Identification and Characterization of *Botrytis fragariae* Isolates on Strawberry in the United States

Madeline E. Dowling, Meng-Jun Hu, and Guido Schnabel,[†] Department of Plant and Environmental Sciences, Clemson University, Clemson, SC 29634

Abstract

Gray mold is a devastating disease on strawberry, and may be caused by several species of *Botrytis*. The goal of this study was to better understand and characterize the species of *Botrytis* with reduced sensitivity to the fungicide Polyoxin D, particularly *Botrytis fragariae*. In total, 78 *Botrytis* isolates of unknown species that were sensitive (28 isolates; S), moderately sensitive (22 isolates; MS), or reduced sensitive (28 isolates; RS) to Polyoxin-D were collected from commercial strawberry fields of five states in the United States, identified to the species level, and characterized. The majority (75%) of S isolates were *Botrytis cinerea* and the majority (79%) of RS isolates were the recently described species *B. fragariae*, indicating an innate ability of *B. fragariae* to tolerate Polyoxin-D. *B. fragariae* produced fluffy, white mycelium and was less likely to sporulate on potato

dextrose agar than *B. cinerea*. Isolates from a commercial field recovered from blossoms in early spring were all *B. fragariae*, those from leaves of the same plants in late spring were a mixture of *B. fragariae* and *B. cinerea*, and those from fruit in early summer were all *B. cinerea*, indicating that *B. fragariae* may preferentially colonize blossom tissue. A polymerase chain reaction-based assay was developed based on *NEP2* sequence variability to distinguish *B. fragariae* from other *Botrytis* spp. that have been reported on strawberry, including *B. cinerea*, *B. mali*, *B. caroliniana*, and *B. ricini*. None of the isolates collected from Canada, California, or North Carolina nurseries were *B. fragariae*, indicating that the newly described species may not exist or not be widely distributed in planting stock.

The genus *Botrytis* consists of over 30 plant-pathogenic species with a wide variety of life history traits (Fillinger and Elad 2015). Differentiation of these species was originally hindered because the internal transcribed spacer (ITS) regions commonly used for fungal phylogenies lacked the necessary interspecific variation to distinguish *Botrytis* spp. More species were delimited after 2005, when the *glyceraldehyde 3-phosphate dehydrogenase* (*G3PDH*), *RPB2*, and *HSP60* regions were first used to genetically characterize *Botrytis* spp., and, in 2007, the *NEP1* and *NEP2* genes were added (Staats et al. 2005, 2007). The genus *Botrytis* is now known to be made up of two clades, with clade 1 containing pathogens that infect mostly eudicots whereas clade 2 pathogens infect mostly monocots. Though the genus *Botrytis* has vast interspecific genetic diversity, many species are difficult to distinguish morphologically (Fillinger and Elad 2015; Fournier et al. 2005; Walker et al. 2011). Because host ranges of species may overlap, it is sometimes necessary to genetically distinguish isolates for accurate data assessment (Chilvers and du Toit 2006), especially when species have different traits such as fungicide resistance, reproduction mechanism, and lifecycle (Fournier et al. 2005).

One common disease caused by several *Botrytis* spp. is gray mold of strawberry (Li et al. 2012; Plesken et al. 2015). *Botrytis cinerea* was long considered the sole causal agent of this disease but several species may be involved (Amiri et al. 2016; Li et al. 2012; Plesken et al. 2015). Most of these species have not been detected in the southeastern United States or, like *B. caroliniana*, *B. mali*, and *B. ricini*, do not appear to be widespread (Amiri et al. 2016; Dowling and Schnabel 2017; Li et al. 2012). The newly described species, *B. fragariae*, appears to be more widespread on strawberry plants and has already been detected in Germany and South Carolina (Rupp et al. 2017). In Germany, where most of the research involving this pathogen has thus far occurred, 38 isolates were found in nine fields and four different states. Unlike *B. cinerea*, *B. fragariae* appears to be host specific for

strawberry and there is even evidence of tissue preference (Rupp et al. 2017).

As part of a regional resistance monitoring program, we recently detected *Botrytis* isolates with reduced sensitivity to the fungicide Polyoxin-D. These isolates were collected from fields that had never been treated with this fungicide or fungicides of similar mode of action. The isolates exhibited slower growth rates and visibly less spore production compared with *B. cinerea* (Dowling et al. 2016). The goal of the present study was to further characterize these isolates. Specific objectives were to (i) identify the isolates to the species level, (ii) develop a polymerase chain reaction (PCR)-based method to distinguish the new species from others affecting strawberry, (iii) determine potential innate differences in sensitivity to Polyoxin-D between *B. cinerea* and *B. fragariae*, (iv) investigate potential sources of *B. fragariae* inoculum, and (v) gather further evidence for potential host tissue preferences.

Materials and Methods

***Botrytis* sample origin and isolation.** *Botrytis* isolates of unknown species were previously collected from blighted strawberry blossoms and fruit of farms from the states Ohio, Maryland, Virginia, North Carolina, and South Carolina to determine sensitivity to the fungicide Polyoxin-D zinc salt (referred to as Polyoxin-D) (Dowling et al. 2016). We revived 78 of these isolates from storage (filter paper) for this study (Table 1). These isolates were not collected randomly but, instead, were selected to form a subset of locations and states with an even representation of three sensitivity phenotypes to Polyoxin-D: sensitive (S; 28 isolates), moderately sensitive (MS; 22 isolates), and reduced sensitive (RS; 28 isolates). These categories were established based on the isolates' relative growth on Amresco malt extract agar amended with Polyoxin-D at 5 µg/ml: S isolates did not grow, MS isolates grew less than or equal to 70% of the control, and RS isolates grew more than 70% of the control. The isolates were from 20 different farms in 18 counties of five states: Ohio (5 isolates), Maryland (7 isolates), Virginia (1 isolate), North Carolina (13 isolates), and South Carolina (52 isolates) (Table 1).

Isolates from the main nursery stock providers for eastern growers were obtained from Dr. N. Peres, University of Florida, to investigate the source of *B. fragariae* in the strawberry fields we profiled (Table 1). In total, 91 isolates came from nursery strawberry leaf debris at nurseries in North Carolina (28 isolates) and California (35 isolates) in the United States and Ontario (2 isolates) and Nova Scotia (26 isolates) in Canada (Table 1).

In 2016, symptomatic strawberry tissues were collected during early bloom and at last harvest from a commercial field in Mullins,

[†]Corresponding author. E-mail: schnabe@clemson.edu

*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary file is published online.

SC set up in a randomized complete block design with four replicate blocks per treatment to determine a possible correlation between host tissue type and infecting species (Table 1). One treatment consisted of six applications of Polyoxin-D in the form of Ph-D WDG fungicide (Arysta LifeScience North America, LLC, Cary, NC) according to strawberry advisory system recommendations based on weather conditions during the growing season (Montone et al. 2016). No fungicides were applied to the other treatment. From Polyoxin-D-treatment blocks, we obtained three leaf isolates and five fruit isolates. From the untreated blocks, we obtained five leaf and five fruit isolates. In total, 28 blossom isolates were collected prior to fungicide applications, whereas isolates from leaves and fruit were collected thereafter. Single-spore isolates were obtained as described previously (Dowling et al. 2016).

Isolate identification by sequencing. The *G3PDH* gene region was amplified for isolates having unique culture morphology on potato dextrose agar (PDA) using PCR with the primers *G3PDH_fw* and *G3PDH_rev*, as described previously (Plesken et al. 2015; Staats et al. 2005). Products were then sequenced (Arizona State University Core Laboratories) to determine their identity to the species level. The resulting sequences were aligned with a *G3PDH* sequence of *B. fragariae* from Germany (accession number KX429703) and a BLAST search was also performed for all sequences to ensure accurate species identification.

Species-specific primer development. *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* gene fragment sequences of the three *Botrytis*

spp. obtained for a previous study (Rupp et al. 2017) were scanned for polymorphism between species that would allow for differentiation using a PCR assay. The *NEP2* gene was chosen because it contained multiple regions where nucleotide sequence varied between all three species (Fig. 1). The *NEP2* nucleotide sequences of *B. cinerea*, *B. fragariae*, and *B. mali* used to develop these primers are provided in Supplementary File S1. Using the Multiple Primer Analyzer web-site by Thermo Fisher Scientific, we compared potential primer sequence combinations to ensure that there was no dimer formation between potential primers and that melting temperatures were similar enough to be combined in a single PCR. A forward primer common between species and three species-specific reverse primers were designed to distinguish *B. cinerea*, *B. fragariae*, and *B. mali* (Table 2). The primers were initially tested on 15 isolates representing all three species from four states and 13 farms, and results were confirmed by sequencing the *G3PDH* region. Primers were also tested on common contaminants obtained from strawberry fruit and flowers, including *Fusarium*, *Rhizopus*, *Cladosporium*, and *Penicillium* spp., to determine whether nonspecific amplification of these contaminants was possible. Each PCR consisted of a small amount of fungal hyphae, hot start Taq PCR mix (Bioneer, Alameda, CA), 1 µl of each primer (20 µM concentration), and 16 µl of DNase-free sterile water for a total reaction volume of 20 µl. The amplification program began with an initial denaturation step of 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. This was followed by a final extension step of 72°C for 5 min. Gel electrophoresis of PCR products was performed on 1.5% agarose gels run at 110 V for 20 to 30 min.

Primers were also tested on *Botrytis* aerial mycelium growing from inoculated, detached strawberry fruit. Commercially grown strawberry fruit were washed with soap and water, then surface sterilized in a 10% bleach solution for 1 min. After drying, they were inoculated by removing a plug from the side of each strawberry using a sterile cork-borer approximately 5 mm in diameter. The same cork-borer was used to transfer agar plugs with actively growing mycelium of isolates 28-2-15, 8-3-16, and 65-4-15 of *B. cinerea*; 5-5-16, 15-1-16, and 43-1-15 of *B. fragariae*; and 55-6-16, 28-6-15, and 49-10-16 of *B. mali* into the fruit opening. Inoculated strawberry fruit were placed in sealable plastic boxes with moistened paper towels, as described previously (Dowling et al. 2016). After 2 to 3 days or when *Botrytis* mycelium was visible, an amount of hyphae just covering the tip of a toothpick was removed and placed directly into the PCR, as described above. The newly developed primers were then used to screen all isolates not sequenced at the *G3PDH* region, as well as all isolates from nurseries and from Mullins, SC. Mycelium from *B. caroliniana* and DNA from *B. ricini* isolates was also used as a template for the species-specific primers to determine whether any amplicons would be produced.

Statistical analysis. A *t* test was performed using the software JMP 11.0 (SAS Institute, Inc., Cary, NC) with a significance level

Table 1. Origin and species frequency for all isolates examined in this study

Origin		Isolate frequency		
Source, location	Host tissue	<i>Botrytis fragariae</i>	<i>B. mali</i>	<i>B. cinerea</i>
Grower farms ^a				
Maryland	Blossoms	0	1	6
Virginia	Blossoms	0	0	1
North Carolina	Blossoms	4	0	1
	Fruit	0	0	8
South Carolina	Blossoms	34	2	16
Ohio	Blossoms	1	0	4
Grower farm				
Mullins, SC	Blossoms	28	0	0
	Leaves	6	0	2
	Fruit	0	0	10
Nursery				
North Carolina	Leaves	0	0	28
California	Leaves	0	0	35
Ontario	Leaves	0	0	2
Nova Scotia	Leaves	0	0	26

^a Isolates in this location category were revived from a previous study (Dowling et al. 2016).

Fig. 1. Schematic of a 705-bp fragment of the *NEP2* gene showing primer location and size of fragments produced by each primer set for *Botrytis* spp. identification. Each solid black arrow represents a primer and the name of the primer is listed above the arrow. Letters below each arrow indicate the first three or four nucleotides where each primer anneals. Nucleotides that vary between species and allow differentiation are shown in bold. Gray segments indicate introns and black segments represent exons.

of $\alpha = 0.05$ to determine whether different species truly have differing sensitivities to Polyoxin-D. Because only three *B. mali* isolates were identified and they all belonged to the RS category, these were not included in the analysis.

Results

Cultural characteristics and molecular identification of isolates. Sequencing of the *G3PDH* gene was performed to identify isolates to the species level that had colony morphologies on PDA different from *B. cinerea*. These distinguishing morphological characteristics included colony color and shape as well as the isolates' ability to sporulate and produce sclerotia. BLAST search revealed that these morphologically unique isolates represented the three species *B. cinerea*, *B. fragariae*, and *B. mali*. On PDA, *B. cinerea* produced characteristic prolific grayish-brown spores, whereas *B. fragariae* colonies were typically white and sporulation was less obvious (Fig. 2). When *B. fragariae* sclerotia were present on minimal media, they typically formed concentric circles, whereas *B. cinerea* sclerotia placement was random. In rare cases, some *B. fragariae* isolates did produce grayish spores and conidiophores similar to *B. cinerea*. The three *B. mali* isolates obtained were not consistent in morphology but also did not appear to sporulate as readily as *B. cinerea* isolates.

G3PDH, *RPB2*, *HSP60*, *NEP1*, and *NEP2* nucleotide sequence analysis of *Botrytis* isolates revealed high interspecific but low intraspecific variation between and among *B. cinerea*, *B. fragariae*,

and *B. mali* species. Pairwise comparison of sequences revealed that, within each species, there were no more than 2 nucleotide differences out of approximately 900 bp. Between species, however, there were more substantial differences (Table 3).

Development of species-specific primers. Primers were developed to differentiate the three species using *NEP2* gene nucleotide sequence variability (Fig. 1). The primers yielded the expected fragment sizes of 592 bp for *B. cinerea*, 332 bp for *B. fragariae*, and 186 bp for *B. mali* (Fig. 3). The same results were obtained when *Botrytis* aerial mycelium removed directly from detached fruit was used as template. However, no bands were obtained for common media and fruit contaminants *Fusarium*, *Cladosporium*, *Rhizopus*, and *Penicillium* spp. (data not shown). DNA from *B. caroliniana* (Fig. 3) and *B. ricini* used as templates produced no amplicons, though positive controls using *B. caroliniana*-specific primers and ITS of *B. ricini* resulted in amplification of the expected band sizes.

Overall species identification results for isolate collections. Of the 78 isolates selected for this study based on the three Polyoxin-D sensitivity phenotypes, we identified 36 *B. cinerea*, 39 *B. fragariae*, and 3 *B. mali* isolates using species-specific PCR and *G3PDH* sequencing. Most ($n = 70$) isolates were collected from blossoms of 19 different farms, and the majority of these (58.2%) were *B. fragariae*. The remaining eight isolates were from fruit collected from the same location and were all *B. cinerea*.

With regard to Polyoxin-D sensitivity, the vast majority of S isolates ($n = 27$; 96.4%) were *B. cinerea*, the majority of RS isolates ($n = 22$; 78.6%) were *B. fragariae* (Fig. 4), and a statistically significant relationship between species and Polyoxin-D sensitivity was detected ($P < 0.0001$). Most MS isolates ($n = 16$; 72.7%) were *B. fragariae*. All but one of the *B. cinerea* MS isolates had relative growth values below 20% on Polyoxin-D amended medium, whereas all MS *B. fragariae* isolates had relative growth values above 20%. All 91 isolates obtained from various nurseries most likely to provide transplants to the locations we sampled were *B. cinerea* based on species-specific PCR results.

Table 2. Species-specific primer sequences for distinguishing *Botrytis cinerea* from *B. fragariae* and *B. mali*

Name	Nucleotide sequence	Anneals to
CFM_NEP2	5'-GTAGGAACAGTTTATGAG-3'	All three species
C_NEP2	5'-GACCCATTGAGTGATCGACG-3'	<i>B. cinerea</i>
F_NEP2	5'-TAGTTTGATCTGTAAGGAGGTGA-3'	<i>B. fragariae</i>
M_NEP2	5'-ACCACTAAGAAACGTTAGAGACATG-3'	<i>B. mali</i>

Fig. 2. Morphology in form of sporulation capacity (above) and hyphal density (below) of representative isolates of each species on potato dextrose agar after approximately 1 month of growth. Isolates 14.9.15, 36.3.15, and 13.6.15 represented the species *Botrytis cinerea*, *B. mali*, and *B. fragariae*, respectively.

We collected 46 *Botrytis* isolates from a single location in Mullins, SC, to determine host tissue preference of species. All 28 blossom isolates were *B. fragariae* as well as 6 of the 8 isolates collected from leaves (Fig. 5). All 10 isolates collected from fruit later in the season were identified as *B. cinerea* (Fig. 5), indicating that the two species had host tissue preference. A possible limitation of this analysis was that some leaf and fruit samples were treated with Polyoxin-D, which could potentially select for *B. fragariae* because this species is prone to RS to Polyoxin-D. However, none of the 10 fruit samples from control or Polyoxin-D-treated plots were *B. fragariae*, and several leaf samples from the untreated control block were *B. fragariae*, indicating that Polyoxin-D-induced selection for *B. fragariae* did not skew our host tissue preference results.

Table 3. Pairwise comparison showing the average percentage of each sequence that differed between species^a

Species	Compared with	Gene (%)				
		<i>G3PDH</i>	<i>HSP60</i>	<i>RPB2</i>	<i>NEP1</i>	<i>NEP2</i>
<i>Botrytis fragariae</i>	<i>B. cinerea</i>	4.2	4.4	4.7	9.6	1.2
<i>B. cinerea</i>	<i>B. mali</i>	1.4	3.0	4.4	N/A	1.2
<i>B. mali</i>	<i>B. fragariae</i>	3.8	4.7	2.8	N/A	9.6

^a Percentage was obtained by dividing the number of nucleotide differences between species by the total sequence length. N/A = NEP1 gene sequence was not obtained for *B. mali*.

Fig. 3. Amplification of *Botrytis* spp. collected from strawberry using species-specific primers CFM_NEP2, C_NEP2, F_NEP2, and M_NEP2. Lanes 1, 2, and 3 contain polymerase chain reaction (PCR) products amplified from *B. cinerea*, *B. fragariae*, and *B. mali* templates, respectively. Lane 4 contains no amplicon from an attempt to amplify from a *B. caroliniana* template. Lane L = GeneRuler 1-kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA). *B. caroliniana*, *B. ricini* (data not shown), and failed PCR produced no amplicons but leftover PCR materials are visible in the bottom of the lane and may appear as a band, as seen in lanes 1 and 4.

Discussion

In a previous study, *B. fragariae* was reported, for the first time, to exist in the United States (Rupp et al. 2017). These isolates were from three commercial farms in South Carolina. The results of the present study show that *B. fragariae* occurs in at least two more states (North Carolina and Ohio), indicating that this species may be widely distributed in the United States. *B. fragariae* was also detected in Germany at low frequencies in four different states (Rupp et al. 2017). However, our sample size and collection bias to represent equal numbers of Polyoxin-D sensitivity phenotypes does not allow for an evaluation of frequency of *B. fragariae* in U.S. strawberry fields. *B. caroliniana*, a species isolated in 2011 from a single strawberry fruit, and *B. ricini*, detected in 2013 in Florida, were not found in our sample populations (Amiri et al. 2016; Li et al. 2012). Species-specific primers were designed that effectively distinguished isolates of *B. cinerea*, *B. mali*, and *B. fragariae*. Approximately 10% of the isolates did not produce any amplicon when these primers were used. It is possible that these isolates did not contain the introns that the species-specific primers are based on.

B. fragariae isolates were more likely ($P < 0.0001$) to be RS to Polyoxin-D compared with *B. cinerea*. The few *B. fragariae* and *B. cinerea* isolates not following this observation are likely outliers of a normal baseline sensitivity distribution. Innate RS to Polyoxin-D may be caused by constitutive expression of drug efflux pumps, resulting in a higher baseline sensitivity to Polyoxin-D in *B. fragariae* compared with *B. cinerea*. This hypothesis is supported by evidence

Fig. 4. Number of isolates collected from flowers in multiple states and locations within states for three *Botrytis* spp. sensitive (S; 0% relative growth), moderately sensitive (MS; >0 to 70% relative growth), and reduced sensitive (RS; >70% relative growth) to Polyoxin-D. Letters representing statistical differences are valid within each phenotype but not across phenotypes. The number of locations refers to the number of farms where isolates were collected. S isolates were found in all five states (15 locations), MS in four states (12 locations), and RS isolates in three states (13 locations).

Fig. 5. Species distribution of Mullins, SC isolates collected from different strawberry tissues preharvest (blossoms), midharvest (leaves), and postharvest (fruit) to determine whether *Botrytis fragariae* had preference for blossom tissue.

that *B. fragariae* isolates from German strawberry fields have higher basal expression levels of *atrB* than equivalent *B. cinerea* isolates (Rupp et al. 2017). However, whether or not Polyoxin-D is a substrate of *atrB* is not known. *B. fragariae* may constitutively express other drug efflux genes such as the *BMR1* gene encoding an ABC transporter previously correlated with Polyoxin-D efflux at levels higher than *B. cinerea* isolates (Nakajima et al. 2001). Another possibility is that chitin synthetase enzymes of *B. fragariae* and *B. cinerea* differ in amino acid sequence, which may result in differential binding affinity to Polyoxin-D in the two *Botrytis* spp. This type of differential binding affinity resulting in sensitivity differences has already been observed in the pathogen *Mycosphaerella graminicola*, where amino acid sequence differences in the enzyme MgCYP51 may result in decreased binding affinities and reduced sensitivity to demethylation inhibitor (DMI) fungicides (Mullins et al. 2011). All three *B. mali* isolates were RS to Polyoxin-D but the sample size was too small to allow for solid conclusions about innate RS to Polyoxin-D in this species.

Though *B. fragariae* is present in several states of Germany and the United States, the source of *B. fragariae* inoculum is unknown. The isolates used in this study came from plasticulture fields with raised beds that were fumigated prior to planting nursery transplants in the fall. Therefore, it is reasonable to hypothesize that *B. fragariae* may have come from nurseries, especially because spread of *B. cinerea* inoculum from nursery to field through latent infections of nursery stock is well documented (Oliveira and Peres 2014; Peres 2015; Schnabel et al. 2015). Screening of 91 isolates from major nursery stock providers yielded only *B. cinerea*. Because all nursery isolates were obtained from symptomatic leaves, whereas most isolates used for Polyoxin-D sensitivity profiling came from blossoms, a host tissue preference could explain our inability to find *B. fragariae* in nursery stock. Results from a commercial farm in Mullins, SC, indicated that *B. fragariae* prefers blossoms to either leaves or fruit but both species were found in leaves. This indicates that host tissue preference is not the best explanation for the absence of *B. fragariae* in nursery isolates. Though *B. fragariae* was found on leaves in Mullins, SC, it was not found on fruit. In fact, a complete species shift occurred: all isolates recovered from blossoms were *B. fragariae* and all isolates recovered from fruit on the same plants were *B. cinerea* (Fig. 5). Subsequent analysis of our collection from Clemson did reveal instances of *B. fragariae* infecting fruit tissue, but they were rare (data not shown). These results agree with competition studies performed in Germany which revealed that *B. fragariae* is outcompeted by *B. cinerea* on fruit in vitro (Rupp et al. 2017).

Because no *B. fragariae* was found in a collection from nursery stock, inoculum may instead be endemic and originate from overwintering plant debris or alternate hosts near strawberry plantings. Because we found that Polyoxin-D RS isolates produce more sclerotia than S isolates in vitro, overwintering of *B. fragariae* sclerotia between planting beds in the form of sclerotia appears more plausible, especially because *B. fragariae* appears to have a limited host range (Dowling et al. 2016; Rupp et al. 2017).

In conclusion, *B. fragariae* from U.S. strawberry fields differs from *B. cinerea* in sensitivity to Polyoxin-D, cultural characteristics on PDA medium, inoculum source, and host tissue preference. The newly developed primers reported in this study will allow for easy distinction between the species causing gray mold of strawberry.

Acknowledgments

This project was supported by Technical Contribution Number 6525 of the Clemson University Experiment Station and by the United States Department of Agriculture National Institute of Food and Agriculture under project number SC-1700501 and Specialty Crop Research Initiative grant number 2014-51181-22377. We thank C. J. Porter, J. Ballew, N. Peres, M. Oliveira, and A. Zuniga for providing some of the isolates for this study; and K. Bryson, B. Cox, and T. Sroka for technical assistance.

Literature Cited

- Amiri, A., Onofre, R. B., and Peres, N. A. 2016. First report of gray mold caused by *Botryotinia ricini* (*Amphobotrys ricini*) on strawberry in United States. Plant Dis. 100:1007.
- Chilvers, M. I., and du Toit, L. J. 2006. Detection and identification of *Botrytis* species associated with neck rot, scape blight, and umbel blight of onion. Plant Health Prog. doi:10.1094/PHP-2006-1127-01-DG
- Dowling, M. E., Hu, M.-J., Schmitz, L. T., Wilson, J. R., and Schnabel, G. 2016. Characterization of *Botrytis cinerea* isolates from strawberry with reduced sensitivity to Polyoxin D zinc salt. Plant Dis. 100:2057-2061.
- Dowling, M. E., and Schnabel, G. 2017. First report of *Botrytis mali* causing gray mold on strawberry in the United States. Plant Dis. 101:1034.
- Fillinger, S., and Elad, Y. 2015. Botrytis—The Fungus, the Pathogen and its Management in Agricultural Systems. Springer, Cham, Switzerland.
- Fournier, E., Giraud, T., Albertini, C., and Brygoo, Y. 2005. Partition of the *Botrytis cinerea* complex in France using multiple gene genealogies. Mycologia 97: 1251-1267.
- Li, X., Fernández-Ortuño, D., Chai, W., Wang, F., and Schnabel, G. 2012. Identification and prevalence of *Botrytis* spp. from blackberry and strawberry fields of the Carolinas. Plant Dis. 96:1634-1637.
- Montone, V. O., Fraisse, C. W., Peres, N. A., Sentelhas, P. C., Gleason, M., Ellis, M., and Schnabel, G. 2016. Evaluation of leaf wetness duration models for operational use in strawberry disease-warning systems in four US states. Int. J. Biometeorol. 60:1761-1774.
- Mullins, J. G. L., Parker, J. E., Cools, H. J., Togawa, R. C., Lucas, J. A., Fraaije, B. A., Kelly, D. E., and Kelly, S. L. 2011. Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. PLoS One 6:e20973.
- Nakajima, M., Suzuki, J., Hosaka, T., Tadaaki, H., and Akutsu, K. 2001. Functional analysis of an ATP-binding cassette transporter gene in *Botrytis cinerea* by gene disruption. J. Gen. Plant Pathol. 67:212-214.
- Oliveira, M. S., and Peres, N. A. 2014. Strawberry nursery plants as a source of *Botrytis cinerea* isolates resistant to fungicides. Poster 208-P in: 2014 American Phytopathological Society Annual Meeting, Minneapolis, MN.
- Peres, N. A. 2015. 2015 Florida Plant Disease Management Guide: Strawberry. Online publication. University of Florida. <http://edis.ifas.ufl.edu/pg056>
- Plesken, C., Weber, R. W. S., Rupp, S., Lerach, M., and Hahn, M. 2015. *Botrytis pseudocinerea* is a significant pathogen of several crop plants but susceptible to displacement by fungicide-resistant *B. cinerea* strains. Appl. Environ. Microbiol. 81:7048-7056.
- Rupp, S., Plesken, C., Rumsey, S., Dowling, M., Schnabel, G., Weber, R. W. S., and Hahn, M. 2017. *Botrytis fragariae*, a new species causing gray mold on strawberries, shows high frequencies of specific and efflux-based fungicide resistance. Appl. Environ. Microbiol. 83:e00269-17.
- Schnabel, G., Hu, M., and Fernández-Ortuño, D. 2015. Monitoring resistance by bioassay: Relating results to field use using culturing methods. Pages 281-293 in: Fungicide Resistance in Plant Pathogens. H. Ishii and D. W. Hollomon, eds. Springer, Tokyo.
- Staats, M., van Baarlen, P., Schouten, A., van Kan, J. A. L., and Bakker, F. T. 2007. Positive selection in phytotoxic protein-encoding genes of *Botrytis* species. Fungal Genet. Biol. 44:52-63.
- Staats, M., van Baarlen, P., and van Kan, J. A. L. 2005. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol. Biol. Evol. 22:333-346.
- Walker, A.-S., Gautier, A., Confais, J., Martinho, D., Viaud, M., Le Pêcheur, P., Dupont, J., and Fournier, E. 2011. *Botrytis pseudocinerea*, a new cryptic species causing gray mold in French vineyards in sympatry with *Botrytis cinerea*. Phytopathology 101:1433-1445.