HOST PLANT RESISTANCE IN STRAWBERRIES TO ANTHRACNOSE AND COLONIZATION OF CROWN AND ROOT TISSUE BY *VERTICILLIUM DAHLIAE* AND *MACROPHOMINA PHASEOLINA*

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ABSTRACT

Host plant resistance in strawberries to anthracnose and colonization of crown and root tissue by *Verticillium dahliae* and *Macrophomina phaseolina*

Omar Alexander Gonzalez Benitez

Strawberries are considered an important crop in California where in 2018 it was in the top 5 valued fruit and vegetable commodities valued at $2.84 billion accounting for 88% of the total U.S. production. Strawberry production can be severely impacted by soilborne pathogens that can affect strawberry roots, crowns and leaves which can result in plant mortality. As much as 50 to 60% mortality can occur in one field. Pathogens responsible for such losses include *Colletotrichum acutatum (*syn. *C. nymphaeae), Macrophomina phaseolina* and *Verticillium dahliae.* With the phaseout of methyl bromide, host resistance and an understanding of host-pathogen interactions can play an important role in control of these diseases.

A two-year study was conducted in order to evaluate host resistance of anthracnose in 105 cultivars and elite breeding lines developed by six strawberry breeding programs. Cultivars and elite breeding lines were inoculated using three local isolates in both years. All breeding programs provided genotypes that had a wide range of anthracnose susceptibility ranging from 0 to 100% mortality during both years. In both years an average of 78% of all the plant mortality occurred by 1 January. From the 105 cultivars and elite breeding lines, 30 cultivars were common to both years. Of these 30 cultivars, nine of them differed in their disease susceptibility between experiments by more than 20%. This suggests that several years of field evaluation may be necessary to determine susceptibility to anthracnose. Popular cultivars that represent the spectrum of susceptibility are Monterey (susceptible), Festival (moderately resistant), and Sensation (resistant).

A second study was conducted to evaluate pathogen colonization of resistant and susceptible strawberry cultivars, testing interactions among crown and root plant tissue and two sampling timings. These cultivars were challenged with two soilborne pathogens, *Macrophomina phaseolina* and *Verticillium dahliae,* over two years. Existing qPCR protocols for *M. phaseolina* and *V. dahliae* were used in order to quantify how much pathogen DNA was detected in crown and root samples. For the 2016-2017 *V. dahliae* trial there were significant effects for cultivar. Cultivar Benicia had significantly higher pathogen DNA compared to resistant cultivars Marquis, UC-12 and Camino Real. Susceptible cultivar BG 1975 had significantly less pathogen DNA compared to resistant cultivars San Andreas and Petaluma. In the 2017-2018 *V. dahliae* trial pathogen DNA amount was not significantly different based on cultivar, plant part colonization, or the sampling period. In the 2017-2018 *M. phaseolina* trial all three of the fixed factors, cultivars, plant part colonization and sampling period were statistically significant. Cultivar ‘Sweet Ann’ had a significantly higher level of *M. phaseolina* DNA in the early vs. the late sampling.

Keywords: *Fragaria* × *ananassa*, *Colletotrichum nymphaeae*, Charcoal Rot, Verticillium Wilt

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Strawberries are considered an important crop in California where in 2017, 803,000 tons of strawberries were harvested. This accounts for 88% of total U.S. production (California Strawberry Commission, 2018). In 2018, the value of strawberries was in the top 10 valued commodities for California at 2.84 billion dollars (CDFA, 2018). There are approximately 300 strawberry growers located in five distinct growing regions: Watsonville-Salinas, Santa Maria, Oxnard, Orange County-San Diego and the Central Valley (California Strawberry Commission, 2018). Areas like Watsonville-Salinas and Santa Maria are able to harvest fruit from March through November due to day-neutral varieties, typically planted each fall (California Strawberry Commission, 2018). Santa Maria and Oxnard, in addition to the fall-planted crop, produce a second crop planted in May and June which allows for production during the winter months, October to December (California Strawberry Commission, 2018).

Prior to planting for fruit production, high-quality and disease-free transplants are necessary for maximum production. Important diseases that can be plant-borne include Macrophomina charcoal rot, anthracnose and Verticillium wilt as they can cause fields to reach 50 to 60% mortality (Chamorro et al., 2016; Rahman et al., 2015; Wilhelm and Koch, 1956). Nursery stock can be certified as “clean” by voluntary inspection and testing through the Strawberry Registration and Certification program, adopted in 1949 by the CDFA.

Plant production begins with a meristem growing in tissue culture. A single meristem can produce 90 million daughter plants in a five-year period (Larson and Shaw,

1995). The use of pre-plant soil fumigation in nurseries reduces plant mortality due to soilborne disease and increases plant vigor (Wilhelm and Paulus, 1980). Low elevation nurseries are located in the Sacramento and San Joaquin valley while high elevation nurseries are located in the intermountain valleys of Northern California (Strand, 2008).

Transplants used for fruit production are planted onto raised beds that help promote drainage and increase yields (Wilhelm and Sagen 1974). Black polyethylene mulches have been used extensively to manage weeds, conserve soil moisture, and increase fruit quality and yield (Freeman and Gnayem, 2005; Voth and Bringhurst 1959). Currently low-permeability films such as virtually impermeable film (VIF) are also used to reduce emissions of fumigants and improve their efficacy (Qin et al., 2011). Other fumigation films being used include totally impermeable film (TIF) (Fennimore and Ajwa, 2011; Holmes et al., 2020).

Fragaria × *ananassa,* first described by Duchesne in 1766, arose from the hybridization of *F. chiloensis* and *F. virginiana* (Hancock, 1999). The origin of *Fragaria* × *ananassa* is not clear but it was discovered in a garden in Brittany and gardens across Europe. *F. chiloensis* is originally from South America while *F. virginiana* is originally from North America (i.e., eastern Canada and the state of Virginia in the US). The modern strawberry is characterized by the large fruit size of *F. chiloensis* and the hardiness, vigor, productivity and flavor of *F. virginiana* (Hancock, 1999; Wilhelm and Sagen 1974). The hybridization of these two species resulted in an important fruit crop worldwide due to its adaptable nature and attractive flavor (Howard et al., 1992).

The variety of traits present in today's strawberry cultivars can be attributed to breeding programs such as the University of California and the private breeding efforts of

Driscoll Strawberry Associates, Inc. (Guthman, 2019). Since the 1950s, most of the strawberry cultivars grown were developed from these two breeding programs (Wilhelm and Paulus, 1980). Strawberry yields during the 1950s were as high as 49.4 to 74.1 tons/ha but the state average from 1950 to 1960 was only 12.3 to 14.8 tons/ha (Wilhelm and Paulus, 1980). From the 1950s to today, breeding programs such as the public UC Davis program have released more than 60 patented cultivars reaching yields of 74 tons/ha compared to the 14.8 tons/ha in 1950 (Nelson, 2019). Despite genetic advances during a 20-year span, such high yields would not be possible in presence of numerous soilborne pathogens without soil fumigation (Wilhelm and Paulus, 1980).

This literature review will discuss the soilborne pathogens *Macrophomina phaseolina, Verticillium dahliae* and *Colletotrichum acutatum* (synonym *C. nymphaeae*). The taxonomy, host range and distribution will be discussed of each pathogen. After that four control methods: preventative, cultural, host resistance and chemical will be discussed that can help a grower combat the diseases that these pathogens cause on strawberries. The review will conclude with the history and use of fumigants.

1.2 Soilborne pathogens

Soilborne pathogens are pathogens that cause plant diseases to its host via inoculum in soil (Koike et al., 2003). There are a few categories by which soilborne pathogens can be ecologically identified, these include soil inhabitants, soil invaders or soil transients and finally saprobes or saprophytes. Soil inhabitants can survive in the soil for a relatively long time, soil invaders can only survive for a short period of time while saprophytes are organisms that live on decaying organic matter (Koike et al., 2003). This includes fungi, bacteria and nematodes. Examples of soilborne pathogens that can affect

strawberry are *Verticillium dahliae, Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *fragariae.* In strawberry, soilborne diseases can be asymptomatic or symptomatic. Symptoms include damage to roots, crowns, leaves, and at times can lead to collapse of the crop (Paulus, 1990). In order for these diseases to thrive in the field, they require certain environmental conditions (Velasquez et al., 2018). Some of these environmental conditions include temperature, light, water availability and soil fertility.

Strawberry pathogens use a wide range of strategies in order to survive (Maas, 1998). These strategies include bacteria accessing the host through biological cell structures such as stomata and hydathodes. Fungi can enter the plant epidermal cells or access the plant with hyphae extending into, through or between the plant cell walls. Nematodes access the plant by inserting a stylet into the plant cell or through wounds (Amil-Ruiz et al., 2011).

1.3 Colletotrichum acutatum (synonym C. nymphaeae)

Anthracnose caused by *Colletotrichum acutatum* J. H. Simmonds, is a cosmopolitan fungus that causes important economic losses to a broad range of crops. The term “anthracnose” was originally used in Florida to describe the dark-brown oval lesions on the petioles and runners of strawberry plants (Brooks, 1931). At that time the symptoms were only described to be caused by *C. fragariae.* For recent decades the term “anthracnose” has been used to describe similar symptoms caused by all *Colletotrichum* species. Due to the similarity in symptoms, species cannot be characterized by symptoms alone and lab techniques are necessary to identify the species (Curry et al., 2002; Howard et al., 1992).

In strawberries, most *C. acutatum* research focuses on the differentiation between species of the pathogen, population dynamics, chemical treatments and control of the disease (Curry et al., 2002; Eastburn and Gubler, 1990; Peres et al., 2005). In California and Europe, *C. acutatum* is the primary source of infection on strawberry plants and fruit (Garrido et al., 2016; Peres et al., 2005). All parts of the plant are susceptible with symptoms of necrosis and blight. These symptoms are typically seen on leaves, petioles, flowers or even roots resulting in plant mortality of up to 50% in well managed fields (Howard et al., 1992; Turechek et al., 2006; Rahman et al., 2015) (Fig. 1.1). Another primary symptom is the fruit lesions on ripe fruit (Freeman et al., 1998; Peres et al., 2005). Disease symptoms on ripe fruit are orange-salmon or black sunken lesions that make the strawberry fruit unmarketable (Rahman et al. 2013) (Fig. 1.1 B).

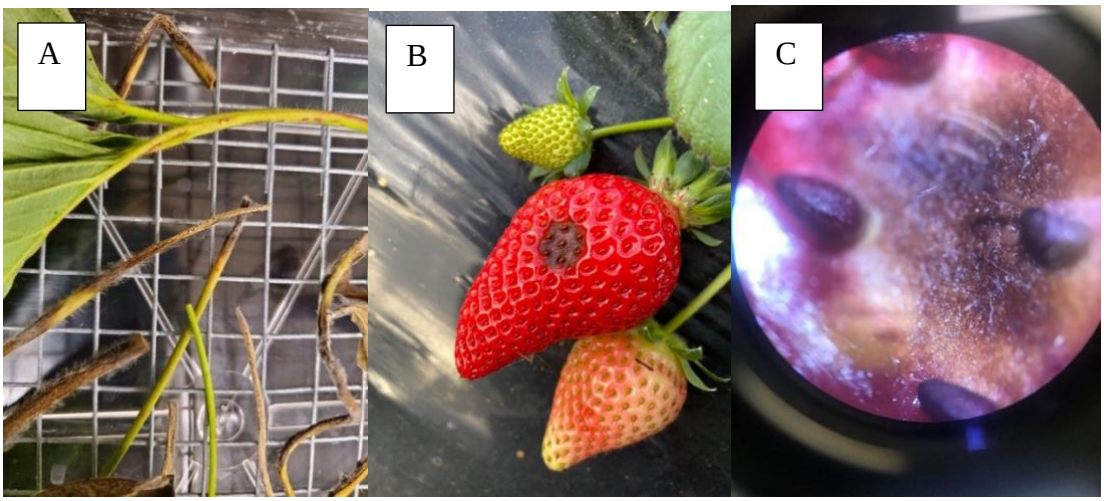

Figure 1. 1 Anthracnose symptoms on strawberry. A, necrosis and blight on petioles; B, fruit lesion on mature fruit; C, micrograph (20X) of acervuli in fruit lesion.

The primary source of infection for *C. acutatum* in fruit production fields is through the introduction of the disease on transplants from the nursery (Delp and Milholland, 1980; Eastburn and Gubler, 1990; Peres et al., 2005). *C. acutatum* is difficult to detect at the nursery if it is in the early stages of development and not producing

visible symptoms (Peres et al., 2005). When *C. acutatum* is introduced into a grower's field it easily spreads through overhead irrigation or through rain-splashed water (Madden et al., 1992; Yang et al., 1992).

1.3.1 Taxonomy, host range and distribution

Colletotrichum acutatum was first described as a distinct species in Queensland, Australia (Simmonds, 1965, 1968). In strawberries, *Colletotrichum* species are major pathogens around the world (Maas, 1998). In recent years, the use of molecular phylogenetic methods as well as morphology have helped to define species within the genus (Cannon et al., 2012). In 2019, Wang et al. rejected the hypothesis that the *C. acutatum* species-complex had increased genetic diversity among contemporary isolates. Based on phylogenetic analyses using four genetic loci of 217 *C. acutatum* species-complex cultures isolated over a 23-year period, Wang et al. (2019) determined that the correct Latin name for this pathogen was *C. nymphaeae*, which accounted for 97.7% of the tested isolates. *C. nymphaeae* is an ascomycete in the Division: Ascomycota, Class: Sordariomycetes, Order: Glomerellales, Family: Glomerellaceae and Genus: *Colletotrichum*. The pathogen was previously described as a *Gloeosporium* species (Arx, 1970). *C. nymphaeae* typically has smooth-walled, septate, branched hyphae, ranging from 1-5.5 μm in diameter (Liu et al., 2018). When on a host, acervuli serve as the primary conidial production for *C. nymphaeae.* Conidia in *C. nymphaeae* are typically ellipsoid. A unique characteristic for conidia in *C. nymphaeae* is that they are fusiform shaped only at one end (Peres et al., 2005). *C. nymphaeae* has the unique characteristic of forming secondary conidia on leaf surfaces (Leandro et al., 2001). Spores on potato-dextrose agar (PDA) are white, salmon pink and orange and will turn black or gray over

time. The colonies when observed on the underside of the culture plates are an orange salmon color with streaks of gray or black (Damm et al., 2012). *C. nymphaeae* has a wide range of hosts and is distributed worldwide. Hosts of *C. nymphaeae* include fruits such as strawberry, blueberry, almond, citrus, apple, olive and peach; ornamental herbaceous crops and conifers (Peres et al., 2005; Sreenivasaprasad and Talhinhas, 2005). *C. nymphaeae* can also be found overwintering on different weed species (Peres et al., 2005).

1.4 Macrophomina charcoal rot

Charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid was first discovered in strawberries in Illinois in 1958 but became more prominent in Florida and California after the phase out of methyl bromide (Koike, 2008; Mertely et al., 2005; Tweedy and Powell, 1958). In California *M. phaseolina* was initially restricted to the southern growing region (Oxnard). By 2010, *M. phaseolina* was present in the northern growing region (Watsonville-Salinas) (Koike., 2008; Koike et al., 2013). During 2015 and 2016, growers in Florida reported up to 30% mortality early in the season, reaching 60% mortality at the end of the season (Chamorro et al., 2016). Reports suggest that *M. phaseolina* has a broad host range having no host preference (Pearson et al., 1987; Zazzeini and Tosi, 1989; Zveibil et al., 2012), but recent studies have shown that there are isolates that have a preference for strawberries (Burkhardt et al., 2018; Koike et al., 2016).

1.4.1 Taxonomy, host range and distribution

Macrophomina phaseolina from the Phylum: Ascomycota, Class: Dothideomycetes, Order: Botryosphaeriales and Genus: *Macrophomina* has a host range

of more than 500 plant species and is distributed worldwide. Host species include soybean, common bean, maize, cotton, cowpea and strawberry (Dhingra and Sinclair, 1977; Mertely et al., 2005; Koike, 2008). On crops like soybean, *M. phaseolina* has been present in North and South America, Australia, Asia Europe and African Continents (McGee, 1991). The pycnidial name of *M. phaseolina* initially was *Macrophoma phaseolina*, Tassi, 1901 but had two name changes in 1905 and 1927, *Macrophoma phaseoli* and *Macrophomina phaseoli* until eventually returning to its original name *Macrophomina phaseolina* by Goidanich (Maublanc, 1905; Ashby, 1927; Goidanich, 1947).

M. phaseolina produces black microsclerotia allowing for it to be long-lived in agricultural soils and can germinate within two days of root presence (Chowdhury et al., 2014). Microsclerotia are the primary source for new infections in strawberry plants as they are protected by fallen crop residues and eventually released after crop residue breakdown (Ramkrishnan, 1955). Microsclerotia are made up of 50 to 200 individual cells that have the ability to germinate (Gupta et al., 2012; Kaur et al., 2012). Infected seedling plants will contain *M. phaseolina* microsclerotia until optimal conditions for disease development, typically when the plant is stressed (Gupta et al., 2012). Ideal conditions for *M. phaseolina* include temperatures of 25 to 30° C, pH between 5 and 7 and multiple days in dry weather (Dhingra and Sinclair, 1978; Fang et al., 2011). Drought and dry weather can increase microsclerotia growth at lower water potential (Olaya and Abawi, 1996). With the loss of methyl bromide and the long persistence in soil of *M. phaseolina*, it is critical to identify alternative control methods for Macrophomina charcoal rot. Typical symptoms caused by Macrophomina charcoal rot are: older leaves

wither and die, while young leaves remain alive, leading to stunted growth (Sanchez et al., 2016). When subjected to stress these plants will eventually fully collapse and die (Sanchez et al., 2016). Crown discoloration also occurred in the internal tissue of the crowns, internal vascular and cortex tissues were dark brown to orange brown (Koike, 2008).

1.5 Verticillium wilt

Verticillium dahliae Kleb, first described in 1931, has been an important soilborne pathogen of strawberries (Tribble Bros, 1912; Willhelm and Koch, 1956). At times fields infested with *V. dahliae* had up to 50% mortality (Thomas, 1932 and Wilhelm and Koch, 1956). Verticillium wilt was so devasting that it was one of the primary considerations when selecting land for strawberry production. The implementation of an annual cropping system rather than multiyear plantings of up to 4 years in southern California and up to 8 years in northern California was another breakthrough in mitigating *V. dahliae* soil infestations (Voth and Bringhurst, 1990). *V. dahliae* has the ability to spread through flowing water, tools and machinery and soil or roots (Ries, 1996).

1.5.1 Taxonomy, host range and distribution

Verticillium spp. from Phylum: Ascomycota, Class: Sordariomycetes, Order: Hypocreales and Genus: *Verticillium* can infect a total of 80 plant genera and more than 410 plant species (Pegg and Brady, 2002). Among *Verticillium* species, *V. dahliae*, along with *V. albo-atrum* cause the most economic losses in crops around the world (Heale, 1988; Pegg and Brady, 2002). *V. dahliae* can be found in agricultural soils throughout California. *V. dahliae* is favored by moist soils and temperatures ranging from 21-27° C.

Verticillium wilt is a monocyclic disease, attacking plant roots to produce inoculum in a single plant growth cycle (Bishop and Cooper, 1983a; Fradin and Thomma, 2006).

Conidiophores are verticillately branched, septate from the base of the terminal whorl (Smith, 1965). Conidia are continuous, hyaline, elliptical and are produced on phialides. Typically, there are 1 to 5 (usually 3 to 4) phialides per whorl (Smith, 1965). *V. dahliae* attacks the xylem, the water-conducting part of the plant. The infection of *V. dahliae* extends into the plant's xylem where it spreads to other parts of the plant as conidia (Fradin and Thomma, 2006). When conidia reach cell walls, they germinate and penetrate into other vessel segments, eventually producing more conidia (Bell, 1992). Hyphal growth in the xylem prevents the transport of water causing the common wilt symptoms on strawberry plants (Berlanger and Powelson, 2000). Microsclerotia are produced by *V. dahliae* and are dense aggregates of dark pigmented, ellipsoid, thick-walled hyphal cells (Fradin and Thomma, 2006). Melanin is present in microsclerotia and can provide protection against the environment, thus increasing survival (Polak, 1989). Microsclerotia can survive in the soil for up to 25 years in the absence of a host (Welch, 1989). Microsclerotia can be resistant to several abiotic stresses that include desiccation, temperature and UV radiation (Jimenez Diaz and Millar, 1988). Crop rotation can be used for reducing microsclerotia in soil, but this only provides limited protection from the disease (Bollen et al., 1989). In the soil, germination and infection by microsclerotia is influenced by root exudates and nutrients being released into the rhizosphere (Lynch and Whipps, 1990; Mol et al., 1995). Microsclerotia germination success is increased due to the ability of individual cells to germinate, which allows for the multicellular microsclerotia to germinate from each cell (Fradin and Thomma, 2006). Infected plants

will wilt, and outer leaves will dry and turn necrotic (Mansoori et al., 1995). Infected plants will collapse, eventually completely wilting and dying. In strawberries, Verticillium wilt will not cause visible damage to the roots or internal discoloration of the crown (Gordon and Subbarao, 2008).

1.6 Pathogen Colonization

Colonization refers to the establishment of a pathogen within a specific host. There are many ways that a fungal pathogen can establish on a specific host. For strawberries, both *Verticillium dahliae* and *Macrophomina phaseolina* are able to colonize on strawberry tissue through germ tubes. Germ tubes are products of germinating microsclerotia in the soil or crop residue. Germ tubes become hyphae that penetrate the cell wall or through natural openings (Bowers and Russin, 1999). In the case of *Verticillium dahliae*, these hyphae grow intercellularly in the cortex and then intracellularly through the xylem resulting in a colonized vascular tissue. For anthracnose, it's primary form of infection and colonization is through conidia produced in acervuli (Peres et al., 2005). Once conidia germinate, appressoria are produced infecting host plants (Agostini et al., 1992). In strawberries several studies have been done on pathogen colonization. The main test that has been used to identify the pathogen on the host tissue has been polymerase chain reaction (PCR). Parikka and Lemmetty (2004) used these techniques and concluded that PCR can confirm a pathogen is present even though the symptoms are not present. Pathogens can interact differently among certain cultivars and different plant parts. Baird et al. (2003) showed that *M. phaseolina* isolation frequencies declined over time, possibly affected by root segment degradation or interactions with other microorganisms in the soil. Studies have been done where

symptom expression was measured among strawberry cultivars (Shaw et al. 2010). Shaw et al. (2010) also showed that 60% of the visual symptom expression variation among genotypes was because of genetic differences. In both Freeman et al. (2001) and Shaw et al. (2010), findings suggest that colonization, resistance and tolerance to colonization can change over the course of a season. Shaw et al. (2010) also observed that when the extent of colonization had a partial genetic correlation with symptom expression, strawberry genotype performance in the presence of *V. dahliae* may be enhanced by both resistance and tolerance mechanisms within genotypes. It was also observed that genetic mechanisms that help prevent systemic infection can be more stable over the growing season.

1.7 Management of Verticillium wilt, Macrophomina charcoal rot and anthracnose

There are a variety of ways that a grower, Pest Control Advisor, plant breeder or researcher can approach a pest problem in the field. Ideally an integrated pest management approach is taken where a sustainable, science-based, decision-making process is used to combine biological, cultural, physical and chemical tools to identify, manage and reduce risk from pests and pest management tools and strategies in a way that minimizes overall economic, health and environmental risks (USDA-ARS, 2018). Controls for strawberry diseases include preventative, cultural, host resistance and chemical controls.

1.7.1 Preventative controls

The first line of defense is using a preventative approach prior to the growing season. A preventative approach involves using factors such managing the crop, surrounding areas and even equipment to prevent the movement of pathogens from entering a site using exclusion (Strand, 2008). Another preventative measure that should be taken is the use of disease-free plants. For anthracnose the main way it gets into a field is on infected transplants. Planting anthracnose-free plants in production fields is the best way to prevent anthracnose from establishing in strawberry fields (McInnes et al., 1992). *C. acutatum* is an issue because when present on strawberry transplants brought from the nurseries, marketable yield can drop by 40% (Daugovish et al., 2009). Planting fields with disease-free plants is the goal and this is often achieved due to strict sanitation and disease management practices in the nursery. Pathogens such as *C. acutatum, V. dahliae* and *M. phaseolina* can infect plants that initially are asymptomatic, making it difficult for workers to identify. Such pathogens can be introduced in soil, plants and equipment that is transported from field to field (Howard et al., 1992; McInnes et al., 1992).

1.7.2 Cultural controls

Cultural controls involve using the production or utilization methods of a commodity (All, 2004). An environment is usually altered by multipurpose technical procedures that either avoid high-risk situations for infestations or develop unfavorable conditions for pests (All, 2004). Alternatives to fumigation are used but are only partly effective in managing disease in strawberry fields. These controls include soil solarization, anaerobic soil disinfestation (ASD), soil amendments and steam application. ASD, also known as biological soil disinfestation, uses organic amendments (e.g., rice

bran, molasses, etc.) to supply labile carbon to soil microbes to create anaerobic conditions in moist and plastic-covered soil (Shrestha et al., 2016). Some trials have shown positive results for diseases like *V. dahliae* but in the case of *M. phaseolina* it did not provide complete control in the field (Shennan et al., 2016). Using ASD involves the addition of a labile carbon source followed by anaerobic conditions, first through application of water to fill soil pore space, and then covering the soil with plastic mulch to prevent oxygen exchange (Shennan et al., 2018). Another primary cultural practice is avoiding the use of fields with cropping history that can harbor the pathogen. In the case of *V. dahliae*, crops like tomato and lettuce in rotation with strawberries can increase the pathogen concentration in the soil (Guthman, 2019; Njoroge et al., 2009). When strawberries are rotated with broccoli, a decrease in disease incidence is seen compared to rotation with lettuce (Njoroge et al., 2009). If a strawberry crop is rotated with a lettuce crop there is a potential increase of *V. dahliae* microsclerotia that can form on lettuce plants (Njoroge et al., 2009; Subbarao et al., 2007; Vallad et al., 2006).

Cultural practices include using drip instead of overhead irrigation in order to avoid any splash dispersal, the primary way *C. acutatum* spores are spread (Madden et al., 1992; Ntahimpera et al., 1998; Smith, 1998; Yang et al., 1990). Lastly, effective sanitation measures should be used in order to remove any infected fruit from the field as it can serve as a source for the spread of anthracnose (Smith, 1998; Yang et al., 1990). Surrounding weeds in nurseries should also be removed or controlled as they could serve as inoculum reservoirs for *C. acutatum* spores (Karimi et al., 2019).

1.7.3 Host resistance

Host resistance can be an important control against soilborne pathogens and a primary replacement to fumigant controls. Host resistance is considered the most cost effective and sustainable control method for crown and root diseases in strawberries (MacKenzie et al., 2006; Partička and Hancock, 2005). Genetic resistance to *V. dahliae* is considered as one of many components in an integrated management system that includes the reduction of pest populations in all growth stages (Shaw et al., 2005). Historically, due to the outstanding control of Verticillium wilt by pre-plant soil fumigation, breeding efforts could focus on fruit production and quality rather than disease resistance (Wilhelm and Paulus, 1980). A positive correlation was documented between fruit firmness and resistance to Verticillium wilt and Macrophomina crown rot (Shaw et al., 1996) and this could have slowed progress in breeding for disease resistance. Early efforts in resistance screening for *V. dahliae* found that in wild germplasm there is extensive variation in susceptibility but none of the genotypes had high resistance levels to withstand high inoculum densities (Bringhurst et al., 1966). High variation in germplasm for resistance to *V. dahliae* has been observed in California (Shaw et al., 1996; Shaw et al., 1997; Holmes et al., 2016). In Russia a segregation of six distinct races in *V. dahliae* was identified due to the resistance of different strawberry varieties indicating a complex host-pathogen interaction between these isolates and cultivars (Govorova and Govorov, 1997). This study supports the claim that multiple sources of resistance to *V. dahliae* were observed across six strawberry cultivars indicating many genetic resources that can be used by breeders (Cockerton et al., 2019).

Varying degrees of resistance to *M. phaseolina* can be found in soybean, cowpea and sorghum (Smith, 1997; Muchero et al., 2011; Diourte et al., 1995). Prior to 2012, no knowledge of resistance to *M. phaseolina* in commercial strawberry cultivars was known (Fang et al., 2012). Fang et al. (2012) showed that 'Albion' was the most resistant cultivar to *M. phaseolina* while 'Camarosa' was the most susceptible. Even though 'Albion' was considered resistant in the Fang et al. trial of 2012, it was found that 'Albion' was susceptible to *M. phaseolina* in a growth chamber trial (Sanchez et al., 2016). The difference in resistance of 'Albion' could be attributed to the inoculation method used by Fang et al. which relates to the biology of microsclerotia; Sanchez et al. (2016) used different isolates than Fang et al.(2012).

Due to the sporadic nature of anthracnose in California no breeding efforts have been made to control or to screen for this disease. Consequently, most research is focused on preventative measures for disease management. At the University of Florida (UF) recurrent phenotypic selection has been used to improve fruit quality and disease resistance to anthracnose fruit rot and anthracnose crown rot (Whitaker et al., 2012). Throughout past decades, most cultivars have shown to be moderately to highly resistant to fruit rot (Chandler et al., 2006; Seijo et al., 2008).

1.7.4 Chemical controls

After the phaseout of methyl bromide, many alternatives have been tested and used for control (Fennimore et al., 2008). Methyl bromide alternatives include chloropicrin, 1,3-dichloropropene, metam sodium and dazomet. Other types of controls include the use of preplant fungicides applied as transplant dips, but this has been effective against anthracnose and not Verticillium wilt or Macrophomina crown rot.

Preplant fungicides have the ability to decrease the incidence of anthracnose crown rot and plant collapse at early plant stages while also increasing fruit yields (Daugovish et al., 2009). Foliar fungicides play an important role in control of anthracnose through a good rotational program to avoid fungicide resistance (Louws et al., 2014). A good fungicide rotational program is important because resistance has been found to the quinone outside inhibitor (QoI) active ingredient azoxystrobin (Haack et al., 2018). Another chemical control approach that can be used is crop termination. Crop termination is another exploratory method of disease management that is used prior to plastic removal from the field to reduce inoculum build up from the survival of any biotrophic plant pathogen, nematode or insect (Holmes et al., 2020; Khatri et al., 2020). Herbicides have been considered for crop termination, but they do not control weed seeds, nematodes or soilborne pathogens (Khatri et al., 2020). With the use of metam sodium, there is the potential that it controls plant pathogens, nematodes and weeds but the efficacy is dependent upon the location of the pest on the bed (Khatri et al., 2020).

Fumigation is important for controlling soilborne diseases. However, given that propagules of *C. acutatum* only survive in the soil for up to 9 months (Eastburn and Gubler, 1992; Freeman et al., 2002), fumigation is rarely a primary means of anthracnose control. In the case for *V. dahliae* and *M. phaseolina*, microsclerotia have shown to live in the soil for many years, making fumigation far more important.

1.8 The use of Fumigants

1.8.1 Chloropicrin and Methyl Bromide

A primary control that has been used for soilborne diseases is soil fumigation. Soil fumigation is the use of volatile compounds applied in the form of a gas to control

plant pathogens and pests that live in the soil and can disrupt plant growth and crop production (Chellemi, 2014). Soil fumigants can provide benefits to growers by controlling a wide range of pests, including nematodes, fungi, bacteria, insects and weeds (Chellemi, 2014).

One of the first soil fumigants used was chloropicrin (Cl_3CNO_2) also called trichloronitromethane. Chloropicrin was first discovered after World War I as one of the war gases that improved soils for plant growth (Russell, 1920). *Verticillium dahliae,* first described in 1931, has been an important soilborne pathogen of strawberries and was one of the main reasons that chloropicrin was developed as a soil fumigant (Tribble Bros, 1912; Thomas, 1939; Wilhelm and Koch, 1956). Verticillium wilt was so devasting that it figured prominently in the selection of land used to grow strawberries. In 1920, the heavy loss of a strawberry field due to *Verticillium dahliae* from the Driscoll-Reiter strawberry organization was believed to be linked to a previous tomato crop (Wilhelm and Paulus, 1980). Soon after, a field where cotton was previously grown in the San Joaquin Valley was said to have the same effects as tomatoes on strawberries (Wilhelm and Paulus, 1980). Over the next years, after the discovery of control with the use of chloropicrin in 1956, more experiments were established to improve such control. The first discoveries that improved fumigation were the use of machine applied chloropicrin rather than hand injected (Wilhelm and Koch, 1956). The improvement of fumigation came with the addition of polyethylene sheeting, leading to the discovery that it was excellent for weed control (Wilhelm et al., 1961). This was important because weeds such as hairy nightshade that serve as an alternative host were linked to *V. dahliae* infestations

(Wilhelm and Paulus, 1980). This weed was important as it didn't show exterior symptoms and symptoms were only visible as vascular discoloration.

The discovery of the combined use of chloropicrin and methyl bromide was important as this helped the strawberry industry in a span of 15 years to be able to produce fruit of 40 to 60 tons/ha by 1972 (Wilhelm et al., 1974). The production in the 1960s was at 12.3 to 14.8 tons/ha (Wilhelm and Paulus, 1980). In 2017 industry-wide average production was at 61.7 tons/ha (California Strawberry Commission, 2018). Methyl bromide (CH_3Br), also known as bromomethane is a colorless odorless gas at room temperature and normal pressure. Methyl bromide is dangerous to humans as it can be readily absorbed through the lungs (Budnik et al., 2012). Methyl bromide is an ozone depleting fumigant because when released into the atmosphere UV light causes the bromine to be released. Bromomethane is readily photolyzed when in the atmosphere and it causes the release of bromine radicals which are more destructive to the stratospheric ozone (Wang et al., 2019).

1.8.2 Methyl bromide phaseout

The Montreal Protocol was established in order to protect the ozone layer by reducing and eliminating the production and consumption of ozone-depleting substances. Both the US Clean Air Act and the Montreal Protocol restricted the use of methyl bromide considering it an ozone-depleting compound. Through the US Clean Air Act, the United States was able to satisfy the obligations of The Montreal Protocol. Starting in 1991 to 2005, methyl bromide was completely banned by the end of a 14-year span (Schneider et al., 2003). Despite its ban in 2005, crops such as strawberries were still able to use methyl bromide by applying for a critical use exemption up until 2016 (Holmes et

al., 2020). Today strawberry nurseries are still able to use methyl bromide through quarantine and preshipment exemption. This exemption authorizes methyl bromide use in order to control the introduction or spread of a pest within the U.S. (Holmes et al., 2020).

1.8.3 Alternative fumigants

The fumigant 1,3-dicholoropropene (1,3-D) was originally developed as a nematicide under the brand name Telone (Dow AgroSciences LLC, Indianapolis, IN). 1,3-D alone controls nematodes and some weeds but has very limited activity against soilborne pathogens and weeds (Noling and Becker, 1994). When 1,3-D is combined with chloropicrin these fumigants can control fungi, bacteria, insects, nematodes and weeds but weed control is limited (Ashworth et al., 2014). In 2014, Ashworth et al. tested several combinations of chloropicrin and 1,3-D showing that in combination, emissions dropped compared to chloropicrin applied alone. Some concerns when using Telone products (Telone II, Telone C-17, and Telone C-35) are potential ground water contamination, worker exposure, air emissions as potential chronic exposure and California Proposition 65 labeling it as a carcinogen (Duniway, 2002).

Other fumigants listed in California include metam sodium or metam potassium. Metam sodium (sodium *N*-methyl dithiocarbamate) and metam potassium are fumigants that breakdown rapidly to methyl isothiocyanate (MITC) (Duniway, 2002). Both of these fumigants work best when applied in sequential applications. MITC is a broad-spectrum fumigant that can control nematodes, weeds, oomycota and fungi (Kreutzer, 1963). Metam sodium uses higher rates and when using VIF, it increases weed control (Ajwa et al., 2010). Metam sodium is typically formulated as 18 to 42% aqueous solutions sold

under trade names such as Metam CLR, Vapam and Sectagon (Carlock and Dotson, 2010).

1.9 Conclusion and objectives

The plant's ability to resist infection or yield reduction when challenged by pathogens has been employed as an important management tactic in agriculture. Host resistance has not been sufficiently utilized by the strawberry industry to manage soilborne pathogens due to the availability and use of effective soil fumigation chemistries. Current fumigant chemistries are effective when distributed efficiently in the soil profile, but this can be difficult to achieve in a field setting, and their availability in the future is uncertain. In the post-methyl bromide era, host resistance will likely be an important tool for managing most soilborne disease in strawberries.

A thorough phenotypic screening of resistance should involve commonly used and available strawberry cultivars, as well as elite breeding selections that are being developed for future use. Disease screening would help growers select resistant cultivars. This could also aid breeding programs in their selection of resistant genotypes. Evaluating pathogen colonization in strawberry cultivars can help future research by providing pathogen-specific management strategies and can help better understand when the disease is more prominent in the growing season.

In Florida, extensive research has been conducted on anthracnose in strawberries. A primary reason for this is that anthracnose is a prominent disease that occurs yearly during the Florida production season. In California, anthracnose is not present in most years but can cause severe yield losses when it does occur. The sporadic appearance of anthracnose in California strawberry production did not give priority to much

anthracnose research for the California crop. With the recent outbreak in the 2015-2016 season, research on anthracnose became a higher priority.

Our first research objective was to determine the susceptibility of cultivars and elite breeding lines to anthracnose. A replicated field trial was conducted to screen commercially available strawberry cultivars and elite breeding lines for their resistance to anthracnose. The results will help plant breeders implement genetics of resistant cultivars into future breeding lines. Research conducted in Florida and other parts of the world can be used and combined in order to have preventive control measures laid out for growers and pest control advisors in the state in preparation for future outbreaks.

Our second research objective was to evaluate colonization of root and crown tissue by *V. dahliae* and *M. phaseolina* in strawberry cultivars with different levels of disease resistance. The overall goal of this objective was to identify host-pathogen interactions between disease resistant and susceptible strawberry cultivars, between plant tissue (crown and root) and over two sampling periods (early- and late-season). The findings will help us understand how and when these pathogens attack their hosts. This research can also provide input to breeders in their continuous characterization of and breeding for disease resistance.

CHAPTER 2: EVALUATING HOST RESISTANCE TO ANTHRACNOSE IN 105 CULTIVARS AND ELITE BREEDING LINES OF STRAWBERRY

2.1 Introduction

Strawberries are considered an important crop in California where in 2017, 1.8 billion pounds of strawberries were harvested (California Strawberry Commission, 2018). With total U.S. production at 2.6 billion pounds, this accounts for 88% of total U.S. production (California Strawberry Commission, 2018). In 2018, the value of strawberries was in the top 5-valued fruit and vegetable agricultural commodities for California at 2.84 billion dollars (California Department of Food and Agriculture, 2018).

Anthracnose caused by *Colletotrichum acutatum* (syn. *C. nymphaeae*) is an economically important disease of strawberry (*Fragaria* × *ananassa*) where in 2002 it caused the California industry millions of dollars (Gaines, 2005). In California and Europe, *C. acutatum* is the primary species causing anthracnose on strawberry plants and fruit (Garrido et al., 2016). All parts of the plant are susceptible, causing necrosis and blight symptoms on tissues such as leaves, petioles, flowers or even roots, resulting in plant mortality (Peres et al., 2005). Another primary symptom is the fruit lesions it causes on ripe fruit (Peres et al., 2005). *C. acutatum* is an issue because when present on strawberry transplants brought from the nurseries, marketable yield can drop by 40% (Daugovish, 2009). A disease like anthracnose will be destructive under the right conditions, especially when grown on black plastic in areas with high rainfall as this provides a springboard for water droplets, encouraging the spread of the disease (Louws, 2014). Due to the low persistence of anthracnose in soil, the main concern and control is at the nurseries (Poling 2008). A grower's main control for anthracnose is not fumigation

and therefore growers rely on disease-free transplants in order to not introduce anthracnose into their fields. During the most recent California outbreak in the 2015-2016 season, it became obvious that cultivars differed widely in their susceptibility to anthracnose (Holmes, *personal communication*). Therefore, host plant resistance to anthracnose could be utilized if susceptibility of strawberry genotypes was widely known. In order to determine susceptibility to anthracnose, a replicated field trial was conducted where 105 cultivars and elite breeding selections were screened. Only 30 of the 105 cultivars and elite breeding selections were evaluated both years.

2.2 Materials and Methods

2.2.1 Strawberry cultivars and elite breeding lines

A total of 105 strawberry cultivars and elite breeding lines were tested over two years (2018 and 2019). A total of 76 strawberry cultivars and elite breeding lines/selections were included in the field evaluation for year one of the trial. For year two, 59 cultivars and elite breeding lines were included. Thirty of the 105 strawberry cultivars and elite breeding lines were tested both years. Day neutral and short-day strawberry genotypes were provided from six public and private breeding programs: University of California Davis (Davis, CA), University of Florida (Wimauma, FL), Driscoll's (Watsonville, CA), Planasa (Red Bluff, CA), Plant Sciences, Inc. (Watsonville, CA) and Lassen Canyon (Redding, CA) (Table 2.1). In year two, no cultivars or elite breeding selections were used from the Planasa breeding program.

2.2.2 Field site and layout

2.2.2.1 Year 1 (2018)

The experiment was conducted on the California Polytechnic State University campus in San Luis Obispo, CA; Field 25, Block 3 (35°18’18.9” N; 120°40’38.3 W). The soil type in block 3 is a Salinas Silty clay and standard irrigation and fertilization practices were used for establishment and maintenance of the crop. The field site selected had previously been cropped for over twenty years with row and vegetable crops (broccoli, lettuce, corn, tomato and squash) prior to its first strawberry planting which occurred in October 2014. Strawberries have been continually grown at this location until the 2019-2020 season. The field was pre-plant, broadcast soil fumigated using Ally 33 (67% AITC + 33% chloropicrin at 55 gal/treated A) on 20 September 2018.

2.2.2.2 Year 2 (2019)

In year two the field trial was conducted on the California Polytechnic State University campus in San Luis Obispo, CA Field 25, Block 8 (35°18'14.2"N 120°40'30.1"W). The soil type in block 8 is the same as block 3 used the previous year. This site is 200 meters distance from the field site in year one with a similar cropping history. The first strawberry planting occurred on 23 October 2019. The field was fumigated via drip lines using the pre-plant soil fumigant Ally 33 (67% AITC + 33% chloropicrin at 55 gal/treated A) on 7 October 2019.

2.3 Experimental design

Raised beds were prepared prior to planting and were constructed as 162 cm center to center, and approximately 30 cm tall. Two lines of drip irrigation (low-flow, 1.2 liter/min/30.4 m at 55 kPa, with 20 cm spacing on emitters) (Tri-Cal®, Hollister, CA) per

bed were buried approximately 5 cm deep in the raised beds, and the beds were covered with 1 mil, black TIF (totally impermeable film) polyethylene mulch. Each bed contained four planting rows which were 25 cm apart, and plants were spaced 40 cm apart within the plant row. Bareroot transplants were planted by hand on 25 October 2018 and on 23 October 2019.

For year one all 76 plots for a single replicate were arranged on three individual strawberry beds. Each plot consisted of 10 plants of the same genotype. In year two, the 60 plots for a single replicate were arranged on two strawberry beds. Again, each plot consisted of 10 plants. Plots were approximately 1 m long and organized in a randomized complete block design (RCBD) with four block replicates plus one non-inoculated replicate.

Immediately prior to planting, transplants were inoculated by adding 100 ml of the prepared inoculum into a 3.8-liter Ziplock bag containing 10 plants and mixed for one minute. The non-inoculated block was separated from the inoculated blocks by a planted bed. The non-inoculated block only contained a single plot per genotype and served as a check for the presence of other pathogens or confounding factors.

2.3.1 Mortality assessments and AUDPC

Visual plant mortality assessments were conducted weekly. Mortality assessments began three weeks after planting (WAP) when symptoms of the disease were observed. Disease diagnostics were conducted on a biweekly basis up until the last ratings in December (8 plants per sampling) and then sporadically sampled throughout the season. Plants with symptoms (50-80% foliar necrosis) were sampled and plated on selective medium acidified corn meal agar, Acidified Potato Dextrose Agar (APDA), P_{10}ARP

(Erwin and Ribeiro, 1996) and Sorensen's NP-10 medium (Sorensen et al., 1991). Symptoms observed when mortality assessments were initiated were necrotic leaves and collapse of plants. After determining initial plant stand, any transplants that failed to produce new leaves were recorded as dead. Weekly assessments to determine plant mortality commenced on 12 November 2018 and continued until 29 April 2019. In year two, weekly assessments began on 11 November 2019 (3 WAP) and continued until 29 April 2020. Percent mortality was calculated for each 10-plant plot.

Mortality assessment data was used to determine the area under disease progress curve (AUDPC). AUDPC is a useful quantitative summary of disease intensity over time (Jeger and Viljanen-Rollinson 2001, Madden et al., 2007). The most common method used for AUDPC is the trapezoidal method, which can discretize the time variable (hours, days, weeks, months, or years) and calculate the average disease intensity between each pair of adjacent time points (Madden et al., 2007). AUDPC is calculated using the following formula:

$$AUDPC = \sum_{i=1}^{N_i-1} \frac{(y_i + y_{i+1})}{2} \times (t_{i-1} - t_i)$$

Where y_i is the percent mortality for the observation number i, t_i is the number of days from the planting date, and N is the total number of observations.

2.3.2 Inoculum production

PDA plates were used to grow *C. acutatum* spores for inoculation. One thousand petioles were collected by cutting them from the plant at the base of each petiole. Trifoliate leaves and stipules were removed from the petioles. Petioles were washed and rinsed three times with deionized (DI) water. Petioles were cut into 2.5 cm segments and

placed into 150 ml plastic containers (Nalgene®, Rochester, NY) with 50 ml of DI water. The containers were autoclaved on two separate days for 30 minutes (STM-E, Market Forge CO., Everett, MA).

Colletotrichum acutatum isolates CA-1, CA-15 and CA-140 used in this study were obtained from diseased strawberry plants in 2015 (CA-1, CA-15) and 2016 (CA-140). These isolates were confirmed and identified as *C. acutatum* based on their morphological and colony characteristics on PDA. The protocol used for the inoculation method in this trial was slightly modified from the protocol used by Haack et al. 2018. *C. acutatum* spores used for transplant inoculations were initially harvested by transferring a 1 × 1 cm agar block from actively growing cultures into a 15 ml water tube. The 15 ml tubes contained 13 ml of autoclaved deionized (DI) water and glass beads for spore separation. The 15 ml tubes were vortexed for 30 s to disperse the fungal hyphae and spores in DI water. *C. acutatum* inoculum (0.5 ml) was spread onto a PDA plate using a sterile glass rod and petiole pieces (9 per plate) were evenly spaced out on the agar surface. Plates were stored at room temperature for a total of 12 days or until over 80% of the plate was covered by *C. acutatum*. After 12 days, 5 ml of autoclaved DI water containing Tween 20 (DI-20) to help disperse spores was added to the PDA plates containing petioles and fungal growth. Spores were harvested by using a glass rod to rub the culture surface, dislodging petiole segments and spores from the agar surface for approximately 30 seconds per plate. The 5 ml of DI-20 water containing spores from the PDA plate were poured through a double layer of cheese cloth (to remove petioles and hyphae). This process was repeated for all three isolates. A total of 8 gallons of inoculum were made by adjusting the spore counts in both years. Year one was adjusted to 6.75 ×

10^5 and year two to 8.25×10^5 conidia/ml for all three isolates (the target concentration was 1×10^6 conidia/ml).

2.3.3 Data analysis

A standard sum of squares analysis of variance (ANOVA) was performed for the single effect of cultivar on percent mortality, and on AUDPC (JMP® pro version 13.1 SAS Institute, Cary, NC). Prior to statistical analysis, AUDPC was calculated using mortality assessments on an Excel spreadsheet for each cultivar. Relative area under disease progress curve (rAUDPC) was calculated showing the proportion of maximum possible disease severity over each year. AUDPC scores were divided by the maximum AUPDC possible over the entire length of the season. Maximum AUDPC score was calculated by calculating the maximum mortality count (i.e., 10) on each assessment date. After calculations, results for rAUDPC will show 0.0 to 1.0, 0.0 (resistant) being no disease severity over time and 1.0 (susceptible) meaning 100 percent disease severity over time. Both trial years were analyzed separately. Significant genotype differences were found based on the F-test, where critical values were calculated at the 5% level of probability ($\alpha = 0.05$). The Student's t-test was used as the multiple comparisons test (*p*-value < 0.0001 for both years).

2.4 Results

2.4.1 Percent mortality by cultivar

2.4.1.1 Year 1

Anthracnose symptoms were initially observed two weeks after planting in December 2018, with the first observations of plant mortality occurring at three weeks after planting. Most plant mortality occurred during the month of December, with 74.8%

mortality occurring before 8 January 2019. Following a rain and increased temperatures in April, 23 additional genotypes showed increased mortality.

There were statistically significant effects (F= 16.6607, $P < 0.0001$) of strawberry genotype on percent mortality observed during the 29 April 2019 assessment. Average mortality of each genotype ranged from 0 to 100% (Figs. 2.1 and 2.3; Table 2.2). The five genotypes with the highest mortality levels were Monterey, UC-9A, Laredo, Victor and Spartan, with 94.7, 95.0, 97.5, 100.0, and 100.0 percent mortality, respectively (Fig. 2.1 and 2.3; Table 2.2). The five strawberry genotypes with the lowest mortality were PS 5016, PSI-9, PSI-10, LC-1, and LC-4, with 0.0. 0.0, 2.5, 5.0 and 5.0 percent mortality respectively (Fig. 2.1 and 2.3).

All breeding programs had genotypes showing a wide range of plant mortality in year one. Variability among replicates measured with standard error showed a range of standard error from 0.0 to 13.1. Among all 46 genotypes listed in year 1, there were eight genotypes with a standard error larger than 10.0. Genotypes from the University of California breeding program had disease ranging from 15.0 (UC-15) to 100% plant mortality (Monterey, UC-9 and UC-10) (Fig. 2.3). Genotypes from the University of Florida breeding program ranged from 15.0% (Sensation) to 60.0% plant mortality (Winterstar) (Fig. 2.3). Genotypes from the Planasa breeding program ranged from 54.7% (PL 3001) to 84.7% plant mortality (PL 12-04R) (Fig. 2.3.). Genotypes from Plant Sciences Inc. ranged from 2.5% (PS 5016 and PSI-10) to 77.5% mortality (BG 3.324) (Fig. 2.3). Genotypes from Lassen Canyon ranged from 10.0% (LC-1) to 70.0% mortality (LC-7) (Fig. 2.3). Genotypes from Driscoll's ranged from 22.5% (Prado) to 100.0% plant mortality (Spartan) (Fig. 2.3). The non-inoculated block maintained a healthy and

vigorous growth throughout the season with the exception of a few genotypes showing up to 20% mortality (Table 2.1) Based on observations comparing inoculated plots with non-inoculated plots, there were some cultivars that were stunted compared to their respective non-inoculated plots (data not presented).

2.4.1.2 Year 2

Symptoms appeared 2 weeks after planting while the first observations of plant mortality began 23 days after planting. Most plant mortality occurred by 1 January 2020, 63 days after planting. At this time, 80.6% of the total mortality occurred. Mortality across all 59 cultivars averaged 58.0%. There were statistically significant effects (F= 18.2975, $P < 0.0001$) of strawberry genotype on percent mortality observed at the 29 April 2020 assessment.

In year 2, a wide range of plant mortality was again observed for the genotypes evaluated. Variability was also seen among replicates for each genotype. For this second year the range in standard error was from 0.0 to 13.4. There was a total of five out of 29 genotypes that had a standard error over 10.0. The five strawberry genotypes exhibiting the highest mortality levels were Lara, UC-9, UC-5, Warrior and Monterey with 97.5, 100.0, 100.0, 100.0 and 100.0% mortality respectively (Fig. 2.2 and 2.4). These five susceptible genotypes were not statistically different from each other. The five strawberry cultivars with the lowest mortality were bg 4.352, bg 4.367, bg 9.3128, Del Rey and pe 7.2054 with 7.5, 7.5, 7.5, 10.0, 10.0 and 12.5% mortality, respectively (Fig. 2.2 and 2.4). These five resistant genotypes with the lowest percent mortality were numerically different but not statistically different from each other. The non-inoculated block maintained healthy and vigorous plants season long. In comparison with the inoculated

plots there were some cultivars that were stunted compared to their respective non-inoculated plots (data not presented).

2.4.1.3 Two-year cultivars

A total of 30 cultivars were tested in both years of the experiment (Fig. 2.5). Breeding programs from Plant Sciences, Driscoll's, University of California, Davis, University of Florida and Planasa had, 8, 7, 7, 6 and 2 genotypes common to both years, respectively. Looking at all genotypes common to both years a total of 21 cultivars had a difference in final plant mortality at or below 20% (Fig. 2.6). Del Rey and Ruby June differed dramatically (>50% mortality) between years. In year one, Ruby June was resistant with 25.0 percent mortality while Del Rey was Moderately susceptible with 66.7 percent mortality (Fig. 2.6). In year two, Ruby June was susceptible with 80.0 percent mortality while Del Rey was resistant with 10.0 percent mortality (Fig. 2.6).

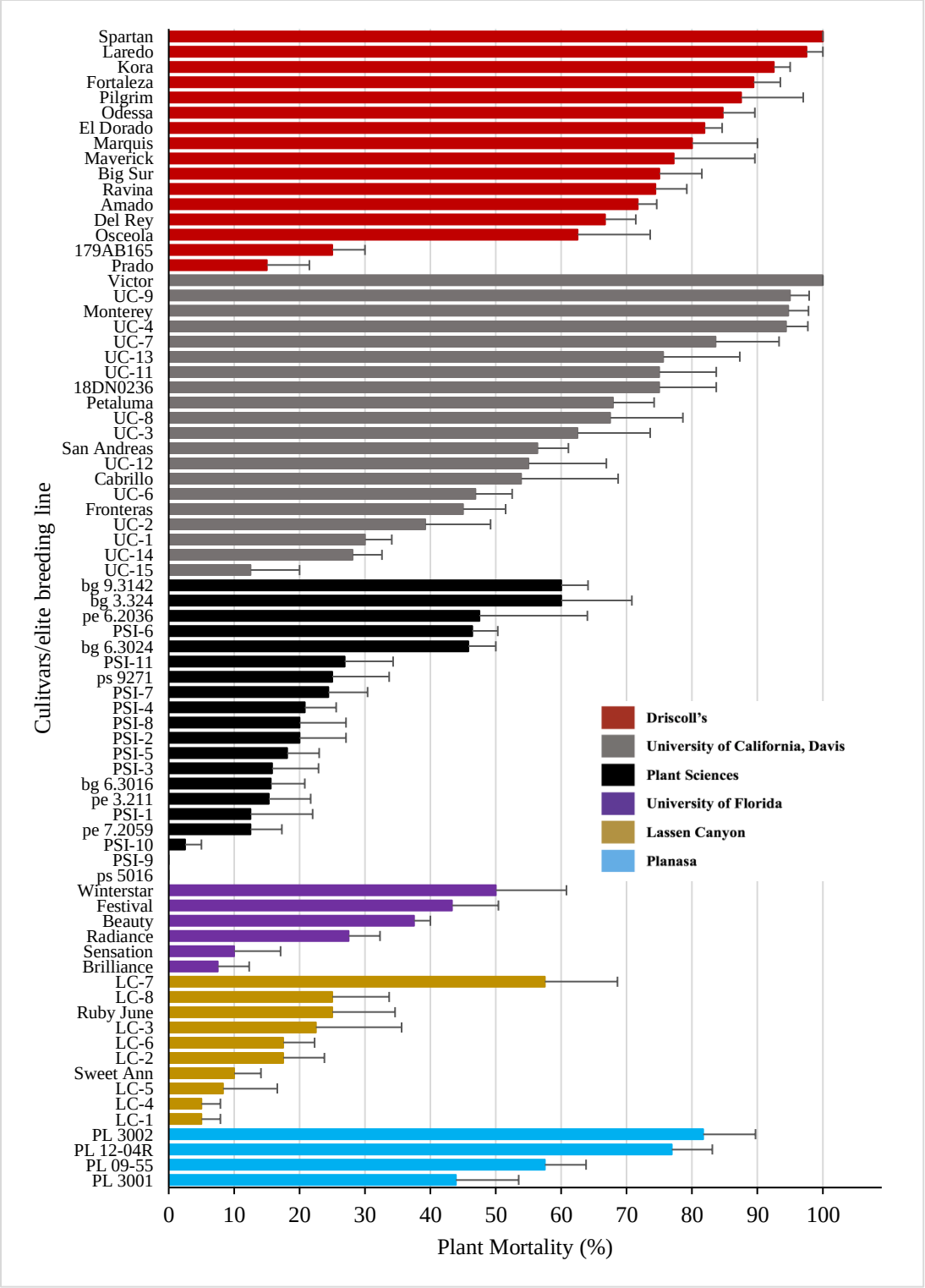

Figure 2. 1 Average percent mortality due to anthracnose as of 29 April 2019, 184 days after inoculation, sorted from highest to lowest within breeding programs. Average values were derived from percent mortality of four replicate plots. Error bars are standard error of the mean.

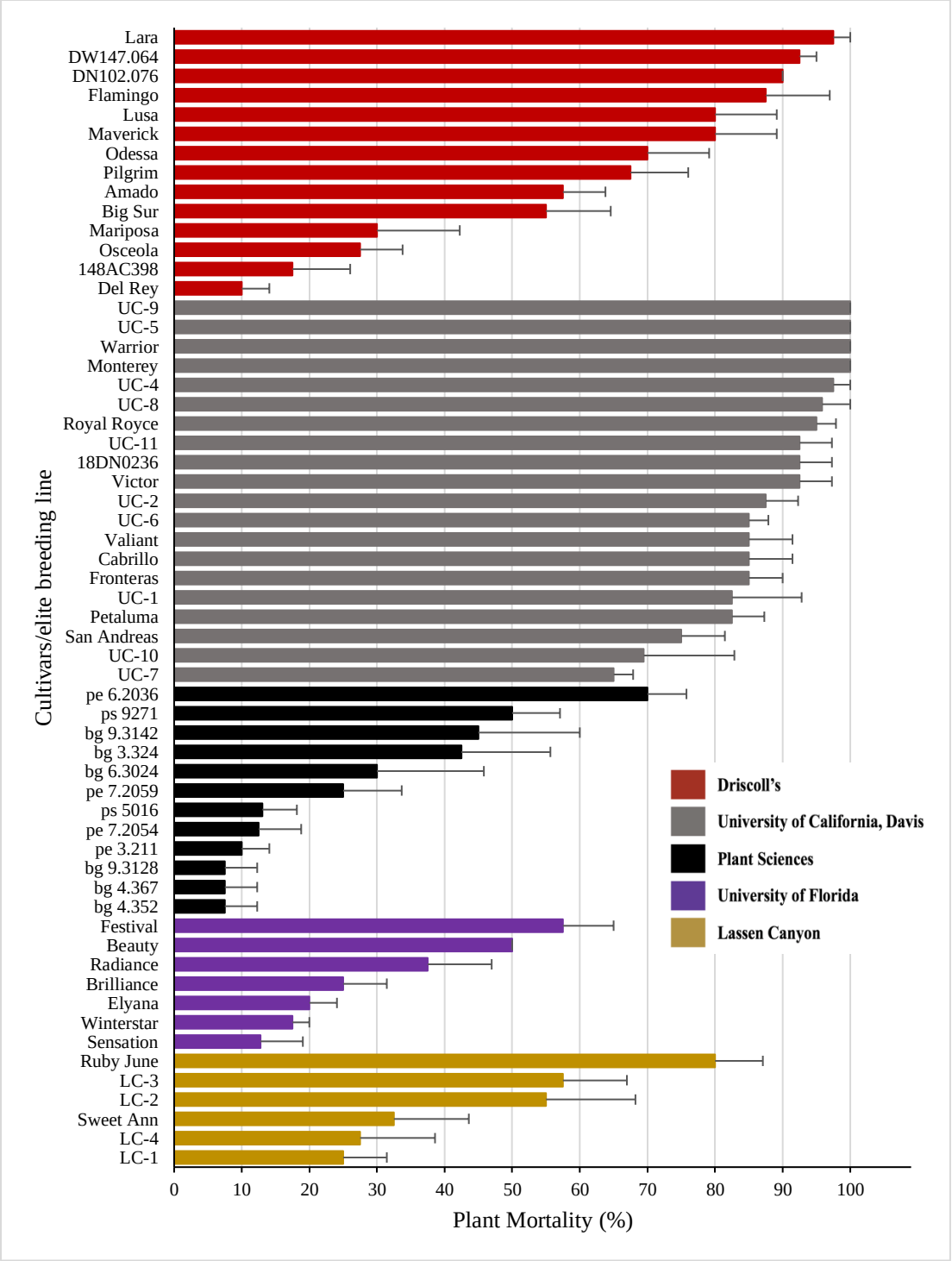

Figure 2. 2 Average percent mortality due to anthracnose as of 29 April 2020, 188 days after inoculation, sorted from highest to lowest within breeding programs. Average values are derived from percent mortality of four replicate plots. Error bars are standard error of the mean.

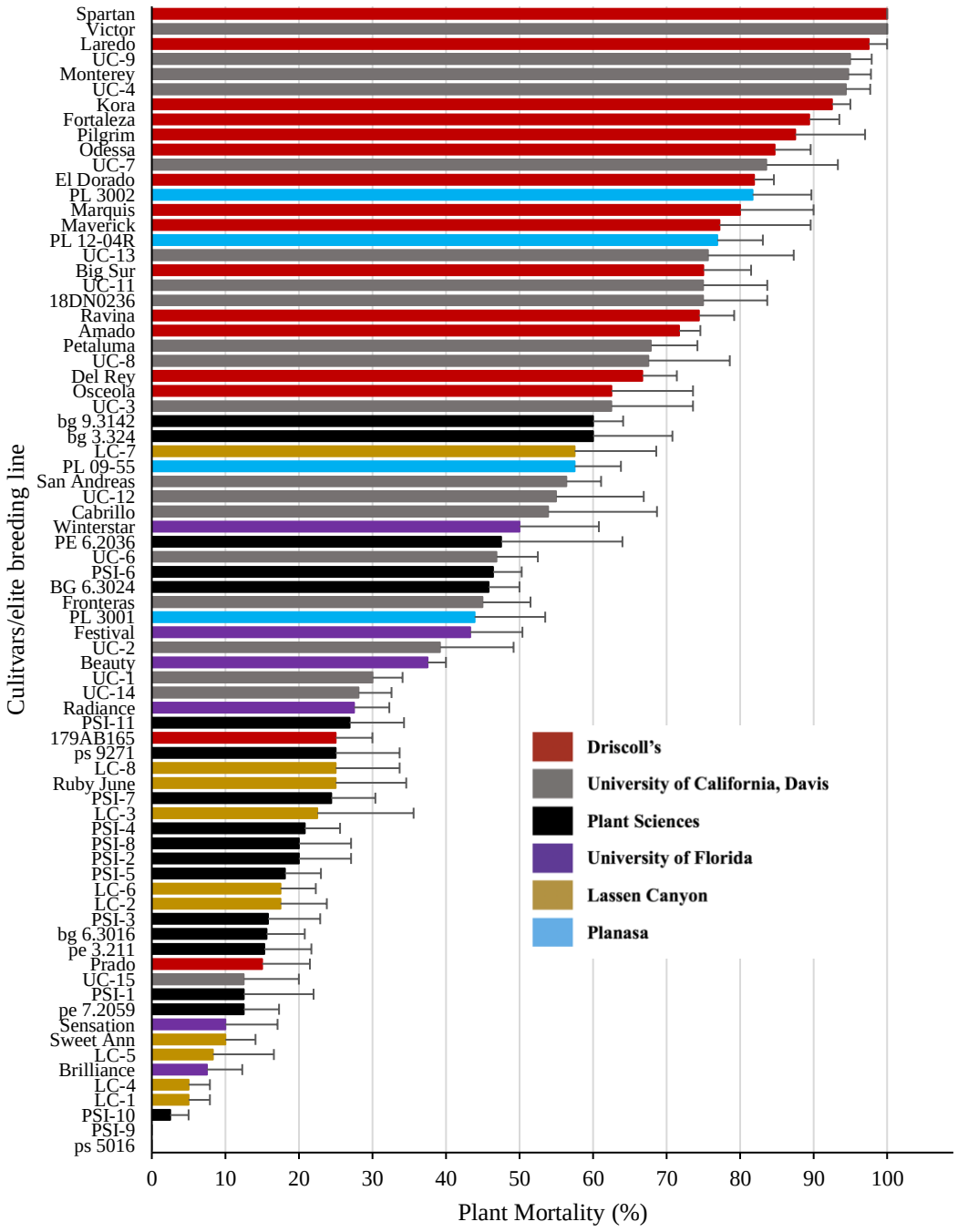

Figure 2. 3 Average percent mortality due to anthracnose as of 29 April 2019, 184 days after inoculation, sorted from highest to lowest. Average values are derived from percent mortality of four replicate plots. Error bars are standard error of the mean.

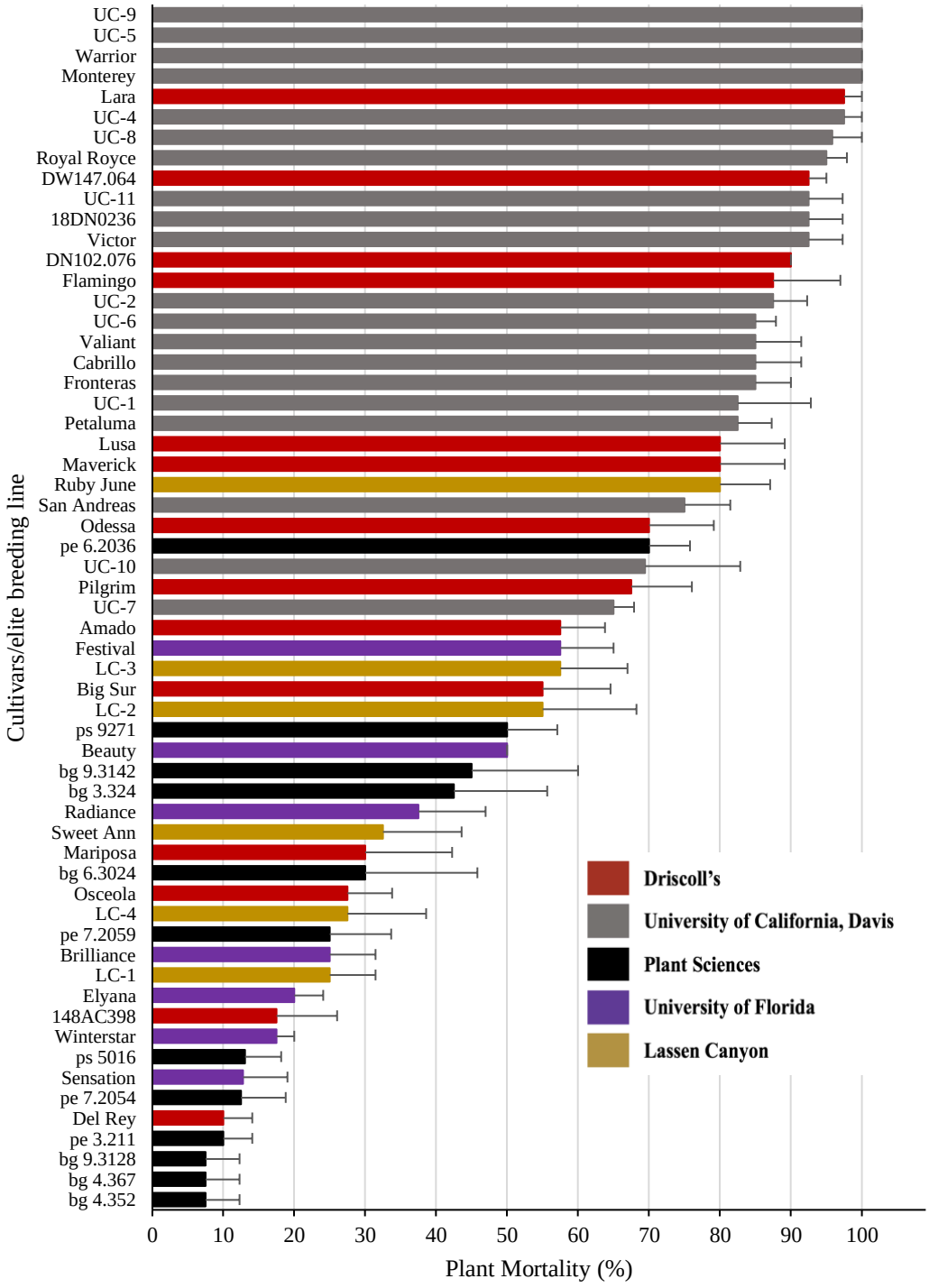

Figure 2. 4 Average percent mortality due to anthracnose as of 29 April 2020, 188 days after inoculation, sorted from highest to lowest. Average values are derived from percent mortality of four replicate plots. Error bars are standard error of the mean.

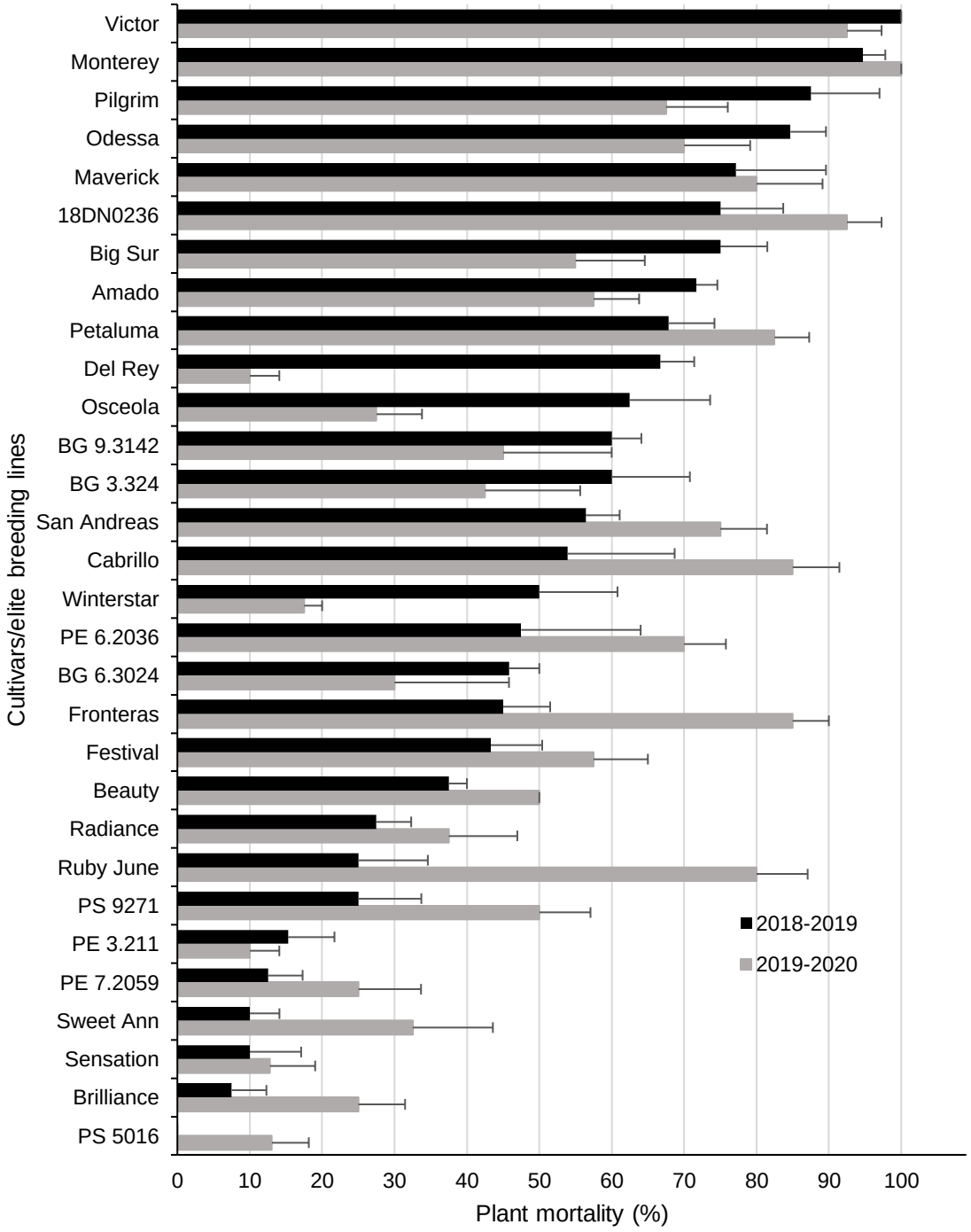

Figure 2. 5 Two-year average percent mortality due to anthracnose as of 29 April 2019 and 29 April 2020 (184 and 188 days after inoculation, respectively). Cultivars and elite breeding lines are sorted from highest to lowest based on results in year one (2018-2019). Average values are derived from percent mortality of four replicate plots. Error bars are standard error of the mean.

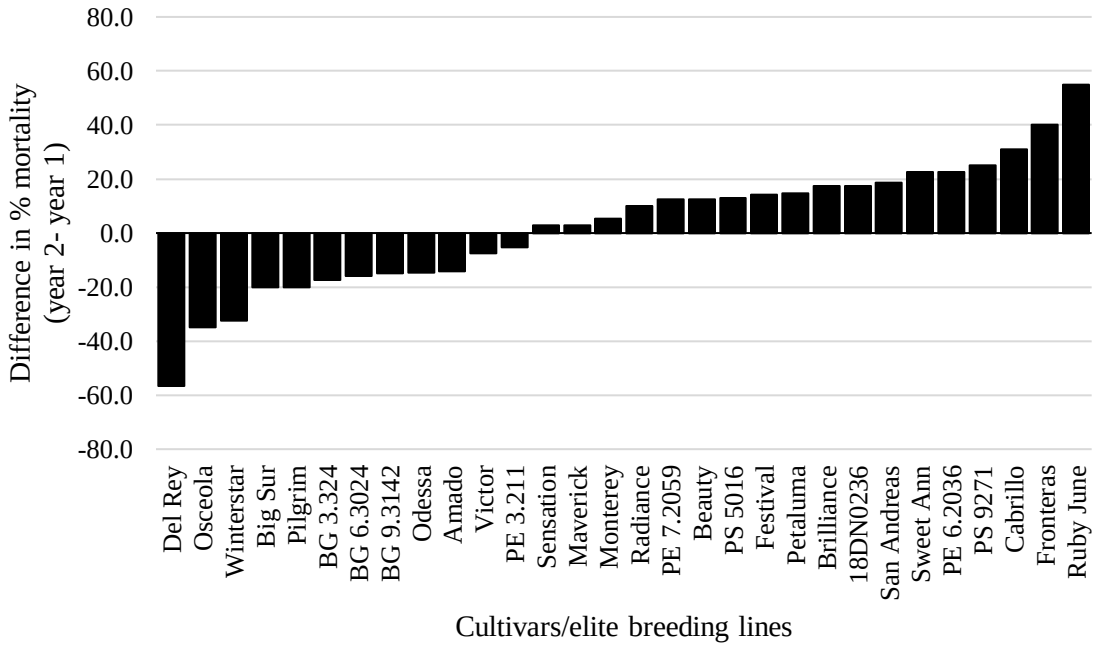

Figure 2. 6 Difference in average percent mortality due to anthracnose between cultivars and elite breeding lines included in both years (Year 2 – Year 1) as of 29 April 2019 and 29 April 2020, 184 and 188 days after planting, respectively. Average values are derived from percent mortality of four replicate plots.

2.4.2 AUDPC by cultivar

2.4.2.1 Year 1

There were statistically significant effects (F= 16.5551, $P < 0.0001$) of strawberry genotype on AUDPC observed for the 29 April 2019 assessment. AUDPC for all 76 genotypes averaged 662, while the total possible AUDPC for the whole season at max mortality for each rating was 1725. Average AUDPC based on plant mortality ranged from 0 to 1709 (Table 2.3). The five strawberry genotypes with the highest rAUDPC were Spartan, Monterey, Laredo, UC-9 and Victor with 0.81, 0.85, 0.87, 0.88 and 0.90 rAUDPC (Table 2.3). All top five susceptible genotypes demonstrated numerical differences in AUDPC, yet they were not statistically different from each other. The five strawberry genotypes with the lowest rAUDPC were PSI-9, PS 5016, PSI-10, LC-1 and

LC-4, with 0.0, 0.0, 0.01, 0.03 and 0.03 rAUDPC, respectively (Table 2.3). These five resistant genotypes with the lowest AUDPC were numerically different but not statistically different from each other. All breeding programs contained genotypes showing a wide range of rAUDPC in year one.

2.4.2.2 Year 2

Symptom development occurred 2 weeks after planting while the first observations of plant mortality began 23 days after planting. Most plant mortality occurred by 1 January 2020, 63 days after planting. At this time, 80.6% of the total mortality occurred. AUDPC across all 59 genotypes averaged 857 AUDPC while the total possible AUDPC for the whole season at max mortality for each rating was 1790. There were statistically significant effects (F= 22.2325, $P < 0.0001$) of strawberry genotype on AUDPC observed at the 29 April 2020 assessment. The five strawberry genotypes exhibiting the highest rAUDPC were Lara, UC-5, UC-4, Warrior and UC-9 with 0.86, 0.89, 0.91, 0.92 and 0.95 rAUDPC (Table 2.3). These five susceptible genotypes demonstrated numerical differences in percent mortality, but they were not statistically different from each other. The five strawberry genotypes with the lowest rAUDPC were Del Rey, bg 9.3128, bg 4.352, pe 3.211 and bg 4.367 with 0.05, 0.05, 0.05, 0.06 and 0.06 rAUDPC (Table 2.3). These five resistant genotypes with the lowest AUDPC were numerically different but not statistically different from each other.

Table 2. 1 Strawberry non-inoculated cultivars and elite breeding lines (genotypes) in ranking order by percent plant mortality as of 29 April 2019.

Cultivar/Elite Breeding Line	Plant Mortality[w] (%)	
	2018-2019	2019-2020
	Mean[x]	Mean[x]
Year 1 & 2		
ps 5016	0	0
Brilliance	0	0
Sensation	0	0
Sweet Ann	0	0
pe 7.2059	0	0
pe 3.211	0	0
ps 9271	0	0
Ruby June	0	0
Radiance	0	0
Beauty	0	0
Festival	0	0
bg 6.3024	0	0
pe 6.2036	0	0
Winterstar	0	0
San Andreas	0	0
bg 9.3142	0	0
Del Rey	0	0
Petaluma	0	0
Big Sur	0	0
18DN0236	0	0
Maverick	0	0
Odessa	0	0
Pilgrim	0	0
Monterey	0	20
Victor	0	0
Fronteras	10	0
Amado	10	10
Cabrillo	10	0
Osceola[y]	11.1	0
Year 1		
PSI-1A	0.0	-
PSI-2A	0.0	-

PSI-3A	0.0	-
PSI-4A	0.0	-
PSI-5A	0.0	-
PSI-6A	0.0	-
PSI-7A	0.0	-
PSI-8A	0.0	-
PSI-9A	0.0	-
PSI-10A	0.0	-
PSI-11A	0.0	-
bg 4.367	0.0	-
bg 6.3016	0.0	-
UC-1A	0.0	-
UC-2A	0.0	-
UC-3A	0.0	-
UC-4A	0.0	-
UC-6A	0.0	-
UC-7A	0.0	-
UC-8A	0.0	-
UC-11A	0.0	-
UC-12A	0.0	-
UC-13A	0.0	-
UC-14A	0.0	-
LC-1A	0.0	-
LC-2A	0.0	-
LC-3A	0.0	-
LC-4A	0.0	-
LC-5A	0.0	-
LC-6A	0.0	-
LC-7A	0.0	-
LC-8A	0.0	-
El Dorado	0.0	-
Kora	0.0	-
Fortaleza	0.0	-
Marquis	0.0	-
Laredo	0.0	-
Spartan	0.0	-
PL 12-04R	0.0	-
PL 09-55	0.0	-
PL 3001	0.0	-
UC-9A	10.0	-

UC-15A	10.0	-
Prado	10.0	-
179AB165	10.0	-
PL 3002	10.0	-
Year 2		
bg 3.324	-	0
bg 4.352	-	0
bg 4.367	-	0
bg 9.3128	-	0
pe 7.2054	-	0
Royal Royce	-	0
Valiant	-	0
Warrior	-	0
UC-1B	-	0
UC-2B	-	0
UC-4B	-	0
UC-5B	-	0
UC-6B	-	0
UC-7B	-	0
UC-9B	-	0
UC-10B	-	0
UC-11B	-	0
LC-1B	-	0
LC-2B	-	0
LC-3B	-	0
LC-4B	-	0
Lusa	-	0
Lara	-	0
Flamingo	-	0
Mariposa	-	0
148AC398	-	0
DW147.064	-	0
DN102.076	-	0
Elyana	-	0
UC-8B[z]	-	16.7

[w] Percent mortality as of 29 April 2019 and 29 April 2020, 184 and 188 days after inoculation, respectively.
[x] Mean values and standard error derived from four plot replicates. Each plot replicate contained 10 plants.
"-" No data available for this year.
[y] Plant mortality for Osceola had an initial plant stand of 9 plants.
[z] Plant mortality for UC-8B had an initial plant stand of 6 total plants

Table 2. 2 Strawberry inoculated cultivars and elite breeding lines (genotypes) in ranking order by percent plant mortality as of 29 April 2019 (year 1).

Cultivar/Elite Breeding Line	Plant Mortality[y] (%)			
	2018-2019		2019-2020	
	Mean[y]	SE[z]	Mean[y]	SE[z]
Year 1 & 2				
PS 5016	0.0	0.0	13.1	5.1
Brilliance	7.5	4.8	25.0	6.5
Sensation	10.0	7.1	12.8	6.3
Sweet Ann	10.0	4.1	32.5	11.1
pe 7.2059	12.5	4.8	25.0	8.7
pe 3.211	15.3	6.4	10.0	4.1
ps 9271	25.0	8.7	50.0	7.1
Ruby June	25.0	9.6	80.0	7.1
Radiance	27.5	4.8	37.5	9.5
Beauty	37.5	2.5	50.0	0.0
Festival	43.3	7.1	57.5	7.5
Fronteras	45.0	6.5	85.0	5.0
bg 6.3024	45.8	4.2	30.0	15.8
pe 6.2036	47.5	16.5	70.0	5.8
Winterstar	50.0	10.8	17.5	2.5
Cabrillo	53.9	14.8	85.0	6.5
San Andreas	56.4	4.7	75.0	6.5
bg 9.3142	60.0	4.1	45.0	15.0
Osceola	62.5	11.1	27.5	6.3
Del Rey	66.7	4.7	10.0	4.1
Petaluma	67.9	6.3	82.5	4.8
Amado	71.7	2.9	57.5	6.3
Big Sur	75.0	6.5	55.0	9.6
18DN0236	75.0	8.7	92.5	4.8
Maverick	77.2	12.4	80.0	9.1
Odessa	84.7	4.9	70.0	9.1
Pilgrim	87.5	9.5	67.5	8.5
Monterey	94.7	3.1	100.0	0.0
Victor	100.0	0.0	92.5	4.8
Year 1				

PSI-9A	0.0	0.0	-	-
PSI-10A	2.5	2.5	-	-
LC-1A	5.0	2.9	-	-
LC-4A	5.0	2.9	-	-
LC-5A	8.3	8.3	-	-
PSI-1A	12.5	9.5	-	-
UC-15A	12.5	7.5	-	-
Prado	15.0	6.5	-	-
bg 6.3016	15.6	5.2	-	-
PSI-3A	15.8	7.1	-	-
LC-2A	17.5	6.3	-	-
LC-6A	17.5	4.8	-	-
PSI-5A	18.1	4.9	-	-
PSI-2A	20.0	7.1	-	-
PSI-8A	20.0	7.1	-	-
PSI-4A	20.8	4.8	-	-
LC-3A	22.5	13.1	-	-
PSI-7A	24.4	6.0	-	-
LC-8A	25.0	8.7	-	-
179AB165	25.0	5.0	-	-
PSI-11A	26.9	7.4	-	-
UC-14A	28.1	4.5	-	-
UC-1A	30.0	4.1	-	-
UC-2A	39.2	10.0	-	-
PL 3001	43.9	9.6	-	-
PSI-6A	46.4	3.9	-	-
UC-6A	46.9	5.6	-	-
UC-12A	55.0	11.9	-	-
PL 09-55	57.5	6.3	-	-
LC-7A	57.5	11.1	-	-
UC-3A	62.5	11.1	-	-
UC-8A	67.5	11.1	-	-
UC-11A	75.0	8.7	-	-
UC-13A	75.6	11.7	-	-
PL 12-04R	76.9	6.2	-	-
Marquis	80.0	10.0	-	-
PL 3002	81.7	8.0	-	-

El Dorado	81.9	2.7	-	-
UC-7A	83.6	9.7	-	-
Fortaleza	89.4	4.1	-	-
Kora	92.5	2.5	-	-
UC-4A	94.4	3.3	-	-
UC-9A	95.0	2.9	-	-
Laredo	97.5	2.5	-	-
Spartan	100.0	0.0	-	-
Year 2				
bg 4.352	-	-	7.5	4.8
bg 4.367	-	-	7.5	4.8
bg 9.3128	-	-	7.5	4.8
pe 7.2054	-	-	12.5	6.3
148AC398	-	-	17.5	8.5
Elyana	-	-	20.0	4.1
LC-1B	-	-	25.0	6.5
LC-4B	-	-	27.5	11.1
Mariposa	-	-	30.0	12.2
bg 3.324	-	-	42.5	13.1
LC-2B	-	-	55.0	13.2
LC-3B	-	-	57.5	9.5
UC-7B	-	-	65.0	2.9
UC-10B	-	-	69.4	13.4
Lusa	-	-	80.0	9.1
UC-1B	-	-	82.5	10.3
Valiant	-	-	85.0	6.5
UC-6B	-	-	85.0	2.9
UC-2B	-	-	87.5	4.8
Flamingo	-	-	87.5	9.5
DN102.076	-	-	90.0	0.0
UC-11B	-	-	92.5	4.8
DW147.064	-	-	92.5	2.5
Royal Royce	-	-	95.0	2.9
UC-8B	-	-	95.8	4.2
UC-4B	-	-	97.5	2.5
Lara	-	-	97.5	2.5
Warrior	-	-	100.0	0.0
UC-5B	-	-	100.0	0.0
UC-9B	-	-	100.0	0.0

[y] Percent mortality as of 29 April 2019 and 29 April 2020, 184 and 188 days after inoculation, respectively.
[z] Mean values and standard error derived from four plot replicates. Each plot replicate contained 10 plants.
"-" No data available for this year.

Table 2. 3 Strawberry inoculated cultivars and elite breeding lines (genotypes) in ranking order by area under disease progress curve (AUDPC) as of 29 April 2019 and 29 April 2020.

Cultivar/Elite Breeding Line	AUDPC[w]					
	2018-2019			2019-2020		
	Mean[x]	SE[y]	rAUDPC[z]	Mean	SE	rAUDPC
Year 1 & 2						
PS 5016	0.0	0.0	0.00	150.5	74.7	0.08
Sensation	94.0	79.7	0.05	166.1	89.9	0.09
Brilliance	116.6	81.3	0.07	376.6	85.6	0.21
pe 3.211	142.9	74.0	0.08	105.6	57.3	0.06
Sweet Ann	159.1	70.9	0.09	402.9	145.9	0.23
pe 7.2059	165.4	65.8	0.10	294.4	70.1	0.16
ps 9271	298.3	90.2	0.17	623.3	131.6	0.35
Ruby June	369.6	144.7	0.21	1258.3	107.5	0.70
Radiance	424.3	90.0	0.25	541.9	121.2	0.30
Beauty	517.6	21.0	0.30	703.0	43.1	0.39
pe 6.2036	538.0	226.2	0.31	778.1	139.9	0.43
Festival	578.1	96.5	0.34	889.8	115.1	0.50
bg 6.3024	593.3	107.2	0.34	413.5	244.8	0.23
Cabrillo	702.5	206.2	0.41	1303.4	109.8	0.73
Fronteras	712.3	112.0	0.41	1382.0	85.9	0.77
Winterstar	779.5	168.2	0.45	270.3	38.0	0.15
Osceola	834.3	146.2	0.48	249.0	39.3	0.14
San Andreas	849.6	92.6	0.49	1195.6	112.0	0.67
Del Rey	901.5	80.5	0.52	87.9	62.5	0.05
Amado	909.1	51.6	0.53	505.4	117.6	0.28
bg 9.3142	910.4	45.1	0.53	471.3	86.3	0.26
Petaluma	936.5	141.2	0.54	1292.8	73.8	0.72
Maverick	1063.1	148.1	0.62	1043.6	139.9	0.58
18DN0236	1168.8	129.2	0.68	1527.0	97.2	0.85
Big Sur	1199.3	132.5	0.70	852.9	157.8	0.48
Odessa	1246.6	108.0	0.72	944.3	114.4	0.53
Pilgrim	1357.0	163.2	0.79	903.8	134.8	0.50
Monterey	1472.8	60.4	0.85	1384.3	20.5	0.77
Victor	1544.5	59.4	0.90	1466.4	79.3	0.82
Year 1						
PSI- 9A	0.0	0.0	0.00	-	-	-
PSI-10A	11.3	11.3	0.01	-	-	-

LC-1A	47.3	27.3	0.03	-	-	-
LC-4A	58.9	34.2	0.03	-	-	-
PSI-1A	68.4	39.7	0.04	-	-	-
PSI-3A	110.8	105.0	0.06	-	-	-
LC-5A	117.6	117.6	0.07	-	-	-
PSI-7A	154.0	62.4	0.09	-	-	-
bg 6.3016	158.6	60.4	0.09	-	-	-
PSI-5A	159.0	58.3	0.09	-	-	-
UC-15A	165.4	96.1	0.10	-	-	-
LC-6A	177.5	62.7	0.10	-	-	-
Prado	186.1	73.2	0.11	-	-	-
PSI-2A	192.1	91.0	0.11	-	-	-
LC-2A	193.4	82.4	0.11	-	-	-
PSI-8A	230.3	88.7	0.13	-	-	-
PSI-4A	267.1	41.9	0.15	-	-	-
LC-3A	288.1	167.2	0.17	-	-	-
179AB165	296.8	44.1	0.17	-	-	-
LC-8A	358.1	130.5	0.21	-	-	-
PSI-11A	365.8	148.5	0.21	-	-	-
UC-1A	414.4	68.0	0.24	-	-	-
UC-14A	426.4	80.9	0.25	-	-	-
UC-2A	572.5	158.6	0.33	-	-	-
PSI-6A	635.3	68.1	0.37	-	-	-
PL 3001	679.8	165.4	0.39	-	-	-
UC-6A	687.6	82.5	0.40	-	-	-
UC-12A	735.8	135.0	0.43	-	-	-
LC-7A	777.4	184.3	0.45	-	-	-
PL 09-55	876.5	116.0	0.51	-	-	-
UC-3A	951.8	181.7	0.55	-	-	-
UC-11A	1101.0	89.6	0.64	-	-	-
Kora	1121.3	44.1	0.65	-	-	-
UC-13A	1124.3	235.0	0.65	-	-	-
PL 12-04R	1141.1	141.0	0.66	-	-	-
UC-8A	1146.8	192.9	0.66	-	-	-
Marquis	1179.1	187.4	0.68	-	-	-
El Dorado	1194.8	60.2	0.69	-	-	-
UC-7A	1195.4	170.7	0.69	-	-	-
PL 3002	1210.1	183.7	0.70	-	-	-
Fortaleza	1246.3	86.0	0.72	-	-	-
UC-4A	1392.8	89.1	0.81	-	-	-

Spartan	1402.0	54.0	0.81	-	-	-
Laredo	1507.1	51.4	0.87	-	-	-
UC-9A	1514.8	22.0	0.88	-	-	-
Year 2						
bg 4.352	-	-	-	95.6	64.5	0.05
bg 9.3128	-	-	-	95.6	58.5	0.05
bg 4.367	-	-	-	111.8	68.6	0.06
148AC398	-	-	-	144.9	63.5	0.08
pe 7.2054	-	-	-	192.1	95.0	0.11
Elyana	-	-	-	243.4	94.9	0.14
LC-1B	-	-	-	323.5	91.0	0.18
LC-4B	-	-	-	409.3	188.3	0.23
Mariposa	-	-	-	415.5	160.3	0.23
bg 3.324	-	-	-	639.8	181.4	0.36
LC-3B	-	-	-	753.6	140.0	0.42
LC-2B	-	-	-	841.5	218.4	0.47
UC-8B	-	-	-	994.3	77.8	0.56
UC-7B	-	-	-	1001.5	26.1	0.56
UC-10B	-	-	-	1044.4	187.0	0.58
Flamingo	-	-	-	1080.9	76.5	0.60
Lusa	-	-	-	1180.3	177.9	0.66
Valiant	-	-	-	1272.8	70.4	0.71
UC-1B	-	-	-	1330.5	224.9	0.74
DN102.076	-	-	-	1346.6	45.9	0.75
DW147.064	-	-	-	1370.5	18.4	0.77
UC-6B	-	-	-	1422.1	47.6	0.79
UC-2B	-	-	-	1453.4	76.1	0.81
Royal Royce	-	-	-	1467.5	64.7	0.82
UC-11B	-	-	-	1502.5	60.3	0.84
Lara	-	-	-	1542.1	71.1	0.86
UC-5B	-	-	-	1592.5	76.8	0.89
UC-4B	-	-	-	1623.8	39.2	0.91
Warrior	-	-	-	1650.5	5.0	0.92
UC-9B	-	-	-	1699.4	16.1	0.95

[w] AUDPC = Area under disease progression curve calculated over all season ratings (18 observation events).
[x] Percent mortality as of 29 April 2019 and 29 April 2020, 184 and 188 days after inoculation, respectively.
[y] Mean values and standard error derived from four plot replicates. Each plot replicate contained 10 plants
"-" No data available for this year.
[z] rAUDPC = refer to materials and methods for calculation of rAUDPC

2.5 Discussion

The objective of this experiment was to identify resistance of strawberry cultivars and elite breeding lines (genotypes) to anthracnose caused by *C. acutatum.* Among all six breeding programs, genotypes showed a wide range of plant mortality allowing us to characterize their susceptibility to anthracnose. Cultivars such as Festival, Radiance and Elyana have been identified as moderately resistant and resistant (Seijo et al., 2008). In this experiment cultivars 'Festival,' 'Radiance' and 'Elyana' had average mortality of 50.4, 32.5 and 20.0%, respectively. For 'Festival' and 'Radiance' average mortality was based on 2 years of data while 'Elyana' was based on one year of data.

Based on Fig. 5, only three of the 30 cultivars and elite breeding lines included in both years can be classified as "resistant": PS 5016, Sensation, and PE 3.211. Resistance is classified using average percent morality and the standard error that was derived from four replicate plots in each year. If a cultivar was resistant, average percent mortality was below 25% and resistance was also confirmed based on AUDPC (Table 2.3). Cultivars such as Ruby June, Fronteras, Cabrillo, Winterstar, Osceola and Del Rey were cultivars that had a difference of at least 30 percent mortality when comparing data for both years. One factor that could have led to this could have been confounding results from another pathogen being the cause of mortality. A good example for this could be cultivar Ruby June, which is susceptible to other pathogens such as *M. phaseolina.* Ruby June was one of the cultivars sampled for further analysis in the lab and no other pathogens besides *C. acutatum* was found. Another factor that could have led to this disparity might be mislabeling or misidentification of cultivars at planting. In this case we believe this was not a factor since the breeding programs had their plants labeled and bagged prior to

arrival. Furthermore, in year one, the Driscoll's breeding program did genotypic screening on their cultivars and showed that plot labels and plant genetics matched.

For several decades breeding programs have tested genotypes at the seedling stage and at different locations over several years (Galletta, 1980). Results with such differences between years demonstrates that disease screening trials such as this, it is important to do several years of evaluation. A cultivar should be evaluated at least three to four years per location with local isolates in order to determine accurate susceptibility to a disease. Multiple year trials can help identify outliers and add confidence to the characterization of resistance attributes.

Breeding programs can identify markers using molecular tools as a way to help decrease time taken to develop resistant cultivars. For anthracnose, SCAR markers such as Rca 2 and FaRCg1 have been identified and tested for resistance to anthracnose (Lerceteau-Kohler et al., 2005; Salinas et al., 2020). Both these markers differ at which pathogenicity group (PG) the markers control anthracnose. A pathogenicity group is a group that is classified by different disease reactions to different cultivars with different resistance genes (Dusabenyagasani and Fernando, 2008). For Rca 2 it is most effective against PG-2 isolates of *C. acutatum* while FaRCg1 is most effective against PG-1 isolates (Salinas et al., 2020).

The inoculation method in this experiment was successful in providing high disease severity for susceptible and resistant cultivars. In both years the majority of death occurred by the beginning of January, 73 and 69 days after planting for year one and two, respectively. In year one, total mortality of 74.8% occurred by 8 January 2019. Total death of 80.6% for year 2 occurred by 1 January 2020. The average percent mortality in

year one was at 56.8% while for year two it was 59.0%. Overall the disease pressure was similar with the exception of an increase of 6% when comparing the total plant mortality by January. Throughout both years, an increase in observed visible symptoms on fruit were observed after sporadic rain events (data not presented). Typical symptoms included water-soaked spots, and firm, sunken brown lesions on fruit (Freeman et al., 1998; Peres et al., 2005). These observations support claims on how *C. acutatum* spores easily spread through overhead irrigation or through rain (Madden et al., 1992; Yang et al., 1992). Irrigation practices at the experimental plots for this experiment followed industry standards and used overhead irrigation for plant establishment for the first four weeks after planting.

Anthracnose has a low persistence in the soil; therefore, the main concern and control is at the nurseries (Strand, 2008; Poling 2008). Understanding host resistance can allow for nurseries to identify susceptible and resistant cultivars to anthracnose allowing them to modify irrigation practices and controls if suspected of having anthracnose in the field. Breeding programs can also benefit from these results by incorporating them into breeding decisions regarding resistance to anthracnose. By doing this categorization, breeding programs can market current cultivars and guide breeding efforts for maximum benefits. Since we saw that there were some cultivars that were stunted compared to their respective non-inoculated plots (no data presented), for future research plant measurements between the non-inoculated and inoculated should be taken to better address the disease occurrence and presence (Salinas et al., *unpublished*).

CHAPTER 3: COLONIZATION OF STRAWBERRY CROWN AND ROOT TISSUES BY MACROPHOMINA PHASEOLINA AND VERTICILLIUM DAHLIAE IN SELECTED GENOTYPES

3.1 Introduction

California is one of the top strawberry producing areas of the world, accounting for 88% of total U.S. production, totaling 1.8 million tons of harvested fruit (California Strawberry Commission, 2018). In 2018, the value of strawberries was in the top 5 valued commodities for California at 2.84 billion dollars (CDFA, 2018). Prior to commercial growers receiving transplants, production of high-quality and disease-free plants is necessary. Production of strawberries can be limited by pathogens like *Verticillium dahliae* and *Macrophomina phaseolina*. It is important for a plant to be able to recognize and identify a pathogen as there are many defense mechanisms such as cell wall reinforcement, production of reactive oxygen species, and pathogenesis-related protein accumulation (Amil-Ruiz et al., 2011). *V. dahliae*, first described in 1931, has been an economically important soilborne pathogen of strawberries (Thomas, 1939; Willhelm and Koch, 1956). After the phase out of methyl bromide in 2005, *M. phaseolina* was first reported as an economically emerging soilborne threat to strawberries (Koike, 2008). *M. phaseolina* was initially restricted to California's southern growing region but by 2010 *M. phaseolina* was present in the northern growing region (Koike et al., 2013). *V. dahliae* has a broad host range and can cause significant losses in susceptible cultivars (Zazzeini and Tosi, 1989; Zveibil et al., 2012; Pearson et al., 1987). Recent studies have shown that there are *M. phaseolina* isolates that have a preference for strawberries (Burkhardt et al., 2018; Koike et al., 2016).

There are many ways that a fungus can become established on a specific host. For strawberries, both *V. dahliae* and *M. phaseolina* are able to colonize strawberry tissue through germ tubes. Germ tubes are produced when microsclerotia germinate in the soil or in crop residue. Germ tubes become hyphae and for *M. phaseolina* can form appressoria that penetrate the cell wall or through natural openings (Bowers and Russin, 1999). In the case of *V. dahliae,* these hyphae grow intercellularly in the cortex and then intracellularly through the xylem resulting in colonized vascular tissue. In strawberries, several studies have been done on pathogen colonization. Through these studies it has been shown that pathogens can interact differently among certain cultivars and different plant parts (Baird et al., 2003; Shaw et al., 2010). Baird et al. (2003) showed that *M. phaseolina* isolation frequencies from plant tissue declined over time, possibly affected by root segment degradation or interactions with other microorganisms in the soil. Studies have been done where the symptom expression was measured among strawberry cultivars (Shaw et al., 2010). Shaw et al. (2010) also showed that 60% of the visual symptom expression variation among genotypes was because of genetic differences. In both Freeman et al. (2001) and Shaw et al. (2010), findings suggest that colonization, resistance and tolerance to colonization can change over the course of a season. Shaw et al. (2010) also observed that when the extent of colonization had a partial genetic correlation with symptom expression, strawberry genotype performance in the presence of *V. dahliae* may be enhanced by both resistance and tolerance mechanisms within genotypes. It was also observed that genetic mechanisms that help prevent systemic infection can be more stable over the growing season.

The use of fumigation in nurseries attributes to reduced plant mortality and increased plant vigor for commercial growers (Wilhelm and Paulus, 1980). Historically a pathogen like *V. dahliae* played an important role in the discovery and use of the soil fumigant chloropicrin as a control measure (Tribble Bros, 1912; Thomas, 1939; Willhelm and Koch, 1956). Fumigants like chloropicrin and methyl bromide were important as they helped the strawberry industry in a span of 15 years to produce fruit of 40 to 60 tons/ha by 1972 (Wilhelm et al., 1974). In 2017 statewide average yield was at 61.7 tons/ha (California Strawberry Commission, 2018). The implementation and addition of new fumigants like methyl bromide and annual planting systems helped to control Verticillium wilt (Wilhelm and Koch, 1956; Voth and Bringhurst, 1990).

M. phaseolina produces black sclerotia allowing for it to be long-lived in agricultural soils and can germinate within two days of root presence (Chowdhury et al., 2014). Microsclerotia are the primary source for new infections in strawberry plants as they are protected by fallen crop residues and eventually released after crop residue breakdown (Ramakrishnan, 1955). Microsclerotia are also produced by *V. dahliae* and are dense aggregates of dark pigmented, ellipsoid, thick-walled hyphal cells (Fradin and Thomma, 2006). Melanin is present in microsclerotia and can provide protection from degrading environmental conditions, thus increasing survival (Polak, 1989).

Host resistance can be an important control tactic for soilborne pathogens and a primary replacement to fumigant controls. Host resistance is considered the most cost effective and sustainable control method for crown and root diseases in strawberries (Partička and Hancock, 2005; MacKenzie et al., 2006). Genetic resistance to *V. dahliae* is considered as one of many components in an integrated management system that includes

the reduction of pest populations in all growth stages. Due to improved yield with fumigation efforts, this allowed for breeding efforts to focus on fruit production and quality rather than from disease resistance breeding (Wilhelm and Paulus, 1980). Fruit firmness and productivity began increasing over time leading to a belief that resistance to *V. dahliae* and *M. phaseolina* is positively correlated with fruit firmness (Shaw et al., 1996). Breeding efforts were slow at first but in recent years host resistance trials have been conducted in an effort to find resistant cultivars. Cultivar Albion was considered resistant in the Fang et al. trial of 2012, but it was found that 'Albion' was susceptible to *M. phaseolina* under a growth chamber trial (Sanchez et al., 2016). The difference in resistance of 'Albion' could be attributed to the inoculation method used by Fang et al. which relates to the biology of microsclerotia; in the case of Sanchez et al. they used different isolates compared to what Fang et al. used (Fang et al., 2012; Sanchez et al., 2016). Host resistance can also be attributed to host genetics as it plays a key role in the selection of the microbial communities associated with the roots (Smith and Goodman, 1999; Wissuwa et al., 2008; Andreote et al., 2010). The presence of certain microbes in the rhizosphere can play an important role as when *V. dahliae* is present in the field the microbiome is dominated by populations of *Pseudomonas* spp. (Berg et al., 2005). Soil moisture and temperature play an important role in survival of the microsclerotia in *V. dahliae* and *M. phaseolina.* For *V. dahliae,* a low soil matric water potential (0.001 bar to air dry) and a high soil temperature (28 °C) cause a rapid decline of microsclerotia present in the soil (Green, 1980). As for *M. phaseolina,* the environment where microsclerotia best thrive is in dry soil and high temperatures (Dhingra, 1975; Pratt, 2006).

Early efforts in resistance screening for *V. dahliae* found that in wild germplasm extensive variation in susceptibility was found but none of the genotypes had sufficiently high resistance levels to withstand high inoculum densities (Bringhurst et al., 1966). High variation in germplasm for resistance to *V. dahliae* has been observed in California (Shaw and Gubler, 1996; Shaw et al., 1997; Holmes et al., 2016). Segregation of six distinct races in *V. dahliae* have been proposed due to the resistance of different strawberry varieties indicating a complex host-pathogen interaction between these isolates and cultivars (Govorova and Govorov, 1997). In this experiment we evaluated pathogen colonization in different strawberry cultivars, plant parts (i.e., crowns and roots) and timings (i.e., early- and late-season). Our objective was to see how much pathogen DNA was present in crowns and roots. Crowns and roots were the two plant parts studied due to the biology of the pathogens. Identified molecular techniques for each pathogen were used to quantify how much pathogen DNA was present. Our *V. dahliae* trial from 2016-2017 is a continuation of the thesis of J. Winslow (2019) where he assessed cultivars resistant to *M. phaseolina.*

3.2 Materials and Methods

3.2.1 Field layout

Two field experiments for *V. dahliae* were carried out in two consecutive field seasons at field 25 block 3 (35°18’18.9” N; 120°40’38.3 W) at California Polytechnic State University. During year one, 90 strawberry cultivars and elite breeding lines were evaluated for host resistance to *V. dahliae.* In this experiment 90 strawberry genotypes were evaluated for susceptibility to Verticillium wilt. Genotypes were arranged randomly into 5 blocks; four blocks were in soil naturally infested with *V. dahliae* and

one block was in soil fumigated with Ally 33 (67% allyl-isothiocyanate + 33% chloropicrin at 55 gal/treated A) as a control. Cultivars for this trial were selected from a non-fumigated block. The same location was used in year two. In 2017-2018 cultivar replications were all on one bed in the fumigated block. For this trial a randomized block design determined the positions of planting for each plant. A total of 3 inoculated replications were laid out for each of the three cultivars.

In the 2017-2018 *M. phaseolina* trial a similar experiment with a similar plot layout was established in field 35B (35°18'20.5"N 120°40'23.8"W) at California Polytechnic State University. For this trial a total of 3 inoculated replications were laid out for each of the three cultivars in the trial. For both fields and for both years, standard irrigation, fertilizer application, and pest management practices were used.

3.2.2 Strawberry genotypes

3.2.2.1 2016-2017

For the 2016-2017 season 90 strawberry cultivars and elite breeding lines were evaluated for resistance to *V. dahliae* (Holmes et al., 2016). Strawberry germplasm was selected from six breeding programs: University of California (UC), University of Florida, Plant Sciences, Driscoll's, Planasa and Lassen Canyon. Ten out of the 90 genotypes were selected to determine the colonization of *V. dahliae* in the crowns and roots of individual plants. The 10 selected cultivars consisted of five susceptible: BG 1975, Festival, BG 4.367, Benicia and Odessa; and five resistant: San Andreas, UC-12, Marquis, Petaluma and Camino Real. Susceptibility was determined through previous germplasm screening trials (Holmes et al., 2016).

3.2.2.2 2017-2018

For the 2017-2018 season three cultivars were selected to determine colonization for *V. dahliae* in the crowns and roots of individual plants. Selected genotypes consisted of two resistant cultivars (Marquis and Sweet Ann) and one susceptible cultivar (Festival).

During the 2017-2018 season, a total three cultivars were selected to determine colonization for Macrophomina crown rot caused by *M. phaseolina*. Cultivars selected consisted of two resistant (Manresa and Marquis) and one susceptible (Sweet Ann). Data presented for the *M. phaseolina* trial is only from the 2017-2018 season.

3.2.3 Field site and layout

3.2.3.1 Planting

Standard grower practices from the southern regions (Oxnard and Santa Maria) were used. Transplants were planted into beds 162 cm between centers with four plant rows spaced 25 cm apart. Plants were spaced 41 cm apart. Strawberry beds were 30 cm high and 2 rows of Tri-Cal low-flow drip irrigation tape (1.2 liter/30.4 m, 20 cm between emitters) were laid 2 to 3 cm below the soil surface. Beds were covered with a totally impermeable film (TIF), polyethylene mulch, which was 1 mil thick, black on top and white on the reverse side.

3.2.3.1.1 Verticillium dahliae trial

For the 2016-2017 season, bare-root transplants were planted on 18 Oct 2016 located on the California Polytechnic State University campus in San Luis Obispo, CA Field 25, block 3 (35°18’18.9” N; 120°40’38.3 W). This field was naturally infested with approximately 20 colony forming units of *V. dahliae* per gram of soil. The trial consisted

of a randomized complete block design of 20-plant plots replicated four times, with a fifth control replicate plot. The fifth control replicate plot was planted in an area fumigated with 350 lb/A methyl bromide (50%) + chloropicrin (50%) in fall of 2014.

For the 2017-2018, bare-root transplants were planted on 23 October 2017 Field 25, block 3. This field was naturally infested with approximately 20 colony forming units of *V. dahliae* per gram of soil. The trial consisted of a complete randomized block design consisting of 20-plant plots replicated four times, with a fifth block planted in fumigated soil as a control.

3.2.3.1.2 Macrophomina phaseolina trial

For the 2017-2018 season, bare-root transplants were planted on 23 October 2017 at field 35b located on the California Polytechnic State University campus in San Luis Obispo, CA. Two weeks later each plant was inoculated with 5 grams of cornmeal-sand-*Macrophomina* inoculum. The inoculum was prepared by making a homogenized 1.1:0.4:0.4 sand:cornmeal:deionized water mixture over a one-month incubation period as described by Mihail (1992) and Winslow (2019). Once colonized, the cornmeal-sand-inoculum was applied by adding 5 g of inoculum to the crown of the strawberry plant. Inoculum was directly placed in contact with the crown and root zone in the upper inch of soil. The trial consisted of 20-plant plots replicated five times, with the fifth replicate plot was not inoculated.

3.2.4 In field plant sampling

Plant sampling was conducted twice per pathogen of interest i.e. early season (4 months after planting) and late season (7 months after planting). Plants were dug up using a garden trowel and roots were slightly shaken to remove soil. Plant samples were placed

in 3.7 liter plastic bags and in a styrofoam cooler for transport (Ziplock®, C. Johnson & Son, Inc., Racine, WI). Between plant samples the garden trowel was cleaned and sanitized using 5.5% O-benzyl-p-chlorophenol (Lysol®, Reckitt Benckiser, Parsippany, NJ).

3.2.4.1 2016-2017

A random selection of five plants per cultivar were harvested on 14 July 2017 in order to determine colonization by *V. dahliae*. Each cultivar had five crowns and five roots. For this sampling there were 50 crown samples and 50 root samples.

3.2.4.2 2017-2018

There were two sampling dates for the *V. dahliae* trial and the *M. phaseolina* trials, 27 February and 30 May 2018; 27 February and June 27, 2018, respectively. On 27 February 2018 both the *V. dahliae* and the *M. phaseolina* trials had the same sampling process. Three plants were sampled from each control plot and four plants were collected from each inoculated/infested plot. For each cultivar there were 3 control plant samples and 12 inoculated plant samples. For the non-inoculated plots there were three crown samples and three root samples. For each cultivar there was a total of 12 crown samples and 12 root samples. For the entire sampling of the early harvest there were 9 non-inoculated crown samples and 9 non-inoculated root samples and there were 36 inoculated crown samples and 36 inoculated root samples.

In the late sampling of the *V. dahliae* trial, plants were sampled on 30 May 2018 while the *M. phaseolina* sampling took place on 27 June 2018. The same plant sampling techniques from the early sampling were used. For this sampling period four plants were harvested from the non-inoculated plots and from the inoculated plots of each cultivar.

For each cultivar there were 12 non-inoculated plant samples and 16 inoculated/infested plant samples. For the entire sampling of the late harvest there were 36 non-inoculated crown samples, 36 non-inoculated root samples and there were 48 inoculated crown samples and 48 inoculated root samples.

3.2.5 Missing Plant Samples

For the late harvest *V. dahliae* trial there were 73 crown and root samples missing (Table 3.1). For the late harvest *M. phaseolina* trial there was one plant sample missing from the non-inoculated plots. Statistical analysis was conducted using the samples and data collected. Cultivar Festival was removed from the late harvest sampling date in the *V. dahliae* trial due to missing samples.

Table 3. 1 Number of crown and root samples for the *V. dahliae* trial cultivars in the late season harvest, 30 May 2018. A total of four plant samples were harvested for non-inoculated and inoculated plants.

		Total Plants		
Cultivar	**Replicate**	**Crown**	**Root**	**Resistance to *V. dahliae***
Sweet Ann, Fumigated	1	4	2	
Sweet Ann, Fumigated	2	4	4	
Sweet Ann, Fumigated	3	2	2	
Sweet Ann	1	4	4	Resistant
Sweet Ann	2	4	3	
Sweet Ann	3	4	4	
Sweet Ann	4	4	4	
Marquis, Fumigated	1	2	3	
Marquis, Fumigated	2	4	4	
Marquis, Fumigated	3	4	4	
Marquis	1	3	3	Resistant
Marquis	2	4	3	
Marquis	3	3	3	
Marquis	4	1	2	
Festival, Fumigated	1	0	1	
Festival, Fumigated	2	0	0	
Festival, Fumigated	3	1	0	
Festival	1	0	0	Susceptible
Festival	2	0	0	
Festival	3	1	0	
Festival	4	0	0	

3.2.6 Plant Processing

Plants were rinsed using tap water in order to remove soil. Once rinsed, plants were aseptically sectioned into crowns and roots. For both seasons, roots were cut into 5 to 8 cm segments. For the 2016-2017 samples, crowns were cut into 0.2 cm × 0.2 cm pieces. For the 2017-2018 season excess root tissue was removed from crowns and were not cut into smaller pieces but were stored for future processing. Crown samples included the vascular tissue, cortex and pith. Roots and crowns were separated and placed into

labeled 4 mil poly bags (10.16 cm × 12.7 cm, ULINE, Pleasant Prairie, WI). Plant samples were then stored in a -20° C freezer for future freeze drying.

3.2.7 DNA Extractions

Plant crowns and roots were lyophilized soon after being removed from the -20°C freezer. Samples were lyophilized at -50°C, 0.039 mBar for 18-24 hours (Freezone® 4.5, Labconco® corporation, Kansas City, MO). Lyophilized tissues were first crushed with three impacts of a hammer then pulverized for 1 min using a hand roller tissue homogenizer (Agdia Inc., Elkhart, IN). Subsamples (≤ 20 mg) were taken for each sample and weights were recorded. Stainless steel spatulas were used to measure sample weights and subsamples were transferred to 2 ml bead beating tubes (Lysing Matrix I, MP Biomedicals, Solon, OH) containing 2 mm yellow zirconia beads and 4 mm white ceramic spheres.

Plant DNA extractions were completed using a modified version of a commercial extraction kit (DNeasy® Plant Mini Kit, Qiagen Inc., Valencia, CA). This modified version followed a version from Dr. Alexis Pasulka's lab at the California Polytechnic State University, San Luis Obispo. The modifications included adding a freeze thawing procedure after adding 400 µl of AP1 buffer. The buffer and sample were freeze-thawed three times in liquid nitrogen and a water bath (65°C) until completely thawed. The sample in 2 ml bead beating tube was then bead beaten for 45 seconds at 4.5 meters per second (FastPrep FP120, ThermoSavant). After bead beating a proteinase-K was added to each tube and incubated at 55°C for 1 hour. Samples were mixed by inverting five times every 15 minutes. After this addition, the original Qiagen Plant Kit protocol was followed, and DNA was eluted with 50 µl of AE buffer.

In order to get pure fungal DNA of *V. dahliae* and *M. phaseolina* for standard curve generation, a modified version of a manufacturer's protocol was used (DNeasy® UltraCleanKit® Microbial Kit Qiagen Inc., Valencia, CA). The procedure was modified at step 4 where a bead beater was used to shake the PowerBead tube for 45 s at 4.5 m/s.

3.2.8 Standard Curve

The standard curve for *M. phaseolina* was created using a five-point serial dilution from pure *M. phaseolina* DNA (isolate Mp 21). DNA was extracted and its concentration was measured following a Qubit™ 4 fluorometer protocol (Applied Biosystems, Foster City, CA). Serial point dilutions using pure *M. phaseolina* DNA were, 1, 0.1, 0.01, 0.001, and 0.0001 ng μL^{-1}. The slope of standard curve was -3.5 with an efficiency of 94.9% and an R^2 of 0.93.

The standard curve for *V. dahliae* was created by using a five-point serial dilution from pure *V. dahliae* DNA (isolate Vd 5). DNA was extracted and its concentration was measured following a Qubit™ 4 fluorometer protocol (Applied Biosystems, Foster City, CA). Serial point dilutions used from *V. dahliae* DNA were, 1, 0.1, 0.01, 0.001, and 0.0001 ng μL^{-1}. The slope of standard curve was -3.3 with an efficiency of 100% and an R^2 of 0.96.

3.2.9 Single-tube nested TaqMan assay

Colonization of root and crown tissues by *M. phaseolina* and *V. dahliae* were determined using quantitative polymerase chain reaction (qPCR) of the plant DNA extractions. The qPCR assay used for *M. phaseolina* followed the procedure outlined by Burkhardt et al. (2018). The qPCR assay used for *V. dahliae* followed the procedure outlined by Bilodeau et al. (2012). Both assays use a TaqMan molecular single-tube

nested assays and the Burkhardt et al. (2018) procedure uses an internal control (IC) that was developed by Bilodeau et al. (2012). The purpose of this IC is to monitor for the presence of PCR inhibitors.

Quantitative PCR assays for *M. phaseolina* were performed in a 25 µl reaction with 5X PerfeCTa® Multiplex qPCR ToughMix (Quantabio, Beverly, MA), and 1 µL of DNA extract. Primers and probes in the reaction included Mps_TaqMan forward (5'-CCT CGG CAA ATC CCT ATA G-3') and Mps_TaqMan reverse (5'-CTT TAC CCT CTC TCT ATT CC-3') primers at 400 nM, Mps_TaqMan_External forward (5'-CTA AAC TGG CTT AAT ACT AAT TTA GCG CCG GCG AAT C-3') and Mps_TaqMan_External reverse (5'-CTA AGC CTT ACC GCA CTA GAA CTA AGG CTA AGA TCG-3') primers at 20 nM, Mps_TaqMan_Probe (5'-TAMRA-CTA TTT GCT TAA CCC CTA CTC GCT TAG ACT-BHQ2-3') at 200 nM. The IC was included in the reaction mixture with the following concentrations Vdf929-PPF1F (5'-CCT TTC CCC TTA CTC TTC T-3') and Vdr1076-PPF1R (5'-GGA TTT CGG CCC AGA AAC T-3') at 1000 nM, and probe Vdhrc-FAM (5'-FAM-CAC CGC AAG CAG ACT CTT GAA AGC CA-BHQ1-3') at 400 nM, and 32 fg *V. dahliae* purified DNA. The nested thermocycling parameters for *M. phaseolina* were 1 cycle of 3 min at 95°C, 20 cycles of 15 s at 95°C and 20 s at 70°C with a plate read, followed by 50 cycles of 15 s at 95°C and 30 s at 62°C with a plate read.

The qPCR assays for *V. dahliae* were performed in a 25 µl reaction with 5X PerfeCTa® Multiplex qPCR ToughMix (Quantabio, Beverly, MA), and 1 µL of DNA extract. Primers and probes included Vdf929-PPF1F (5'-CCTTTCCCCTTACTCTTCT-3') and Vdr1076-PPF1R (5'-GGATTTCGGCCCAGAAACT-3') at 1000 nM, and probe

Vdhrc-FAM (5'-FAM-CACCGCAAGCAGACTCTTGAAAGCCA-BHQ1-3') at 400 nM, 32 fg *V. dahliae* purified DNA, and Vert IC probe (VIC-AAACTAAGCTTATCGATACCCTCGACCT-QSY) at 2µM. For *V. dahliae*, the thermocycling parameters were 1 cycle of 2 min at 95°C, followed by 55 cycles of 15 s at 95°C and 30 s at 62°C with a plate read. For *V. dahliae* C_t values were taken from the amplification cycle and readings for *M. phaseolina* C_t values were taken from the second amplification cycle.

3.2.10 Statistical Analysis

For the 2016-2017 season, data was tested for normality and multiple transformations were attempted to normalize the data using JMP® pro statistical software (version 14.2 SAS Institute, Cary, NC). Due to there being no normality, data was analyzed using the Wilcoxon/Kruskall-Wallis test. The fixed factors tested were cultivars and plant part sampled (crown and root). For the 2017-2018 season, data was tested for normality and multiple transformations were used to try and meet normality assumptions. Wilcoxon/Kruskall-Wallis test was performed to measure cultivars, plant part colonization (crown and root), and sampling period. A Fisher's least significant differences (LSD) post hoc analysis at a *p*-value of 0.05 was used to test factor means from the main effect cultivar.

3.3 Results

3.3.1 Colonization by Verticillium dahliae

Wilcoxon/Kruskall-Wallis test for the amount of *V. dahliae* DNA present among cultivars in 2016-2017 were statistically significant (*p*-value = 0.0090, Chi-square = 21.96). Mean amount of *V. dahliae* in different cultivars was significantly different (*p*-

value = 0.0090). When comparing the mean amount of *V. dahliae* between plant part we saw no differences between roots and crowns (*p*-value = 0.98, Chi-square = 0.024). The average *V. dahliae* DNA amount from all cultivars was 68.721 mg/g dry strawberry tissue (Fig. 3.1). Among the 10 cultivars within the 2016-2017 trial there were means that were numerically smaller than the others in ng/g dry strawberry tissue between susceptible and resistant cultivars. Susceptible cultivars had an average *V. dahliae* DNA amount of 83.724 mg/g dry strawberry tissue while resistant cultivars had an average of 53.719 mg/g dry strawberry tissue.

For the 2017-2018 *Verticillium* samples (Fig. 4.2) the fixed factors of cultivar, plant part colonization, and sampling period all had *p*-values higher than 0.05 and we were not able to reject the null hypothesis stating that all the mean ranks for the groups are the same. P-values for cultivar, plant part colonization and sampling period were 0.16, 0.87 and 0.43, respectively. Chi-squared values for cultivar, plant part colonization and sampling period were 3.65, 0.0269, and 0.61, respectively.

3.3.2 Colonization by Macrophomina phaseolina

Significance was found between all three of the fixed factors for the cultivars used to measure colonization by *M. phaseolina* (*p*-value = 0.0027, Chi-square = 11.84), plant part colonization (*p*-value = 0.0342, Chi-square = 4.49) and sampling period (*p*-value <0.0001, Chi-square = 39.88). Cultivar Sweet Ann had an average of *M. phaseolina* DNA of 25.2 pg/g dry strawberry tissue, Manresa had an average of *M. phaseolina* DNA of 0.89 pg/g dry strawberry tissue and Marquis had an average of *M. phaseolina* DNA of 0.56 ng/g dry strawberry tissue. The mean amount of *M. phaseolina* DNA was significantly different among cultivars (*p* value = 0.0027). The means of the early and

late sampling period were 0.19 pg/g and 4.26 pg/g dry strawberry tissue, respectively. Only Sweet Ann had a significantly higher level of *M. phaseolina* between the early and late sampling.

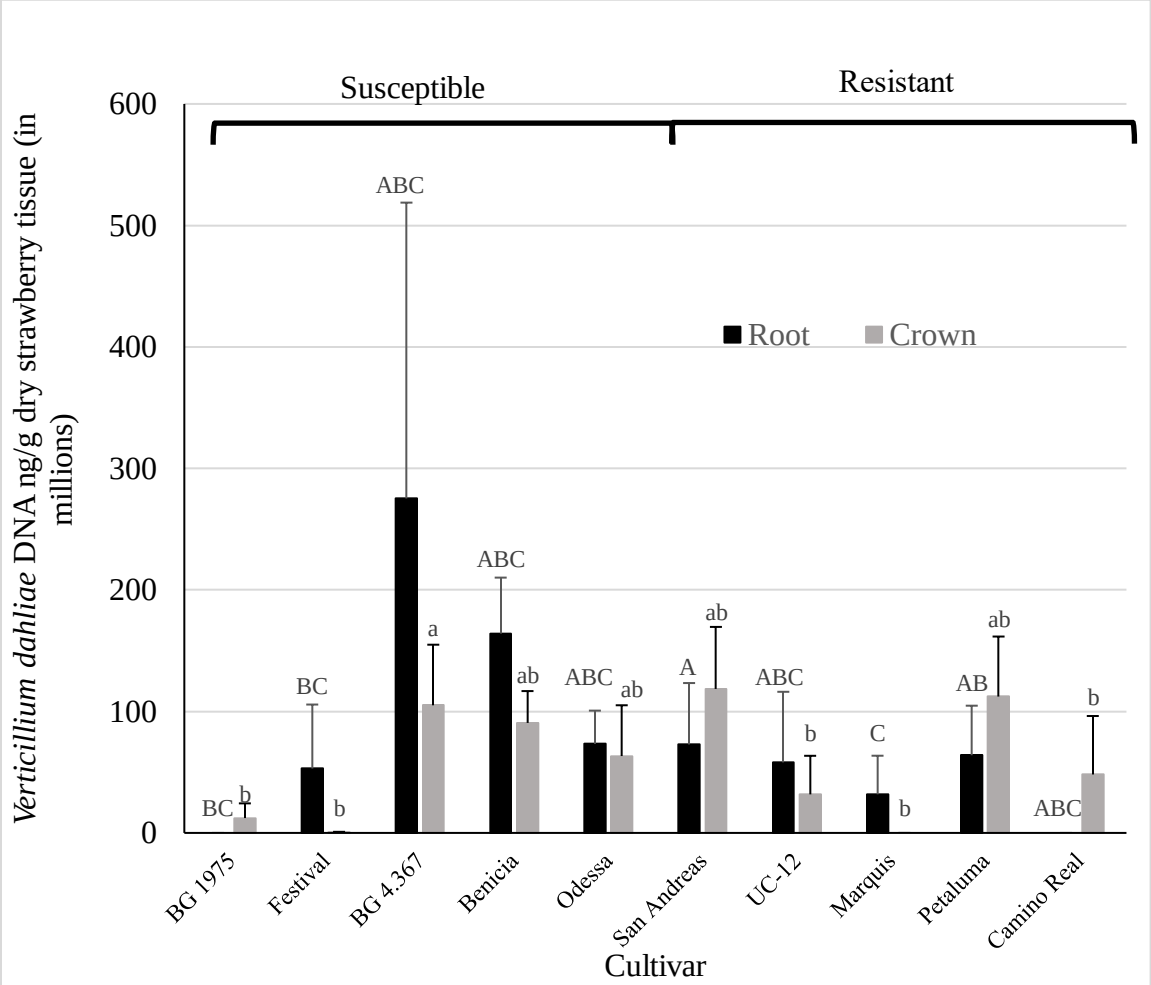

Figure 3. 1 Total average value of *V. dahliae* DNA detected in root and crown tissue for the year 2016-2017 with n = 5 and error bars represent standard error of mean. Uppercase letters are for root means comparison and lowercase letters are for crown means comparison. Means designated with the same letter are not significantly different (P > 0.05) as determined by Fishers's LSD. Resistance and susceptibility were determined with phenotypic assessments of plant mortality as of 14 July 2017 (Table 3.2). Plant samples were collected on 14 July 2017.

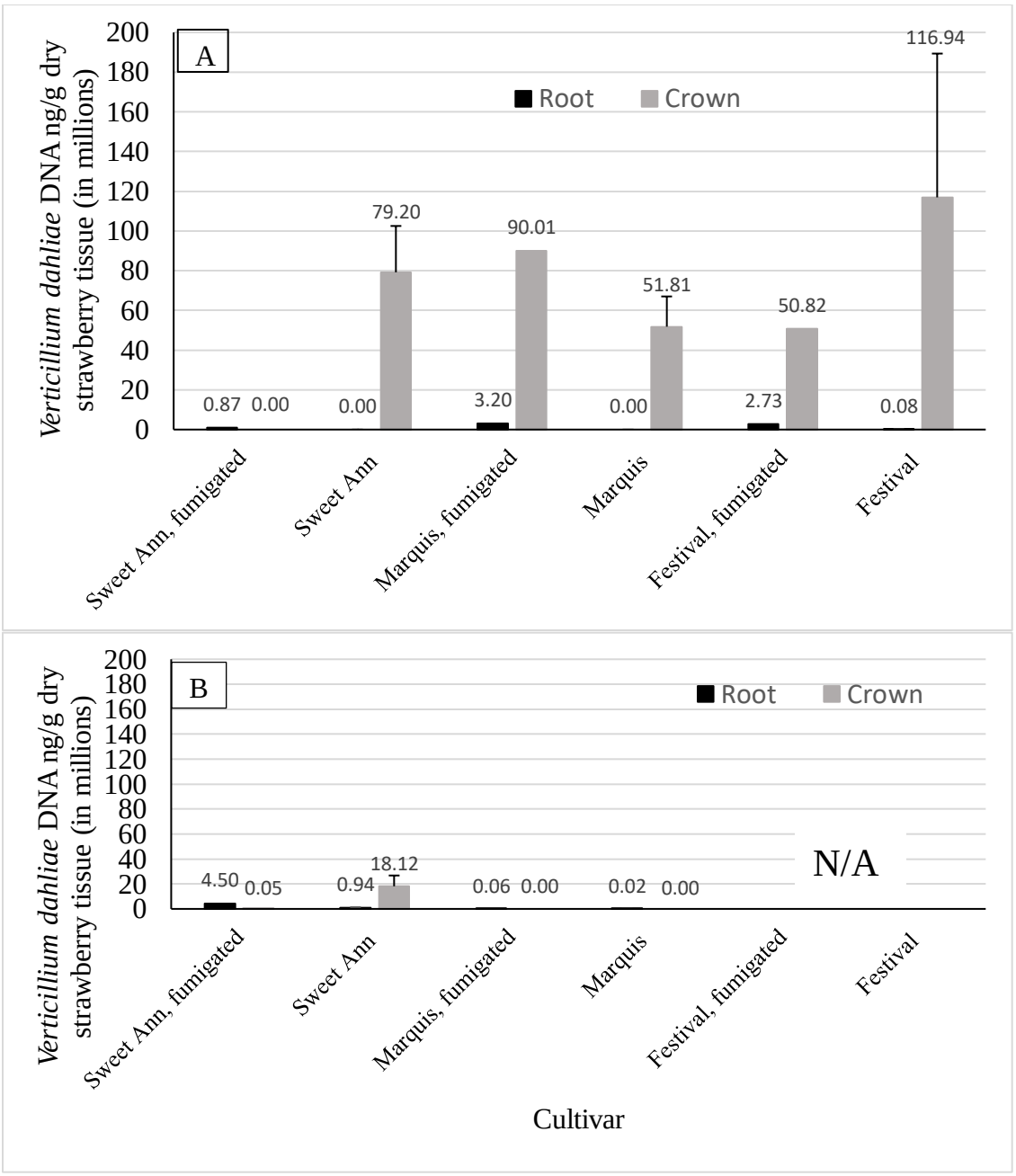

Figure 3. 2 Total average value of *V. dahliae* DNA detected in root and crown samples for early and late sampling dates. Averages determinized among three cultivars. Resistance was determined with phenotypic assessments of plant mortality by 14 July 2017 (Table 3.2). Early samples (A) were collected on 27 February 2018 and late samples (B) were collected 30 May 2018. Error bars represent the standard error of the means. No error bars are shown for the non-inoculated reps as they only represent one replication. The fixed factors for cultivar, plant part colonization, and sampling period all had *p*-values higher than 0.05 and we were not able to reject the null hypothesis. Symbol "N/A" represents no data shown for the missing plant samples not analyzed.

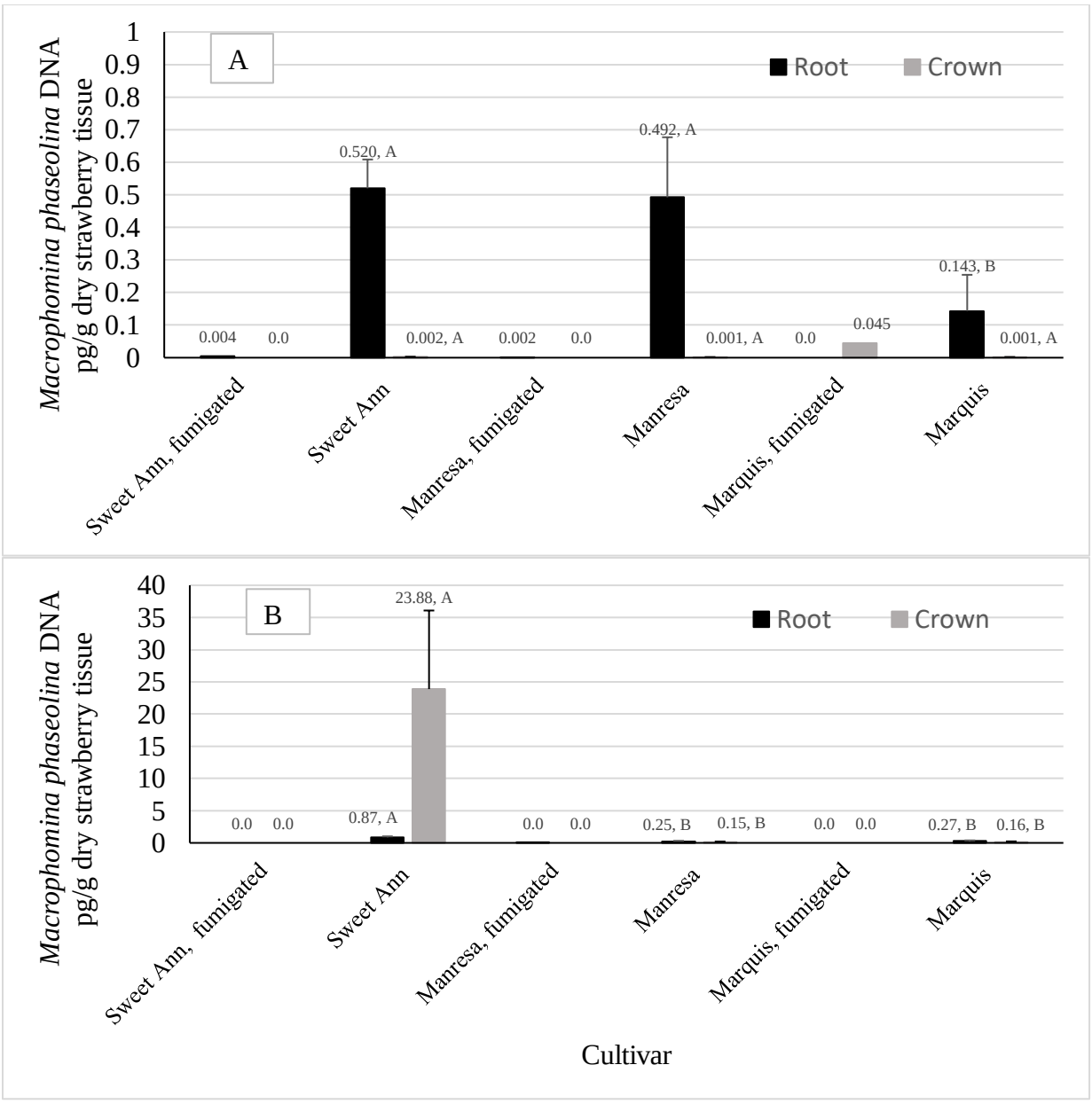

Figure 3. 3 Average values of *M. phaseolina* DNA (n = 16) detected in root and crown tissue for early (A) and late (B) sampling dates in the year 2017-2018; error bars represent standard error of mean. Means designated with the same letter are not significantly different (P > 0.05) as determined by Fishers's LSD.

Table 3. 2 Cultivars selected for this trial and their respective percent mortality at the date of sampling.

Verticillium dahliae

Cultivar	Year	Sample Date	Percent Mortality Mean
Year 1			
BG 1975	2016-2017	14-Jul-17	45.0 ± 7.1
Festival	2016-2017	14-Jul-17	46.3 ± 10.9
BG 4.367	2016-2017	14-Jul-17	57.5 ± 1.4
Benicia	2016-2017	14-Jul-17	68.8 ± 9.9
Odessa	2016-2017	14-Jul-17	40.0 ± 12.7
San Andreas	2016-2017	14-Jul-17	6.3 ± 2.4
UC-12	2016-2017	14-Jul-17	1.3 ± 1.3
Marquis	2016-2017	14-Jul-17	2.5 ± 1.4
Petaluma	2016-2017	14-Jul-17	3.8 ± 3.8
Camino Real	2016-2017	14-Jul-17	2.5 ± 1.4
Year 2			
Festival	2017-2018	27-Feb-18	0.0
Sweet Ann	2017-2018	27-Feb-18	1.3 ± 1.3
Marquis	2017-2018	27-Feb-18	4.1 ± 2.5
Festival	2017-2018	30-May-18	4.1 ± 4.1
Sweet Ann	2017-2018	30-May-18	2.6 ± 2.6
Marquis	2017-2018	30-May-18	4.1 ± 2.5

Macrophomina phaseolina

Manresa	2017-2018	27-Feb-18	0.0
Marquis	2017-2018	27-Feb-18	0.0
Sweet Ann	2017-2018	27-Feb-18	0.0
Manresa	2017-2018	27-Jun-18	0.0
Marquis	2017-2018	27-Jun-18	5.0 ± 3.5
Sweet Ann	2017-2018	27-Jun-18	36.3 ± 8.5

3.4 Discussion

Susceptibility of a plant to a pathogen is defined as a compatible interaction, while resistance of a plant to a pathogen is defined as an incompatible interaction (Fang et al., 2012). For this trial, all cultivars had colonization by the pathogen in both crown and root tissues (Figs. 3.1, 3.2, 3.3). In a *V dahliae* trial by Shaw et al. (2010), 11 resistant strawberry genotypes were tested to characterize the relationship between symptom expression and plant infection levels. The results showed that resistance scores and percentage of pathogen-free petioles decreased from the first sample to the second. For this trial our susceptible cultivar (Table 4.1) Sweet Ann in the *M. phaseolina* trial showed that disease incidence increased from the early sample period to the late sample period. *M. phaseolina* symptoms usually appear when the plant has been established, at the beginning of harvest or when exposed to water stress and high temperatures (Sanchez et al., 2016). For Macrophomina charcoal rot, cultivar Sweet Ann showed an increase in crown colonization in the late harvest compared to the early harvest. In sorghum Diourte et al. (1995) showed that *M. phaseolina* increased the disease development under environmental stresses such as drought and increased temperatures among resistant and susceptible cultivars. Potential future observations to support these claims can be adding multiple sampling events in the season. Evaluating colonization too early can result in overestimation of resistance in a cultivar or if evaluating colonization too late it can miss a critical time of tuberization (Pasche et al., 2013).

There are detection methods used for *V. dahliae* that are soil based or by plating on agar plates while counting microsclerotia (Goud and Termorshuizen, 2003; Kabir et al., 2004; Bilodeau et al., 2012). In recent years, Babu et al. (2007) reported about

conventional polymerase chain reaction (PCR) based on the internal transcribed spacer (ITS) for *M. phaseolina*. Mercado-Blanco et al. (2003) found that the extent of the pathogen colonization does not clearly determine the virulence phenotype. Another observation made was that pathogen DNA was present both in the stem and root tissue before visual symptoms had fully developed.

In strawberries several studies have been done on pathogen colonization. The main method used to identify the pathogen on the host tissue has been PCR. Freeman et al. (2001) used this technique and showed that the pathogen is present even though symptoms are not. For this trial the qPCR technique identified the pathogen in all cultivars and plant parts. Pathogens can interact differently in certain cultivars and different plant parts. Baird et al. (2003) showed that *M. phaseolina* isolation frequencies declined over time, possibly affected by root segment degradation or interactions with other microorganisms in the soil. Studies have been done where symptom expression was measured in strawberry cultivars (Shaw et al., 2010). Shaw et al. (2010) also showed that 60% of the visual symptom expression variation among genotypes was because of genetic differences. In both Freeman et al. (2001) and Shaw et al. (2010), findings suggest that colonization, resistance and tolerance to colonization can change over the course of a season. In this trial susceptible cultivar Sweet Ann (*M. phaseolina)* and resistant cultivars, Sweet Ann and Marquis (*V. dahliae)* both showed how resistance and tolerance to colonization changed between early- and later-season. Shaw et al. (2010) also observed that the presence of *V. dahliae* may be enhanced by both resistance and tolerance mechanisms among genotypes. It was also observed that genetic mechanisms that help prevent systemic infection can be more stable over the growing season.

In the *V. dahliae* trial from 2016-2017 (Fig 3.1) there was little distinction between pathogen DNA among susceptible and resistant cultivars. In this trial there was one susceptible cultivar (Benicia) that had significantly higher pathogen DNA compared to resistant cultivars Marquis, UC-12 and Camino Real. Susceptible cultivar BG 1975 had significantly less pathogen DNA compared to resistant cultivars San Andreas and Petaluma. This difference could have could be due to a difference in virulence among *V. dahliae isolates*, as shown in *M. phaseolina* isolates by Mercado-Blanco et al. (2003).

We saw the same trend in the *M. phaseolina* trial with the exception of cultivar Sweet Ann that had higher quantity in the crown and even had an increase from the early to the late sampling period. In the *V. dahliae* trial from 2017-2018 we observed that during the early sampling season the crowns were more readily colonized compared to the late sampling season. Potential reasons for this could be that primary sites of infection for *V. dahliae* from germinating microsclerotia are the root tip, the root elongation zone and the points where lateral roots emerge (Soesanto, 2000). Fang et al. described how spores of *F. oxysporum* f. sp. *fragariae* were settled on grooves and between epidermal cell grooves on roots and some on root hairs. After the spores germinated, germ tubes penetrated roots through depressions and junctions of epidermal cells but only in the susceptible cultivar did the tube directly penetrate the epidermal cell by hyphae swollen at the penetration sites (Fang et al., 2012). For *V. dahliae*, microsclerotia are stimulated to germinate by root exudates of host and non-host plants (Berlanger and Powelson, 2000). For *M. phaseolina*, once the microsclerotia germinate, germ tubes become hyphae and can form appressoria that penetrate the host epidermal cell walls (Bowers and Russin, 1999).

By understanding host and pathogen interactions we can help develop pathogen-specific management strategies. Host and pathogen interactions involve colonization of the plant. Colonization refers to the establishment of a pathogen on a specific host. For fungal pathogens there are many ways that a fungus can establish on a specific host. For strawberries, both *V. dahliae* and *M. phaseolina* are able to colonize strawberry tissues through germ tubes, hyphae and appressoria. It is important for a plant to be able to recognize and identify a pathogen as there are many defense mechanisms such as, cell wall reinforcement, production of reactive oxygen species, and pathogenesis-related protein accumulation (Amil-Ruiz et al., 2011).

A combination of disease suppressive cultural practices and resistant cultivars can lead to alternatives to the use of methyl bromide. Strawberry cultivars can respond to pathogens in many ways. These results show that cultivar Sweet Ann in the *M. phaseolina* trial had an increase in pathogen DNA from the early- to the late-season sampling dates. Unfortunately, we did not have data on 'Festival' as this could have provided some valuable information.

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