EVALUATION OF HOST RESISTANCE AND THE UTILIZATION OF ORGANIC AMENDMENTS TO MANAGE MACROPHOMINA CROWN ROT OF STRAWBERRY IN CALIFORNIA

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ABSTRACT

Evaluation of host resistance and the utilization of organic amendments to manage Macrophomina crown rot of strawberry in California

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The production of strawberries can be severely limited by soilborne plant pathogens, insects and weeds. *Macrophomina phaseolina* is a problematic soilborne fungal pathogen in California strawberry production inciting the disease Macrophomina crown rot. When established, the pathogen can cause extensive plant decline and mortality. Host resistance will be a critical tool for managing this disease and guiding breeding programs in the post methyl bromide era. Evaluation of host resistance in strawberry germplasm to *M. phaseolina* was evaluated through phenotypic assessments of disease incidence. A total of 90 strawberry cultivars and elite selections were included in a replicated field trial conducted in artificially inoculated soils to assess host resistance. Significant differences in levels of resistance and susceptibility were observed among genotypes tested in this trial. The five most resistant strawberry genotypes from highest to lowest in percent plant mortality were: UC-R, UC-G, UC-V, Manresa, and Osceola. The five most susceptible strawberry genotypes with the highest percent mortality in ranking order from highest to lowest were: UC-J, Ruby June, Festival, UC-Y, and UC-A. Of the genotypes tested in this trial UC-V, Manresa and Osceola could be characterized as highly resistant, but no complete resistance was observed.

An additional study was conducted to correlate host symptom expression with the extent of pathogen colonization in different strawberry tissues, and to determine if resistant germplasm can contribute to secondary inoculum production in the field. An established qPCR method was utilized to quantify *M. phaseolina* colonization of strawberry tissues. There were significant effects for cultivar ($P < 0.0001$) as well as a significant two-way interaction of cultivar x sample time ($P = 0.0083$) on the concentration of *M. phaseolina* DNA detected in strawberry tissues. Expression of the resistant phenotype in strawberry cultivars was associated with limited plant colonization by *M. phaseolina*. The extent of colonization of a specific cultivar by *M. phaseolina* was dependent on the sample time after inoculation with the pathogen. In addition, the roots and crowns of a specific strawberry cultivar were equally colonized on a per plant tissue weight basis, but this provides only speculation into the mechanisms conferring host resistance.

A third study was conducted to integrate host resistance of strawberry genotypes with the use of organic amendments in effort to mutually enhance the efficacy of each factor for the control of Macrophomina crown rot. Artificially inoculated potting substrate was amended with *Brassica juncea* mustard seed meal at a rate of 4.94 tons ha^{-1} (MSM), and anaerobic soil disinfestation utilizing rice bran at a rate of 22.24 tons ha^{-1} (ASD) were compared to a non-amended (UTC) and steam controls. The soil assay indicated that the ASD and steam treatments were able to reduce the CFU g^{-1} potting substrate of *M. phaseolina* by 99.7-100%. In addition, there were significant effects of soil treatment on the fresh biomass of weed seedlings recovered from the potting substrate. However, disease severity and host colonization of multiple strawberry

cultivars by *M. phaseolina* was not reduced when grown in the treated potting substrate. The effect of strawberry cultivar on the extent of pathogen colonization was highly significant ($P < 0.0001$), in which cultivars characterized as resistant from phenotypic screenings possessed lower concentrations of *M. phaseolina* DNA. The suppression of *M. phaseolina* in response to organic amendments was limited but this study supports findings from previous experiments that genotype specific host resistance minimizes host colonization and reduces the production of secondary inoculum.

Keywords: Strawberry Cultivar, Host Resistance, Organic Amendment, Anaerobic Soil Disinfestation, Plant Pathogen, *Macrophomina phaseolina*

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1
Literature Review

1.1 *Introduction*

Commercial agricultural systems of California produce more than half of the fruits, vegetables, and nuts consumed in the United States (U.S.). California is the world's largest producer of strawberries, with 14,730 ha in production reported for 2016 (USDA-NASS). Moreover, in 2017 California produced over 89% of the U.S. market share of strawberries (USDA-NASS). In 2015, the California strawberry industry represented a value of $2.5 billion, ranking it 5th in market value among top agricultural commodities in California (CDFA 2016). It is estimated that currently about 1.3 million tons of strawberries are produced in California annually, grown by more than 55,000 industry workers (CSC 2017; USDA-NASS). Consumer demands for fresh produce, both nationally and internationally, has been rising with demands for strawberries steadily increasing. The berry category is the top-selling produce category in California and strawberries ranked the highest in volume and value of all berry crops (CSC 2015). Considering its value, the strawberry industry in California is of vital importance to the agricultural economy.

The success of the California strawberry industry can be attributed to intensive public and private breeding programs, along with the optimization of planting systems and specialized cultural practices (Voth and Bringhurst 1990). The production of strawberries can be severely limited by soilborne plant pathogens, insects and weeds. If left unchecked, pathogens can cause complete crop failure and affect the success of subsequent crops (Straus and Kluepfel 2015). Since 1957, conventional growers of strawberries and other specialty crops have relied on the use of soil fumigants such as

methyl bromide (MeBr) and chloropicrin to manage a broad spectrum of soilborne diseases (Wilhelm and Paulus 1980). This in turn has led to increased yields and reduced crop rotations (Butler et al., 2014). Such a profitable industry in California has been made possible by the fumigation technologies developed, as nearly all conventional strawberry production occurs in fumigated soils (Ajwa et al. 2002; Wilhelm and Paulus 1980). The phaseout of MeBr under the Montreal Protocol due to its stratospheric ozone depleting nature has left many crop systems with less effective and potentially more toxic alternatives (Momma et al. 2006; Oka 2010). Therefore, it is imperative to identify alternative, non-fumigant methods for managing soilborne pathogens that are adaptable to current agricultural production systems.

1.2 The cultivated strawberry

The modern cultivated strawberry (*Fragaria* x *ananassa* Duchesne) is a member of the rose (*Rosaceae*) family. The famed qualities of the strawberry are traceable to hybrid crosses of three American species, *Fragaria chiloensis* L. from the west coast of south America, *Fragaria virginiana* of the east coast of North America, and *Fragaria chiloensis* from the central coast of California (Johnson 1990). Strawberries have been harvested and cultivated from a range of wild species dating to antiquity, but the modern cross bore superior fruit size from *F. chileonsis* and vigorous foliage and palatable fruit from *F. virginiana* (Wilhelm and Sagen 1974). The American species were brought from the New World to Europe in the 18th century, where they were crossed to make modern hybrids (Galletta and Maas 1990). Early American colonists brought with them seeds and gardening skills where they continued to make crosses with the flourishing native *F. virginiana* (Wilhelm and Sagen 1974). It is proposed that early strawberry crosses were

brought to California with the gold rush in San Francisco where the endemic *F. chiloensis* was cross pollinated with existing varieties (Wilhelm and Sagen 1974). These crosses made in California provided the ever-bearing nature of the strawberry where plants would bear fruit any month of the year (Wilhelm and Sagen 1974).

By 1856 there was nearly fifty commercial strawberry varieties available to growers, many specific and adapted to California soils and climates (Johnson 1990). In the early 20th century strawberry breeding efforts for cultivar development was taken on by the University of California as well as the Driscoll Strawberry Associates, Inc. (DSA) in Watsonville California; since about 1950 nearly all strawberries grown are from cultivars they developed (Wilhelm and Paulus 1980). These cultivars constitute the genetic framework for present day cultivars with potential for high productivity and qualities of fruit for market. Improved cultivars generated through traditional breeding allowed for the optimization of planting systems by means of additional horticultural practices to realize their genetic potential.

1.3 California strawberry production and horticultural practices

Strawberry planting systems have become highly specialized in comparison to the cultural practices first utilized in the 19th century. Strawberry plants are produced in nursery fields separate from fruit production fields. Transplant nurseries are located in both high and low elevations where they are propagated vegetatively and indexed for viruses (Galletta and Bringhurst 1990). High and low elevation nurseries experience photoperiod and temperature factors that benefit plant growth and fruit development of the transplants for high yields (Galletta and Bringhurst 1990). Transplants are then

moved to coastal regions where climates are suitable for year-round fruit production (Strand 2008).

The majority of planting for fruit production in California typically occurs in the fall for short day varieties, and some in the summer for day neutral varieties. Fall planting in the coastal regions allows for several months of vegetative growth over the winter so plants can produce vigorous foliage prior to fruit production. Transplants are planted into raised beds which promotes soil drainage and boosted yields (Wilhelm and Sagen 1974). Raised beds are still employed but they are now tightly covered in polyethylene mulch. Polyethylene mulch warms bed temperatures, which benefit root development in the winter and reduces evaporative losses of irrigation during the warm seasons; opaque mulches can aid in suppressing weeds (Strand 2008; Voth and Bringhurst 1990). As noted previously strawberry cultivar developments contributed to the greatest improvements in fruit productivity and quality (Voth and Bringhurst 1990). The advent and employment of pre-plant soil fumigation revolutionized production practices as a broad spectrum of pests were now easily managed.

1.4 Soilborne plant pathogens

Soilborne phytopathogenic fungi are problematic for strawberry production worldwide and can potentially infect any component of the plant: roots, crown, petiole, leaves, flowers and fruit (Paulus 1990). Of the fungal pathogens that infect strawberries, soilborne phytopathogenic fungi are the most devastating. During early periods of strawberry production in California around the turn of the 20th century, it was observed that strawberries grown on lands following tomatoes were almost certain to fail (Wilhelm and Paulus, 1980). Growers of DSA suffered heavy losses on sites following tomato

production where their strawberry plants became stunted, then wilted and collapsed. These plant symptoms occurred during periods when plants typically would be in peak fruit production beginning in the late spring (Paulus 1990). Wilting and plant collapse symptoms observed were first attributed to *Verticillium dahliae* Kleb by Thomas in 1931. *Verticillium dahliae* exists around the world and has a wide host range; it can persist in soil for long periods of time as a resting structure termed 'microsclerotia' (Bhat and Subbarao 1999; Paulus 1990). With production costs of strawberries exceeding $150,000 per ha, damages to a crop due to soilborne pathogens during or before peak production would diminish the economic viability of the enterprise (Bolda et al. 2016).

Other lethal soilborne fungal pathogens of strawberry exist and have been identified in California. These include *Phytophthora* spp., *Fusarium oxysporum* f. sp. *fragariae*, *Colletotrichum acutatum* and *Macrophomina phaseolina* (Martin and Bull 2002; Koike et al. 2013). The listed fungal pathogens have wide to specific host ranges, and resilient resting body structures (microsclerotia) allowing them to persist in the soil for long periods of time (Koike et al. 2013; Paulus 1990; Martin and Bull 2002). Their life cycle strategies and host range render crop rotations ineffective (Martin and Bull 2002). In addition, several non-lethal soilborne species have been identified that stunt strawberry plant growth and reduce yields (Paulus 1990; Martin and Bull 2002). Complexes of several fungi including *Pythium* spp., *Rhizoctonia solani*, binucleate *Rhizoctonia* spp., and *Cylindrocarpon destructans* have been identified (Martin 2000; Martin and Bull 2002; Martin 2003). While these pathogens are generally non-lethal to strawberries, they have been documented to cause yield reductions of 25 to 85% in non-fumigated grounds (Martin and Bull 2002). Of the numerous soilborne fungal pathogens

that attack strawberries in California, some are of larger concern to growers than others depending on a multitude of factors. The pathogen host range, extent of distribution, ability to persist in soil, virulence, and resilience to eradication can render a pathogen as meddlesome to highly problematic. *Macrophomina phaseolina* represents an emerging pathogen in California strawberry production that is of increasing concern to growers because of its continued establishment in additional fields every year.

1.5 Macrophomina phaseolina

1.5.1 Taxonomy, host range and distribution

Macrophomina phaseolina (Tassi.) Goidanich is a soilborne fungus of the Botryosphaeriaceae family, that incites a disease called charcoal rot (Kaur et al. 2012). The genus *Macrophomina* is monotypic, containing only one species "*phaseolina"* (Sutton 1980). It has one of the widest reported host rages for a phytopathogenic fungus and is documented to readily infect over 500 different plant species (Su et al. 2001; Wang et al. 2004). Both wild and cultivated plant species can be damaged, including many notable economically important crops such as legumes, vegetables and fruits (Kaur et al. 2012). The fungus has a vast geographical distribution and can be found on every inhabited continent (Kaur et al. 2012). *M. phaseolina* appears to be a larger threat in tropical and subtropical regions with arid and semi-arid climates (Kaur et al. 2012; Wrather et al. 1997. When identified, *M. phaseolina* is typically found distributed in clusters or patchy regions of a field and is concentrated in the top 0-20 cm depth of soil (Campbell and van der Gaag 1993).

1.5.2 Host-interaction biology and symptomology

M. phaseolina is heterogeneous in host specificity and is also polyphagous in its host tissue selection ranging from seeds (Pun et al. 1998), post-emergent seedlings (Kaur et al. 2012), roots (Ammon et al. 1974), crown (Koike 2008), stem (Su et al. 2001) and can be saprophytic (Beas-Fernández et al. 2006). Despite the general perception of *M. phaseolina's* wide host range recent reports suggest some host specificity on strawberry and an analysis of over 460 isolates infecting strawberry plants were grouped in a single clade (Burkhardt et al 2018). In light of new reports of the genetic relatedness of *M. phaseolina* isolates having some host specificity little information has been generated on the host-pathogen interaction of *M. phaseolina* and strawberry. Many studies of host resistance to this pathogen have been conducted with other crops in the U.S., including but not limited to soybean (*Glycine max* [L.] Merril), corn (*Zea mays* L.), sorghum (*Sorghum bicolor* [L.] Moench), and cotton (*Gossypium hirstutum* L.) (Papavizaas and Klag 1974; Mayek-Perez et al. 2002; Su et al. 2001). Such work has led to the identification of some key components of the biology of *M. phaseolina*, how it infects its host, and its epidemiology.

When present in the soil *M. phaseolina* produces vegetative hyphae, in which germ tubes produce specialized appressoria (Kaur et al. 2012). The hyphae containing appressoria can penetrate host epidermal cells with cell wall-degrading enzymes, or through natural openings or wounds (Islam et al. 2012; Wang et al. 2004). Typically, infection first occurs in the roots or hypocotyl of common bean, then the infected epidermal and cortex cells collapse and become necrotic (Mayek-Perez et al. 2002). Hyphae of *M. phaseolina* then extend intercellularly and colonize the vascular cambium,

phloem cells and xylem cells as reported in chickpea and common bean (Mayek-Perez et al. 2002; Singh et al. 1990). As the fungus spreads through the taproot and into the vascular tissues, plugging occurs and disruption of water flow to the above tissues can be observed, and vascular cells become necrotic (Wyllie 1998). Toxin production by *M. phaseolina* and enzymatic degradation of host cells can also exacerbate wilt symptoms (Bowers and Russin 1999). Upon dissection, a reddish-brown discoloration of the vascular tissues can typically be observed. Plants closer to flowering stages begin to wilt and collapse, greatly diminishing yields.

Hyphae of *M. phaseolina* eventually produce microsclerotia, and very rarely pycnidia. As vegetative hyphae congregate, they become honey to brown colored eventually resulting in black melanized microsclerotia. Microsclerotia produced by *M. phaseolina* are clusters of 50 to 200 individual cells and are variable in size, typically ranging 50-150 μm (Kaur et al. 2012; Koike 2008). Microsclerotia genesis is regulated by many factors including moisture and nutrient availability, and oxidative stress of the environment (Georgiou et al. 2006). Microsclerotia are highly resilient to desiccation and can persist in host tissues and in soil for very long periods of time. Reports of microsclerotia survival range from 2 to 15 years irrespective of host tissue species (Baird et al. 2003). Survival of microsclerotia was most influenced by environmental conditions including temperature, moisture, and chemical composition. In general, conditions that adversely affected the survival of *M. phaseolina* microsclerotia in soil were freezing and thawing of soils (Short et al. 1980), high soil moisture (Dhingra and Sinclair 1975), and low carbon to nitrogen ratios (Dhingra and Sinclair 1975). Microsclerotia readily germinated in temperatures ranging from 28-35°C, but vegetative hyphae could persist in

bulk soils no longer than seven days (Mihail 1989). Microsclerotia in bulk soils and in host tissues are considered the primary *M. phaseolina* inoculum in the disease cycle.

1.5.3 Macrophomina and strawberry

While *M. phaseolina* was first identified to infect strawberries by Tweedy and Powell (1958) it was not reported as a significant pathogen in California strawberry production until 2005 (Koike 2008). In strawberries, disease symptoms incited by *M. phaseolina* are referred to as a crown rot and root rot rather than charcoal rot (Zveibil and Freeman, 2005). This is due to the lack of visible exterior lesions or gray 'ashy' mycelium produced, as typically seen on other plant or crop types such as soybean (Zveibil and Freeman, 2005). In strawberry, plants become stunted and the infected vascular tissue becomes necrotic, causing the plant to wilt and collapse (Koike 2008). By 2010, *M. phaseolina* had been identified in all major strawberry fruit growing regions of California and was associated with necrosis of older leaves and wilted plants (Koike et al., 2013). The dramatic increase in incidence and severity of *M. phaseolina* coincides with reductions in use of the soil fumigant MeBr (Chamorro et al., 2016). In recent years *M. phaseolina* has moved past the status of an emerging pathogen into that of a long-term problem for the California strawberry industry (Koike et al. 2013). New information about the biology *M. phaseolina* in the strawberry pathosystem and methods for its management are needed.

Little information exists about the biology and epidemiology of *M. phaseolina* in strawberry, thus few conclusions can be made about its population dynamics and the relationship between inoculum density and disease incidence. In addition, control action thresholds for *M. phaseolina* have not been determined. If there is any cropping history

with incidence of *M. phaseolina*, control measures must be employed, or the disease will only increase over time as strawberries are cultivated (Koike et al. 2013).

1.6 Management of soilborne pathogens in strawberry

Fresh market vegetable and strawberry production systems are designed around the single application of a broad-spectrum biocide to disinfest soils before planting. MeBr in mixtures with chloropicrin had been used since the 1960s on nearly all strawberry production acreage. It was unsurpassed in the ability to control a myriad of pathogens and was cost effective over a range of soil conditions and production systems (Chellemi 2002). In the height of MeBr use, nearly 20,000 metric tons were applied annually in the U.S. ranking it one of the highest used pesticides in the country (Ristaino and Thomas 1997). In 1993 the U.S. Environmental Protection Agency (EPA) classified MeBr as a Class I Stratospheric Ozone Depleting Substance (Carpenter et al. 2000). In concordance with the United Nations' Montreal Protocol, the U.S. capped MeBr production to 1991 levels and issued a phaseout of its production overtime (Duniway 2002). By December 31, 2016, all critical use exemptions had expired except for use in nursery production systems. Due to the paucity of data and information for the control of *M. phaseolina* in strawberry more theoretical approaches to its management will be further discussed. As summarized by Sylvia and Chellemi (2001), management of soilborne plant pathogens can be categorized into three divergent approaches: a proactive approach, a single tactic approach, and integrated pest management (IPM) approaches.

1.6.1 Chemical management

Fumigation of the soil prior to planting is the most common method for managing soilborne pathogens in California strawberry production. Soil fumigation also promotes a

positive plant growth and yield response even in the absence of soilborne pathogens (Chamorro et al. 2016). This single tactic approach can be applied in two primary methods, by tractor pulled shank injection or through drip fumigation administered through the tape used for irrigation (Qin et al. 2011). Multiple MeBr alternatives exist and are registered, including chloropicrin, 1,3-dichloropropene, and methyl isothiocyanate (Ajwa et al. 2003).

1.6.1.1 Chloropicrin

Chloropicrin (trichloronitromethane) also known as 'pic,' is the most widely used preplant fumigant, initially introduced to control *V. dahliae* (Martin 2003). This product has strong fungicidal activity but limited efficacy on nematodes and weeds, so historically it was applied with MeBr at a mixture of 2 MeBr : 1 pic at a rate of 390 $kg \cdot ha^{-1}$ (Shaw and Larson, 1999). When pic was applied as the sole treatment in a broadcast shank injection at a rate of 336 $kg \cdot ha^{-1}$, strawberry yields were 4-6% less than those treated with MeBr:pic mixtures (Duniway 2002). However, application with this high rate of pic alone is above the registered rates for use in strawberry and fail to control weeds (Martin 2003). Pic is still widely used in combinations with other fumigant chemistries.

1.6.1.2 1,3-Dichloropropene

The fumigant 1,3-dichloropropene (Telone®, Dow AgroScience) (1,3-D) had first been identified as a nematicide but has also been recognized for its fungicidal properties. Traditionally applications of 1,3-D can be applied alone, but most typically in mixtures with pic at 83% 1,3-D (Telone C17) or 65% 1,3-D (Telone C35) (Martin 2003), at rates of 476 $kg \cdot ha^{-1}$. In Florida, strawberry yields following Telone C17 and C35 achieved

comparable levels to MeBr:pic (Ajwa et al. 2003). As a fumigant 1,3-D is characterized by relatively low vapor pressure when compared to MeBr:pic so there have been some reports of its potential for poor distribution in the soil profile (Ajwa et al. 2003). For this reason, an emulsified formulation has been developed for administration through the drip tape system which has been shown to be more effective (Ajwa et al. 2002). It has also been demonstrated that the use of plastic mulch tarps has increased the efficacy of 1,3-D (Ajwa et al. 2003). There has been a history of regulatory concerns with 1,3-D contamination of groundwater and air quality, and it is listed as a carcinogen in California (Duniway 2002).

1.6.1.3 Methyl Isothiocyanate

Metam sodium (sodium *N*-Methyl dithiocarbamate) is one of several fumigant products that degrades into biocidal methyl isothiocyanate (MITC). MITC is the primary active ingredient of metam sodium (Vapam HL, Amvac Chemical Corp.), which has broad spectrum activity against plant pathogenic nematodes, weeds, oomycetes and fungi (Duniway 2002). The use of MITC in strawberries has been limited compared to pic and 1,3-D due to its inconsistent control of pests (Martin 2003). This has been investigated and it is hypothesized to be due to non-optimal distribution in the treated soil profile (Duniway 2002). Results of its efficacy have been variable and generally strawberry yields following its use are significantly lower than achieved with MeBr:pic (Duniway 2002). Due to these complications, metam sodium has developed a reputation of being unreliable and its use has been limited.

1.6.1.4 Alternative fumigants

Despite reports of their efficacy, alternative fumigants fail to achieve the same range of broad-spectrum control across the same range of soil types and conditions as MeBr (Chellemi 2002). Since the phase out of MeBr, there has been increasing demand for alternative fumigants. However, these alternative fumigants have been under constant review due to increased awareness of their toxicology. With increased regulatory scrutiny over concerns of environmental contamination, worker exposure, as well as township caps on their production and usage, combined with rising costs of these alternative fumigants, new non-fumigant methods for managing soilborne pathogens are desirable but not readily available.

1.6.2 Cultural management

Cultural strategies for disease management in strawberry encompasses a wide range of techniques involving preventive approaches and integrated pest management options. Typically, cultural techniques can benefit both conventional and organic strawberry systems. In theory, cultural practices that reduce plant stress, such as maintaining proper soil moisture and reduced soil salinity, will allow for a more vigorous plant which is more capable of combating disease even if the pathogen is present (Strand 2008). The success of cultural management strategies is often constrained by the necessity of optimal conditions. Cultural management techniques typically have variable and somewhat inconsistent effects regarding disease management especially pertaining to soilborne pathogens in strawberries. Considering the inconsistencies, studying the conditions where their efficacy was the greatest can help growers utilize these techniques more efficiently and effectively.

1.6.2.1 Preventative and sanitary practices

Cultural practices are an important component of disease management in strawberries. While in general they lack the effectiveness of the single-tactic approach of soil fumigation they can be incorporated into crop production as preventive measures, or as integrated pest management techniques (Chellemi 2002). Preventive or proactive pest management can be effective in the avoidance of disease outbreaks (Chellemi 2002). These approaches involve factors that prevent the movement of pathogens from entering a site by exclusion or with the use of disinfecting methods or products (Strand 2008). Sourcing high-quality transplants from certified, virus indexed nurseries can reduce the chance of pathogens moving with planting material (Strand 2008). Some nurseries use hot water bath treatments of bare root transplants to reduce some fungal pathogens, but mostly for *Colletotrichum acutatum* (Strand 2008). Certified transplants are guaranteed to be free of viruses but are not guaranteed to be free of other fungal or bacterial pathogens. Soilborne pathogens such as *M. phaseolina*, *F. oxysporum*, and *P. cactorum* can move with soils on equipment or on transplants (Pastrana et al. 2017). Pastrana et al. (2017) identified the presence of both *M. phaseolina* and *P. cactorum* in soils from nurseries of both fumigated and non-fumigated plots in Spain. Mother and runner plants were also tested for the presence of *M. phaseolina* and *P. cactorum* in which they only detected *P. cactorum* (Pastrana et al. 2017). It is proposed that soils from nurseries and transplants can be sources of inoculum of *M. phaseolina* and *P. cactorum* for fruiting fields, and infected transplants can be a source for *P. cactorum*, *F. oxysporum* f. sp. *Fragariae*, and *Rhizoctonia fragariae* AG-I from systemic infections in transplants (Nam et al. 2011; Koike and Gordon 2015; Mazzola personal communication). Power-washing of

equipment to remove soil between fields is encouraged though the efficacy of this tactic is lacking in literature (Koike and Gordon 2015). Despite employing numerous proactive measures which avoid the movement of soilborne pathogens they continue to move from nursery fields to fruiting fields, and between fruiting fields. For this reason, additional integrated pest techniques need to be employed for the non-chemical management of *M. phaseolina*.

1.6.2.2 Crop rotation

Crop rotation has been explored as a management technique for soilborne pathogens of strawberry. Alternating non-host crops with strawberries has the ability to reduce the incidence of soilborne pathogens (Xiao et al. 1998), from the breakdown products of the crops and the incorporation of their residues (Subbarao et al. 2007), or through manipulation of the soil microbial communities (Mazzola et al. 2017). Most data generated in using crop rotation or cover crops for suppression of soilborne pathogens has focused on *V. dahliae* in strawberry, but little exists for *M. phaseolina*. Rotations with broccoli and the incorporation of its residues reduced *V. dahliae* microsclerotia in soil, and reduced wilt severity to levels achieved by metam sodium (Subbarao et al. 1999). Mazzola et al. (2017) identified that cropping with wheat (*Triticum aestivum*) cv. 'Lewjawin,' and 'Penawawa' reduced soil inoculum of *M. phaseolina* and incidence of infection in strawberry but it did not abolish symptoms. In breaking the pattern of continuous monocropping of strawberry, and with specific rotational crops, the disease incidence of soilborne pathogens can be reduced; but rotational techniques cannot achieve the level of control of a single tactic approach.

1.6.2.3 Organic amendments

The incorporation of organic amendments of crop residues or agricultural byproducts into the soil can be a useful management strategy for diseases in strawberry production. Only recently have plant pathologists been studying their effects for disease management (Lazarovits et al. 2001). It has been identified that the addition of organic amendments into the soil profile alters the microbial composition of bulk and rhizosphere soils (Mazzola et al. 2015). Changes in the microbial composition of the soil have been associated with mediating the incidence of disease and increasing the suppressiveness of a soil regarding plant infection (Mendes et al. 2011). This is exemplified when amendments added to pasteurized soils consistently fail to reduce soil inoculum of disease or plant symptoms compared to non-pasteurized soils (Hewavitharana and Mazzola 2016; Mazzola et al. 2017). In addition, it has been observed that the disease control due to the addition of organic amendments can depend on the substrate used and control efficacy may be pathogen specific (Hewavitharana and Mazzola 2016). Some organic amendments such as rice bran have suppressed *Pratylenchus penetrans* but increased the soil densities of *Pythium ultimum* (Hewavitharana et al. 2014; Mazzola et al. 2017). In a strawberry field trial rice bran increased the amount of *Fusarium oxysporum* DNA in the soil and increased the incidence of disease (Mark Mazzola, unpublished). The utilization of organic amendments will rely on knowledge of the pathosystem to which they are being applied; a specific substrate effective in controlling a specific pathogen will likely be required for the treatment to be cost effective at a commercial level.

1.6.2.4 Host resistance

The employment of cultivars resistant to *M. phaseolina* would be the easiest and potentially most effective method for managing the disease. Unfortunately, due to the effectiveness and widespread use of MeBr, breeding programs for strawberries focused on the development of horticultural traits rather than host resistance to soilborne pathogens (Martin 2003). In addition, little information exists about the potential resistance of available strawberry cultivars to *M. phaseolina*. In a screening of seven cultivars in field and controlled settings, Fang et al. (2014) observed different levels of resistance to *M. phaseolina*. The authors determined that cv. Albion and Aromas were the most resistant to *M. phaseolina*, and cv. Camarosa was the most susceptible when conducting root and crown disease incidence assays. There appears to be some conflicting responses of host resistance regarding specific cultivars to soilborne pathogens. For example, Fang et al. (2014) rated cv. Albion as resistant to *M. phaseolina* rather than susceptible as determined by Koike et al. (2013). This might be due to the cryptic nature of soilborne pathogens and the difficulty in their diagnostic identification or difficulty in finding fields with natural infestations of a single pathogen for cultivar resistance screening. To date, no comprehensive studies have been conducted that comparatively determine disease resistance of the dozens of strawberry cultivars currently on the market, in particular for *M. phaseolina*. Identifying host resistance of strawberry cultivars to soilborne pathogens could aid the strawberry industry in several ways. Growers could select cultivars resistant to the soilborne pathogens specifically present at their production sites, and breeders could begin to select for cultivars containing genetic resistance to multiple pathogens.

1.6.2.5 Anaerobic soil disinfestation

The flooding of fields and subsequent reduced effects in the oxidation-reduction potential of soils has long been associated with plant pathogen suppression (Cook and Baker, 1983). A novel technique described as anaerobic soil disinfestation (ASD), also referred to as biological soil disinfestation (BSD), has been proposed as a potential pre-plant soil treatment to control soilborne pathogens and limit yield decline in many agricultural production systems (Shinmura et al. 1999; Blok et al., 2000). Initial research by Shinmura et al. (1999) and Blok et al. (2000) identified that the methods of ASD depend on inducing anaerobic conditions by incorporating a labile carbon rich amendment (C-source) into the soil, moistening the soil, and preventing the resupply of oxygen from entering the system by covering the soil with a plastic film for a short duration, two to fifteen weeks (Rosskopf et al., 2015). The following procedures have been demonstrated to reduce soilborne pathogens and boost crop yields to levels achieved with MeBr fumigation (Butler et al., 2012). Butler et al. (2014) utilized techniques described as ASD combined with solarization to greatly increase yields of fresh bell pepper crops in Florida in a research plot naturally infested with *Phytophthora capsici* and root-knot nematode (*Meloidogyne incognita)*. ASD treatments resulted in yields that were significantly higher than that attained in untreated plots, however solarization may not work in cool coastal CA conditions (Butler et al., 2014). While the basic techniques of ASD are straightforward and viable at the field scale, inconsistencies in crop yield performance from ASD warrant further inspection of the mechanisms functional in phytopathogen control and increased yields, as well as optimization to the strawberry

pathosystem. To implement such a system for extensive adoption at the regional scale, consistent and predictable levels of pathogen control must be obtained.

1.7 Summary and objectives

M. phaseolina is a problematic soilborne pathogen affecting strawberry production in California. There is a paucity of information concerning the specific management of *M. phaseolina* in strawberry. Pre-plant chemical fumigation of the soil can be effective in managing soilborne pathogens but increasing concerns of fumigant availability and use into the future demand new non-fumigant management techniques. Studies were therefore undertaken to evaluate non-fumigant approaches to managing *M. phaseolina* in strawberry, and to elucidate the biology of host-pathogen interactions. The specific objectives of these studies were to: i) screen commercial cultivars and elite selections for resistance to *M. phaseolina* in an artificially infested field setting; ii) correlate host symptom expression with the extent of pathogen colonization in different strawberry tissues to determine if resistant germplasm can contribute to secondary inoculum production in the field; and iii) examine the combination of strawberry host resistance and organic amendments as an integrated approach for the control of Macrophomina crown rot.

CHAPTER 2

Evaluating Host Resistance of Strawberry Genotypes to Macrophomina Crown Rot

2.1 Introduction

The ability of a plant to resist infection or yield reduction due to pathogens has been employed as management tactic in agriculture. However, host resistance has been underutilized by the strawberry industry to manage soilborne pathogens due to the availability and use of effective soil fumigation chemistries. While current fumigant chemistries are effective when distributed efficiently in the soil profile, this can be difficult to achieve in a field setting (Chamorro et al. 2016), and their availability into the future is uncertain (Mazzola et al. 2017). In the post-methyl bromide era, host resistance will be a critical tool for managing Macrophomina crown rot of strawberry.

Differential phenotypic resistance of strawberry germplasm to Macrophomina crown rot has been observed. Preliminary observations of strawberry cultivar (cv.) resistance to Macrophomina crown rot indicate promise for the use of host resistance in managing the disease. In western Australia, Fang et al. (2014) determined that cv. Albion and Aromas were the most resistant to *M. phaseolina*, and cv. Camarosa was the most susceptible when measuring root and crown disease incidence of seven cultivars, of which only two are regularly grown in California (Fang et al. 2014). At present no comprehensive studies testing a range of existing cultivars has been completed. A thorough phenotypic screening of resistance should involve commonly used and available strawberry cultivars, as well as elite selections that are being developed for future production. A comparative screening would aid growers in selecting resistant cultivars if their field had a history of Macrophomina crown rot and could aid breeding programs in their selection of resistant genotypes or phenotypes. Therefore, a replicated field trial was

conducted to screen a wide selection of commercially available strawberry cultivars and elite selections for their relative resistance to *M. phaseolina*.

2.2 Materials and methods

2.2.1 Strawberry genotypes

A total of 90 strawberry cultivars and elite selections was included in the field evaluation. Day neutral and short-day strawberry germplasm was provided from six public and private breeding programs: University of California (Davis, CA), University of Florida (Wimauma, FL), Driscoll's (Watsonville, CA), Plant Sciences, Inc. (Watsonville, CA), Planasa (CA) and Lassen Canyon (Redding, CA) (Table 2.1).

2.2.2 Inoculum production

M. phaseolina isolates Mp8, Mp21, and Mp22 used in this study were obtained from diseased strawberry plants in 2014 (Mp8) and 2015 (Mp21, Mp22). These isolates were confirmed to be *M. phaseolina* due to their morphological characteristics on PDA and by sequencing of the ITS region (data not shown).

Macrophomina cornmeal-sand inoculum was produced from a modified procedure outlined by Mihail (1992). A homogenized 1.1:0.4:0.4 sand:cornmeal: deionized water mixture was autoclaved for one hour on two separate days in separate 250 mL containers (Nalgene®, Rochester, NY). After autoclaving and cooling to room temperature the containers were inoculated with a single isolate of Mp8, Mp21, or Mp22 per container. Inoculated cornmeal-sand containers were then incubated at 30°C for two weeks and shaken vigorously by hand daily to aid in rapid colonization. After incubation the colonized cornmeal-sand inoculum was spread over a metal tray to air dry for five

days. Once dry, this inoculum was stored in the dark at room temperature for two weeks before being deployed in the field.

2.2.3 Field trial

The field trial was conducted on the campus of California Polytechnic State University in San Luis Obispo, CA Field 35b (35°18'20.21' N; 120° 40'23.39'). The field site selected had previously been cropped for over twenty years with row and forage crops (corn, alfalfa and triticale) prior to strawberry planting. The field was fumigated with the pre-plant soil fumigant Tri-Con 50/50® (50% MeBr/ 50% chloropicrin) at a rate of (350 lbs/acre) on 23 May 2015 since the history of soilborne pathogens was unknown. One summer plant season of strawberries had been established in this field and removed prior to planting of this experiment.

Strawberry plants were grown using standard commercial practices typical of the southern growing districts of CA. Raised beds were prepared prior to planting and were constructed as 162.56 cm center to center, and approximately 30.48 cm tall. Two lines of drip irrigation (low-flow, 0.34 gal/min/100 ft at 8 psi, with 8 in. 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 25.4 cm apart, and plants were spaced 40.64 cm apart within the row. Bare root transplants were set by hand on 17 October 2016.

Beds of the experimental plot were split into subplots, with each subplot containing 20 plants of a single strawberry genotype. The subplots were approximately 2 m long, with each bed containing 18 sub plots. The subplots were organized in a

randomized complete block design (RCBD) with four block replicates. Each block replicate consisted of five full length beds containing 90 subplots of each cultivar.

Two weeks after planting, plants were inoculated within the four blocks by infestation with cornmeal-sand *M. phaseolina* inoculum. The plants were inoculated by spreading 5 g of the inoculum mixture at the base of the crown and exposed upper region of the root zone then covered with soil. A fifth replicated block was included in the study that was not inoculated with *Macrophomina* cornmeal-sand inoculum. This replicate block was separated from the inoculated replicates by a three-bed buffer zone. The non-inoculated block only contained a single subplot per cultivar type and served as a check for the presence of other pathogens or confounding factors.

2.2.4 Mortality assessments and disease incidence

Strawberry plant mortality assessments were conducted to determine the disease incidence of genotypes due to *M. phaseolina*. After determining initial plant stand, any transplants that failed to produce new trifoliates were removed from the trial and not included in disease incidence. Monthly assessments to determine plant mortality commenced on 1 December 2016, and continued until 5 May 2017, when assessments started on a biweekly basis until the trial was terminated on 24 July 2017. The assessments completed on 5 April 2017 were used as the starting baseline for the number of live plants per subplot. The date of 5 April 2017 was chosen because up until this point it was confirmed that plant death that occurred was not incited by *M. phaseolina*. Mortality was defined as the point at which plant foliage was completely necrotic. The assessment completed on 24 July 2017 served as the final plant mortality assessment. Percent mortality of the plot was derived by calculating the number of dead plants due to

Macrophomina crown rot on 24 July 2017, divided by the number of alive plants on April 5th, 2017. Percent mortality for the 20 plant subplots was considered the percent mortality of a single replicate and used in the statistical analysis of data.

Area under the disease progression curve (AUDPC) was calculated for each entry. AUDPC is a quantitative metric used to summarize disease incidence over the course of an entire growing season rather than from a single point in time (Jeger and Viljanen-Rollinson 2001) and was calculated from the mortality assessment data according to the following formula from Jeger and Viljanen-Rollinson (2001):

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

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.

2.2.5 Data analysis

Mean values of percent mortality and AUDPC were derived using JMP® pro statistical software (version 13.1 SAS Institute, Cary, NC). A standard sum of squares analysis of variance (ANOVA) was performed for the single effect of cultivar on percent mortality, as well as cultivar on AUDPC. Global treatment differences due to cultivar were found significant based on the F-test, where critical values were calculated at the 5% level of probability ($\alpha = 0.05$). A square root transformation of AUDPC was completed to meet the assumption of the ANOVA test of homogeneity of variance and normality of error. After the square root transformation of AUDPC a Shapiro-Wilk W test was conducted to test the goodness of fit of normality on the distribution of the residuals, in which the probability < W = 0.0694. With the goodness of fit test, we

concluded the error was not statistically different for a normal distribution after the transformation, satisfying the assumptions of the ANOVA analysis. Post-hoc pairwise comparisons of genotypes were completed using a Tukey's HSD for percent mortality and AUDPC. All statistical analyses were performed with JMP® pro statistical software.

2.3 Results

2.3.1 Percent mortality by strawberry genotype

Symptom development due to Macrophomina crown rot were initially observed in late April, with the first observations plant mortality occurring in early May. These dates were approximately 210 days after planting. The greatest increase in plant mortality occurred after 10 June 2017 during a week of daily temperatures exceeding 30°C. There were statistically significant effects ($F_{89,358} = 9.607$, $P < 0.0001$) of strawberry genotype on percent mortality observed during the 24 July 2017 assessment. Mortality across all 90 accessions averaged 36.2 percent. Average mortality ranged widely over the accessions tested. The five strawberry genotypes exhibiting the highest mortality levels were UC-J, Ruby June, Festival, UC-Y, and UC-A, with 92.9, 89.9, 81.5, 78.8, and 76.3 percent mortality respectively (Figure 2.1). While these five genotypes demonstrated numerical differences in percent mortality, they were not statistically different from each other, but were statistically different from the grand mean, with all five having a parameter estimate $P < 0.0001$. The five strawberry genotypes with the lowest incidence of mortality were UC-R, UC-G, UC-V, Manresa, and Osceola with 7.5, 6.1, 5.0, 3.3, and 1.3 percent mortality respectively (Figure 2.1). Similarly, the five cultivars with the lowest percent mortality were numerically different but not statistically different from

each other. All five of the cultivars with the lowest percent mortality were statistically different from the grand mean having a parameter estimate $P < 0.0001$.

All breeding programs contained germplasm exhibiting a diversity of plant mortality in this trial, ranging from low to high percent mortality. Accessions from The University of California breeding program had mortality incidence ranging from 5.0 (UC-V) to 92.9 percent mortality (UC-J) (Figure 2.1). The University of Florida breeding program only had two cultivars tested ranging from 61.3 (Radiance) to 81.5 percent mortality (Festival) (Figure 2.1). Plant Sciences Inc. had a range of 7.5 (PSI-D) to 48.8 percent mortality (PE 7.2059) (Figure 2.1). Planasa breeding program had germplasm with a range of 16.4 (PL 02-32) to 55.0 percent mortality (PL 09-49). Lassen Canyon germplasm had a range of 37.5 (LC-F) to 89.9 percent mortality (Ruby June) (Figure 2.1). Driscoll's germplasm had a range of 1.3 Osceola) to 68.8 percent mortality (El Dorado) (Figure 2.1).

2.3.2 AUDPC by Genotype

There was a statistically significant effect ($F_{89,358} = 10.232$, $P < 0.0001$) of genotype on AUDPC. The five genotypes with the highest AUDPC in ranking order from highest to lowest: Ruby June, UC-J, Odessa, LC-A, and Festival (Table 2.1). While these five demonstrated numerical differences in the percent mortality, they were not statistically different from each other, but were statistically different from the grand mean (351.2), with all five having a parameter estimate $P < 0.0001$. The five cultivars with the lowest AUDPC in ranking from higher to lowest were: UC-R, UC-G, Manresa, UC-V, and Osceola with AUDPC (Table 2.1). Again, the five cultivars with the lowest AUDPC

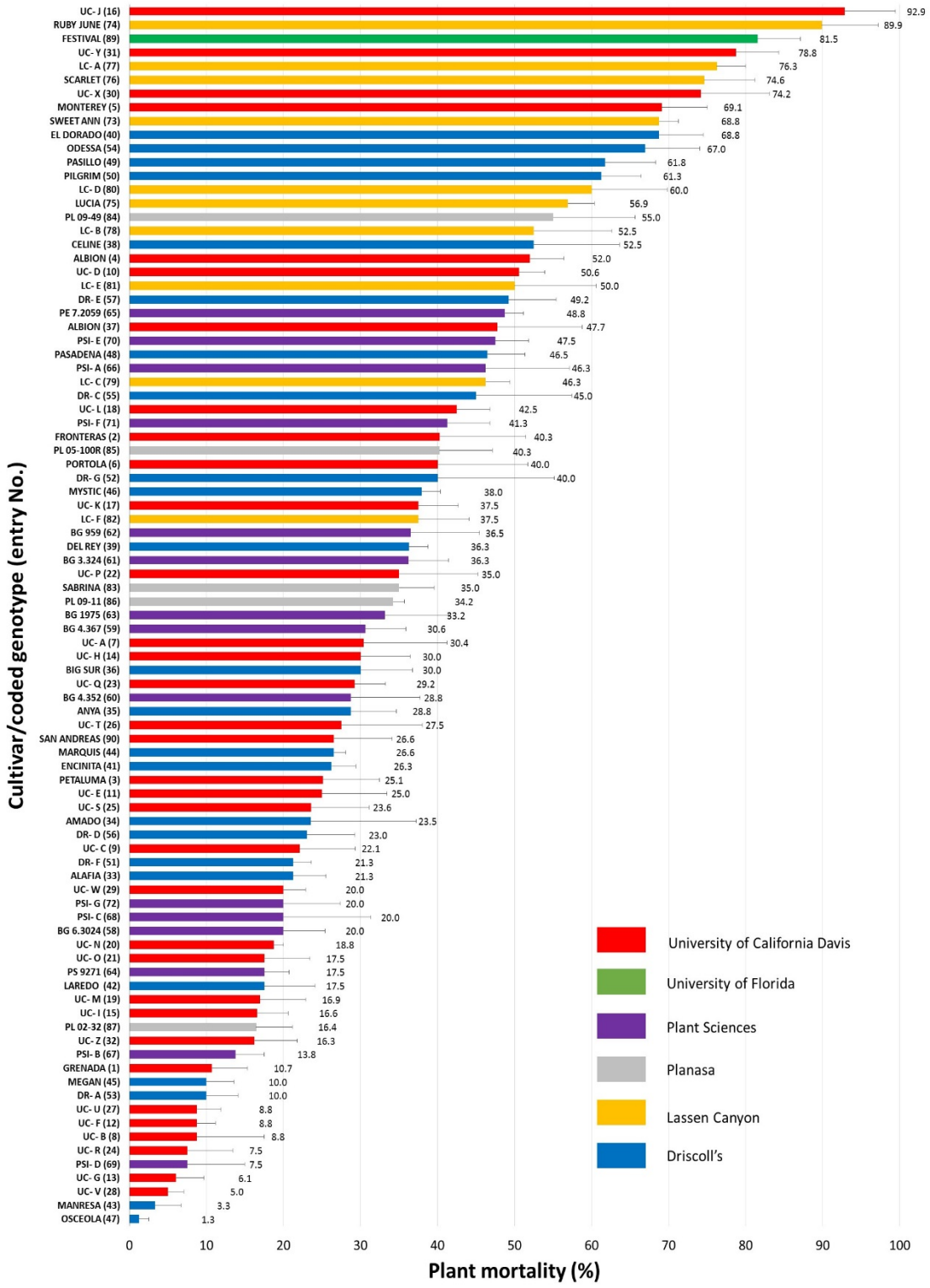

Figure 2.1. Average percent mortality due to Macrophomina Crown Rot as of 24 July 2017, 266 days after inoculation, in ranking order from lowest to highest. Average values are derived from percent mortality of four replicate plots. Color indicated by the legend correspond to the breeding program that developed the cultivar/coded genotype. Error bars are one standard error of the mean.

Table 2.1. Cultivars and coded genotypes of strawberry plants tested in ranking order by area under the disease progression curve (AUDPC), and their respective percent mortality at the end of the growing season 24 July 2017.

Cultivar/Coded Genotype	Rank by AUDPC	AUDPC[x]		Percent Mortality [y]	
		Mean[z]	Standard Error	Mean[z]	Standard Error
Ruby June	1	2126.1	85.4	89.9	6.6
UC- J	2	1870.6	250.4	92.9	5.1
Odessa	3	1733.9	230.5	67.0	5.7
LC- A	4	1598.8	73.8	76.3	5.5
Festival	5	1517.7	206.1	81.5	7.4
UC- X	6	1429.2	233.6	74.2	6.6
Scarlet	7	1390.8	62.2	74.6	3.7
Radiance	8	1367.5	319.1	61.3	6.6
LC- D	9	1143.1	191.9	60.0	9.8
Pilgrim	10	1116.9	239.2	61.3	5.2
UC- Y	11	1093.8	124.9	78.8	5.5
Monterey	12	1080.2	193.7	69.1	8.9
El Dorado	13	1006.3	168.2	68.8	2.6
Sweet Ann	14	971.3	76.1	68.8	5.9
LC- B	15	910.0	334.8	52.5	10.1
PL 09-49	16	861.3	226.9	55.0	10.6
Albion	17	852.3	266.3	47.7	11.0
Lucia	18	804.5	130.3	56.9	3.4
PE 7.2059	19	796.3	129.7	48.8	2.4
PL 05-100R	20	704.6	116.8	40.3	6.8
UC- D	21	669.2	186.5	50.6	3.3
Pasadena	22	668.2	189.2	46.5	4.9
Albion	23	661.6	107.1	52.0	4.3
DR- E	24	654.0	85.2	49.2	6.2
Celine	25	631.9	183.7	52.5	11.1
Pasillo	26	627.9	154.2	61.8	7.1
UC- L	27	612.5	170.6	42.5	4.3
Fronteras	28	598.8	129.9	40.3	11.2
LC- F	29	567.5	199.2	37.5	6.6
UC- Q	30	560.0	121.9	29.2	4.0
LC- E	31	550.6	70.3	50.0	10.6
Mystic	32	550.3	79.7	38.0	2.4
PSI- E	33	542.5	73.6	47.5	4.3
LC- C	34	516.3	129.7	46.3	3.1
BG 1975	35	502.1	118.5	33.2	8.3
BG 959	36	500.6	80.2	36.5	8.9
PSI- A	37	498.8	66.1	46.3	10.9
San Andreas	38	485.3	115.8	26.6	7.5
Sabrina	39	474.4	131.6	35.0	4.6
DR- C	40	472.5	146.1	45.0	12.4
UC- A	41	467.0	200.3	30.4	10.8
BG 6.3024	42	466.9	79.1	20.0	5.4
Portola	43	455.0	162.3	40.0	11.7
Del Rey	44	431.6	28.3	36.3	2.5
PSI- F	45	428.8	80.0	41.3	5.5
BG 3.324	46	428.8	43.8	36.3	5.2
Amado	47	424.5	283.3	23.5	13.7
UC- T	48	420.0	194.4	27.5	10.5
UC- H	49	402.5	115.6	30.0	6.5
DR- D	50	383.5	113.1	23.0	6.2
Petaluma	51	370.3	97.6	25.1	7.3

Table 2.1. (*continued from preceding page*)

Cultivar/Coded Genotype	Rank by AUDPC	AUDPC[x]		Percent Mortality [y]	
		Mean[z]	Standard Error	Mean[z]	Standard Error
UC- P	52	367.5	93.2	35.0	10.2
Big Sur	53	367.5	70.7	30.0	6.8
DR- G	54	350.0	106.0	40.0	15.1
UC- K	55	350.0	62.3	37.5	5.2
PL 09-11	56	348.2	72.5	34.2	1.5
UC- E	57	332.5	105.5	25.0	8.4
BG 4.352	58	331.9	124.4	28.8	9.0
Anya	59	306.3	52.3	28.8	5.9
Encinita	60	288.8	16.8	26.3	3.1
Alafia	61	271.3	93.0	21.3	4.3
BG 4.367	62	266.9	61.2	30.6	5.2
UC- C	63	253.4	77.9	22.1	7.2
PSI- C	64	245.0	179.0	20.0	11.4
Marquis	65	220.9	33.0	26.6	1.6
Grenada	66	215.0	35.7	10.7	4.6
PSI- G	67	210.0	74.3	20.0	7.4
UC- S	68	204.0	42.5	23.6	7.5
DR- F	69	201.3	16.8	21.3	2.4
PSI- B	70	201.3	64.5	13.8	3.8
UC- W	71	196.9	54.1	20.0	2.9
Megan	72	194.4	59.7	10.0	3.5
UC- N	73	183.8	33.1	18.8	1.3
DR- A	74	176.9	110.6	10.0	4.1
PS 9271	75	175.0	42.9	17.5	3.2
UC- M	76	171.1	60.0	16.9	5.9
PL 02-32	77	167.6	25.2	16.4	4.7
Laredo	78	157.5	46.3	17.5	6.6
UC- O	79	157.5	41.7	17.5	6.0
UC- I	80	134.5	44.3	16.6	4.0
UC- Z	81	131.3	29.9	16.3	5.5
UC- B	82	96.3	96.3	8.8	8.8
UC- F	83	96.3	41.4	8.8	2.4
PSI- D	84	87.5	87.5	7.5	7.5
UC- U	85	78.8	36.1	8.8	3.1
UC- R	86	70.0	58.9	7.5	6.0
UC- G	87	60.0	35.6	6.1	3.6
Manresa	88	46.7	46.7	3.3	3.3
UC- V	89	35.0	14.3	5.0	2.0
Osceola	90	8.8	8.8	1.3	1.3

[x]AUDPC = (Area under disease progression curve), AUDPC calculated from 10 observation events, see materials and methods for calculation formula.
[y] Percent mortality as of 24 July 2017, 261 days after inoculation
[z]Mean values and standard error derived from four plot replicates. Each plot replicate contained 20 plants

were numerically different but not statistically different from each other. All five of the cultivars with the lowest AUDPC were statistically different from the grand mean having a parameter estimate $P < 0.0001$.

2.4 Discussion

While previous studies have tested a select few of the most commonly grown cultivars for susceptibility/resistance to *M. phaseolina* (Fang et al. 2014), this study was the first to compare a multitude of strawberry genotypes from various breeding programs within a single field study. A wide range of host resistance to *M. phaseolina* was observed among the strawberry genotypes examined. Cultivars UC-R, UC-G, UC-V, Manresa, and Osceola expressed low levels of plant mortality, but none demonstrated complete resistance to the pathogen. Cultivars UC-J, Ruby June, Festival, UC-Y, and UC-A were highly susceptible to *M. phaseolina* exhibiting high levels of plant mortality. In contrast with the previous study completed by Fang et al. (2014), cv. Albion was classified as susceptible to *M. phaseolina* based upon AUDPC and plant mortality whereas it had previously been characterized as resistant. In total, only three percent of the 90 strawberry cultivars and coded genotypes tested could be characterized as highly resistant, but complete resistance was not observed.

The ranking order of cultivars from most susceptible to the most resistant did not change greatly when comparing ranks of percent plant mortality at the final assessment to AUDPC. This likely occurred for two reasons. First, AUDPC calculates disease over time, in which plots with more percent mortality that occurred earlier in the growing season would have higher AUDPC values than plots with the same percent mortality occurring later. Plant mortality was not expressed until later in the growing season after May, with the most mortality occurring after June, thus, AUDPC was most influenced by end of season plant mortality. The plant response observed in this trial agreed with Zveibil et al. (2012) which reported that damage due to Macrophomina crown rot

occurred later in the season and was exacerbated by warm temperatures during summer months. Second, percent plant mortality was used to calculate AUDPC, if an additional metric of a plant health scoring was used, greater differences of AUDPC values could have been realized. In addition, because of the high standard error values of percent plant mortality across replicate plots it was difficult to detect statistical differences among means when percent plant mortality was close. However, statistical differences between the most resistant and most susceptible strawberry genotypes was detected.

Roughly half of the strawberry genotypes examined in this study expressed high sensitivity to infection by *M. phaseolina* and a smaller proportion of the total tested were characterized as highly resistant, yet no complete resistance was observed. While resistant genotypes have been identified that could be of benefit when cultivating fields infested with *M. phaseolina,* additional testing of these putative resistant strawberry genotypes, over multiple years are necessary to warrant their recommendation by to growers. Furthermore, additional testing to determine the extent of *M. phaseolina* colonization of resistant cultivars would demonstrate the potential for pathogen infestation and corresponding contributions of secondary inoculum to the soil produced in infested plants over time. This phenotypic screening could also be used as the basis for further genetic testing of resistant genotypes to identify specific R genes or to ascertain the mechanisms conferring resistance to *M. phaseolina*.

CHAPTER 3

Characterizing *Macrophomina phaseolina* Colonization of Strawberry Roots and Crowns as Affected by Host Genotype

3.1 Introduction

Macrophomina crown rot of strawberry (*Fragariae* x *ananassa* Duch.) is incited by the fungus *Macrophomina phaseolina* (Tassi) Goidanich and is a problematic soilborne pathogen in strawberry production systems worldwide (Chamarro et al. 2015). Macrophomina crown rot epidemics have been recently been reported in both California (Koike et al. 2016) and Florida (Mertely et al. 2005), which are the two most productive strawberry regions in the U.S. (Zveilbil et al. 2012). Control of *M. phaseolina* in strawberry can be difficult to achieve in part due to its production of resilient resting structures called microsclerotia, as well as inconsistent distribution of fumigants in the soil profile (Chamarro et al. 2015; Koike 2008; Zveilbil et al. 2012). Phenotypic host resistance of strawberries to Macrophomina crown rot has been observed and shows promise as a management strategy for growers at field sites with a history of the disease (Gupta et al. 2012; Fang et al. 2014). The deployment of resistant cultivars is considered the most efficient and environmentally sustainable strategy for the control of soilborne diseases in strawberry production systems and could be applied to the management of Macrophomina crown rot (Smith and Carvil 1997; Fang et al. 2012; Gupta et al. 2012).

The susceptibility of a plant, and more specifically a cultivar, to a pathogen can be defined as a compatible interaction which allows for the colonization of the host plant by the pathogen, while resistant plants can be defined as incompatible with decreased pathogen colonization, especially in relation to plant host genetic resistance (Fang et al. 2012). In the case of soybeans, numerous cultivars were demonstrated as resistant to *M.*

phaseolina and contained reduced levels of colonization in roots and lower stem segments when compared to susceptible cultivars (Gupta et al. 2012). In the instance of strawberries, Fang et al. (2012) identified that cultivars resistant to *Fusarium oxysporum* f. sp. *fragariae* impeded host colonization at both the root surface and within the roots by preventing hyphae from entering the cortex and vascular tissue. However, little information exists about the host-pathogen biology of *M. phaseolina* in strawberry. Furthermore, no studies exist examining the extent of colonization of resistant and susceptible strawberry genotypes by *M. phaseolina*.

Remarkably, pathogen susceptibility is often assessed through a subjective scoring of disease symptoms, which been demonstrated to not always correlate with actual pathogen colonization (Brouwer et al. 2003). To infer true host resistance for the long-term management of Macrophomina crown rot, a strawberry host plant would need to limit or impede colonization of its tissues to prevent the production of secondary inoculum. In an effort to more effectively manage Macrophomina crown rot over time by reducing amplification of secondary inoculum, a study was conducted to examine the extent of colonization of several susceptible and partially resistant strawberry cultivars. Specifically, the concentration of *M. phaseolina* DNA was measured over time in root and crown tissues of susceptible and partially resistant strawberry genotypes grown in an artificially infested field.

3.2 Materials and methods

3.2.1 Field plot, strawberry genotypes, inoculum production and inoculation

A field experiment was established with the primary objective to evaluate the host resistance of 90 strawberry genotypes to Macrophomina crown rot. The field experiment

was conducted at the campus of California Polytechnic State University in San Luis Obispo, CA in field 35b (35°18'20.21' N; 120° 40'23.39'). Refer to the Materials and Methods section of Chapter 2. of this thesis for more details regarding the experiment.

Due to an unknown soilborne disease history at the planting site the field was fumigated with the pre-plant soil fumigant Tri-Con 50/50® (50% MeBr/ 50% chloropicrin) at a rate of (350 lbs acre^{-1}) on 23 May 2015. The soil was fumigated to minimize any confounding effects due to the presence of any established soilborne strawberry pathogens. The soil type of field 35b is classified as a Salinas silty clay loam, with an organic matter content of 3%, ECe of 1.6 dS m^{-1}, CEC of 17.4 meq 100 grams^{-1}, and 6.8 pH (A&L Laboratories, Modesto, CA).

Table 3.1. List of strawberry genotypes selected for characterization of colonization by *Macrophomina phaseolina* as well as their relative resistance to *M. phaseolina*

Cultivar	**Resistance to *M. phaseolina*** *	**Breeding Program**
Ruby June	Susceptible	Lassen Canyon
Festival	Susceptible	University of Florida
Odessa	Susceptible	Driscoll's
Monterey	Susceptible	University of California
Albion	Susceptible	University of California
Fronteras	Resistant	University of California
Grenada	Resistant	University of California
Del Rey	Resistant	Driscoll's
Petaluma	Resistant	University of California
BG.959	Resistant	Plant Sciences

*Relative susceptibility and resistance to *M. phaseolina* was determined by phenotypic field evaluations described in Chapter 2.

Ten cultivars in total out of 90 entries were selected for sampling to determine the extent of colonization by *M. phaseolina.* The cultivars were selected based on the mean percent mortality recorded in May 2017 (Chapter 2). The series of genotypes consisted of

five “resistant” cultivars (Fronteras, Grenada, Del Rey, Petaluma, and BG 959) and five “susceptible” cultivars (Ruby June, Festival, Odessa, Monterey, and Albion; Table 3.1).

3.2.2 Sample collection and processing for DNA extractions

Plants were harvested from the replicated field plots and collected on two separate dates (3 July 2017, 1 August 2017) for the detection of *M. phaseolina* in roots and crowns. The sampling collected on 3 July 2017 are denoted as mid-season samples and those collected on 1 August 2017 are denoted as late-season samples. For each sampling event, two asymptomatic plants were randomly selected and removed from each replicate plot. On the second sampling event, all plants of the susceptible cultivars were symptomatic and were sampled with symptomology recorded. A total of eight plants of each genotype from the inoculated field plots and two plants from the non-inoculated plot were collected on each sampling date. A small trowel was used to carefully excavate the intact root system; the trowel was disinfected between each plant sampled with 5.5% O-benzyl-p-chlorophenol (Lysol®, Reckitt Benckiser, Parsippany, NJ). Once removed, the root system was lightly shaken to remove loose rhizosphere soil and bulk soils, and the entire plant was placed into plastic bags (Ziplock®, C. Johnson & Son, Inc., Racine, WI) and then placed into a styrofoam cooler for transportation. Each plant sample was rinsed thoroughly with running tap water to remove residual soil. The rinsed plants were aseptically sectioned into roots and crowns. Cutting boards and instruments were disinfested with a 10% bleach solution of 8.25% sodium hypochlorite in between each plant sample (Clorox®, Oakland, CA). Once the roots were completely removed from the crown they were cut into fine pieces (approximately 1 mm to 5 mm long) and thoroughly mixed. From a single plant, root segment (< 2 g fresh weight) subsamples were taken and

weighed. The crowns were cut into smaller pieces (approximately 2 mm x 2 mm), including vascular tissue, cortex, and pith, but excluding any root tissue. The small crown segments of a single plant were thoroughly mixed by hand and (< 2 g fresh weight) subsamples were taken and weighed for subsequent processing. All fresh plant tissues that had been subsampled and weighed were transferred into individual plastic bags (12 cm x 15 cm, Agdia Inc., Elkhart, IN) then frozen and stored at -20 C until freeze drying. The number of samples taken and tested for extent of colonization by *M. phaseolina* are tabulated in. A total of 200 plants were sampled from field plots and sectioned into roots and crowns equating to a total of 400 tissue samples processed for DNA extraction and subsequent molecular assays.

3.2.3 DNA extractions from plant and fungal tissues

3.2.3.1 Plant tissue preparation

To isolate total DNA from plant tissues a multi-step process was conducted as follows. Frozen root and crown tissues were lyophilized (model: Freezone® 4.5, Labconco® corporation, Kansas City, MO) prior to DNA extractions. using the following parameters: -52 C, 0.021 mBar, for 18 hrs. Lyophilized tissues were then mechanically disrupted and homogenized in individual sample bags with a hand roller tissue homogenizer (Agdia Inc., Elkhart, IN) for 1 min per sample. The cell disrupted tissues were subsampled (≤ 20 mg) and weights were recorded. A stainless-steel lab spatula instrument was used for weighing and was cleaned with a 10% bleach solution of 8.25% sodium hypochlorite between each sample. The subsamples were transferred to sterile 1.5 mL tubes (Fisherbrand™ premium microfuge, ThermoFisher Scientific, Waltham, MA) for total DNA extractions.

3.2.3.2 Fungal tissue preparation and DNA extractions

DNA was extracted from *M. phaseolina* isolates of Mp8, Mp21, and Mp22, the same isolates used in generating inoculum. The resulting DNA was used to generate standard curves in qPCR assays. Sterile vials containing 10 mL of potato dextrose broth (PDB) were inoculated with two plugs (2 mm) of a single isolate of *M. phaseolina* in a laminar flow hood. Plugs of *M. phaseolina* had been previously cryogenically stored in a 50% glycerol 50% DI H2O solution. The inoculated PDB vials were incubated at 30 C in the dark for 7 days. After incubation the samples were centrifuged at 14,000 rpms for 1 min to remove the supernatant. The remaining mycelial tissue was frozen at -20 C, until being lyophilized. The harvested mycelial tissue was lyophilized for 5 hrs. Subsamples of 20 mg were weighed and the cells of the tissue were mechanically disrupted by hand with a polypropylene mortar (Sigma-Aldrich®, St. Louis, MO). The disrupted tissues were then used for subsequent DNA extractions.

Both plant and fungal DNA extractions were completed with a commercial extraction kit following a modified version of the manufacturer's instructions (DNeasy® Plant Mini Kit, Qiagen Inc., Valencia, CA). The DNeasy plant mini kit utilizes a silica spin column technology that consistently yielded higher concentrations of DNA when testing the absorbance at 260 nm (A_{260}) with a spectrophotometer (Nanodrop™ 2000, ThermoFisher Scientific, Waltham, MA) and with improved purity (ratio of absorbance 260 nm to 280 nm, A_{260}/A_{280}) when compared to lab made buffer-extraction protocols (data not shown) (Brouwer et al. 2003). The procedure was modified at step 11, in which 50 µL of AE buffer was used per elution step for a final elution volume of 100 µL. Eluted DNA extractions were stored at -20 C.

3.2.4 Quantitative polymerase chain reaction (qPCR) for the detection of M. phaseolina

3.2.4.1 Single-tube nested TaqMan assay

The extent of colonization of strawberry tissues by *M. phaseolina* was determined by qPCR of plant DNA extracts. The qPCR assay employed the procedure outlined by Burkhardt et al. (2018). The assay utilizes is a single-tube nested approach, with TaqMan chemistry, while utilizing a standard curve of quantified concentrations of *M. phaseolina* DNA to extrapolate unknown concentrations contained in plant extracts. The reaction is multiplexed with an internal control (IC) developed by Bilodeau et al. (2012) to monitor for the presence of PCR inhibitors.

Quantitative PCR was performed in 25 µL volume reactions using a QuantStudio™ 3 Real-Time PCR system (Applied Biosystems, Foster City, CA). The single-tube nested reaction contained primers and probes at the following concentrations: Mps_TaqMan forward (5'-CCTCGGCAAATCCCTATAG-3') and reverse (5'-GTTTACCCTCTGTCTATTCC-3') primers at 400 nM, Mps_TaqMan_External forward (5'-CTAAAGTGGCTTAATACTAATTTAGCGCCGGCGAATC-3') and reverse (5'-GTAAGCCTTACCGCACTAGAAGTAAGGGTAAGATCG-3') primers at 20 nM, Mps_TaqMan_Probe (5'-TAMRA-CTATTTGGTTAACCCCTACTCGCTTAGACT-BHQ2-3') at 200 nM. The internal control was included in the reaction mixture with the following concentrations Vdf929-PPF1F (5'-CGTTTCCCGTTACTCTTCT-3') and Vdr1076-PPF1R (5'-GGATTTCGGCCCAGAAACT-3') at 1000 nM, and probe Vdhrc-FAM (5'-FAM-CACCGCAAGCAGACTCTTGAAAGCCA-BHQ1-3') at 400 nM, and 32 fg *Verticillium dahliae* purified DNA. The list of *M. phaseolina* primers and probe sequences and concentrations, as well as IC primers, probe, and DNA sequences are

summarized in (Table 3.2). The reaction was performed with 5X PerfeCTa® Multiplex qPCR ToughMix (Quantabio, Beverly, MA), and 1 µL of DNA extract. All reactions were performed in duplicate technical replications with each plant extract being amplified in two wells, except for the generation of the standard curve which was completed in triplicate. The nested thermocycling parameters were 1 cycle of 95°C for 3 min, 20 cycles of 95°C for 15 sec and 70°C for 30 sec with a plate read, followed by 50 cycles of 95°C for 15 sec and 62°C for 30 sec with a plate read.

Table 3.2. Primer and probes used for the single-tube nested qPCR TaqMan assay along with the internal control for the detection of *Macrophomina phaseolina*.

Primer/Probe Name[*]	**Sequence**[y]
Mps_TaqMan Forward	CCTCGGCAAATCCCTATAG
Mps_TaqMan Reverse	GTTTACCCTCTGTCTATTCC
Mps_TaqMan_External Forward	CTAAAGTGGCTTAATACTAATTTAGCGCCGGCGAATC
Mps_TaqMan_External Reverse	GTAAGCCTTACCGCACTAGAAGTAAGGGTAAGATCG
Mps_TaqMan_Probe	TAMRA-CTATTTGGTTAACCCCTACTCGCTTAGACT-BHQ2[z]
Vdf929-PPF1F	CGTTTCCCGTTACTCTTCT
Vdr1076-PPF1R	GGATTTCGGCCCAGAAACT
Vdhrc-FAM	FAM-CACCGCAAGCAGACTCTTGAAAGCCA-BHQ1[z]

[y]All sequences are listed in 5' to 3'
[z]TAMRA and FAM = fluorescent reporter of the probe, BHQ = black hole quencher the non-fluorescent quencher of the probe.
[*]Primer/probes for Mps were adopted with permission from Burkhardt et al. (2018) and Vd from Bilodeau et al. (2012).

The C_t values were taken from the second amplification phase of 50 cycles.

3.2.4.2 Standard curve

A seven-point serial dilution was used to generate a standard curve for the qPCR analysis and quantification of *M. phaseolina* from DNA extractions. The dilution series used following concentrations of extracts from *M. phaseolina*: 2 ng uL^{-1}, 0.2 ng uL^{-1}, 0.02 ng uL^{-1}, 0.002 ng uL^{-1}, 0.001 ng uL^{-1}, and 0.005 ng uL^{-1}. For plot of the C_t values versus the logarithm of the initial concentration a regression line was fit, and the

amplification efficiency was calculated with the slope of the curve which was within the acceptable norms for qPCR assays (Efficiency = 100.64 %; R^2 = 0.989 for *M. phaseolina*). The default amplification threshold for the generation of the standard curve was ΔRn = 16,054 and this value was used in all subsequent reactions of plant samples assayed. The initial concentration of DNA from *M. phaseolina* extracts was quantified with a Qubit™ 4 fluorometer (Applied Biosystems, Foster City, CA) following the manufacture's protocol.

3.2.4.3 Quality control of DNA extractions

If the C_t values of the internal control were more than two standard deviations from the grand mean C_t for the 96 well plate, then the qPCR reaction was run again on the same extract. If amplification of the IC failed, then the DNA was re-extracted on the same lyophilized plant sample and run in a new qPCR reaction. In total, 12 DNA extracts had to be run in new qPCR reactions, and 12 plant samples had to be re-extracted and run in new qPCR reactions; this amounted to 6% of the total samples having unsatisfactory amplification of the internal control.

3.2.5 Data and statistical analysis

A multiple factor analysis of variance with ordinary least squares method was conducted using JMP® pro statistical software (version 13.1 SAS Institute, Cary, NC) to test the effects of 1) cultivar, 2) plant tissue type, 3) tissue type x cultivar interaction, 4) time of sampling and 5) the block the plot was sampled from on the concentration of *M. phaseolina* DNA detected. To assess the effects of each factor, a linear mixed effects model was fit to the data with plot nested in sample time, and tissue type nested in cultivar; all factors were treated as fixed factors. A $\log_{10}$ transformation was performed

on the concentration of *M. phaseolina* DNA to meet the assumption of the ANOVA test concerning the homogeneity of variance and normality of error. After the $\log_{10}$ transformation of the data, a Shapiro-Wilk W test was conducted to test the goodness of fit of normality on the distribution of the residuals, in which the probability < W = 0.13. With the goodness of fit test, I concluded the error is not statistically different for a normal distribution after the transformation, satisfying the assumptions of the ANOVA analysis. A Tukey's honest significant differences (HSD) post hoc analysis at $P < 0.05$ was used to test differences between factor means for the cultivar x sample event date interaction. A Fisher's least significant differences (LSD) post hoc analysis at $P < 0.05$ was used to test differences between factor means for the main effect of cultivar. The data from the non-inoculated control plot was not presented and were excluded from the analysis. All mean values presented are back-calculated from the $\log_{10}$ transformation.

3.3 Results

3.3.1 Comparison of genotypes by sample event date

There were statistically significant effects for cultivar ($F_{9,260} = 7.759$, $P < 0.0001$) and sampling date ($F_{1,260} = 24.426$, $P < 0.0001$), as well as a significant two-way interaction of cultivar x sample time ($F_{9,260} = 2.549$, $P = 0.0083$) on the concentration of *M. phaseolina* DNA detected in strawberry samples (Table 3.3). Quantity of *M. phaseolina* DNA detected was not always elevated in the late-season sampling event relative to the initial sampling event. In some instances, the concentration of *M. phaseolina* DNA in roots and crowns of the strawberry plants was higher at the first sampling event, but this was cultivar-dependent (Figure 3.1). The plants sampled in the mid-season had average concentrations of *M. phaseolina* DNA (pg g^{-1} dry strawberry

tissue) of: Grenada = 9.45, BG. 959 = 41.94, Del Rey = 41.99, Odessa = 138.64, Monterey = 186.21, Petaluma = 265.01, Festival = 267.54, Fronteras = 333.59, Ruby June = 426.91, and Albion = 612.40 (Figure 3.1). The plants sampled in the late-season had average *M. phaseolina* DNA concentrations of (pg g^{-1} dry sample strawberry tissue) of: BG. 959 = 5.36, Petaluma = 21.86, Fronteras = 25.33, Grenada = 28.16, Odessa = 70.58, Del Rey = 75.89, Festival = 227.41, Ruby June = 249.42, Albion = 282.93 and Monterey = 580.05 (Figure 3.1). The average concentration of *M. phaseolina* DNA detected across all strawberry tissue types and cultivars was higher for the mid-season

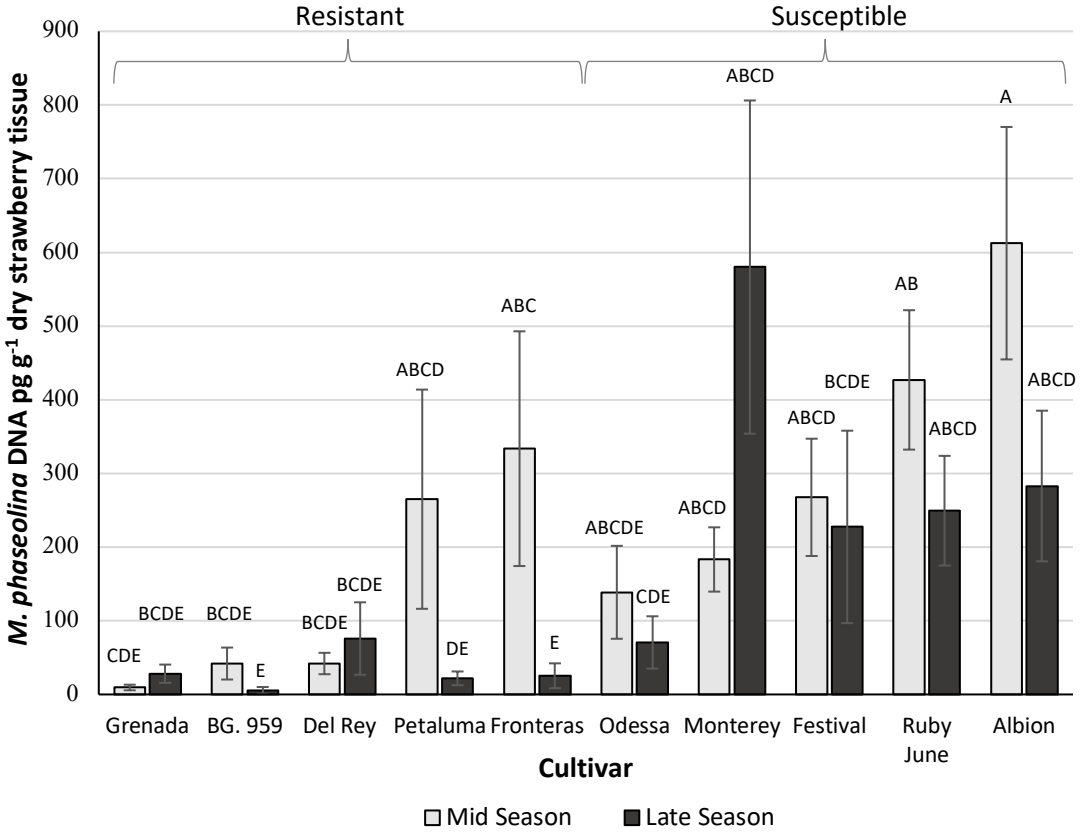

Figure 3.1. Average values of the concentration of *Macrophomina phaseolina* DNA detected in roots and crown tissues of strawberry plants of ten cultivars. Resistance and susceptibility were determined by phenotypic assessments of plant mortality (Chapter 2). Mid-season plants were sampled on 3 July 2017 and late season plants were sampled on 1 August 2017. Error bars represent the standard error of the means. Values that share the same letter are not statistically different from each other by Tukey's honest significant differences pairwise comparisons ($\alpha = 0.05$).

sampling event (233.27 pg g^{-1} dry wt) than for late-season samples (157.24 pg g^{-1} dry wt). While the time of plant sampling had a statistically significant main effect on the average concentration of *M. phaseolina* DNA detected in strawberry plant tissues it had a significant interaction with the cultivar tested. Plant samples of cv. Fronteras contained statistically significantly higher concentrations of *M. phaseolina* DNA in the mid-season when compared to the late-season sampling ($P = 0.0083$) demonstrating that Fronteras was the primary cv. driving the significant interaction between sample time x cultivar interaction.

Table 3.3. Analysis of variance for the concentration of *Macrophomina phaseolina* DNA detected in roots and crowns of ten strawberry cultivars detected at two different sampling event dates.

Source of Variance[z]	df	F statistic	p-value
Cultivar	9	7.759	<0.0001
Sample Time	1	24.426	<0.0001
Field Block [Sample Time]	6	1.67	0.1296
Tissue Type [Cultivar]	10	1.219	0.2798
Cultivar x Sample Time	9	2.549	0.0083
Residual	225		
Total	260		

[z]Analysis of variance was completed on $\log_{10}$ transformation of the concentration of *M. phaseolina* DNA (pg *M. phaseolina* DNA g^{-1} dry strawberry tissue)

3.3.2 Comparison of roots and crown tissue of strawberry cultivars

The main effects of plant tissue type for each cultivar and field block replicate were not statistically significant with regard to the concentration of *M. phaseolina* DNA detected ($P = 0.2798$ and $P = 0.1296$ respectively) (Table 3.3). Any differences in *M. phaseolina* DNA concentration detected in the strawberry samples between root and crown of a single cultivar type were not statistically significant and could not be supported by the data collected. However, the main effects of cultivar on quantity of pathogen DNA detected in plant tissue were significant ($F_{9,260} = 7.759$, $P < 0.0001$ (Table

3.3). Pairwise comparisons are completed only for the main effects of cultivar (Figure 3.2). The cultivars of Grenada, BG. 959, and Del Rey had the lowest average concentration of *M. phaseolina* DNA detected in the strawberry roots and crowns and values were significantly lower than that of the three cultivars possessing the highest *M. phaseolina* DNA concentration, Monterey, Albion, and Ruby June (Figure 3.2).

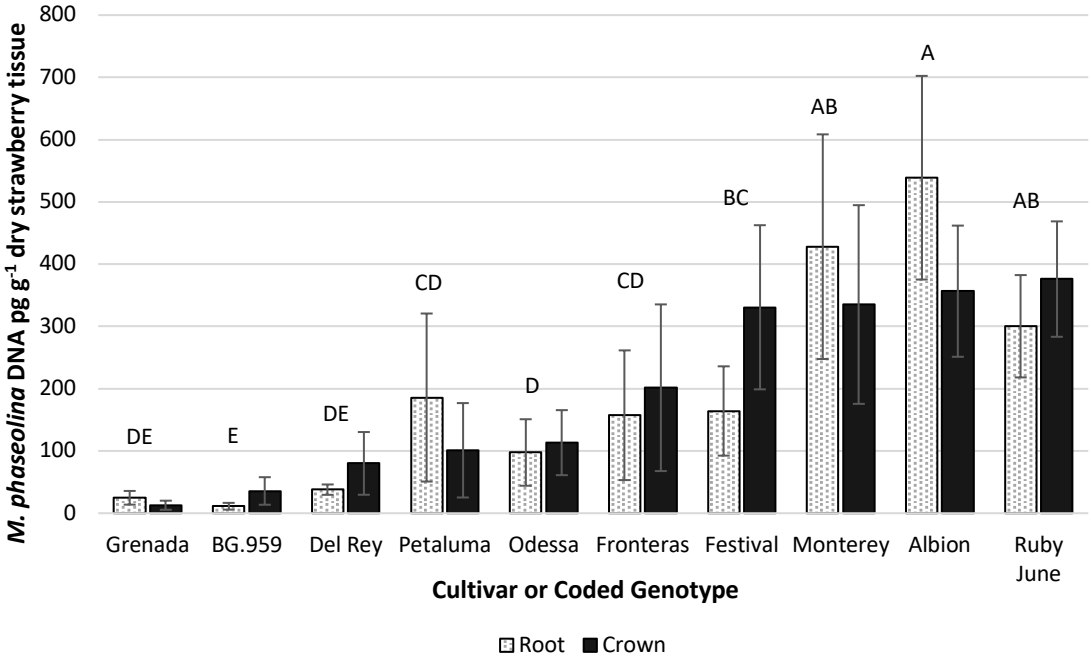

Figure 3.2. Average values of the concentration of *Macrophomina phaseolina* DNA detected in roots and crown tissues of strawberry plants of ten cultivars averaged for both sampling times. Resistance and susceptibility were determined by phenotypic assessments of plant mortality (Chapter 2). Error bars represent the standard error of the means. Values that share the same letter are not statistically different from each other by Fisher's least significant differences pairwise comparisons ($\alpha = 0.05$).

3.3 Discussion

Phenotypic host resistance of strawberry genotypes to Macrophomina crown rot has been previously described (Fang et al. 2014; Sánchez et al. 2016). Limitation of pathogen colonization in a strawberry cultivar specific manner has been explored in the case of *Fusarium oxysporum* f. sp. *fragariae* (Fang et al. 2012), but such information about *M. phaseolina* does not exist. This study sought to explore the extent of colonization of different strawberry cultivars inoculated with *M. phaseolina*. Our results

demonstrate that even strawberry genotypes showing phenotypic signs of resistance to Macrophomina crown rot in the form of reduced disease severity are colonized by *M. phaseolina*. Furthermore, phenotypic resistance in strawberry do not correspond with limiting pathogen progression from the root to vascular tissue, and eventually the pathogen can enter the crown of both resistant and susceptible plants. However, phenotypic host resistance of strawberry cultivars does limit the amount of host tissue colonization by *M. phaseolina* since significant less pathogen DNA was detected in resistant cultivars compared to the highly susceptible cultivars.

While colonization of resistant cultivars was limited but present, the observed concentrations of *M. phaseolina* DNA matched previous characterizations of relative tolerance. The concentrations of *M. phaseolina* DNA of cultivars tested here were significantly positively correlated with phenotypic percent mortality observed in chapter 2 (data not show). Cultivars Grenada, BG. 959, Del Rey and Odessa, which were previously characterized as resistant, were significantly less colonized when compared to susceptible cultivars Festival, Monterey, Albion, and Ruby June. Cultivars Petaluma and Fronteras demonstrated limited colonization when inoculated with *M. phaseolina* compared to the most susceptible cultivars Monterey, Albion and Ruby June but should be characterized as a moderate resistance compared to cultivar BG. 959. While phenotypic studies conducted in chapter two were able to identify a relative resistance of cultivars to wilt severity caused by *M. phaseolina* they were unable to illuminate the gradient of tolerances and resistance to colonization that was demonstrated here with molecular assay techniques.

Intuitively, and as we observed, the time of sampling had significant effects on the amount of *M. phaseolina* DNA detected in the different cultivars. Different genotypes likely have different physiological and physical responses to pathogen challenge. In this study pathogen DNA was detected at higher quantities in roots and crowns of strawberry for plants sampled in July relative to August. However, the significant interaction between cultivar and sample time indicates that progression of pathogen colonization of the host through a growing season will differ among strawberry cultivars.

The observed variation and significant differences in the amount of *M. phaseolina* DNA due to sampling time can be influenced by both sampling methodology as well as be driven by biological causes. The sampling methodology utilized here potentially lacked technical replication in which randomly selected plants in the mid-season by chance were more colonized then the plants randomly selected in the late-season samples. In addition, improper homogenization of the plant tissues during the grinding process could have produced poorly mixed samples where subsequent subsamples were not representative of the plant sample. Both notions are supported by the observed variance and standard error observed especially in the circumstances of cvs. Petaluma, Fronteras, Monterey and Albion (Figure 3.2). Increased subsamples from each plant replicate for molecular analysis would potentially minimize variance of *M. phaseolina* DNA observed for each cultivar. A systematic longitudinal study composed of many sampling events over time would derive a more thorough understanding of the extent of colonization of host tissues over time. Utilizing a sampling methodology that included more sampling events across the growing season and with increased plant replication would increase the

statistical power of the analysis and ascertain when each cultivar experiences a peak colonization.

Significant differences in the amount of *M. phaseolina* detected by sample time could in fact be associated with biological causes. It likely that when plants inoculated with *M. phaseolina* are asymptomatic that the pathogen is in a vegetative growth stage where most of the fungus biomass consists of mycelium rather than the resistant spore form of microsclerotia. For example, all plants were asymptomatic at the time of sampling for the mid-season sample event. This contrasted to the late-season sampling, where susceptible cultivars, were either symptomatic or necrotic at the time of sampling. While unexplored, in this study there is likely differences in nucleic acid extraction efficiencies of the two tissue types of *M. phaseolina* when considering mycelium compared to microsclerotia that would be present in symptomatic or necrotic plants (Mahuku and Platt 2002). Thus, the increase in concentration of *M. phaseolina* DNA observed during the July sampling event compared to August can be caused by increased DNA extraction efficiency from asymptomatic plants compared to those of highly symptomatic plants. While out of the scope of this paper, future studies focusing on determining the extraction efficiency of the two tissue types of *M. phaseolina* would allow for more accurate comparisons of plants sampled over time regardless of symptomology Though it has been demonstrated that the amount of DNA of a target fungus derived from a qPCR assay from soil was significantly correlated with the initial amount of microsclerotia tested, this has not been demonstrated for *M. phaseolina* (Tellenbach et al. 2010). . In addition, future studies correlating detected concentrations of *M. phaseolina* DNA with the number of viable infectious cells would aid in

determining pathogen threshold densities in soil that warrant management strategies (Lievens et al. 2006).

While the present study was able to demonstrate that resistant cultivars are being colonized less by the pathogen than susceptible cultivars, the fact that the pathogen is present throughout the roots and crown offers only some insight into the mechanisms of resistance. Reduced symptom development of resistant cultivars, despite being colonized, results from a multitude of mechanisms inferring observed phenotypic resistance many of which can be mediated by host genetics that vary with cultivar. These resistance genetics ((R) genes) could allow for a multitude of transcriptional products and pathways limiting or localizing colonization by the pathogen (Hammond-Kosack and Jones 1996). Now that phenotypic screening of strawberry genotypes for resistance to *M. phaseolina* has been completed, comparative genetic analysis could be used to assist in the identification of pathways or processes that confer resistance. Quantitative trait loci (QTL) mapping has been successfully used to identify such (R) genes in the pathogen-host interaction of late blight of potato (Young 1996), but no literature of this for strawberries and *M. phaseolina* exists. Another hypothesized mechanism of resistance is the hypersensitive reaction of the plant to the pathogen plant in which induced cell death limits the nutritional resources available to the pathogen thus localizes its infection and reduces observable symptom development (Goodman and Novacky 1994). The gradient of colonization observed in this study suggests that there is no presence of a binary on-off gene or gene for gene relationship of resistance between host and pathogen as would be expected if there were only resistant or susceptible cultivars. Another potential mechanism of reducing colonization would be through rhizosphere mediated inhibition, where cultivar specific

roots would influence the rhizosphere microbial community of the rhizosphere in a way that would inhibit infection (Pérez-Jaramillo et al. 2015). Microbially mediated tolerance against *M. phaseolina* is possible and supported by the notion that once in a resistant cultivar's tissue there is little inhibition of the pathogen moving throughout the plant, but there could be less of the pathogen entering the root system in total. Regardless of the mechanism resistant plants are less colonized then susceptible cultivars and produce less secondary inoculum in their tissues.

CHAPTER 4

Integration of Host Resistance and Organic Soil Amendments as a Strategy for Management of Macrophomina Crown Rot in Strawberry

4.1 Introduction

Strawberry production systems are designed around the single application of a soil fumigant to disinfest soils of fungal pathogens, weeds and pests before planting. A mixture of methyl bromide (MeBr) and chloropicrin was the most effective and commonly used soil fumigant by the strawberry industry in California (CA), but MeBr use currently restricted by the Montreal Protocol (Mazzola et al. 2017). Alternative soil fumigants registered for use in strawberry production exist and currently used CA, however their efficacy relative to MeBr is reduced. The practical implementation of alternative fumigants has been challenging, where poor distribution in the soil profile has been attributed to their decreased efficacy (Chamorro et al. 2015). Increasing concerns of the toxicology of alternative fumigants and their potential for emissions could warrant regulatory actions limiting their use on a statewide to regional basis (Duniway 2002; Mazzola et al. 2017). In addition, the use of alternative soil fumigant chemistries has been associated with a rise in soilborne pathogens throughout CA (Koike et al. 2013). One important disease is Macrophomina crown rot incited by the fungal soilborne pathogen *Macrophomina phaseolina* (Zveibil and Freeman 2005; Koike 2008). It has been suggested that the increased incidence of Macrophomina crown rot in strawberry production is due to inconsistent control by alternative fumigation technologies (Zveibil et al. 2012; Chamorro et al. 2016; Mazzola et al. 2017).

Current and potential restrictions in the use of soil fumigants has led to numerous studies exploring new non-chemical approaches to managing soilborne disease in

strawberries (Subbarao et al. 2007; Muramoto et al. 2014; Fang et al. 2014; Mazzola et al. 2017; Shennan et al. 2017). The incorporation of organic amendments into the soil profile with compost, plant residues, or carbon-based substrates have been linked to some disease control of soilborne pathogens (Leandro et al. 2007, Subbarao et al. 2007, Muramoto et al. 2014). The soil incorporation of Brassicaceae plant residues, more specifically seed meals derived from mustard plants (MSM) *Brassica napus Brassica juncea* [L.] or *Sinapis alba* [L.], have been widely explored for their effects on soilborne pathogens and plant growth responses (Smolinska et al. 1997; Fennimore et al. 2014; Hewavitharana et al. 2014; Mazzola et al. 2015; Neubauer et al. 2015; Mazzola et al. 2017). The efficacy of MSM amendments against soilborne pathogens have been mainly attributed to containing biologically active compounds of glucosinolates which hydrolyze into isothiocyanates (ITCs) (Smolinska et al. 1997). However more recent studies have demonstrated that the mechanisms of pathogen suppression from the soil incorporation of MSM are predominantly biological and microbially mediated, but the predominant mechanisms of pathogen suppression may differ from pathogen to pathogen (Mazzola et al. 2007). In addition, the relative level of pathogen suppression and plant responses to MSM can differ with host genotype (Mazzola et al. 2009). A study by Mazzola et al. (2017) demonstrated that the soil incorporation of *B. juncea* and *S. alba* MSM did not significantly reduce soil inoculum of *M. phaseolina* but did significantly reduce root infection of strawberries cv. 'Camarosa' even without reducing pathogen soil density.

The flooding of fields and subsequent reduced effects in the oxidation-reduction potential of soils has long been associated with plant pathogen suppression (Cook and Baker, 1983). A novel technique described as anaerobic soil disinfestation (ASD) also

referred to as biological soil disinfestation (BSD), utilizes induced soil anaerobicity to generate reduced soil conditions. It has been employed as a pre-plant soil treatment to control soilborne pathogens and limit yield decline in several agricultural pathosystems (Shinmura et al. 1999; Blok et al., 2000). Initial research by Shinmura et al. (1999) and Blok et al. (2000) identified that the methods of ASD depend on inducing soil anaerobic conditions by incorporating a labile carbon rich amendment (C-source) into the soil, moistening the soil, and preventing the resupply of oxygen from entering the system by covering the soil with a plastic film for a short duration (two to fifteen weeks) (Rosskopf et al., 2015). In some circumstances these outlined procedures have been demonstrated to reduce soilborne pathogens and boost crop yields to levels achieved with MeBr fumigation (Butler et al., 2012; Mazzola et al. 2018). Butler et al. (2014) utilized techniques described as ASD combined with solarization to increase yields of fresh bell pepper crops in Florida, in a research plot naturally infested with *Phytophthora capsici,* and root-knot nematode *Meloidogyne incognita.* Lemars et al. (2014) outlined that ASD was used on over a dozen cropping systems throughout the world including vegetables, cut flowers, fruit and strawberries, utilizing a range of carbon substrates against nine distinct fungal pathogens and four plant parasitic nematodes. Despite the past and present research initiatives, further experimentation is required to optimize the procedure on a regional and pathosystem basis. Enhanced and consistent efficacy of ASD will require knowledge of the primary lethal plant pathogen, utilizing a specific carbon substrate for the pathosystem, and will differ with soil characteristics and temperatures.

Mechanisms of pathogen control during the ASD process are complex and not fully understood. It has been determined that there are multiple mechanisms that can lead

to pathogen control. The process can be microbially mediated through the production of organic acids and volatile organic compounds (Momma et al. 2006; Hewavatharana et al. 2014), induced changes in the soil microbial community that are not conducive to pathogen infection (Strauss and Kluepfel 2015; Mazzola et al. 2018), and the potential for the stimulation of antagonistic microbes (Hewavitharana and Mazzola 2016; Strauss et al. 2017; Mazzola et al. 2018). The efficacy of ASD depends on the carbon substrate used and can differ from pathogen to pathogen; for example, rice bran substrate ASD was effective at reducing the soil inoculum density of *Verticillium dahliae* in strawberries, but unable to control *Fusarium oxysporum* f. sp. *fragariae* (Shennan et al. 2017; Mazzola et al. 2018). Muramoto et al. (2016) was able to double strawberry yields compared to the grower standard in a field naturally infested with *M. phaseolina* while employing ASD with rice bran at 9 tons acre^{-1}, however Macrophomina crown rot in this field was not abolished. ASD is currently used on over 2000 acres in commercial strawberry production with rice bran as a carbon substrate (personal communication: Stefanie Bourcier, Farm Fuel Inc.). Despite reports of ASD matching net returns to MeBr fumigation, as well as currently used alternative fumigants of chloropicrin-1,3-dichloropropene mixtures to strawberry, there has been limited adoption of ASD in CA conventional commercial strawberry production in CA (Fennimore and Goodhue 2016). This is likely due to inconsistencies of disease control by ASD.

The objectives of this study were to i) combine host resistance with the use of organic soil amendments in effort to mutually enhance the efficacy of control of Macrophomina crown rot; and ii) determine whether the integration of these two methods

altered the level of disease severity and extent of *M. phaseolina* colonization of multiple strawberry genotypes.

4.2 Materials and methods

4.2.1 Inoculum production and quantification

M. phaseolina inoculum was produced according to the Materials and Methods section in Chapter 2 of this thesis.

The density of viable microsclerotia of the cornmeal-sand inoculum was enumerated with a direct plating culture method. In a 250 mL Erlenmeyer flask 10.0 g of sieved (1 mm) cornmeal-sand inoculum was added to 100 mL of sterile DI H_2O, in the presence of 1% (w:v) sodium hexametaphosphate (Sigma-Aldrich®, St. Louis, MO). The mixture was homogenized with a magnetic stirbar at 300 rpm for 5 minutes. Because *M. phaseolina* microsclerotia do not solubilize the stir bar was kept moving when a subsample was taken for a serial dilution. A serial dilution was completed from 10^0 to 10^{-4} solutions. From each dilution, 100 µL was pipetted and spread over the surface of a Petri dish containing NP-10 medium (Kabir et al. 2004). Ten replicate plates of each dilution were used. The plates were incubated in the dark at 30°C for 7 days before counting colony forming units (CFU). *M. phaseolina* CFU were resolved and enumerated with a stereo microscope at 5X magnification. Estimates of the total CFU g^{-1} of inoculum ranged from the 10^{-2} dilution estimate of 50,300 CFU g^{-1} inoculum, to the 10^{-3} dilution estimate of 70,000 CFU g^{-1}. To be conservative and not underestimate inoculation density of potting substrates the low estimate was used to calculate the amount of inoculum needed for infestation of the potting substrate at 100 CFU g^{-1} potting substrate.

4.2.2 Soil assay

4.2.2.1 Soil potting-substrate mixture

The potting substrate used in this study was a mixture of field soil, coconut coir, and course texture perlite a ratio of 1:1:1 (v:v:v). The field soil was obtained from a research plot located on California Polytechnic State University in San Luis Obispo, CA Field 35b (35°18'20.21' N; 120° 40'23.39'). The soil was collected from a region of the field where strawberry plants had not been cultivated previously. The soil type of field 35b is classified as a Salinas silty clay loam, with an organic matter content of 3%, ECe of 1.6 dS m^{-1}, CEC of 17.4 meq 100 $grams^{-1}$, and 6.8 pH (A&L Laboratories, Modesto, CA). The soil was sieved through a standard 4.75 mm sieve and mixed before being used in the mixture. The soil, coconut coir, and perlite were mixed in a cement mixer by adding 1 L of each item and repeating. *M. phaseolina* cornmeal sand inoculum was added to the mixing substrate to achieve 100 CFU *M. phaseolina* g^{-1} potting substrate. This was achieved by adding 0.23% (w:w) of inoculum which had 50,300 CFU *M. phaseolina* g^{-1} cornmeal sand inoculum to the total potting substrate batch. The inoculated potting substrate mixture was then split into four batches to mix in the respective soil treatment. A slow release fertilizer 14-14-14 (Osmocote®, The Scotts Company, Marysville, OH) was amended to the soil potting-media mixture of the inoculated unamended control (UTC), and steam soil treatment mixtures at a target of 360 lbs N $acre^{-1}$, or 5.89 g per potting container.

4.2.2.2 Soil treatments and amendments

The single batch of inoculated potting substrate mixture was split into four separate batches respective for the four different soil treatments. The UTC batch was amended

with slow release fertilizer as listed above, then was left unamended. The MSM soil treatment was amended with a milled fine-course particle *B. juncea* cv. 'Pacific Gold' seed meal (Farm Fuel Inc., Watsonville, CA) at a rate of 2 tons acre^{-1}. This was achieved by mixing 9.2 g MSM in each container of potting substrate at a rate of 0.7% (w:w), (MSM:potting substrate). Glucosinolate concentrations of the MSM were not quantified. The ASD batch was amended with finely milled rice bran (Pajaro Valley Gold 1.0, Farm Fuel Inc., Watsonville, CA) at a rate of 9 tons acre^{-1}. This was achieved by mixing 41.3 g of rice bran into each container potting substrate, at a rate of 3.1% (w:w). The steam soil treatment was also amended with slow release fertilizer as described above. The inoculated potting substrate for the steam treatment was injected with 98.3°C steam for 8 hrs in a sealed metal container. The temperature of the potting substrate during the steam treatment was monitored with three analog soil thermometers that were 50 cm long inserted into the middle of the substrate. Temperatures were recorded hourly for eight hours from the commencement of the steam treatment. Values presented are the mean temperature from the three replicate thermometers. The steamed soils were allowed to cool and air dry for one week before being used in potting containers.

4.2.2.3 Anaerobic phase

The dry amended soil treatments were placed into 3.8 L pots (1 ga trade-pots) (300 series elite nursery containers [17.5 cm tall x 16 cm diameter] McConkey & Co., Sumner, WA). The containers were transferred into the greenhouse and placed on the bench in a completely randomized design. All the pots were then irrigated with an overhead sprinkler for 30 mins, allowed to drain for 10 mins, and then irrigated again with an overhead sprinkler. The potting containers were allowed to drain for 10 mins more to

achieve field capacity of the potting substrate before being placed into specimen plastic storage bags (Saranex™ [40.6 cm long x 40.6 cm wide], ThermoFisher Scientific, Waltham, MA) to simulate conditions obtained in field ASD under plastic mulch. The specimen bags were sealed with zip lock tops and incubated in the greenhouse for a total of 22 days. The 22-day duration of the anaerobic phase was following the typical incubation time outlined by Shennan et al. (2017). Soil conditions of the anaerobic soil phase are outlined below. At completion of the incubation phase all pots were removed from the specimen bags and allowed to air dry for 1 week before planting.

4.2.2.5 Soil sampling and estimating inoculum density

Prior to the anaerobic phase potting substrate samples from each soil treatment were collected to assess the change in density of *M. phaseolina* inoculum due to soil treatments. A probe with 2 cm inner diameter was used to randomly collect a soil sample from each container to a 15 cm depth. Four soil cores were collected for each soil treatment. The soil sample was mixed by hand and air dried for 10 days. Dried soils were hand ground with a mortar and pestle for 5 mins for each sample. Soil samples were plated on NP-10 media (Sorenson et al. 1991, Kabir et al. 2004) using a modified two stage Anderson air sampler (Fisherbrand™, ThermoFisher Scientific, Waltham, MA) (Butterfield and DeVay 1977). For each soil sample three replicates were used consisting of 0.5 g of soil spread over six 100 mm Petri plates. The plates were incubated at 30°C in the dark for 15 days. After incubation, the surface of the plates was washed by hand under running tap water and examined with a stereo microscope for the presence of *M. phaseolina* colonies with distinct microsclerotia. Colonies were enumerated and a mean CFU g^{-1} potting substrate was calculated from the three replicates of a single potting

substrate sample. After the anaerobic phase and before planting soil core samples were taken again from the same potting containers and the same process was repeated.

4.2.2.6 Measuring soil Eh

Soil redox potential (E_h) was monitored during the anaerobic phase with oxidative reduction potential (ORP) sensors (S500-CD-ORP-HT; Sensorex Inc., Garden Grove CA) as outlined by Shennan et al. (2017). The ORP sensors were inserted into the soil at the 15 cm depth after the soil treatments were irrigated and before sealing the containers with the specimen bags. Four replicate ORP sensors where set for each soil treatment. ORP mV values were recorded daily with a handheld pH/ORP meter (WD-35614, Oakton, Vernon Hills, IL). Due to the AuCl internal reference of the probe the addition of 199 mV to the reading value was completed to adjust mV to E_h (Shennan et al. 2017). A threshold value of E_h under 200 mV were considered as anaerobic under the assumption that soil pH levels were 6.6 (Butler et al., 2012).

Temperature sensors were also installed in potting containers along with the ORP sensors to monitor air and soil temperatures. Three temperature sensors with associated data loggers (HOBO Pro v2; Onset Co., Bourne, MA) were exposed to ambient air temperatures, and three temperature sensors were buried in random UTC soil treatment containers at a 10 cm depth. A temperature reading for both soil and air was recorded every 15 mins for the duration of the anaerobic phase. Reported temperatures are averages of the three sensors for both soil and air respectively.

4.2.3 Cultivars

The cultivars used in this trial were selected based upon relative resistance or susceptibility as reported Chapter 2 and Chapter 3. Three “resistant” cultivars consisting

of cvs. Fronteras, Del Rey, and Petaluma and three "susceptible" cultivars consisting of cvs. Festival, Monterey and Albion were selected. The strawberry plants were obtained as bareroot stock and stored at 4ºC prior to transplanting.

4.2.4 Experimental design

The experiment was a two factor completely randomized design (CRD). The first factor of soil treatment consisted of four levels including an inoculated unamended control (UTC), ASD with rice bran at 9 tons $acre^{-1}$, MSM at 2 tons $acre^{-1}$, and a steam control. The second factor was strawberry cultivar which consisted of six levels including cv. Del Rey, Fronteras, Petaluma, Monterey, Albion, and Festival. The full factorial design had 24 treatments total, in which there were 10 single plant replicates per treatment with a total of 240 plants. All plants were grown in a greenhouse

4.2.5 Weed seed measurements

At the completion of the anaerobic phase, when potting containers were removed from the specimen bags samples of the weed seedlings that had germinated during the anaerobic phase were collected from each soil treatment. Four potting containers per soil treatment were randomly selected for collection of weed seedling samples. The weed seedlings were carefully removed including root systems and all soils were brushed off to maintain fine roots. The number of weed seedlings per container were enumerated and total fresh biomass in weight of weed seedlings including root systems of each container were recorded (Fennimore et al. 2003).

4.2.6 Plant health assessments

A scoring metric was used to assess disease severity of the inoculated strawberry cultivars over time. Plant health assessments were completed monthly from December

2018 until April 208 when, thereafter, assessments were completed every two weeks. Plant health scoring was completed with a 0 to 5 scoring system as follows: 0 = healthy plant, 1 = <10% necrotic foliage, 2 = <25% necrotic foliage, 3 = severe stunting and <50% necrotic foliage, 4 = <90% necrotic foliage, 5 = >90% necrotic foliage or plant death. A total of eight plant health assessments were completed by the conclusion of the trial on 25 June 2018.

Plant health assessments were used to derive AUDPC values for each plant replicate using a modified formula outlined in Chapter 2. The same formula was used except y_i was represented with the 0 to 5 scoring value.

4.2.7 Plant sampling and DNA extractions

Four plants from each treatment were randomly sampled for processing and subsequent DNA extractions. The plants were carefully removed from the potting containers and the roots and foliage were removed. Once reduced to a complete crown they were transferred to individual plastic bags (12 cm x 15 cm, Agdia Inc., Elkhart, IN) and lyophilized for 24 hrs at -54°C, 0.018 mBar. The lyophilized crowns were hand ground with a hand roller tissue homogenizer (Agdia Inc., Elkhart, IN) for 1 min per sample.

Total DNA extractions from strawberry crowns were completed according to the Materials and Methods section in Chapter 3 of this thesis. DNA extractions were conducted with a commercial extraction kit following a modified version of the manufacturer's instructions with a final elution volume of 100 µL (DNeasy® Plant Mini Kit, Qiagen Inc., Valencia, CA).

4.2.8 Quantitative polymerase chain reaction (qPCR) for the detection of M. phaseolina

qPCR testing for the extent of pathogen colonization in crown tissue was completed as outlined in chapter 3. The procedure was modified to only process crowns of the strawberry plants.

4.2.9 Statistical analysis

A single factor analysis of variance with ordinary least squares method was conducted on the percent reduction of *M. phaseolina* CFU g^{-1} potting substrate. A multi factor analysis of variance with ordinary least squares method was conducted on AUDPC, and qPCR quantification of the concentration of *M. phaseolina* from crown tissue (pg *M. phaseolina* DNA g^{-1} dry strawberry tissue). Prior to analysis the AUDPC data was square root transformed to meet the requirements of the assumptions of homogeneity of variance the test. A $\log_{10}$ transformation of the concentration of the concentration of *M. phaseolina* DNA was completed to meet the assumption of the ANOVA test concerning the homogeneity of variance and normality of error. All pairwise comparisons of mean separation were completed with a Tukey's HSD test, except for comparisons of weed seedling fresh biomass and AUDPC which utilized a Fisher's LSD test, both with $\alpha = 0.05$. All analyses were completed using JMP® pro statistical software (version 13.1 SAS Institute, Cary, NC).

4.3 Results

4.3.1 Soil assay

4.3.1.1 Steam treatment and anaerobic phase

The steam treatment of the potting substrate achieved temperatures ranging from 207ºF to 209ºF (97.2ºC to 98.3ºC) for 6 hrs (Figure 4.1). After the steam apparatus was

turned off the soils cooled to 188.5ºF for the seventh hour of steam treatment and after 27 hrs after steaming the potting substrate had cooled to ambient temperatures (Figure 4.1).

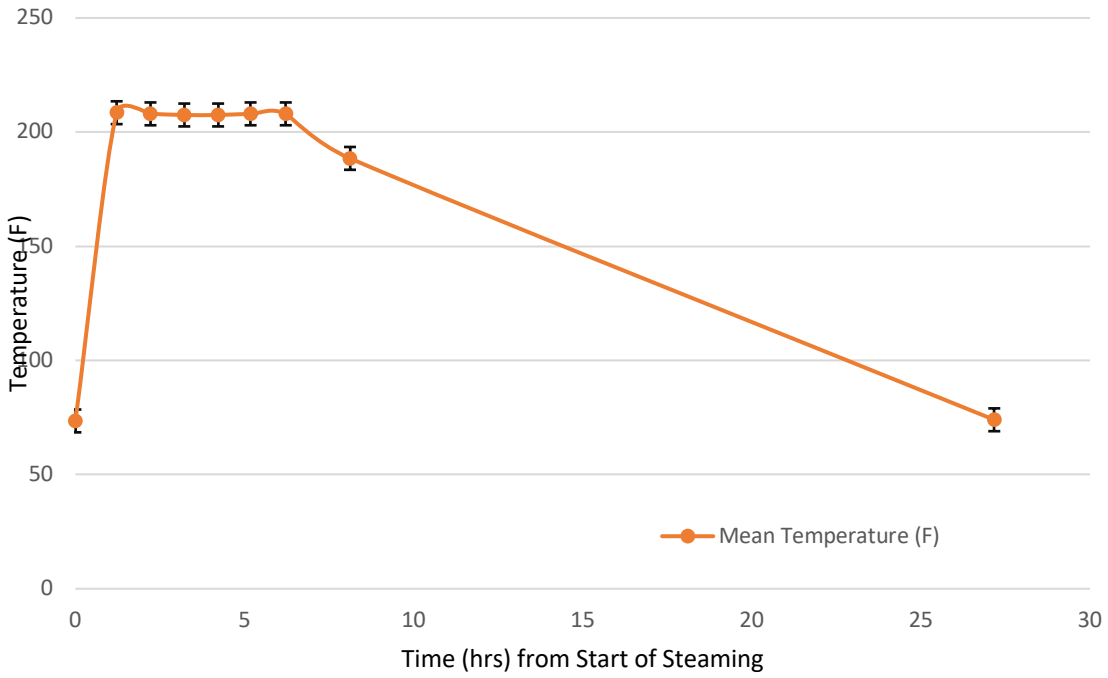

Figure 4.1. The average temperature of the potting substrate when undergoing the steam treatment. Temperatures were measured with a thermometer located in the middle of the potting substrate. A total of three thermometers were used in which values presented are the mean, and the error bars represent one standard error of the mean.

During the anaerobic phase of the soil treatments only the ASD soil treatment achieved reduced anaerobic soil conditions. The ASD rice bran treated soil experienced 18 days under moderate to strong anaerobic soil conditions with ORP values of $E_h < 200$ mV (Figure 4.2) (Shennan et al. 2017). In contrast, the UTC, MSM and steam treatments did not undergo any time under this threshold maintaining aerobic soil conditions (Figure 4.2). During the anaerobic phase air temperatures in the greenhouse ranged from 17.5°C to 37.8°C and soil temperatures ranged from 18.1°C to 31.4°C. The average air temperature in the greenhouse for the 22 days during the anaerobic phase was 23° C and soil was 23.2° C (Figure 4.3).

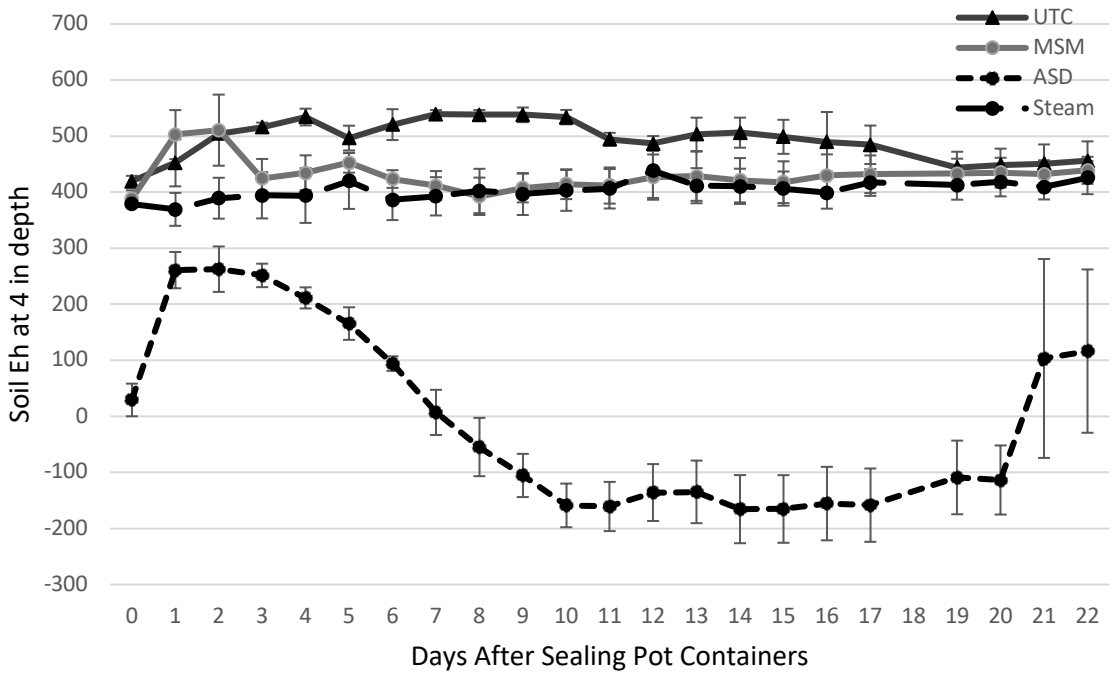

Figure 4.2. Soil E_h of the anaerobic phase of soil treatments completed on *Macrophomina phaseolina* infested potting substrate, measured by oxidative reduction potential (ORP) sensors. Threshold for anaerobic conditions of soil is $E_h < 200$ mV. Mean values are derived from four ORP probes per soil treatment. Error bars represent one standard error from the mean.

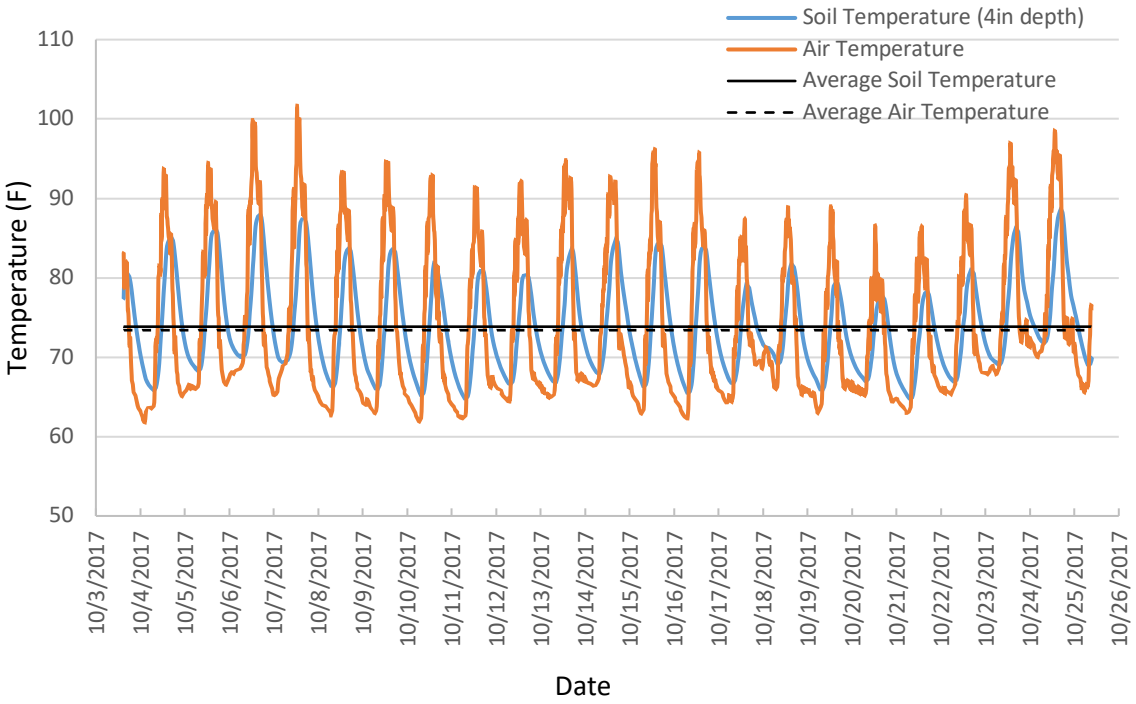

Figure 4.3. Air and soil temperatures at 10 cm depth of greenhouse and potting substrate containers respectively throughout the anaerobic phase of soil treatments. Values plotted are the mean of three air temperatures and three soil temperatures. Temperatures were recorded every 15 mins by HOBO Onset data loggers.

4.3.1.2 M. phaseolina inoculum density

There was a statistically significant effect of soil treatment ($F_{2,11} = 42.2$, $P < 0.0001$) on the percent reduction of *M. phaseolina* colony forming units (CFU). In the UTC there was a 65.1% reduction of *M. phaseolina* CFU, the MSM treatment had an average percent reduction of 70%, which was not statistically different from UTC, whereas the ASD treatment had a 99.7% reduction and was significantly different from the UTC ($P <$ 0.0001) (Figure 4.4). The steam soil treatment had a 100% reduction of *M. phaseolina* CFU g^{-1} potting substrate, as there was no *M. phaseolina* detected in the potting substrate of the steam soil treatment after the anaerobic phase thus had no variance around the mean and was excluded from statistical analysis.

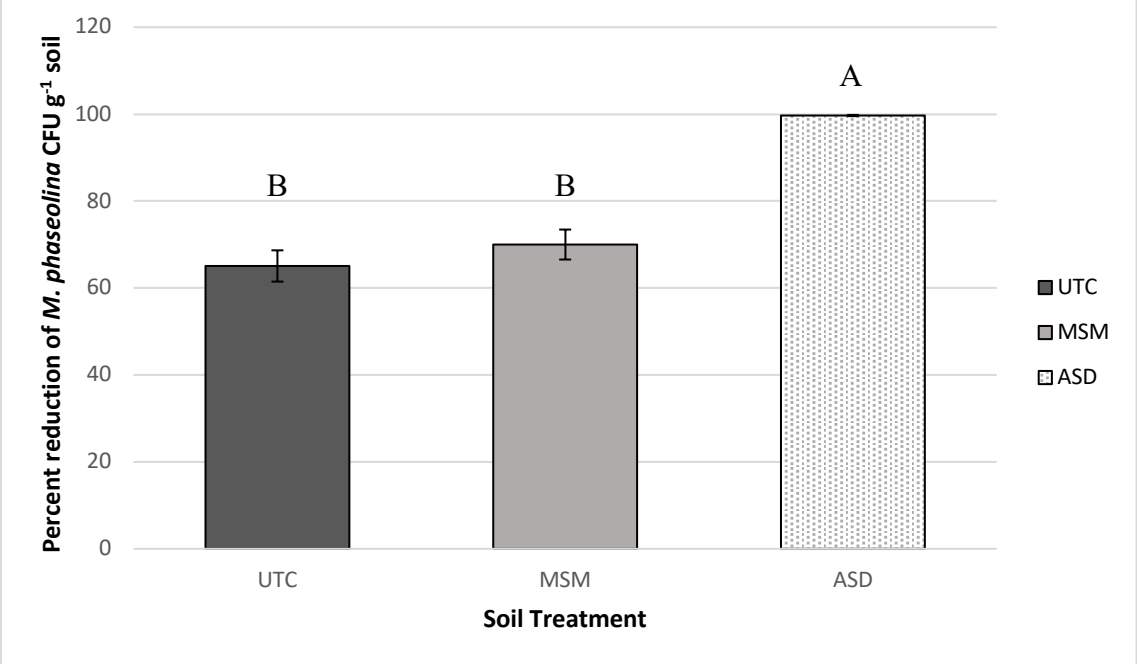

Figure 4.4. The percent reduction of *Macrophomina phaseolina* CFU g^{-1} soil potting mixture in response to soil treatments. Potting substrate was infested with the pathogen at a rate of 100 CFU g^{-1} soil potting mixture prior to treatment. UTC = inoculated but non-amended control, MSM = *B. juncea* mustard seed meal applied at 4.94 tons ha^{-1}, ASD = rice bran at 22.24 tons ha^{-1}. Error bars represent one standard error of the mean. Pairwise comparisons were completed with a Tukey's HSD ($\alpha = 0.05$). Steam was not included in the analysis because of 100% reduction of viable CFU.

4.3.1.3 Weed seedling

There was a significant effect of soil treatment on weed seedling fresh biomass ($F_{3,15} = 7.9$, $P = 0.0036$) recovered from the potting substrate after the anaerobic phase. The UTC soil treatment had an average weed seedling fresh biomass of 4.87 g, which was statistically similar ($P > 0.05$) to the fresh biomass of weed seedlings recovered from the MSM soil treatment of 2.6 g (Figure 4.5). A weed seedling fresh biomass of 0.01 g was recovered from the ASD soil treatment and there were no weed seedlings recovered from the steam soil treatment (Figure 4.5). Both the ASD and the steam soil treatments were significantly ($P < 0.05$) lower than the UTC and the MSM soil treatments (Figure 4.5).

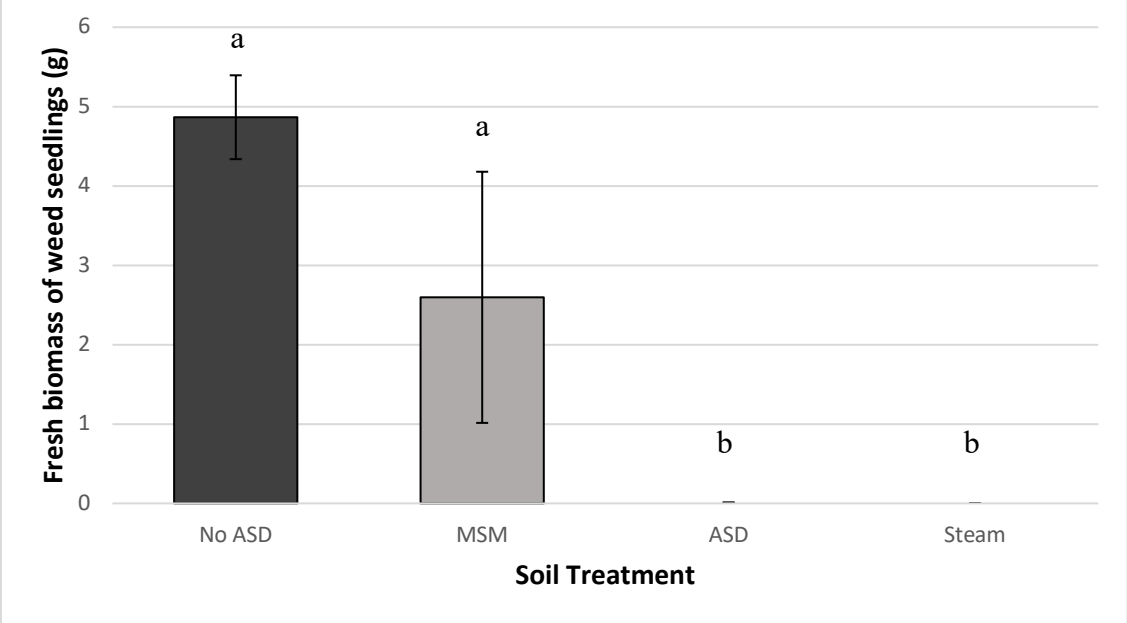

Figure 4.5. Weed seedling fresh biomass recovered from potting substrate after the anaerobic phase of soil treatments. UTC = inoculated but non-amended control, MSM = *B. juncea* mustard seed meal applied at 4.94 tons ha^{-1}, ASD = rice bran at 22.24 tons ha^{-1}. The values are average fresh weight from four replicate containers per soil treatment. Error bars represent one standard error of the mean. Pairwise comparisons were completed with a Fishers LSD ($\alpha = 0.05$)

4.3.2 Plant assay

4.3.2.1 Area under disease progress curve

Strawberry cultivar had a significant effect ($F_{5,234} = 11.9$, $P < 0.0001$) on the AUDPC values of plants grown in potting substrate infested with *M. phaseolina*. The factor of soil treatment was not statistically significant, nor was the strawberry cultivar x soil treatment interaction of the two factors (Table 4.1). The cultivar Festival had the lowest AUDPC value of 154.2, which was not statistically different from Del Rey, Monterey, or Albion having AUDPC values of 155.9, 156.6 and 183.4 respectively (Figure 4.6). Cultivar Petaluma had the highest AUDPC value of 284.2, which was statistically Significantly different from all cultivars tested ($P < 0.05$), followed by Fronteras with an AUDPC value of 209.8 which was significantly higher ($P < 0.05$) then Festival, Del Rey, and Monterey (Figure 4.6).

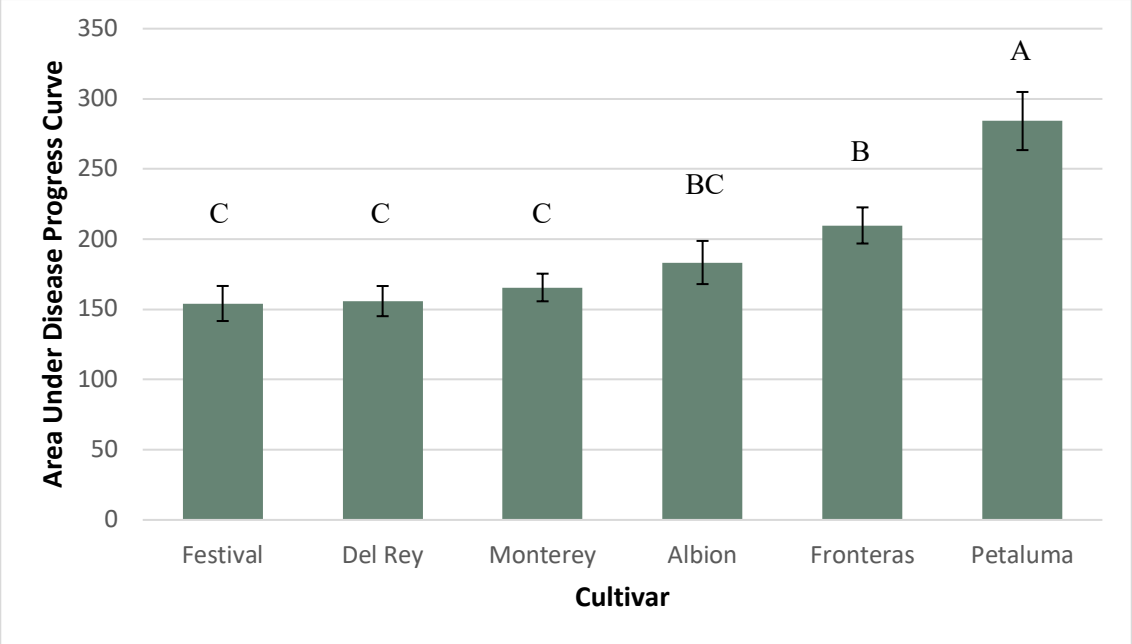

Figure 4.6. Disease severity expressed as the area under disease progress curve (AUDPC) of six strawberry cultivars. AUDPC is calculated from a plant disease severity rating with 0 = healthy plant, 1 = <10% necrotic foliage, 2 = <25% necrotic foliage, 3 = severe stunting and <50% necrotic foliage, 4 = <90% necrotic foliage, 5 = >90% necrotic foliage or plant death. Eight assessments were conducted. Error bars represent one standard error of the mean. Pairwise comparisons were completed with a Tukey's HSD ($\alpha = 0.05$).

Table 4.1. Analysis of variance of soil and plant assays conducted in greenhouse trials using *Macrophomina phaseolina* infested potting substrate and strawberry plants.

Assay	Effect	df	F value	Pr > F
Andersen Sampler	Soil Treatment	2	42.2	<0.0001
Disease Severity[y]	Cultivar	5	11.9	<0.0001
	Soil Treatment	3	1.3	0.2858
	Cultivar*Soil Treatment	15	1.2	0.2735
qPCR detection of *M. phaseolina* in Crown[z]	Cultivar	5	4.2	0.0051
	Soil Treatment	2	0.01	0.9857

[y]Disease severity AUDPC values were square root transformed prior to the analysis
[z]The concentrations of *M. phaseolina* DNA detected in crowns was $\log_{10}$ transformed prior to analysis

4.3.3 Host colonization

Strawberry cultivar had a significant effect ($F_{5,37} = 4.22$, $P = 0.0051$) on the concentration of *M. phaseolina* DNA recovered from strawberry crown tissue samples (Table 4.1). Soil treatment had no significant effect on the concentration of *M. phaseolina* DNA recovered from the crowns (Table 4.1). Of the 96 plants tested with the qPCR assay only 38 crown samples had positive detection of the *M. phaseolina* target DNA despite having amplification of the internal control. The remaining samples were determined to have no detection of *M. phaseolina* in the strawberry crowns and greatly limited the degrees of freedom error in the analysis. Because of the limited degrees of freedom of error, the cultivar x soil treatment interaction analysis could not be completed.

The cultivars Del Rey, Petaluma and Fronteras had the significantly ($P < 0.05$) lowest concentration of *M. phaseolina* DNA in their crowns with 7.1 pg g^{-1}, 160.6 pg g^{-1}, and 201.8 pg g^{-1} respectively (Figure 4.7). Cultivars Monterey and Albion followed which were statistically similar to Del Rey, Petaluma, and Fronteras but numerically higher with concentrations of 339.2 pg g^{-1} and 1889.2 pg g^{-1} respectively (Figure 4.7). The cultivar Festival had significantly ($P < 0.05$) higher concentrations of *M. phaseolina*

DNA in their crowns compared to the other five cultivars containing 3423.6 pg g^{-1} (Figure 4.7).

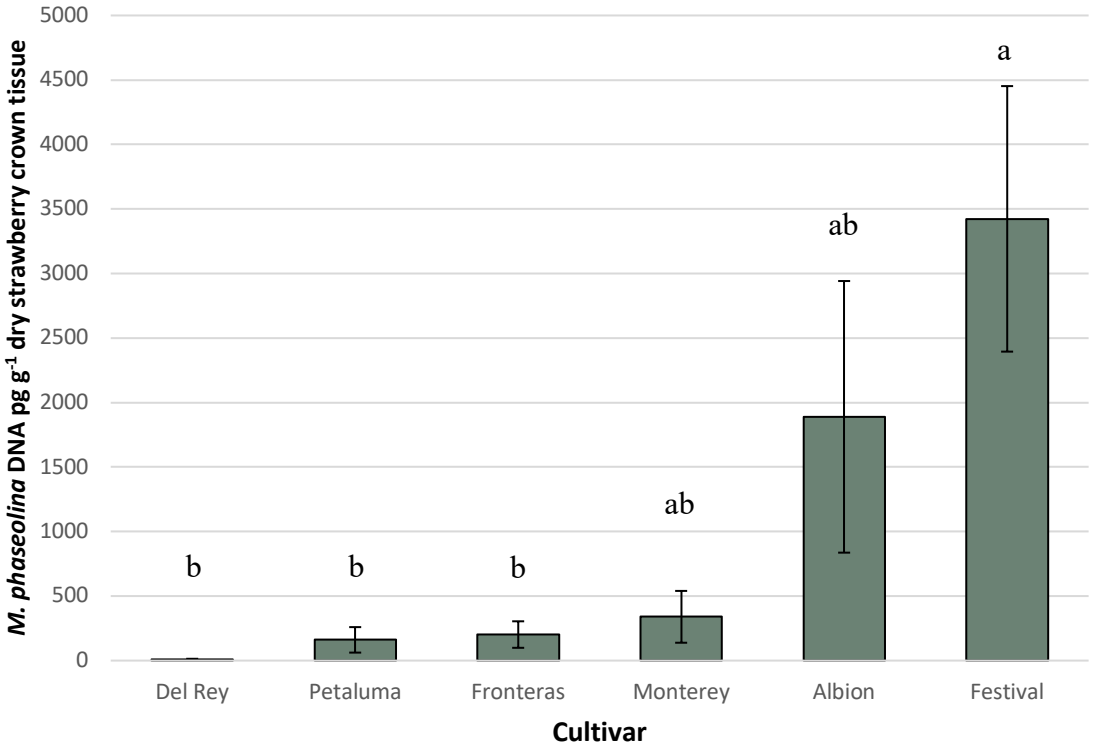

Figure 4.7. Comparison of the mean quantity of *Macrophomina phaseolina* DNA detected in strawberry crowns of different cultivars grown in inoculated potting substrate. Mean values are derived from twelve plant samples per cultivar. Error bars represent one standard error of the mean. Pairwise comparisons were completed with a Tukey's HSD ($\alpha = 0.05$).

Despite a lack of statistical evidence of differences in the cultivar x soil treatment interaction, numerical trends exist for the more colonized cultivars of Albion and Festival. The cultivars Monterey, Albion and Festival had numerically lower concentrations of *M. phaseolina* DNA in their crowns with the addition of organic amendments albeit not statistically significant (Figure 4.8). These same cultivars had the lowest concentrations of *M. phaseolina* DNA in the ASD soil treatments (Figure 4.8). There was no detection of *M. phaseolina* in strawberry crowns grown in steam treated soils for any of the cultivars signifying these plants were not colonized at all by the pathogen.

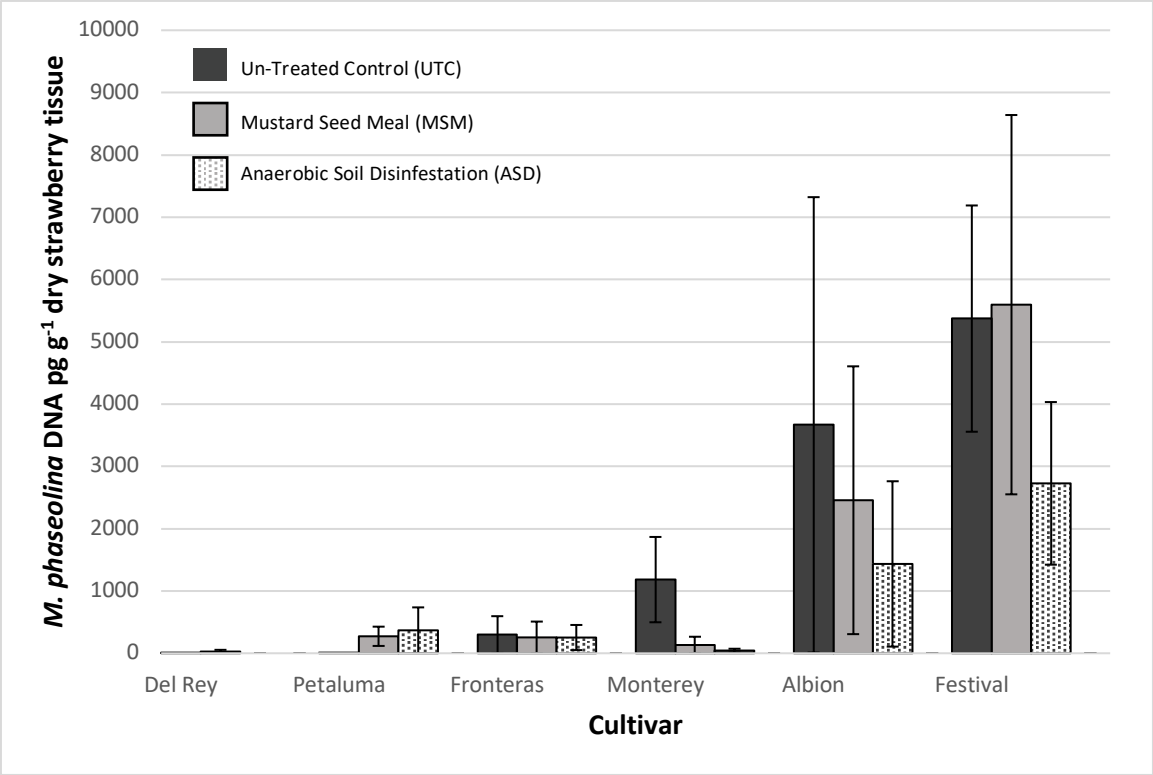

Figure 4.8. The mean quantity of *Macrophomina phaseolina* DNA detected in strawberry crowns of different cultivars by soil treatment. Strawberry plants were grown in inoculated potting substrate. Error bars represent one standard error of the mean. Pairwise comparisons were not completed due to lack of statistically significant differences. Steam treatment is not included due to lack of detection of *M. phaseolina* DNA.

4.4 Discussion

The results of the parameters measured during the anaerobic phase and soil assay were in accordance with literature in regard to induced anaerobic conditions generated from the soil incorporation of rice bran followed by wetting and sealing the soil with plastic (Rosskopf et al. 2015; Muramoto et al. 2016; Shennan et al. 2017). The average soil temperatures achieved in the potting substrate in this study were higher than temperature thresholds required during ASD to consistently eliminate *Verticillium dahliae* established by Shennan et al. 2017. Subsequently, the soil plating assay indicated that the ASD and steam treatments were able to reduce the CFU g^{-1} potting substrate of *M. phaseolina* by 99.7 and 100% respectively. However, disease severity and

host colonization of multiple strawberry cultivars was not reduced when grown in the potting substrate treated with organic amendments. All considered soil E_h measurements of anaerobicity likely do not properly assess the complex interactions of mechanisms contributing to disease suppression in a predictable manor (Strauss et al. 2017).

Organic amendment control of plant symptoms due to *M. phaseolina* was incomplete, which is consistent with literature for the use of MSM (Mazzola et al. 2017) but conflicted with literature that ASD was able to suppress Macrophomina crown rot (Muramoto et al. 2016; Shennan et al. 2017). The disease severity of the strawberry cultivars grown in steam treated potting substrate was not reduced when compared to non-inoculated controls, but plants grown in steam treated potting substrate were not colonized according to molecular diagnostics. This suggests that the necrotic foliage of older leaves observed throughout the plant health assessments in this trial could have not been due to Macrophomina crown rot. Necrosis of older foliage and plant stunting of strawberry can also be attributed to abiotic stresses of inadequate soil moisture (Yuan et al. 2004) or high soil salinity (Barroso and Alvarez 1997) which could be confused with disease symptoms caused by *M. phaseolina*. Additional soil parameter measurements of potting substrate moisture content and salinity content would benefit subsequent studies by reducing the potential of confounding effects of abiotic stresses with those caused by Macrophomina crown rot.

The incomplete suppression of disease symptoms on strawberry plants and *M. phaseolina* colonization of crowns by the pathogen from the organic amendment soil treatments in this study could occur for multiple reasons. The production of organic acids (Momma et al. 2006) and volatile fatty acids (Hewavatharana et al. 2014) from microbial

metabolism are important components of disease suppression resulting from the soil incorporation of carbon sources. It is possible that despite the generation of strong anaerobic conditions the production of these acids needed for complete disease suppression were not realized. Similarly, the production of AITC from *B. juncea* MSM was fungistatic in *in vitro* assays with *M. phaseolina* but after dispersal of AITC the fungus continued to grow (Mazzola et al. 2017). The glucosinolate content of the *B. juncea* MSM used in this study could have been at suboptimal levels but the lack of suppression of Macrophomina crown rot observed in this study is in concordance with literature (Mazzola et al. 2017). Additional studies utilizing increased rates of MSM soil treatment has potential to increase its efficacy, but increased rates may render the soil treatment as economically unviable to growers. The data presented suggests that MSM might not be efficacious in managing Macrophomina crown rot, but additional studies are warranted.

Similar to the previous study in the Results section of Chapter 3 this study exhibits agreement that the strawberry cultivar or genotype grown has the most significant effect over the extent of colonization of the plants by *M. phaseolina*. The cultivars Grenada, and Fronteras in both field and greenhouse trials exhibit phenotypic host resistance and reduced colonization by *M. phaseolina*. Alternatively, across multiple experiments Festival and Albion exhibit susceptibility and extensive colonization by *M. phaseolina*. These cultivars would be good candidates for additional comparative studies in the future.

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