Sources of Inoculum and Survival of *Macrophomina phaseolina* in Florida Strawberry Fields

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Abstract

Macrophomina phaseolina, the causal agent of charcoal rot, affects strawberry crowns, inducing plant collapse. The fungus survives in the soil through the production of microsclerotia and is usually controlled by preplant fumigation of soil. However, in the 2016 to 2017 Florida strawberry season, even after soil fumigation, about 30% plant mortality still occurred in plastic-covered beds that were used for a second season and where crop residue (mainly old strawberry crowns) was disposed of between beds. Therefore, this study was conducted to determine if *M. phaseolina* can survive on strawberry debris over summer in Florida and if so, verify whether strawberry debris might act as a source of inoculum for new transplants. Crowns from the previous season were collected from commercial farms where charcoal rot had been reported, and *M. phaseolina* was recovered from all samples. In a research field, infected crowns were buried in the soil at different depths and retrieved

every 2 weeks during the summer. After 8 weeks, *M. phaseolina* could be recovered at all depths. Moreover, inoculation of strawberry plants by drenching the soil, dipping roots, or spraying leaves with a *M. phaseolina* microsclerotial suspension from pure cultures or infected crowns produced symptoms with differences in incubation periods depending on cultivar susceptibility. Furthermore, infected crowns disposed of in the aisles between beds or buried next to new transplants of cultivars Strawberry Festival, Florida Beauty, and Winterstar induced charcoal rot, with the level of aggressiveness depending on the cultivar susceptibility and inoculum placement in the field.

Keywords: disease development and spread, disease management, epidemiology, fruit, fungi, pathogen survival, small fruits

Florida is the largest winter producer of strawberry (*Fragaria x ananassa* Duch) in the world. In contrast to other regions where the crop is perennial, strawberry in Florida is produced in an annual system. Transplants are obtained from nurseries in the northern United States or Canada and transplanted about late September and mid-October on plastic mulch raised beds (Brown 2003; Whitaker et al. 2017a). Fruit production extends from November to March, and early harvests (November to January) are the most profitable because of the limited market competition. At the end of the season, plants are usually destroyed by mowing off the leaves, herbicide sprays, and/or fumigant applications (Noling 2015; Strand 1994).

From April to June, some growers reuse the plastic-mulched beds to cultivate secondary crops, such as cucurbits, eggplant, or pepper, whereas others remove the plastic, till the soil, and plant a cover crop (e.g., *Crotalaria juncea*) to add organic matter to the soil (Yu et al. 2018). It has become popular for some strawberry growers in Florida to reuse the plastic-mulched bed for a second strawberry crop. In this case, the plants from the previous season are maintained on the beds until new transplants are ready to be planted in the following season. Remaining debris from the previous strawberry crop is maintained alongside of new strawberry transplants on the beds or disposed of in the aisles between beds (Nyoike and Liburd 2014).

Charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goidanich, can cause severe losses of early season plantings and can account for >80% plant mortality by the end of the season when

conditions are favorable and proper disease management is not adopted (Peres et al. 2018). The disease has become a new challenge for the strawberry industry since methyl bromide was phased out (Mertely et al. 2005). For instance, during the 2017–2018 strawberry season, *M. phaseolina* was recovered from symptomatic plants on about 40 farms in central Florida and accounted for 21% of the strawberry samples received by the Gulf Coast Research and Education Center (GCREC) Diagnostic Clinic compared with <10% in the previous season (N. A. Peres, unpublished data).

M. phaseolina is pathogenic to >500 species of plants, and it is distributed worldwide (Mihail 1992). In strawberry, the fungus usually colonizes vascular tissues, producing reddish brown necrotic areas along the margins and the vascular ring in the crowns. Symptoms are initially characterized by water stress, wilting, and death of older leaves with eventual plant collapse (Avilés et al. 2008; Mertely et al. 2005), which are symptoms similar to those caused by other crown rot pathogens, such as *Colletotrichum* and *Phytophthora* species. *M. phaseolina* produces microsclerotia, which are resistant long-lived structures that are usually formed during the parasitic phase in infected strawberry tissues. Microsclerotia are released into the soil on decomposition of the host, and eventually, they become the primary and most important source of inoculum to new strawberry transplants (Dhingra and Sinclair 1975; Zveibil et al. 2012). High air and soil temperatures (28 to 35°C), sandy soil, and continuous growth of susceptible hosts for several seasons are common conditions during strawberry production in Florida, and they are conducive to pathogen survival and disease development (Zveibil et al. 2012). Moreover, charcoal rot occurs soon after planting, and disease incidence usually remains stable throughout the winter until temperatures start rising in the spring.

Management of charcoal rot is challenging, especially because of heat tolerance and production of resilient survival structures by *M. phaseolina*. In Florida strawberry production, preplant soil fumigation in the fall is the main method to reduce levels of *M. phaseolina* sclerotia and manage charcoal rot (Baggio et al. 2017; Chamorro et al. 2016). Since the broad-spectrum soil fumigant methyl bromide was phased out, growers have transitioned to alternative products that have not been as effective, especially because of their higher boiling points and lower vapor pressures that can limit their movement and

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diffusion throughout the soil profile (Baggio et al. 2018; Porter et al. 2004). Even on a farm where fumigants were shown to have controlled *M. phaseolina* populations in the soil, plant mortality was observed, and the fungus was still recovered from most of the dying plants (N. A. Peres, unpublished data). On this same farm, plastic-mulched beds were reused for a second strawberry season, and strawberry residue (mainly strawberry crowns) from the previous season was disposed of between beds.

In contrast to *Colletotrichum acutatum* and *Phytophthora cactorum*, which are introduced into strawberry production fields via quiescently infected transplants and do not survive in the soil or on strawberry debris in Florida summer conditions (Howard et al. 1992; Pettitt and Pegg 1994), *M. phaseolina* sclerotia survive in the soil for months and are favored by high temperatures (Zveibil et al. 2012). Although plant debris may remain in the soil until the following season (Freeman and Gnyem 2005), information about the fungal survival on strawberry debris, primarily strawberry crowns, is not known, especially during the summer between strawberry seasons in central Florida. Furthermore, these infected crowns could still potentially disseminate *M. phaseolina* and infect newly transplanted strawberries.

Based on the reduction of *M. phaseolina* population in fumigated soils and the continued recovery of the fungus from symptomatic strawberry plants collected in those fields, the objectives of this study were to (i) evaluate survival of *M. phaseolina* sclerotia on strawberry crowns over summer in Florida, (ii) determine potential sources of *M. phaseolina* inoculum for strawberry, and (iii) determine whether strawberry debris can act as source of inoculum to infect new transplants.

Materials and Methods

Survival of *M. phaseolina* in strawberry debris. To determine the viability of *M. phaseolina* inoculum on strawberry crowns over summer in Florida, surveys of commercial farms and evaluations at the research facility were conducted.

Commercial strawberry field survey. During the 2016–2017 strawberry season, specifically in December 2016, strawberry crowns from the 2015–2016 season that were disposed between reused plastic-mulched beds were sampled in two commercial fields (Floral City and Dover, Florida) that had reported charcoal rot for 3 to 5 years. Strawberry crowns were also recovered from the surface of tilled soil on the same farm in Floral City at the end of the 2016–2017 season (March 2017) and at the beginning of the 2017–2018 season (September 2017). In the 2017–2018 season (December 2017) at the strawberry farm in Dover, strawberry crowns from the previous season were collected from old debris kept on the beds adjacent to the newly planted strawberry. In the beginning of the 2017–2018 season (September 2017), strawberry crowns were sampled from the surface of tilled soil on a commercial farm in Plant City. All crown samples were brought to the laboratory and processed using a coffee grinder. One gram was disinfectant in 0.5% NaOCl (bleach) for 3 min in a 50-ml tube and shaken periodically. The samples were poured through a 325-mesh sieve to retain *M. phaseolina* microsclerotia and washed with sterile distilled water to remove residual bleach. The sample retained by the sieve was collected in the tube and plated diluted to 10 and 1% on RB semiselective medium (39 g of potato dextrose agar [PDA] medium [Oxoid], 1 ml of tergitol, 0.1 g of rifampicin, 1 g of sodium carbonate, and 0.494 ml of Ridomil Gold [45.3% mefenoxam] in 1 liter of distilled water). Plates were incubated at 30°C for 7 days in the dark, and *M. phaseolina* populations were calculated as CFU per gram of crown (Chamorro et al. 2016).

Field trials at the research facility. Three experiments were conducted in strawberry fields at the GCREC in Wimauma, Florida during the spring/summer of 2017 and 2018. The survival of *M. phaseolina* inoculum in strawberry debris over time was evaluated by arbitrarily placing artificially inoculated strawberry crowns on the surface or burying at 7.6- or 20.3-cm depth in the soil of strawberry fields that had been tilled at the end of the strawberry season. Crowns were tied with different color strings representing each burial

depth. In the first and second experiments, crowns were buried on 12 and 13 June 2017, respectively, and in the third experiment, they were buried on 6 June 2018. The soil type was a Myakka fine sand 160 (Siliceous Hyperthermic Oxyaquic Alorthod) with a pH range of 6.1 to 6.8 and an organic matter content of 0.9 to 1.5%. The strawberry crowns came from plants previously inoculated with an *M. phaseolina* sclerotia suspension and were stored in paper bags at room temperature for up to 1 month in the laboratory until the start of the experiments. After burying in the soil, crowns were retrieved every 2 weeks for 2 months until the field was prepared for the following season. Each depth-time of recovery combination was composed of four crowns (replications). Crowns were processed, and populations of *M. phaseolina* were calculated as described above.

Sources of inoculum and methods of inoculation of *M. phaseolina* on strawberry. To evaluate whether old strawberry crowns can serve as a source of inoculum to infect new plants and whether infections can occur from above ground or only from below ground, two experiments were conducted in a greenhouse located at the GCREC during the spring of 2017. The effects of four sources of inoculum of *M. phaseolina* (two isolates and strawberry debris from two locations) and three methods of inoculation (drenching the soil, dipping roots, and spraying leaves) on disease incubation period were evaluated on strawberry cultivars with different levels of susceptibility to charcoal rot (cultivars Strawberry Festival, Florida Beauty, and Winterstar). Two pathogenic *M. phaseolina* isolates, collected in 1995 and 2016, were revived from our culture collection, transferred to PDA plates, and kept at 30°C for 10 to 14 days in the dark to produce microsclerotia. Inoculum was prepared by adding two plates of fungal culture to 200 ml of sterile deionized water in a blender, and suspension was adjusted to 6.10^3 sclerotia per milliliter. The other two sources of inoculum consisted of strawberry crowns infected with *M. phaseolina* recovered from the same two commercial farms surveyed during the 2016–2017 season. Crowns were processed separately using a coffee grinder, and 1 g was added to 300 ml of sterile DI water. Three inoculation methods were used: dip, spray, or drench. In the first method, strawberry plants with trimmed roots were dipped into the inoculum suspension (prepared in 0.15% water-agar medium) for 2 min before transplanting into 1-liter plastic pots filled with potting soil (Metro-Mix 820; Sun Gro Horticulture). For the spray and drench methods, plants were transplanted first, and the inoculum suspension (prepared in water) was sprayed over the leaves or added (50 ml) to the potting soil. Noninoculated plants were transplanted into the potting soil and used as the control. Treatments were arranged in a complete randomized factorial design, with five replications of each inoculum-inoculation method combination. Plants were irrigated with tap water through misting nozzles for 10 to 12 days (6 s every 10 min for 8 h/day); then, they were irrigated twice per day through a drip system and fertilized weekly (Miracle-Gro 24:8:16; Scotts). Strawberries were monitored for development of typical charcoal rot symptoms for 12 weeks, and disease incubation period was recorded for each plant. Reisolations from symptomatic plants were performed to confirm the causal agent.

Strawberry debris as a source of *M. phaseolina* inoculum under field conditions. To test the ability of infected crop residues, particularly strawberry crowns, to serve as a source of inoculum for newly transplanted strawberry on second-year, plastic-mulched beds, strawberry crowns infected with *M. phaseolina* were placed between beds in the aisles (“aisles” treatment) or buried next to new transplants (“buried” treatment). As a positive control, trimmed roots of strawberry transplants were dipped into a sclerotial suspension of three pathogenic *M. phaseolina* isolates from our culture collection (“positive control” treatment). Noninoculated plants were used as negative controls (“negative control” treatment). Cut-top bare root transplants of three strawberry cultivars (Strawberry Festival, Florida Beauty, and Winterstar) were used. Treatments were arranged in a split-plot design that considered cultivar as the whole plot and source of inoculum as the split plot. Beds were 91.4 cm long, 71 cm wide, and 15 and 18 cm high at the edges and the center, respectively. The distance between the centers of the beds was 1.2 m, and transplants

were placed 30 cm apart in two rows. Each replication consisted of 10 plants, and each treatment was replicated three times. Plants were overhead irrigated for 10 days, and then, they were irrigated and fertilized daily through drip irrigation. Plants were evaluated weekly for 12 weeks for development of typical symptoms and disease incidence. Reisolations from symptomatic plants were performed to confirm the causal agent.

Three experiments were conducted in areas with different *M. phaseolina* inoculum density levels within the GCREC strawberry fields during the spring of 2017 and 2018. Before the installation of each experiment, soil was sampled to monitor *M. phaseolina* population in the soil. Three soil samples with three subsamples each were collected from each replication (strawberry bed) from the bed surface to a 15-cm depth using a vertical calibrated drill. Soil samples were processed as previously described, with the exception that 5 g of soil per sample was used. Samples were plated nondiluted and diluted to 10 and 1% on RB semiselective medium, and *M. phaseolina* populations were calculated as CFU per gram of soil. In each of the three experiments, *M. phaseolina* was detected; means were 0.0 CFU/g for Area 1 (2017), 0.2 CFU/g for Area 2 (2018), and 4.8 CFU/g soil for Area 3 (2017), which were considered as areas with low, moderate, and high levels of inoculum, respectively.

Data analysis. *Survival of M. phaseolina on strawberry debris.* Trials were analyzed separately to obtain additional information on the effects of time and depth on *M. phaseolina* population under different scenarios of location and temperature variation. The response variable (CFU per gram crown) of each experiment was fitted into a Poisson distribution and analyzed in a factorial fashion with two factors (depth and weeks) to investigate the significance ($P \leq 0.05$) of the factors and the interaction between them. The interaction between the two fixed factors was not significant at $\alpha = 0.05$. Thus, the significance of depth was analyzed separately for each time (week of inoculum recovery), and the P values of the time significance within depths were presented by using the slice function of the lsmeans statement of the PROC GLIMMIX function within SAS statistical software version 9.4 (SAS Institute).

Sources of inoculum and methods of inoculation of M. phaseolina on strawberry. Trials were conducted and analyzed as a complete randomized factorial design with two fixed factors (inoculum and method of inoculation). Five plants per inoculum-method combination were used for disease incubation period data. This period corresponded to the number of days between inoculation and plant wilting. Asymptomatic plants received an incubation period value longer than the longevity of the trial. Data were fitted in a Poisson distribution, and an analysis of variance was conducted to determine the significance ($P \leq 0.05$) of the fixed factors and their interaction (Table 1). When models were significant, Fisher's least significant difference ($\alpha = 0.05$) test was used for mean separation of the factor inoculum within each inoculation method. If the interaction between inoculum and method was significant, the slice function was used to determine the differences between inoculation methods within each inoculum. Analyses were performed using the procedure PROC GLIMMIX on the statistical software SAS version 9.4 (SAS Institute).

Strawberry debris as source of M. phaseolina inoculum under field conditions. The three experiments were analyzed separately to quantify the effects of *M. phaseolina* inoculum sources on plant mortality in areas with different levels of inoculum density. Because of the intrinsic characteristics of the pathosystem of this study, a monomolecular model was used to describe the disease progress of each treatment (source of inoculum) for three cultivars with different levels of susceptibility to charcoal rot. A linearized version of the monomolecular model was used to estimate the rate and intercept parameters of the treatments applied to each cultivar (Campbell and Madden 1990).

We used the PROC MIXED procedure of the statistical software SAS version 9.4 (SAS Institute) to conduct a repeated measures analysis, with $\ln\{1/(1 - y)\}$ as our response variable. Time (evaluation day), inoculum source, and their interaction were analyzed as fixed factors, whereas repetition was considered to be random. Within

the repeated function of PROC MIXED, our subject was the interaction between repetition and inoculum source, and we used the compound symmetry type of covariance structure. The estimate function was used to estimate the rate and intercept of disease progress and conduct a t test to determine the significance of the contrasts between these parameters of each treatment applied to each cultivar.

Results

Survival of *M. phaseolina* in strawberry debris. *Commercial strawberry field survey.* *M. phaseolina* was recovered from all of the crown samples collected from commercial farms during the 2016–2017 and the 2017–2018 seasons at 35 to 7,753 CFU/g crown. During the 2016–2017 strawberry season, 834 to 5,880 and 580 to 7,753 *M. phaseolina* CFU/g crown were recovered from strawberry crowns (2015–2016 season) collected arbitrarily in the aisles of plastic-mulched beds in the commercial fields in Dover and Floral City, respectively. At the end of the 2016–2017 season (March 2017), crowns sampled at the surface of tilled soil from the same farm in Floral City contained 35 to 3,028 *M. phaseolina* CFU/g crown, whereas at the beginning of the 2017–2018 season, this number ranged from 1,418 to 2,914 CFU/g crown. The number of colonies of *M. phaseolina* recorded in crowns collected at the beginning of the 2017–2018 season from tilled soil in a commercial field in Plant City ranged from 157 to 292 CFU/g crown. For strawberry crowns (2016–2017 season) that were kept alongside of new strawberry transplants on raised beds that reused the plastic mulch in the 2017 to 2018 season, counts varied from 1,633 to 2,843 CFU/g crown.

Field trials at the research facility. In each of the three experiments, *M. phaseolina* was detected in all infected strawberry crown samples regardless of time and depth of burial (Fig. 1). In experiment 1, the mean number of colonies of *M. phaseolina* recovered from infected crowns placed on the field soil surface or buried at 7.6 or 20.3 cm varied from 787 to 1,664 CFU/g crown at 2 weeks, from 742 to 861 CFU/g crown at 4 weeks, from 279 to 680 CFU/g crown at 6 weeks, and from 808 to 1,327 CFU/g crown at 8 weeks after recovery (Fig. 1A). In experiment 2, sclerotial viability ranged from 712 to 2,385, from 600 to 949, from 490 to 2,028, and from 461 to 1,852 CFU/g crown at 2, 4, 6, and 8 weeks after recovery, respectively (Fig. 1B). In experiment 3, 383 to 4,341, 223 to 4,753, 175

Table 1. Type III test of fixed effects of the combination of two greenhouse experiments conducted during the spring of 2017 testing different *Macrophoma phaseolina* inoculation methods on strawberry cultivars with different levels of susceptibility to charcoal rot

Effect ^w	Numerator DF ^x	Denominator DF ^y	F value	Pr > F ^z
Cultivar Strawberry Festival				
Method	2	2.27	7.64	0.0982
Inoculum	3	3.554	42.35	0.003
Method × inoculum	6	85.35	3.89	0.0018
Cultivar Florida Beauty				
Method	2	2.943	1.23	0.41
Inoculum	3	3.035	26.69	0.0111
Method × inoculum	6	84.96	0.81	0.5615
Cultivar Winterstar				
Method	2	92	18.81	<0.0001
Inoculum	3	16	14.26	<0.0001
Method × inoculum	6	92	2.91	0.012

^w The effects of method (inoculation method [dip, drench, and spray]) and inoculum (isolates 09-95 and 16-369 and *M. phaseolina*-infected strawberry crowns from two commercial farms in Florida) along with their interaction were evaluated in a two-factor factorial design for three strawberry cultivars (Strawberry Festival, Florida Beauty, and Winterstar) with different susceptibility levels to charcoal rot caused by *M. phaseolina*.

^x Numerator degrees of freedom (DFs) of the type III test of fixed effects.

^y Denominator DFs computed with the Kenward–Roger approximation.

^z Probability of a greater F value; effects with $P < 0.05$ were considered significant ($\alpha = 5\%$).

to 4,554, and 202 to 935 CFU/g crown of *M. phaseolina* were viable at 2, 4, 6, and 8 weeks after recovery, respectively (Fig. 1C). Despite the high variability, the interaction between depth and time on the pathogen sclerotial viability was not significant at $\alpha = 0.05$ for any experiment (Fig. 1). The effect of depth for each time was significantly different in all of the situations, except at 4 weeks in experiment 1 (Fig. 1A). However, burying the crowns did not reduce the survival of sclerotia except in experiment 3 (Fig. 1C), where the mean number of colonies of *M. phaseolina* recovered from crowns placed on the soil surface was higher than in buried crowns. However, the effect of time within depths was not significantly different ($P > 0.05$), and no difference in *M. phaseolina* survival was observed over the 8-week period of the experiments.

Sources of inoculum and methods of inoculation of *M. phaseolina* on strawberry. Symptoms of charcoal rot were observed on

Fig. 1. Viability of *Macrophomina phaseolina* sclerotia (CFU per gram crown) recovered from infected strawberry crowns after placement on soil surface (black bars) or buried at 7.6 cm (gray bars) or 20.3 cm (white bars) and retrieved every 2 weeks. **A**, Experiment 1 (performed in 2017). **B**, Experiment 2 (performed in 2017). **C**, Experiment 3 (performed in 2018). Probability of greater *F*-value effects with $P < 0.05$ is considered significant. Bars followed by different letters within a same time of recovery (weeks) are significantly different according to Fisher's protected LSD test ($\alpha = 0.05$), and ns indicates not significantly different.

strawberry plants from Strawberry Festival, Florida Beauty, and Winterstar, and *M. phaseolina* was isolated from symptomatic crown tissues in both greenhouse experiments regardless of inoculum source and methods of inoculation, with differences in incubation period. The shorter the incubation period, the faster the disease symptoms were observed. Effects of inoculum source (isolates 09-95 and 16-369 and strawberry crowns from two commercial farms) were significant on the three cultivars evaluated ($P \leq 0.05$), whereas inoculation method (dip, drench, and spray) was significant only for Winterstar. However, the interaction between inoculum and inoculation method was significant for Strawberry Festival and Winterstar (Table 1).

For Strawberry Festival, within different sources of inoculum, disease incubation periods were different depending on the inoculation method used, and in most of the cases, the spray method had shorter incubation periods. However, regardless of the method of inoculation, incubation periods were shorter on strawberry transplants inoculated with pure *M. phaseolina* isolates than those inoculated with infected strawberry crowns (Table 2). For Florida Beauty, there were no differences among the methods of inoculation used within the same source of inoculum; however, the *M. phaseolina* isolate from 2016 (16-369) was the most aggressive when the dip method was used, with the shortest incubation period (Table 2). For Winterstar, incubation periods were the longest among the cultivars, and differences among inoculation methods were not observed for strawberry plants inoculated with infected crowns from commercial farms, whereas the spray method was usually the most effective for plants inoculated with pure cultures of *M. phaseolina* (Table 2). The isolate 16-369 was equally or more aggressive than the isolate collected in 2009 (09-95) for all inoculation methods, sources of inoculum, and cultivars (Table 2).

Strawberry debris as source of *M. phaseolina* inoculum under field conditions. Charcoal rot symptoms were observed over time in strawberry transplants regardless of the source of *M. phaseolina*

Table 2. Incubation period after inoculation of *Macrophomina phaseolina* using different inoculation methods applied to strawberry cultivars with different levels of susceptibility to charcoal rot (two greenhouse experiments were conducted during the spring of 2017)

Inoculum	Incubation period (days) ^w and inoculation method			Pr > F ^x
	Dip	Spray	Drench	
Cultivar Strawberry Festival				
FF	95.8 a	99 a	75.2 ab ^y	0.0171
EM	87.3 a	63.4 b	83.9 a	0.017
09-95	62.2 b	34.3 c	58 b	0.0052
16-369	41 c	19.6 c	37.8 c	0.0353
P =	<0.0001	0.0015	<0.0001	—
Cultivar Florida Beauty				
FF	95.9 a	92	89.1	0.8088
EM	78.5 a	70.6	89.3	0.2243
09-95	83.2 a	69.1	73.1	0.3973
16-369	45.5 b	34.4	49.2	0.3568
P =	<0.0001	0.0996	0.067	—
Cultivar Winterstar				
FF	99	87.5 a	95.7 a	0.3375
EM	92.7	78.3 a	94.7 a	0.0868
09-95	82.5	66.3 ab	89.2 a	0.0157
16-369	87.4	37.5 b	65.2 b	<0.0001
P = ^z	0.2542	0.0384	0.0007	—

^w Incubation period in days: period between inoculation and plant collapse.

^x Probability associated with test of effect slices for inoculum $\times$ method interaction sliced by inoculum.

^y Treatments followed by the same letter within a column are not significantly different according to Fisher's protected LSD test ($\alpha = 0.05$).

^z Probability associated with type III test of fixed effects for inoculum (FF and EM correspond to processed *M. phaseolina*-infected crowns collected on two commercial farms in Florida, and 09-95 and 16-369 correspond to two *M. phaseolina* isolates) within each inoculation method (dip, spray, or drench).

inoculum used, including in the noninoculated control treatment ("negative control"), and the monomolecular model adequately described the observed disease incidence progress curves (Fig. 2).

In the areas with low (Area 1) and moderate (Area 2) inoculum levels, disease progress rates of the treatment in which strawberry transplants were inoculated with sclerotial suspensions of *M. phaseolina* ("positive control") were significantly higher than the other treatments for Florida Beauty (Fig. 2A and B and Table 3) and Strawberry Festival (Fig. 2D and E and Table 3). Disease progress rates were not significantly different among the other sources of inoculum used. For Winterstar, charcoal rot progress rates were not different among the different treatments in the area with low inoculum level (Fig. 2G and Table 3). Nonetheless, when the level of inoculum was moderate (Area 2), the "positive control" treatment differed from the others, and the treatment in which strawberry crowns infected with *M. phaseolina* were buried next to new transplants ("buried") differed from the treatment in which plants were not inoculated ("negative control") (Fig. 2H and Table 3). For Florida Beauty, in the area with high inoculum levels (Area 3), disease progress rate for the treatment in which strawberry crowns were placed between beds in the aisles ("aisles") was higher than in the other treatments. However, the "buried" treatment did not differ from either the "positive control" or the "negative control," which were different from each other (Fig. 2C and Table 3). For Strawberry Festival, the "positive control" progress rate was lower than that for the "negative control." However, the intercept parameter that considers disease incidence at time $t = 0$ was

higher (3.615 against 0.862), meaning that disease symptoms were observed earlier. Progress rate of the "aisles" treatment was also lower than "buried" and "negative control" treatments; however, the intercept of "aisles" was higher (4.605 against 3.768 and 0.862, respectively) (Fig. 2F and Table 3). For Winterstar, disease progress rate of "aisles" was significantly higher than "positive control" and "buried" treatments and did not differ from the "negative control," whereas the progress rate of "buried" treatment was lower than the "negative control" treatment but did not differ from the "positive control" (Fig. 2I and Table 3).

Discussion

M. phaseolina can survive in strawberry crowns over summer under central Florida conditions through the production of resistant structures (microsclerotia). Under natural conditions in commercial fields, pathogen inoculum survived for >8 months between strawberry seasons (March to November). In experiments conducted at a research field, we showed that, over the two hottest months of the year (June to August), there was no reduction of inoculum in the debris. Previous studies have demonstrated that the viability of *M. phaseolina* microsclerotia in the soil had declined to negligible levels after 6 to 9 months under controlled temperature (25 and 30°C) or fluctuating (18 to 32°C) greenhouse conditions and declined more slowly at 30°C (Zveibil et al. 2012). Although our studies were conducted in the field under noncontrolled environmental conditions, air and soil temperatures were recorded at different locations, and it was

Fig. 2. Linearized monomolecular model describing the progress of charcoal rot incidence. Experiments were conducted in areas with **A, D, and G**, low, **B, E, and H**, moderate, and **C, F, and I**, high *Macrophomina phaseolina* inoculum densities in the soil using strawberry cultivars **A to C**, Florida Beauty, **D to F**, Strawberry Festival, and **G to I**, Winterstar. Strawberry crowns infected with *M. phaseolina* were placed between beds in the aisles (dotted lines) or buried next to new transplants (dashed lines). As positive control, strawberry transplant trimmed roots were dipped into sclerotial suspension of three pathogenic *M. phaseolina* isolates from our culture collection (solid lines). Noninoculated plants were used as negative controls (dot-dash lines).

very common to observe temperatures >30°C throughout the experiment, which might explain the lack of reduction of inoculum over the 2 months.

Zveibil et al. (2012) showed a 40% reduction in inoculum in strawberry crowns placed 30 cm deep in the soil compared with infected crowns maintained in the laboratory at 25°C, which they attributed to the elevated soil temperatures under the plastic mulch; however, no additional evaluations over time were performed, such as we did in our trials. Moreover, our results showed that, in commercial fields with the reuse of plastic for a second season (thus maintaining the plastic over summer), *M. phaseolina* inoculum was still recovered from strawberry crowns of old plants kept on the plastic between strawberry seasons. In soybean, *M. phaseolina* can survive in residue under growing season conditions in mid-Missouri; the viability of microsclerotia in debris on the soil surface increased threefold from October to July, and then, it declined for the next 9 months (Short et al. 1980). In contrast, pathogen inoculum in soybean residue

buried 10 to 20 cm deep was relatively stable, and depth had little effect on sclerotial survival, which was similar to the results observed in our trials for crowns buried at 7.6 or 20.3 cm.

The use of fumigants at crop destruction (end of the season) or pre-plant (beginning of the season) reduces *M. phaseolina* inoculum in the soil, because exposed microsclerotia might be more vulnerable to the effect of the chemicals. However, fumigants have had little effect on controlling inoculum present within infected strawberry crowns, probably because of limitations related to product penetration of the crowns (Chamorro et al. 2016). If this remnant source of inoculum is not reduced, fields are continually reinoculated over the years; as Short et al. (1980) observed in soybean production areas, an increase in viable sclerotial populations from soil and crop residue could occur over consecutive years of planting soybeans and corn.

Additionally, we have demonstrated that infected crowns can serve as a source of inoculum for new transplants when they were

Table 3. Analysis of contrasts between the disease progress rates of different *Macrophomina phaseolina* dissemination methods on strawberry cultivars with different levels of susceptibility to charcoal rot

Contrast ^x	Estimate ^y	SE	<i>t</i> value	<i>P</i> value ^z	95% Confidence interval
Area 1					
Cultivar Florida Beauty					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0745	0.0067	11.15	<0.0001	0.0612–0.0878
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0785	0.0067	11.75	<0.0001	0.0652–0.0918
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0836	0.0067	12.52	<0.0001	0.0703–0.0969
<i>r</i> (aisles) – <i>r</i> (buried)	0.0040	0.0067	0.60	0.5509	–0.0093–0.0173
<i>r</i> (aisles) – <i>r</i> (neg cont)	0.0091	0.0067	1.36	0.1756	–0.0041–0.0224
<i>r</i> (buried) – <i>r</i> (neg cont)	0.0051	0.0067	0.77	0.4455	–0.0081–0.0184
Cultivar Strawberry Festival					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0297	0.0074	3.99	0.0001	0.0149–0.0445
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0335	0.0074	4.49	<0.0001	0.0187–0.0482
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0394	0.0074	5.29	<0.0001	0.0246–0.0542
<i>r</i> (aisles) – <i>r</i> (buried)	0.0038	0.0074	0.51	0.6140	–0.0110–0.0186
<i>r</i> (aisles) – <i>r</i> (neg cont)	0.0097	0.0074	1.30	0.1962	–0.0051–0.0245
<i>r</i> (buried) – <i>r</i> (neg cont)	0.0059	0.0074	0.80	0.4282	–0.0089–0.0207
Cultivar Winterstar					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0093	0.0071	1.30	0.1962	–0.0049–0.0235
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0078	0.0071	1.09	0.2788	–0.0064–0.0220
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0126	0.0071	1.77	0.0800	–0.0015–0.0268
<i>r</i> (aisles) – <i>r</i> (buried)	–0.0015	0.0071	–0.21	0.8323	–0.0157–0.0127
<i>r</i> (aisles) – <i>r</i> (neg cont)	0.0033	0.0071	0.47	0.6407	–0.0108–0.0175
<i>r</i> (buried) – <i>r</i> (neg cont)	0.0049	0.0071	0.68	0.4979	–0.0093–0.0190
Area 2					
Cultivar Florida Beauty					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0456	0.0071	6.38	<0.0001	0.0314–0.0597
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0400	0.0071	5.60	<0.0001	0.0258–0.0542
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0359	0.0071	5.03	<0.0001	0.0217–0.0501
<i>r</i> (aisles) – <i>r</i> (buried)	–0.0056	0.0071	–0.78	0.4376	–0.0197–0.0086
<i>r</i> (aisles) – <i>r</i> (neg cont)	–0.0097	0.0071	–1.35	0.1785	–0.0238–0.0045
<i>r</i> (buried) – <i>r</i> (neg cont)	–0.0041	0.0071	–0.58	0.5664	–0.0183–0.0100
Cultivar Strawberry Festival					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0594	0.0083	7.12	<0.0001	0.0428–0.0759
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0547	0.0083	6.55	<0.0001	0.0381–0.0712
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0471	0.0083	5.64	<0.0001	0.0305–0.0636
<i>r</i> (aisles) – <i>r</i> (buried)	–0.0047	0.0083	–0.56	0.5738	–0.0213–0.0118
<i>r</i> (aisles) – <i>r</i> (neg cont)	–0.0123	0.0083	–1.47	0.1437	–0.0288–0.0042
<i>r</i> (buried) – <i>r</i> (neg cont)	–0.0076	0.0083	–0.91	0.3655	–0.0241–0.0090

(Continued on next page)

^x Contrasts between the rate of disease progress (*r*) of the treatments (aisles, buried, positive control, and negative control) on the linearized monomolecular scale. Aisles and buried indicate treatments in which strawberry crowns infected with *M. phaseolina* were placed in between plastic-mulched beds in the aisles or buried next to new transplants, respectively. Pos cont indicates the positive control in which strawberry transplant trimmed roots were dipped into sclerotial suspension of three pathogenic *M. phaseolina* isolates from our culture collection. Neg cont indicates the negative control in which noninoculated plants were used. Area 1 indicates the experiment performed in the spring of 2017 in an area with low *M. phaseolina* inoculum densities. Area 2 indicates the experiment performed in the spring of 2018 in an area with moderate *M. phaseolina* inoculum densities. Area 3 indicates the experiment performed in the spring of 2017 in an area with high *M. phaseolina* inoculum densities.

^y Estimate indicates the estimated mean contrast between the rates of disease progress. SE is the standard error of the estimated mean rate. The *t* value is the *t* statistic from the contrasts. *P* value indicates the probability value or significance level of the contrast between treatments.

^z *P* values in bold were significant (*P* ≤ 0.05).

inoculated with suspensions of ground crowns collected from infected commercial fields. However, inoculation with microsclerotia directly from pure culture of *M. phaseolina* resulted in earlier disease symptoms compared with inoculation with the crowns. This could be explained by bare microsclerotia being more available when growing in artificial culture media than on residue pieces (Short et al. 1980; Zveibil et al. 2012) or by differences in inoculum concentration, because inoculum on infected crowns was not quantified.

Although the three strawberry cultivars used in this study were infected by *M. phaseolina*, they differ in susceptibility to charcoal rot: Strawberry Festival is considered highly susceptible, Florida Beauty is moderately susceptible, and Winterstar is moderately resistant (Peres et al. 2018; Whitaker et al. 2017b, 2018). Overall, for the moderately susceptible and resistant cultivars, the type of inoculation method did not affect disease development. However, for susceptible cultivars (e.g., Strawberry Festival), when the leaves of the plants were sprayed with *M. phaseolina* sclerotial suspension, the disease was more aggressive than when the roots were dipped or when the soil was drenched. These findings demonstrate that *M. phaseolina*, a known soilborne pathogen that infects plants through the root system (Dhingra and Sinclair 1978; Mihail 1992), can also infect and colonize strawberry crowns through the aerial parts. For instance, in strawberry fields that reuse the plastic for a second season and dispose of old crowns from the previous season between beds, overhead irrigation water used immediately after planting for plant establishment might disperse inoculum present on old, infected crowns, and this will infect new transplants on the beds. This dissemination method is usually more common with pathogens that affect aerial parts of the plants, such as *C. acutatum*, which is often disseminated by water splash from infected neighboring plants (Freeman 2008; Smith 2008).

Therefore, given that spray-inoculated plants developed charcoal rot symptoms, we conducted studies in the fields and confirmed that infected crowns disposed of in the aisles of raised beds or buried

next to new strawberry transplants were able to cause charcoal rot symptoms in the new transplants. In some of the experiments, disease was more severe when plastic was reused. In fact, it has already been shown that reusing plastic mulch might increase the incidence of fungal diseases, such as *Colletotrichum* crown rot caused by *Colletotrichum gloeosporioides*, especially in highly susceptible cultivars, such as Strawberry Festival (Nyoike and Liburd 2014). In addition to the inoculum present in the soil, infections can also occur because of proximity to the source (i.e., buried or tilled crowns) or by aerial and water splash dispersal (i.e., crowns in aisles). Moreover, as also noticed on commercial strawberry farms, it has been observed that colonization by *M. phaseolina* can start from the top to the bottom portion of the strawberry crowns, indicating that infection might have occurred through the aerial part instead of through the soil and root system (Supplementary Figs. S1, S2, S3, and S4).

In an area with low and moderate inoculum density in the soil, the addition of infected crowns did not result in increased disease. This demonstrates that, even if a field containing infected crowns from the previous season is fumigated in the following season to reduce the amount of inoculum in the soil, those crowns might not contribute to an increase of charcoal rot, regardless of the cultivar used. However, in an area with a high level of inoculum, early presence of inoculum from discarded crowns resulted in earlier initiation of an epidemic. In the case of a resistant cultivar (Winterstar), infected crowns left on beds posed less risk than those disposed of in the bed aisles.

Nevertheless, symptoms of charcoal rot in areas with different levels of *M. phaseolina* inoculum in the soil without the addition of external sources of inoculum needs additional investigation, because the combination of inoculum concentration in the soil and cultivars with different levels of susceptibility might play an important role in understating disease development in different situations. Therefore, *M. phaseolina* inoculum density in a strawberry field, which can be determined by soil sampling, and choice of cultivar to be used in that area might help to determine management strategies. These

Table 3. (Continued from previous page)

Contrast ^x	Estimate ^y	SE	<i>t</i> value	<i>P</i> value ^z	95% Confidence interval
Cultivar Winterstar					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0124	0.0022	5.53	<0.0001	0.0079–0.0169
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0163	0.0022	7.24	<0.0001	0.0118–0.0207
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0103	0.0022	4.59	<0.0001	0.0059–0.0148
<i>r</i> (aisles) – <i>r</i> (buried)	0.0038	0.0022	1.71	0.090	–0.0006–0.0083
<i>r</i> (aisles) – <i>r</i> (neg cont)	–0.0021	0.0022	–0.94	0.3506	–0.0066–0.0023
<i>r</i> (buried) – <i>r</i> (neg cont)	–0.0059	0.0022	–2.65	0.0093	–0.0104–0.0015
Area 3					
Cultivar Florida Beauty					
<i>r</i> (pos cont) – <i>r</i> (aisles)	–0.0326	0.0087	–3.75	0.0003	–0.0500–0.0153
<i>r</i> (pos cont) – <i>r</i> (buried)	0.0156	0.0087	1.80	0.0756	–0.0016–0.0330
<i>r</i> (pos cont) – <i>r</i> (neg cont)	0.0200	0.0087	2.29	0.0242	0.0027–0.0373
<i>r</i> (aisles) – <i>r</i> (buried)	0.0483	0.0087	5.54	<0.0001	0.0310–0.0656
<i>r</i> (aisles) – <i>r</i> (neg cont)	0.0526	0.0087	6.04	<0.0001	0.0353–0.0699
<i>r</i> (buried) – <i>r</i> (neg cont)	0.0043	0.0087	0.49	0.6219	–0.0130–0.0216
Cultivar Strawberry Festival					
<i>r</i> (pos cont) – <i>r</i> (aisles)	0.0033	0.0057	0.59	0.5593	–0.0080–0.0146
<i>r</i> (pos cont) – <i>r</i> (buried)	–0.0089	0.0057	–1.57	0.1200	–0.0202–0.0024
<i>r</i> (pos cont) – <i>r</i> (neg cont)	–0.0138	0.0057	–2.44	0.0167	–0.0251–0.0026
<i>r</i> (aisles) – <i>r</i> (buried)	–0.0122	0.0057	–2.16	0.0338	–0.0235–0.0010
<i>r</i> (aisles) – <i>r</i> (neg cont)	–0.0172	0.0057	–3.02	0.0032	–0.0285–0.0059
<i>r</i> (buried) – <i>r</i> (neg cont)	–0.0049	0.0057	–0.87	0.3878	–0.0162–0.0063
Cultivar Winterstar					
<i>r</i> (pos cont) – <i>r</i> (aisles)	–0.0313	0.0094	–3.32	0.0013	–0.0500–0.0126
<i>r</i> (pos cont) – <i>r</i> (buried)	–0.0075	0.0094	–0.80	0.4255	–0.0263–0.0112
<i>r</i> (pos cont) – <i>r</i> (neg cont)	–0.0278	0.0094	–2.95	0.0041	–0.0465–0.0091
<i>r</i> (aisles) – <i>r</i> (buried)	0.0238	0.0094	2.52	0.0135	0.0050–0.0425
<i>r</i> (aisles) – <i>r</i> (neg cont)	0.0035	0.0094	0.37	0.7100	–0.0152–0.0223
<i>r</i> (buried) – <i>r</i> (neg cont)	–0.0202	0.0094	–2.15	0.0345	–0.0390–0.0015

strategies include the use of adequate fumigants for crop destruction and preplanting fumigation, crop residue removal, and use of new plastic mulches for the next season.

We showed that *M. phaseolina*-resistant structures can survive in strawberry crowns over summer in Florida and that the pathogen viability was not reduced over time. In addition, *M. phaseolina* can be dispersed above ground by water splash regardless of the source of inoculum, and it can cause charcoal rot in strawberries. Furthermore, infected crowns from the previous strawberry season can act as a source of inoculum and infect new strawberry transplants when plastic-mulched beds are reused in the next season; management strategies should consider removal of strawberry debris from known infected fields at the end of the season.

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