STRAWBERRY POWDERY MILDEW CAUSED BY *PODOSPHAERA APHANIS*: FUNGICIDE
RESISTANCE AND HOST PLANT RESISTANCE

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ABSTRACT

STRAWBERRY POWDERY MILDEW CAUSED BY *PODOSPHAERA APHANIS*: FUNGICIDE RESISTANCE AND HOST PLANT RESISTANCE

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Strawberry powdery mildew, caused by *Podosphaera aphanis*, affects leaves, fruit, and runners of strawberry plants. Infected leaves have reduced photosynthetic capability and infected fruit become unmarketable. Both of these factors translate to economic loss for the grower and therefore merit taking measures to control the disease. One objective of this study was to evaluate the resistance developed in populations of strawberry powdery mildew to chemical control measures. A fungicide assay was developed to evaluate the efficacy of six treatments (penthiopyrad, quinoxyfen, myclobutanil, trifloxystrobin, cyflufenamid, fluopyram + trifloxystrobin) for control of the disease. Nineteen isolates of strawberry powdery mildew were collected from Balico, Salinas, Watsonville, San Luis Obispo, Santa Maria, Ventura, and Oxnard CA and tested through the assay. The number of isolates resistant to each treatment was: penthiopyrad (7), quinoxyfen (6), myclobutanil (7), trifloxystrobin (2), cyflufenamid (1), fluopyram + trifloxystrobin (0). This documents resistance in *P. aphanis* to multiple chemicals used for its control. Documentation of any resistance is novel in California and novel worldwide with resistance to Fungicide Resistance Action Committee (FRAC) codes 7 and 13. Another objective of this study was to evaluate host plant resistance to strawberry powdery mildew. Twelve cultivars were evaluated in a winter greenhouse trial, sixteen cultivars in a summer

greenhouse trial, and the ten cultivars shared in both trials were also evaluated in two fields. The cultivars found to be most susceptible to mildew infection were BG 3.324 and Royal Royce. The cultivars found to be the least susceptible to mildew infection were Fronteras, San Andreas, and Sweet Ann. The cultivars evaluated represent more than 55% of the state's acreage and the host plant resistance information will be a valuable tool to growers looking to culturally control powdery mildew.

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CHAPTER 1

Literature Review

1.1 Introduction

The California strawberry industry produces 800 million kilograms of fruit every year and is the sixth-largest economic contributor to agriculture in the state (CSC 2018b). This industry faces problems that are both unique to the crop and the climate in which it is grown. Due to the high value of the crop, losses to pests are a significant cost to the industry. Additionally, strawberries are susceptible to infection by many pathogens including *Podosphaera aphanis*, the causal agent of strawberry powdery mildew (SPM). SPM can reduce yield through infecting leaves and inhibiting photosynthesis as well as infecting fruit directly, rendering the product unmarketable (Bolda and Koike 2015). This high loss potential provides justification for growers and pest control advisors to utilize fungicides to control the pest. Though these applications are often effective, they place a heavy selection pressure on populations of *P. aphanis* leading to the development of field resistance. This literature review will discuss strawberry production, powdery mildew and its control, and fungicide resistance.

1.2 The Strawberry

Strawberry plants are herbaceous and grow trifoliate leaves, runners, and reproductive structures from a central crown. Wild strawberry plants are well adapted to sandy soils and different species can be found inhabiting a diverse range of habitats from costal dunes to high alpine mountain sides. The fruit of these wild varieties are typically very small. The modern strawberry (*Fragaria* x

ananassa) was first bred in the 1760s from an accidental cross of the two wild types *Fragaria chiloensis* and *Fragaria virginiana*. This cross was first botanically described in 1766 by Antione Nicolas Duchense. All commercially grown cultivars today stem from this first cross and have thicker leaves with a waxy cuticle and bear larger fruit. Botanically, the strawberry fruit is an aggregate of achenes. Though it is often thought of as a fruit with seeds attached externally, the "fruit" is actually a swollen receptacle while the "seeds" are the hardened fruit (achenes). Though they can be grown from seed, strawberries are most often propagated as runners (both commercially and in the wild). This means that genetic uniformity is well-maintained within populations and cultivated fields of strawberry plants (Darrow 1966).

1.2.1 The California Strawberry Industry. As mentioned above, billions of pounds of strawberries are produced in California annually, making the crop the second most economically significant fruit crop in the state, behind grapes. Over 34,000 acres were dedicated to growing strawberries in 2018 and produced 88% of the country's crop (CSC 2018b). Strawberries in California are grown in three major regions: Oxnard/Ventura, Santa Maria, and Salinas/Watsonville. All plants in these regions are propagated as bare root transplants. These transplants are produced in high densities by nurseries with low-elevation plantings in Turlock and Manteca, CA and high-elevation plantings in Macdoel, CA. The low-elevation plantings supply plants that go to the high-elevation plantings as well as other nurseries throughout the United States. Most planting for fruit production takes place in October with harvesting occurring from winter to

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early fall. Fruit production peaks in the southern regions first and moves north as the season progresses. The southern regions also have summer plantings that are planted in May and June and harvested October through December. All harvesting is done by hand. After harvest, fruit is rushed to the cooler where it is cooled and stored at 34°F.

Cultural practices vary among regions, but all commonly utilize raised beds of sandy loam soil covered in plastic mulch to promote soil drainage and manage weeds. Drip irrigation is used to precisely manage the plants' water needs while conserving water and limiting the spread of water-loving diseases such as Phytophthora root rot. Pest management programs also vary regionally but seek to mitigate crop losses due to weeds, insects, viruses, nematodes, bacteria, oomycetes, and fungi (CSC 2018b).

1.2.2 Fungal Pest Management in Strawberry. Of the above-mentioned pests, fungal diseases pose some of the greatest economic threat to strawberry production. Fungal diseases can be soilborne, airborne, or both and can affect the plant superficially and/or systemically (Maas 1984). Economically important soilborne diseases include: Verticillium wilt, Fusarium wilt, Macrophomina crown rot, and Phytophthora crown rot. Soilborne pathogens are most often managed with crop rotation, host resistance, and soil fumigation. Methyl bromide was the industry standard for fumigation until its phase out from commercial use ending in December 2016. Alternative fumigants and non-chemical treatments are being further evaluated for control of diseases.

Significant aerial diseases include: Botrytis gray mold, Rhizopus leak, Pestalotiopsis leaf spot, Anthracnose fruit rot, and SPM. Management tactics for foliar and fruit diseases vary more as each pathogen infects different parts of the plant and favors a range of environmental conditions. Diseases of above-ground plant parts are often managed with host resistance, environmental modification (i.e., drip irrigation instead of overhead), and fungicide applications (Koike et al. 2018).

1.3 Strawberry Powdery Mildew

SPM is caused by *Podosphaera aphanis* Wallr. (formerly *Sphaerotheca macularis*). It is a member of the family *Erysiphaceae* in the order Ascomycota (Bélanger et al. 2002). All powdery mildews are obligate biotrophs, meaning they require living host tissue to complete their life cycle. Powdery mildews also exhibit host specificity with each species only affecting a single to a few hosts. Obligate biotrophism and host specialization in powdery mildews may have evolved with and adapted to specific angiosperm host species during a boom in host range and diversity 70 million years ago (Takamatsu 2018). This holds true for *P. aphanis*, as strawberry is its only reported host.

Podosphaera aphanis is heterothallic and produces sexual ascospores contained within a chasmothecium (Gadoury et al. 2010). Ascospores, serve as the primary inoculum of the disease. These structures utilize specially formed appendages to attach to leaf litter or plant crowns and overwinter. *P. aphanis* also produces asexual spores known as conidia. Conidia grow basipetally in long chains from a conidiophore attached to the leaf surface. These are the secondary

inoculum (primary inoculum in the absence of chasmothecia) and begin forming four to five days after infection (Amsalem et al. 2006). Conidia are primarily wind dispersed but will also disperse with any sort of disturbance to an infected leaf.

Since strawberries are grown as an annual crop in California, no overwintering of the pathogen is necessary and consequently infection of the plant occurs primarily from conidia. Germination of conidia requires temperatures of 15-25°C and relative humidity ≥75% (Amsalem et al. 2006; Miller et al. 2003). Unlike spores of most other fungal pathogens, these spores will not germinate in free water. Conidia most often germinate on young unfurled leaves and on reproductive structures prior to the white/pink fruit stage (Asalf et al. 2014; Asalf et al. 2016). Once germinated, an appressorium is produced and builds pressure in order to drive a penetration peg through the surface of the leaf. Haustoria then infect the epidermal layer of leaf tissue, though the majority of the colony grows superficially on the leaf (Bélanger et al. 2002). Conidiophores form from the established colony and bear conidia. Established colonies can mature with leaves and fruit and will only die as the infected tissue has fully senesced.
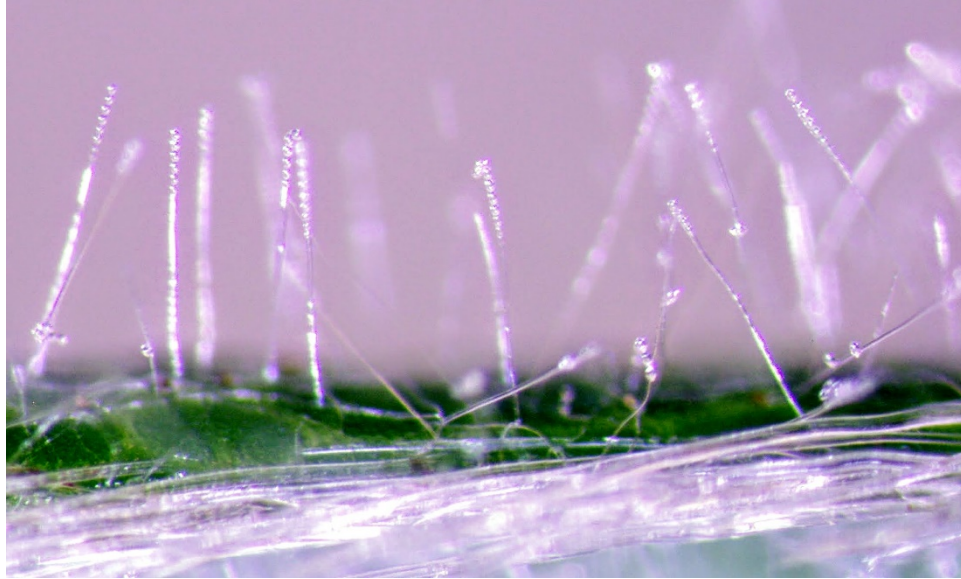
Figure 1. Conidia on conidiophores produced by *Podosphaera aphanis* (photo by M. Palmer).

Symptoms of infection are often inconspicuous, but the fungus produces signs such as white fuzzy colonies on the undersides of leaves. Signs on fruit are similar and can first be seen in the depression between the achene and swollen tissue of the receptacle. Colonies on both leaves and fruit will increase in size and can eventually cover the entire surface of the infected plant part. Severe infections on leaves can also cause upward curling as well as purple-brown blotching of the leaf surface (Maas 1984).
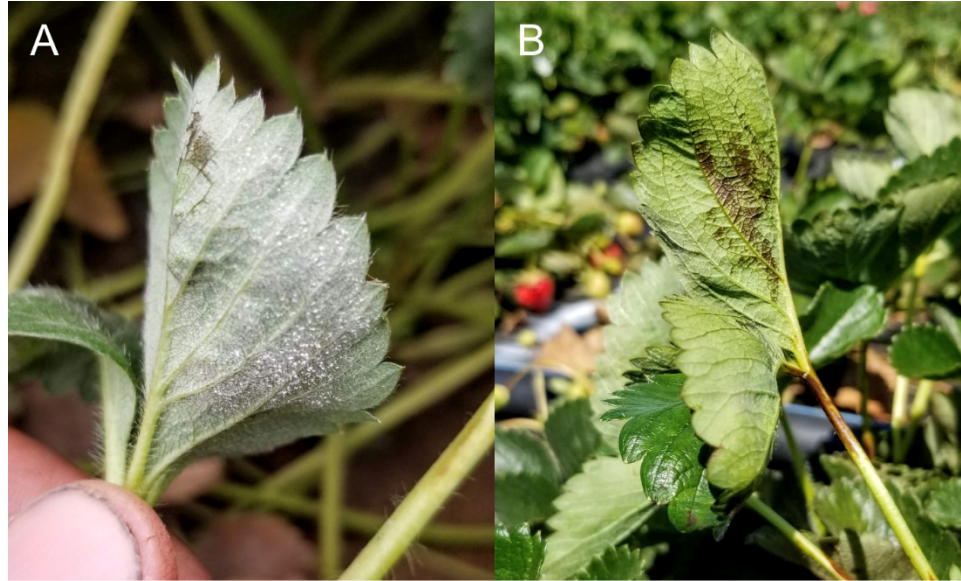
Figure 2. A) Mature powdery mildew colony on a strawberry leaf (photo by M. Palmer). B) Upward curling and purple-brown blotching caused by powdery mildew infection (photo by M. Palmer).

1.3.1 Management. Management of SPM can be achieved through cultural and chemical methods. Cultural methods primarily focus on host and environmental manipulation to minimize pathogen infection and spread. Chemical methods are a direct action taken against the disease, typically after infection has occurred. Integrated pest management (IPM) principles recommend taking cultural control measures prior to chemical control (Koike et al. 2018). These different control measures will be expanded upon in the sections below.

Making the proper management decision requires proper monitoring of environmental conditions and disease progress. However, monitoring the progress of SPM infection is particularly difficult as developing colonies are inconspicuous, typically hidden on the undersides of young leaves within the plant canopy. It is also difficult to find pre-sporulating colonies as they are shrouded among trichomes and bear no identifying structures (Miller et al. 2003).

Mature colonies are visible to the naked eye and identifiable with a hand lens. However, this identification can still be difficult as mature colonies are visually similar to pesticide residue and the waxy secretions produced by whitefly (Koike et al. 2018). If cultural and preventive measures are not taken, the difficulty of monitoring often necessitates chemical control to manage existing infections that have surpassed the grower's action threshold.
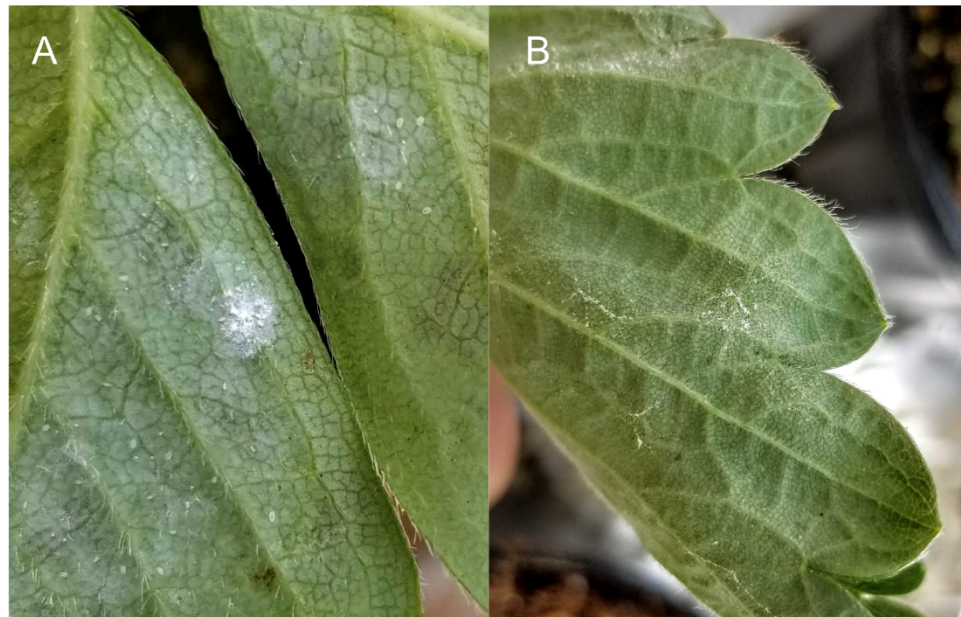
Figure 3. A) Whitefly and whitefly secretions on a strawberry leaf (photo by M. Palmer). B) Powdery mildew colony on a strawberry leaf (photo by M. Palmer).

  1.3.2 Cultural Management. There are a few different methods that will provide cultural control against infection from *P. aphanis*. First is selecting a cultivar with host resistance to powdery mildew. However, it is difficult for growers to prioritize this trait when considering other important factors such as yield and postharvest fruit quality. Factors that contribute to host resistance and its evaluation will be discussed below.

Modifying the growing environment to create conditions less conducive to SPM infection is also a tool used by growers. An increasingly common method of environmental modification is using tabletop production under high plastic tunnels. Growers will use these structures to extend the season, protect from rainfall, increase yields, and improve harvest working conditions. However, it has been reported that powdery mildew infection increases in high tunnel production systems (Xiao et al. 2001). Overhead irrigation creates an environment unfavorable to powdery mildew as conidia will not germinate in free water, but does not have the efficiency and fertigation potential offered with drip irrigation. Additionally, prolonged periods of wetness from overhead irrigation from sprinklers creates favorable conditions for another significant disease of strawberry, Botrytis gray mold (Sosa-Alvarez et al. 1995). Recent research has shown, however, that using short bursts of overhead irrigation I addition to a drip irrigation system can inhibit powdery mildew development without favoring gray mold development (Asalf et al. 2020).

Curative cultural treatment can be implemented in the form of UV-C applications followed by a four-hour dark period. The dark period is required as UV light is also required to stimulate the process of repairing damaged cells (Janisiewicz et al. 2016). Though effective on the colonies exposed to the light, it is difficult for UV-C to reach every part of the plant canopy. Finally, there is preliminary research showing efficacy of heat-treating strawberry transplants in a sauna before planting (Dias Da Silva et al. 2019). However, this treatment is not yet scaled for commercial use.

Host resistance to powdery mildew in any pathosystem was first identified in barley to *Blumeria graminis* in 1942 (Freisleben and Lein 1942) and was later characterized as broad-spectrum resistance conferred by mutations in the *Mildew Locus O (MLO)* protein (Jørgensen 1992). This protein is found in the plasma cell membrane of the plant and is essential for mildew infection to occur. Mutations in this protein in grapevine have also been reported to confer resistance to infection from *Erysiphe necator* (Pessina et al. 2016). *MLO* genes in strawberry have been identified (Cockerton et al. 2018; Tapia et al. 2020) but specific mutations conferring disease resistance have not. The strength of *MLO* resistance is cumulative and therefore contradicts the established model of gene for gene resistance. Mutations that comply with the gene for gene model have been identified in barley (Azevedo et al. 2002), but none have been found in strawberry.

Since *MLO* genes have only recently been identified in strawberry, all evaluations of host resistance have been done phenotypically on live plants. Studies evaluating host resistance have been done in Canada (Carisse et al. 2013), Florida (Kennedy et al. 2013), and California (Nelson et al. 1995; Nelson et al. 1996). These studies found a range of resistance among cultivars from highly resistant to highly susceptible. All four studies also found that ratings of cultivars were highly correlated across different growing environments. Though the information from these studies is important, they do not provide any host resistance information on currently grown cultivars in California.

1.3.3 Chemical Management. The above measures to prevent mildew infection are often not taken as the benefits of cultivar selection based on increased yield, postharvest quality, labor costs, and resistance to more economically significant diseases often outweigh the potential losses from an SPM outbreak (Koike et al. 2018). Therefore, growers turn to chemical control to manage SPM. Sulfur is most commonly applied for control of the disease, though these applications are best made as a preventative measure (Peres and Mertely 2018). After sulfur there are many conventional products registered in California that offer effective treatment of the disease. These chemicals can be costly but are relatively inexpensive compared to many of the preventative measures taken against the disease. Chemical control must not be overused though, as repeated applications of a single mode of action can contribute to development of resistant populations of *P. aphanis* (Sombardier et al. 2009*)*.

Table 1. List of fungicides labeled for control of strawberry powdery mildew (caused by *Podosphaera aphanis*) in California.

Active ingredient(s)/common name	Example trade name	FRAC*
Azoxystrobin	Abound	11
Bacillus spp.	Double Nickel	NC
Boscalid + Pyraclostrobin	Pristine	7 + 11
Cyflufenamid	Torino	U6
Cyprodinil + Fludioxonil	Switch	9 + 12
Fluopyram + Pyrimethanil	Luna Tranquility	7 + 9
Fluopyram + Trifloxystrobin	Luna Sensation	7 + 11
Fluoxastrobin	Evito	11
Flutriafol	Rhyme	3
Fluxapyroxad + Pyraclostrobin	Merivon	7 + 11
Isofetamid	Kenja 400	7
Myclobutanil	Rally 40 W	3
Penthiopyrad	Fontelis	7
Polyoxin-D	Ph-D	19
Potassium salts or fatty acids	M-Pede	NC
Propiconazole	Bumper/Tilt	3
Pyraclostrobin	Cabrio	11
Pyriofenone	Prolivo 300SC	U8
Quinoxyfen	Quintec	13
Sulfur	Microthiol	M2
Tetraconazole	Mettle	3
Thiophanate-methyl	Topsin	1
Trifloxystrobin	Flint	11
Triflumizole	Procure	3

*Fungicide Resistance Action Committee. Active ingredients that share the same FRAC code are susceptible to cross resistance by a pathogen.

1.4 Fungicide Resistance

Fungicide resistance has become a major problem in modern agriculture. Thousands of years ago the Sumerians and Chinese began using sulfur-based compounds to control pests with a multi-site mode of action. The 1960's saw the development of the first site-specific fungicides and emergence of resistant

fungal populations soon after (Brent and Hollomon 2007). Targeted fungi gain resistance through mechanisms such as: altering target site, synthesizing enzymes that function as the targeted enzyme, overproducing the fungicide target, reducing uptake of the fungicide, or metabolizing the fungicide (Ma and Michailides 2005). These mutations happen naturally in all fungi, but only give a competitive advantage when they are selected for by chemical control. Repeated applications of the same single-site chemical will allow the resistant population to survive and reproduce. Fitness levels vary among resistant populations and can contribute to the reproductive success of a population (Rallos et al. 2014), though they are often not as important as selection from repeated fungicide use (Brent and Hollomon 2007).

Though many fungicides used today target a single site in the pathogen's cell, the specific site or process targeted differs among active ingredients. For example, active ingredients such as myclobutanil inhibit production of key compounds in the cell membrane while azoxystrobin interrupts cellular respiration. Different active ingredients that target the same process are considered to have the same mode of action (FRAC 2020). Given that mutations in fungal populations occur at very low and random rates to overcome a mode of action, using a mixture or rotation of products with different modes of action will be more effective as the chances of a population developing two specific beneficial mutations is significantly lower (Brent and Hollomon 2007).

The Fungicide Resistance Action Committee (FRAC) is the world authority on the classification of fungicide modes of action and resistance risk. The

committee organizes active ingredients with similar modes of action into different groups and assigns them a code. Once a population mutates to overcome a certain mode of action, it is likely that this mutation will confer the same resistance against products with similar modes of action. Therefore, FRAC states that active ingredients assigned the same code are susceptible to cross-resistance. FRAC also makes recommendations on proper use of fungicides in order to mitigate resistance. Though these recommendations are given specific to crop type or chemical mode of action on their website, the general practices recommended are rotating or mixing products with different FRAC codes, reducing frequency of applications of products with the same FRAC code, and applying all products at the maximum labeled rate (FRAC 2020).

1.4.1 Fungicide Resistance in Strawberry Powdery Mildew and Other Powdery Mildews. Fungicide resistance in SPM has not been extensively researched. This is partially due to the pathogen only affecting strawberry and not posing the same economic threat or host diversity as other diseases like Botrytis gray mold. It is also likely due to the obligate biotrophy of the pathogen and consequent difficulty to work with in a lab setting. The few studies that do exist have addressed methods to test for fungicide sensitivity (Okayama et al. 1995) or have tested the sensitivity of multiple isolates to FRAC group 3 active ingredients (Pertot et al. 2007; Sombardier et al. 2009). Pertot et al (2007) tested isolates against FRAC group 11 active ingredients as well. None of these studies included isolates collected from the United States.

Fungicide resistance in mildews of other pathosystems has been researched more extensively. Although these are different pathogen species affecting other crops, they are closely related to *P. aphanis* (Takamatsu 2018). *Erysiphe necator* is the causal agent of grapevine powdery mildew and merits frequent fungicide applications for its control. This, along with *Podosphaera xanthii* and *Blumeria graminis* (the causal agents of cucurbit and cereal powdery mildews, respectively) are the most researched powdery mildews. Resistance in these mildews to the most common control agents has been characterized and will be expanded upon in the next sections.

1.4.2 Sterol Biosynthesis Inhibitors. Group 3 fungicides are known as the sterol biosynthesis inhibitors. Within group 3, all fungicides registered for control of strawberry powdery mildew are in the subgroup demethylation inhibitors (DMIs). DMI fungicides were first synthesized and labeled for use in the late 1980s. They are single-site systemic fungicides working by inhibiting the C14 demethylation step within fungal sterol biosynthesis, a key step in cell membrane production (Stevenson et al. 2019). Because all DMI fungicides share the same target site at C14 demethylase (cyp51), they are all labeled as susceptible to cross-resistance. All DMI fungicides are labeled by FRAC as having a medium risk for resistance development. Resistance to group 3 active ingredients has been characterized in grape (Gubler et al. 1996; Miller and Gubler 2004; Colcol et al. 2012), cucurbit (McGrath et al. 2001; McGrath and Shishkoff 2001; Lopez-Ruiz et al. 2010), barley (Brent et al., 1989), and strawberry (Sombardier et al. 2009) powdery mildew. The grape and cucurbit studies found the greatest

resistance to triadimefon while the strawberry study found resistance to myclobutanil and penconazole. Pertot et al. (2007) also found some resistance to active ingredients in the triazole family in two separate populations of SPM from Italy and Israel.

Several resistance mechanisms to DMIs have been identified, most of which are mutations in the cyp51 gene. These mutations accumulate to reduce sensitivity and no one mutation has been correlated with total resistance. Therefore, the chance of total resistance developing is lower than that of resistance to groups such as QoIs where only a single point mutation is required. Additionally, DMI resistance is not conferred with resistance to other chemical classes within group 3 (Stevenson et al. 2019; FRAC 2020).

1.4.3 Succinate Dehydrogenase Inhibitors. Group 7 fungicides are known as succinate dehydrogenase inhibitors (SDHIs). SDHIs target succinate dehydrogenase, a compound playing a crucial role in the mitochondrial electron transport chain. The absence of this compound also prevents the conversion of succinate into fumarate and therefore inhibits the completion of the Krebs cycle (Stevenson et al. 2019). Active ingredients in this group are represented by a wide range of chemistries and can have contact or systemic activity. SDHIs are all single-site inhibitors and are labeled with a medium to high risk for resistance by FRAC.

Multiple point mutations conferring resistance to SDHIs have been identified, but only a single point mutation is required for high to total resistance (Avenot and Michailides 2010). Resistance to SDHIs has been reported in

mildew populations affecting grape (Colcol and Baudoin 2016), cucurbit

(McGrath and Miazzi 2008), and wheat (Kleczweski et al. 2020). Fungicide

sensitivity of SPM to active ingredients in this group has not been characterized.

1.4.4 Strobilurins. Group 11 fungicides are known as strobilurins or

quinone outside inhibitors (QoIs). Active ingredients in this group work by

inhibiting the Qo site in cytochrome B1 in complex III of mitochondrial activity.

These fungicides were all derived from **β**-methoxacrylic acid, a compound

produced by basidiomycete wood-rotting fungi. The first fungicides of this group

were developed in the early 1990s and first sold in 1996 (Bartlett et al. 2002).

QoIs are absorbed into the plant at varying rates and are xylem systemic once

absorbed. QoIs are all single-site inhibitors and are labeled with a high risk for

resistance by FRAC.

The most common mechanism conferring resistance to active ingredients

in this group is the G143A mutation in cytochrome B. This is a single point

mutation and confers complete resistance. The earliest report of this resistance

was on powdery mildew of wheat by (Reschke 1999), just a few years after the

first QoIs were labeled for commercial use. Additionally, it has been found that

populations of mildew with this mutation can remain competitive with sensitive

isolates even in the absence of QoI fungicide applications (Rallos et al. 2014).

Molecular methods for confirming the presence of the G143A mutation have

been developed for grape (Dufour et al. 2011), wheat (Fraaije et al. 2002), and

cucurbit (Ishii et al. 2007; Vielba-Fernández et al. 2018) powdery mildew. No

methods of G143A molecular detection have been developed for strawberry powdery mildew.

Resistance to QoI fungicides has since been characterized in grape (Colcol and Baudoin 2016; Miles et al. 2012; Miller and Gubler 2004), cucurbit (Fernández-Ortuño et al. 2006; Fernández-Ortuño et al.; 2008, Ishii et al. 2007; McGrath 2001), sugar beet (Heick et al. 2019), and wheat (Kleczweski et al. 2020; Reschke 1999) powdery mildew. All the above reports cite the mutation at G143A for conferring resistance except for Fernández-Ortuño et al. (2008) as no mutations in cytochrome B were found. No evidence of resistance has been documented in SPM, though Pertot et al. (2007) did include fungicides from this group in their 2007 assessment of Italian and Israeli SPM populations' sensitivity to chemical control.

1.4.5 Azanaphthalenes. Group 13 fungicides are known as the azanaphthalenes. Only two active ingredients make up this group: quinoxyfen and proquinazid. The mode of action of this group is not completely understood, though it is theorized that active ingredients in this group disrupt early cell signal transduction (Wheeler et al. 2003). Group 13 fungicides were first used in the late 1990s and are only registered for control of powdery mildew. FRAC labels active ingredients in this group as having a medium risk of resistance as there have been reports in grape (Colcol and Baudoin 2016) and cucurbit (McGrath 2017) powdery mildew.

1.4.6 Phenyl-acetamides. Group U6 fungicides are known as the phenyl-acetimides. The only active ingredient in this group is cyflufenamid. The mode of

action of this group is unknown. FRAC recommends using tactics to mitigate

resistance development as there have already been reports in powdery mildew of

cucurbit (Pirondi et al. 2014; McGrath and Sexton 2018).

      1.4.7 Sulfur-based Compounds. Group M2 fungicides is comprised of

sulfur-based compounds. These compounds were among the first fungicides

used and have a multi-site inhibitory mode of action. No resistance has been

reported to sulfur due to this multi-site mode of action. However, sulfur is typically

only applied as a preventative measure and its use is not recommended at

temperatures exceeding 30°C (Peres and Mertely 2009).

1.5 Conclusion

      The risk of resistance development, as reported by FRAC, and the studies

listed above illustrate that resistance in strawberry powdery mildew to commonly

used fungicides can occur. While there are published reports of fungicide

resistance in several powdery mildews in the US and internationally, this data

cannot be directly applied to the current state of fungicide resistance in

populations of *P. aphanis* in California. The causal agents of grape and cucurbit

powdery mildews are close relatives to *P. aphanis*, but are not genetically

identical (Takamatsu 2018). The studies conducted on *P. aphanis* provide

excellent reference but cannot provide a full characterization of current disease

and host plant resistance in California. However, the findings of the above

studies, size of the California strawberry industry, and premium price of the crop

provide a strong justification to assess fungicide resistant populations of *P.*

aphanis and cultural practices used to prevent outbreaks of the disease in the state.

The objective of this thesis is to address knowledge gaps regarding management of SPM in California. Specifically host plant resistance to powdery mildew and fungicide resistance in populations of *P. aphanis* will be evaluated.

CHAPTER 2

Fungicide Sensitivity in Strawberry Powdery Mildew caused by

***Podosphaera aphanis* in California**

2.1 Abstract

Field observations suggest that reduced fungicide sensitivity exists in field

populations of *Podosphaera aphanis,* the causal agent of strawberry powdery

mildew (SPM). SPM 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 (%) after 14 days.

Pathogen growth on the treated leaflets constituted a measure of insensitivity to

the fungicide. The six fungicide treatments and the average disease incidence

resulted from the 19 isolates are penthiopyrad (51.4%), quinoxyfen (41.5%),

myclobutanil (39.8%), trifloxystrobin (19.8%), cyflufenamid (19.3%), and

fluopyram + trifloxystrobin (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 > 66.6%). Two isolates collected from organic production systems were sensitive to all fungicides. This documents that *P. aphanis* in California can become resistant to most of the fungicides currently used for its control.

2.2 Introduction

Strawberry powdery mildew (SPM) is caused by the obligate parasite *Podospheara aphanis* Wallr. (syn. *Sphaerotheca macularis* f. sp. *fragariae*). and affects all fruit, leaves, and stolons of the strawberry plant. SPM 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 are 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).

Powdery mildew infection is difficult to detect and easy to control at early stages, but it 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 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 (see chapter 1) and fungicide residue which further complicates early detection (Koike et al. 2018).

Conducive conditions throughout the season combined with difficulties in early detection often leads growers to control SPM with curative chemical measures. During peak production (6-8 weeks of the season) some growers will make biweekly fungicide applications to control SPM (personal communication). The most common chemical classes used to control SPM are: Fungicide Resistance Action Committee (FRAC) codes 3 (demethylation inhibitors (DMIs)), 7 (succinate dehydrogenase inhibitors (SDHIs)), 11 (quinone outside inhibitors (QoIs)), 13, and unknown (U6). Fungicides in some of these chemical classes are also used to control a more economically significant disease, gray mold 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 of the pathogen. 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 (Peres and Mertely 2018).

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. Random mutations in fungal populations occur at a low frequency (Brent and Hollomon 2007). However, once a mutation occurs that allows a pathogen to overcome a single-site mode of action, repeated applications of chemicals with that mode of action will select for that mutant and allow it to reproduce and accumulate within 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. 2007; Sombardier et al. 2009). Though Pertot et al. (2007) 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. One *P. aphanis* isolate studied in the lab assay was also used in a potted-plant greenhouse spray trial to confirm accuracy of the lab assay results.

2.3 Materials and Methods

2.3.1 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. All material from a given field was labeled as a single isolate. 'Monterey' strawberry leaflets of ontogenic stage three (Asalf et al. 2016) or younger were collected from an outdoor field at the Cal Poly Strawberry Center. These leaflets were used in the assay as they are most susceptible to

infection at early ontogenic stages and 'Monterey' is considered to be a susceptible cultivar. Leaflets were sterilized for three minutes in 0.5% NaClO and Tween 20 (0.1 mL/L). Each isolate was brought back to the lab and brushed onto the disease-free leaflets using a camelhair brush and Andersen sampler (Andersen 1958). 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 (0.5 g/L) water agar and stored in a growth chamber at 20°C and 16/8 hours light/dark for 14 days to allow for a uniform growth of the pathogen (Fig. 4).

Figure 4. A) Sample of infected plant material, clean plant material, and the tools used to do the initial inoculation of the fungicide assay. B) Brushing sporulating strawberry powdery mildew onto susceptible leaflets using a camelhair brush and Andersen sampler (the same process was used to transfer 1 cm^2 lesions from the inoculated leaflets onto leaflets treated with fungicide). C) Incubation of inoculated leaflets, both untreated and treated, on water agar in the growth chamber at 20°C and 16/8 hours light/dark.

2.3.2 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 fungicide treatments. Each leaflet was dipped (1 sec) into a treatment (Table 2) three times in succession and placed on a paper towel to dry. After drying, leaflets were placed on a benzimidazole-amended water agar Petri dish. A treatment was composed one dish containing three leaflets, replicated three times (1 rep/Petri dish).

Table 2. List of treatments used in fungicide 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	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.

Treated leaflets were removed from the dish and placed onto the Andersen sampler and inoculated. Each rep of three leaflets was inoculated by brushing a 1 cm^2 sporulating colony from the previously incubated leaflets through the sampler. The inoculated and fungicide treated leaves were then placed back onto the Petri dish and stored in the growth chamber at the conditions described above for 14 days (Fig. 4). A negative control of three plates containing three non-inoculated leaflets each was also stored in the growth chamber at this time to ensure no cross-contamination was occurring.

2.3.3 Data collection and analysis. After 14 days the leaflets were evaluated for disease incidence with the aid of a dissecting microscope at five

times magnification. 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 separation of means in JMP 14 (SAS Institute Inc. Cary, NC).

2.3.4 Greenhouse fungicide evaluation. Bareroot 'Monterey' strawberry transplants were established in 2 L 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 an 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 control plots and processed through the lab fungicide assay described above. Disease incidence was compared within the trial and within the assay using a one-way ANOVA and Tukey HSD separation of means. Correlation

between the lab assay and greenhouse trial was determined using Pearson correlation.

2.4 Results

2.4.1 Fungicide assay. Resistance to each treatment varied across the 19 isolates processed (Table 3). The non-treated control had the highest disease incidence with a mean of (93.6% ± 1.8) and was significantly different from all other treatments. Penthiopyrad, quinoxyfen, and myclobutanil were less effective with mean disease incidences of 51.4% ± 6.6, 41.5% ± 7.5, and 39.8% ± 7.3, respectively. Trifloxystrobin, cyflufenamid, and fluopyram + trifloxystrobin were the more effective with disease incidence at 19.8% ± 5.6, 19.3% ± 6.1, and 3.5% ± 1.9 respectively (Fig. 5). Disease incidence for the penthiopyrad treatment was significantly higher than all "more effective" treatments. Disease incidence for the fluopyram + trifloxystrobin treatment was significantly lower than all "less effective" treatments. All negative control leaflets showed no symptoms or signs of infection.

Table 3. Disease incidence (%) of 19 strawberry powdery mildew isolates for six fungicide treatments in the lab fungicide assay.

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

Treatment means that do not share the same letter are significantly different according to Tukey HSD separation of means.

*Isolate collected from organic production.
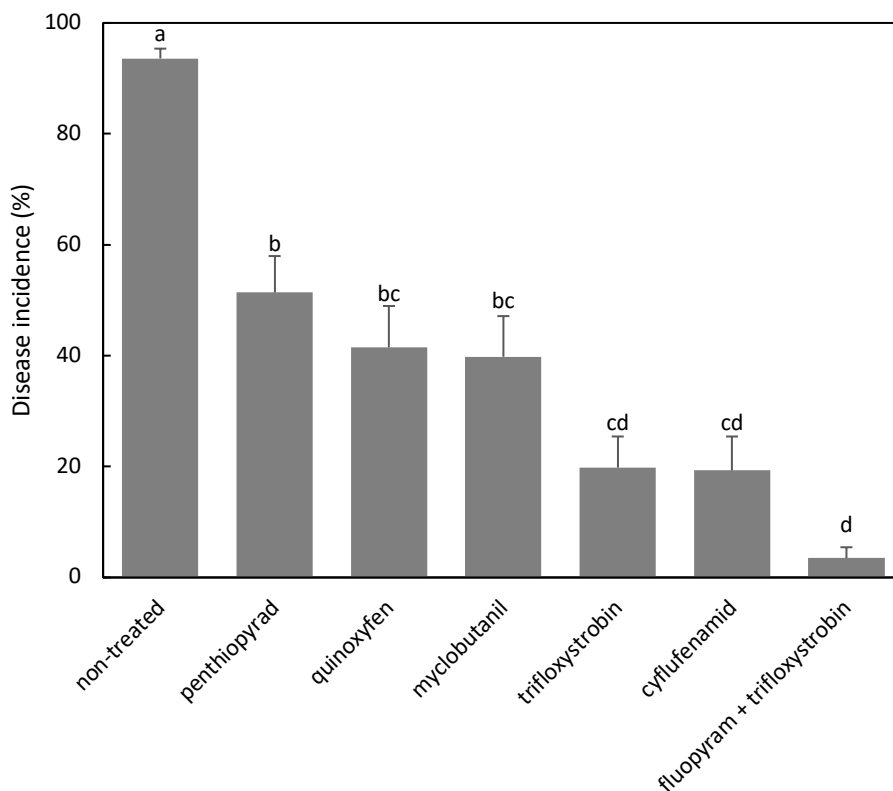
Figure 5. Average disease incidence (%) of each fungicide treatment for 19 isolates of strawberry powdery mildew according to the lab fungicide assay. Treatments that do not share a letter are significantly different according to Tukey HSD separation of means. Error bars represent standard error of the mean.

Isolates were placed into one of five categories depending on their disease incidence for each treatment: entirely sensitive (0%), sensitive (0.1 – 33.2%), somewhat sensitive (33.3 – 66.6%), resistant (66.7 – 99%), and entirely resistant (100%). The number of resistant isolates for each treatment was: penthiopyrad (7), myclobutanil (7), quinoxyfen (6), trifloxystrobin (2), cyflufenamid (1), fluopyram + trifloxystrobin (0). Two isolates from organic production systems (SPM8 and SPM13) were sensitive to all treatments. Two isolates (SPM15 and SPM19) were found to be entirely resistant to penthiopyrad and two isolates

(SPM1 and SPM15) to quinoxyfen. The fluopyram + trifloxystrobin treatment had

no individual isolates with a disease incidence >11%.

Average disease incidence was significantly higher for each treatment for

isolates collected from the Santa Maria region than isolates collected from the

Salinas/Watsonville region. Average disease incidence was significantly higher

for each treatment in the Oxnard/Ventura regions than average disease

incidence in the Salinas/Watsonville region with the exception of the

trifloxystrobin treatment. Oxnard/Ventura had significantly higher disease

incidence averages than Santa Maria for the penthiopyrad, myclobutanil, and

cyflufenamid treatments (Table 4).

Table 4. Average fungicide resistance (%) for each treatment in the lab fungicide
assay grouped by region. Regions that do not share the same letter within each
treatment are significantly different according to Tukey HSD separation of
means. Isolates from organic production systems are excluded from the regional
groupings.

Location	n	non-treated	trifloxy-strobin	penthio-pyrad	fluopyram + trifloxy-strobin	quinoxy-fen	myclo-butanil	cyflufen-amid
Oxnard/Ventura	3	96.3 b	18.4 AB	81.9 a	7.3 A	63.1 a	70.6 A	59.3 a
San Luis Obispo	2	100 a	16.5 B	38.8 c	0 B	11.2 c	38.8 AB	0 c
Santa Maria	8	93.1 b	33.3 A	61 b	5.5 A	59.8 a	52.8 B	22.2 b
Salinas/Watsonville	3	92.7 b	7.3 B	40.3 c	0 B	29.2 b	3.7 C	0 c

2.4.2 Greenhouse fungicide evaluation. The disease incidence rated

two weeks after the final fungicide application of the live plant fungicide

evaluation showed varying efficacy among the treatments (Fig. 6). The non-

treated control 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 penthiopyrad and

quinoxyfen treatments. The myclobutanil treatment was only significantly different

from the fluopyram + trifloxystrobin treatment and the non-treated control.
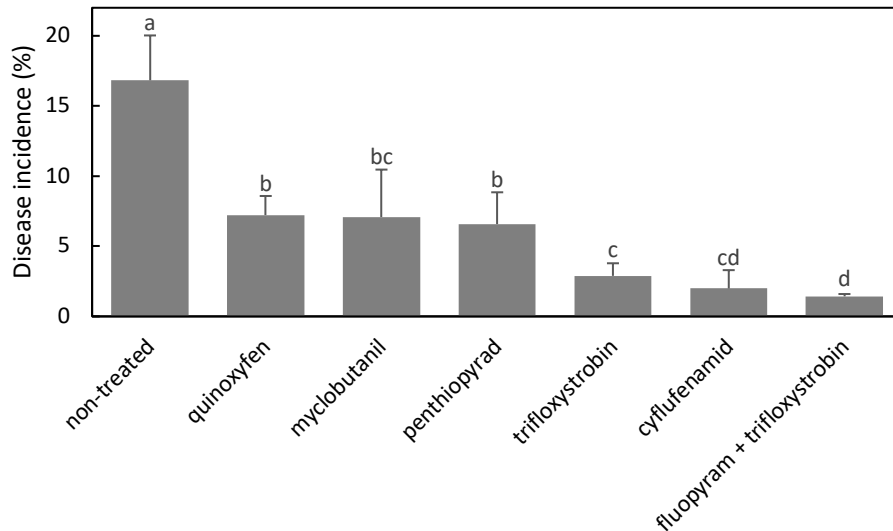
Figure 6. Average disease incidence (%) of strawberry powdery mildew for each treatment in the live plant greenhouse trial. Treatments that do not share a letter are significantly different according to Tukey HSD separation of means. Values represent mean disease incidence of four replicates and error bars represent standard error of the mean.

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 and P value of 0.0039. 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 was only significantly different

from the non-treated control in the lab assay. (Fig. 7).
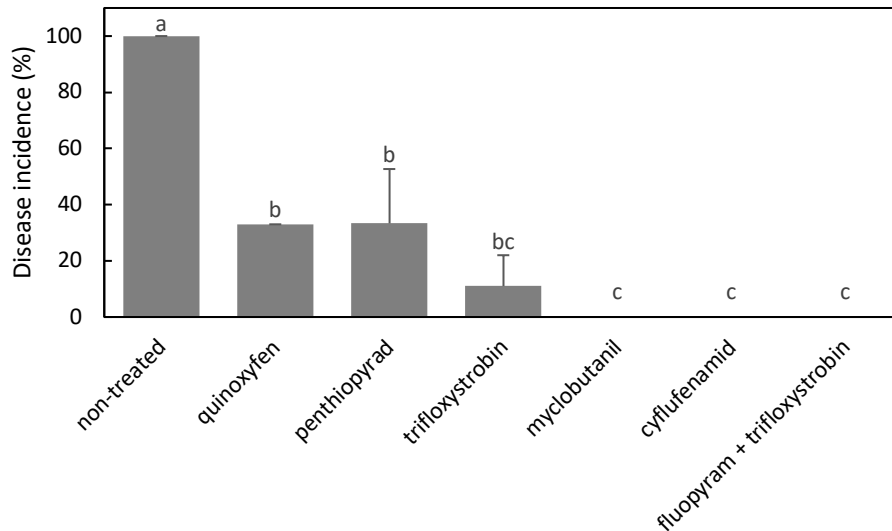
Figure 7. Average disease incidence (%) of the greenhouse strawberry powdery mildew isolate for each treatment when evaluated using the lab assay. Treatments that do not share a letter are significantly different according to Tukey HSD separation of means. Values represent mean disease incidence of three replicates and error bars represent standard error of the mean.

2.5 Discussion

Fungicide resistance has been characterized in various different 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 in SPM populations (Pertot et al. 2007; Sombardier et al. 2009) in Europe and Israel. This study adds to the documentation of fungicide resistance in powdery mildews.

The treatment means from the fungicide assay show a well-established range of efficacy among various fungicides. We can determine from this that there is reduced efficacy in some fungicides used to control SPM in California. The multiple isolates with disease incidence greater than or equal to 66.7% to

myclobutanil, penthiopyrad, and quinoxyfen suggests that SPM in California is capable of becoming resistant to FRAC groups 3, 7, 13. The resistance documented to group 3 fungicides is consistent with the work of Pertot et al. (2007) and Sombardier et al. (2009). Our work is the first to document resistance in SPM to fungicides in groups 7 and 13 anywhere in the world. Though resistant isolates were characterized with trifloxystrobin and cyflufenamid, more resistant isolates need to be characterized to document fungicide resistance to these products.

Fluopyram + trifloxystrobin had the least recorded resistance of all treatments in the fungicide assay. It was also the only product with two active ingredients with two different FRAC codes. 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 assay were sensitive to all treatments. This was to be expected as the SPM collected from these fields should not have recently been exposed to the fungicides used in the assay as they are all conventional products. It also supports the conclusion that fungicide

resistance is a locally developed trait. This has not been observed before in SPM

and could indicate that *P. aphanis* conidia may not survive relatively short

distances of dispersal due to their ephemeral nature. There is no research

directly addressing this claim, however, SPM conidia have been documented to

be sensitive to UV light (Janisiewicz et al. 2016). This finding could also suggest

that isolates of *P. aphanis* with a mutation that confers fungicide resistance have

a lower fitness than *P. aphanis* without the mutation and will be outcompeted in

the absence of selection pressure from fungicides. Both of these scenarios are

supported by the fact that chasmothecia are observed very infrequently in

California and therefore the fungus is not thought to use these overwintering

structures as primary inoculum. Instead, the disease is likely introduced into new

fields from neighboring fields or on plant material from nurseries. (Bolda and

Koike 2015). Nurseries use conventional fungicides to control mildew even when

supplying plants to organic growers because of the need to reduce disease

spread on the plant material. This study opens the door to future studies aiming

to answer questions of conidia dispersal range, within-season resistance

development, and fitness and survival time of resistant individuals in the absence

of selection from fungicides.

   The regional differences in disease incidence among treatments suggest

that fungicide resistance could become a more prevalent issue in some

strawberry production regions based on fungicides used and application

frequency. Since the isolates collected from organic production fields were

sensitive to fungicides, this may suggest that traits in *P. aphanis* populations are

localized and do not disperse much beyond the field level. More isolates would need to be tested to confirm these observed differences along with a deeper comparison of pesticide use records and cultural practices of each region.

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 and results obtained from the greenhouse fungicide evaluation. Additionally, the 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 (2007) 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 to future studies. The high efficacy of fluopyram + trifloxystrobin 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 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 chemical control

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.

CHAPTER 3

Characterization of Strawberry Host Plant Resistance to Powdery Mildew caused by *Podosphaera aphanis*

3.1 Abstract

Host plant resistance is an essential tool in plant disease management worldwide. Evaluations of strawberry cultivar resistance to powdery mildew have been done previously in California, but many new cultivars have been released since the last evaluation in 1996 and merit evaluation for today's growers. Two studies were conducted over the winter and summer of 2020 evaluating 12 and 16 commonly grown cultivars, respectively. Powdery mildew-free plants were established in six-inch pots under high plastic tunnels and after three weeks moved into a greenhouse where an active mildew epidemic was present. These plants were at the four- to five-leaf stage and showed no visible symptoms or signs of powdery mildew. Evaluations took place at 40 (winter) and 41 (summer) days after transfer to the greenhouse. Plants were evaluated for disease incidence (%) and severity (%) from which an overall foliar disease severity was calculated. Significant differences were found in foliar disease severity among cultivars, but none were totally free of disease. Moderately resistant cultivars were 'Fronteras', 'San Andreas', and 'Sweet Ann'. Highly susceptible cultivars were 'BG 3.324', 'Royal Royce', and 'Warrior'. A field evaluation of the 10 shared cultivars from both greenhouse trials confirmed the observed relative differences in host resistance under field conditions. This information is valuable to California

strawberry growers who select cultivars based in part on their susceptibility to economically important diseases such as powdery mildew.

3.2 Introduction

Strawberry powdery mildew (SPM) is caused by *Podosphaera aphanis* Wallr. (syn. *Sphaerotheca macularis* f. sp. *fragariae*). *P. aphanis* affects all above-ground parts of the plant including leaves, fruit, flowers, petioles, and stolons. Infected leaves have reduced photosynthetic capacity and can lead to yield loss (Bolda and Koike 2015). Infected fruit is unmarketable (Horn et al. 1972). SPM infection is favored by cool temperatures (15-25°C) and high relative humidity (>35%) (Miller et al. 2003). These conditions occur throughout the season in every major fruit production region in California (Bolda and Koike 2015). SPM only infects leaves at early ontogenic stages (Asalf et al. 2016) and thrives in dense plant canopies, making it difficult to detect before leaves mature and become heavily colonized. Early-stage colonies can look very similar to secretions left by whiteflies (see chapter 1) as well as pesticide residues, adding another hurdle to early detection.

Yield losses to SPM can be mitigated by cultural and chemical control measures. Though growers often turn to curative chemical control, it creates high selection pressure for resistant populations and can lead to reduced efficacy (Pertot et al. 2007, Sombardier et al. 2009). Integrated pest management practices recommend taking cultural control measures prior to chemical control (Koike et al. 2018). Perhaps the most important cultural control is the selection of a resistant cultivar. Host plant resistance is a key quality for nursery production

as well, since producing disease-free transplants is crucial to establishing healthy plants for fruit production (Bolda and Koike 2015).

Though SPM resistance is typically not the primary factor influencing cultivar selection, it is still an important consideration for many growers and breeding programs. These considerations are best informed by data gathered from replicated evaluations of host plant resistance rather than anecdotal observations. Previous evaluations have shown that cultivars can range from entirely resistant to highly susceptible. These evaluations have been done in the past in Florida (Kennedy et al. 2013), and California (Nelson et al. 1996). In California strawberry production, most cultivars are only grown for about 5 to 10 years before being replaced by newer cultivars. Thus, the most common cultivars bred and grown in present-day California have not been evaluated in replicated studies for their susceptibility to SPM.

This study aims to evaluate host plant resistance to SPM in some of cultivars commonly grown in California today. Evaluations were done in both greenhouse and field environments to establish a robust measure of susceptibility.

3.3 Materials and Methods

3.3.1 Greenhouse trials. Strawberry cultivars were evaluated for their susceptibility to SPM in a greenhouse environment at the California Polytechnic State University (Cal Poly) Crops Unit in San Luis Obispo, California. The initial evaluation (12 cultivars) took place in January and February 2020 (winter) and the repeat evaluation (16 cultivars) in May and June 2020 (summer) (Table 5).

Table 5. List of cultivars used in the greenhouse trials and field evaluations.

Cultivar	Breeding Program	Winter Trial	Summer Trial	Field Evaluations
Albion	UC	✓	✓	✓
BG 3.324	Plant Sciences	✓	✓	✓
BG 4.367	Plant Sciences	✓	✓	✓
Cabrillo	UC	✓	✓	✓
Driscoll's 1	Driscoll's	✓		
Driscoll's 2	Driscoll's	✓		
Fronteras	UC		✓	
Monterey	UC	✓	✓	✓
Petaluma	UC	✓	✓	✓
R858	Lassen Canyon		✓	
Royal Royce	UC	✓	✓	✓
Ruby June	Lassen Canyon	✓	✓	✓
San Andreas	UC	✓	✓	✓
Sangria	Lassen Canyon		✓	
Sweet Ann	Lassen Canyon	✓	✓	✓
Valiant	UC		✓	
Victor	UC		✓	
Warrior	UC		✓	

Plants were established in three-liter, six-inch pots in a planting medium composed of equal parts peat, perlite, and coconut coir. The mix was amended with a nutrient mix of lime (2.8 g/L), potassium nitrate (0.86 g/L), triple phosphate (0.57 g/L). Fifteen milliliters of Osmocote Plus (Scotts Miracle-Gro Company, Marysville, OH) was added to each pot two weeks after planting. Plants were grown under high plastic tunnels and overhead irrigated daily. At the four- to five-leaf stage, plants were transferred into a greenhouse where an active SPM

epidemic was established on mature plants. Prior to transferring into the greenhouse, each plant was inspected to ensure that no SPM was present.

Plants of the same cultivar were arranged into plots of four plants; each plot was replicated four times in a randomized complete block design. A single infected plant was placed between each plot as a source of inoculum. A highly susceptible cultivar (BG 3.324) was used for the inoculum source plants. These plants were started in the greenhouse and exposed to SPM continuously for four weeks prior to transferring disease-free experimental plants. All plants were irrigated four times per day with 0.13 L of water delivered via stake emitters (NaanDanJain, Kibbutz Na'an, Israel).

Cultivar evaluations for SPM susceptibility began 14 days after transfer into the greenhouse and were done weekly for the following eight weeks. The single ratings that best illustrated differences in cultivar susceptibility were determined to be at 40 and 41 days after transfer into the greenhouse in the winter and summer trials, respectively. These were the ratings described in the results section. Plots were evaluated for disease incidence and severity. Disease incidence was calculated by counting the number of infected trifoliates and dividing by the total number of trifoliates in each plot. Disease severity was recorded for each infected trifoliate as the percent of the total leaf surface area colonized by SPM. Foliar disease severity was calculated for each plot by multiplying disease incidence and mean disease severity for the plot. Disease score means were statistically evaluated in JMP 14 (SAS Institute Inc. Cary, NC) using one-way ANOVA and Tukey HSD separation of means.

3.3.2 Field evaluations. Ten strawberry cultivars were evaluated in each of two fields located at Cal Poly. The cultivars evaluated were included in both the first and second greenhouse trials. These cultivars were part of a larger trial assessing host resistance to Macrophomina crown rot (Field A) and Verticillium wilt (Field B) in the two respective fields. The fields were planted on 23-Oct-2019. The Macrophomina field had four inoculated replications and the Verticillium field had four naturally inoculated replications. All SPM inoculum was naturally occurring in these fields. No fungicides were applied to control SPM in either field. The results from each field are reported separately.

Five leaves from mature plants were rated from each of the ten plots and selected with a preference toward those showing symptoms of SPM. Each leaf was scored for disease severity as described above. Evaluations were done on 05-Jul-2020.

3.4 Results

3.4.1 Winter greenhouse trial. Disease severity scores ranged widely among the cultivars evaluated (Fig. 8). Both 'BG 3.324' and 'Royal Royce' were highly susceptible to SPM and had disease severity scores of 19.4% and 12.2%, respectively. 'San Andreas' and 'Sweet Ann' and were the least susceptible cultivars with disease severity scores of 1.5% and 1.9%, respectively. These were the only two cultivars significantly different from both of the most susceptible cultivars according to Tukey HSD separation of means. All the other cultivars evaluated were only significantly different from 'BG 3.324' and are considered moderately susceptible.

Figure 8. Foliar disease severity for each cultivar evaluated in the winter greenhouse trial. Cultivars that do not share the same letter are significantly different according to Tukey HSD separation of means. Error bars represent the standard error of the mean.

3.4.2 Summer greenhouse trial. There were significant differences among disease severity scores of the cultivars evaluated (Fig. 9). 'BG 3.324' and 'Warrior' were the most susceptible cultivars and had disease severity scores of 10.0% and 8.9%, respectively. 'Fronteras' and 'Valiant' were the least susceptible cultivars with disease severity scores of 1.4% and 2.2%, respectively. The range of scores was 8.6% which was lower than the range of 17.9% in the winter greenhouse trial. The severity scores of the ten shared cultivars in the winter and summer greenhouse trials were significantly correlated (Table 6).
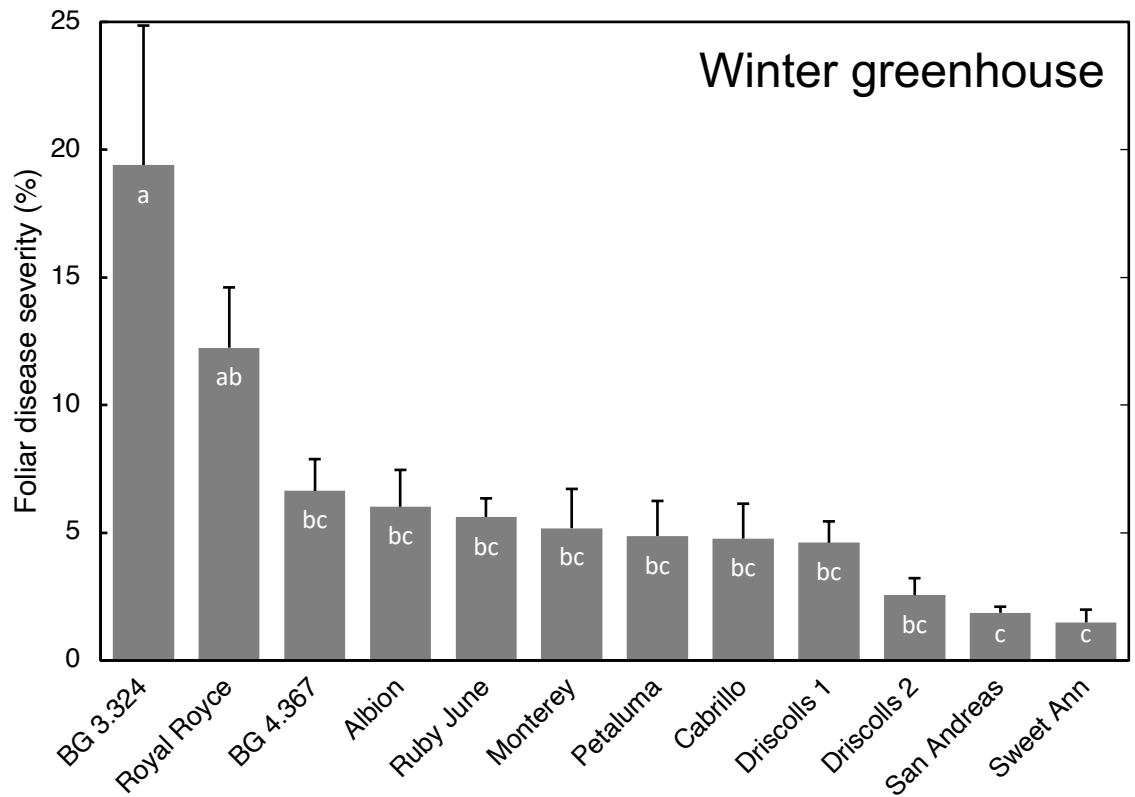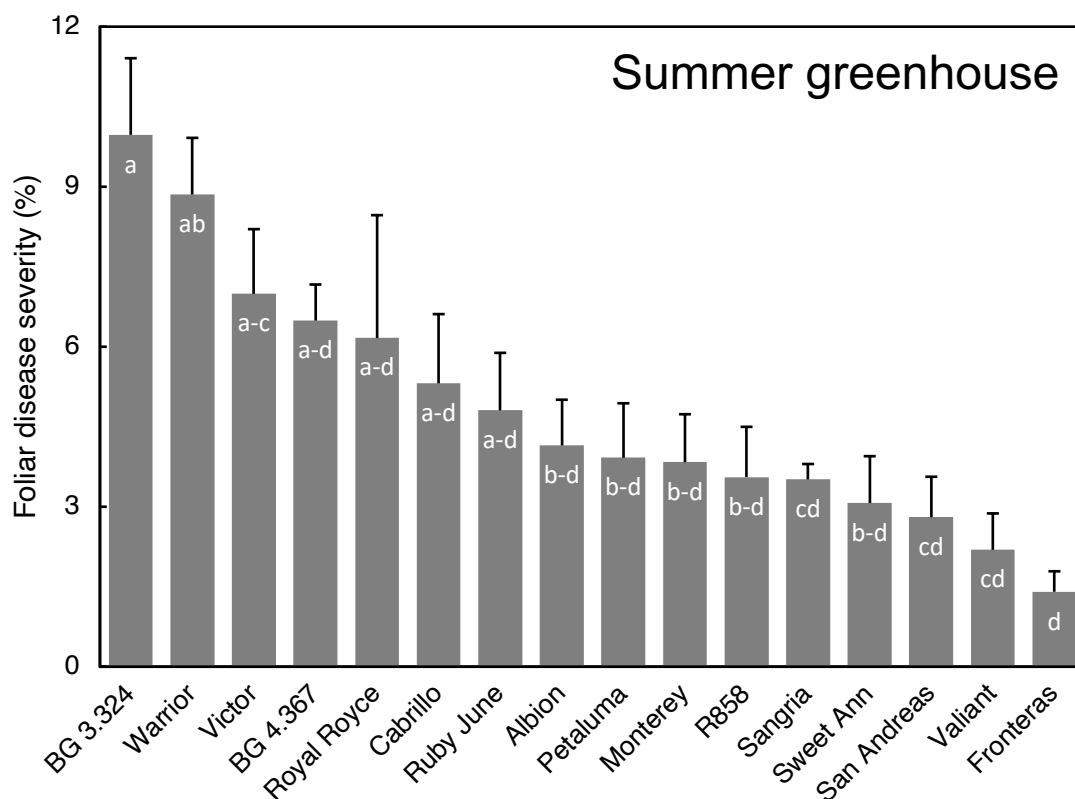
Figure 9. Foliar disease severity for each cultivar evaluated in the summer greenhouse trial. Cultivars that do not share the same letter are significantly different according to Tukey HSD separation of means. Error bars represent the standard error of the mean.

Table 6. Pearson correlation coefficients and subsequent P values generated by comparing each pair of disease severity scores in four separate experiments. Pairs that have a P value less than the established alpha of 0.05 are considered to be significantly correlated.

Experiment	By experiment	Pearson coefficient	P value
Winter greenhouse	Summer greenhouse	0.669	< 0.0001
Field A	Field B	0.597	< 0.0001
Winter greenhouse	Field B	0.565	0.0001
Summer greenhouse	Field B	0.488	0.0014
Winter greenhouse	Field A	0.388	0.0135
Summer greenhouse	Field A	0.281	0.0794

3.4.3 Field evaluations. The cultivar with the highest average disease severity in both Field A and Field B was 'BG 3.324' at 4.8% and 7.5%, respectively. The cultivar with the lowest disease severity in both Field A and Field B was 'Sweet Ann' at 0.8% and 1.8%, respectively (Fig. 10). The disease severity scores from both fields were significantly correlated. The scores from both fields were also significantly correlated with the scores of the ten shared cultivars from the winter and summer greenhouse trials with the exception of the Field A – summer greenhouse pairing (Table 6).
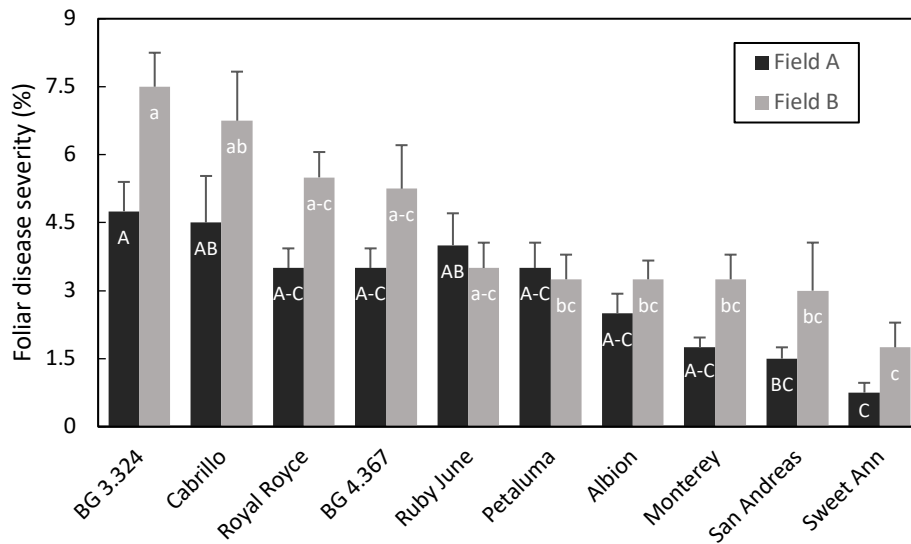
Figure 10. Foliar disease severity for each cultivar evaluated in the field evaluations separated by field. Cultivars that do not share the same letter as others in the same field are significantly different according to Tukey HSD separation of means. Error bars represent the standard error of the mean.

3.5 Discussion

The cultivars in the greenhouse trials and the field evaluations all showed variation in susceptibility to SPM with statistically significant differences. This was expected since previously published work has put host plant resistance to

powdery mildew on a continuous spectrum rather than a binary scale (Kennedy et al. 2013, Nelson et al. 1996). These results confirm the findings of Cockerton et al. (2018) that strawberry host plant resistance to powdery mildew is controlled by multiple genes and not defined by the gene-for-gene model.

No cultivars were found to be entirely free of SPM in any of the evaluations in this study. Our findings are in contrast with those from Kennedy et al. (2013) where some completely resistant cultivars were characterized but confirms findings of other host resistance evaluations of SPM where no disease-free cultivars were observed (Darrow et al. 1954; Peries 1962; Nelson et al. 1996). The contrast from the findings of Kennedy et al. (2013) is most likely attributed to a discrepancy in sampling methods. The 2013 study randomly selected five mature leaves from each plot under field and high plastic tunnel environments. This raises the likelihood of recording plots without disease incidence since selecting five leaves from a plot with hundreds of leaves could overlook the little disease present in less susceptible cultivars.

The significant correlation among the different trials and evaluations suggests that host resistance in a controlled setting such as a greenhouse with purposeful inoculation is comparable to host resistance in a field setting with natural inoculum. This is important as it would allow for evaluations of host plant resistance to SPM to be done in a controlled setting when natural field conditions are not conducive to SPM development. Greenhouse evaluations are also typically done on a smaller scale and are less expensive to establish. The ease of establishing and moving uninfected plants into the greenhouse also allows for

multiple evaluations in a single year. Finally, the closed environment of the greenhouse contains the disease and reduces pathogen spread to nearby fields.

The results produced by this study will be valuable to California strawberry growers as no SPM host resistance studies have been done in California for over 20 years (Nelson et al. 1996). Aside from evaluating some proprietary and newer varieties, this study evaluated six cultivars that make up a majority of the state's acreage: 'Monterey' (31.7%), 'San Andreas' (13.1%), 'Fronteras' (6.1%), 'Cabrillo' (4.6%), 'Petaluma' (1.2%), and 'Sweet Ann' (1.2%) (CSC 2018a). Though host plant resistance to powdery mildew is not often the first consideration for growers, it is still a valuable trait that is now documented and will contribute to a more informed cultivar selection process.

CHAPTER 4

Detection of the G143A Mutation Conferring Resistance to QoI Fungicides

in *Podosphaera aphanis*

4.1 Introduction

One of the main chemical classes used to provide control of powdery

mildews as well as many other phytopathogenic fungi are the site-specific

strobilurin fungicides. These fungicides work by inhibiting the quinone outside

inhibitors (QoIs) site in cytochrome B1 in complex III of fungal cell mitochondrial

activity. These fungicides were developed in the early 1990s from β-

methoxacrylic acid, a compound produced by basidiomycete wood-rotting fungi

(Bartlett et al. 2002). Though they are a relatively new class of fungicides, QoIs

were incredibly popular immediately after their registration in 1996 and are still

the second most widely used class of agricultural fungicides (Xiao et al. 2014).

Resistance has been well documented to QoI fungicides and reports of

reduced efficacy began soon after their registration. The earliest official report of

this resistance was on powdery mildew of wheat by Reschke (1999). Growers in

Japan also reported reduced efficacy of QoIs on cucurbit powdery mildew around

this same time (Ishii et al. 2001). Many reports of resistance have been made

since in powdery mildews, other phytopathogenic fungi, and oomycetes. These

reports have all contributed to the Fungicide Resistance Action Committee

(FRAC) labeling chemicals in this group (group 11) as a high risk for resistance.

The rate and frequency of resistance to QoIs has prompted studies to identify the point-mutations that occur within a fungal population to overcome the fungicidal mode of action. So far, three amino acid substitutions within the cytochrome B1 complex have been identified in phytopathogenic fungi and oomycetes: from phenylalanine to leucine at position 129 (F129L), from glycine to arginine at position 137 (G137R), and from glycine to alanine at position 143 (G143A) (Ferńandez-Ortuño et al. 2008; Gisi et al. 2002). F129L and G137R mutations have been found to confer partial resistance whereas G143A confers higher resistance (Sierotzki et al. 2000).

It is this higher resistance that has pushed the need for the development of detection tools for the G143A mutation in many powdery mildews. The most common method of detection is using polymerase chain reaction (PCR). This requires extraction of DNA, development of primers to amplify the cytochrome b region of the DNA, annealing and further amplification of that region, and gel electrophoresis of the amplified DNA. This process allows detection of mutations within hours. This is substantially less time than other methods of resistance detection in obligate pathogens, as methods such as the fungicide leaf assay detailed in chapter two, which takes three to four weeks to produce results. Using PCR assays, one could process hundreds of isolates to be processed as opposed to dozens with the fungicide leaf assay. Though PCR can be done on any region of any DNA, specific primers and annealing and amplification temperatures must be developed for each fungus and region of the genome. These have been developed for causal agents of powdery mildews of grape

(Dufour et al. 2011), wheat (Fraaije et al. 2002), and cucurbit (Ishii et al. 2007; Vielba-Fernández et al. 2018). The cytochrome b region has not been sequenced in *P. aphanis* and consequently no methods of G143A molecular detection have been developed for the fungus. *Podosphaera xanthii* (causal agent of cucurbit powdery mildew) is the closest relative to *P. aphanis* with primers developed to isolate its cytochrome b region. The purpose of this experiment was to see if the methods used to detect the G143A mutation in *P. xanthii* could be used to detect the mutation in the pathogen causing strawberry powdery mildew.

4.2 Materials and Methods

Strawberry (*Fragaria × ananassa*) and spaghetti squash (*Cucurbita pepo* L.) leaves with sporulating powdery mildew colonies were collected to isolate DNA of *P. aphanis* and *P. xanthii*, respectively. The DNA extraction and PCR follow exactly the published work from Vielba-Fernández et al. (2018). The processed DNA from each species was separately extracted using the DNEasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions provided with the kit. The cytb genes were isolated using primers F3cytb-Px(5'-AGCAATGCATTACAACCCTAGC-3')/R3cytb-Px (5'-CTATTCATGGTATAGCGCTC-3'). PCR was done in a 50 µL reaction mixture containing 1.25 U of GoTaq G2 Flexi DNA Polymerase (Promega), 10 µL Green GoTaq Flexi Buffer, 0.2 mM each dNTP, 0.2 mM of each primer, 3 mM MgCl2, and 20 ng of fungal genomic DNA. PCR amplification was achieved following an initial preheating of 2 min at 95°C followed by 35 cycles consisting of 30 s

denaturation at 95°C, 30 s annealing at 51.5°C, and 30 s extension at 72°C; and a final extension stage of 10 min at 72°C. PCR product (5 μL) was combined with 3 μL loading buffer and separated in GelRed-stained agarose (1 g/L) gel using 100 volts. The gel was exposed to UV light to visualize DNA fragments.

4.3 Results

DNA fragments were identified at ~300 bp in the lanes loaded with PCR product containing DNA isolated from the sporulating lesions on spaghetti squash leaves (*P. xanthii*). No fragments were identified in the negative control lane or the lanes loaded with PCR product containing DNA isolated from the sporulating lesions on strawberry leaves (*P. aphanis*) (Fig. 11). This verifies that the specific PCR process outlined by Vielba-Fernández et al. (2018) is able to detect cytochrome b in *P. xanthii*, but not in *P. aphanis*.
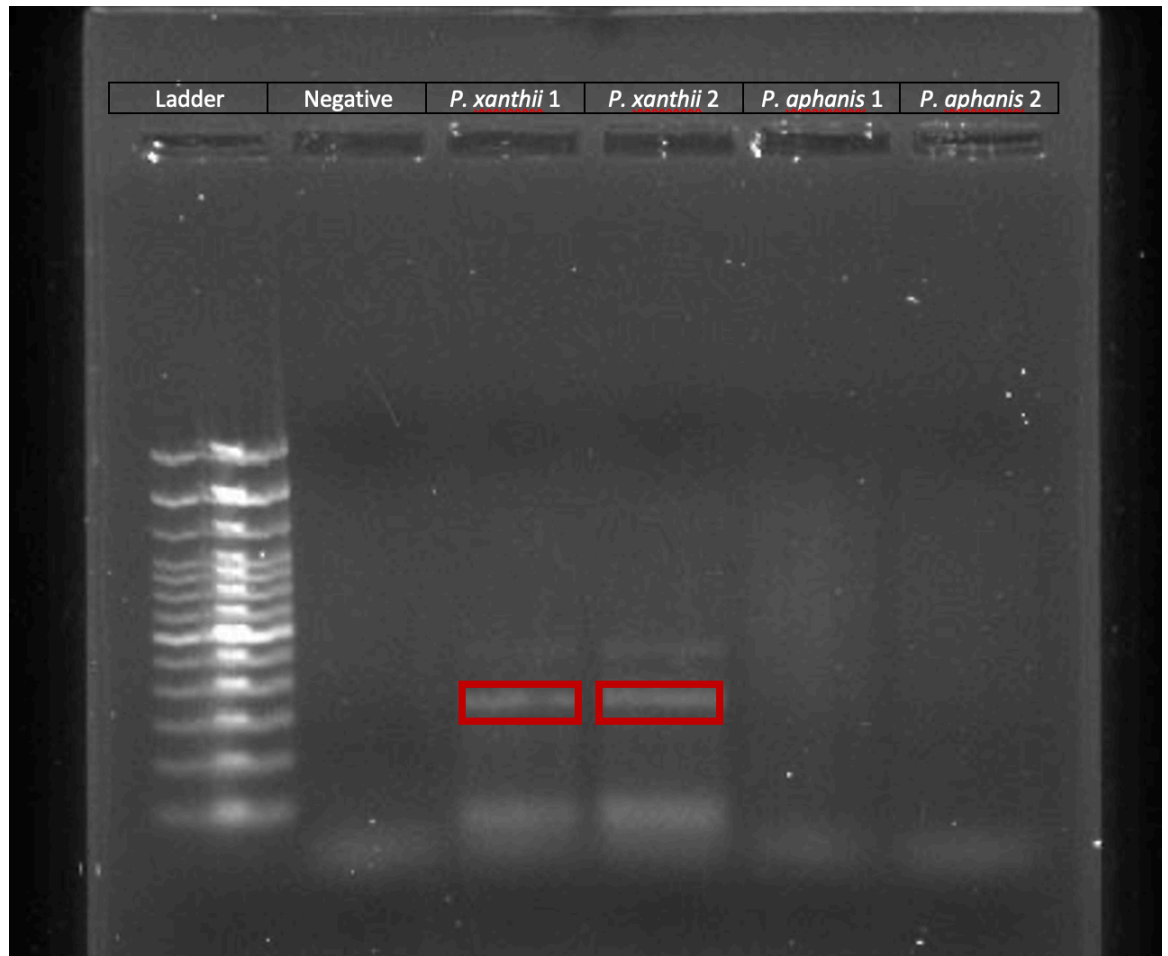
Figure 11. PCR for the cytb G143A alleles of *Podosphaera xanthii* and *Podosphaera aphanis*. Amplified DNA fragments appeared in the lanes containing *P. xanthii* DNA (lanes 3 and 4 outlined by the red boxes) but no fragments were observed in the lanes containing PCR product with *P. aphanis* DNA (lanes 5 and 6).

4.4 Discussion

This experiment confirmed that a method used to detect the G143A

mutation in *P. xanthii* would not work to detect the same mutation in *P. aphanis*.

P. xanthii is the only fungus in the same genus with a detection methodology

developed so it can be assumed that detecting the mutation in *P. aphanis* with a

methodology used for another powdery mildew causal agent would not have a

higher likelihood of success. To successfully isolate the cytochrome b region will

take a set of primers developed specifically for *P. aphanis*. There are programs

that can assist in primer development such as Geneious (Kearse et al. 2012), but

the genomic sequence of the fungus is required. The sequenced genome of *P.*

aphanis has not yet been published. This means that the detection of the G134A

mutation in *P. aphanis* is more appropriate for a long-term project that is allotted

the time and resources to accomplish the tasks of genome sequencing and

primer development.

CHAPTER 5

First Report of *Aspergillus tubingensis* Causing Strawberry Fruit Rot in

California

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As part of a Botrytis gray mold fungicide performance evaluation, 2,304 ripe strawberry fruit (*Fragaria × ananassa* Duch., cultivar Monterey) were picked from the field at the Cal Poly farm in San Luis Obispo and placed in incubation chambers to determine the amount of decay caused by *Botrytis cinerea*. Of these, five fruit (0.2%) developed black fungal sporulation on the surface. Each lesion was surrounded by a white advancing margin. Spores were removed from the fruit and streaked onto potato dextrose agar (PDA) to obtain pure cultures. Resulting colonies produced the same black sporulation. Conidia from a pure culture were suspended in a 0.5% Tween-water solution and used to inoculate individual fruit. Sterile toothpicks were dipped in the conidial suspension and used to wound-inoculate six sound ripe fruit. Six wounded but non-inoculated fruit were used as a control. Inoculated fruit were kept at 24 to 26°C in moist chambers, and lesions developed after 3 days. Sporulating lesions on all

inoculated fruit phenotypically matched those observed on fruit collected from the

field (Fig. 12). No symptoms developed on the controls. The fungus was then

reisolated from inoculated fruit by streaking conidia onto PDA. The resulting

colonies phenotypically matched the colonies obtained from the originally

diseased fruit, thus completing Koch's postulates. This experiment was repeated

once. Micromorphological features of the fungus include smooth, globose conidia

(1.4 to 1.9 μm diameter), metulae (7 to 7.3 μm), phialides (8 to 8.2 μm), brown-

black conidial heads (57 to 69 μm diameter), and smooth, hyaline stalks (7.5 ×

320 to 380 μm). Colonies grown on Czapek's agar after 10 days in the dark at 24

to 26°C were 4.1 to 4.5 cm in diameter, dark olive to gray, and compact white

basal mycelium extending beyond the sporulating portions; reverse was

uncolored to yellow. These measurements match previously published

descriptions of *Aspergillus tubingensis* and *A. niger* (Klich 2002; Raper and

Fennel 1965). Total genomic DNA was extracted using a DNeasy Plant Mini Kit

(Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region

(ITS1/ITS4 primers), β-tubulin (Bt2a/Bt2b primers), and calmodulin gene

(Cmd5/Cmd6 primers) were amplified using polymerase chain reaction (PCR)

(Henry et al. 2000; Yaguchi et al. 2007). The PCR products were purified and

sequenced. The sequences were processed using BLAST (Basic Local

Alignment Search Tool) in the National Center for Biotechnology, and results

showed 99, 100, and 100% homology with the ITS (MG551283), β-tubulin

(KY990215), and calmodulin (KX231825) sequences of isolates for *A.

tubingensis*, respectively. The sequences were deposited in GenBank with

accession numbers MK598817 (ITS), MK636652 (β-tubulin), and MK636653

(calmodulin). This disease is of minor significance on strawberry, because only a

small percentage of the incubated fruit were infected. Of greater significance is

that fruit were collected during a week of unusually hot weather (daytime highs:

32 to 36°C), which favors fungi such as *Aspergillus*.

Figure 12. Strawberry fruit showing typical symptoms of Aspergillus rot caused by *Aspergillus tubingensis*. Specimen showed no signs of decay in the field, but developed symptoms and signs during incubation for 4-6 days at 24-26°C.

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