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Response of Strawberry Cultivars Inoculated with *Macrophomina phaseolina* in Australia

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ABSTRACT

Macrophomina phaseolina causes charcoal rot in strawberry. The pathogen has a wide host range, is favored by high soil temperatures, and current fumigants are not as effective as methyl bromide. Breeding strawberry cultivars resistant to *M. phaseolina* has become an important focus. Eleven cultivars were evaluated in a glasshouse trial for resistance to an isolate of *M. phaseolina*. Plants were inoculated by drenching the potting medium with a suspension of microsclerotia. Plant mortality was recorded for up to 23 weeks. Based on plant mortality and survival analyzes, 'Albion' was similarly susceptible as 'Camarosa' and a number of historical and current cultivars showed tolerance and/or resistance to the pathogen. The preliminary findings in this study can assist in the development of new strawberry genotypes against *M. phaseolina*.

KEYWORDS

Pathogenicity; charcoal rot; incidence; breeding; resistance

Introduction

Macrophomina phaseolina is a soil-borne fungal pathogen that causes the strawberry crown rot disease charcoal rot. Within Australia, charcoal rot in strawberry has been reported in commercial farms in Queensland, Victoria and Western Australia (Golzar et al., 2007; Hutton et al., 2013). These three states account for 88% of the strawberry production in Australia, where the industry is valued at A$445M (Hort Innovation, 2019). Apart from Australia, the disease has also been reported in other strawberry producing countries including Argentina (Baino et al., 2011), Chile (Sanchez et al., 2013), France (Baudry and Morzieres, 1993), Iran (Sharifi and Mahdavi, 2012), Israel (Zveibil and Freeman, 2005), Spain (Aviles et al., 2008), Tunisia (Hajlaoui et al., 2015) and the United States (Koike, 2008a; Mertely et al., 2005). Sanchez et al. (2016) described *M. phaseolina* as an emerging and devastating pathogen of strawberry.

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At least 500 plant species are known hosts of *M. phaseolina* (Wyllie, 1988) including cotton where the disease is also known as charcoal rot (Ghaffar and Erwin, 1969), on soybeans where it causes ashy-stem blight (Dhar and Sarbhoy, 1987) and in rice, causing dry root rot (Than et al., 1991). Sorghum is also a known host of *M. phaseolina* in Australia (Ryley et al., 2008). This is a particular concern as sorghum is commonly used as a cover crop between strawberry seasons in many of the production areas in Australia. In 2015, *M. phaseolina was* isolated from sorghum grown as a cover crop in Wamuran, Queensland and was subsequently found to be pathogenic to strawberry (Gomez, unpublished data). Similarly, in Israel, isolates of *M. phaseolina* from other host plant species rotated with strawberry were found to be pathogenic to strawberry and thus highlighted the importance of avoiding rotation of crops that may host the pathogen (Zveibil et al., 2012). Weeds may also serve as alternative hosts as was reported in a study in which various weeds found in mung bean fields in Queensland were also hosts of *M. phaseolina* (Fuhlbohm et al., 2012).

Strawberry plants affected with charcoal rot show progressive symptoms of wilting of foliage, drying and death of older leaves with the younger leaves persisting initially but later succumbing to complete plant collapse leading to plant death (Koike et al., 2013). When affected crowns are cut open longitudinally, dark brown necrotic areas in the internal cortex and vascular tissues are observed (Koike et al., 2013).

The pathogen produces round to irregularly shaped structures, called microsclerotia that are made up of aggregations of hyphae held together by a melanized rind (Dhingra and Sinclair, 1978; Gangopadhyay and Wyllie, 1974). These are resting structures that allow the pathogen to survive in soil or in infected material as it senesces (Short and Wyllie, 1978). In soil, microsclerotia may allow persistence of the fungus for up to 15 years (Short et al., 1980).

M. phaseolina microsclerotia remained viable in bean crop residue buried in soil for 21 months (Songa and Hillocks, 1998), and 18 and 16 months on corn and sorghum, respectively (Cook et al., 1973). It is not known how long the pathogen survives in buried strawberry crop residues, but incorporating infected strawberry material into the soil may increase the microsclerotium concentration in the soil.

Microsclerotia are considered resistant to environmental variables (Olaya and Abawi, 1996). Soil temperature and moisture content are the two most important factors that can affect the survival of microsclerotia (Papavizas, 1977); however, Zveibil et al. (2012) demonstrated that survival of microsclerotia of *M. phaseolina* was dependent on soil temperatures with soil moisture only having limited effects. High soil temperatures (greater than 27°C) have been shown to promote charcoal rot (Persley et al., 2010). Such high temperatures occur in the Australian strawberry growing seasons. In 2016, the monthly maximum air temperatures in the production period in

South East Queensland (winter) and Victoria (summer) ranged from 27°C to 31°C and 33°C to 42°C, respectively (Bureau of Meteorology, 2017). In addition, the use of black-colored plastic mulch can raise soil temperatures. Under this mulch at a depth of 5 cm, soil temperatures can be 6.5°C to 9.0°C higher than air temperature (Hutton and Gomez, 2006). This may create more favorable conditions for the development of charcoal rot.

Historically in Australia, the majority of production fields used methyl bromide as a soil fumigant to control soil-borne pathogens, and charcoal rot was only a very minor disease. In 1997 however, under the Montreal Protocol, a global phase-out of methyl bromide was agreed upon due to the emissions being associated with the degradation of the ozone layer. Consequently, methyl bromide was phased out of Australian strawberry fruit production in 2006. Hutton et al. (2013) reported the association of *M. phaseolina* causing crown rots in fruit production farms in Australia not long after methyl bromide was phased out. Several reports also attributed the increase in charcoal rot incidence in the last decade to the withdrawal of methyl bromide and the ineffectiveness of current soil fumigant alternatives (Koike, 2008b; Mertely et al., 2005; Zveibil and Freeman, 2009). In a field study, Hutton et al. (2013) found soil fumigants chloropicrin and 1, 3-dichloropropene were ineffective in eradicating *M. phaseolina* in buried infected strawberry crowns. A recent study in California showed fungicides alone were not effective at controlling the pathogen (Carter, 2016).

Cultural management of *M. phaseolina* is difficult, with a wide host range, an ability to produce survival structures and withstand high soil temperatures, current fumigants are not as effective as methyl bromide and there are no effective fungicides against the pathogen. The management strategy of developing resistant cultivars has therefore become increasingly important. Identifying and developing pathogen-resistant strawberry cultivars is considered the most cost effective and sustainable strategy for control of crown and root diseases (Mackenzie et al., 2006). Consequently, the attainment of cultivars resistant to *M. phaseolina* is now the focus of many breeding programs internationally, with great importance put on the identification of resistance in existing strawberry genotypes to soil-borne diseases in general (Holmes et al., 2017).

Previous studies characterizing strawberry genotype response against *M. phaseolina* have used the toothpick-inoculated and microsclerotial suspension methods predominantly. Koike et al. (2016) described the *M. phaseolina*-colonized toothpick method to be a very severe inoculation method, and as a result only showed differences in susceptibility early and for a short time after inoculations. Drenching the growing medium with a suspension of microsclerotia was considered a closer representation of what occurs in the field (Zveibil and Freeman, 2005). Aviles et al. (2009) compared the colonized toothpick and microsclerotial suspension methods of inoculation and found that symptoms were expressed earlier in the plants inoculated using the colonized toothpick method, but no cultivar by isolate interactions

were detected. In contrast, with the microsclerotia method, cultivars were found to respond differently depending on the isolate.

Strawberry cultivars that have been reported to show tolerance and/or resistance to *M. phaseolina* include 'Seascape' (Koike, 2008b), 'Albion' (Fang et al., 2012), 'Aromas' (Fang et al., 2012), 'Coral' (Aviles et al., 2012) and 'Splendor' (cited in De Los Santos et al., 2016). Holmes et al. (2017) tested several cultivars from different breeding programs in California and found cultivars that were highly and moderately resistant, as well as those that were susceptible to *M. phaseolina*. However, there is limited information on the response of strawberry cultivars currently grown commercially in Australia to *M. phaseolina* infection. Apart from 'Albion', those that have been reported previously to have resistance are either no longer grown in significant numbers in Australia or were not available for testing.

In this study, 11 strawberry cultivars were screened for resistance to *M. phaseolina* by drenching the potting medium with a microsclerotial suspension of *M. phaseolina* isolate BRIP 66625. Current commercial cultivars grown in Australia and internationally, and a number of historical cultivars from the Australian collection were evaluated. The results of this study will add knowledge of cultivar response to *M. phaseolina* and aid in developing commercial strawberry breeding lines with potential resistance to *M. phaseolina*.

Materials and Methods

M. phaseolina Isolate from Infected Strawberry Plants

M. phaseolina BRIP 66625 (Queensland Plant Pathology Herbarium) was isolated from a strawberry plant (cv. Florida Radiance) showing symptoms of crown rot from Glenview in Queensland in 2009. These symptoms had included an internal necrotic rot in the crown and discolouration along the vascular tissues when cut longitudinally. The isolation was conducted using methods described by Hutton et al. (2013) by placing small crown tissue pieces with necrotic symptoms on a Petri dish containing quarter-strength potato dextrose agar (PDA) amended with 50 ppm streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA). *M. phaseolina* was identified based on production of microsclerotia and colony morphology as described by Holliday and Punithaligam (1970). The isolate, which was hyphal-tipped prior to storage in sterile-deionized water (SDW), was subsequently verified as *M. phaseolina* by DNA sequencing of the internal transcribed spacer region.

Inoculum Preparation

BRIP 66625 was revived from storage by sub-culturing onto PDA amended with streptomycin, as described. The plates were incubated at 24°C in continuous near-

UV light for 3 weeks. A microsclerotial suspension was prepared based on methods described by Aviles et al. (2009) with the following modifications. PDA imbedded with microsclerotia was blended for 45 s (two Petri dishes with 200 mL SDW at a time) to produce a microsclerotial suspension. As indicator of concentration, the final stock of inoculum suspension was mixed thoroughly, and microsclerotia of *M. phaseolina* were counted from 10 samples of 0.1 mL samples of the suspension under a stereo microscope. This was repeated an additional two times to obtain an average of 1.4×10^3 microsclerotia per mL.

Strawberry Cultivars

Current commercially grown cultivars 'Albion', 'Camarosa', 'Strawberry Festival', 'Florida Radiance', 'Red Rhapsody', 'Rubygem' and 'Suncoast Delight' along with historical cultivars 'Earlibrite', 'Kabarla', 'Phenomenal' and 'Sweet Charlie' were utilized in the trials. Runners of 'Albion' and 'Camarosa' were purchased as certified commercial runners from Toolangi Certified Strawberry Runner Grower's Co-operative Ltd., Victoria, Australia. Runners of all other cultivars were produced as part of the Australian National Strawberry Varietal Improvement Program at Maroochy Research Facility, Nambour. All plants were grown in 1:1 sterile peat: sand mix in 100 mm Spacesaver® pots and maintained prior to pathogen inoculations.

Screening of Cultivars

To study the response of strawberry cultivars to *M. phaseolina*, 50 mL of the microsclerotial suspension was poured into each pot containing a strawberry plant. Ten plants of each cultivar, with the exception due to limited availability of 'Kabarla' (nine plants), were inoculated. Plants from different cultivars were inoculated in a randomized block design and afterward placed in the same order in an evaporatively cooled glasshouse set to a maximum of 40°C. The pots were placed on a heated bench set at 30°C. Non-treated controls consisted of ten plants each of 'Camarosa' and 'Albion', treated with 50 mL of SDW per pot and located separate from the inoculated plants in the same glasshouse and were not included in the analysis. The two cultivars were chosen based on reported susceptibility and resistance respectively in a study in Western Australia (Fang et al., 2012).

All plants were assessed weekly for up to 23 weeks post inoculation during which time the incidence of plant mortality, represented by the symptom of complete plant collapse or wilt due to *M. phaseolina* isolate BRIP 66625, was recorded. Those plants showing symptoms were collected and isolations made for recovery of the pathogen, using the method of Hutton et al. (2013) as described previously. When *M. phaseolina* and/or a mixed culture with *M. phaseolina* was present on the PDA plate, it was considered that

plant death was due to *M. phaseolina*. If *M. phaseolina* was not recovered it was assumed the plant died from other causes and any such plants were eliminated from the final analysis. Control plants were similarly monitored for development of any symptoms and were also not included in the final analysis. Assessments were carried out in November to April at the Maroochy Research Facility, Nambour.

Data Analysis

First, an initial analysis of the mortality of plants from each cultivar to BRIP 66625 was performed based on whether plants were alive or dead at the final time point (week 23). The mortality data were coded as 0 (alive) and 1 (dead) for each plant. The standard analysis approach for such data is to fit a Generalized Linear Model (GLM) assuming a binomial distribution and logit link (logistic regression), however there were some cultivars with 0% mortality and others with 100% mortality showing complete separation (Albert and Anderson, 1984). A logistic regression model was therefore fitted, applying Firth's correction to the likelihood (Firth, 1993). The R (R Core Team, 2015) package *logistf* (Heinze and Schemper, 2002) was used for the analysis.

In the second analysis, strawberry plant survival data over time was analyzed using the Cox Proportional Hazards model (Cox, 1972) using the *Survival* package (Therneau, 2015) in R (R Core Team, 2015). The data consisted of a vector of times (time to event for each plant) and a vector indicating which times where deaths and which were censored (plant still alive at the end of the trial). The survival analysis was based on the hazard function for each cultivar. The hazard function is the predicted instantaneous risk of death at time t, conditional on survival to that time. It may vary over time but the proportional hazards model assumes the hazard for one genotype is a constant proportion of the hazard in other genotypes, and this proportion is called the hazard ratio (Duerden, 2014). Hazard ratios were predicted for each cultivar relative to the hazard of a standard reference cultivar. For this study, 'Camarosa' was used as the standard susceptible reference cultivar, based on reported susceptibility to *M. phaseolina* (Fang et al., 2012; Koike, 2008a; Sanchez et al., 2013). A hazard ratio for a cultivar equal to or greater than 1 suggests equal or greater susceptibility than 'Camarosa', i.e. higher risk of plant deaths. Hazard ratios less than 1 suggest lower susceptibility (therefore lower risk of death at any given time) than 'Camarosa'. Cultivars that had 0% plant mortality were not included in the analysis, and would have a nil hazard. Predicted survival plots, which show the proportion of plants that survive at each time, were presented for 'Camarosa' and the other cultivars for resistance against *M. phaseolina.*

Results

Yellowing and early stage of necrosis of leaves were first observed on plants 2 weeks after inoculation. The first signs of plant wilt due to *M. phaseolina* were recorded on an 'Albion' plant 10 weeks following inoculation. At the final time point (23 weeks after inoculation), plant mortality varied significantly between cultivars ($P < .001$) based on Firth's method for logistic regression. The predicted proportion dead at week 23 for each cultivar is given in Table 1. Based on confidence intervals, the proportion dead at the final time point for 'Albion' and 'Camarosa' were significantly higher than that of 'Phenomenal', 'Earlibrite' and 'Kabarla'. 'Sweet Charlie' and 'Suncoast Delight' have significantly lower mortality than 'Albion' but not 'Camarosa'. The proportion dead for 'Red Rhapsody' is significantly lower than 'Albion'. The proportion dead for 'Albion' at the final time point was higher than 'Camarosa', but not statistically different.

Hazard ratios from the survival analysis for each cultivar are shown in Figure 1. 'Phenomenal', 'Kabarla' and 'Earlibrite' had 0% mortality, therefore hazard ratios of zero. 'Sweet Charlie' and 'Suncoast Delight' had significantly lower hazard ratios than the reference cultivar 'Camarosa' ($P \geq 0.05$). The hazard ratios of other cultivars were not significantly different from 'Camarosa'. 'Albion' had a hazard ratio greater than 1 (1.447) however was not significantly different from 'Camarosa'.

The predicted survival proportion for each cultivar over time is presented in Figure 2. As expected, for the reference (susceptible) cultivar 'Camarosa', the analysis predicted a very low proportion of survival at 23 weeks after

Table 1. Regression coefficients, standard errors and 95% confidence intervals (CI) together with predicted proportion dead (%) on the backtransformed original scale for each cultivar at week 23 after inoculating with *M. phaseolina* isolate BRIP 66625 from the logistic regression model using Firth's method.

			Confidence intervals		Predicted proportion dead	
Cultivar	Coefficients	Standard errors	Lower 0.95	Upper 0.95	(%)	
Albion	2.833	1.543	0.752	7.695	94	a
Camarosa	0.956	0.789	−0.407	2.649	72	ab
Strawberry Festival	0.368	0.643	−0.826	1.652	59	abc
Rubygem	0.201	0.670	−1.059	1.514	55	abcd
Florida Radiance	−0.368	0.643	−1.652	0.826	41	abcd
Red Rhapsody	−1.099	1.033	−3.398	0.587	25	bcd
Suncoast Delight	−1.609	0.949	−3.859	−0.091	17	bcd
Sweet Charlie	−1.609	0.949	−3.859	−0.091	17	bcd
Kabarla	−2.708	1.561	−7.574	−0.604	6	cd
Earlibrite	−2.833	1.543	−7.695	−0.752	5	cd
Phenomenal	−2.944	1.529	−7.804	−0.882	5	d

Coefficients are prediction (proportion dead at week 23), with standard errors for the estimate. The CI (lower and upper 0.95) gives the 95% confidence interval for the predictions. If the 95% CI for one prediction does not overlap the 95% CI of another prediction, then cultivars are significantly different (i.e. cultivars with same subscript are not significantly different). The predicted proportion dead is the predicted proportion at week 23, e.g. it is predicted that by week 23, 94% of 'Albion' plants will be dead.

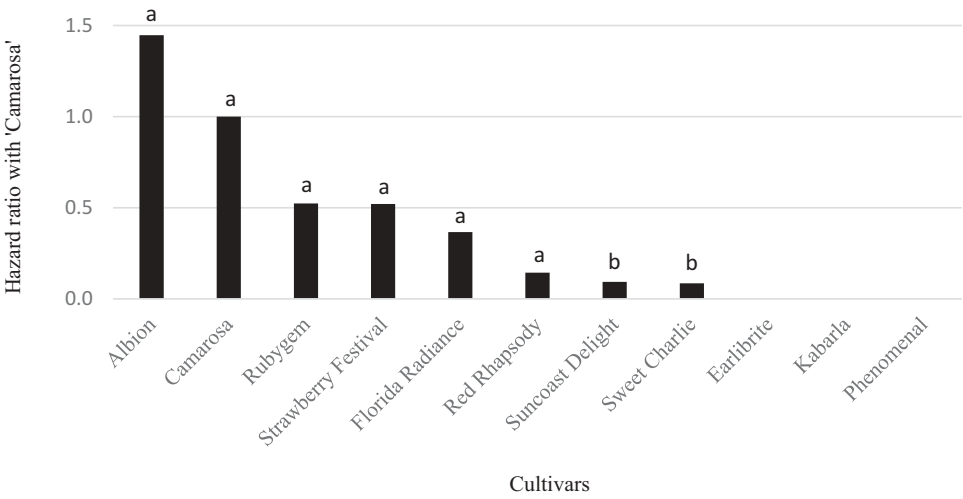

Figure 1. Hazard ratios for cultivars tested compared to the reference cultivar 'Camarosa' when challenged with *M. phaseolina* isolate BRIP 66625. A hazard ratio of 1 or more implies equal or greater susceptibility than the control 'Camarosa'. Hazard ratios less than 1 imply greater resistance than 'Camarosa'. Columns with the same subscript are not significantly different in response to the pathogen ($P \geq 0.05$).

inoculation. The survival prediction for 'Albion' was similar to 'Camarosa'. Predicted survival proportions for 'Florida Radiance', 'Strawberry Festival' and 'Rubygem' ranged between 0.4 and 0.6 at 23 weeks. 'Suncoast Delight' and 'Sweet Charlie' had a predicted survival proportion of over 0.8, followed closely by 'Red Rhapsody'. With 0% mortality in this study, the predicted survival for 'Phenomenal', 'Kabarla' and 'Earlibrite' was 1.

Discussion

In this study, cultivars 'Camarosa' and 'Albion' were the most susceptible to *M. phaseolina*. As described earlier, 'Camarosa' was used as a reference cultivar based on several reports of susceptibility to *M. phaseolina*. One of those was a study by Fang et al. (2012) in Western Australia, where the authors also reported that 'Albion' was resistant. This however differs from the results observed here where 'Albion' was rated as having similar susceptibility to 'Camarosa'. Reports from California (Koike et al., 2016) and Chile (Sanchez et al., 2016) also showed that 'Albion' plants exhibited a high mortality rate of 100% and 60%, respectively, when challenged with *M. phaseolina*. Additionally in California, Holmes et al. (2017) ranked 'Albion' as highly susceptible to *M. phaseolina* after screening several existing cultivars and breeding lines from different strawberry breeding programs. The result for 'Albion' in this glasshouse study is supported by observations in Queensland, Australia's Granite Belt region during summer production, where high plant losses of

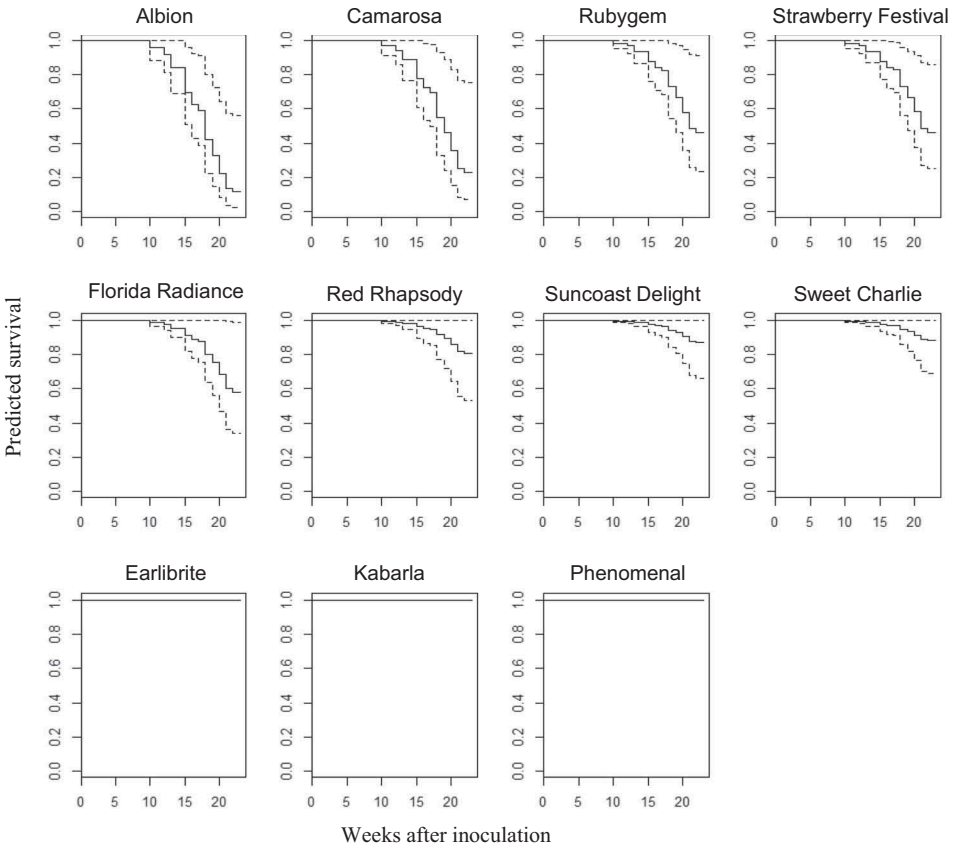

Figure 2. Predicted survival plots for the 11 cultivars tested showing the predicted proportion of plants alive at each time after inoculation with *M. phaseolina* isolate BRIP 66625. Predicted survival proportion range from 0 to 1, where 0 = no plants survived and 1 = all plants survived.

'Albion' in recent years have been attributed to *M. phaseolina* (Gomez, unpublished data). In Western Australia, *Fusarium oxysporum* f. sp. *fragariae* is regarded as the major pathogen associated with crown rot diseases (Fang et al., 2011). However, *M. phaseolina* has been isolated from 'Albion' from a Western Australian fruit farm (Gomez, unpublished data). Assessment of 'Albion' in Western Australia warrants further investigation to determine if current isolates may be able to overcome the resistance and so exhibit a shift in the pathogen profile, or if indeed *M. phaseolina* from Western Australia is distinct from that in the eastern states of Australia.

Based on the hazard ratios, 'Strawberry Festival', 'Rubygem', 'Florida Radiance' and 'Red Rhapsody' were not statistically different to the reference cultivar 'Camarosa'. A study by Sanchez et al. (2016) reported 'Florida Radiance' and 'Strawberry Festival' plant mortality greater than 70% and 40%, respectively, when inoculated with *M. phaseolina*. Aviles et al. (2012) also found 'Florida Radiance' was one of the most susceptible cultivars

to *M. phaseolina* in Spain. Mertely et al. (2005) in Florida reported 100% mortality in 'Strawberry Festival' when inoculated with *M. phaseolina*. 'Rubygem' is a cultivar developed by the national breeding project in Australia (Herrington et al., 2007), made available commercially by mid-2000s, and is currently grown for winter production. The pathogen has previously been isolated from wilting plants of this cultivar from commercial farms in the Sunshine Coast, Queensland (Gomez, unpublished data). 'Rubygem' is also grown in other parts of the world, such as Turkey. *M. phaseolina* has been reported in Turkey (Yildiz et al., 2010), but it is not known if the pathogen has an association with 'Rubygem' in production fields outside Australia at the time of this study.

Interestingly, no plant mortality was recorded due to *M. phaseolina* isolate BRIP 66625 on the historical cultivars 'Phenomenal', 'Kabarla' and 'Earlibrite'. 'Phenomenal' was developed in Queensland in the early 1900s and by 1946 was the basis for the strawberry industry in Queensland (Barnes et al., 2017). 'Kabarla' and 'Earlibrite' were available commercially between 1990 and early 2000. All three cultivars are no longer available commercially, but are in the Australian National Strawberry Varietal Improvement Program germplasm collection. In contrast, cultivars 'Suncoast Delight' and 'Red Rhapsody' are currently grown commercially for the Australian winter production. Both of these cultivars were developed by the Australian varietal improvement program and while the hazard ratios of each cultivars were low, it was only 'Suncoast Delight that was significantly lower, implying greater resistance to charcoal rot, than 'Camarosa'. 'Sweet Charlie' also showed high resistance, and according to our pedigree charts is a distant ancestor of both 'Suncoast Delight' and 'Red Rhapsody'. 'Phenomenal' is a progenitor of both 'Kabarla' and 'Red Rhapsody' (Barnes et al., 2017). This may suggest that possible resistance from the oldest cultivar 'Phenomenal' may have been inherited through the crossings and development of past and current cultivars.

Differences in cultivar responses in this study compared with other studies may be due to the inoculation methods and the resistance levels of the cultivars to one local isolate of *M. phaseolina*. As described earlier, considering the very severe nature of jabbing a *M. phaseolina*-colonized toothpick into the crown of a strawberry plant, the method used in this study was to drench the growing medium with a suspension of microsclerotia, which is considered to be a closer representation of natural infection. The use of one strawberry isolate of *M. phaseolina* from Queensland may also explain the difference between responses of 'Albion' in this study compared with the study done in Western Australia (Fang et al., 2012). It is feasible that the two isolates differ in virulence.

Hence, further work is needed to investigate variation in virulence to strawberry by using a more extensive range of *M. phaseolina* isolates obtained throughout Australia. This includes testing a wide range of isolates originating from strawberry and non-strawberry (alternative) hosts.

Host-pathogen studies are integral to understanding the behavior of existing cultivars in breeding programs to develop *M. phaseolina* resistant genotypes (Sanchez et al., 2016). This preliminary study has demonstrated that current strawberry cultivars grown in Australia, including cultivars now in the germplasm collection, have varying degrees of resistance to *M. phaseolina*. Cultivars that showed the lowest hazard ratio and high predicted survival proportion could be used in future crossing strategies to develop new elite breeding lines with resistance to *M. phaseolina* in Australia, to help minimize economic losses to charcoal rot.

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