Emergence of Stemphylium Leaf Blight of Onion in New York Associated With Fungicide Resistance

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Abstract

A complex of foliar diseases affects onion production in New York, including Botrytis leaf blight (*Botrytis squamosa*), purple blotch (*Alternaria porri*), Stemphylium leaf blight (SLB; *Stemphylium vesicarium*), and downy mildew (*Peronospora destructor*). Surveys were conducted in 2015 and 2016 to evaluate the cause of severe premature foliar dieback in New York onion fields. SLB was the most prevalent disease among fields with the greatest incidence, surpassing downy mildew, purple blotch, and Botrytis leaf blight. Sequencing of the internal transcribed spacer region of ribosomal DNA and the *glyceraldehyde-3-phosphate dehydrogenase* and *calmodulin* genes identified *S. vesicarium* as the species most commonly associated with SLB. *S. vesicarium* was typically associated with a broad range of necrotic symptoms but, most commonly, dieback of leaf tips and asymmetric lesions that often extended over the entire leaf. Because of the intensive use of fungicides for foliar disease control in onion crops in New York, the sensitivity of *S. vesicarium* populations to various fungicides with site-specific modes of action was evaluated. Sensitivity of *S. vesicarium* isolates collected in 2016 to the quinone outside inhibitor (QoI) fungicide, azoxystrobin, was tested using a conidial germination assay. Isolates representing a broad range of QoI sensitivities were selected

for sequencing of the *cytochrome b* gene to evaluate the presence of point mutations associated with insensitivity to azoxystrobin. The G143A mutation was detected in all 74 *S. vesicarium* isolates with an azoxystrobin-insensitive phenotype (effective concentrations reducing conidial germination by 50%, EC₅₀ = 0.2 to 46.7 µg of active ingredient [a.i.]/ml) and was not detected in all 31 isolates with an azoxystrobin-sensitive phenotype (EC₅₀ = 0.01 to 0.16 µg a.i./ml). The G143A mutation was also associated with insensitivity to another QoI fungicide, pyraclostrobin. Sensitivity to other selected fungicides commonly used in onion production in New York was evaluated using a mycelial growth assay and identified isolates with insensitivity to boscalid, cyprodinil, and pyrimethanil, but not difenoconazole. The frequency of isolates sensitive to iprodione, fluxapyroxad, and fluopyram was high (93.5 to 93.6%). This article discusses the emergence of SLB as dominant in the foliar disease complex affecting onion in New York and the complexities of management posed by resistance to fungicides with different modes of action.

Keywords: disease management, fungi, vegetables, yield loss and economic impacts

New York is the fifth largest producer of onion bulbs in the United States, producing 142,246 tons from 2,832 hectares (U.S. Department of Agriculture National Agricultural Statistics Service 2017). The majority of onions are grown in localized regions on muck (high organic matter) soils, where foliar disease management is a major determinant of onion productivity and profitability (Stivers 2001). The main foliar diseases deleteriously impacting green leaf area include Botrytis leaf blight (*Botrytis squamosa*), purple blotch (*Alternaria porri*), Stemphylium leaf blight (SLB; *Stemphylium vesicarium*), and downy mildew (*Peronospora destructor*) (Stivers 2001). In the United States, SLB epidemics on onion were first reported in Texas in 1976 (Miller et al. 1978). In New York, SLB was first reported in 1985 (Shishkoff and Lorbeer 1989) and occurred in almost all onion fields surveyed throughout New York in 1990, with severe foliar dieback observed in some fields (Lorbeer 1993). However, SLB

epidemics were sporadic in 1991 and 1992 and rare in 1993 (Lorbeer 1993). Therefore, SLB traditionally has been considered of minor importance in the foliar disease complex affecting onion in New York.

SLB lesions are initially small, tan to brown, and may be water soaked, becoming spindle shaped to ovate-elongate or diffusate spots, which often coalesce and extend the length of the leaves (Miller and Schwartz 2008). Lesions turn light brown to tan at the center, becoming dark olive brown to black as profuse production of conidia occurs (Miller and Schwartz 2008). The disease, therefore, contributes to the loss of green leaf area, which may affect bulb size and quality, and results in the inability for plant tops to lodge naturally when the bulbs are fully matured (Brewster 2008).

Little is known of the dominant primary inoculum sources for SLB epidemics in onion. Pseudothecia of the sexual morph (*Pleospora allii*) are produced in SLB lesions on onion leaves, and 3 to 6 months are required for the production of asci containing ascospores (Miller and Schwartz 2008). A single *S. vesicarium* isolate from onion in New York was reported to produce pseudothecia and ascospores in culture (Shishkoff and Lorbeer 1989), suggesting homothallism. Pseudothecia are commonly observed in onion leaves in New York fields, but ascospores have not been observed within these structures (F. S. Hay, unpublished data). Hence, the role of ascospores in the long-distance (between-field) dispersal of the pathogen in New York is unknown. *S. vesicarium* invades dead and dying tissue or healthy leaves directly or through stomata and, under analogous conditions, may be as virulent on onion as *A. porri* (Suheri and Price 2000). Conidia may be disseminated by wind and water splash, and multiple infection cycles can occur within the cropping season, resulting in polycyclic epidemics (Miller and Schwartz 2008). Fungicides are applied regularly and prophylactically in New York for control of the foliar disease complex, with the aim of reducing the rate of epidemic progress (Madden et al. 2007).

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In intensive onion production in muck fields in New York, the majority of the onion acreage is not rotated away from onion (Stivers 2001). This monoculture system of onion production may perpetuate build up of primary inoculum associated with infested onion debris from the previous years. There is also no highly effective resistance to any of the foliar diseases affecting onion within commercially acceptable cultivars for this region (Stivers 2001). Regular application of fungicides is therefore critical for foliar disease management in New York onion fields. Although best management practices for the use of fungicides are routine in this region, such as rotation of fungicides with different single-site modes of action, repeated exposure of foliar pathogens to specific fungicides within and between cropping seasons is inevitable. Fungicides that are central to the New York onion program include quinone outside inhibitors (QoIs) belonging to Fungicide Resistance Action Committee (FRAC) group 11, anilinopyrimidines (FRAC group 9), demethylation inhibitors (DMIs; FRAC group 3), dicarboximides (FRAC group 2), and multiple members of the carboxamide group (FRAC group 7) (Fungicide Resistance Action Committee 2018). QoI fungicides bind to the quinol oxidation site of the cytochrome *bc₁* complex, resulting in ATP inhibition (Bartlett et al. 2002; Fisher and Meunier 2008). The QoI fungicides pose a high risk of resistance development (FRAC 2018), and shifts in sensitivity within a pathogen population are quantitative (McGrath 2004; Reddy 2012). Resistance to QoIs has been attributed to several point mutations in the *cytochrome b* gene (*cytb*), of which the most common are substitution of glycine with alanine (G143A) and phenylalanine with leucine (F129L) (Fernández-Ortuño et al. 2010; Gisi et al. 2002; Kim et al. 2003; Reddy 2012). DMIs have broad efficacy and act by interrupting ergosterol biosynthesis by inhibiting demethylation at the 14- α carbon of lanosterol (Köller and Scheinpflug 1987). Resistance to DMIs is qualitative (McGrath 2004). Common mechanisms of resistance associated with DMIs are point mutations within the target *CYP51A1* gene, leading to reduced fungicide binding affinity (Asai et al. 1999; Stammer et al. 2009); overexpression of ATP-binding cassette and major facilitator superfamily transporters (Hayashi et al. 2002; Hulvey et al. 2012); and overexpression of the *CYP51A1* gene leading to increased copy number (Hamamoto et al. 2000; Schnabel and Jones 2001; Villani et al. 2016). Carboxamide fungicides act on ubiquinone binding sites within the mitochondrial succinate dehydrogenase, leading to respiration inhibition (Sierotzki and Scalliet 2013). Carboxamides used in New York onion production include boscalid, fluopyram, and fluxapyroxad, either on their own or within premixed formulations. Resistance to this group of fungicides is relatively complex and diverse, including amino acid exchanges within the *SdhB*, *SdhC*, or *SdhD* proteins (e.g., Avenot and Michailides 2010; Avenot et al. 2008, 2012; Mallik et al. 2014).

The objectives of this study were as follows: (i) to quantify the prevalence of SLB among fields and the SLB incidence within fields, and the contribution of SLB to the foliar disease complex affecting onion crops in New York; (ii) to identify the *Stemphylium* spp. associated with SLB epidemics in New York; and (iii) to evaluate the sensitivity of *S. vesicarium* isolates to commonly used fungicides in onion production with site-specific modes of action.

Materials and Methods

Prevalence and incidence of foliar diseases affecting onion. Surveys were conducted in New York onion fields to characterize the prevalence of foliar diseases among fields and the disease incidence within fields. Leaves were collected from selected high- and low-input onion fields. High-input fields were broad-acre monocultures that received conventional agricultural practices including regular applications of single-site mode-of-action fungicides (at least six per season). Low-input fields were smaller in size and located on mixed vegetable farms that received less than three applications of either products approved for use in organic production by the Organic Materials Review Institute or multisite fungicides, including biopesticides or copper-based products. In 2015, diseased leaves were collected from 16 July to 28 August from 10 low-input ($n = 283$ leaves) and 22 high-input ($n = 846$ leaves) fields. High-input

fields were located in Yates, Orange, Oswego, Wayne, and Genesee and Orleans (combined to represent the Elba muck production region) counties. Low-input fields were located in Genesee, Ontario, Yates, Schuyler, Clinton, and Allegany counties. In each field, one diseased leaf was collected from each of 10 to 73 plants selected arbitrarily along up to four 10-m-long transects. In 2016, diseased leaves were collected from nine low-input ($n = 155$ leaves) and 13 high-input ($n = 212$ leaves) fields. High-input fields were in Wayne, Genesee, Orleans, Orange, and Oswego counties. Low-input fields were in Genesee, Schuyler, Ontario, Orange, Allegany, and Seneca counties. In each field, one diseased leaf was collected from each of 10 to 28 plants selected in the same manner as those collected in 2015.

Leaves were stored at 4°C for up to 4 days before they were placed in sealed plastic trays containing damp tissue to maintain high humidity to induce sporulation. Trays were incubated at 20°C and observed at 60 $\times$ magnification for fungal growth after 7 days. Emerging mycelia and conidia were transferred to 2% water agar (WA; Hardy Diagnostics, Santa Maria, CA) with a sterile needle, and pure cultures were obtained by hyphal tip or single-spore transfers to potato dextrose agar (PDA) plates. Fungi were identified to genus and species (where possible) based on morphological characteristics. For long-term storage, colonized PDA plugs were placed in sterile distilled water or transferred to PDA slants in test tubes and kept at 4°C.

The prevalence (number of onion fields in which a fungal or oomycete species was observed/total number of fields sampled $\times$ 100) and incidence in each field of each fungal or oomycete species (number of diseased leaves from which a fungal species was isolated/total number of leaf samples from the field $\times$ 100) were calculated for each year. Disease symptoms were also recorded for each field. The effect of low- and high-input production practices on the distribution of disease incidence among fields was tested using an independent-sample *t* test, assuming unequal sample variances (Genstat version 17.2; VSN International, Hemel Hempstead, UK).

Confirmation of *Stemphylium* spp. identity. A subset of *Stemphylium* spp. isolates collected in 2016 ($n = 39$) was used to confirm the species identity of the isolates of this genus. Isolates were grown on PDA at 20°C for 7 days before two to three mycelial plugs of each were transferred to clarified V8 broth amended with 100 μ g of ampicillin/ml (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature on an orbital shaker at 80 rpm. Fungal mycelia were harvested after 7 days, and genomic DNA was extracted with the Wizard Extraction Kit (Promega Corp, Madison, WI) according to the manufacturer's instructions. DNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), diluted to 25 ng/ μ l, and stored in sterile distilled water at -20°C.

Conventional PCR assays were performed to amplify internal transcribed spacer (ITS) region ITS1-5.8s-ITS4 of the ribosomal DNA (rDNA) and the *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*) and *calmodulin* (*cmdA*) genes using primer pairs ITS1/ITS4, GPD1/GPD2 (Berbee et al. 1999), and CaldF1/CaldR1 (Lawrence et al. 2013), respectively. The ITS rDNA region and the *GPD* and *cmdA* genes were selected because they are phylogenetically informative for species resolution within the *Pleospora* clade (Inderbitzin et al. 2009; Puig et al. 2015). Each PCR reaction consisted of 37.75 μ l of water, 5 μ l of 10 $\times$ PCR buffer (New England Biolabs, Ipswich, MA), 1 μ l of dNTPs (10 μ M), 1 μ l of each primer (10 μ M), 0.25 μ l of 5 U/ μ l Taq DNA polymerase (New England Biolabs), and 4 μ l of diluted DNA. The PCR conditions were as follows: an initial denaturation step at 94°C for 3 min; followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 68°C for 1 min; and a final extension for 3 min at 68°C (T100 Thermal Cycler; Bio-Rad, Hercules, CA). The sizes of the PCR amplicons were estimated by comparing band intensity with a 100-bp DNA ladder (New England Biolabs) on a 1% (wt/vol) agarose gel with 1 $\times$ Tris-acetate-EDTA buffer amended with 0.5 $\times$ (vol/vol) GelRed (Biotium, Freemont, CA). Excess nucleotides and primers were removed by treatment with exonuclease and shrimp alkaline phosphatase (Exo-SAP; Thermo Fisher Scientific). PCR

products were sequenced in both directions at the Cornell University Institute of Biotechnology Genomics Facility (Ithaca, NY) using the PCR primers.

Forward and reverse sequences for each PCR product were aligned in Geneious version R11.1 (<https://www.geneious.com/>) to obtain a consensus sequence for the full length of the product. Initial analyses were done by evaluating similarity within the ITS rDNA region to sequences from reference isolates in GenBank by searching the BLAST nucleotide database. Additional analyses were conducted with isolates that matched with the genus *Stemphylium* based on the BLAST search.

For phylogenetic analyses, the sequences were aligned using MAFFT (Katoh et al. 2002) in Geneious version R11.1. Sequences of the type isolates were retrieved from GenBank and included in the analyses. *A. alternata* isolate GV14-634a1 was used as an out-group. Ambiguously and poorly aligned regions were removed with Gblocks (Castresana 2000). The resulting sequences from different loci were concatenated, and the data were subjected to phylogenetic analysis using a maximum likelihood algorithm implemented in MEGA 7 (Kumar et al. 2016). The maximum likelihood tree was based on a Kimura two-parameter model (Kimura 1980) with a discrete gamma distribution (K+G) to model evolutionary differences among nucleotide sites. To assess the relative stability of the branches, 1,000 bootstrap replicates were conducted.

Sensitivity to QoI fungicides, azoxystrobin and pyraclostrobin. Sensitivity to azoxystrobin was assessed using a conidial germination assay for 105 *S. vesicarium* isolates collected from across New York in this survey. Sixty isolates were from 12 high-input fields in Genesee/Orleans, Oswego, and Wayne counties; 45 isolates were from nine low-input fields in Cattaraugus, Genesee/Orleans, Ontario, Orange, Schuyler, and Seneca counties. Technical-grade azoxystrobin (100 mg; Sigma-Aldrich) was dissolved in warm acetone, made up to 5 ml of a stock solution (20,000 µg of active ingredient [a.i.]/ml), and used to make a dilution series in sterile distilled water with four final concentrations (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}). Aliquots from each dilution of the series were added to 3% WA at 60°C to achieve concentrations of 0.001, 0.01, 0.5, 10, 25, and 100 µg of a.i. azoxystrobin/ml. Salicylhydroxyamic acid (SHAM; Sigma-Aldrich) was added to each fungicide-amended plate of agar medium to inhibit the alternative respiration pathway (Wood and Hollomon 2003). SHAM (0.2 g) was dissolved in warm methanol to prepare 5 ml of 40,000 µg of a.i./ml of stock solution. An aliquot (500 µl) was added to 200 ml of 3% WA to make a final concentration of 100 µg of a.i./ml in each fungicide-amended agar plate and a treatment of agar medium with the SHAM-only control.

After the range of azoxystrobin sensitivities of the *S. vesicarium* isolates ($n = 38$) was determined, they were also tested for sensitivity to another QoI used in New York onion production, pyraclostrobin. A stock solution of 0.2 g of SHAM was first dissolved in 2.5 ml of warm methanol (80,000 µg of a.i./ml). Technical-grade pyraclostrobin (100 mg; Sigma-Aldrich) was dissolved in 2.5 ml of acetone (40,000 µg of a.i./ml) and used to prepare a 10-fold dilution series. Appropriate amounts of pyraclostrobin and SHAM were added to 3% WA to achieve concentrations of 0.005, 0.05, 0.5, 5, and 50 µg of a.i./ml (including SHAM at 100 µg of a.i./ml). A control treatment of medium with SHAM only and a nonamended control treatment with no fungicide or SHAM were also included. Plates were dried in a laminar flow hood at room temperature for 2 h and stored at 20°C in the dark for no longer than 10 days before use. The final concentrations of the solvents used to evaluate azoxystrobin and pyraclostrobin sensitivity in the agar medium did not exceed 1% (vol/vol).

Conidia used in QoI sensitivity assays were generated by culturing *S. vesicarium* isolates on clarified V8 agar (Secor and Rivera 2012) for 14 days under 118-volt/60-Hz fluorescent lights with a 12-h photoperiod/day at 20°C. Plates were each flooded with sterile distilled water, and conidia were detached from the mycelium using a sterile spatula, placed in microcentrifuge tubes in 1-ml quantities, and vortexed at maximum speed for 10 s. Each resultant conidial suspension (30 µl) was then placed onto a 15-mm³ block of 3% WA

mounted on a microscope slide for each of three replicate blocks of agar with each fungicide concentrations, SHAM control treatment, and nonamended control treatment per isolate. Agar blocks were each covered with a glass coverslip, placed in a sealed container in the dark for 7 h, and then maintained at 5°C overnight prior to evaluation of conidial germination at 100 to 200× magnification. Fifty conidia were evaluated from each slide, and the numbers of germinated and nongerminated conidia were recorded. Conidia were considered germinated if the length of any single germ tube was greater than the length of the conidium. The effective fungicide concentration that caused 50% inhibition (EC₅₀) of conidial germination compared with the nonamended control treatment was calculated by probit analysis (PROC PROBIT; Statistical Analysis System SAS version 9.4). The relationship between sensitivity to azoxystrobin and pyraclostrobin within the subset of isolates was quantified using linear regression analysis (Genstat version 17.2).

Sequencing of *cytb*. For 105 *S. vesicarium* isolates, a portion of the *cytb* gene reported to contain mutations associated with resistance to QoIs was amplified with primers SVCBFw-SVCBRe (Alberoni et al. 2010) or KES1999 and 2000 (Graf et al. 2016). For each PCR reaction, the final volume (50 µl) contained 37.84 µl of sterile type I water, 5 µl of 10× PCR buffer (New England Biolabs), 1 µl of dNTPs (10 µM), 1 µl of each primer (10 µM), 0.16 µl of 5 U/µl Taq DNA polymerase (New England Biolabs), and 4 µl of DNA. PCR conditions consisted of an initial denaturation at 95°C for 5 min; followed by 36 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 5 min. The size of each PCR amplicon was estimated visually by comparing band intensity with a 100-bp DNA ladder on a 1% (wt/vol) agarose electrophoresis gel in Tris-acetate-EDTA amended with 0.5× (vol/vol) GelRed. Excess nucleotides and primers were removed with an E.Z.N.A. Cycle Pure Kit and eluted in sterile distilled water according to the manufacturer's instructions.

Bidirectional sequencing of the purified PCR products using the same primers was conducted by the Cornell University Institute of Biotechnology Genomics Facility. Sequences were searched manually for the presence of the G143A (G to C change) and F129L mutations (C to A change). The presence or absence of point mutations associated with QoI resistance guided the establishment of an EC₅₀ threshold characterizing resistant and sensitive isolates for azoxystrobin.

Sensitivity to additional fungicides. Mycelial growth assays were used to quantify the sensitivity of selected *S. vesicarium* isolates to other fungicides commonly used in New York onion production. In 2016, 21 *S. vesicarium* isolates were obtained from eight low-input fields in Allegany, Genesee/Orleans, Ontario, Orange, Schuyler, and Seneca counties; and 25 isolates were obtained from 12 high-input fields in Genesee/Orleans, Oswego, and Wayne counties. Active ingredients were technical-grade formulations (Sigma-Aldrich) of the following: iprodione (dicarboximide; FRAC group 2); difenoconazole (DMI; FRAC group 3); boscalid, fluopyram, and fluxapyroxad (carboxamides; FRAC group 7); cyprodinil and pyrimethanil (anilinopyrimidines; FRAC group 9); and fludioxonil (phenylpyrrole; FRAC group 12).

Mycelial growth inhibition was tested on minimal medium (Myresiotis et al. 2008) poured into 150-mm-diameter × 15-mm Petri plates with a Wheaton OmniSpense Elite pump (DWK Life Sciences, Millville, NJ), to a height of 3 mm in each plate. Sensitivity testing was conducted using an Eddy Jet 2 spiral plater (Neutec Group Inc., Farmingdale, NY). Each fungicide was dispensed at the M3K EXP SLOW setting, resulting in 54.3 µl of a.i./ml of stock solution/140-mm-diameter Petri plate. Technical-grade aliquots of each fungicide were first dissolved in acetone. Stock concentrations of fluopyram (6,400 µg of a.i./ml), cyprodinil (6,300 µg of a.i./ml), iprodione (1,300 µg of a.i./ml), fludioxonil (6,300 µg of a.i./ml), difenoconazole (1,300 µg of a.i./ml), pyrimethanil (6,400 µg of a.i./ml), boscalid (6,300 µg of a.i./ml), and fluxapyroxad (2,000 µg of a.i./ml) were utilized to achieve concentration ranges of 0.50 to 56.53 µg of fluopyram/ml, 0.5 to 56.5 µg of cyprodinil/ml,

0.1 to 11.6 µg of iprodione/ml, 0.5 to 56.5 µg of fludioxonil/ml, 0.1 to 11.4 µg of difenoconazole/ml, 0.51 to 56.99 µg of pyrimethanil/ml, 0.5 to 56.22 µg of boscalid/ml, and 0.16 to 17.7 µg of fluxapyroxad/ml at distances ranging from 20 to 64 mm from the center of each plate. After pouring, agar plates were dried in a laminar flow cabinet for 4 h, stored in the dark at 20°C, and used within 7 days. Immediately prior to use, a 20-mm plug of agar medium was removed from the center of the plate with a sterile cork borer.

To prepare inoculum of each *S. vesicarium* isolate for the mycelial growth assay, each isolate was grown on PDA at 20°C for 10 days. A 10-mm³ colonized agar block was then cut from the edge of the colony, macerated in sterile distilled water, and spread over a PDA plate. Wooden sandwich picks (Diamond, Jarden Brand, IN) were cut to a length of 64 mm, autoclaved in V8 broth, and dried in a laminar flow cabinet. Picks ($n = 15$ to 20) were laid across the surface of the PDA plates, each containing a lawn of *S. vesicarium* mycelia. Plates were sealed and incubated at 20°C for 10 days in the dark for fungal colonization of the picks.

For each fungicide, four colonized wooden picks (one per fungal isolate) were positioned on the agar medium in a radial pattern at 90° angles from each other. For each isolate, colonized picks were placed on each of three replicate plates of agar medium for each fungicide-amended and nonamended control plate. Plates were incubated in the dark at 20°C for 5 days. For each isolate, the width of mycelial growth across the three picks on the control plate was averaged as the baseline for growth in the absence of fungicide. For each isolate on a fungicide-amended plate, the point along the sandwich pick nearest the center of the plate at which the width of mycelial growth was 50% that of the mean for the control plates was marked on the underside of the plate. The distance from the center of the plate to this point was recorded, and the concentration of fungicide at that point was calculated using the ECX software package (Torres-Londoño et al. 2016) with R statistical software version 3.5.1 (R Core Team 2017). For those isolates for which the width of mycelial growth was not <50% that of the mean growth width for the nonamended control plates along the length of the pick, the EC₅₀ was considered greater than the maximum fungicide concentration evaluated on a 20-mm radius. Conversely, for isolates for which the width of mycelial growth was <50% of the mean width for the nonamended

control plates across the length of the pick, the EC₅₀ was considered less than the minimum fungicide concentration on a 64-mm radius.

Results

Prevalence and incidence of foliar diseases affecting onion in New York. *S. vesicarium* was the most prevalent fungus associated with foliar disease in onion, occurring in all fields in 2015 and 2016 (Table 1). *S. vesicarium* was isolated from a broad range of disease symptoms, most commonly dieback from leaf tips that initially was <1 cm of leaf length (Fig. 1A) but later spread down the leaf to cause lesions ≥30 cm long and usually encompassing the leaf asymmetrically (Fig. 1B). Lesions were covered profusely in *S. vesicarium* conidia. Pseudothecia were also commonly observed and tentatively identified as *Pleospora* spp. pseudothecia because of their presence within lesions in association with conidia of *S. vesicarium*, although ascospores were not observed. Other known or potential pathogens of onion, including *A. porri* (purple blotch), *P. destructor*, *Colletotrichum* spp., and *Botrytis* spp., were detected at lower prevalences (≤9.1%). The prevalence of fungal and oomycete species associated with foliar dieback (*A. porri*, *P. destructor*, *Botrytis* spp., and *Colletotrichum* spp.) was substantially greater in low-input than high-input fields in both years (Table 1).

The incidence of onion leaves with SLB was high in both years, ranging from 77.4 to 86.2% in low-input fields and 85.7 to 88.2% in high-input fields (Table 2). The incidence of SLB was significantly greater in high-input than low-input fields in 2016, and the incidence of *Pleospora* spp. was significantly higher in high-input fields in both years. The incidence of leaves with purple blotch and disease symptoms associated with other *Alternaria* spp. was significantly greater in low-input than high-input fields in both years. The incidence of leaves with downy mildew was significantly greater in low-input versus high-input fields in 2015 but incidence was low (≤1.3%) in both types of fields in 2016. The incidence of foliar disease associated with *Botrytis* spp. was ≤4.5% in both years. The incidence of *Colletotrichum* spp. associated with foliar disease was significantly greater in low-input than high-input fields in both years and was ≤8.4% (Table 2). Additional fungal species found on leaves at limited incidence and that were assumed saprophytic included *Helminthosporium* spp., *Humicola* spp., *Fusarium* spp., and *Ulocladium* spp. (data not shown). A limited incidence of *Cladosporium* spp. was also found.

The small number of fields sampled within counties was insufficient to make statistical inferences on geographical differences in SLB prevalence and incidence among counties. However, the incidence of leaves with SLB was ≥72% from all counties in 2015, with the exception of Genesee and Orleans. SLB incidence was ≥69% in all counties in 2016, with the exception of Schuyler (Supplementary Table S1).

Identification of *Stemphylium* spp. PCR amplification of the ITS rDNA region and the *GPD* and *cmdA* genes resulted in sequences of 581, 613, and 742 bp, respectively. Sequences were deposited in GenBank with accession numbers MK652792 to MK652830 (ITS rDNA), MK675668 to MK675706 (*cmdA*), and MK675707 to MK675745 (*GPD*). Initial BLAST analyses using sequences of the ITS rDNA region identified 38 isolates as *S. vesicarium* with 99.8 to 100% similarity and one isolate as *S. vesicarium* with 98.4% similarity. However, phylogenetic analyses with the ITS rDNA and including *GPD* and *cmdA* loci confirmed that 38 isolates grouped together with the matching sequences of the *S. vesicarium* reference isolate CBS311.92, and the remaining isolate grouped with sequences of a *S. beticola* reference isolate CBS 141024 (Fig. 2). The *S. vesicarium* group was further split into two by two single nucleotide polymorphisms within the *cmdA* gene.

Sensitivity to QoI fungicides, azoxystrobin and pyraclostrobin. The inclusion of SHAM at 100 µg of a.i./ml had no significant effect on *S. vesicarium* conidial germination ($P = 0.432$; data not shown). Insensitivity to azoxystrobin was defined operationally as EC₅₀ values >0.2 µg of a.i./ml, and the G143A mutation was present in 53 of 60 isolates collected from all of the high-input fields and in 21 of 45 isolates collected from five of the nine low-input fields. Isolates that

Table 1. Prevalence of fungal and oomycete species associated with foliar disease of onion in low-input and high-input fields in New York in 2015 and 2016

Fungal species	Prevalence (% of fields with fungal species)			
	2015		2016	
	Low input ^a	High input	Low input	High input
<i>Stemphylium vesicarium</i>	100	100	100	100
<i>Pleospora</i> spp. ^b	80	95.5	55.6	84.6
<i>Alternaria porri</i>	50	0	33.3	7.7
Other <i>Alternaria</i> spp.	80	59.1	100	61.5
<i>Peronospora destructor</i>	60	4.5	11.1	0
<i>Botrytis</i> spp.	30	9.1	33.3	7.7
<i>Colletotrichum</i> spp.	30	4.5	44.4	0
Number of fields ^c	10	22	9	13

^a Low-input fields were located on mixed vegetable farms and received less than three applications of either Organic Materials Review Institute-approved or multisite fungicides with multisite modes of action, including biopesticides and/or copper-based products. High-input fields were broad-acre monocultures that received conventional agricultural practices, including regular applications of fungicides with single-site mode-of-action fungicides (at least six per season).

^b *Pleospora* spp. pseudothecia (presumably classifiable as *P. allii*) were observed in diseased tissue but ascospores were not observed in the pseudothecia.

^c Total number of fields included in the survey. Refer to the main text for details on field locations.

were insensitive to azoxystrobin were broadly distributed (Fig. 3). The G143A mutation was detected in 74 *S. vesicarium* isolates that were less sensitive to azoxystrobin, with EC₅₀ values ranging from 0.23 to 46.39 µg of a.i./ml, and was not detected in 31 isolates with EC₅₀ values at least 10-fold less, ranging from 0.01 to 0.16 µg of a.i./ml (Fig. 3). There was a significant ($P < 0.001$) linear relationship between the log EC₅₀ for azoxystrobin and pyraclostrobin, with EC₅₀ values for pyraclostrobin generally greater than those for azoxystrobin (Fig. 4). The G143A mutation was not detected in eight isolates with EC₅₀ values ranging from 0.006 to 0.088 µg of a.i. pyraclostrobin/ml and was present in 30 isolates with EC₅₀ values from 0.72 to 94.03 µg of a.i. pyraclostrobin/ml. Sequences of *cytb* were deposited in GenBank (MK717243 to MK717298).

Sensitivity to selected fungicides in other FRAC groups. A majority of the 46 *S. vesicarium* isolates that were collected in 2016 and tested for sensitivity to difenoconazole, fludioxonil, and iprodione appeared highly sensitive, with 97.8, 95.7, and 93.5% of the isolates showing EC₅₀ values <0.5 µg of a.i./ml, respectively (Fig. 5).

There was evidence of insensitivity for the anilinopyrimidines, as 23.9 and 32.6% of the 46 *S. vesicarium* isolates had EC₅₀ values > 10 µg of a.i./ml for cyprodinil and pyrimethanil, respectively (Fig. 5). Almost all 46 *S. vesicarium* isolates appeared sensitive to fluopyram and fluxapyroxad, as 93.6 and 93.5% had EC₅₀ values < 1 µg of a.i./ml, respectively (Fig. 5). Conversely, a large proportion

of the 46 *S. vesicarium* isolates exhibited insensitivity to boscalid, as only 30.4% of the isolates had EC₅₀ values < 1 µg of a.i./ml and 50% had EC₅₀ values > 10 µg of a.i./ml (Fig. 5).

Differences in sensitivity to some of the fungicides tested were identified between *S. vesicarium* isolates obtained from low- and high-input fields (Table 3). For cyprodinil, 14 of 23 isolates from high-input fields had EC₅₀ values > 5 µg of a.i./ml. Isolates that were insensitive to cyprodinil were from high-input fields in Genesee/Orleans, Oswego, and Wayne counties. Conversely, all isolates from low-input fields were sensitive to cyprodinil (Table 3). For pyrimethanil, 1 of 19 isolates and 16 of 23 isolates from low- and high-input fields, respectively, had EC₅₀ values > 5 µg of a.i./ml. Isolates that were insensitive to pyrimethanil were from Seneca, Genesee/Orleans, Oswego, and Wayne counties. For boscalid, 8 of 21 and 18 of 23 isolates from low-input and high-input fields, respectively, had EC₅₀ values > 5 µg of a.i./ml and were obtained from Allegany, Genesee/Orleans, Schuyler, Seneca, Oswego, and Wayne counties. One isolate from a low-input field in Allegany County was insensitive to fluxapyroxad (EC₅₀ > 5 µg of a.i./ml) (Table 3).

Discussion

SLB caused by *S. vesicarium* was the dominant disease affecting foliar health in onion crops surveyed across New York in 2015 and 2016, providing insights into the previously unexplained increase

Fig. 1. A, Stemphylium leaf blight (SLB) lesion on an onion leaf. B, An SLB disease focus in a commercial onion field in New York.

Table 2. Effect of production practice (low input compared with high input) on the incidence of fungi and oomycetes associated with foliar disease in onion in New York in 2015 and 2016

Fungal species	Incidence (%)					
	2015			2016		
	Low input (n = 283) ^a	High input (n = 846)	t (P value) ^b	Low input (n = 155)	High input (n = 212)	t (P value)
<i>Stemphylium vesicarium</i>	86.2	85.7	0.02 (0.887)	77.4	88.2	6.9 (0.009)
<i>Pleospora allii</i> ^c	35.6	62.1	59.6 (<0.001)	35.5	53.8	11.3 (<0.001)
<i>Alternaria porri</i>	12.0	0	105.2 (<0.001)	25.8	0.5	55.4 (<0.001)
<i>Alternaria</i> spp.	57.6	5.7	384.1 (<0.001)	85.2	42.0	67.9 (<0.001)
<i>Peronospora destructor</i>	11.3	0.1	93.7 (<0.001)	1.3	0	–
<i>Botrytis</i> spp.	2.5	0.4	–	4.5	0.5	–
<i>Colletotrichum</i> spp.	8.1	0.5	52.5 (<0.001)	8.4	0	16.06 (<0.001)

^a n = total number of leaves sampled.

^b t-test (probability values). Dashes indicate that differences were not tested as a result of inadequate disease incidence.

^c *Pleospora* spp. pseudothecia (presumably classifiable as *P. allii*) were observed in diseased tissue but ascospores were not observed in the pseudothecia.

in foliar disease severity of concern in conventional production on muck soils in this region. Foliar disease severity continued to be high in subsequent seasons (2017 and 2018). The result was similar to recent findings in onion muck production in Ontario, Canada, where SLB occurred sporadically in 2008, but by 2010 was in all fields scouted at high incidences and was associated anecdotally with yield

losses (M. R. McDonald, University of Guelph, Ontario, Canada, *personal communication*). SLB is also of increasing importance in asparagus production in Ontario, Canada (Foster et al. 2019). The effect of SLB on onion yields in New York is likely substantial. SLB has been associated with severe disease outbreaks, leading to premature and almost complete loss of foliage in New York onion fields.

Fig. 2. Phylogenetic relationships between isolates of *Stemphylium vesicarium* obtained from diseased onion in New York. The tree was inferred by using the maximum likelihood method based on the Kimura two-parameter model along with a discrete gamma distribution to model evolutionary rate differences among sites (K2+G). The tree was based on combined sequences from the internal transcribed spacer region and the *glyceraldehyde-3-phosphate dehydrogenase* and *calmodulin* genes, which resulted in 1,498 positions in the final data set. Bootstrap values are given when above 70% (1,000 replicates). *Alternaria alternata* was used as an outgroup. The scale bar shows the number of substitutions per site.

Artificial defoliation of onion plants of as little as 20% during bulb growth can lead to yield losses between 10 and 15% (Muro et al. 1998). Clough (2004) demonstrated that artificial defoliation of onions by 50, 75, or 100% at the 12-leaf growth stage resulted in marketable yield losses of 74.5, 67.6, and 54.8% compared with nondefoliated plants, respectively, and significantly reduced bulb weights by 24.4 to 37.8%. In two field trials in New York, SLB control with applications of selected fungicides led to increases of 32.5 to 39.6% in the 100-bulb weight over that of bulbs harvested from non-treated control plots (Hoepting 2018a, b) and to a 28.7% increase in the number of jumbo-grade bulbs (>7.62 cm in diameter) (Hoepting 2018b). Moreover, severe leaf senescence associated with SLB may have deleterious effects on the ability of bulbs to be stored after harvest. Application of the sprout inhibitor, maleic hydrazide, close to harvest requires the presence of five to eight green leaves to allow sufficient translocation to the bulb (Brewster 2008).

Although *S. vesicarium* was shown to be the dominant species associated with SLB in onion in New York in this study, other *Stemphylium* spp. have been associated with foliar diseases of allium

Fig. 3. Sensitivity of *Stemphylium vesicarium* isolates from foliar disease of onion in New York in 2016 to azoxystrobin (effective concentration required to reduce conidial germination by 50% [EC_{50}], in μg of active ingredient/ml). Triangles and circles denote *S. vesicarium* isolates with and without the G143A mutation within the cytochrome *b* gene, respectively. Locations 1 to 4, Genesee County; 5 to 9, Oswego County; 10 to 12, Wayne County; 13, Cattaraugus County; 14 and 15, Genesee County; 16 and 17, Ontario County; 18 and 19, Orange County; 20, Schuyler County; and 21, Seneca County. Locations 1 to 12 are high-input fields. Locations 13 to 21 are low-input fields.

Fig. 4. Relationship between the effective concentration to reduce conidial germination by 50% (EC_{50}) for azoxystrobin and pyraclostrobin for *Stemphylium vesicarium* isolates ($n = 38$) collected from diseased onion leaves in New York. SEEy = standard error of the estimate of y .

crops, including *S. eturmiunum* on onion in Puerto Rico (Fernández and Rivera-Vargas 2006) and *S. solani* on garlic in China (Zheng et al. 2008, 2010). The saprophytic species *S. botryosum* is also commonly found on onion leaves and has been reported in New York (Shishkoff and Lorbeer 1989). Multilocus sequencing of a subset of *Stemphylium* spp. isolates associated with SLB epidemics in New York in this study identified all but one of the *Stemphylium* isolates as *S. vesicarium*. A single isolate from an organic farm in Orange County grouped with a reference isolate of *S. beticola*. Attempts to infect onion with this isolate in a greenhouse were not successful (F. S. Hay and S. Sharma, unpublished data), suggesting a saprophytic lifestyle of that species on onion.

Insensitivity within the *S. vesicarium* populations from onion in New York to fungicides in multiple FRAC groups was detected using in vitro assays. Resistance to QoI fungicides has been reported for *S. vesicarium* populations from pear orchards in Italy, where a discriminate dose of 0.5 $\mu\text{g}/\text{ml}$ was established, above and below which isolates were considered insensitive or sensitive, respectively (Alberoni et al. 2010). In this onion study, a slightly lower discriminatory dose of 0.2 μg of a.i. azoxystrobin/ml was identified, as the G143A mutation was present in isolates with EC_{50} values ranging from 0.23 to 46.39 μg of a.i./ml and absent when EC_{50} values ranged from 0.01 to 0.16 μg of a.i./ml. Azoxystrobin (formulated as Quadris; Syngenta Corporation) was first labeled for foliar disease control in onion in New York in 2003. The detection of isolates insensitive to QoI fungicides in vitro in this study may explain poor foliar disease control achieved with QoI fungicides in various field trials in New York (Hoepting 2017). Isolates of *S. vesicarium* insensitive to azoxystrobin occurred at much greater frequency in high-input than low-input onion fields in this study. However, insensitive isolates were detected in several low-input fields despite the absence of azoxystrobin applications in these low-input fields and, usually, spatial separation of these fields from high-input fields. This may have resulted from inoculum movement over long distances, on seed or transplants. *S. vesicarium* has been shown to be seed transmitted in onion (Aveling et al. 1993), which was suspected, although not confirmed, as a potential inoculum source associated with the SLB epidemic in onion crops in New York in 1990 (Lorbeer 1993). Ascospores may be an additional means of dispersal between fields but mature pseudothecia with ascospores have not been observed in New York onion fields (F. S. Hay, unpublished data).

There was differential sensitivity within the *S. vesicarium* isolates to the selected carboxamide fungicides tested, with a high frequency of insensitivity to boscalid and a high level of sensitivity to

Fig. 5. Frequency of *Stemphylium vesicarium* isolates collected from diseased onion leaves in New York in various ranges of effective concentrations to reduce mycelial growth by 50% for the fungicides difenoconazole, fludioxonil, iprodione, cyprodinil, pyrimethanil, fluopyram, boscalid, and fluxapyroxad.

fluopyram and fluxapyroxad. In the absence of baseline data from a known sensitive population, it is difficult to elucidate whether the insensitivity to boscalid reflected naturally occurring sensitivity in the population or occurred as a result of selection pressure from fungicide use. *S. vesicarium* populations in New York onion crops have been exposed to boscalid as a result of use of this fungicide in onion production in the commercial formulations Pristine (boscalid plus pyraclostrobin; BASF Corporation) and Endura (boscalid; BASF Corporation) since 2005. In contrast, the active ingredient, fluxapyroxad, in the product Merivon (fluxapyroxad plus pyraclostrobin; BASF Corporation) was first labeled for use in onion crops in New York in 2015, and the active ingredient fluopyram in Luna Tranquility (fluopyram plus pyrimethanil; Bayer Crop Science) was only registered in onion crops in New York in 2016. Therefore, there has been a substantially longer potential period of exposure of the *S. vesicarium* isolates to boscalid than fluopyram or fluxapyroxad. In a New York onion field trial in 2016, Endura was highly efficacious for SLB control (Hoepting 2017), but in a 2017 field trial, the product was significantly less efficacious than products containing fluopyram or fluxapyroxad (Hoepting 2018b). Although isolates insensitive to boscalid were in low-input fields in this survey, they were less frequent than in high-input fields, providing further evidence that fungicide use patterns may have contributed to the development of insensitivity to boscalid within the *S. vesicarium* population. Currently, there is no information on mutations within the *Sdh* genes in *S. vesicarium* that may be responsible for conferring resistance to carboxamide fungicides (Sierotzki and Scalliet 2013).

Some of the New York onion *S. vesicarium* isolates were insensitive to cyprodinil and pyrimethanil. Fungicides containing these active ingredients have been used for several years within onion production in New York. For example, Scala (pyrimethanil; Bayer Crop Science), Switch (fludioxonil and cyprodinil; Syngenta Corporation), Inspire Super (cyprodinil and difenoconazole; Syngenta Corporation), and Luna Tranquility (fluopyram and pyrimethanil; Bayer Crop Science) were labeled in 2005, 2006, 2012, and 2016, respectively, for use in onion crops in New York. There is also no information on the genetic basis of resistance to anilinopyrimidine fungicides in *S. vesicarium*. In the absence of baseline data from an unexposed population, it is not possible to elucidate whether these results reflect naturally occurring incidences of insensitivity in the fungal population or result from selection through fungicide exposure. However,

the presence of isolates with EC₅₀ values > 10 µg of a.i./ml only in the high-input fields surveyed suggested that fungicide use may have contributed to selection for insensitivity to cyprodinil and pyrimethanil. The presence of insensitivity in the onion *S. vesicarium* population also concurs with poor or only moderate efficacy of SLB control in field trials using products containing cyprodinil and pyrimethanil (Hoepting 2016, 2017, 2018b).

Resistance to the dicarboximide fungicide procymidone (FRAC group 2) and associated control failures in field trials have been reported for brown spot caused by *S. vesicarium* in pear orchards in Italy, along with cross-resistance to iprodione (Alberoni et al. 2005; Collina et al. 2002). In the study by Alberoni et al. (2005), *S. vesicarium* isolates were classified as follows: (i) sensitive (EC₅₀ values < 1 µg of a.i./ml for procymidone and iprodione), (ii) resistant R₁ (251 to 9,690 µg of a.i. procymidone/ml and 3 to 29.84 µg of a.i. iprodione/ml), or (iii) resistant R₂ (1,370 to 11,500 µg of a.i. procymidone/ml and 64.8 to 2,120 µg of a.i. iprodione/ml). In this study, 93.5% of isolates had EC₅₀ values < 1 µg of a.i. iprodione/ml, suggesting that the population was highly sensitive despite Rovral (FMC Corporation) being used in New York onion production since 2003. However, 2.2% of isolates of *S. vesicarium* sampled from onion fields in New York had EC₅₀ values between 5 and 10 µg of a.i. iprodione/ml, suggesting a low incidence of insensitive isolates. Insensitivity to the DMI difenoconazole was not detected despite the use of this fungicide in New York onion crops since 2010 in the pre-mixed formulations Quadris Top (azoxystrobin plus difenoconazole; Syngenta Corporation) and Inspire Super (cyprodinil and difenoconazole; Syngenta Corporation).

The detection of widespread insensitivity of *S. vesicarium* in onion crops in New York to QoI (azoxystrobin and pyraclostrobin), carboxamide (boscalid), and anilinopyrimidine (cyprodinil and pyrimethanil) fungicides representing three FRAC groups is concerning and represents a substantial threat to the durability of fungicide-based control of this foliar disease in onion in New York. The insensitivity profiles of isolates quantified in vitro aligns with efficacy results from field trials (Hoepting 2017, 2018a, b). Currently, difenoconazole, fluopyram, and fluxapyroxad are the most efficacious single-site mode-of-action fungicides for SLB control, and iprodione, cyprodinil, and pyrimethanil offer only moderate efficacy (Hoepting 2016, 2017, 2018a, b). These findings substantially limit rotational options for best management practices for fungicide use and rotation.

Table 3. Frequency (number of isolates) in the range of sensitivity of *Stemphylium vesicarium* isolates associated with foliar disease in high- and low-input onion fields across New York in 2016 to selected fungicides based on the effective concentration required to cause 50% inhibition of mycelial growth (EC₅₀) calculated using a spiral plater assay

Fungicide (FRAC group) ^a	Onion production system ^b	Fungicide concentration range at which EC ₅₀ was detected (µg of a.i./ml)					
		<0.5	0.5 to 1.0	1.0 to 5	5.1 to 10.0	10.1 to 50.0	>50.0
Difenoconazole (3)	Low	21	0	0	0	0	0
	High	24	0	1	0	0	0
Fludioxonil (12)	Low	21	0	0	0	0	0
	High	23	0	2	0	0	0
Iprodione (2)	Low	20	0	1	0	0	0
	High	23	0	1	1	0	0
Cyprodinil (9)	Low	21	0	0	0	0	0
	High	8	0	3	3	8	3
Pyrimethanil (9)	Low	19	0	1	1	0	0
	High	6	0	3	1	5	10
Fluopyram (7)	Low	10	8	3	0	0	0
	High	16	9	0	0	0	0
Boscalid (7)	Low	9	1	1	2	4	4
	High	2	2	3	3	9	6
Fluxapyroxad (7)	Low	18	1	1	1	0	0
	High	21	3	1	0	0	0

^a FRAC = Fungicide Resistance Action Committee.

^b Low-input fields were located on mixed vegetable farms and received less than three applications of either Organic Materials Review Institute-approved or multisite fungicides, including biopesticides and/or copper-based products. Twenty-one *S. vesicarium* isolates from low-input fields were tested for sensitivity to each fungicide. High-input fields were broad-acre monocultures that received conventional agricultural practices, including regular applications of single-site mode-of-action fungicides (at least six per season). Twenty-five *S. vesicarium* isolates were tested for sensitivity to each fungicide from these high-input fields.

The detection of low incidences of *S. vesicarium* isolates with insensitivity to difenoconazole, iprodione, fluxapyroxad, and fluopyram is also concerning, as it suggests the potential for *S. vesicarium* to develop resistance to these fungicides and highlights the need for strict adherence to resistance management strategies to preserve their efficacy. Fludioxonil has been registered for use in onion crops in New York since 2006 but only within a premixed formulation with cyprodinil (Switch; Syngenta Corporation). Although these results demonstrated that fludioxonil was able to inhibit mycelial growth of *S. vesicarium* at low concentrations in vitro, field trials have only demonstrated poor SLB control from Switch (Hoepting 2017), suggesting that other factors may be important in the performance of this product against SLB, such as adjuvant selection. It is imperative that the New York onion industry develop, and adhere to, a fungicide resistance management strategy to preserve the efficacy of the remaining FRAC groups for SLB control. Continued monitoring of spatiotemporal changes in fungicide resistance profiles within the *S. vesicarium* population is a high priority. Information on the relative contributions of primary inoculum sources and the role of the sexual morph in SLB epidemiology would also be beneficial.

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