Improving Strawberry Gray Mold Management through Identification of Ineffective Sprays

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Summary

Gray mold of strawberry, caused by *Botrytis cinerea*, is a very destructive pre- and post-harvest fruit rot and is routinely managed by foliar fungicide applications. Outside of California, resistance of *B. cinerea* has been reported to every single-site active ingredient currently labelled for use against gray mold. We characterized the resistance profiles of 888 *B. cinerea* isolates to ten active ingredients. Isolates were collected from 47 California strawberry fields during the early-season (zero to eight fungicide applications) and late-season (16 to 26 fungicide applications) of 2016.

Sensitivity of each isolate was determined for each fungicide using the following discriminatory dosages (µg/ml): boscalid (75), cyprodinil (4), fenhexamid (50), fludioxonil (0.5), fluopyram (10), iprodione (10), isofetamid (5), penthiopyrad (5), pyraclostrobin (10), and thiophanate-methyl (100). The percentage of conventional field isolates (early-season/late-season) resistant to each active ingredient was: boscalid (12/35), cyprodinil (12/46), fenhexamid (53/91), fludioxonil (1/4), fluopyram (2/7), iprodione (24/8), isofetamid (0/1), penthiopyrad (8/25), pyraclostrobin (77/98), and thiophanate-methyl (81/96). Resistance between early-season and late-season increased for all active ingredients except for iprodione, likely due to its restricted use. The observed shift in resistance to most fungicides within one season demonstrates the rapid, within-season selection of resistant subpopulations.

Introduction

Strawberry gray mold, caused by *Botrytis cinerea*, is responsible for yield losses in strawberries as well as many other crops (Williamson et al., 2007). Fungicides are typically applied at regular intervals throughout the season to manage gray mold; however, populations of *B. cinerea* with multiple chemical class resistances (CCR) are a rising concern (Fernández-Ortuño et al., 2014; Konstantinou et al., 2015; Saito et al., 2016).

All site-specific fungicides labeled for gray mold of strawberry belong to eight chemical classes. Fungicide Resistance Action Committee (FRAC) codes 1, 2, 7, 9, 11, 12, 17, and 19 represent the methyl benzimidazole carbamate (MBC), dicarboxamide (DC), succinate dehydrogenase inhibitor (SDHI), anilinopyrimidine (AP), quinone outside inhibitor (QoI), phenylpyrrole (PP), hydroxyanilide (HA), and polyoxin chemical classes, respectively. Active ingredients are classified according to their cross-resistance behavior (FRAC 2017). In the U.S., *B. cinerea* isolates collected from strawberries have been reported to be resistant to fungicides within every chemical class except for the polyoxins (Dowling et al., 2016; Fernández-Ortuño et al., 2015). In the southeastern U.S., individual isolates of *B. cinerea* have been found to be resistant to seven chemical classes (7CCR) (Fernández-Ortuño et al., 2015). Although 5CCR isolates have been found to be competitively disadvantaged compared to sensitive isolates, these populations may persist because of selection by the application of multiple site-specific fungicides associated with these resistances every season (Chen et al., 2016; Hu et al., 2016a).

Reports of resistance of *B. cinerea* to fungicides changing between seasons and within a season demonstrate that resistance management strategies have the potential to prevent or delay fungicide resistance development (Adaskaveg and Gubler 2006; Fernández-Ortuño et al., 2014). With a limited number of chemical classes available and recent reports of multi-fungicide resistance, the objective of this study was to (i) determine the frequency of resistance in California to boscalid, cyprodinil, fenhexamid, fludioxonil, fluopyram, iprodione, isofetamid, penthiopyrad, pyraclostrobin, and thiophanate-methyl at two times within one season, and to (ii) investigate how fungicides applied during the same season affect the resistance observed at each sampling time.

Materials and Methods

Isolate collection. A total of 930 isolates of *B. cinerea* were collected from 37 conventional and 10 organic fields dispersed throughout California's southern (310 isolates), central (210 isolates), and northern (410 isolates) strawberry growing districts. Isolates were randomly sampled from a five to 10 acre block in each field by brushing an individually wrapped, sterile applicator stick (Fisher Scientific, Hampton, NH) against a sporulating lesion of gray mold. If sporulating fruit could not be found, green strawberry fruit were collected, surface sterilized, and incubated in the lab until signs of gray mold appeared as described previously (Fernández-Ortuño et al., 2014). The same five to 10 acre block in each field was sampled twice: once before most fungicides had been applied that season (early-season) and once after most fungicides had been applied that season (late-season). About 10 isolates were collected per field at each sampling time.

Mycelial growth assay. An assay was conducted that used a single fungicide dose mixed in appropriate media capable of differentiating sensitive from resistant isolates to each fungicide tested (Table 1). Fungicide dosages from a previous study were used (Fernández-Ortuño et al., 2014; Weber and Hahn, 2011). Sterile toothpicks were used to transfer spores of each bulk conidial isolate upon the different fungicide amended media in wells [0.59 in (15 mm) in diameter] of 24-well plates (Thermo Fisher Scientific). Inoculated plates were incubated at room temperature (22 C) for four days and diametric colony growth was visually assessed in each well: sensitive (S) for less than 20% diametric growth, and resistant (R) for more than 20% diametric growth compared with the 15 mm-well diameter.

Table 4. Discriminatory concentrations and media used in the mycelial growth assay to monitor resistance in *Botrytis cinerea*[z]

Example trade name	Active ingredient (FRAC code)	Concentration (µg/ml)	Medium
Non-amended	N/A	N/A	CZA
Vangard	Cyprodinil (9)	4.0	CZA
Scholar SC	Fludioxonil (12)	0.5	MEA
Elevate	Fenhexamid (17)	50.0	MEA
Rovral	Iprodione (2)	10.0	MEA
Topsin M	Thiophanate-methyl (1)	100.0	MEA
Cabrio	Pyraclostrobin (11)	10.0	MEA + SHAM at 100 µg/ml
Endura	Boscalid (7)	75.0	YBA*
Luna Privilege	Fluopyram (7)	10.0	YBA
Fontelis	Penthiopyrad (7)	5.0	YBA
Kenja	Isofetamid (7)	5.0	YBA

[z]Fungicide concentrations and media were previously described by Weber and Hahn (2011). FRAC = Fungicide Resistance Action Committee, CZA = CzapeK-Dox agar medium, MEA = malt extract agar, SHAM = the alternative oxidase. inhibitor salicyl hydroxamic acid, YEA = yeast bacto acetate agar; this media avoids the interference of sugars with the assay (Weber and Hahn, 2011).

Fungicide use survey. In California, all pesticide use is reported to the California Department of Pesticide Regulation. This reporting is facilitated by the Agricultural Commissioner offices in each county. In 2017, a public records request for pesticides applied in 2016 was submitted to the counties of the southern (Ventura county), central (San Luis Obispo and Santa Barbara counties), and northern (Monterey and Santa Cruz counties) strawberry growing districts. Fungicide use data of products labeled for gray mold of strawberry in 32 of the sampled conventional ranches was analyzed.

Results

The diametric mycelial growth on fungicide-amended media was visually assessed and 888 of 930 isolates were pure or viable. The percentage of all *B. cinerea* isolates that were resistant to pyraclostrobin, thiophanate-methyl, fenhexamid, cyprodinil, boscalid, penthiopyrad, iprodione, fluopyram, fludioxonil, and isofetamid was 90, 85, 66, 26, 23, 15, 14, 4, 2 and 1%, respectively. Resistance generally increased from the early-season to the late-season for all active ingredients except for iprodione. Resistance was generally lower in organic fields and did not increase as dramatically from the early-season to the late-season (data not shown). Frequencies of resistance varied from district to district (Figure 1). In the southern district, resistance to boscalid, cyprodinil, and penthiopyrad increased over 40% from the early-season to the late-season. Resistance to isofetamid was only observed in the late-season in the northern district. Resistance varied greatly from field to field even within the same district.

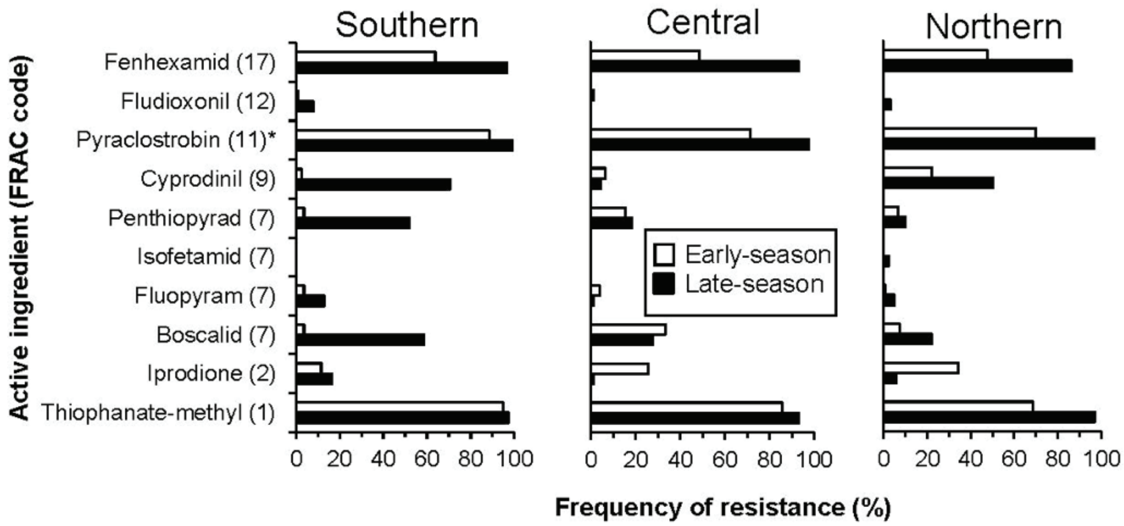

Figure 2. Early-season and late-season frequencies of resistance to ten active ingredients from *Botrytis cinerea* isolates collected from conventional fields in the Northern, Central, and Southern strawberry growing districts of California
*Fewer isolates were tested for resistance to pyraclostrobin.

Resistance phenotypes. Only isolates that tested resistant to pyraclostrobin are included in this section. Of all isolates collected, there were 45 distinct resistance patterns. The most common resistance pattern was isolates resistant to fenhexamid, pyraclostrobin, and thiophanate-methyl. The ten active ingredients in this study are within seven chemical classes. The most common chemical class resistance phenotype was CCR3 both in the early-season and late-season collections. There was a general shift towards isolates being resistant to more chemical classes from the early-season to the late-season. In the early-season, no 7CCR isolates were observed, but in the late season 7CCR isolates were found in the Northern district (Figure 2). No 7CCR isolates were found in any other regions.

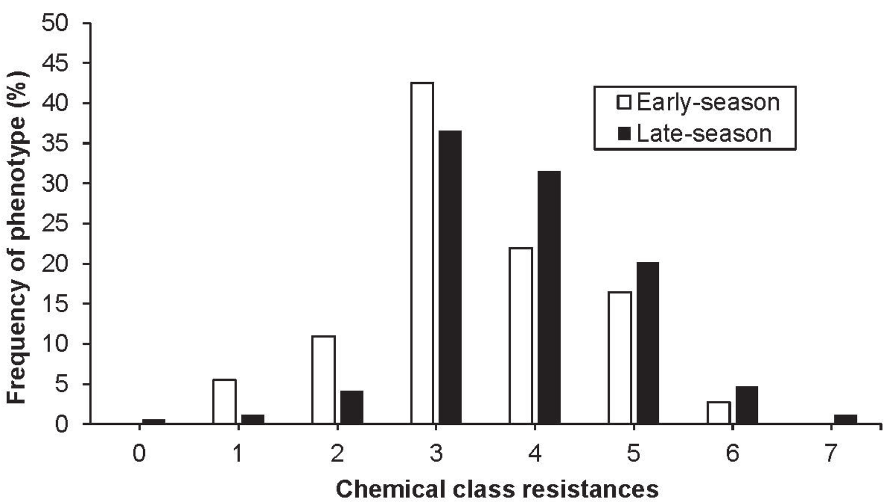

Figure 2. Early-season and late-season frequencies of chemical class resistance (CCR) phenotypes from *B. cinerea* isolates collected in the northern, central, and southern strawberry growing districts of California.

Fungicide use survey. Between the early-season and late-season sampling dates, there was an average of 15 applications of fungicides labeled for gray mold of strawberry at an average interval of 12 days. The practice of rotating modes of action with every application was universally employed. Captan, a multi-site fungicide, was the most often applied fungicide, while thiram was rarely used (Figure 3). Switch (cyprodinil + fludioxonil), Pristine (boscalid + pyraclostrobin), and Elevate (fenhexamid) were the most frequently applied products in the 32 surveyed ranches.

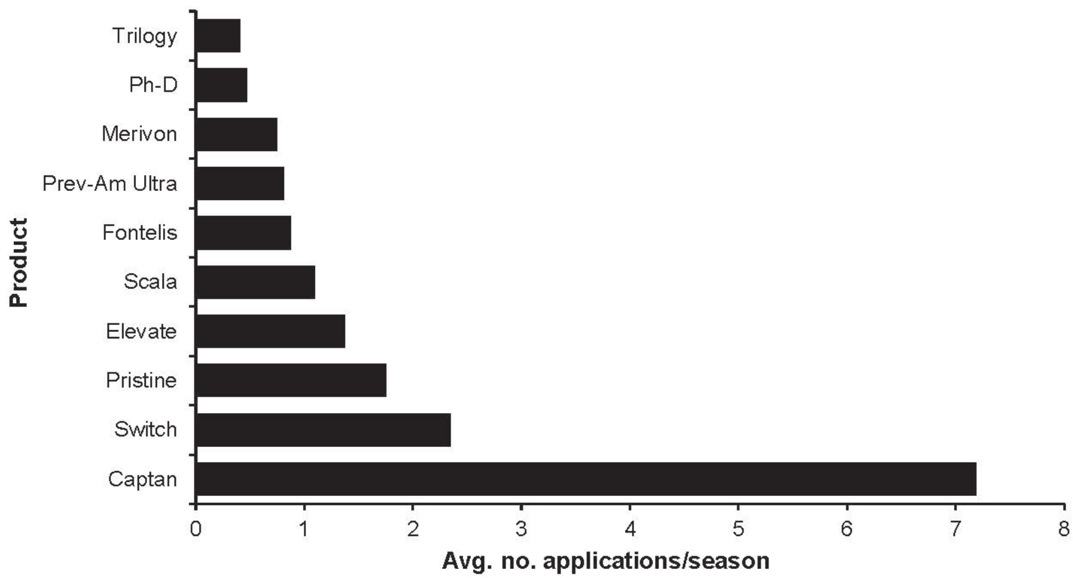

Figure 3. Average number of applications/season of the top ten most frequently applied fungicides labeled for gray mold of strawberry in 32 California strawberry farms in 2016.

DISCUSSION

Resistance of *B. cinerea* to fungicides has been previously described in California (Mercier et al., 2010; Adaskaveg and Gubler 2006), but this study is more comprehensive in that it documents resistance to all the active ingredients currently used and does so using a large number of isolates from all the fruit growing regions of the state. Resistant populations are widespread in California strawberry production. High frequencies of resistance to fenhexamid, pyraclostrobin, and thiophanate-methyl was found in most fields. Our survey showed that although the use of MBCs in fruit production fields is rare, resistance remains high. For *B. cinerea*, resistance to MBCs does not entail a significant fitness cost, and other studies in the United States have also observed high frequencies of resistance (Fernández-Ortuño et al., 2014; Johnson et al., 1994; Raposo et al., 1996). Because of very high levels of resistance (85%) to thiophanate-methyl, this product should not be used for gray mold management, but may be effective for other diseases.

We documented a within-season shift in fungicide resistance and found that an average of 15 applications of fungicides labeled for gray mold were used within the same season. The shift in resistance was towards an increased frequency of resistance to all fungicides except for iprodione. The 15 fungicide applications may be the selection pressure responsible for the shift (Adaskaveg and Gubler, 2006). We observed a considerable increase in resistance to the frequently used fungicides cyprodinil, boscalid, fenhexamid, however, the PP fungicide, fludioxonil, remained robust. Similar to recent studies, fludioxonil resistance was rare, supporting the evidence that the fludioxonil resistance may not be stable (Chen et al., 2016; Fernández-Ortuño et al., 2014). Resistance even tended to increase to thiophanate-methyl, which was rarely used in the 2016 season. This may be attributed to selection by association, a phenomenon that suggests that fungicide resistance determinants can 'piggy back' alongside unrelated fungicide resistance genes even though there is no direct selection pressure (Hu et al., 2016a). Resistance to iprodione may have decreased because this fungicide tends to only be applied in nurseries, and resistance to this active ingredient may entail a fitness cost, although previous studies show mixed results (Johnson et al., 1994; Raposo et al., 1996).

There was a general shift towards isolates being resistant to more chemical classes. Studies have shown CCR to fungicides changing from year to year (Fernández-Ortuño et al., 2014). The results of this study show a rapid, within-season shift in resistance to fungicides. Fungicides that may have been effective at the beginning of the season, may not control gray mold at the end of the season. More research needs to be conducted to observe how fungicide applications affect frequencies of resistance changing within a field.

The SDHI fungicides tested in this study had varying frequencies of resistance. Specific mutations within the sdhB region are associated with different resistance patterns among the SDHI fungicides (Fernández-Ortuño et al., 2017; Hu et al., 2016b). The older and more used SDHIs (boscalid and penthiopyrad) had the highest frequencies of resistance. SDHI active ingredients are sold under six different trade names currently labeled for gray mold of strawberry. For this reason, rotation among fungicide chemical classes (FRAC codes), not just among different active ingredients or trade names, should continue to be emphasized and practiced.

None of the fungicides tested in this study are labeled for use in organic strawberry production, yet a similar resistance profile was observed in organic fields to that of early-season conventional fields. Resistant subpopulations of *B. cinerea* can be introduced into organic fields from wind-dispersed spores from nearby crop fields (Fitt et al., 1985). Infected strawberry transplants can also be a vehicle for disseminating resistant subpopulations of *B. cinerea* (Oliveira et al., 2017). Strawberry nurseries typically don't apply fungicides for gray mold management, however similar chemical classes are used for other diseases and non-target selection for resistant subpopulations of *B. cinerea* is possible. Resistance management strategies should also be implemented in nurseries.

The site-specific fungicides in this study are critical for gray mold management during high disease pressure. Resistance management strategies are key for their sustained efficacy. The multi-site fungicides captan or thiram should be tank mixed whenever a site-specific fungicide is applied (Hu et al., 2016a). A weather based disease warning tool, the Strawberry Advisory System, has shown that when disease pressure is low, a fungicide may not need to be applied at all (Bulger et al., 1987; Pavan et al., 2011). Cultural methods can also be effective ways to decrease gray mold incidence (Janisiewicz et al., 2016; Legard et al., 1997, 2000; Xiao et al., 2001). Since polyoxin-D is a member of a new chemical class to strawberries and is already popular, it needs to be used with care to prevent resistance development (Dowling et al., 2016).

Acknowledgements

This project was co-funded by the California Strawberry Commission and the California Department of Food and Agriculture Specialty Crop Block Grant Program. We thank the multitude of cooperative growers, Randy Widerburg, Sage Finch, Mark Edsall, Jason Sharrett, Dan Chellemi, Driscoll's, Naturipe, and the Schnabel lab.

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