Mature watermelon vine decline: Suppression with fumigants of a soil-borne problem and association with *Rhizopycnis vagum*

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**Abstract**

Mature watermelon vine decline (MWVD) is a late-season disease of unknown etiology, characterized by vine collapse and discoloration, much reduced root systems. While several new disorders in watermelon have been reported recently, this problem appears specific to the Midwest. To test for a biological cause and methods to suppress this disorder, soil was collected from two southern Indiana fields with histories of MWVD for microplot and greenhouse experiments. Watermelons grew best in microplots treated with methyl bromide or methyl iodide. Roots of plants growing in the untreated MWVD soil were severely necrotic; those from the fumigation treatments appeared healthy. In heating experiments, watermelon seedlings were grown in autoclaved soil mixed with 10% MWVD soil that was either untreated or heated at 40, 50, 60, 70 or 80 °C for 30 min. Watermelon plants in the untreated controls and the 40 and 50 °C treatments had reduced top dry weights with decreased fibrous roots and associated discoloration in contrast to treatments of equal to and greater than 60 °C. Representative cultures from necrotic roots were identified as *Rhizopycnis vagum* by morphological analysis and sequencing the ITS region of the ribosomal DNA. PCR analysis with specific primers confirmed that *R. vagum* was present in roots from untreated MWVD soil but not in symptomless roots. This is the first report of *R. vagum* associated with plants suffering from MWVD in Indiana and indicates that this fungus occurs much farther north in the U.S. than known previously.

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1. Introduction

Several soil-borne pathogens have been identified during the past two decades that cause vine declines with severe yield losses in watermelon (*Citrus lanatus* (Thunb.) Matsum. and Nakai) and other cucurbits in the United States and other parts of the world (Martyn and Miller, 1996). Many of these disorders have similar symptoms manifested by yellowing and wilting of the plants followed by rapid plant death. Probably the most notorious soil-borne pathogens on cucurbits in the United States and other parts of the world are the following two decades that cause vine declines with severe yield losses in watermelon (*Citrus lanatus* (Thunb.) Matsum. and Nakai) and other cucurbits in the United States and other parts of the world (Martyn and Miller, 1996). Many of these disorders have similar symptoms manifested by yellowing and wilting of the plants followed by rapid plant death. Probably the most notorious soil-borne pathogens on cucurbits in the United States and other parts of the world are the following.

Other late-season vine declines are caused by a squash bug-transmitted bacterium or an insect-transmitted virus (Bruton et al., 2003; Adkins et al., 2007).

Since 1989, an apparently new disease, mature watermelon vine decline (MWVD), has been observed on watermelon crops in southwestern Indiana (Egel et al., 2000). This disorder appears similar to other late-season vine declines because the entire canopy collapses rapidly. Preliminary analyses showed that this disorder was specific to watermelon and did not affect other cucurbit species such as muskmelon (*Cucumis melo*) (Harikrishnan et al., 2002). Although various fungal pathogens, herbicide residues or the practice of transplanting have been implicated as possible causes of MWVD (Egel et al., 2000; Egel and Latin, 2001; Makam et al., 2005), the disease has not been associated with any of the known pathogens and its etiology remains uncertain.

Indiana is the largest producer of watermelon in the Midwest, and this crop is an important agricultural commodity for the region. During 2005, Indiana growers produced watermelon on ca. 3000 ha,
with a value approaching $20 million (Anonymous, 2006). Melon production is concentrated in counties in southwestern Indiana with coarse-textured soils that are notoriously infested with Meloidogyne spp. (Kruger et al., 2007). MWVD was first observed in Indiana primarily in low, poorly drained areas of fields, but entire fields also were affected (Egel et al., 2000). Symptomatic plants typically had watery, often brown-pinkish lesions and reduced numbers of secondary fibrous roots compared to those with healthy root systems (Egel et al., 2000). Watermelon plants affected with MWVD collapse about two weeks prior to harvest, increasing the risk of sunburn and preclude water and nutrients from reaching the maturing fruit (Egel et al., 2000). During 2000, qualitative and quantitative yield losses due to MWVD occurred widely and severely in Indiana, resulting in a crop and value loss of ca. 20% (D.S. Egel, personal communication).

Some possible pathogens, specifically Fusarium spp. and Pythium spp., have been isolated from roots showing symptoms of MWVD (Egel et al., 2000), but these species often are encountered when isolating from roots of agricultural crops and their pathogenicity is unclear (Westphal, unpublished). Other known pathogens such as M. cannonballus were not found. Particular attention was paid to R. vagum because this species has been described as a pathogen of cucurbits relatively recently (Farr et al., 1998; Armengol et al., 2003). This fungus has primarily been found on muskmelon from southern states of the U.S., Central America and South Europe in Mediterranean climates (Miller et al., 1996; Bruton and Miller, 1997a,b; Armengol et al., 2003; Ghignone et al., 2003; Chiolisi et al., 2008) and in the U.S., has not been reported as far north as Indiana. In general, the fungus sometimes caused mild or no symptoms in pathogenicity tests on melons when inoculated alone (Aegerter et al., 2000; Chiolisi et al., 2008) and usually seems to be considered a minor pathogen.

The objectives of this project were to: (1) test the hypothesis that MWVD can be suppressed by fumigation and heat treatments; and (2) test whether specific, potentially pathogenic organisms, including R. vagum, were consistently associated with the watermelon roots affected with MWVD. These hypotheses were tested in field microplot and greenhouse experiments. A PCR-based method was developed and used for identifying and detecting R. vagum in infected watermelon roots. A preliminary report of part of this study was published (Westphal and Egel, 2003).

2. Materials and methods

2.1. Microplot experiments with soil fumigants

During 2002 and 2003, microplot experiments were conducted at the Southwest Purdue Agricultural Center (SWPAC) at Vincennes, IN, to investigate the effects of fumigant treatments on MWVD. During the spring of 2002, soil was collected from representative areas of two fields with histories of MWVD in a grid pattern to a depth of 15 cm. The soil samples were passed through a coarse sieve (<1.2 cm aperture), and then placed in polyethylene drainage pipes of 45-cm-diameter (N12; Advanced Drainage Systems Inc., Hilliard, OH) that had been inserted perpendicularly into the ground to a depth of 55 cm. In the first field (Soil 1: 70% sand, 20% silt, 10% clay, pH 7.0, 1.2% O.M.), an annual rotation of muskmelon and watermelon had been in place more than 10 years during summer with rye cover crops during winter for the previous 5 years. In the second field (Soil 2: 72% sand, 18% silt, 10% clay, pH 6.7, 1.3% O.M.), watermelon had been cropped continuously in sequence with corn and soybean for many years.

Before treatment, pre-soaked seeds of nutsedge (Cyperus rotundus L.) were placed in cotton sampling bags (8.9 × 12.7 cm) (Hutchinson Bag Corporation, Hutchinson, KS) at a depth of 15 cm in all plots for monitoring of fumigation effectiveness. Treatments were arranged in a randomized complete block design with four replications. The following treatments were applied each year: (1) untreated; (2) methyl bromide (392 kg/ha); (3) methyl iodide (586 kg/ha); and (4) 1,3-dichloropropene (1,3-D) - chloropicrin mixture in Telone C-35 (63% 1,3-D, 35% chloropicrin, 2% inert; Dow AgroSciences, Indianapolis, IN; 327 L/ha). Treatments were made on 26 April 2002 (soil temperature: 21 °C) and 29 April 2003 (soil temperature: 18 °C). The methyl iodide and methyl bromide treatments were at the same molar equivalent. During 2003, soil from the same origin was removed from all plots within one block, mixed thoroughly, and placed back into the plots to reconstitute MWVD problem soils in all plots. Fumigants were transported and stored in sample cylinders (Swagelok Company, Solon, OH) and applied through a 0.64-cm O.D./0.43-cm I.D. polyethylene tube with a 10-ml gas-tight syringe (Hamilton Company, Reno, NV) with its open end at a depth of 25 cm. Immediately after dispensing the material, the plots were covered with 0.1-mm thick black polyethylene sheeting that was sealed to the upper rim of the microplot. After 8 (2002) or 5 (2003) days, the plastic tarps were removed and the monitoring bags recovered. The nutsedge nutlets were planted individually into single cells of plastic seeding trays in a soil-less potting mix (Metro mix 510, Scotts Company, Marysville, OH) in individual in the greenhouse to allow for germination. After sufficient time of incubation, indicated by germination of the control nutlets, germination of all nutlets was enumerated.

On 21 May 2002 and 22 May 2003, plots were fertilized with commercial N–P–K fertilizer and potassium chloride at 112 kg N, 49 kg P and 186 kg K/ha. Each plot was then planted with two 3- to 4-week-old seedlings of watermelon ‘Royal Sweet’, and watered in with 100 (2002) or 50 (2003) ml per plot of starter nutrient solution (10 g of Miracle Gro per 3.79 L of water, 15% N, 13.2% P, 12.5% K and micronutrients). Watermelon plants were maintained under standard agricultural conditions following a standard fungicide and insecticide spray program (Foster et al., 2002). Watermelon vines were trellised around two pairs of stakes adjacent to each plant within the plots. On 17 July 2002 and 6 August 2003, canopies of each trellised plant were measured for length, height and width, and the cubic volume was calculated. At harvest, fruits were weighed, and top fresh weights were determined. Roots were excavated to a depth of 30 cm, washed, weighed and rated for percent of necrosis typical of MWVD as described by Egel et al. (2000). Data were tested for homogeneity using the Box–Cox procedure (Box et al., 1978), and transformed using either log10(x), arcsine(√(x/total)), or √x before entering into ANOVA. Mean separation was done with the LSD procedure at P = 0.05. Procedures in SAS software (Gary, NC) were used for all statistical analyses.

2.2. Selective heat treatments in greenhouse experiments

During the fall of 2001, soil was collected from field 1 (Soil 1) for the microplot experiments. Part of the soil was autoclaved (twice for 2 h with 24 h between autoclavings) and a second part was subjected to selective heat treatments as suggested for other soilborne microbial complexes (Baker and Roistacher, 1957; Westphal and Becker, 2001; Westphal et al., 2002). In a water bath, soil samples in excess of the 10% of the final pot volume were placed in double-envelope polyethylene bags, and were treated at 40, 50, 60, 70 or 80 °C for 30 min after reaching the target temperature. Each of these treated soils plus an untreated control were mixed into autoclaved soil at 1:10 (vol/total vol) and placed into 3-L pots. Additional controls were 100% MWVD soil and unamended autoclaved soil. Four-week-old seedlings of watermelon ‘Royal Sweet’ were transplanted into each pot. Two experiments were arranged on greenhouse benches in randomized complete block designs (first experiment: 3 replications; second experiment: 5
replicates). Both tests were incubated in a greenhouse with a 16–8 h day–night cycle at 25 °C until plants in the untreated controls (100% MWVD soil) started to senesce. At harvest (8 and 13 weeks after transplanting for the first and second experiments, respectively), tops were cut and oven-dry weights were determined. Root systems were washed and inspected for MWVD symptoms of discoloration and necrosis. Data were tested for homogeneity of variances using the Box–Cox procedure (Box et al., 1978) and analysed by ANOVA after appropriate transformation.

2.3. Isolation and identification of potential fungal pathogens

Ten or twenty representative root sections from treatments of the different experiments were surface sterilized by placing in 70% ethanol for 30 s followed by either 10 or 40 min in 10% bleach solution (adjusted to pH 7), depending on the experiment, then were plated onto water agar or potato dextrose agar (PDA), both amended with 100 ppm rifampicin, or onto a selective medium for oomycetes (PARP; Browne and Viveros, 1999) at ten pieces per plate. Fungal growth was determined and representatives of the major morphological groups were subcultured.

Identification of fungal cultures was facilitated by sequencing of the internal transcribed spacer (ITS) region of the fungal DNA. Mycelial pieces from seven isolates representing the major morphological groups were inoculated into 100-ml of YSB broth (10 g of yeast extract, 10 g of sucrose and 50 mg of kanamycin per liter) and grown at room temperature in 250-ml Erlenmeyer flasks shaken at 150 rpm on an orbital shaker. After 6 days of growth, mycelia were harvested by centrifugation and lyophilized overnight. The lyophilized mycelia were ground in liquid nitrogen over night. The lyophilized mycelia were ground in liquid nitrogen and DNA was extracted according to the directions of the AquaPure DNA Extraction Kit (Bio-Rad Laboratories, Hercules, CA, USA). The DNA was then extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, and dissolved in 50 μl of water. Purified DNA was quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and diluted to 12.5 ng μl⁻¹ for a working solution.

The ITS region of each isolate was amplified with the universal primers ITS4 and ITS5 (White et al., 1990), and the amplification products were sequenced as described above to test whether they were from R. vagum or not from other species.

2.4. Testing for R. vagum in watermelon roots

Watermelon root systems were removed to a depth of 30 cm from microplots or from fields without a history of MWVD, root pieces were excised randomly from areas adjacent to necrotic root parts, and stored at −80 °C. Frozen root segments were placed in mortars, covered with liquid nitrogen and ground to a powder with a pestle after the nitrogen had evaporated. DNA was extracted from the powdered samples with Qiagen's QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA) according to the instructions provided by the manufacturer. Purified DNA was quantified with a NanoDrop Spectrophotometer and diluted to a working concentration of 12.5 ng μl⁻¹. PCR amplification of the DNA from each watermelon root sample was with the R. vagum-specific ITS primers as described above with the following parameters: 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 7 min. Amplifications with water-only and with universal primers ITS4 and ITS5 were included in each experiment as negative and positive controls, respectively. Amplification products from four samples were cloned and sequenced as described above to test whether they were from R. vagum.

3. Results

3.1. Microplot experiments with soil fumigants

Data for canopy volume, root fresh weight, and root necrosis could be averaged over both years and soils because the error variances of both trials were homogeneous. The canopy volume increased 4-fold after methyl bromide fumigation, and 3-fold after treatment with methyl iodide compared to the untreated control; means of both treatments were significantly greater than that of the untreated control (Table 1). Canopy volume of the 1.3-D+chloropicrin treatments was not significantly different from that of the methyl iodide treatment, but was significantly greater than that in the untreated control (Table 1). Root fresh weights were similar among the treatments. Root necrosis was greater in the untreated and the 1.3-D+chloropicrin plots than in those treated with methyl bromide or methyl iodide (Table 1).

Trends of the top biomass in response to fumigation were similar in 2002 and 2003; weights were greatest in the methyl bromide-treated plots for both years. In 2002, the tops of plants from methyl iodide-treated plots weighed less than those from methyl bromide-treated plots, but top weights were the same for these two treatments in 2003 (Table 2). In both years, these two fumigants gave greater top weights than the control and 1.3-D+chloropicrin treatments (Table 2). The methyl bromide and methyl iodide treatments also had the greatest fruit yield; treatment with 1.3-D+chloropicrin had lower yields than the other fumigant treatments in both years but had higher yields than the control in 2002 and the same as the control in 2003 (Table 2). The nutseed used to bioassay the fumigation effects only grew in the untreated control, but not in any of the

| Table 1 | Effect of three fumigants on growth of watermelon 'Royal Sweet' grown in soils from two fields with histories of mature watermelon vine decline (MWVD) in 45-cm-diameter microplots at Vincennes, IN.† |
| --- | --- | --- | --- |
| Treatment | Rate [kg ha⁻¹, ml [litre/plant] | Canopy volume [m³/plant] | Root weight [g/plant] | Root necrosis [% root system] |
| Untreated | | 49.3 ± 10.1c | 19.8 ± 1.9 | 59.4 ± 5.4a |
| Methyl bromide | 932 | 200.7 ± 17.5a | 27.2 ± 3.1 | 7.1 ± 3.4b |
| Methyl iodide | 586 | 149.6 ± 13.4b | 25.0 ± 2.9 | 13.3 ± 5.4b |
| 1,3-D + chloropicrin | 278.2 | 80.4 ± 9.1b | 21.1 ± 2.2 | 65.4 ± 6.9a |
| P treatment | < 0.01 | 0.54 | < 0.01 |
| P soil × treatment | 0.67 | 0.88 | 0.94 |

† Data were averaged over both years (2002 and 2003) and both soils (Soil 1 and Soil 2). Effects of treatment and soil × treatment were tested with the pooled error: year × soil × treatment = treatment × soil × treatment. Statistical analyses were done on transformed values according to the Box–Cox test. Numbers within a column followed by the same letter were not significantly different (P = 0.05); means ± SE of the means of original data are presented.

The original data were based on a cubic canopy volume (Vol = height × length × width); the log-transformed [log(Vol)] data of the average per plant in each plot were used for statistical analyses.

The root fresh weight was determined and the non-transformed data were analysed.

Roots were rated for percentage root discoloration/necrotic appearance; arc sine-transformed [arcsine(√%)] data were analysed.
the other treatments (data not shown). There were no significant effects on any of the parameters due to the main factor-soil origin.

3.2. Selective heat treatment greenhouse experiment

Watermelon plants in the untreated soil treatments (100% and 10% MWVD soil) and the 40 and 50 °C heat treatments had reduced top dry weights compared to treatments at 60 °C or above, and the autoclaved control (Table 3). Root fresh weights varied somewhat in the different treatments, but were at a similar level; only the 100% MWVD-amended soil had significantly reduced weights of roots in Study 1 (Table 3). The percentages of healthy secondary fibrous roots were greatly reduced in all treatments <60 °C compared to soil heated to ≥60 °C (Table 3).

3.3. Isolation and identification of potential fungal pathogens

A number of fungi was isolated from watermelon roots. Isolates that could be identified to genus morphologically included Fusarium spp., Pythium spp., Acremonium spp., Rhizoctonia spp., and R. vagum. In the first microplot experiment, R. vagum was isolated commonly from diseased watermelon roots but rarely from those that were healthy. This species was not obtained as commonly with diseased watermelon roots (data not shown). These primers amplify products ranging in size from approximately 550 to 1000 bp (data not shown). BLAST analysis (Altschul et al., 1997) of the ITS sequences obtained from each isolate indicated that four were 99% identical to the ITS region of R. vagum, one was a species of Fusarium, and the remaining two were similar to sequences from species of Cercophora, Zoophiella, Adopus and Pedospora. These sequences were deposited into GenBank under accession numbers HQ610503—HQ610509.

Table 2

Effect of three fumigants on top biomass and total fruit yield per plot of watermelon ‘Royal Sweet’ grown in soils from two fields with histories of mature watermelon vine decline (MWVD) in 45-cm-diameter microplots at Vincennes, IN, in 2002 and 2003.a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate</th>
<th>Top biomass [g/plant]b</th>
<th>Total fruit yield [kg/plot]c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[kg ha⁻¹; ml plot⁻¹]</td>
<td>2002</td>
<td>2003</td>
</tr>
<tr>
<td>Untreated</td>
<td>−</td>
<td>29±50c</td>
<td>21±30b</td>
</tr>
<tr>
<td>Methyl bromide</td>
<td>392</td>
<td>13±29a</td>
<td>20±57a</td>
</tr>
<tr>
<td>Methyl iodide</td>
<td>586</td>
<td>59±54b</td>
<td>19±25a</td>
</tr>
<tr>
<td>1,3-D + chloropicrin</td>
<td>278±152a</td>
<td>24±8c</td>
<td>23±48b</td>
</tr>
<tr>
<td>P treatment</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P soil × treatment</td>
<td>0.03</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

Table 3

Effect of heat treatments of soil from a field with a history of mature watermelon vine decline (MWVD) on growth of seedlings of watermelon ‘Royal Sweet’.a

<table>
<thead>
<tr>
<th>Soil [%]</th>
<th>Heat [°C]</th>
<th>Average weight [g]d</th>
<th>Root fresh weight [g]e</th>
<th>Fibrous roots [% healthy]f</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Study 1g</td>
<td>Study 2h</td>
<td>Study 1g</td>
</tr>
<tr>
<td>100</td>
<td>−</td>
<td>8.6 ± 0.6b</td>
<td>3.1 ± 0.3c</td>
<td>10.3 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>9.4 ± 0.6b</td>
<td>3.0 ± 0.28</td>
<td>6.7 ± 1.65</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>9.4 ± 0.6b</td>
<td>3.0 ± 0.28</td>
<td>6.7 ± 1.65</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>9.4 ± 0.6b</td>
<td>3.0 ± 0.28</td>
<td>6.7 ± 1.65</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>7.7 ± 1.6b</td>
<td>4.6 ± 0.38</td>
<td>9.7 ± 1.46</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>8.1 ± 1.6b</td>
<td>4.7 ± 0.38</td>
<td>9.7 ± 1.46</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>8.2 ± 1.6b</td>
<td>4.7 ± 0.38</td>
<td>9.7 ± 1.46</td>
</tr>
<tr>
<td>0</td>
<td>ATC</td>
<td>8.2 ± 1.6b</td>
<td>4.7 ± 0.38</td>
<td>9.7 ± 1.46</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.01</td>
<td>−0.01</td>
<td>0.06</td>
<td>0.60</td>
</tr>
</tbody>
</table>

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a Data were averaged across the soils. Effects of treatment and soil × treatment were tested with the experimental error. Statistical analyses were done after transformation of the data according to the Box—Cox test. Numbers within one column followed by the same letter were not significantly different (P = 0.05); means ± SE of the original data are presented. Original data are presented.

b The log-transformed [log10(x)] data were used for statistical analyses and means were separated using the LSD procedure (P = 0.05).

c All fruits per plot whether marketable or non-marketable. Data were square root-transformed [√x] prior to analysis.

d Treatment effects were tested using the LSD test at P = 0.05. Numbers followed by the same letter within one column were not significantly different; analysis of top dry weights was combined for both studies; root measures were presented separately for each study.

e Soil mixes were generated from autoclaved MWVD soil with 10% portions of raw MWVD soil that was treated in various ways before mixing: − : untreated; heated at different temperatures [°C] for 30 min; or ATC: autoclaved twice for 2 h each with 24 h between autoclavings.

f Top dry weight after oven-drying averaged for study 1 and 2.

g Top dry weight after oven-drying for study 1 and 2.

h Weight after washing free of soil.

i Percent of healthy, non-discolored fibrous roots on a scale of 0—100%; mean separation from 100% healthy roots was done on arc sine-transformed values [arc sine(√x/100)]; original values are presented. Numbers indexed with * were significantly different from 100% (=1.57 as transformed value; P = 0.05). Values with zero error variances were eliminated from the analysis.

j Two experiments were conducted in a greenhouse with a 16–8 h day—night cycle at 25 °C until plants in the untreated controls (100% MWVD) started to senesce. Study 1: three replications, harvested 8 weeks after transplanting; study 2: five replications, harvested 13 weeks after transplanting.
the complete 18S intron from *R. vagum* plus 6 bases of the 18S rRNA gene. Specificity was further tested *in silico* by *blastn* analyses of the primer sequences. Primer Rhizo 5R was not specific and will amplify intron sequences from many other fungi (data not shown). However, primer Rhizo 7F only had identical matches to other sequences from *Rhizopycnis* spp., and was at least 4 bp different from other fungi in GenBank. Therefore, it is expected to provide highly specific amplification.

### 3.4. Testing for *R. vagum* in watermelon roots

During the 2002 microplot experiment, the *R. vagum*-specific 18S intron band amplified strongly in DNA from watermelon roots grown in both MWVD soils (Soil 1 and Soil 2) except for Rep B of Soil 2 (Fig. 1a). Little or no amplification was seen in roots of plants grown in fumigated soils, except for Rep D of Soil 1, in which a band of intermediate intensity was present (Fig. 1a). All samples amplified with the ITS4/ITS5 universal primers used as positive controls except for the Rep B sample from Soil 2 (data not shown). A second round of PCR with this sample did give amplification showing that *R. vagum* was present (data not shown).

During the 2003 microplot experiments, amplification products specific for *R. vagum* were generated from DNA extracted from the necrotic watermelon roots in all four replications when plants were grown in untreated MWVD soils and no amplification occurred from plant roots grown in the fumigated MWVD soil (Fig. 1b). The weak amplifications seen in the lanes for untreated Soil 1, replication A and untreated Soil 2, replication D were confirmed by additional PCR reactions (data not shown). Sequencing of the amplification products from four MWVD-affected watermelon roots representing the four replicates confirmed that they were from *R. vagum*.

When watermelon roots from a field without a history of MWVD were tested with the PCR procedure no amplification was found except for the positive controls, suggesting that no *R. vagum* was present in those roots (Fig. 1c).

### 4. Discussion

These experiments demonstrated that MWVD in southwestern Indiana can be eliminated from soil by fumigation and heat treatment at $\geq 60^\circ$C for 30 min. Therefore, the soil-borne problem appears to be biological in nature. The disease-causing principal was transferable when 10% of the problem soil was added to autoclaved equivalents. Transfer of a soil-intrinsic phenomenon with small portions of soil has been used to demonstrate the biological nature of soil-borne disorders or beneficial functions (Menzies, 1959; Shipton et al., 1973; Westphal and Becker, 2000). MWVD was eliminated by various fumigant treatments, such as methyl bromide, methyl iodide or, to a limited extent, by treatments with 1,3-
D+-chloropicrin mixtures, as was used to demonstrate the biological nature of other soil-borne complexes (Westphal and Becker, 1999). When a range of heat treatments was applied to the MWVD soil, the problem was removed after heating to ≥60 °C for 30 min, a treatment temperature that was found to be selective for several hyphomycete fungi (Baker and Roistacher, 1957; Rouxel et al., 1977; Westphal and Becker, 2001; Westphal et al., 2002).

A number of hyphomycete fungi and watermolds was isolated from watermelon roots. Relatively high frequencies of Pythium and Fusarium species were isolated, which confirmed earlier findings in this disease problem (Egel et al., 2000; R. Harikrishnan and D.S. Egel, personal communication). Because these organisms were present on asymptomatic as well as symptomatic roots their importance in the etiology of MWVD is uncertain. More importantly, R. vagum was isolated frequently from symptomatic, but not from asymptomatic, roots. Identity of this pathogen was confirmed by sequencing the ITS region of the ribosomal DNA. PCR primers specific to an intron near the 3′ end of the 18S rRNA gene confirmed that R. vagum was present in all samples of diseased watermelon roots tested. Diseased roots were associated with strong amplification of the specific DNA product from R. vagum, whereas no amplification was detected in DNA from roots grown in soils that had been cured from the disorder by fumigation, or in fields without a history of MWVD. These results indicate that this fungus may be a contributing factor to MWVD in southwestern Indiana. This is the first report of R. vagum associated with MWVD in Indiana and indicates that this fungus occurs much farther north in the US than known previously.

The ecology of R. vagum remains uncertain, and it often is reported as an endophyte as well as a pathogen (Girlanda et al., 2002; Posada et al., 2007; Armengol et al., 2003). Isolates of R. vagum were placed with other dark, sterile, mycelia-forming fungi that colonised roots of Pinus halepensis or Rosmarinus officinalis in the Mediterranean; these fungi were grouped by having a morphotype that is characterized by “bulgily-growing” hyphae, quite distinct from other hyphomycetes (Girlanda et al., 2002). This fungus, called Stagonospora-like before its official naming (Farr et al., 1998), has been reported on muskmelon (C. melo) roots in Guatemala, Honduras, Spain, and frequently in the U.S. (Brunton and Miller, 1997a,b; Armengol et al., 2003). In northern California, this fungus was frequently isolated from melon roots with dry-corky symptoms before its of a root rot (Aegerter et al., 2000). In a survey in South Texas, a fungus was frequently isolated from melon roots with dry-corky symptoms before its of a root rot (Miller et al., 1996).

The Indiana isolates of R. vagum may represent a population that is specifically adapted to watermelon. Previously, this fungus was isolated commonly from diseased roots of cantaloupe (C. melo and its varieties), and its pathogenicity to muskmelon was demonstrated in the southern U.S. (Biernacki and Bruton, 2001). In the greenhouse studies of this current study, no root necrosis was detected on muskmelon plants that had been included as controls, only some nematode-induced galling was observed in contrast to the gall-free watermelon plants (data not shown). Lack of symptoms on muskmelon roots in experiments of this project, and absence of necrosis or other symptoms on muskmelon plants grown in fields that are affected with MWVD when watermelon is grown may indicate a host-specific population or physiological race of R. vagum in Indiana. In numerous pathogenicity tests with Indiana isolates of R. vagum grown on millet seeds, watermelon plants did not exhibit the typical root symptoms of MWVD seen in the field; only in one experiment was the fungus detected by PCR in fumigated and re-infested microplots (Westphal, unpublished). This possibly indicates that the re-infestation was not effective, because the 10% MWVD soil-amended controls had the typical symptoms in these tests. Lack of symptom expression has been observed with this pathogen in other trials (Chilosi et al., 2008) and indicated that R. vagum may be a weak pathogen. Possibly, the efficiency of the artificial inoculations was much lower and did not lead to disease compared to infections from infested soil. We hypothesize that R. vagum may be a contributor to MWVD in southwestern Indiana, but that other factors in addition to the fungus may be necessary to increase disease severity to detectable levels on watermelon plants.

The PCR primers developed here provide an additional tool for detection and identification of R. vagum. These primers are specific for an intron near the 3′ end of the 18S rRNA gene and complement primers in the ITS1 and ITS2 regions that were published previously (Ghignone et al., 2003). New primers were developed because the other ones developed by Ghignone et al. (2003) were not available at the time this work was initiated.

Late-season vine collapse caused by various organisms is a severe problem of watermelon production. Cucurbit production relies on plastic mulch as ground cover for weed suppression and to support higher soil temperatures during the early part of the growing season (Hochmuth et al., 2001). The benefits of this transplanting system are offset by poorer root architecture compared to plants grown under direct-seeded cultivation (D.S. Egel, personal communication). In our experiments, transplanting into fumigated soil did not lead to the late-season disease. The MWVD was clearly a soil-inherent problem.

Earlier reports of the disease pattern in the field were consistent with chemical damage; entire fields were affected irrespective of horizontal soil type patterns or topography. During 2003, the pattern fit more closely to that expected of an infectious disease, occurring first in areas of watermelon fields that were moister or in general more conducive to soil-borne diseases and then spreading over time. In the current study, we did not find evidence for the involvement of herbicide breakdown products as suggested earlier (Makam et al., 2005), since the effects were consistently observed in the field and in the greenhouse without additional herbicide applications to soil. At the same time it was unlikely that the fumigants eliminated breakdown products of the herbicide.

In summary, we conclude that MWVD is a soil-borne disease of watermelon that can be eliminated by soil fumigation with methyl bromide and by heat ≥60 °C. The disease in Indiana is associated with R. vagum but other biological factors may be necessary to cause full symptom development on watermelon crops. Additional experiments are needed to test the hypothesis that R. vagum is a primary or contributing pathogen of MWVD, or whether it is just a secondary colonizer of necrotic tissue.

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