points, and

with dolipore septa. Aggregations of monilioid cells, 25 to 50 by 15 to 30 μm , formed loose, cream to brown sclerotia on plant tissue and in culture. In culture, the mycelium was white to various shades of brown and with or without aerial hyphae. Tape mounts of the mycelium on the base of inoculated plants revealed hyphae matching the description of the original field isolates, as well as holobasidia of the

teleomorph. Basidia were 15 by 8 to 10 μm , discontinuously distributed on short hyphal branches arising from a basal hyphal layer, hyaline, barrel-shaped or subcylindrical, and typically had four sterigmata. Sterigmata were typically broader at the base than at the tip, and 5 to 13 by 2 to 4 μm . Basidiospores were 4 to 7 by 4 to 5 μm , smooth, hyaline, oblong to ellipsoid, unilaterally flattened, and prominently

apiculate. This morphology is consistent with that of *R. solani* and teleomorph *T. cucumeris* (4,31).

Isolate characterization by DNA sequencing and phylogenetic analyses. Because all ITS rDNA sequences generated for the tomato foliar blight isolates characterized in this study were identical, only one sequence (862 nucleotides in length) was submitted to GenBank. Based on phylogenetic relatedness, the NC tomato foliar blight isolates formed a single group with other isolates of *R. solani* AG-3 from tomato in Japan (Fig. 2). All tomato isolates were more closely related to isolates of *R. solani* AG-3 from potato than tobacco. When an expanded phylogeny was created, the tomato foliar blight isolates grouped together with members of *R. solani* AG-3 and separately from members of all other *R. solani* AGs (*data not shown*).

Isolate characterization by hyphal anastomosis. All tomato foliar blight isolates paired with *R. solani* AG-3 tester isolates Rhs1AP and Rs161 showed a C2 interaction, with anastomosis followed by cell death as described by Carling (*data not shown*; 5). All self-pairings displayed a C3 interaction, with fusion of cell walls and cytoplasm that did not result in cell death. No anastomoses were observed between tomato foliar blight isolates and AG-4 tester isolates (*data not shown*). This supports the placement of these isolates in AG-3. The anastomosis reactions observed between 12 of the tomato foliar blight isolates paired with each other were either C2 or C3 (*data not shown*). These results support the placement of all tomato foliar blight isolates within the same AG.

Isolate characterization by macroscopic somatic hyphal interactions. Tomato foliar blight isolates showed both compatible and incompatible somatic interactions when paired on PDA with 1% activated charcoal (Table 2). All self-pairings displayed a compatible somatic interaction. Data for somatic hyphal interactions were congruent with data for anastomosis interactions. Paired isolates that displayed a compatible somatic interaction

Fig. 2. Consensus tree based on phylogenetic analyses with Phylogenetic Analysis Using Parsimony (32) of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) from tomato foliar blight isolates and other isolates of *Rhizoctonia solani* anastomosis group 3 (AG-3), with an AG-1-IA isolate defined as the outgroup taxon. Branch lengths indicate the number of nucleotide changes between the sequences of each isolate. The relative support for each clade is indicated by bootstrap values on branches.

Table 2. Macroscopic interaction between paired tomato foliar blight isolates of *Rhizoctonia solani* anastomosis group 3 on potato dextrose agar with 1% activated charcoal^a

Isolates	Tom7c	Tom19a	Tom19b	Tom21f	Tom22a	Tom22b	Tom22c	Tom23a	Tom23b	Tom23c	Tom23d	Tom23e
Tom7c	+	—	—	+	+	+	+	—	—	+	—	—
Tom19a	...	+	+	—	—	—	—	—	—	—	—	—
Tom19b	...	...	+	—	—	—	—	—	—	—	—	—
Tom21f	...	...	...	+	+	+	+	—	—	+	—	—
Tom22a	...	...	...	...	+	+	+	—	—	+	—	—
Tom22b	...	...	...	...	...	+	+	—	—	+	—	—
Tom22c	...	...	...	...	...	...	+	—	—	+	—	—
Tom23a	...	...	...	...	...	...	...	+	—	—	—	+
Tom23b	...	...	...	...	...	...	...	...	+	—	+	—
Tom23c	...	...	...	...	...	...	...	...	...	+	—	—
Tom23d	...	...	...	...	...	...	...	...	...	...	+	—
Tom23e	...	...	...	...	...	...	...	...	...	...	...	+

^a Interaction categories: + = somatic compatibility showing merging reaction in the hyphal interaction zone between two paired colonies and — = somatic incompatibility showing border line in the hyphal interaction zone between paired isolates (11,22).

also displayed a C3 microscopic anastomosis interaction, and isolates that displayed an incompatible somatic interaction also displayed a C2 anastomosis interaction, with one exception. The pairing between isolates Tom19A and Tom21F displayed an incompatible somatic interaction but a C3 anastomosis interaction.

Demonstration of pathogenicity. Mycelia were observed on tomato stem tissue in the growth chamber within 5 to 7 days after inoculation of soil with colonized oat grains, whereas basidia and basidiospores were observed on stems 2 weeks after inoculation. When infected tomato stem tissue with basidia was used to inoculate healthy tomato plants, necrosis and blighting of leaves similar to that observed in the field appeared within 2 weeks. Symptoms began as small, circular necrotic lesions 5 to 15 mm in diameter, often with target-like zonation. As the lesions expanded, they appeared water soaked with irregular borders, eventually causing disintegration of leaf tissue. Entire leaf blighting was also observed. Cultures reisolated from symptomatic leaf tissue were morphologically similar to and somatically compatible with the isolates used to generate inoculum. This technique was successful for inoculations with both single and paired isolates in repeated runs of the experiment.

DISCUSSION

Morphological characteristics of the tomato foliar blight isolates indicated that *R. solani* AG-3 was the causal agent of this disease. However, the typical symptoms of *R. solani* infection on this host do not include foliar blight, which has been reported only once from Japan (13,20). Blighting of tomato leaves under natural conditions in the field did not occur on the lower foliage but was only observed in the mid- to upper canopy. This location is unlikely to be reached by splash-dispersed sclerotia or mycelial fragments from the soil surface. This pattern of symptom expression was most likely the result of infection by aerially dispersed basidiospores of the teleomorph of *R. solani* AG-3.

Although basidiospores are believed to be the primary inoculum for several foliar diseases caused by *R. solani*, their role as infective propagules seldom has been confirmed empirically. Sexual spores are rarely used as inoculum in pathogenicity studies because previously described techniques to induce basidia and basidiospore formation may not be effective for certain isolates of *R. solani* (1,28,33,35). However, the use of basidiospores as inoculum would increase the applicability to natural field situations for investigations into pathosystems where sexual spores are known to play a role in the disease.

In order to accurately demonstrate Koch's postulates for this foliar pathogen, a novel method was developed for the production of basidiospore inoculum on

tomato stems. Few experimental protocols currently exist to induce the sexual stage of *R. solani* AG-3 under controlled laboratory conditions (8). Attempts to induce the sexual stage of tomato foliar blight isolates using previously described methods such as the nutrient step-down and soil over culture methods were unsuccessful (*data not shown*) (1,28,33,35). The method developed in this study is a modification of the technique used by Naito for *R. solani* AG-2-2 on sugar beet (25). This technique was successfully used to induce basidiospore production of at least three different isolates of *R. solani* AG-3 and to demonstrate their pathogenicity on tomato. This in planta method may potentially be useful for producing the teleomorph of other isolates of *R. solani* when demonstrating pathogenicity.

Phylogenetic analysis of ITS rDNA sequences grouped tomato foliar blight isolates from NC together with tomato isolates from Japan and separate from *R. solani* AG-3 potato and tobacco isolates from NC. The isolates analyzed in this study were differentiated based on host rather than geography, despite the fact that several *R. solani* AG-3 potato isolates caused damping off and canker diseases on tomato in greenhouse pathogenicity trials (*data not shown*). This agrees with the results obtained by other investigators, which have indicated subdivision within populations of *R. solani* AG-3 based on host specificity (9,10,19). The *R. solani* AG-3 tomato isolate subgroup identified in this study was more closely related to isolates from potato than those from tobacco. This is interesting given that the epidemiology of the tomato isolates is more similar to those from tobacco, which also infect the foliage of their host via aerially dispersed basidiospores. This phylogenetic separation of potato and tobacco isolates has been shown in previous studies, and supports the conclusion that tobacco isolates are not pathogenic on potato and vice versa (9,19). The identity of the tomato foliar blight isolates as *R. solani* AG-3 is supported by ITS rDNA sequences, traditional hyphal anastomosis tests, and nuclear staining. Although the ITS rDNA sequences of the tomato isolates from NC were identical, the observation of incompatible macroscopic somatic interactions indicates that they are probably not of clonal origin.

It is not known whether *R. solani* AG-3 has a homothallic or heterothallic mating system (34). For this reason, inocula consisting of both single and paired foliar blight isolates were used in various combinations when attempting to induce the sexual stage on tomato stems in this study. Basidia were formed on plants inoculated with both single and paired isolates, and basidiospores from both types of treatments were capable of causing infections. Cultures reisolated from symptomatic leaf

tissue were tested for somatic compatibility with the isolates used to generate inoculum. In all cases when basidiospores were formed from a paired isolate inoculation, the reisolated culture was somatically compatible with only one of the two progenitor isolates (*data not shown*). These results indicate that the isolates used in this study do not require a compatible mating partner to initiate meiosis. However, a more thorough study would be necessary to adequately address the hypothesis that the fungi belonging to *R. solani* AG-3 have a homothallic mating system.

Tomato foliar blight disease symptoms and signs were observed when weather conditions were warm and humid, providing extended periods of leaf wetness on tomato plants in fields that had not received preventative fungicide applications. However, additional studies are required to determine the effects of environmental variables on symptom expression and the specific climatic conditions required for disease development. When healthy tomato plants were inoculated with field isolates under controlled conditions in the growth chamber, disease symptoms developed that were similar to those originally observed in the field. During pathogenicity experiments, symptoms first appeared as small, necrotic lesions, often with a target-like appearance, eventually expanding to cause entire leaf blighting and disintegration. Leaf blighting was observed in the field without small, necrotic target lesions. It was likely that blight symptoms developed from coalesced target spot lesions that were not seen due to the timing of symptom observation in the field. Therefore, more observations are required to determine the range of symptoms for this disease and the temporal sequence of their expression under natural field conditions. To our knowledge, this is the first comprehensive report of tomato foliar blight caused by *Rhizoctonia solani* AG-3 in North America.

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