ABSTRACT

BARTZ, FAITH ELIZABETH. Modulation of the Phenylacetic Acid Metabolic Complex by Quinic Acid Alters the Disease-causing Activity of *Rhizoctonia solani* on Tomato. (Under the direction of Marc A. Cubeta and Margaret E. Daub).

A tomato foliar blight disease of unknown etiology was observed in North Carolina (NC) during 2005-2006. The morphology of isolates from symptomatic leaves was consistent with that of the soil fungus *Rhizoctonia solani* Kühn. 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, all of which supported placement of the isolates in *R. solani* anastomosis group 3 (AG-3). To our knowledge, this is the first detailed report of tomato foliar blight caused by *R. solani* AG-3 in North America.

R. solani produces phenylacetic acid (PAA), hydroxy (OH-), and methoxy (MeO-) derivatives of PAA with demonstrated phytotoxic activities. However, limited information is available on the specific role that these compounds play in the development of *Rhizoctonia* disease symptoms and concentration(s) required to induce a host response. Recent reports that PAA inhibits the growth of *R. solani* conflict with the established ability of the fungus to produce and metabolize PAA. Experiments were conducted to clarify the role of the PAA metabolic complex in *Rhizoctonia* disease by determining the concentrations of PAA, and derivatives of PAA required to induce development of root necrosis and stem cankers on tomato in the absence of the fungus, and the concentration at which PAA inhibits the growth of *R. solani*. The effects of exogenous PAA and derivatives of PAA on tomato seedling

growth were also investigated. PAA and derivatives of PAA induced root necrosis and stem cankers, and reduced root growth. *R. solani* biomass was reduced when PAA was added to the growth medium. Gas chromatography-mass spectrometry (GC-MS) was used to quantify PAA and derivatives of PAA from the culture medium of 14 genetically diverse isolates of *R. solani* belonging to three anastomosis groups. Correlation analyses revealed relationships between the production of PAA and derivatives of PAA and the disease causing activities of *R. solani* isolates on tomato seedlings.

The metabolic control of plant growth regulator production by *R. solani* and consequences associated with the parasitic and saprobic activity of the fungus were investigated by growing isolates of *R. solani* on Vogel's minimal media with and without the addition of a 25 mM quinic acid (QA) source of carbon. The effect of QA on fungal biomass was determined by measuring the dry weight of mycelia produced under each growth condition. QA stimulated growth of 13 of 14 isolates of *R. solani* examined. The production of PAA and derivatives of PAA on the two different media was compared by GC-MS. The presence of QA in the growth medium of *R. solani* altered the PAA production profile, limiting the conversion of PAA to derivative forms. The effect of QA on the ability of *R. solani* to cause disease was examined by inoculating tomato plants with 11 isolates of *R. solani* AG-3 grown on media with and without the addition of 25 mM QA. Mean percent survival of tomato plants inoculated with *R. solani* was significantly higher when the fungal inoculum was generated on growth medium containing QA. The results of this study support the hypotheses that utilization of QA by *R. solani* leads to reduced production of the plant growth regulators belonging to the PAA metabolic complex which can suppress plant disease development.

Modulation of the Phenylacetic Acid Metabolic Complex by Quinic Acid Alters the
Disease-causing Activity of *Rhizoctonia solani* on Tomato

by
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BIOGRAPHY

Faith is a native of Milwaukee, Wisconsin who decided to study plants after her first attempt at gardening failed. She earned her Bachelor's degree in plant pathology and horticulture from the University of Wisconsin – Madison in 2001. It was there that she gained her first research experience in the nematology lab of Dr. Ann MacGuidwin under the direction of Dorith Rotenburg. Her time spent interviewing farmers in Trinidad as an exchange student at the University of the West Indies in 2000 led to the formation of her career objective. She hopes to strengthen the lines of communication between agricultural researchers and the agricultural industry, increasing the availability of accurate and unbiased information to producers, especially in developing countries without well-supported extension systems.

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TABLE OF CONTENTS

LIST OF TABLES _____	ix
LIST OF FIGURES _____	x
CHAPTER 1. An <i>in planta</i> method for assessing the role of basidiospores in	
Rhizoctonia foliar disease of tomato _____	1
Abstract _____	1
Introduction _____	3
Materials and methods _____	5
Examination of diseased samples and isolation _____	5
Isolate characterization by DNA sequencing and phylogenetic analyses ____	6
Isolate characterization by hyphal anastomosis _____	8
Isolate characterization by macroscopic somatic hyphal interactions _____	9
Demonstration of pathogenicity _____	10
Results _____	11
Examination of diseased samples and isolation _____	11
Isolate characterization by DNA sequencing and phylogenetic analyses ____	12
Isolate characterization by hyphal anastomosis _____	12
Isolate characterization by macroscopic somatic hyphal interactions _____	13
Demonstration of pathogenicity _____	13
Discussion _____	14

Acknowledgments	17
Literature Cited	19
Interpretive summary	23
CHAPTER 2. Elucidating the role of phenylacetic acid and hydroxy- and methoxy- phenylacetic acid derivatives in the pathogenic activity of <i>Rhizoctonia solani</i> AG-3	
<i>Rhizoctonia solani</i> AG-3	30
Abstract	30
Introduction	31
Materials and methods	34
Tomato seedling root response to exogenous PAA and derivatives of PAA	34
Tomato stem canker response to exogenous PAA and derivatives of PAA	35
Rhizoctonia culture maintenance	36
Rhizoctonia growth response to exogenous PAA and derivatives of PAA	37
Quantification of PAA and derivatives in Rhizoctonia culture medium	38
Pathogenicity assay	41
Correlation analysis	42
Results	42
Tomato seedling root response to exogenous PAA and derivatives of PAA	42
Tomato stem canker response to exogenous PAA and derivatives of PAA	45
Rhizoctonia growth response to exogenous PAA and derivatives of PAA	45
Quantification of PAA and derivatives in Rhizoctonia culture medium	45

Pathogenicity assay	46
Correlation analysis	46
Discussion	47
Acknowledgments	52
Literature Cited	53
 CHAPTER 3. Modulation of the phenylacetic acid metabolic complex by	
quinic acid alters the disease-causing activity of <i>Rhizoctonia solani</i> on tomato	65
Abstract	65
Introduction	66
Results and Discussion	68
QA stimulates the growth of <i>R. solani</i>	68
QA modulates the production of PAA and OH and MeO derivatives of	
PAA by <i>R. solani</i>	69
Disease-causing activity of <i>R. solani</i> in response to QA	71
Conclusions	71
Experimental	73
Maintenance of fungal isolates	73
<i>R. solani</i> growth response to QA	73
Extraction of <i>R. solani</i> growth medium	74
Chemical analysis of <i>R. solani</i> growth medium	74

Bioassay to assess the disease-causing activity of <i>R. solani</i> in response to QA	76
Acknowledgments	77
Literature Cited	78
APPENDIX. Rhizoctonia Foliar Blight	85
Introduction	86
Symptoms	86
Causal organism	88
Disease cycle	89
Control	90
Selected References	91

LIST OF TABLES

Table 1.1. Source, anastomosis group (AG), and accession numbers of sequences deposited for isolates of *Rhizoctonia solani* used in this study_____25

Table 1.2. Macroscopic interaction between paired tomato foliar blight isolates of *Rhizoctonia solani* anastomosis group 3 (AG-3) on potato dextrose agar with 1% activated charcoal_____27

Table 2.1. Anastomosis group (AG) affinity, plant host, geographic origin, and source of isolates of *Rhizoctonia solani* used in this study_____58

Table 2.2. Multivariate pairwise correlation values for disease-causing activity and production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) derivatives of PAA by isolates of *Rhizoctonia solani* AG-3. Numbers followed by an asterisk indicate level of significance for the correlation analysis (all with $\alpha= 0.05$)_____59

LIST OF FIGURES

Figure 1.1. A, Blight symptoms on a tomato leaf infected by <i>Rhizoctonia solani</i> AG-3. B, Necrotic lesions and mycelial growth on abaxial side of <i>R. solani</i> AG-3 infected tomato leaves	28
Figure 1.2. Consensus tree based on phylogenetic analyses with PAUP (32) of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) from tomato foliar blight isolates and other isolates of <i>Rhizoctonia solani</i> AG-3, with an AG-1-IA isolate defined as the outgroup taxon	29
Figure 2.1. Tomato seedling growth response to phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) PAA derivatives in the growth medium. A. Mean percent area of root system necrotic per plant. B. Mean total root system length per plant. C. Mean total length of lateral root system per plant. D. Mean number of lateral roots per plant. E. Mean shoot length per plant	60
Figure 2.2. Tomato stem canker length in response to injection of stems with phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) PAA derivatives	61

Figure 2.3. Reduction in mycelial growth of *Rhizoctonia solani* after 3 wk in potato dextrose agar amended with phenylacetic acid_____ 62

Figure 2.4. Mean production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) PAA derivatives by isolates of *Rhizoctonia solani* in Vogel's minimal medium after 3 wk of incubation. A. Production of PAA. B. Production of 3-MeO-PAA. C. Production of 2-OH-PAA. D. Production of 3-OH-PAA. D. Production of 4-OH-PAA_____ 63

Figure 2.5. Tomato seedling symptom expression 28 d post inoculation with isolates of *Rhizoctonia solani* AG-3. A. Percentage of planted seeds that emerged. B. Percentage incidence of post-emergence damping-off. C. Length of root necrosis (cm). D. Length stem of cankers (cm). E. Percentage of planted seeds that survived for the duration of the experiment_____ 64

Figure 3.1. *Rhizoctonia solani* mycelial dry wt after three weeks of growth in Vogel's medium amended with quinic acid (QA)_____ 81

Figure 3.2. Differential production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) derivatives of PAA by *Rhizoctonia solani* isolates after three weeks of growth in Vogel's medium N amended with quinic acid (QA)_____ 82

Figure 3.3. Production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) derivatives of PAA by *Rhizoctonia solani* isolates after three weeks of growth in Vogel's medium N amended with quinic acid (QA)_____83

Figure 3.4. Percent survival of tomato plants relative to control plants three weeks after inoculation with *Rhizoctonia solani* grown in half-strength potato dextrose agar medium amended with or without quinic acid (QA)_____84

Figure 4.1. Necrotic lesions and mycelia on abaxial side of infected tomato leaves_____87

Figs. 4.2 and 4.3. *Rhizoctonia* foliar blight symptoms_____87

Figure 4.4. Microscopic image of mycelium stained with lactophenol blue from the abaxial side of an infected tomato leaf_____90

Figure 4.5. Microscopic image (400X) of basidium with sterigmata stained with lactophenol blue on tomato stems_____90

CHAPTER 1

***An in planta* method for assessing the role of basidiospores in Rhizoctonia foliar disease of tomato¹**

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ABSTRACT

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A tomato (*Solanum lycopersicum* L.) foliar blight disease of unknown etiology was observed in North Carolina (NC) during 2005-2006. 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 *Rhizoctonia 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.

Additional key words: Basidiomycota, ITS, rDNA, Solanaceae

INTRODUCTION

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. 1.1A), and signs of white mycelial growth were observed on the underside of infected leaves (Fig. 1.1B). 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 (AG) 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 beta-tubulin genes support the traditional AG classification and suggest that the AGs of *R. solani* share a common evolutionary history (16, 17). While 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 and/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 non-cultivated 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 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: 1) to verify that *R. solani* was the causal agent of the tomato foliar blight observed in NC from 2005-2006 by demonstrating Koch's postulates using a recently developed *in planta*-based method to produce basidiospores as inoculum, and 2) to characterize tomato foliar blight isolates based on phylogenetic analysis of 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 North Carolina in 2005 to 2006. Disease incidence in each field ranged from 5 to 15 percent 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 de-ionized 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, Detroit, MI) 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 *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk) were isolated and stored for further examination in this study (Table 1.1). 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 (PD) broth (Difco, Detroit, MI) and incubated for 48 h at 25°C. A 500 µl aliquot of sterile 50% glycerol (Sigma Aldrich, St. Louis, MO) 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 regions (ITS) of the ribosomal DNA (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, Tom23e. For each isolate, three plugs (5 mm diameter) of actively growing mycelium on PDA were transferred to a 10 cm diameter Petri dish containing 15 ml of PD broth and incubated at 25°C in the dark for 5 to 7 d, until colonies had reached the edge of the dish. The mycelia were separated from the broth by vacuum filtration onto Whatman #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 de-ionized 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 comprised of 1 µl extracted DNA, 1 µl each of 10 µM solutions of primers ITS5 and ITS4b (Integrated DNA Technologies, Coralville, IA), 12.5 µl 2x PCR master mix (Fermentas, Glen Burnie, MD), and 9.5 µl DNase-free water (Fermentas, Glen Burnie, MD) (15, 36). Amplifications were performed with a

Mastercycler epgradient 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 TOPO vector was added to 5.5 µl total volume of each cloning reaction mixture. Vectors were transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA). Three transformant colonies were picked per isolate and grown in LB 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, Germantown, MD). 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 BigDye v.3.1 cycle sequencing terminator (Applied Biosystems, Foster City, CA), 1.5 µl BigDye sequencing buffer (Applied Biosystems, Foster City, CA), and 1 µl of 10 µM M13 primer solution (Integrated DNA Technologies, Coralville, IA). Vector map and primer sequences are available through Invitrogen (Invitrogen, Carlsbad, CA). 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 sec, annealing at 50°C for 15 sec, and extension at 60°C for 4 min. The sequences were determined using an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). 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 1.1).

Sequences were compared to 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 (Rhs1AP [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, St. Louis, MO) (30). For each paired isolate, one mycelial plug (5 mm 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, St. Louis, MO). Each slide was observed using a light microscope at 100x and 400x 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 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 d 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-diameter, 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 de-ionized 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 4-5 days. For each isolate, four 5-mm-diameter mycelial plugs were added to a 100-ml glass jar containing 20 g of sterile oats. The oat grains had been moistened by 20 ml of de-ionized H₂O before autoclaving. The oat grain inocula were incubated at 25°C in the dark for at least 10 d, until the oats 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 de-ionized 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 400x 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 de-ionized H₂O after inoculation and incubated in the dark for 25-h before resuming the 12-h photoperiod. Re-isolation 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. Re-isolated 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 x 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 x 8 to 10 µm, discontinuously distributed on short hyphal branches arising from a basal

hyphal layer, hyaline, barrel-shaped or sub-cylindrical, and typically had four sterigmata. Sterigmata were typically broader at the base than at the tip, and 5 to 13 x 2 to 4 μm . Basidiospores were 4 to 7 x 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.

Since 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. 1.2). 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* anastomosis groups (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 1.2). 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 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 re-isolated 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 *Rhizoctonia 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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Interpretive Summary

An unknown disease of tomato was observed in fields in western North Carolina in 2005 and 2006. The disease appeared as brown spots on leaves on the upper part of the plant and the fungus *Rhizoctonia solani* was subsequently isolated from diseased leaves. A method was developed to produce sexual spores of the fungus and eject them onto leaves of healthy tomato plants to determine whether the fungus could cause disease. The fungus caused symptoms on tomato leaves similar to those observed in the field and was

confirmed as the organism responsible for the disease on tomato. This research represents the first report of a leaf disease of tomato caused by *Rhizoctonia solani* in North America. An increased understanding of the disease causing activity of the fungus will aid in the development of appropriate and effective management practices to reduce crop damage in the future.

Table 1.1. 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	US	AG-3	AB000001
30	Tobacco	US	AG-3	AB000002
1600	Tobacco	US	AG-3	AB000004
1614	Tobacco	US	AG-3	AB000005
4Rs (ATCC 14006)	Potato	US	AG-3	AF354064
5Rs (ATCC 44660)	Potato	US	AG-3	AF354107
6Rs (ATCC 14701)	Potato	US	AG-3	AF354106
ST3-1	Potato	Japan	AG-3	AB000041

^aIsolate 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, etc). ^bGenBank 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.

Table 1.1. (Continued)

Isolate^a	Host	Location	AG/ subgroup	GenBank accession number^b
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	US	AG-1-IA	AF354097

^aIsolate 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, etc). ^bGenBank 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.

Table 1.2. Macroscopic interaction^a between paired tomato foliar blight isolates of *Rhizoctonia solani* anastomosis group 3 (AG-3) on potato dextrose agar with 1% activated charcoal.

	Tom 7c	Tom 19a	Tom 19b	Tom 21f	Tom 22a	Tom 22b	Tom 22c	Tom 23a	Tom 23b	Tom 23c	Tom 23d	Tom 23e
Tom 7c	+	-	-	+	+	+	+	-	-	+	-	-
Tom 19a		+	+	-	-	-	-	-	-	-	-	-
Tom 19b			+	-	-	-	-	-	-	-	-	-
Tom 21f				+	+	+	+	-	-	+	-	-
Tom 22a					+	+	+	-	-	+	-	-
Tom 22b						+	+	-	-	+	-	-
Tom 22c							+	-	-	+	-	-
Tom 23a								+	-	-	-	+
Tom 23b									+	-	+	-
Tom 23c										+	-	-
Tom 23d											+	-
Tom 23e												+

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

Fig. 1.1. A, Blight symptoms on a tomato leaf infected by *Rhizoctonia solani* AG-3. B, Necrotic lesions and mycelial growth on abaxial side of *R. solani* AG-3 infected tomato leaves.

Fig. 1.2. Consensus tree based on phylogenetic analyses with PAUP (32) of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) from tomato foliar blight isolates and other isolates of *Rhizoctonia solani* 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.

CHAPTER 2

Elucidating the role of phenylacetic acid and hydroxy- and methoxy- phenylacetic acid derivatives in the pathogenic activity of *Rhizoctonia solani* AG-3¹

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ABSTRACT. The soil fungus *Rhizoctonia solani* produces phenylacetic acid (PAA), hydroxy (OH-), and methoxy (MeO-) derivatives of PAA with demonstrated phytotoxic activities. However, limited information is available on the specific role that these compounds play in the development of *Rhizoctonia* disease symptoms and concentration(s) required to induce a host response. Recent reports that PAA inhibits the growth of *R. solani* conflict with the established ability of the fungus to produce and metabolize PAA. Experiments were conducted to clarify the role of the PAA metabolic complex in *Rhizoctonia* disease by determining the concentrations of PAA, OH-, and MeO- PAA derivatives required to induce development of root necrosis and stem cankers

¹ This chapter has is formatted in the style of the journal *Mycologia*

on tomato in the absence of the fungus, and the concentration at which PAA inhibits the growth of *R. solani*. The effects of exogenous PAA and derivatives of PAA on tomato seedling growth were also investigated. Growth of tomato seedlings in medium containing PAA, OH-, or MeO- derivatives of PAA at concentrations of 0.1 – 7.5 mM induced necrosis of up to 85% of root system area. Canker development resulted from the injection of tomato seedling stems with 7.5 mM PAA, 3-OH-PAA, or 3-MeO-PAA. *R. solani* biomass was reduced when PAA was added to the growth medium, with 50% reduction observed at 7.5 mM. Gas chromatography-mass spectrometry was used to quantify PAA and OH- and MeO- derivatives of PAA from the culture medium of 14 genetically diverse isolates of *R. solani* belonging to three distinct anastomosis groups. The quantities of these compounds produced by *R. solani* were compared to the concentrations experimentally determined to be toxic to the plant. Correlation analyses revealed relationships between the production of PAA and derivatives of PAA and the disease causing activities of *R. solani* isolates on tomato seedlings.

INTRODUCTION

Rhizoctonia solani Kühn (teleomorph=*Thanatephorus cucumeris* (A. B. Frank) Donk) is a species complex composed of genetically and ecologically diverse soil-associated fungi that can function as saprobes, mycorrhizal symbionts, and pathogens of plants (Cubeta and Vilgalys 2000; Ogoshi 1987). Members of this species complex are placed into anastomosis groups (AG) based on their somatic hyphal interactions with tester strains (Anderson 1982; Carling 1996; Matsumoto, Yamamoto, Hirane 1932). The

delineation of these groups has been supported by more recent multilocus sequence analyses, which suggest that the AGs of *R. solani* share a common evolutionary history (Gonzalez and others 2001; Rodriguez-Carres and others 2010). Although pathogenic fungi in the *R. solani* species complex can infect more than 500 genera of plants, host range is generally more limited within specific AG (Farr and others 1989; Ogoshi 1987). For example, *R. solani* AG-3 is predominantly pathogenic on members of the family Solanaceae that includes eggplant, pepper, potato, tobacco, and tomato (*Solanum lycopersicum* L.) (Bartz and others 2010b; Date and others 1984; Johnk and others 1993; Ogoshi 1987). Even within specific AG, isolates may vary in the type of symptoms caused after infection. Typical symptoms of Rhizoctonia infection on tomato include reduced seedling emergence from soil, post-emergence damping-off (resulting in decay of the entire seedling), root necrosis, and stem cankers (Stevenson 1991). The death of seedlings can result in significant stand reduction and yield losses (Baker 1970).

The observation that symptom development in host tissue often preceded hyphal colonization led to the hypothesis that symptoms result from the production of phytotoxic substances by *R. solani* (Boosalis 1950; Wyllie 1962). The ability of *R. solani* to produce phenylacetic acid (PAA), three hydroxy (OH-), and a methoxy (MeO-) derivative of PAA by has been reported, and is believed to be important in the parasitism and infection process on plants (Aoki, Sassa, Tamura 1963; Chen 1958; Iacobellis and Devay 1987; Kohmoto and others 1973; Mandava and others 1980). The plant growth regulating activity of PAA, which is classified as an auxin, was described before the compound was known to occur naturally (Haagen-Smit and Went 1935; Zimmerman and Wilcoxon

1935). PAA and 4-OH-PAA were later found to be present in the tissues of algae and higher plants (Abe, Uchiyama, Sato 1974; Okamoto, Isogai, Koizumi 1967; Onishi and Yamasaki 1955; Wightman and Rauthan 1974). There are numerous reports of plant growth-regulating activity and phytotoxicity of PAA and its derivatives on various species of plants (Chamberlain and Wain 1971; Wheeler 1977). Conflicting reports and methodological limitations of early studies have left the specific role that PAA plays in the development of Rhizoctonia disease symptoms and the concentration required to induce these host responses unclear (Betancourt and Ciampi 2000; Frank and Francis 1976; Iacobellis and Devay 1987). Furthermore, limited information is available regarding the role of OH- and MeO- derivatives of PAA in plant growth and development or in Rhizoctonia disease. It has also been shown that PAA has antimicrobial properties, and reports that it inhibits the growth of *R. solani* conflict with reports that PAA is produced and metabolized by the fungus (Ding and others 2008; Hwang and others 2001; Lima Mendonça and others 2009; Mao and others 2006; Yoon and others 2004).

The primary objective of this study was to clarify the role of the PAA metabolic complex in Rhizoctonia disease using 11 genetically distinct isolates of *R. solani* AG-3 and tomato as a model pathosystem. Two additional isolates of *R. solani* AG1-IA and AG-4 were also critically examined for production of PAA. Experiments were conducted to test the hypotheses that 1) PAA, OH-, and MeO- derivatives of PAA will induce disease symptoms on tomato in the absence of the fungus, 2) isolates of *R. solani* will vary in their ability to produce PAA, OH-, and MeO- derivatives of PAA in vitro, 3) higher concentrations of PAA than those that cause damage to tomato will be needed to

inhibit growth of the fungus, and 4) the relationship between production of PAA and disease causing activity of *R. solani* will be correlated. Preliminary results have been reported (Bartz and others 2010a).

MATERIALS AND METHODS

Tomato seedling root response to exogenous PAA and derivatives of PAA.—Seeds of tomato cv. Micro-Tom (PanAmerican Seed Co., West Chicago, Illinois) were surface disinfested by placing in 95% EtOH for 1 min, followed by immersion in 50% Clorox (2.5% NaOCl) for 1 min. Seeds were rinsed 3X with sterile deionized H₂O (di H₂O) and spread evenly over a sterile Whatman #1 filter paper in a glass Petri dish moistened with 3 mL of di H₂O. The seeds were incubated in the dark at 24 C for 3–4 d, until the length of the emerging radical had reached 1–4 mm.

Murashige and Skoog medium (Murashige and Skoog 1962) obtained from Caisson Laboratories (North Logan, Utah) was prepared by adding 10 g L⁻¹ agar (Apex Bioresearch Products, San Diego, California), balanced to pH 5.5 and autoclaved in a Steris Amsco Century SV-120 (Steris, Mentor, Ohio) at 121 C and approximately 16 psig for 45 min. The molten medium was amended with aqueous solutions of either phenylacetic acid (PAA), 2-hydroxy-phenylacetic acid (2-OH-PAA), 3-hydroxy-phenylacetic acid (3-OH-PAA), 4-hydroxy-phenylacetic acid (4-OH-PAA), or 3-methoxy-phenylacetic acid (3-MeO-PAA) (Sigma Aldrich, St. Louis, Missouri) standardized to pH 5.5 and sterilized by passing through a 0.22 µm membrane filter (Millipore, Billerica, Massachusetts). The final concentration for each compound in each

medium was 0.1, 0.5, 1.0, 3.0, or 7.5 mM. Non-amended Murashige and Skoog medium served as the control. For each treatment and concentration level, 100 mL volumes of media were dispensed into four replicate Magenta GA-7 culture vessels (Magenta Corp., Chicago, Illinois). Five pre-germinated tomato seedlings were placed into each vessel. Vessels were arranged in a randomized complete block design at room temperature (21 C) under a 40 w fluorescent light emitting $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation set to a photoperiod of 14 h light and 10 h dark for 12–13 d.

After incubation, seedlings were carefully removed from each vessel and placed on a white light transilluminator with the root system gently teased apart. An image was taken using a Nikon D50 digital SLR camera and imported into the APS Assess 2.0 software program (The American Phytopathological Society, St. Paul, Minnesota) for measuring root and shoot length, number and length of lateral roots, and percent necrosis of total root area for each seedling. The experiment was conducted twice. Statistical analyses were carried out using JMP 7 software (SAS Institute, Inc., Cary, North Carolina). Linear models were constructed using the restricted maximum likelihood approach to determine the concentration of each compound that increased the mean percent area of root necrosis significantly over that of the control. For this analysis, data were square root transformed to achieve homoscedasticity.

Tomato stem canker response to exogenous PAA and derivatives of PAA.—Seeds of tomato cv. Micro-Tom were spread evenly over a sterile Whatman #1 filter paper in a glass Petri dish, moistened with 3 ml of 2% KNO_3 (Sigma Aldrich, St. Louis, Missouri), and incubated in the dark at 24 C for 24 h. Two seeds were planted in each plastic pot (10

cm diam, 7-cm deep) containing a 1:1 mixture of pasteurized peat and sand. The pots were placed in a 22 C growth chamber illuminated by fluorescent bulbs emitting 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation set to a photoperiod of 14 h of light. Plants were watered as needed with di H₂O for 2 wk.

Aqueous solutions of either PAA, 2-OH-PAA, 3-OH-PAA, 4-OH-PAA, 3-MeO-PAA, or an equimolar mixture of PAA and each of the four derivatives were standardized to pH 5.5 and filter-sterilized. All solutions were prepared to contain a final total concentration of 7.5 mM. Fifty μL volumes of each solution were injected into the base of the tomato stems using a sterile 1 cc syringe and 26 gauge needles (Becton, Dickinson and Co., Franklin Lakes, NJ). Control plants were injected with di H₂O or were punctured by the needle with no di H₂O or PAA solution injected. There were four replicate pots for each treatment. The pots were arranged in a randomized complete block design. Plants were not watered for a 24 h period after injection, then were watered as needed with di H₂O. After 9 d, plants were removed from the pots and measurements of root and shoot length, number of lateral roots, incidence of root necrosis, and length of stem canker for each plant were recorded. The experiment was conducted twice. Statistical analysis was carried out using JMP 7 software. A linear model was constructed to determine if the compounds tested had a significant effect on tomato stem canker length. A Student's t-test was conducted to identify significant differences between least-squared mean canker lengths for each treatment.

Rhizoctonia culture maintenance.—The fourteen field isolates of *Rhizoctonia solani* used in this study (TABLE 2.1) were stored cryogenically as follows. Five mm diam plugs of

mycelia were excised from 1 cm behind the leading edge of an actively growing colony on potato dextrose agar (PDA) (Difco, Detroit, Michigan) and placed in 2.0 ml cryogenic vials (Corning, Corning, NY) containing 500 μ l of potato dextrose (PD) broth (Difco, Detroit, Michigan) and incubated for 48 h at 25 C. A 500 μ L aliquot of sterile 50% glycerol (Sigma Aldrich, St. Louis, Missouri) was added to each cryogenic vial before storing at -80 C. Isolates were retrieved from cryogenic preservation at -80 C by placing the mycelial plug in a Petri dish containing 20 mL of PDA and incubating in the dark at 24 C.

Rhizoctonia growth response to exogenous PAA and derivatives of PAA.—PD broth medium was balanced to pH 5.5 and autoclaved at 121 C and approximately 16 psig for 45 min, then amended with a filter-sterilized aqueous solution of PAA standardized to pH 5.5. Amended media were prepared to contain the following final concentrations of PAA: 0.1, 0.5, 1.0, 3.0, or 7.5 mM. Non-amended PD broth served as the control. Two field isolates of *R. solani* AG-3 were selected based on pathogenicity test described below: an isolate that caused minimal tomato symptom development designated as Bs69, and an aggressive isolate designated Rs114. For each fungal isolate and PAA concentration level, 100 mL volumes of media were dispensed into three replicate 250 mL Erlenmeyer flasks. Each flask was inoculated with one 5 mm diam plug of mycelium from an actively growing culture of *R. solani* isolate Bs69 or Rs114. Triplicate flasks of PD broth containing 0.5 mM PAA served as non-inoculated controls. Flasks were arranged in a randomized complete block design and incubated in the dark at 24 C.

After 3 wk incubation, the mycelium was separated from the broth onto pre-tared grade 103 qualitative filter papers (Millipore, Billerica, Massachusetts) using a vacuum pump. The mycelia and filter papers were placed in a drying oven at 60 C. After 2 d, mycelia and filter papers were equilibrated to room temperature in a desiccator for 15 min and mycelial dry weight measurements were taken. The experiment was conducted twice. Statistical analyses were carried out using JMP 7 software. A linear model was constructed using the restricted maximum likelihood approach to determine the concentration of PAA that caused a significant decrease in the mycelial dry weight for each isolate compared to that of the control.

Quantification of PAA and derivatives in Rhizoctonia culture medium.—Fourteen isolates of *Rhizoctonia solani* were stored and recovered from cryogenic preservation as described above (TABLE 2.1). For each isolate, one 5 mm diam plug of actively growing mycelium from PDA was placed into each of three replicate Erlenmeyer flasks containing 100 mL of Vogel's minimal medium N prepared without the addition of sucrose (Vogel 1956). Non-inoculated flasks were used as controls. Flasks were arranged in a randomized complete block design and incubated in the dark at 24 C. After 3 wk incubation, the mycelium was separated from the broth medium and mycelial dry weight measurements were obtained as described above. The production values for each analyte were normalized by dividing by the mycelial dry weight from each flask. Aliquots of the broth were dispensed into 2.0 ml cryogenic vials and stored at -20 C until processing for chemical analysis.

PAA and its derivatives were quantified from the broth using gas chromatography-mass spectrometry (GC–MS). Duplicate 0.5 mL aliquots from each flask of broth were mixed with 0.5 mL of 1N HCl (Mallinckrodt Baker, Inc. Phillipsburg, New Jersey) saturated with NaCl (Thermo Fisher Scientific, Waltham, Massachusetts) and extracted 3X with 0.5 mL volumes of methyl tert-butyl ether (Alfa Aesar, Ward Hill, Massachusetts) containing 5% toluene (Mallinckrodt Baker, Inc. Phillipsburg, New Jersey). The duplicate extracts for each sample were combined in a 2 mL glass autosampler vial (National Scientific Company, Rockwood, Tennessee) with 25 μ L pyridine (Alfa Aesar, Ward Hill, Massachusetts) containing 2.5 ng μ L⁻¹ 1-octadecanol (Sigma Aldrich, St. Louis, Missouri) as an internal standard. The contents of each vial were evaporated under air flow at approximately 40 °C. The extracts were resuspended in 100 μ L of acetonitrile (Honeywell Burdick and Jackson, Morristown, NJ) containing 1% pyridine and 0.5% trifluoroacetic acid (EMD Merck KGaA, Darmstadt, Germany) and 25 μ L of either 1:1 *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide with hexamethyldisilazane or *N,N*-dimethyltrimethylsilylamine (Sigma Aldrich, St. Louis, Missouri) and incubated at approximately 40 °C for 15 min to convert PAA and its derivatives to the trimethylsilyl (TMS) derivatives.

GC–MS was performed with a Thermo Scientific Trace GC Ultra gas chromatograph coupled to a Trace DSQII mass-selective detector (Thermo Fisher Scientific, Waltham, Massachusetts). Chromatographic separations were achieved with a Thermo TR-5 MS (5% phenyl polysilphenylene–siloxane) column (30 m length, 0.25 mm inside diameter, 0.25 μ m film thickness). The carrier gas was helium, set at a

1 mL min⁻¹ constant flow (40 cm s⁻¹ linear velocity). One µL of each sample was injected into a PTV injector operated in splitless mode at a constant temperature of 240 C. The initial column oven temperature of 60 C was held for 30 sec, then ramped 8 C min⁻¹ to a 360 C final temperature held for 4 min. The MS, equipped with an electron impact source (EI) in positive mode, was programmed for selected ion monitoring (SIM) of ions characteristic of the analytes (m/z 164, 192-194, 223, 238, 251-253, 280-282, 296) and the internal standard (m/z 327).

A series of reference standards were analyzed concurrently with fungal broth culture extracts, and quantitation was achieved by comparing reference standard peak areas with those of the monitored ions from experimental samples. Variation in instrument response was corrected for when necessary using calculations of the peak area ratios of analytes to the internal standard. The chromatographic data were processed using Thermo Scientific Xcalibur software (Thermo Fisher Scientific, Waltham, Massachusetts). The minimum limit of quantitation under these conditions was estimated to be 6.25 ng mL⁻¹ broth culture. The percent recovery mean and standard error were calculated from broth containing 62.5 ng mL⁻¹ of each analyte run concurrently with the fungal culture extracts: 104 ± 10% for PAA, 107 ± 11% for 2-OH-PAA, 111 ± 13% for 3-OH-PAA, 126 ± 17% for 3-MeO-PAA, and 110 ± 14% for 4-OH-PAA. The experiment was conducted twice. Statistical analyses were carried out using JMP 7 software. A linear model was constructed using the restricted maximum likelihood approach to determine if the PAA and derivative production varied with isolate.

Pathogenicity assay.—Eleven isolates of *Rhizoctonia solani* AG-3 were stored and recovered from cryogenic preservation as described above. Inoculum of each isolate was transferred two times to half-strength PDA (pH 5.5). Plates were incubated in the dark at 24 C for 4-7 d. After the second growth period, 5 mm diameter plugs of mycelia were used to inoculate tomato plants.

Seeds of tomato cv. Micro Tom were placed in petri dishes between two sheets of Whatman #1 filter papers moistened with 3 ml of 2% KNO₃ solution containing 1 µl Allegiance (Bayer CropScience, Research Triangle Park, NC) and incubated in the dark at 24 C for 24 h. Ten seeds were planted in each plastic pot (10 cm diam, 7 cm deep) containing a 1:1 mixture of pasteurized peat and sand. Two 5 mm diam plugs of *R. solani* mycelium were placed under each seed as it was planted. Four replicate pots were prepared for each isolate and QA treatment. Plugs of sterile media were placed under the seeds of control plants.

The pots were arranged in a randomized complete block design in a 22 C growth chamber illuminated by fluorescent bulbs emitting 450 µmol m⁻² s⁻¹ of photosynthetically active radiation set to a photoperiod of 14 h of light. Plants were watered as needed with de-ionized H₂O. After 3 wk, final stand count was assessed by recording the number of surviving plants in each pot. During the course of the experiment, pots were observed daily for seedling emergence from soil and symptom development. After 3 wk, the following measurements were recorded: the percent of planted seeds that emerged, the percent of emerged seeds that developed post-emergence damping-off, the length of root necrosis on each plant, the length of stem cankers on each plant, and the percentage of

planted seeds that germinated and survived until the end of the experiment. The experiment was repeated three times. Statistical analyses were carried using JMP 7 software. Linear models were constructed using the restricted maximum likelihood approach to determine if isolates varied in their ability to cause each of the observed symptom types on tomato seedlings.

Correlation analysis.—To determine whether the production quantity of PAA and derivatives of PAA from a given isolate is related to its ability to cause disease symptoms on tomato seedlings, a multivariate correlation analysis was performed using JMP software. Least-squared mean values of PAA production quantities for each isolate were compared to least-squared mean values of tomato disease symptom response to inoculation by that isolate.

RESULTS

Tomato seedling root response to exogenous PAA and derivatives of PAA.—The relationship between the concentration of PAA, OH- , and MeO- PAA derivatives and the percent area of root necrosis of tomato seedlings is summarized in FIG. 2.1A. At the lowest concentration tested, 0.1 mM, root necrosis of plants treated with PAA or 4-OH-PAA was significantly higher than root necrosis of control plants ($P < 0.0001$ in both cases, $\alpha = 0.05$). At 1 mM concentration, root necrosis of plants treated with each of the tested compounds was significantly higher than that of control plants ($P < 0.0001$ in all cases, $\alpha = 0.05$). The mean percent area root necrosis exceeded 30% at 0.5 mM PAA, and

at 1 mM 3-MeO-PAA. This level of root necrosis was not achieved below 7.5 mM for the other derivatives tested.

The relationship between the concentration of PAA, OH- , and MeO- PAA derivatives and total tomato seedling root system length is summarized in FIG. 2.1B. Root length did not vary significantly between seedlings treated with each compound ($P=0.5349$). However, the effect of concentration on root length varied significantly between seedlings treated with each compound ($P=0.0002$). Root length was significantly reduced at the lowest concentration tested (0.1 mM) for all compounds ($P<0.0001$ for PAA, 3-MeO-PAA, 2-OH-PAA, and 3-OH-PAA; $P=0.0161$ for 4-OH-PAA), and remained significantly lower than control for all increasing concentration levels tested.

The relationship between the concentration of PAA, OH- , and MeO- PAA derivatives and lateral root system length of tomato seedling is summarized in FIG. 2.1C. Lateral root system length did not vary significantly between seedlings treated with each compound ($P=0.5728$). However, the effect of concentration on lateral root system length varied significantly between seedlings treated with each compound ($P=0.0097$). Plants had significantly reduced lateral root system length when treated with PAA at 0.5 mM ($P<0.0001$); with 3-MeO-PAA at 0.1 mM ($P=0.0044$); with 2-OH-PAA at 0.5 mM ($P=0.0232$); and with 3-OH-PAA at 7.5mM ($P=0.0299$) and all higher concentrations for each compound tested.. Plants had significantly reduced lateral root system length when treated with 4-OH-PAA at 1 mM and 3 mM ($P=0.0248$ and 0.0144, respectively).

The relationship between the concentration of PAA, OH- , and MeO- PAA derivatives and number of lateral roots per tomato seedling is summarized in FIG. 2.1D. Number of lateral roots did not vary significantly between seedlings treated with each compound ($P=0.3745$). However, the effect of concentration on number of lateral roots varied significantly between seedlings treated with each compound ($P=0.0111$). Plants had significantly less lateral roots when treated with PAA and 3-MeO-PAA at the lowest concentration tested (0.1 mM, $P=0.0004$ for both compounds); with 2-OH-PAA at 0.5 mM ($P=0.0063$) and with 4-OH-PAA at 1 mM ($P=0.0048$), and all higher concentrations of each compound tested. Plants treated with 3-OH-PAA at the lowest concentration tested (0.1 mM) had significantly more lateral roots ($P=0.0059$). At higher concentrations of 4-OH-PAA, plants had reduced numbers of lateral roots, with a significant reduction at ≥ 3 mM ($P=0.0265$).

The relationship between the concentration of PAA, OH- , and MeO- PAA derivatives and tomato seedling shoot length is summarized in FIG. 2.1E. Shoot length varied significantly between seedlings treated with each compound ($P=0.0267$), and the effect of concentration varied significantly between compounds ($P=0.0055$). For plants treated with PAA, 3-MeO-PAA, and 4-OH-PAA, shoot length was significantly lower than control plants at the lowest concentration tested ($P=0.0002$, 0.0445, and 0.0165, for each compound respectively) and all higher concentrations. For plants treated with 2-OH-PAA, shoot length was significantly lower than control plants at 0.5 mM ($P=0.0062$) and all higher concentrations. For plants treated with 3-OH-PAA, shoot length was

significantly lower than control plants at 3 mM ($P=0.0055$) and all higher concentrations.

Tomato stem canker response to exogenous PAA and derivatives of PAA.—The mean canker length of control plants and plants injected with each compound is summarized in FIG. 2.2. The identity of the compound had a significant effect on canker length ($P=0.0073$, $\alpha=0.05$). The mean canker length was significantly higher than the control for plants treated with PAA, 3-MeO-PAA, or 3-OH-PAA. Treatment with a mixture of PAA compounds did not increase stem canker symptoms compared to compounds applied individually to tomato stems.

Rhizoctonia growth response to exogenous PAA and derivatives of PAA.—The percent reduction in mycelial dry weight relative to the control for each isolate at each PAA concentration is summarized in FIG. 2.3. There was no significant difference in the growth response of the two isolates to PAA ($P=0.5049$, $\alpha=0.05$). PAA concentration had a significant effect on mycelial dry weight for both isolates ($P<0.0001$, $\alpha=0.05$), with the mean percent reduction in mycelial dry weight exceeding 50% at the 7.5 mM PAA concentration for both isolates.

Quantification of PAA and derivatives in Rhizoctonia culture medium.—The mean production of PAA and each derivative across all fungal isolates is summarized in FIG. 2.4. The differences in production amounts of each compound did not vary significantly among the isolates tested ($P=0.2331$ for PAA, 0.5853 for 3-MeO-PAA, 0.9432 for 2-OH-PAA, 0.2448 for 3-OH-PAA, and 0.1927 for 4-OH-PAA). The concentration values calculated from the growth medium of *R. solani* ranged from 34-678 nM PAA, 40-59 nM

2-OH-PAA, 40-429 nM 3-OH-PAA, 33-244 nM 3-MeO-PAA, and 35-100 nM 4-OH-PAA.

Pathogenicity assay.—The tomato seedling disease symptom responses to inoculation with 11 isolates of *R. solani* AG-3 are summarized in FIG. 2.5. FIG. 2.5A summarizes the mean percent emergence, which varied significantly with isolate ($P < 0.0001$). The following eight isolates reduced emergence significantly compared to the control: Rhs1AP, Rs113, Rs114, Rs182, Rs183, Rs191, Rs88, and Tom7. FIG. 2.5B summarizes the mean percent incidence of damping-off, which varied significantly with isolate ($P < 0.0001$). The following seven isolates increased post-emergence damping-off significantly compared to the control: Rhs1AP, Rs113, Rs114, Rs138, Rs182, Rs183, and Rs88. FIG. 2.5C summarizes the mean length of root necrosis per plant, which varied significantly with isolate ($P < 0.0001$). The following eight isolates increased root necrosis length significantly compared to the control: Rhs1AP, Rs113, Rs114, Rs138, Rs182, Rs183, Rs88, and Tom7. FIG. 2.5D summarizes the mean length of shoot cankers per plant, which varied significantly with isolate ($P < 0.0001$). The following four isolates increased shoot canker length significantly compared to the control: Rs114, Rs138, Rs88, and Tom7. FIG. 2.5E summarizes the mean final stand (as percent of planted seeds that survived until the end of the experiment), which varied significantly with isolate ($P < 0.0001$). All isolates decreased final stand significantly compared to the control with the exception of Bs69 and Rhs1A1.

Correlation analysis.—TABLE 2.2 summarizes the correlation values, and their statistical significance, between in-vitro production of PAA (and derivatives of PAA) and disease-

causing activities of *R. solani* AG-3 isolates. Production of PAA and all derivatives was negatively correlated with both measures of plant survival (seedling emergence and final stand). This correlation was significant for PAA, summed total derivatives of PAA, and summed total PAA plus all derivatives. Incidence of post-emergence damping-off was positively correlated with production of PAA and all tested derivatives, with a significant correlation for PAA and summed total PAA plus all derivatives. Canker incidence was negatively correlated with production of PAA and all tested derivatives, with a significant correlation for 3-OH-PAA. No significant correlations were observed between root necrosis length and production of PAA or any derivative of PAA.

DISCUSSION

The implication of PAA as playing a role in the infection process of Rhizoctonia on plants has led to a number of studies investigating the production of PAA by isolates of *R. solani* from various AGs on a range of host plant species. There have been many reports of the effects of PAA on plant growth, but few studies have directly linked PAA or a derivative of PAA to the development of symptoms typical of Rhizoctonia infection. Furthermore, reports that PAA inhibits the growth of *R. solani* conflict with reports that the fungus produces and metabolizes PAA to other chemically related compounds. In this study, experiments were conducted to determine the concentrations of PAA, OH-, and MeO- derivatives of PAA required to induce root necrosis and stem cankers of tomato in the absence of the fungus, the concentration at which PAA inhibits the growth *R. solani*,

the amounts of PAA and derivatives of PAA produced by *R. solani* and the relationship between PAA and production and disease symptom development on tomato.

Results of this study demonstrated that PAA and OH- and MeO- PAA derivatives can damage tomato seedlings by causing both root and shoot necrosis. These symptoms, typical of *Rhizoctonia* infection, are induced on the host in the absence of the fungus. To our knowledge, this is the first report that these derivatives of PAA induce necrosis of tomato roots. This finding is consistent with the reports that necrosis occurs in potato roots treated with PAA and in nodule cells of soybean treated with 3-OH-PAA and 3-MeO-PAA (Frank and Francis 1976; Orellana and Mandava 1983). In this study necrosis was observed when PAA, 3-OH-PAA, and 3-MeO-PAA were injected into tomato stems at concentrations of 7.5 mM. This is consistent with previously described tissue collapse of tomato stem cuttings dipped in 0.1-0.2 mM PAA solutions (Evidente and others 2005). Interestingly, no necrosis was observed when 0.1-1 mM solutions of PAA were injected into bean hypocotyls or tobacco leaves (Iacobellis and Devay 1987), or when tomato stems were injected with 75-225 mM concentrations of PAA (Zimmerman and Wilcoxon 1935).

Treatment of tomato plants with PAA and each of the derivatives in this study also reduced root and shoot length, and these results are consistent with the demonstrated inhibitory effects of PAA on the growth of tomato as well as lettuce and soybean (Iacobellis and Devay 1987; Mandava and others 1980; Orellana and Mandava 1983). Although reduced lateral root formation was observed up to 7.5 mM PAA in this study with no observed adventitious roots formation upon stem injection, Zimmerman and

Wilcoxon (1935) reported increased adventitious root formation on tomato stems injected with 75-225 mM concentrations of PAA. Though there have been reports that PAA and 3-MeO-PAA inhibit seed germination of various plant species (e.g., cress, lettuce, cotton, corn, soybean), neither PAA nor any derivative of PAA reduced the germination of tomato seeds in this study (data not shown) (Nair and Burke 1988; Orellana and Mandava 1983; Siddiqui and Shaukat 2005).

Although this study confirmed the reports that PAA can inhibit the growth of *R. solani* (Mao and others 2006), 50% growth reduction of was not achieved below 7.5 mM PAA. Our results differ from the findings of Hwang et al (Hwang and others 2001) that the growth of *R. solani* was completely inhibited by 0.4 mM PAA. The detection of 131 nM as the average quantity of PAA in the culture medium of *R. solani* supports the initial hypothesis that production levels are below concentrations inhibitory to fungal growth.

PAA has been quantified from *R. solani* culture medium (Aoki, Sassa, Tamura 1963; Danson, Wasano, Nose 1999; Iacobellis and Devay 1987; Kohmoto, Nishimura, Hiroe 1970), detected in sclerotia (Aliferis and Jabaji 2010), and quantified from plants and soils colonized by the fungus (Kohmoto and others 1973). Comparison of the quantities determined in each study is complicated by the use of isolates belonging to different AGs, as well as differences in growth media formulations, incubation times, methods of quantification, and units used to express results. The quantities of PAA and the OH- and MeO- derivatives of PAA from the growth medium of *R. solani* isolates belonging to AG-3, AG-4 and AG-1-IA were determined in this study to range from 628 nM to levels below the limit of detection. A previous study found that isolates of AG-4

produce concentrations of total PAA and OH- derivatives of PAA up to 22 μ M in broth culture without the addition of phenylalanine, 3-MeO-PAA was not quantified (Iacobellis and Devay 1987). Consistent with the results of this investigation, PAA and 3-OH-PAA were present in higher concentrations than other derivatives of PAA.

The concentrations of PAA and the OH- and MeO- derivatives of PAA produced by *R. solani* in this study were lower than the concentrations required to induce necrotic symptoms typical of Rhizoctonia disease on tomato (0.1-0.5 mM). The levels of production measured in this study are likely to represent conservative estimates based on the use of a minimal medium without supplementation with PAA precursors such as phenylalanine. Medium formulation has been demonstrated to affect the amount of PAA and its OH derivatives produced by *R. solani* (Danson, Wasano, Nose 1999). Furthermore, production under these growth conditions may not be reflective of production levels on a plant host. Quantification of PAA and derivative production by *R. solani in planta* was not undertaken in the study due to the difficulty of distinguishing fungal from plant-derived PAA, but would be a valuable contribution to the understanding of this system.

Breeding for resistance to Rhizoctonia is complicated by a high degree of variability in disease causing activity of *R. solani*, making selection of an appropriate isolate for screening of germplasm challenging (Banville and Carling 2001; Leach and Garber 1970; Panella and Ruppel 1996). Several investigators have suggested that the plant growth regulator phenylacetic acid (PAA) be used in lieu of the pathogen for plant germplasm resistance screening (Betancourt and Ciampi 2000; Frank and Francis 1976;

Mandava and others 1980). In this study, correlation was observed between the quantity of PAA and or derivatives of PAA produced in culture and the development of several types of symptoms on tomato infected by a given isolate of *R. solani*. This conflicts with the findings of previous investigations with isolates from AG-3 on potato and AG-4 on cabbage (Betancourt and Ciampi 2000; Iacobellis and Devay 1987), but is consistent with the findings of another study with isolates from AG-3 on potato (Tavantzis and others 1989). The results of this investigation add to the body of evidence that the PAA metabolic complex is involved in Rhizoctonia disease development, but do not indicate production of these compounds is the primary or the only determinant of pathogenicity. The intricacy associated the PAA metabolic complex in *R. solani*, which involves multiple related compounds, further limits the utility of these compounds in developing a simple, straightforward method for screening plant germplasm for resistance to the fungus.

The documented antimicrobial properties of PAA suggest that the compound might affect the disease ecology of Rhizoctonia via multitrophic interactions, conferring a competitive advantage to *R. solani* over other microbes present in the rhizosphere. The possibility that PAA and its derivatives might facilitate the infection of hosts at concentrations below those demonstrated to be phytotoxic also justifies further investigation. A study designed to determine if infection by *R. solani* is aided by prior exposure of tomato to PAA or its OH- and MeO- derivatives would contribute to our understanding of this system.

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TABLE 2.1. Anastomosis group (AG) affinity, plant host, geographic origin, and source of isolates of *Rhizoctonia solani* used in this study.

Isolate	AG	Host	Source	Collector/ Provider
Bs69	AG-3	<i>Solanum tuberosum</i>	Alaska	D. Carling
Cs-Ka (ATCC# 76121)	AG-1-IA	<i>Oryza sativa</i>	Hokkaido, Japan	S. Kuninaga
Rhs1A1	AG-3	<i>S. tuberosum</i>	Maine	S. Tavantzis
Rhs1AP (ATCC# MYA-4579)	AG-3	<i>S. tuberosum</i>	Maine	S. Tavantzis
Rs29 (ATCC# 48802)	AG-4	<i>Picea glauca</i>	Canada	E. Butler
Rs88	AG-3	<i>S. tuberosum</i>	Maine	S. Tavantzis
Rs113	AG-3	<i>S. tuberosum</i>	Washington Co., North Carolina	P. Ceresini
Rs114	AG-3	<i>S. tuberosum</i>	Washington Co., North Carolina	P. Ceresini
Rs138	AG-3	<i>S. tuberosum</i>	Tyrrell Co., NC	P. Ceresini
Rs182	AG-3	<i>S. tuberosum</i>	Hyde Co., North Carolina	P. Ceresini
Rs183	AG-3	<i>S. tuberosum</i>	Hyde Co., North Carolina	P. Ceresini
Rs191	AG-3	<i>S. tuberosum</i>	Camden Co., North Carolina	P. Ceresini
T2	AG-3	<i>Nicotiana tabacum</i>	North Carolina	D. Shew
Tom7b (ATCC# MYA-4649)	AG-3	<i>S. lycopersicum</i>	Rowan Co., North Carolina	M. Lancaster

TABLE 2.2. Multivariate pairwise correlation values for disease-causing activity and production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) derivatives of PAA by isolates of *Rhizoctonia solani* AG-3. Numbers followed by an asterisk indicate level of significance for the correlation analysis (all with $\alpha=0.05$).

Production ($\mu\text{g}/\text{mg}$ mycelial dry wt)	Emergence %	Damping-off incidence %	Root necrosis length (cm)	Shoot canker length (cm)	Final stand %
PAA	-0.6135	0.8899***	0.3519	-0.2669	-0.8047**
3-MeO-PAA	-0.3130	0.4440	0.2164	-0.1814	-0.4238
2-OH-PAA	-0.3079	0.4558	-0.4506	-0.5039	-0.4001
3-OH-PAA	-0.2323	0.3444	0.0332	-0.5766	-0.3131
4-OH-PAA	-0.9109	0.9836	0.9918	0.9663	-0.9609
Total derivatives	-0.6727**	0.4915	0.1207	-0.2897	-0.6183*
Total PAA + derivatives	-0.7393***	0.6138*	0.2988	-0.1468	-0.7277**

* $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$

FIG 2.1. Tomato seedling growth response to phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) PAA derivatives in the growth medium. Bars represent the standard error of the mean response for each treatment. A. Mean percent area of root system necrotic per plant. B. Mean total root system length per plant. C. Mean total length of lateral root system per plant. D. Mean number of lateral roots per plant. E. Mean shoot length per plant.

FIG 2.2. Tomato stem canker length in response to injection of stems with phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) PAA derivatives. Bars represent the standard error of the mean canker length for each treatment. Lengths with different letters are significantly different ($\alpha=0.05$).

FIG. 2.3. Reduction in mycelial growth of *Rhizoctonia solani* after 3 wk in potato dextrose agar amended with phenylacetic acid. Bars represent the standard error of the mean percent reduction in growth for each treatment compared to the control.

FIG 2.4. Mean production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) PAA derivatives by isolates of *Rhizoctonia solani* in Vogel's minimal medium after 3 wk of incubation. Normalized production values are expressed as μg produced per mg mycelial dry weight. Bars represent the standard error of the mean production for each isolate. A. Production of PAA. B. Production of 3-MeO-PAA. C. Production of 2-OH-PAA. D. Production of 3-OH-PAA. D. Production of 4-OH-PAA.

FIG 2.5. Tomato seedling symptom expression 28 d post inoculation with isolates of *Rhizoctonia solani* AG-3. Bars represent the standard error of the mean response for plants inoculated with each isolate. A. Percentage of planted seeds that emerged. B. Percentage incidence of post-emergence damping-off. C. Length of root necrosis (cm). D. Length stem of cankers (cm). E. Percentage of planted seeds that survived for the duration of the experiment.

CHAPTER 3

Modulation of the phenylacetic acid metabolic complex by quinic acid alters the disease-causing activity of *Rhizoctonia solani* on tomato¹

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Abstract

The metabolic control of plant growth regulator production by the plant pathogenic fungus *Rhizoctonia solani* Kühn (teleomorph=*Thanatephorus cucumeris* (A. B. Frank) Donk) and consequences associated with the parasitic and saprobic activity of the fungus were investigated. Fourteen genetically distinct isolates of the fungus belonging to anastomosis groups (AG) AG-3, AG-4, and AG-1-IA were grown on Vogel's minimal media with and without the addition of a 25 mM quinic acid (QA) source of carbon. The effect of QA on fungal biomass was determined by measuring the dry weight of mycelia produced under each growth condition. QA stimulated growth of

¹ This chapter has been formatted in the style of the journal Phytochemistry

13 of 14 isolates of *R. solani* examined. The production of phenylacetic acid (PAA) and chemically related derivatives 2-hydroxy-PAA, 3-hydroxy-PAA, 4-hydroxy-PAA, and 3-methoxy-PAA on the two different media was compared by gas chromatography-mass spectrometry. The presence of QA in the growth medium of *R. solani* altered the PAA production profile, limiting the conversion of PAA to derivative forms. The effect of QA on the ability of *R. solani* to cause disease was examined by inoculating tomato (*Solanum lycopersicum* L.) plants with 11 isolates of *R. solani* AG-3 grown on media with and without the addition of 25 mM QA. Mean percent survival of tomato plants inoculated with *R. solani* was significantly higher when the fungal inoculum was generated on growth medium containing QA. The results of this study support the hypotheses that utilization of QA by *R. solani* leads to reduced production of the plant growth regulators belonging to the PAA metabolic complex which can suppress plant disease development.

1. Introduction

The soil fungus *Rhizoctonia solani* Kühn (teleomorph=*Thanatephorus cucumeris* (A. B. Frank) Donk) possesses a unique combination of characteristics that enable it to function as both a pathogen of plants and a competitive saprobe in soil (Papavizas, 1970; Cubeta and Vilgalys, 2000). The production of the plant growth regulator phenylacetic acid (PAA) and four hydroxy (OH) and methoxy (MeO) derivatives of PAA has been implicated as an important component of the *R. solani* infection processes on plants (Aoki et al., 1963; Frank and Francis, 1976; Mandava et al., 1980). The primary objective of this study was to critically examine the production of plant growth regulators in the

PAA metabolic complex by *R. solani* and to understand the role of these compounds in disease-causing activity.

PAA and the OH and MeO derivatives of PAA are synthesized by *R. solani* from phenylalanine through the shikimate pathway (Lakshman et al., 2006; Kohmoto and Nishimura, 1975; Kohmoto et al., 1975; Kohmoto et al., 1973). This pathway shares two metabolic intermediates with the quinic acid (QA) carbon metabolism pathway, which is induced by the presence of QA in the growth substrate as it is released from lignin in decaying plant material (Giles et al., 1967; Hawkins et al., 1982; Herrmann and Weaver, 1999). The switch between parasitic and saprobic phases of the life history of this fungus may be controlled via interplay between these metabolic pathways. It has been recently demonstrated that the induction of the QA pathway leads to sequestration of intermediates from the shikimate pathway, which reduces production of the PAA precursor phenylalanine in *R. solani* and in other filamentous fungi (Lamb et al., 1992; Liu et al., 2003a). Induction of the QA pathway in inoculum of one isolate of *R. solani* AG-3 has also been shown to reduce symptom development on potato (Liu et al., 2003a; Liu et al., 2003b). It has been suggested that the manipulation of metabolic pathways within the fungus by the application substrates containing QA could potentially reduce the disease causing activity of *R. solani* in cultivated crops (Liu et al., 2003b). This *R. solani* disease management strategy aimed at altering fungal metabolism represents a novel approach, since current practices such as fungicide use are primarily focused on reducing populations of the fungus (Leach and Garber, 1970; Sweetingham, 1996).

In this study three hypotheses related to the addition of QA to the fungal growth environment were tested: 1) QA will not negatively alter mycelial growth of *R. solani*; 2) QA will reduce production of PAA and the 2-OH, 3-OH, 4-OH and 3-MeO derivatives of PAA by *R. solani*; and 3) QA will reduce disease symptom development on tomato (*Solanum lycopersicum* L.) inoculated with *R. solani*. Fourteen isolates belonging to three different anastomosis groups (AG) of *R. solani* were examined. The results of preliminary experiments have been previously published (Bartz et al., 2010a).

2. Results and Discussion

2.1. *QA stimulates the growth of R. solani*

Fig. 3.1 summarizes the fungal biomass of 14 isolates of *R. solani* in response to the addition of 25 mM quinic acid to minimal medium N (Vogel, 1956). The mycelial dry wt was significantly increased by amendment of the medium with QA for of all isolates except CsKa ($p = 0.3819$ for CsKa, $\alpha = 0.05$). Although mean biomass of isolates Bs69, Rs113, Rs182, Rs183, and Rs88 differed significantly between experimental runs, the increase in mycelial dry wt upon QA treatment was significant for both experimental runs of each isolate (Bs69 $p = 0.022$ and 0.0036 , Rs113 $p = 0.0052$ and <0.0001 , Rs182 $p = 0.0043$ and 0.0007 , Rs183 $p = 0.0014$ and 0.0003 , and Rs88 $p < 0.0001$ and <0.0001 for the first and second experimental run for each isolate respectively; $\alpha = 0.05$). There were no significant differences in biomass between experimental runs for the remaining isolates, all of which displayed a significant increase in mycelial dry wt upon QA ($p = <0.0001$, 0.0008 , 0.0014 , 0.0005 , 0.0127 , <0.0001 , <0.0001 , and 0.0037 , for Rhs1A1,

Rhs1AP, Rs114, Rs138, Rs191, Rs29, T2, and Tom7b, respectively; $\alpha = 0.05$). Because the addition of QA to the growth medium of *R. solani* stimulates growth, any inhibition of disease causing activity or PAA production resulting from QA treatment cannot be attributed to reduced biomass.

2.2. *QA modulates the production of PAA and OH and MeO derivatives of PAA by R. solani*

The production of PAA, 2-OH-PAA, 3-OH-PAA, 4-OH-PAA, and 3-MeO-PAA by 14 isolates of *R. solani* grown in Vogel's minimal medium N with and without the addition of 25 mM QA was compared by GC-MS. The effect of QA on production of PAA differed from the effect on the production of derivatives of PAA (Fig. 3.2). While more PAA was produced in the medium containing QA, the mean production of each of the tested derivatives decreased ($p < 0.0001$ for each compound, $\alpha = 0.05$).

When QA was added to the growth medium of *R. solani*, the resulting difference in the mean total production of PAA and PAA derivatives ranged from 6-50% (Fig. 3.3). The magnitude of the response to QA varied with isolate ($p < 0.0001$, $\alpha = 0.05$). The effect of medium amendment with QA on the mean total production of PAA and derivatives was not significant for isolates Bs69, CsKa, Rhs1A1, Rs138, and T2 ($p = 0.9975$, 0.2497 , 0.9996 , 0.9972 , and 0.0924 , respectively; $\alpha = 0.05$). For isolates Rs182, Rs191, Rs88, and Tom7b, there was a significant interaction effect between QA and PAA compound due to the differential effects of QA on PAA and its derivatives ($p = 0.0002$, < 0.0001 , $= 0.0206$, and 0.006 for each isolate respectively; $\alpha = 0.05$). For isolate Rs182, medium amendment with QA resulted in increased production of PAA and decreased production of 2-OH-

PAA and 4-OH-PAA ($p = 0.027$, 0.0146 , and 0.0013 , respectively; $\alpha = 0.05$). For isolate Rs191, medium amendment with QA resulted in increased production of PAA and decreased production of 3-MeO-PAA and 4-OH-PAA ($p = 0.0017$, 0.01 , and 0.019 , respectively; $\alpha = 0.05$). For isolate Rs88, medium amendment with QA resulted in decreased production of 4-OH-PAA ($p = 0.0228$, $\alpha = 0.05$). For isolate Tom7b, medium amendment with QA resulted in increased production of PAA ($p = 0.0022$, $\alpha = 0.05$). There were no significant interaction effects between QA treatment and the mean total production of PAA and the OH and MeO derivatives for the remaining five isolates (Rhs1AP, Rs113, Rs114, Rs183, and Rs29) with the presence or absence of QA being the only significant factor affecting production ($p = 0.0332$, 0.0168 , <0.0001 , 0.001 , and 0.0009 , for each isolate respectively; $\alpha = 0.05$).

Although results revealed variability in the specific effects of QA on the production of PAA and derivatives of PAA by individual isolates of *R. solani*, the average effect across all isolates was clear. The presence of the substrate of the QA metabolic pathway in the growth medium of *R. solani* altered the PAA production profile, limiting the conversion of PAA to derivative forms. This is consistent with the finding that QA pathway induction in *R. solani* can reduce the production of the PAA precursor phenylalanine (Liu et al., 2003a). It was also shown that 3-OH-PAA is present in greater quantities than PAA in *R. solani* growth medium supplemented with phenylalanine, but PAA is present in higher quantities in non-supplemented medium (Iacobellis and Devay, 1987). The results presented here extend our understanding of the

metabolic responses to QA downstream of phenylalanine in a more diverse range of isolates of *R. solani*.

2.3. Disease-causing activity of *R. solani* in response to QA

The effect of QA on the ability of *R. solani* to cause disease was examined using 11 isolates of *R. solani* AG-3, a group whose host range is generally limited to the family Solanaceae (Date et al., 1984; Ogoshi, 1987; Johnk et al., 1993; Bartz et al., 2010b). Inoculum of each isolate was prepared on growth media with and without the addition of 25 mM QA. Isolates varied in their abilities to reduce tomato plant survival relative to the control treatments ($p < 0.0001$, $\alpha = 0.05$) (Fig. 3.4). However, the significant positive effect of QA on the survival of inoculated plants ($p = 0.0006$, $\alpha = 0.05$) did not vary with isolate ($p = 0.7279$, $\alpha = 0.05$). While the mean percent survival of tomato plants inoculated with *R. solani* was 31% lower than control plant survival, this value increased to 23% below control plant survival when the fungal inoculum was generated on growth medium containing QA. These results are consistent with the previous finding that the presence of QA in the growth medium of an AG-3 isolate of *R. solani* reduced the severity of potato stem lesions (Liu et al., 2003a; Liu et al., 2003b), and demonstrates a similar effect on a closely related host with a more genetically diverse range of isolates.

3. Conclusions

The results of this study support the hypotheses that utilization of QA by *R. solani* as a growth substrate leads to reduced production of the plant growth regulators in the PAA metabolic complex, and can suppress plant disease development. The GC-MS SIM

method employed in this study enabled comparative quantitation of PAA, 2-OH-PAA, 3-OH-PAA, 4-OH-PAA, and 3-MeO-PAA at nM levels in the fungal culture medium at the time of analysis, but did not allow for the identification of other compounds present. It would be of interest to determine if QA affects other components of the biochemical profile of *R. solani*, and to investigate the temporal dynamics of the metabolic responses of *R. solani* to QA.

The importance of developing novel methods to combat this pathogen is highlighted by the wide geographic distribution of the *R. solani* species complex (Ogoshi, 1987; Farr et al., 1989) combined with a lack of practical host resistance and the ineffectiveness of crop rotation to manage Rhizoctonia disease (Leach and Garber, 1970; Panella and Ruppel, 1996; Banville and Carling, 2001). An increased understanding of the metabolism of *R. solani* may facilitate the development of more effective and novel strategies to suppress disease and improve plant health. The results of this study suggest that QA metabolism may be the mechanism behind the observed effectiveness of mature plant-based composts (generally rich in QA) towards Rhizoctonia disease suppression (Hoitink and Boehm, 1999), and justify further investigation into whether QA content of a soil amendment can be of predictive value regarding the potential to suppress Rhizoctonia disease.

4. Experimental

4.1. *Maintenance of fungal isolates*

Fourteen field isolates of *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk) were used in this study. Twelve isolates belonged to *R. solani* anastomosis group 3 (AG-3), and were designated as Bs69, Rhs1A1, Rhs1AP (ATCC# MYA-4579), Rs88, Rs113, Rs114, Rs138, Rs182, Rs183, Rs191, T2, and Tom7b (ATCC# MYA-4649). An isolate of *R. solani* AG-1-IA, designated as CsKa (ATCC# 76121), and an isolate of *R. solani* AG-4, designated as Rs29 (ATCC# 48802), were also included in this study.

All isolates were stored cryogenically at -80 °C as five mm diam plugs of mycelia in 2.0 ml cryogenic vials containing 500 µl of potato dextrose (PD) broth and 500 µl of sterile 50% glycerol. Isolates were retrieved from cryogenic preservation at -80 °C by placing the mycelial plug in a Petri dish containing 20 ml of potato dextrose agar (PDA) and incubating in the dark at 24 °C.

4.2. *R. solani* growth response to QA

Fourteen isolates of *Rhizoctonia solani* were stored and recovered from cryogenic preservation as described in section 4.1. A five mm diam plug of mycelium of each isolate was placed into each of three replicate Erlenmeyer flasks containing 100 ml of Vogel's minimal medium N prepared without the addition of sucrose and balanced to pH 5.5 (Vogel, 1956), and three replicate flasks containing pH 5.5 Vogel's medium with 25 mM QA (based on the formulation of quinate minimal medium) (Hawkins et al., 1982). Controls consisted of non-inoculated flasks. Flasks were arranged in a randomized

complete block design and incubated in the dark at 24 °C for 3 wk. The fungal biomass was separated by filtration and mycelial dry weight measurements were obtained. Aliquots of the broth were stored at -20 °C in 2.0 ml cryogenic vials until processing for chemical analysis. This experiment was conducted twice. Statistical analyses were carried out using JMP 7 software. Linear models were constructed using the restricted maximum likelihood approach to determine if the addition of QA to the growth medium affected the mycelial dry wt of each isolate.

4.3. *Extraction of R. solani growth medium*

Duplicate 0.5 ml aliquots from each flask of broth were mixed with 0.5 ml of NaCl satd. 1 N HCl and extracted 3X with 0.5 ml volumes of methyl tert-butyl ether containing 5% toluene. The extracts from each sample were combined with 25 µl pyridine with 2.5 ng µl⁻¹ 1-octadecanol as an internal standard in glass autosampler vials. The contents of each vial were evaporated under air flow at ca. 40 °C. Trimethylsilyl (TMSi) derivatives were formed by adding 100 µl of acetonitrile containing 1% pyridine and 0.5% TFA and 25 µl of either 1:1 *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide with hexamethyldisilazane or *N,N*-dimethyltrimethylsilylamine to each vial and incubating at ca. 40 °C for 15 min.

4.4. *Chemical analysis of R. solani growth medium*

TMSi derivatives of PAA, 3-MeO-PAA, 2-OH-PAA, 3-OH-PAA and 4-OH-PAA were quantified from the broth extracts with a Thermo Scientific Trace GC Plus gas chromatograph coupled to a Trace DSQII mass-selective detector. A Thermo TR-5 MS (5% phenyl polysilphenylene-siloxane) column (30 m length; 0.25 mm inside diameter;

0.25 μm film thickness) performed chromatographic separations. The carrier gas was helium at a constant flow of 1 ml min^{-1} (ca. 40 cm s^{-1} linear velocity). One μl of each sample was injected into a PTV injector operated in splitless mode at a constant temperature of $240\text{ }^{\circ}\text{C}$. Column oven temperature was set to $60\text{ }^{\circ}\text{C}$ initial temp. held for 30 sec, increased $8\text{ }^{\circ}\text{C min}^{-1}$ to a $360\text{ }^{\circ}\text{C}$ final temp. held for 4 min. The MS, equipped with an electron impact source (EI) in positive mode, was programmed for selected ion monitoring (SIM) of ions characteristic of the analytes (m/z 164, 192-194, 223, 238, 251-253, 280-282, 296) and the internal standard (m/z 327).

A series of reference standards were analyzed concurrently with fungal broth culture extracts, and quantitation was achieved by comparing reference standard peak areas with those of the monitored ions from experimental samples. Variation in instrument response was corrected for when necessary using calculations of peak area ratios of analytes to the internal standard. The chromatographic data were processed using Thermo Scientific Xcalibur software. The experiment was conducted twice. The mean peak area for each analyte from the growth medium of each isolate was calculated separately for each run. The percent difference from the mean peak area was calculated for each sample. Statistical analyses were carried out using JMP 7 software. Linear models were constructed using the restricted maximum likelihood approach to determine if growth medium amendment with QA affected *in vitro* production of PAA or hydroxy and methoxy derivatives of PAA by each isolate of *R. solani*.

The ability to detect differences in analyte quantities was examined by preparing a composite of all experimental samples. Volumes of 0.5, 0.75, 1, and 1.5 ml were

extracted in triplicate as described in section 4.3. Peak areas were significantly different when extracted volumes of 2-OH-PAA differed by 50%. Peak areas were significantly different when extracted volumes of PAA, 3-MeO-PAA, 3-OH-PAA, or 4-OH-PAA differed by 25%.

4.5. Bioassay to assess the disease-causing activity of *R. solani* in response to QA

Eleven isolates of *Rhizoctonia solani* AG-3 were stored and recovered from cryogenic preservation as described in section 4.1. Inoculum of each isolate was transferred two times to half-strength PDA (pH 5.5) with and without 25 mM QA. Plates were incubated in the dark at 24 °C for 4-7 days. After the second growth period, 5 mm diameter plugs of mycelia were used to inoculate tomato plants.

Seeds of tomato cv. Micro Tom were placed in Petri dishes between two sheets of Whatman #1 filter papers moistened with 3 ml of 2% KNO₃ soln. containing 1 µl Allegiance (Bayer CropScience, Research Triangle Park, NC) and incubated in the dark at 24 °C for 24 hr. Ten seeds were planted in each plastic pot (10 cm diam, 7-cm deep) containing a 1:1 mixture of pasteurized peat and sand. Two 5 mm diameter plugs of *R. solani* mycelium were placed under each seed as it was planted. Four replicate pots were prepared for each isolate and QA treatment. Plugs of sterile media were placed under the seeds of control plants.

The pots were arranged in a randomized complete block design in a 22 °C growth chamber illuminated by fluorescent bulbs emitting 450 µmol m⁻² s⁻¹ of photosynthetically active radiation set to a photoperiod of 14 h of light. Plants were watered as needed with de-ionized H₂O. After 3 wk, final stand count was assessed by recording the number of

surviving plants in each pot. The mean percent survival was calculated for control plants, and the difference from this value was calculated for each inoculated plant. The experiment was conducted three times. Statistical analyses were carried out on average values for each pot using JMP 7 software. A linear model was constructed using the restricted maximum likelihood approach to determine if conditioning *R. solani* inoculum on QA increased the survival of inoculated plants.

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Fig. 3.1. *Rhizoctonia solani* mycelial dry wt after three weeks of growth in Vogel's medium amended with quinic acid (QA). Bars indicate standard error of the mean dry wt for each isolate and QA treatment.

Fig. 3.2. Differential production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) derivatives of PAA by *Rhizoctonia solani* isolates after three weeks of growth in Vogel's medium N amended with quinic acid (QA). Production was measured by GC-MS peak area and normalized by mycelial dry wt, bars indicate standard error.

Fig. 3.3. Production of phenylacetic acid (PAA) and hydroxy (OH) and methoxy (MeO) derivatives of PAA by *Rhizoctonia solani* isolates after three weeks of growth in Vogel's medium N amended with quinic acid (QA). Production values (μg) were normalized by mycelial dry wt, bars indicate standard error.

Fig. 3.4. Percent survival of tomato plants relative to control plants three weeks after inoculation with *Rhizoctonia solani* grown in half-strength potato dextrose agar medium amended with or without quinic acid (QA). Bars indicate standard error of the mean.

APPENDIX

Rhizoctonia Foliar Blight¹

In: Jones, J. B., Zitter, T. A., Momol, T. M., and Polston, J. E. (Eds.)

Compendium of Tomato Diseases, 2nd Ed.

American Phytopathological Society, St. Paul, MN. (in press)

(Prepared by Faith Bartz, Marc Cubeta and Kelly Ivors)

Rhizoctonia foliar blight disease of tomato has been recently identified in Japan and North America. There are also undocumented reports of this disease occurring in Latin America.

The causal relationship of *Rhizoctonia solani* Kühn anastomosis group 3 (AG-3) and this disease on tomato was not demonstrated by fulfillment of Koch's postulates until 2009, although foliar diseases caused by *R. solani* AG-3 and teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk have been reported from other solanaceous hosts including eggplant, potato, and tobacco.

Symptoms

Symptoms observed in the field on naturally infected plants included brown colored necrotic lesions and blighting of leaves (Figures 4.1-4.3). White mycelial growth on the underside of infected tomato leaves was also observed (Figures 4.1 and 4.3).

¹ This chapter is currently in press, and is formatted in the style of Compendium of tomato diseases

Fig. 4.1. Necrotic lesions and mycelia on abaxial side of infected tomato leaves.

Fig. 4.2 *Rhizoctonia* foliar blight symptoms.

Fig. 4.3. *Rhizoctonia* foliar blight symptoms

Causal Organism

The soil fungus *Rhizoctonia solani* Kühn exists as a heterogeneous species whose current classification is based on hyphal anastomosis (fusion) to established anastomosis groups (AGs). *Rhizoctonia* foliar blight on tomato is caused by *R. solani* anastomosis group 3 (AG-3) (teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk). The identity of the fungus is based on hyphal anastomosis criteria and sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) repeat. Isolates of *R. solani* AG-3 from tomato are phylogenetically related to isolates of the same AG from potato more so than tobacco, although their disease biology (epidemiology) is more similar to the target spot pathogen of tobacco .

Hymenium may be white to various shades of brown, resupinate with or without aerial hyphae. Hyphae (Fig. 4.4) are 5 - 13 µm wide, hyaline to brown, without clamp connections, typically with slight constrictions at branch points, with dolipore septa, and multinucleate. Aggregations of monilioid cells form loose, cream to brown sclerotia on plant tissue and in culture. Holobasidia of the teleomorph are formed discontinuously on short, vertically branching hyphae arising from a basal hyphal layer. Basidia are 15 x 8 - 10 µm, hyaline, barrel-shaped or sub-cylindrical, and typically have four sterigmata (Fig. 4.5). Sterigmata are 5 - 13 x 2 - 4 µm, typically broader at the base than at the tip. Basidiospores are 4 - 7 x 4 - 5 µm smooth, hyaline, oblong to ellipsoid, can be unilaterally flattened, and prominently apiculate.

Disease cycle

Little is known about the disease cycle. This disease was initially identified in North America in 2005 from a commercial tomato field (variety Mountain Fresh Plus) located in Rowan County, North Carolina (NC) and was subsequently observed in four fields in two additional NC counties (Henderson and Polk) in 2006. In these fields, there was no history of fungicide use and the disease was observed during warm and humid weather conditions with extended periods of leaf wetness. Currently, the economic impact and importance of *Rhizoctonia* foliar blight disease is not known.

When foliar isolates of this fungus were used to infest soil in greenhouse inoculations, root necrosis developed on both tomato and eggplant seedlings. Basidia and basidiospores were observed in tape mounts taken from infected tomato stem tissue after infesting soil with oat grains colonized with mycelium of the fungus in growth chamber experiments (Figures 4.4 and 4.5).

Since disease symptoms in the field were observed on leaves in the mid and upper plant canopy, the primary inoculum is assumed to be aerially dispersed basidiospores. No disease symptoms have been observed on tomato leaves lower in the canopy, a pattern likely to result from splash dispersal of fungal propagules from soil.

Fig. 4.4. Microscopic image of mycelium stained with lactophenol blue from the abaxial side of an infected tomato leaf.

Fig. 4.5. Microscopic image (400X) of basidium with sterigmata stained with lactophenol blue on tomato stems.

Control

Rhizoctonia foliar blight has only been identified on plants not exposed to foliar fungicides. This indicates that some fungicides, such as mancozeb- and chlorothalonil-containing products, may be effective at preventing this pathogen from colonizing leaf surfaces and causing infection.

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