Improving Strawberry Gray Mold Management through Identification of Ineffective Sprays

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Summary

Botrytis cinerea can cause a devastating fruit rot on strawberries called gray mold. This disease can be prevented by the application of fungicides. However, recent reports have shown that California's strawberry fields contain strains of *B. cinerea* that are resistant to many currently registered fungicides. Resistance management strategies such as rotating modes of action and tank-mixing have been proposed as ways to delay resistance development. We evaluated these strategies under field conditions to determine their effect on the rate of resistance development to the fungicides boscalid, fenhexamid, and fludioxonil.

The initial population of *B. cinerea* in our field had low frequencies of resistance to boscalid (7%) and fludioxonil (3%), and a moderate frequency of resistance to fenhexamid (21%). Before, during, and after the application of various fungicide treatments, the frequencies of resistance to boscalid and fludioxonil remained unchanged over the course of the trial. In contrast, the frequency of resistance to fenhexamid was more variable. Resistance frequency increased greatly in strawberry plots exposed to fenhexamid, even if fenhexamid was rotated with other modes of action or tank-mixed. After a 27 and 48-day period following the last application of fenhexamid, the frequency of resistance to fenhexamid decreased. This decrease could be due to fenhexamid-resistant *B. cinerea* being less fit than fenhexamid-sensitive *B. cinerea*. This trial gives insight into how *B. cinerea* responds to fungicide selection pressure in a real-world scenario, and to the effectiveness of resistance management methods.

Introduction

Modern site-specific fungicides have been used to manage diseases caused by *Botrytis cinerea*, including strawberry gray mold, since the late 1960s (Leroux et al., 2002). Multiple applications of fungicides are made per season to prevent strawberry gray mold because *B. cinerea* can infect and rot fruit throughout the five- to six-month harvest season (Cosseboom et al., 2018). Many of the most effective fungicides are synthetic compounds that have a site-specific mode of action, which inhibits the function of a critical protein within a fungal pathogen (Ma and Michailides, 2005). These chemicals are very effective at low concentrations and are often non-toxic to mammals. However, resistance has been reported to every effective chemical class (Fernández-Ortuño et al., 2014). Resistance to a site-specific fungicide typically arises from an advantageous, single point mutation in the gene that codes for the target protein of that fungicide (Ma and Michailides, 2005). Multisite fungicides have not shown signs of resistance development despite their extensive use since their introduction decades ago. These fungicides are usually not as effective as site-specific fungicides in regard to *B. cinerea* (FRAC, 2017). Multisite fungicides are often used in rotation or in mixture (tank-mixed) with site-specific fungicides to prevent gray mold while decreasing selection pressure for resistance to the site-specific fungicides (Northover and Matteoni, 1986). Two multisite fungicides, captan and thiram, are labeled for gray mold of strawberry. The site-specific fungicides labeled for gray mold have been placed into eight chemical classes according to their cross-resistance behavior: anilinopyrimidines (APs), dicarboximides (DCs), hydroxyanilides (HAs), methyl benzimidazole carbamates (MBCs), phenylpyrroles (PPs), polyoxins, quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs) (FRAC, 2017).

A multitude of theoretical models gives evidence that applying a high resistance-risk (site-specific) fungicide consecutively will select for more resistance to that chemical class than applying that high-risk fungicide with a low-risk (multisite) fungicide in a mixture or rotation (van den Bosch et al., 2014). Yet, a study of solo, rotation, and tank-mixing spray programs in strawberries showed that a tank-mix or rotation of a DC and multisite fungicide did not result in less resistance than a DC alone. Furthermore, resistance to MBCs and DCs was correlated with the number of applications of those chemical classes (Hunter et al., 1987; Johnson et al., 1994; Northover and Matteoni, 1986). In the absence of selection pressure from a fungicide, the frequency of resistance to that fungicide may recede over time if resistance to that fungicide entails a fitness cost. *In vitro* fitness cost studies can conflict on whether resistance to a chemical class like the SDHIs is associated with reduced fitness (Karaoglanidis et al., 2010; Lalève et al., 2014; Veloukas et al., 2014; Yin et al., 2011). Resistance to fungicides such as DCs, HAs, PPs, and polyoxins has been associated with measurable fitness costs in vitro, while resistance to APs, MBCs and QoIs has not (Bardas et al., 2008; Billard et al., 2012; Dowling et al., 2016; Raposo et al., 2000; Ren et al., 2016; Yourman et al., 2001). *In vivo* studies provide practical evidence of the effects of a resistance-associated fitness cost on a population. In the absence of exposure, DC-resistant field populations of *B. cinerea* have declined in frequency of resistance (Hunter et al., 1987; Johnson et al., 1994; Katan, 1982).

The objective of this study was to monitor frequencies of fungicide resistance in populations of *B. cinerea* before, during, and after rotation, tank-mix + rotation, or consecutive solo fungicide treatments. Active ingredients in the four most frequently applied fungicides labeled for gray mold of strawberry in California were tested: boscalid (SDHI) captan (multisite), fenhexamid (HA), and fludioxonil (PP) (Cosseboom et al., 2018).

MATERIALS & METHODS

Field Trial Design. The field trial was conducted in Field 25, block 3 at California Polytechnic State University in San Luis Obispo, California. The trial consisted of nine plastic mulch-covered, raised beds that were 48 in. wide, 150 ft long, 12 in. high, and 64 in. between bed centers. Every other bed was planted with cereal rye (*Secale cereale* L.) 'Merced'. The four beds interspersed between the cereal rye planted beds contained eight plots of 30 strawberry plants 'San Andreas' (112 in.), a 16 in. buffer, and a 72 in. barrier planting of cereal rye, thus surrounding each strawberry plot with cereal rye on all four sides. Strawberries were transplanted on November 21, 2016 and cereal rye was planted December 1, 2016. The cereal rye was used to prevent inter-plot interference from wind-dispersed *B. cinerea* spores (conidia). A single microsprinkler emitter (Netafim SuperNet #50 green nozzle (13.2 gph at 30 psi), Netafim USA, Fresno, CA) was placed in the center of each plot of 30 strawberry plants. Immediately following planting, the strawberry plants were overhead-irrigated via microsprinklers for one month to facilitate plant establishment.

Treatments were arranged in a randomized complete block design with four blocks and seven treatments replicated once in each block. Fungicides used for the field trial were boscalid + pyraclostrobin (Pristine®; BASF Corporation, Ludwigshafen, Germany), captan (Captan 80WDG; Arysta LifeScience, Cary, NC), cyprodinil + fludioxonil (Switch® 62.5WG; Syngenta Crop Protection, Basel, Switzerland), and fenhexamid (Elevate® 50WDG; Arysta LifeScience, Cary, NC). The experiment included a non-treated control, and six other treatments applied for six consecutive weeks (Table 1). Each treatment was applied with a CO_2 pressurized backpack spray system and handheld boom.

Table 1. Field trial fungicide treatments evaluated for resistance response in *Botrytis cinerea*.

Treatment Name	Trade Name	Treatment Sequence	Rate
Non-treated		n/a	n/a
Boscalid + pyraclostrobin (P)	Pristine®	P, P, P, P, P, P	23 fl oz/acre
Cyprodinil + fludioxonil (S)	Switch® 62.5WG	S, S, S, S, S, S	14 fl oz/acre
Fenhexamid (E)	Elevate® 50WDG	E, E, E, E, E, E	1.5 lb/acre
Captan (C)	Captan 80 WDG	C, C, C, C, C, C	3.75 lb/acre
Rotation		P, S, E, P, S, E	P, S, E
Tank-mix + rotation		P + C, S + C, E + C, P + C, S + C, E + C	C + low (P, S, E)[a]

[a] The tank-mix + rotation used low rates of P (18.5 fl oz/acre), S (11 fl oz/acre), and E (1 lb/acre).

Isolate Collection And Laboratory Assay. Depending on the weather, microsprinklers were employed intermittently throughout the experiment to promote disease development in strawberry flowers and fruit. Isolates of *B. cinerea* were collected at five times throughout this experiment. At each collection time, four isolates were collected per strawberry plot yielding 112 isolates per collection date. Isolates were collected in 21-day intervals starting at one day before the first fungicide application. Isolates were collected by brushing a sterile, individually wrapped cotton swab against a sporulating lesion of gray mold. If not enough sporulating fruit could be found in the field, green fruit were collected, frozen overnight, and surface sterilized for one minute in 1% sodium hypochlorite. These fruits were then incubated at room temperature in humidity chambers until sporulation occurred.

Isolates were tested for resistance to boscalid, fenhexamid, and fludioxonil using a visually assessed mycelial growth assay. Each isolate was transferred with a sterile toothpick from the swab to four wells (0.59 in. diameter) of 24-well plates (Thermo Fisher Scientific, Waltham, MA). The four wells contained a discriminatory dosage of fungicide-amended growing medium: non-amended malt extract agar (MEA), 75 ppm boscalid in yeast bacto acetate agar (YBA), 50 ppm fenhexamid in MEA, and 0.5 ppm fludioxonil in MEA. Formulated products containing only boscalid (Endura®; BASF Corporation, Ludwigshafen, Germany), fenhexamid (Elevate® 50WDG; Arysta LifeScience, Cary, NC), and fludioxonil (Scholar® SC; Syngenta Crop Protection, Basel, Switzerland) were used. Infested 24-well plates were incubated at 72° F for four days and diametric colony growth was visually assessed in each well as: sensitive (S) for less than 20% diametric growth, and resistant (R) for more than 20% diametric growth with respect to the well diameter. A Bonferroni adjusted, one-tailed, Fisher's exact test was used to compare the frequency of resistance observed between collection I and collection III and between collection III and collection V for each treatment. A repeat experiment is currently underway.

Results

Isolates collected prior to fungicide applications (collection I), 7, 21, and 3% of isolates were resistant to boscalid, fenhexamid, and fludioxonil, respectively. Collection I frequencies of resistance were relatively similar among treatments. Resistance to boscalid and fludioxonil remained relatively steady throughout the trial, while resistance to fenhexamid was more variable (Figure 1). In plots treated with fenhexamid, resistance to fenhexamid increased from collection I to collection II, and again until collection III (6 days after the last application). At 27 (collection IV) and 48 days (collection V) after the last application of fenhexamid, resistance to fenhexamid decreased. Frequency of resistance significantly increased from collection I to collection III in the fenhexamid, tank-mix, and tank-mix + rotation treatments. Frequency of resistance significantly decreased from collection III to collection V in the rotation treatment only. Fenhexamid resistance frequency remained relatively constant in the non-treated plots throughout the trial; however, fenhexamid resistance frequency did erratically change in plots treated with fungicides besides fenhexamid.

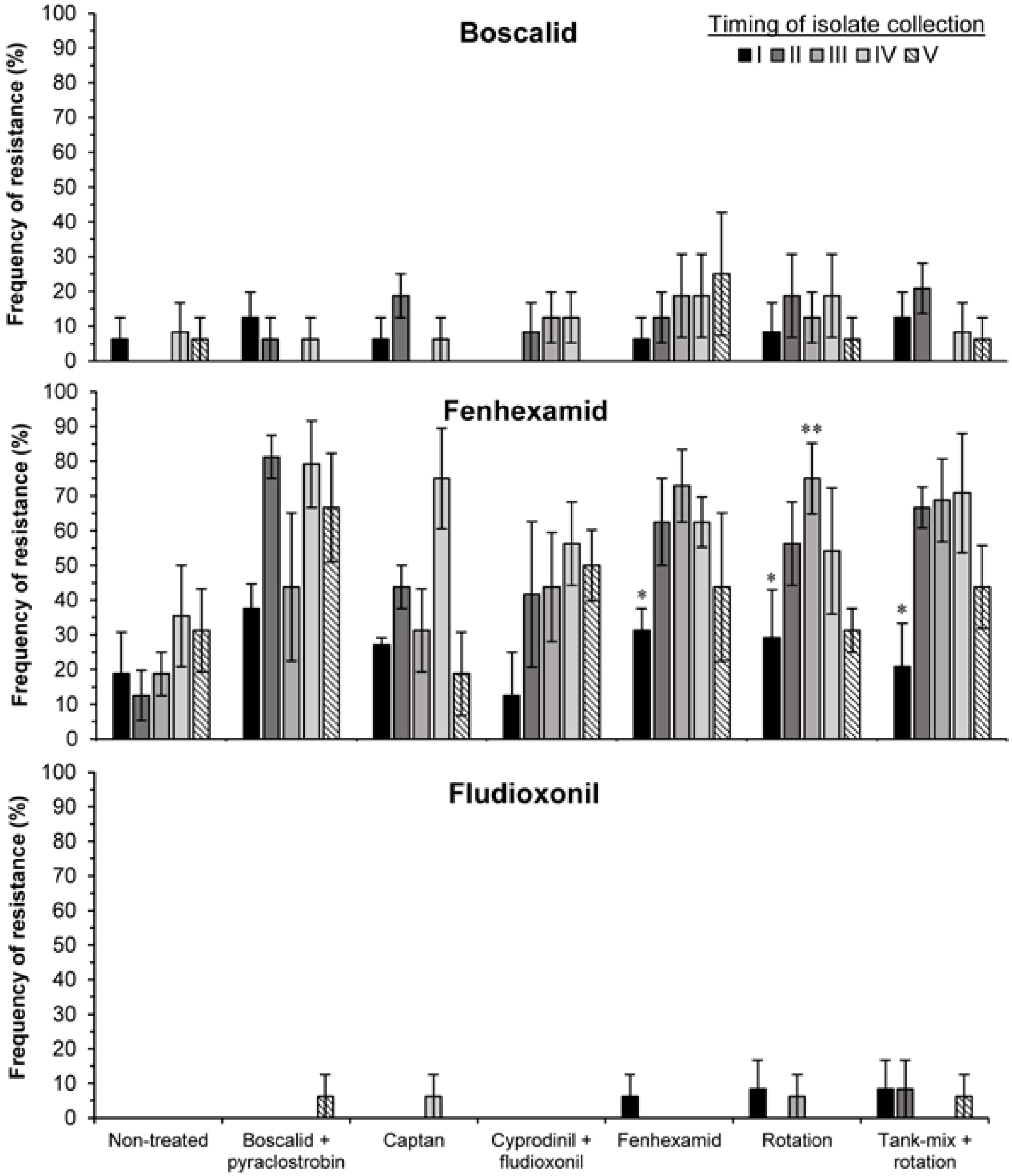

Figure 1. Frequencies of resistance of *Botrytis cinerea* to boscalid (top), fenhexamid (middle), and fludioxonil (bottom) observed in plots of strawberries treated with repeated applications of fungicides at five collection points. Collections timings: (I) one day before first fungicide application, (II) six days after third fungicide application, (III) six days after sixth fungicide application, (IV) 27 days after sixth fungicide application, and (V) 48 days after sixth fungicide application. Statistical significance of frequency of resistance between I and III is represented by an asterisk (*), and III and V by a double asterisk (**) ($p<0.05$).

Discussion

The *B. cinerea* population in field 25 contained fungicide resistant phenotypes that were initially rarely resistant to boscalid (7%) and fludioxonil (3%) but were commonly resistant to fenhexamid (21%). This is a scenario similar to fungicide resistance monitoring surveys in California (Cosseboom et al., 2018), the Eastern United States (Fernández-Ortuño et al., 2014), and Spain (Fernández-Ortuño et al., 2016); however, boscalid resistance was less frequent in our test site. This could be because resistance is variable between different strawberry fields (Fernández-Ortuño et al., 2014). Despite repeated applications of boscalid and fludioxonil, resistance to these fungicides did not increase or change regardless of the fungicide treatment or rotation program. The initial level of resistance to both of these fungicides may have been too low to observe a meaningful shift in resistance in 12 weeks. Although microsprinklers were utilized to create longer wetness periods and thereby increase gray mold incidence, gray mold incidence was often so low that fruit needed to be incubated for gray mold to develop, especially at the later collection dates. A population in a field with greater disease pressure may have been more prone to selection pressure.

The populations of *B. cinerea* within the plots treated with fenhexamid displayed a response in frequency of resistance, tending to increase with selection pressure, and tending to decrease without it. The high level of resistance to fenhexamid in the first collection may have made the population shift more rapidly towards high resistance frequency. The fungicide rotation or tank-mix + rotation treatments did not appear to delay the selection for resistance to fenhexamid. By collection III, the fenhexamid treatment plots were exposed to six applications of fenhexamid, while the rotation and tank-mix + rotation treatment plots were only exposed to two applications of fenhexamid. Other studies have also observed that tank-mixing a multisite fungicide with a site-specific fungicide may not sufficiently delay resistance development to the site-specific fungicide (Hunter et al., 1987; Northover and Matteoni, 1986). This may be due to the number of applications per season in a crop like strawberries (van den Bosch et al., 2014) and the limited efficacy of multisite fungicides like captan and thiram (Adaskaveg et al., 2017).

Inter-plot interference is a common issue in similar fungicide resistance studies (Northover and Matteoni, 1986). The planting of cereal rye appeared to prevent this phenomenon because the frequency of resistance to fenhexamid in the non-treated plots stayed relatively constant throughout the trial. However, resistance to fenhexamid was variable in other treatment plots that were not exposed to fenhexamid. Even though this variability was not observed in the non-treated plots, this may still be attributable to inter-plot interference. The thick stand of cereal rye appeared to block wind from blowing spores between plots horizontally, but the plots were exposed to spores dropping into plots from above. Selection by association, also known as "genetic hitchhiking", could also contribute to this shift (Hu et al., 2016). The application of a fungicide such as boscalid may have selected for isolates that are dual resistant to boscalid and fenhexamid. The replication of this trial will help illuminate if this is truly the case. Analyzing the phenotypes of single-spore isolates collected in these plots throughout the trial would also help characterize this as selection by association.

The decrease in resistance in the absence of selection pressure exhibits the effects of the fitness cost associated with fenhexamid resistance (Billard et al., 2012; Saito et al., 2014). In a field with a high level of fungicide resistance (with a resistance-associated fitness cost), reducing or eliminating its use for a period of time may yield a restoration of its efficacy. Yet, this study shows that a population may become effectively resistant after exposure to only one or two applications of a high-risk chemical class. Therefore, use of DC (iprodione) and HA (fenhexamid) use should be limited. Alternatively, fungicides with persistent resistance issues (e.g., QoIs and MBCs) should not be considered for gray mold management for the foreseeable future.

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References

- Adaskaveg, J.E., D. Gubler, and T.J. Michailides. 2017. Fungicides, bactericides, and biologicals for deciduous tree fruit, nut, strawberry, and vine crops. Online publication. University of California Division of Agriculture and Natural Resources. http://ipm.ucanr.edu/PDF/PMG/fungicideefficacytiming.pdf
- Bardas, G.A., C.K. Myresiotis, and G.S. Karaoglanidis. 2008. Stability and fitness of anilinopyrimidine-resistant strains of *Botrytis cinerea*. Phytopathology 98:443–450.
- Billard, A., S. Fillinger, P. Leroux, H. Lachaise, R. Beffa, and D. Debieu. 2012. Strong resistance to the fungicide fenhexamid entails a fitness cost in *Botrytis cinerea*, as shown by comparisons of isogenic strains. Pest Manag. Sci. 68:684–691.
- Cosseboom, S.D., G. Schnabel, P.K. Bryson, K.L. Ivors, and G.J. Holmes. 2018. Within-season shift in fungicide resistance profiles of *Botrytis cinerea* in California strawberry fields. Plant Dis. *In press*.
- Dowling, M.E., M.-J. Hu, L.T. Schmitz, J.R. Wilson, and G. Schnabel. 2016. Characterization of *Botrytis cinerea* isolates from strawberry with reduced sensitivity to polyoxin D zinc salt. Plant Dis. 100:2057–2061.
- Fernández-Ortuño, D., A. Grabke, P.K. Bryson, A. Amiri, N.A. Peres, and G. Schnabel. 2014. Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven southern U.S. states. Plant Dis. 98:825–833.
- Fernández-Ortuño, D., J.A. Torés, M. Chamorro, A. Pérez-García, and de A. Vicente. 2016. Characterization of resistance to six chemical classes of site-specific fungicides registered for gray mold control on strawberry in Spain. Plant Dis. 100:2234–2239.
- Fungicide Resistance Action Committee (FRAC). 2017. FRAC code list: fungicides sorted by mode of action. Online publication. Fungicide Resisance Action Committee. http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2017-final.pdf?sfvrsn=2
- Hu, M.-J., K.D. Cox, and G. Schnabel. 2016. Resistance to increasing chemical classes of fungicides by virtue of “selection by association” in *Botrytis cinerea*. Phytopathology 106:1513–1520.
- Hunter, T., K.J. Brent, G.A. Carter, and J.A. Hutcheon. 1987. Effects of fungicide spray regimes on incidence of dicarboximide resistance in grey mould (*Botrytis cinerea*) on strawberry plants. Ann. Appl. Biol. 110:515–525.
- Johnson, K.B., T.L. Sawyer, and M.L. Powelson. 1994. Frequency of benzimidazole-resistant and dicarboximide-resistant strains of *Botrytis cinerea* in western Oregon small fruit and snap bean plantings. Plant Dis. 78:572–577.
- Karaoglanidis, G.S., Y. Luo, and T.J. Michailides. 2010. Competitive ability and fitness of *Alternaria alternata* isolates resistant to QoI fungicides. Plant Dis. 95:178–182.
- Katan, T. 1982. Resistance to 3,5-dichlorophenyl-N-cyclic imide (“dicarboximide”) fungicides in the grey mould pathogen *Botrytis cinerea* on protected crops. Plant Pathol. 31:133–141.
- Lalève, A., S. Fillinger, and A.-S Walker. 2014. Fitness measurement reveals contrasting costs in homologous recombinant mutants of *Botrytis cinerea* resistant to succinate dehydrogenase inhibitors. Fungal Genet. Biol. 67:24–36.
- Leroux, P., R. Fritz, D. Debieu, C. Albertini, C. Lanen, J. Bach, M. Gredt, and F. Chapeland. 2002. Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest Manag. Sci. 58:876–888.

- Ma, Z., and T.J. Michailides. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853–863.
- Northover, J. and J.A. Matteoni. 1986. Resistance of *Botrytis cinerea* to benomyl and iprodione in vineyards and greenhouses after exposure to the fungicides alone or mixed with captan. Plant Dis. 70:398–402.
- Raposo, R., V. Gomez, T. Urrutia, and P. Melgarejo. 2000. Fitness of *Botrytis cinerea* associated with dicarboxamide resistance. Phytopathology 91:1246–1249.
- Ren, W., W. Shao, X. Han, M. Zhou, and C, Chen. 2016. Molecular and biochemical characterization of laboratory and field mutants of *Botrytis cinerea* resistant to fludioxonil. Plant Dis. 100:1414–1423.
- Saito, S., L. Cadle-Davidson, and W.F. Wilcox. 2014. Selection, fitness, and control of grape isolates of *Botrytis cinerea* variably sensitive to fenhexamid. Plant Dis. 98:233–240.
- Van den Bosch, F., N. Paveley, F. van den Berg, P. Hobbelen, and R. Oliver. 2014. Mixtures as a fungicide resistance management tactic. Phytopathology 104:1264–1273.
- Veloukas, T., P. Kalogeropoulou, A.N. Markoglou, and G.S. Karaoglanidis. 2014. Fitness and competitive ability of *Botrytis cinerea* field isolates with dual resistance to SDHI and QoI fungicides, associated with several sdhB and the cytb G143A mutations. Phytopathology 104:347–356.
- Yin, Y.N., Y.K. Kim, and C.L. Xiao. 2011. Molecular characterization of boscalid resistance in field isolates of *Botrytis cinerea* from apple. Phytopathology 101:986–995.
- Yourman, L.F., S.N. Jeffers, and R.A. Dean. 2001. Phenotype instability in *Botrytis cinerea* in the absence of benzimidazole and dicarboximide fungicides. Phytopathology 91:307–15.