Resistance of Strawberry Powdery Mildew to Fungicides in California

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Summary

Field observations of poor fungicide performance suggest that reduced fungicide sensitivity exists in field populations of *Podosphaera aphanis*, the causal agent of strawberry powdery mildew (SPM). Strawberry powdery mildew is one of the most common diseases in strawberry production and is controlled using foliar fungicide applications. This study characterizes the sensitivity of 19 *P. aphanis* isolates to the most common fungicides used against SPM in California. Isolates were collected from commercial fruit production fields in Oxnard, Ventura, Santa Maria, Salinas, and Watsonville, and from a plant nursery in Balico, California. Healthy, unfurled strawberry leaves (cv. 'Monterey') free of any visual disease symptoms were removed from actively growing plants and treated with one of six commercially formulated fungicides using the minimum labeled rate and inoculated with conidia of *P. aphanis*. Inoculated leaves were incubated at 20°C under 16/8 hours of day/night lighting and assessed for disease incidence (%) at 14 days. Pathogen growth on the treated leaflets constituted a measure of insensitivity to the fungicide. The six fungicide treatments and the average resulting disease incidence for the 19 isolates are: penthiopyrad (Fontelis®) (51.4%), quinoxyfen (Quintec®) (41.5%), myclobutanil (Rally® 40WSP) (39.8%), trifloxystrobin (Flint®) (19.8%), cyflufenamid (Torino®) (19.3%), and fluopyram + trifloxystrobin (Luna® Sensation) (3.5%). The average disease incidence for the trifloxystrobin treatment was raised significantly by two isolates considered to be resistant to the product (disease incidence > 66%) Two isolates collected from organic production systems were sensitive to all fungicides. Although some level of resistance exists to most of the currently used fungicides, high levels of fungicide efficacy remain for some active ingredients. The remaining efficacy among fungicides should be managed to improve efficacy and reduce selection pressure towards resistance.

Introduction

Strawberry powdery mildew (SPM) is caused by the obligate parasite *Podospheara aphanis* and affects all above-ground parts of the strawberry plant (i.e., fruit, leaves, and stolons). Strawberry powdery mildew infections can reduce yield through reducing the photosynthetic capabilities of leaves as well as infect fruit directly and render it unmarketable (Horn et al., 1972). SPM infection and development is favored by cool temperatures (15-25°C) and high relative humidity (>35%) (Miller et al., 2003). These conditions occur throughout the strawberry growing season in all major coastal production regions in California (Bolda and Koike, 2015).

Mildew infection is difficult to detect and easy to control at early stages and becomes easier to detect and more difficult to control as the infection advances. Leaves and fruit are most susceptible to infection at early growth stages (Asalf et al., 2016). Therefore, early infections are difficult to detect as these leaves and fruit are typically somewhat hidden within the plant canopy. Signs of infection also first show on the abaxial side of the leaf and can be hard to see among trichomes and lighter coloration. Additionally, developing colonies are visually similar to whitefly secretions and fungicide residue which further complicates early detection (UC IPM 2020).

Conducive conditions throughout the season combined with difficulties in early detection often lead growers to control SPM with curative fungicide applications. During peak production (six to eight weeks of the season) some growers will make biweekly fungicide applications to control SPM (*personal communication*). The most common fungicide groups used to control SPM are: Fungicide Resistance Action Committee (FRAC) groups 3 - demethylation inhibitors (DMI), 7 - succinate dehydrogenase inhibitors (SDHI), 11 - quinone outside inhibitors (QoI), 13 – azanaphthalenes (AZN), and unknown (U6). Fungicides in these chemical classes are also used to control a more economically significant disease, Botrytis fruit rot caused by *Botrytis cinerea*, and can select for resistant populations of SPM even if it is not the targeted disease. Each of these chemical classes has a single-site mode of action and targets a specific process in cellular development or respiration. The only chemical with a multi-site mode of action used to control SPM is sulfur, however it is a contact fungicide used for preventative control (CDPR 2017).

Despite efforts to follow effective integrated pest management practices and guidelines outlined by FRAC, frequent applications of single-site fungicides are still made and can lead to selection of resistant populations. Mutations in fungal populations occur at low and random rates (Brent and Hollomon, 2007). However, once a mutation occurs that allows a population to overcome a single-site mode of action, repeated applications of a chemical with that mode of action will select for that population and allow it to reproduce, thus increasing its proportion in the population. Resistance to the above listed FRAC codes has been reported in powdery mildews of grape (Gubler et al., 1996; Miller and Gubler, 2004; Colcol et al., 2012), cucurbits (McGrath 2001; Vielba-Fernández et al., 2018; Pirondi et al., 2014), and wheat (Fraaije et al., 2002). Resistance to DMI fungicides has also been reported in populations of SPM in France, Italy, and Israel (Pertot et al., 2005; Sombardier et al., 2010). Though Pertot et al., (2005) also studied SPM sensitivity to QoI fungicides, no resistance has been documented in SPM to fungicide chemical classes other than FRAC group 3. To the best of the authors' knowledge, no characterization of fungicide resistance in SPM has been done in California or the United States.

The aim of this work was to characterize fungicide sensitivity of *P. aphanis* in California strawberry production to commonly used fungicides. A fungicide assay was developed to process multiple isolates of *P. aphanis* and determine their sensitivity to fungicides from a diverse range of FRAC codes. A single isolate studied in the lab assay was also used in a potted plant, greenhouse spray trial to verify if lab results would predict performance in the greenhouse.

Materials and Methods

Isolate collection and preparation. Leaves and/or fruit showing signs of powdery mildew were collected from commercial production fields in Oxnard, Ventura, Santa Maria, Salinas, and Watsonville, CA, as well as a nursery production field in Balico, CA. No more than a single isolate was collected from an individual field. 'Monterey' strawberry leaflets in early (not yet unfolded) growth stages were collected from an outdoor field at Cal Poly. These leaflets were used in the assay as they are most susceptible to infection at early growth stages and 'Monterey' is considered to be a susceptible cultivar. Leaflets were sterilized for three minutes in 0.5% NaOHCl and Tween 20 (0.01 fl oz/gal). Each isolate was brought back to the lab and brushed onto the disease-free leaflets using a camelhair brush and Andersen spore cascader. The entire surface of the infected leaves or fruit was brushed over to ensure maximum inoculum transfer. Inoculated leaflets were placed onto petri dishes of benzimidazole-amended (0.07 oz/gal) water agar and stored in a growth chamber at 20°C and 16/8 hours light/dark for 14 days to allow for uniform growth of the pathogen.

Lab fungicide assay. Detached leaflets were again collected and sterilized as described above. After sterilization, leaves were rinsed with deionized water and treated with one of six fungicides. Each leaflet was dipped (1 second) into a fungicide suspension (Table 1) three times in succession and placed on a paper towel to dry. After drying, leaflets were placed on a Petri dish containing benzimidazole-amended water agar. A treatment composed one Petri dish containing three leaflets, replicated three times (one rep/Petri dish).

Each replication of three treated leaflets was placed onto the Andersen spore cascader and inoculated by brushing a 0.16 in^2 sporulating colony from the previously incubated leaflets aided by a vacuum. The inoculated, fungicide treated leaves were then placed onto the Petri dish with water agar and stored in a growth chamber at the conditions described above for 14 days.

Table 1. List of fungicides used in the resistance assay.

Active ingredients	Trade name	AI % by weight	FRAC code(s)	Rate	Resistance risk *
trifloxystrobin	Flint®	50	11	0.15 g/L	high
penthiopyrad	Fontelis®	20.4	7	1.25 mL/L	high
fluopyram + trifloxystrobin	Luna® Sensation	21.4, 21.4	7 + 11	0.312 mL/L	N/A
quinoxyfen	Quintec®	22.58	13	0.312 mL/L	medium
myclobutanil	Rally® 40WSP	40	3	0.187 g/L	medium
cyflufenamid	Torino®	10	U6	0.265 mL/L	reported in *Sphaerotheca*

*Resistance risk reported from FRAC Code List 2020.

Data collection and analysis. After 14 days, leaflets were evaluated for disease incidence with the aid of a dissecting microscope. Disease incidence was defined as the presence of a sporulating colony on a leaflet. A Petri dish containing three leaflets was assigned a disease incidence score of 0%, 33%, 67%, or 100%, if there were sporulating colonies on zero, one, two, or three leaflets, respectively. Disease incidence for each treatment averaged over all isolates was compared using a one-way ANOVA and means were separated by Tukey HSD post hoc testing in JMP 14 (SAS Institute Inc. Cary, NC)**.**

Greenhouse fungicide evaluation. Bareroot 'Monterey' strawberry transplants were established in 6-inch pots in a mixture of peat (35%), perlite (15%), bark (25%), and coconut coir (25%) under high plastic tunnels. After four weeks, the disease-free plants were moved into a greenhouse where a SPM epidemic was present on mature plants. Plants were arranged into plots of four with an infected spreader plant between each plot. Each treatment had four replicates arranged in a randomized complete block design. Four weeks after transferring plants into the greenhouse, each plot was sprayed with its assigned fungicide treatment. This was repeated weekly for the next five weeks for a total of six applications. Two weeks after the final application, each plot was rated for disease incidence (number of infected leaves per plot/total leaf count of plot).

At the end of the experiment, infected leaves were collected from the non-treated plots and processed through the lab fungicide assay described above. Mean disease incidence was compared within the trial and within the assay using a one-way ANOVA and Tukey HSD post-hoc testing. Results from the greenhouse evaluation and lab assay were used to generate a Pearson correlation coefficient.

Results

Lab fungicide assay. Resistance to each treatment varied greatly across the 19 isolates assayed (Table 2). The non-treated had the highest disease incidence with a mean of 93.6% and was significantly different from all other treatments. Penthiopyrad, quinoxyfen, and myclobutanil were less effective with disease incidences of 51.4%, 41.5%, and 39.8%, respectively. Trifloxystrobin, cyflufenamid, and fluopyram + trifloxystrobin were the more effective with disease incidence at 19.8%, 19.3%, and 3.5%, respectively (Figure 1). Penthiopyrad was significantly different from all treatments categorized as less effective. Fluopyram + trifloxystrobin was significantly different from all treatments categorized as more effective. An isolate with >66% disease incidence to a treatment was considered to be resistant to that treatment. The number of resistant isolates for each treatment was: penthiopyrad (7), myclobutanil (7), quinoxyfen (5), trifloxystrobin (2), cyflufenamid (1), fluopyram + trifloxystrobin (0).

Two isolates collected from organic production systems (8 and 13) were sensitive to all treatments. Two isolates (15 and 19) were found to be entirely resistant (disease incidence 100%) to penthiopyrad and one isolate (1) to quinoxyfen. Fluopyram + trifloxystrobin had no individual isolates with a disease incidence >11%.

Table 2. Disease incidence (%) of 19 isolates for six fungicide treatments. Treatment means that do not share the same letter are significantly different according to Tukey HSD post-hoc testing.

Isolate	Date Collected	Location	non-treated	trifloxy-strobin	Penthio-pyrad	fluo-pyram + trifloxy-strobin	quinoxy-fen	myclo-butanil	cyflufen-amid
1	6 Mar 2019	Santa Maria, CA	100.0	0.0	66.7	0.0	100.0	66.7	44.3
2	4 Apr 2019	San Luis Obispo, CA	100.0	22.0	44.3	0.0	22.3	77.7	0.0
3	20 Nov 2019	Santa Maria, CA	89.0	89.0	44.3	11.0	55.7	77.7	0.0
4	20 Nov 2019	Santa Maria, CA	89.0	11.0	44.3	0.0	11.0	22.3	22.3
5	26 Nov 2019	Balico, CA	89.0	0.0	44.3	0.0	11.0	33.0	11.0
6	24 Jan 2020	Oxnard, CA	100.0	0.0	78.0	11.0	67.0	44.7	44.3
7	24 Jan 2020	Oxnard, CA	89.0	11.0	67.7	0.0	55.7	89.0	78.0
8*	24 Jan 2020	Ventura, CA	77.7	0.0	0.0	0.0	0.0	0.0	0.0
9	27 Feb 2020	San Luis Obispo, CA	100.0	11.0	33.3	0.0	0.0	0.0	0.0
10	26 Mar 2020	Watsonville, CA	100.0	11.0	22.0	0.0	11.0	0.0	0.0
11	26 Mar 2020	Watsonville, CA	89.0	11.0	55.7	0.0	43.3	0.0	0.0
12	26 Mar 2020	Salinas, CA	89.0	0.0	43.3	0.0	33.3	11.0	0.0
13*	22 Apr 2020	Watsonville, CA	100.0	0.0	0.0	0.0	0.0	0.0	0.0
14	27 Apr 2020	Santa Maria, CA	77.7	33.3	33.0	11.0	33.3	66.7	55.7
15	27 Apr 2020	Santa Maria, CA	100.0	67.0	100.0	11.0	100.0	67.0	0.0
16	11 Jun 2020	Santa Maria, CA	89.0	33.3	66.7	0.0	66.7	55.7	0.0
17	6 Jul 2020	Santa Maria, CA	100	11	44.3	11	78	33.3	0
18	9 Jul 2020	Santa Maria, CA	100	22	89	0	33.3	33.3	55.3
19	13 Jul 2020	Oxnard, CA	100	44.3	100	11	66. 7	78	55. 7
Average			**93.6**	**19.8**	**51.4**	**3.5**	**41.5**	**39.8**	**19.3**
			a	cd	b	d	bc	bc	cd

*Isolate collected from organic production.

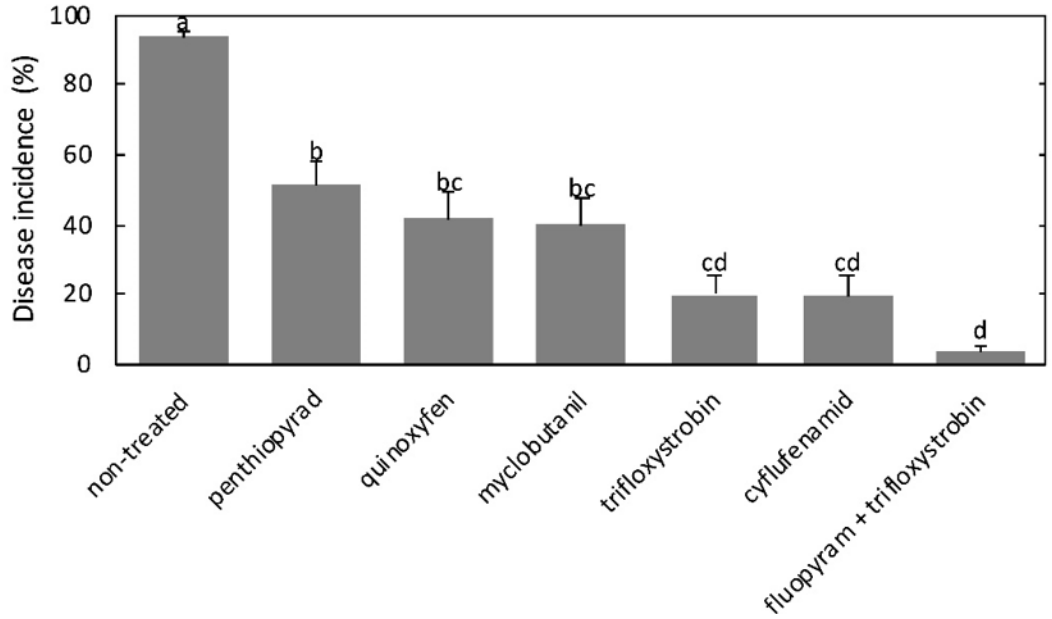

Figure 1. Average disease incidence (%) of each treatment for 19 isolates of SPM. Treatments that do not share a letter are significantly different according to Tukey HSD post-hoc testing.

Greenhouse fungicide evaluation. The mean disease incidence scores taken two weeks after the final spray application of the greenhouse fungicide evaluation showed varying efficacy among the treatments (Figure 2). The non-treated was significantly different from all treatments. Penthiopyrad and quinoxyfen were the treatments with the highest disease incidence. The trifloxystrobin, cyflufenamid, and fluopyram + trifloxystrobin treatments had lower disease incidence and were significantly different from the two penthiopyrad and quinoxyfen treatments. The myclobutanil treatment was only significantly different from the fluopyram + trifloxystrobin treatment and the non-treated control.

The results from the greenhouse fungicide evaluation were significantly correlated with the results from the lab assay with a Pearson correlation coefficient of 0.6. A notable exception was the myclobutanil treatment in the lab assay having a disease incidence of 0% and being significantly different from the penthiopyrad and quinoxyfen treatments. Another exception was the trifloxystrobin treatment which was only significantly different from the non-treated (Figure 3).

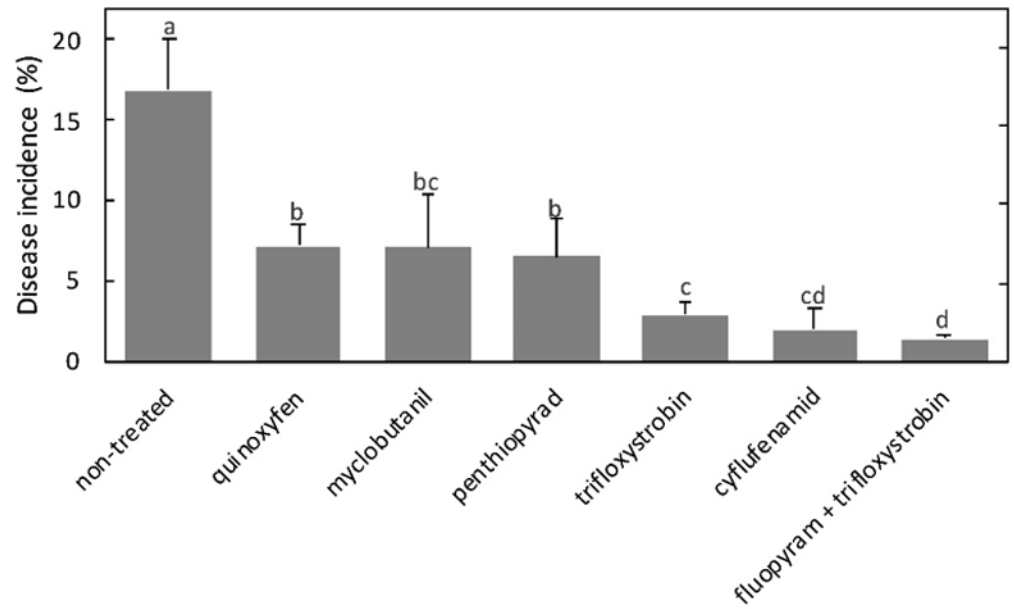

Figure 2. Average disease incidence (%) of SPM for each treatment in the greenhouse fungicide evaluation. Treatments that do not share a letter are significantly different according to Tukey HSD post-hoc testing.

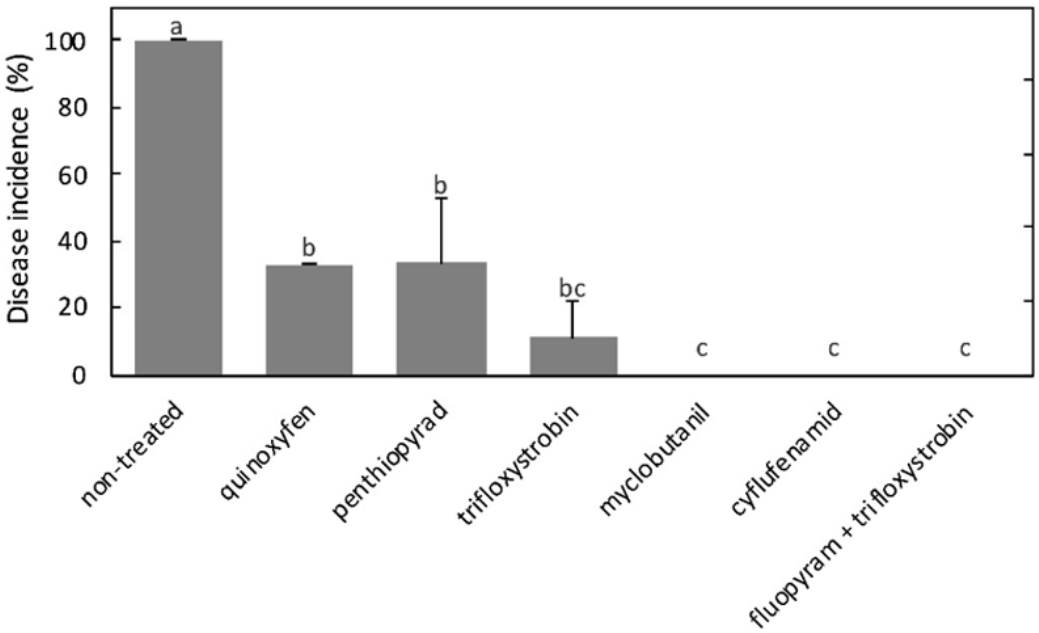

Figure 3. Average disease incidence (%) of the greenhouse SPM isolate for each treatment in the lab assay. Treatments that do not share a letter are significantly different according to Tukey HSD post-hoc testing.

Discussion

Fungicide resistance has been characterized in various species of powdery mildew to fungicides in FRAC groups 3 (Gubler et al., 1996; McGrath et al., 2001), 7 (Colcol and Baudoin, 2016; Kleczwesk et al., 2020), 11 (Fraaije et al., 2002; Vielba-Fernández et al., 2018), 13 (Colcol and Baudoin, 2016), and U6 (Pirondi et al., 2014). There is documented resistance to DMIs in SPM populations (Pertot et al., 2005; Sombardier et al., 2010) in Europe and Israel. This study adds to the documentation of fungicide resistance in powdery mildews and shows that SPM in California has developed resistance to fungicides used for its control.

Fungicide sensitivity among the 19 isolates show a range of efficacy among the six fungicides. Reduced sensitivity in *P. aphanis* was documented in some isolates to all fungicides except fluopyram + trifloxystrobin. Several isolates showed disease incidence greater than or equal to 66.7% to myclobutanil, penthiopyrad, and quinoxyfen (FRAC groups 3, 7 and13, respectively). Although isolates resistant to trifloxystrobin and cyflufenamid were found, they were few and suggest a low level of resistance in the industry.

Fluopyram + trifloxystrobin had the least recorded resistance of all treatments in the lab fungicide assay. It was also the only product with two active ingredients of different FRAC groups. Both fluopyram + trifloxystrobin and trifloxystrobin treatments shared the same active ingredient, yet fluopyram + trifloxystrobin was more effective. This implies that the increased efficacy is attributed to either the other active ingredient, fluopyram, or the mixture of two modes of action. Seeing that penthiopyrad, the other treatment with a FRAC group 7 active ingredient, was not as effective on its own, the latter conclusion is more likely. This finding adds to the established knowledge base of proper fungicide use in supporting the principle that using products with multiple modes of action helps prevent resistance development (Brent and Hollomon, 2007).

The two organic isolates processed in the lab fungicide assay were sensitive to all treatments. This was to be expected as the SPM collected from these fields should not have been exposed to the fungicides used in the assay as they are all conventional products. It may also indicate that fungicide resistance development is a local phenomenon. This could indicate that *P. aphanis* conidia may not survive dispersal over relatively short distances due to their ephemeral nature.

As an obligate pathogen, *P. aphanis* is difficult to work with in a lab setting. The fungicide assay developed for this study proved to be a viable method for evaluating resistance of multiple isolates of SPM. This finding is supported by the significant correlation of results obtained from the lab assay with those obtained from the greenhouse fungicide evaluation. Additionally, the lab assay details a process of propagating SPM at a high success rate without cross-contamination. This can be of use to those looking to study the disease, especially if working with multiple isolates and limited space. Conidial germination on glass slides has also been used to evaluate fungicide resistance in powdery mildews (Miles et al., 2012), but due to the complex nature of host-pathogen interaction in powdery mildews, stronger conclusions can be drawn from a process involving both host and pathogen. This is supported by the work of Pertot et al., (2005) comparing results from a glass slide germination assay and leaf assay.

This study is novel in characterizing fungicide resistance in SPM in California and therefore opens the door for future studies. The high efficacy of Luna® Sensation raises the question of efficacy of the product being attributed to each individual active ingredient or the combination of two different modes of action. This could be determined by designing an experiment evaluating efficacy of products with multiple modes of action and comparing that to the efficacy of treatments of the individual active ingredients.

It would also be of use to design a study characterizing resistance of SPM to multiple active ingredients within the same FRAC code to determine presence of mutations that confer cross resistance. Finally, the differences in sensitivities to fungicides should be further evaluated in conventional and organic production systems. Findings from a study like this could be used to draw conclusions not only about the development of fungicide resistance in SPM populations, but about mobility of SPM conidia in general.

The findings of this study will help growers make more informed decisions on managing SPM using fungicides. Grower observations that fungicide efficacy has been compromised by resistance development is supported by this research. Strawberry growers should avoid the use of fungicides where high levels of resistance were found and judiciously use those fungicides with the lowest levels of resistance. Rotating fungicidal modes of action or use of fungicide premixes (i.e., with two active ingredients) should help to mitigate resistance development in the future.

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