Final Report

National Strawberry Varietal Improvement Program

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Content

Summary

The Australian strawberry industry has an estimated farm gate value of $420 million, with approximately 200 commercial producers and 60 opportunistic producers (Hort Innovation, 2017). For the national industry to remain profitable the ongoing development and availability of improved strawberry varieties is required. Opportunities exist for locally adapted varieties with superior production and consumer traits.

This project (BS12021) was initially established in 2013 to develop and commercially release superior strawberry varieties adapted for production in subtropical climatic regions in Australia. In 2015 the scope of the project was expanded to additionally include the development of improved strawberry varieties for Australian temperate and Mediterranean production regions. As part of this expansion we began management of the existing Victorian Strawberry Breeding Program at this time. Individual breeding targets for each production region were defined in consultation with local industry in each area with an overall goal of supporting a sustainable and profitable national strawberry industry. Traits included, among other targets, developing varieties with improved resistance to the crown wilt diseases *Macrophomina phaseolina* (charcoal rot), *Colletotrichum gloeosporioides* and *Fusarium oxysporum* f. sp. *fragariae*. The general breeding strategy built upon the successful pipeline developed in the previous iteration of the project (BS09013) and was further enhanced by the incorporation of quantitative genetic analysis and economic modelling of fruit and plant traits to guide crossing and selection decisions.

The project developed and positioned for commercialisation 12 new strawberry varieties, locally adapted to Australia's growing regions and satisfying Australian consumer expectations. These included six varieties targeted for subtropical production: 'Red Rhapsody', 'Parisienne Kiss', 'Sundrench', 'Scarlet Rose-ASBP', 'Sunglow ASBP', 'Meadowsong', and 'Venus-ASBP'; two varieties targeted for temperate production: 'Summer Song', and 'Scarlet-silk'; and three varieties targeted for Mediterranean production: 'Rosalie-ASBP', 'Jubilee-ASBP', and 'Fanfare-ASBP'. Sixteen peer reviewed journal articles were published, along with four conference posters and eight publications in the national strawberry industry magazine Simply Red. Eighteen presentations were delivered at conferences, industry meetings and field days, and four breeding trial tours given. The project was also covered extensively in the media following the official release and outstanding success of its variety 'Red Rhapsody'.

In 2017, varieties from the Australian Strawberry Breeding Program (ASBP) captured 44% of the subtropical market, and 19% of the national market. These plantings had a farm gate value of approximately $78 million and contributed ~2700 jobs in production. Indications from runner growers suggest that 2018 sales of ASBP varieties will be substantially higher than 2017 levels.

Based on current industry and marketing trends, it is recommended that key directions for future breeding should include varieties for alternative production systems, specifically substrate culture and protected cropping, as well as higher value varieties for product diversification e.g. different fruit colours and flavours. Additionally, DNA based breeding tools such as marker assisted selection represent a significant opportunity that should be incorporated into future strawberry breeding programs.

Keywords

1. Introduction

The Australian strawberry industry has an estimated farm gate value of $420 million, with approximately 200 commercial producers and 60 opportunistic producers (Strawberry Strategic Investment Plan 2017-2021). For the national industry to remain profitable the ongoing development and availability of improved strawberry varieties is required. This feeds into Outcome 3 in the Strawberry Strategic Investment Plan 2017-2021, of increased farm productivity (marketable yield per hectare) by an average of 10 per cent by 2021. New varieties need to be locally adapted to each of Australia's growing regions and satisfy Australian consumer expectations. Furthermore, these need to be developed through a pipeline that can adapt to changing market and environmental demands.

Under project BS12021, the Australian Strawberry Breeding Program (ASBP) aimed to develop and commercially release superior strawberry varieties adapted for production in temperate, subtropical and Mediterranean climatic regions in Australia. Initially instigated as a breeding program for the subtropical production region only, a variation to project BS12021 was implemented in December 2014. This variation aligned the existing Southern (temperate) Strawberries Australia Inc. and Hort Innovation breeding program with the subtropical program managed by the Queensland Department of Agriculture and Fisheries. At this time some breeding additionally commenced targeting the Mediterranean production region in Western Australia, in order to provide a national scope covering all major Australian production regions.

The new national focus of this project allowed the optimisation of breeding targets to encourage a more unified, profitable national strawberry industry. Breeding targets were defined in consultation with local industry in each region with an overall goal of supporting a sustainable and profitable national strawberry industry. Traits included, among other targets, suitability for substrate culture and improved disease resistance.

Over the course of the project, 12 varieties catering for subtropical, temperate and Mediterranean production regions were commercialised. In 2017 ASBP varieties captured 19% of the national industry, and 44% of the subtropical industry. These plantings had a farm gate value of approximately $78 million and contributed ~2700 jobs in production alone. The number of plants from this project grown across Australia is predicted to increase substantially in 2018.

This report covers the outputs from project BS12021, specifically in regard to breeding trials undertaken for all three targeted production regions and varieties commercialised, and findings from disease screening trials conducted for three major crown wilt diseases: *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *Fragariae*, and *Macrophomina phaseolina* (charcoal rot).

2. Subtropical trials and selection

Jodi Neal and Mark Herrington

2.1. Introduction

The subtropical production region is primarily located in South East Queensland, Bundaberg, and northern New South Wales. Of these, production is concentrated in the Beerwah and Sunshine Coast regions in Queensland (Strawberry Strategic Investment Plan 2017-2021). The subtropical production season runs each year from approximately May to October. Specific challenges for this region include typically good punnet prices early in the season but low prices late season, and decreasing punnet prices overall due to increasing fruit production - driven largely by greater plant numbers - with relatively static demand. The number of subtropical strawberry plants planted rose from approximately 35 million to over 50 million between 2016 and 2017 seasons. As with all targeted productions regions, the overall aim of the subtropical breeding program is to provide varieties that encourage a profitable and environmentally, economically and socially sustainable strawberry industry.

2.2. Methods

The breeding methodology applied for the subtropical production region is outlined below (Sections 2.2.1 to 2.2.8). All stages of this process were undertaken simultaneously each year for seedlings and clonal selections at each phase.

2.2.1. Breeding objectives

Definition of breeding objectives for the subtropical production region was undertaken in consultation with the subtropical strawberry industry and Strawberry Variety Steering Committee (SVSC). The process was ongoing over the life of the project, with breeding objectives refined and adapted as necessary to ensure that they were in line with any changes in environmental or market forces. The general breeding objective for the subtropical production region has been to develop and make available varieties that provide increased profitability for producers while satisfying consumer and market expectations. Each of these two objectives are discussed in more detail below.

The traits important for increasing production profitability were estimated using an economic model developed by Mark Herrington in consultation with industry. The model allowed informed selection decisions to be made, weighting the value of plant and fruit traits to estimate the profitability of each selection as a variety. Taken into account were the effects of traits such as fruit size, bruise resistance, and ease of picking on the time expended to pick and pack fruit, and therefore labour cost. Also predicted were punnet prices each month based on yield quantity and yield distribution over the season, thereby estimating income.

Consumer expectations were also weighted heavily in developing subtropical breeding objectives, in order to help create the demand necessary to support the growing industry. Among the traits deemed important for consumer acceptance and repeat purchasing were: reliable good flavour (lower acid), good shelf life, and attractive appearance.

Pre-breeding objectives were also defined for resistance to the diseases *Macrophomina* (charcoal rot), *Fusarium* and *Colletotrichum*, as well as specific flavour traits and specialty fruit colours: white/pale pink and very dark red.

2.2.2. Generating genetic variation

Parents with desirable traits as defined by the breeding objectives were selected using breeding values calculated from quantitative genetic analysis of trial data. Crosses were generally designed to improve specific traits whilst maintaining overall acceptability of underlying traits. Cross design in 2017 was additionally informed using estimated maximum and minimum profitability of offspring from each potential parent pair, based on combining the best and worst traits from each in an economic model. Prior to their use in crosses, all parents were molecularly virus indexed to ensure that they were likely virus-free. This was undertaken as some viruses are seed-transmitted, and clean-up of infected plant material is expensive and can take several years. The number of

seedling families (unique parent pairs) and constituent parents planted in trials each season are shown in Table 2.1.

Table 2.1 For each subtropical production season: the number of seedling genotypes planted, number of seedling families and parents used, and the number of clones tested in early, advanced, and on-farm trials.

Location	Season	Seedlings	Families	Parents	Early clones	Advanced clones	1st stage on-farm clones	2nd stage on-farm clones
Nambour	2013	3626	121	80	104	97	2	6
	2014	7885	66	66	69	44	6	2
	2015	8027	84	41	146	23	0^1	0^1
	2016	7657	67	52	127	31	6	2
	2017	6084	73	51	216	13	3	2
Bundaberg	2013	924	5	4	0^2	0	0	0
	2014	1410	18	13	0^2	0	0	0
	2015	1491	18	19	0^2	0	0	0
	2016	3219	21	18	0^2	0	0	0
	2017	4334	34	28	0^2	0	0	0
Substrate	2015	0	0	0	0	5	0	0
	2016	0	0	0	0	5	0	0
	2017	152	7	4	12	6	0	0

[1] No 2015 on-farm trials due to disease.

[2] Bundaberg seedling selections retrieved annually for clonal testing at Nambour.

Crosses were conducted in insect-free glasshouse conditions, using a paint brush to apply pollen from dehisced anthers of the pollen parent to emasculated flowers of the maternal parent (Figure 2.1). Fruit were harvested once mature, blended with water in a blender, and then strained to separate out the seed. Due to recurrent issues with low germination success of seeds from the subtropical germplasm, seed was germinated in-vitro in tissue culture (see Section 12.2) in October each year. In December the germinated seedlings were transferred into 60 cell plug plant trays in 50/50, peat /vermiculite (size 3) mix and grown on under misters in a glasshouse for one month. Following this time they were transferred to a shadehouse for sun-toughening until being planted out in trials.

Figure 2.1 Transferring pollen from pollen parent flower (left) to emasculated maternal parent flower (right).

2.2.3. Assessment of genetic variation and selection of seedlings

Seedlings were planted into field trials each year at the Maroochy Research Facility, Nambour, Queensland (Figure 2.2), and Bundaberg Research Facility, Bundaberg, Queensland. In 2017 seedlings were also included in a small substrate (soilless culture) trial established at the Maroochy Research Facility. Approximately 10,000 seedlings were planted each year across all subtropical locations, with two plots per family per location. Seedling numbers planted in each trial in each season are shown in Table 2.1. Planting was undertaken in early March each year in Bundaberg, and mid-March for both field and substrate trials in Nambour. These timings are earlier than commercial plantings in order to drive the breeding objective of varieties with earlier fruit production.

Figure 2.2 Newly planted seedlings at Maroochy Research Facility, March 2018.

Assessment of seedling performance was undertaken via individual plant assessments in the field for highly heritable traits. These traits included fruit colour, firmness, shape, and display. Field assessments were conducted at three to four weekly intervals to allow a snapshot of current and future yield (via flower and bud production) for that time period to be estimated. At each assessment time plants thought to be performing well were flagged with a pin tag. At the end of the season plants with multiple pin tags were likely to have performed well across the season and were considered for further testing in early-stage clonal trials. Approximately 100-200 seedlings were selected each year.

In 2017, 152 seedlings were additionally planted in a small protected substrate trial at the Maroochy Research Facility. These were assessed similarly to the field trials described above.

2.2.4. Evaluation of progeny in early-stage clonal trials

The 100-200 seedlings selected for further testing each year across all subtropical trials were clonally propagated via runners in December/January each year. These were planted out as plugs into early-stage clonal trials at Nambour in mid-March of each year. Trials were randomised and replicated, with two reps of six plant plots per selection.

From the beginning of March each year fruit from all plots were harvested weekly. Fruit were counted and weighed, and assessed multiple times per season for the traits described in Table 2.2. Each week data was electronically entered and checked for consistency to ensure high data quality. At the end of the season all data was entered into a Katmandoo database containing all strawberry breeding trial data since 2004.

Table 2.2 Flower, fruit and plant traits assessed in field trials, and number of times traits were assessed per season for early and advanced stage clonal trials.

Trait group	Trait	Description	Unit	Approx. times assessed per season	
				Early clonal trials	Advanced clonal trials
Flower traits	Flower angle	Angle of flower emergence	Score 1-9[1]	1	1
	Flower display	Position (extension) of flowers relative to foliage	Score 1-9[1]	1	1
Fruit traits	Plot yield	Weight of harvested fruit per plot	g	Weekly	Weekly
	Plot fruit count	Count of harvested fruit per plot	Count	Weekly	Weekly
	Sugar (Brix)	Soluble sucrose content	°Brix	2	3
	Acid	Acid measurement	% Citric acid equivalent[2]	2	3
	Flavour	Flavour rating	Score 1-9[1]	3	3
	Appearance	Fruit appearance acceptability	Score 1-9[1]	4	4
	Fruit shape	Fruit shape code	Score 1-9[1]	2	2
	External colour	External colour rating (light to dark)	Score 1-9[1]	3	3
	Internal colour	Internal colour rating (light to dark)	Score 1-9[1]	2	3
	Gloss	External gloss rating	Score 1-9[1]	2	1
	Impact	Score of good visual impact after 2 days storage at room temperature	Score 1-9[1]	Weekly	Weekly
	Cull	Score of poor visual impact after 2 days storage at room temperature	Score 1-9[1]	Weekly	Weekly
	Bruise resistance	Fruit firmness measurement	0-100[3]	3	3
	Abrasion resistance	Abrasion resistance rating	Score 1-9[1]	3	3
	Rain damage resistance	Proportion of fruit undamaged by rain	Proportion	As available	As available
	Black spot resistance	Proportion of fruit unaffected by black spot	Proportion	As available	As available
	Sunburn resistance	Proportion of fruit undamaged by sunburn[4]	Proportion	As available	As available
	Ease of pick (roll)	Ease of fruit detachment using roll technique	Score 1-9[1]	1	1
	Fruit display	Position (extension) of fruit relative to foliage	Score 1-9[1]	1	1
	Truss type	Truss branching rating	Score 1-9[1]	1	1
Plant traits	Bush size	Bush size rating	Score 1-9[1]	1	1
	Leaf disease	Leaf disease rating	Score 1-9[1]	As available	As available
	Mite tolerance	Mite severity rating	Score 1-9[1]	As available	As available
	Runner number	Runner number rating	Score 1-9[1]	1	1
	Plant counts	Count of plants per plot	Count	4	4

[1] 1 = low/poor, 9 = high/good
[2] pH end point = 8.1
[3] 0 = very soft, 100 = very hard
[3] Assessed in temperate trials only

Data was analysed using a Quantitative Genetic approach to estimate breeding and clonal values. This approach incorporates plant pedigree, so that shared genetics between selections can be used to more accurately predict performance. It also removes small-scale environmental effects across the field that may obscure estimation of genetic potential. This technique allows the assessment of a selection's performance both as a clone (variety) and as a parent (the genetics it can contribute to its offspring) for each trait. Thresholds were established for each trait comparative to leading commercial varieties, and clonal values for each selection were run through the economic model to identify those most likely to have increased profitability for producers. Using this approach, 10-20 selections were chosen each year to progress to advanced-stage clonal trials.

2.2.5. Evaluation of progeny in advanced-stage clonal trials

Plant material for 10-20 selections from early-stage clonal trials were clonally propagated via runners in December/January each year. These were planted out into advanced-stage clonal trials at Nambour in mid-March of each year. Trials were randomised and replicated, with three reps of six plant plots per selection. Data collection and selection decisions were made as described in Section 2.2.4 and in Table 2.2.

2.2.6. 'On-farm' evaluation

Virus free plant material of selections from advanced-stage clonal trials (1-10 selections) were supplied most years to two producers to assess in first-stage on-farm trials (see Table 2.1). Based on these evaluations, plants of one to five of these selections were subsequently supplied to four to five producers the following year for second-stage on-farm trialling. Data was collected by producers on total and marketable yield per harvest (fruit number and weight), plant habit, fruit quality traits, and growing conditions. At the end of the season the breeding team provided all involved producers with a report anonymously summarising the results and outcomes from all collaborating farms.

2.2.7. Pre-commercialisation

A major barrier to the rapid release of new varieties is the time needed to certify disease-free status, requiring a minimum of three years before material can be distributed to runner growers. To reduce the time needed we routinely undertook molecular and biological virus indexing of all new advanced selections and established them in tissue culture prior to sending to Agribio for testing and certification. This substantially reduced the amount of testing and time required in the system before commercial deployment - from three years down to two - and presented significant cost savings. Virus indexing occurred simultaneously with the preparation of selections for testing in on-farm trials. Indexed material at the pre-commercialisation stage was also distributed to runner growers under Material Transfer Agreements (MTAs) for early bulking of plant numbers to speed commercial deployment.

2.2.8. International germplasm exchange

For the ASBP to remain cutting edge, collaboration and information exchange with international strawberry breeding programs and scientists is essential. In 2003 a seed exchange agreement was established between the Department of Agriculture and Fisheries and the University of Florida Strawberry Breeding Program. Each year approximately 1,000 seed are exchanged between the two breeding programs. This agreement has been extremely beneficial for maintaining genetic variation within our subtropical breeding program.

2.3. Results and discussion

Over the course of project BS12021 five subtropical strawberry varieties were commercially released. These were 'Red Rhapsody', 'Parisienne Kiss', 'Sundrench', 'Scarlet Rose-ASBP', and 'Sunglow ASBP'. Four additional varieties were accepted for PBR (Part I) in March 2018: 'Meadowsong', 'Venus-ASBP', 'Summer Song' and 'Scarlet-silk'.

The variety Red Rhapsody (breeding ID 2010-114, Figure 2.3) was accepted for Part I Plant Breeders Rights (PBR) in 2013 and PBR granted in 2016. This variety has had exceptional uptake by the subtropical strawberry industry and has been described as a "game changer" (Bugs for Bugs Berry Bulletin, October 2017). In 2017 plants of 'Red

Rhapsody' were first available in substantial numbers from runner growers and all plants were sold out. Plants of 'Red Rhapsody' represented 24% of all subtropical varieties grown in Australia in 2017. In 2018 this proportion is expected to further increase. This variety was the first to be selected using economic modelling of plant and fruit traits, and its success suggests that this approach is useful for selecting varieties with a profitable balance of production traits.

Figure 2.3 Fruit of commercialised variety Red Rhapsody.

There was initially some concern that the external fruit colour of 'Red Rhapsody' would be too dark to gain consumer acceptance. In June 2017 project co-lead Jodi Neal, Subtropical Industry Development Officer Jennifer Rowling and Queensland Strawberry Growers Association (QSGA) president Luigi Coco met with Woolworths representatives to discuss retailer and consumer education on the darker colour of 'Red Rhapsody' fruit. Despite some early concerns, Woolworths reported no issues with the variety over the course of the season, and were very pleased with its performance. They reported discard losses of only 1% for 'Red Rhapsody' compared with an internationally expected 7% for strawberries generally.

In 2015 the varieties Parisienne Kiss and Sundrench (breeding IDs 2011-049 and 2011-214, Figure 2.4) were accepted for PBR (Part I), and PBR was granted in 2016. At the current time these varieties have only been available with runner growers in small numbers, however uptake by industry, particularly of 'Parisienne Kiss', has been encouraging. In 2017 approximately 1,090,000 plants of 'Parisienne Kiss' were planted, and 360,000 plants of 'Sundrench'.

Figure 2.4 Plants and fruit of commercialised varieties Parisienne Kiss (left) and Sundrench (right).

The varieties Scarlet Rose-ASBP and Sunglow ASBP (breeding IDs 2013-055 and 2013-027, Figure 2.5) were entered into PBR (Part I) and had PBR granted in 2017. Neither of these varieties were available in commercial numbers in 2017, however early reports for the 2018 season show good uptake.

Figure 2.5 Fruit of commercialised varieties Scarlet Rose-ASBP (left) and Sunglow ASBP (right).

In March 2018 the varieties Meadowsong (breeding ID 2014-162), Venus-ASBP (2014-167), Summer Song (2015-237), and Scarlet-silk (2015-240) were submitted for Part I PBR (Figure 2.6). The latter two varieties were produced from crosses of subtropical germplasm and selected from a small trial at Applethorpe Research Facility, Applethorpe, Queensland. These two varieties also possess good resistance to charcoal rot (*Macrophomina phaseolina*). Small numbers of all four varieties are currently with runner growers and will be available in greater numbers over the next few years.

Figure 2.6 Varieties Meadowsong (top left), Venus-ASBP (top right), Summer Song (bottom left), and Scarlet-silk (bottom right).

In 2016 the University of Florida Strawberry Breeding Program released the variety Florida Beauty (Whitaker et al., 2017) (Appendix 1). This variety originated as seed produced at the Maroochy Research Facility and sent to Florida under the seed exchange agreement. 'Florida Beauty' has demonstrated adaptation to early planting, with excellent fruit quality.

2.4.   Conclusions

This project has produced excellent outcomes for the subtropical production region. Variety uptake from the project is extremely high, with 44% of all commercial subtropical plantings being ASBP varieties. Similarly Queensland strawberry industry support for the project is very high. In an email dated 24/10/2017 QSGA wrote: "QSGA congratulates the DAF breeding team, yourself [Mark] and Jodi on producing world class varieties for strawberry growers in Queensland and Australia. We doubt that there is any other adapted material available globally which can now match the top shelf varieties coming out of the ASBP subtropical node. We look forward to seeing the next tranche of genotypes, but are aware the bar has been set extremely high."

2.5.   References

Strawberry Strategic Investment Plan 2017-2021. Horticulture Innovation Australia, Sydney, Australia.

Whitaker, V.M., L.F. Osorio, N.A. Peres, Z. Fan, M. Herrington, M.C.d.N. Nunes, A. Plotto and C.A. Sims. 2017. 'Florida Beauty' Strawberry. HortScience 52:1443-1447.

3. Temperate trials and selection

Jodi Neal and Mark Herrington

3.1. Introduction

Temperate strawberry production is concentrated in the Yarra Valley in Victoria, the Adelaide Hills region in South Australia, Albany in Western Australia, and the Stanthorpe region in Queensland. Victoria has the highest production of these, contributing around 36% of national production in 2014/15 (Strawberry Strategic Investment Plan 2017-2021). The temperate season runs from approximately October to May each year. Specific challenges for temperate production include high summer temperatures, especially in Victoria, and a high incidence of plant deaths in the field from charcoal rot (*Macrophomina phaseolina*). The latter of these is exacerbated by susceptibility of the dominant commercial variety Albion to this disease (see Chapter 9). With the absence of effective disease control since the deregistration of the fungicide methyl bromide, a small but growing number of producers have been moving to production in substrate on tabletops.

In 2015 the Australian Strawberry Breeding Program (ASBP) began management of the existing Victorian Strawberry Breeding Program based at the Wandin Strawberry Research Centre, Victoria. This merging involved fusing the staff capacity, skills and germplasm of the Strawberries Australia Inc. (SAI) and Hort Innovation program centred in Victoria with the staff capacity, skills and germplasm of the subtropical program centred in Queensland and led by Queensland Department of Agriculture and Fisheries (DAF). Merging the programs has increased capability and efficiency, and built on the strong foundations laid by the Victorian program and its grower collaborators in Victoria, South Australia, Tasmania, Western Australia, and New South Wales/Australia Capital Territory.

3.2. Methods

The methods employed in the temperate breeding program were very similar to those described in Chapter 2. The strategy undertaken is outlined in Sections 3.2.1 to 3.2.7 below.

3.2.1. Streamlining the breeding cycle

From observations and discussions with the temperate industry we identified that the key requirement to ensure that advanced and advantageous germplasm from the temperate breeding program gets competitively into commerce, was to shorten the cycle from selection to deployment into commercial availability. The main constraint to reducing the time was identified as an extremely high level of virus infection among selections. In 2015 94% of plants tested were virus infected, and 50% of these had dual infections of Strawberry mild yellow edge virus (SMYEV) plus Strawberry mottle virus (SMoV). All nucleus plants made available to runner growers must first be certified as disease-free (including virus), and cleaning up of virus infected material can add up to five years to the time from selection to commercial deployment as well as significant expense. Limiting new virus infections within the breeding populations therefore became a key focus of the temperate breeding activities.

The key strategy employed to reduce virus incidence in the breeding population was to break the infection cycle across generations. Specific activities included the following:

- Removal of possible sources of infection (i.e. any plants from previous generations) from the field environment.
- Isolation (via quarantine into a screen house) of all known virus infected but required plants.
- Elimination of virus vectors i.e. transmitting insects.
- Rapid removal of non-selected plants and poor performing clonal selections from field trials throughout the trial season.
- Increased spacing of field plants to allow better spray penetration. This reduced the total number of plants that could be assessed but made evaluation more efficient.
- Termination of field trials as soon as selection decisions had been made. This involved removal of all remaining field plants and culled selections in runner production beds.

- Molecular virus screening of selections, and disposal of infected plants or isolation in vector-proof cages.

Historic germplasm was maintained, as much as possible, with the assumption that the previous program's more recently developed germplasm would be more advanced and desirable than older germplasm. Additionally all on-farm trials were initially suspended to avoid distributing virus to commercial farms in trial plants.

3.2.2. Breeding objectives

Breeding objectives for the temperate production region were developed based on consultation with the temperate industry and Strawberry Variety Steering Committee (SVSC). These discussions were ongoing over the course of the project to ensure that breeding objectives remained relevant to any changes in production environment or consumer expectations. Similar to the subtropical production region, the major aim for the temperate region was the development and availability of new varieties with increased profitability of production that met consumer needs.

Many of the traits identified as important for profitability in the subtropical economic model (Section 2.2.1) are also relevant for production under temperate conditions, however in discussion with industry some differences were identified. For in-ground production in Victoria especially, increased heat tolerance and fruit sunburn resistance are likely to have a large impact on yield and fruit quality, and so have been incorporated into the breeding strategy. Additionally, a number of temperate producers, particularly in Tasmania, have changed from in-ground production to substrate culture on tabletops. Plant and fruit requirements for this environment is subtly different to those for in-ground production (e.g. longer trusses and a more pressing need for high bruise resistance). These have now been accounted for in breeding objectives.

3.2.3. Generating genetic variation

Parents were selected each year based on breeding values for each trait generated through Quantitative Genetic Analysis of trial data. High performing parents were combined in crossing strategies aimed at producing offspring with traits matching the temperate breeding objectives. Seed was produced using the method described in Section 2.2.2. Seeds were soaked in 98% technical grade sulphuric acid for eight minutes, then rinsed in water and sown into shallow trays of seedling mix (sand with some peat moss and brown coal). Once germinated, seedlings were transferred to potting mix in 42 cell plug plant trays (Figure 3.1). The number of families and parents used in crosses each year are shown in Table 3.1.

Figure 3.1 Young seedlings being raised for the 2018/19 Wandin seedling trial.

Table 3.1 For each temperate production season: the number of seedling genotypes planted at the Wandin Strawberry Research Centre, number of seedling families and parents used, and the number of clones tested in early, advanced, and on-farm trials. On-farm trials were suspended until 2017 to avoid distributing virus to commercial farms in trial plants.

Season	Seedlings	Families	Parents	Early clones	Advanced clones	1st stage on-farm clones	2nd stage on-farm clones
2015-16	12069	89	21	115	11	0	0
2016-17	13739	23	12	127	4	0	0
2017-18	12979	21	15	85	11	14	0

3.2.4. Assessment of genetic variation and selection of seedlings

In April to May each year 12,000-14,000 seedlings from 20-90 families were planted into a seedling trial at the Wandin Research Centre (Table 3.1, Figure 3.2). Both short day and day neutral plants were planted at the same time, but slightly earlier than day neutrals are planted in commercial farms. This was done to drive selection for plants that have both the early yield of short day varieties as well as the consistent production of day neutral varieties. Families were randomised and planted into two replicate plots per trial.

Assessment of seedling trials began in October each year and continued until the end of February. While the commercial production season continues until May, the selection of trial material in early March allowed the strategic management of virus control and plant propagation. Selections were made using the strategy described in Section 2.2.3.

Figure 3.2 2016/17 seedling trial and early- and advanced-stage clonal trial (top right) at the Wandin Strawberry Research Centre.

3.2.5. Evaluation of early- and advanced-stage progeny in clonal trials

In April to May of each year, bare-rooted plants of 80 to 130 early-stage selections and 4-11 advanced-stage selections were planted into randomised, replicated clonal trials at the Wandin Research Centre (Table 3.1). Two replicate plots of six plants each were included for each of the early selections, and three replicate six plant plots for each advanced selection. Data collection, quality management, storage, and analysis was undertaken as described in Section 2.2.4 and Table 2.2. A simplified economic model was applied to the temperate selections based on their yields and Melbourne Market punnet prices over the trial season. Thresholds were also established for each trait comparative to the leading commercial variety 'Albion' to help guide selection decisions.

3.2.6. 'On-farm' evaluation

Evaluation of elite selections in on-farm trials was temporarily suspended in 2015 until virus-free material could be made available to distribute to commercial producers. In 2017, virus-free plants of 10 of the 14-series selections were distributed to four Victorian producers (the Temperate Node Reference Group).

In 2017 SAI also nationally distributed four earlier SAI/Hort Innovation selections for on-farm trials following ASBP's positioning of plants to facilitate this. Three of these selections were from the 05-series and one from the 08-series, and are discussed in more detail in Section 3.3.2 below.

3.2.7. Pre-commercialisation

Pre-commercialisation activities for the temperate material were undertaken as described in Section 2.2.7.

3.3. Results and discussion

3.3.1. Virus management

Significant progress has been made in reducing new virus infections in the temperate plant material since mitigation activities were first undertaken. In 2015, 94% of plants tested were infected, with 50% of these infected with two viruses (SMYEV plus SMoV). Under the strategies put in place, infections in 2016 were reduced to 33% and none of these were dual infections. In 2017 virus incidence was only 7%. Access to virus-free plants allowed on-farm trials to resume in 2017 for the 14-series selections, and this material is already positioned with runner growers and ready for commercialisation. By avoiding expensive virus clean-up the project has reduced the time from cross to potential commercial deployment from 10-13 years for the 05- and 08-series, to 4 years for the 14-series.

3.3.2. SAI/Hort innovation historic selections

The program facilitated the progress of four SAI/Hort Innovation selections made prior to 2015 which were at various stages in the testing and commercialisation process. Three of the selections are from the 05-series (short day: 05-027-001 and 05-028-055, day neutral: 05-069-063) and one is a day neutral from the 08-series (08-042-022) (Figure 3.3). Final virus testing of these selections was progressed by the ASBP. Positioning of these selections onto three commercial runner producers by the project has resulted in up to 6000 runners to be available for the 2017 planting season. These selections were distributed nationally by SAI for trial on producer's farms in the 2017/18 season, and may be progressed by SAI and Hort Innovation towards commercial release in the near future.

Figure 3.3. Selection 08-042-022, one of the SAI and Hort Innovation Victorian Breeding Program temperate selections positioned for commercialisation.

Following discussions with those familiar with SAI/Hort Innovation selections of the 07-series that were part way though virus testing, two of the three were withdrawn from the process 'as not likely to give an advantage'. The continuing selection 07-012-17 (short day) was subsequently found to be virus positive on the final test, and withdrawn from the positioning process.

3.3.3. ASBP selections

Ten advanced, 14-series selections are currently being trialled on farms in the 2017/18 season for assessment by producers (Figure 3.4). These 10 selections were chosen in collaboration with the industry's Temperate Node Reference Group, and feedback from producers collected at a field day at the Wandin Research Centre in November 2016. While trialling and selection decisions for the 14-series were made by ASBP, this material was crossed under the previous Victorian Breeding Program and is therefore the intellectual property of SAI and Hort Innovation. At present feedback from the Temperate Node Reference Group suggests that four of these selections may have some commercial potential. We have recommended that SAI and Hort Innovation pursue commercialisation of these as soon as possible to make them commercially available for the 2019/20 season.

Figure 3.4. Fruit from 'Albion' and one of the temperate 14-series selections currently being trialled on-farm and recommended for commercialisation.

Eleven advanced selections were made from the 15-series in March 2018 (Figure 3.5). These selections are currently being clonally propagated for distribution for on-farm trialling in the 2018/19 season.

Figure 3.5 One of the 15-series selections to be included in on-farms trials in the 2018/19 season.

3.4. Conclusions

Through effective virus management, the project has been successful in reducing the time from cross to potential commercial deployment from 10-13 years for earlier selections to 4 years for the 14-series. This will allow advantageous material to be released competitively into commerce, and has significantly reduced the cost of commercialisation. The project has facilitated the progress of eight SAI/Hort Innovation varieties to the point of commercialisation, and have recommended that SAI and Hort Innovation commercialise these as soon as possible to have them available for sale by runner growers in the August 2018 plant order period.

3.5. References

Strawberry Strategic Investment Plan 2017-2021. Horticulture Innovation Australia, Sydney, Australia.

4. Mediterranean trials and selection

Jodi Neal and Mark Herrington

4.1. Introduction

Mediterranean strawberry production is primarily located in the Wanneroo/Bullsbrook region north of Perth, Western Australia. While international export of strawberries is only around 4% nationally, approximately 90% of this is from Western Australia (Strawberry Strategic Investment Plan 2017-2021). Given the existing expertise and markets for export in the Mediterranean region, breeding for this area focused on designing varieties for international export. It was expected that this focus would allow capacity building and later inclusion of the subtropical and temperate production regions into a consolidated, integrated national export market.

The Australian Strawberry Breeding Program (ASBP) first began varietal development for the Mediterranean production region in 2015. The initial objective was to develop procedures for the successful establishment and assessment of seedling and clonal trials in the Wanneroo region, as well as retrieval and transport of selections back to the Maroochy Research Facility (MRF), Nambour, Queensland for virus testing, initiation into tissue culture, and potential distribution to runner growers and Agribio for disease-free certification.

4.2. Methods

4.2.1. Breeding objectives

The overarching breeding objective for the Mediterranean production region was to develop varieties suitable for international export. The traits necessary for this to be achieved were selected via discussion with Mediterranean industry members and the Strawberry Variety Steering Committee (SVSC), and included good shelf life, bruise resistance, and bright red fruit colour.

4.2.2. Generating genetic variation

High performing parents were combined in crossing strategies aimed at producing offspring with traits matching the Mediterranean breeding objectives. Seed and seedlings were produced at MRF using the method described in Section 2.2.2.

4.2.3. Assessment and selection of progeny from seedling and early-stage clonal trials

In 2015, 546 seedlings (Table 4.1) were transported from MRF to a producer's farm in the Wanneroo region for assessment. Seedlings were inspected at approximately monthly intervals over the production season (approximately April to October), and five selections made using the strategy described in Section 2.2.3. These selections all died subsequent to being transported back to MRF for clonal propagation, and an alternative strategy was devised for the 2016 season to avoid this issue.

The following season (2016), 1,054 seedlings were clonally duplicated prior to sending to Western Australia, and copies maintained at MRF for the duration of the season. Ten clonal selections from subtropical trials thought to have some potential for the Mediterranean region were also duplicated and sent to Western Australia (Table 4.1). Seedlings were assessed and selected as described above. Clonal plants were planted into two replicate plots of six plants each, and each plot harvested, weighed and fruit counted weekly. Fruit were also assessed on shape, firmness, flavour, and general commercial acceptability. At the end of the season 18 seedlings were selected to progress for further testing. None of the clones were deemed to have commercial potential and were therefore discontinued.

In 2017, 1,893 seedlings were sent from MRF to Western Australia and established in a seedling trial. Clonal plants of the 18 seedling selections from the previous year were also sent for trial (Table 4.1). Seedling and clonal selections were made as described above. Three clones were selected as being sufficiently promising to progress

to an advanced clonal trial and on-farm trials in 2018.

Table 4.1 For each Mediterranean production season: the number of seedling genotypes planted in Wanneroo, number of seedling families and parents used, and the number of clones tested in early, advanced, and on-farm trials. Due to issues with plant retrieval following selection, 2018 will be the first year that on-farm trials of Mediterranean selections will be conducted.

Season	Seedlings	Families	Parents	Early clones	Advanced clones	1st stage on-farm clones	2nd stage on-farm clones
2015	546[1]	18	13	0	0	0	0
2016	1054	7	9	10	0	0	0
2017	1893	17	15	18	0	0	0

[1] *Selections from this trial died following transit back to the Maroochy Research Facility for clonal propagation.*

While clonal duplication of seedling plants worked well the previous year, the resources required for this approach were prohibitive, and so a third alternative strategy was trialled. Seedling and clonal selections from the 2017 Mediterranean trials were retrieved and sent to a new Western Australia runner grower, Trandos Hydroponic Growers (THG), for clonal propagation for 2018 trials.

4.2.4. Pre-commercialisation

Pre-commercialisation activities for the three selections from the 2017 Mediterranean clonal trial were undertaken as described in Section 2.2.7.

4.3. Results and discussion

In order to provide an early outcome for the Mediterranean production region, the three clonal selections from the 2017 clonal trial were fast-tracked to 2018 on-farm trialling, and also submitted and accepted for PBR (Part I) in March 2018. These selections were 'Rosalie-ASBP', 'Jubilee-ASBP' and 'Fanfare-ASBP' (Figure 4.1). Small numbers of these varieties are currently with runner growers and will be available in greater numbers over the next few years.

Figure 4.1 Mediterranean varieties Fanfare-ASBP (left), Jubilee-ASBP (top right), and Rosalie-ASBP (bottom right).

4.4. References

Strawberry Strategic Investment Plan 2017-2021. Horticulture Innovation Australia, Sydney, Australia.

5. Screening for resistance to *Colletotrichum gloeosporioides*

Jodi Neal, Hian-Lien Ko, Apollo Gomez, Joanne De Faveri and Mark Herrington

5.1. Introduction

Anthracnose crown rot caused by the pathogen *Colletotrichum* is a major disease in strawberry (*Fragaria ×ananassa* Duch.) production worldwide, resulting in crown collapse and death of strawberry plants (Lewers et al., 2007, Mangandi et al., 2015). While several *Colletotrichum* species are known to cause anthracnose crown rot, the most common species observed infecting strawberry plants in Australia in recent years is *C. gloeosporioides* (Penz) (*Cg*) (Gomez et al., 2011, Mackenzie et al., 2009).

Cg is often referred to by its teleomorph name *Glomerella cingulata* (*Gc*), which indicates a sexual stage of *Cg*, and *Gc2* is the sub-group responsible for most of the recent infections in Australia (Gomez et al., 2012, Mackenzie et al., 2009). For simplicity both stages will be referred to as *Cg* in this report. In the field the source of this fungal disease is commonly other infected strawberry plants, however it has a broad host range including various commercially grown fruit and nut crops (Freeman, 2008, Lewers et al., 2007, Xiao et al., 2004) and weed species (Neal et al., 2017). Early work suggests that at least some isolates from alternative hosts can also infect strawberry (Neal et al., 2017). The pathogen is typically splash-dispersed, and frequent rainfall and overhead irrigation are therefore regarded as exacerbating dispersion (Ntahimpera et al., 1999, Simpson et al., 1994).

Several cultural practices and chemical treatments are available to reduce losses in strawberry crops, however these are only partially effective, and options are limited due to the number of permitted sprays per season (Hutton et al., 2009). Large numbers of plant deaths can occur in the field if susceptible cultivars are planted, potentially causing devastating economic losses (Hutton et al., 2010). The development and availability of strawberry cultivars with increased resistance to *Cg* is therefore desirable.

Several sources of resistance to *Colletotrichum* have been identified and reported, including a wild *F. virginiana* ssp. g*rayana* genotype 'NC95-19-1' (Lewers et al., 2007) and several breeding lines from Mississippi, USA (Galletta et al., 1993). High levels of crown rot resistance in the early Australian cultivar Phenomenal has also been observed (Neal et al., 2017). None of these lines are suitable for cultivation under today's standards, however they may represent a valuable resource for incorporating resistance into breeding programs.

Different strains of *Cg* with varying levels of pathogenicity have been found in different parts of the world. A local isolate may not have the same level of pathogenicity to a local cultivar compared to a different genotype under different conditions (Mackenzie et al., 2009).

The response of various strawberry genotypes, including commercial cultivars, clonal breeding lines, and seedling families resulting from controlled crosses, to Australian *Cg* isolates was determined in a series of experiments undertaken over a period of six years. Variation in pathogenicity among isolates and the interaction of strawberry genotype × *Cg* isolate were also evaluated. Times to plant collapse and death, and survival rates of the plants were monitored for 5-10 weeks per experiment. Introducing *Colletotrichum* resistance through breeding programs is a long-term disease management strategy and sources of resistance are useful for improving genetic material.

5.2. Methods

5.2.1. Experimental design

A series of 22 glasshouse inoculation trials were conducted between 2012 and 2017. These trials included three separate categories of experiments, namely *Cg* resistance screening of clonal strawberry genotypes (experiments 1-19), *Cg* resistance screening of seedling families (experiments 4, 5, 7, 9, 20-22), and isolate pathogenicity assessment.

Isolate pathogenicity assessment was undertaken using six *Cg* isolates (Table 5.1). Five to ten plants of each of eight genotypes ('Camarosa', 'Earlisweet', 'Kabarla', 'Phenomenal', 'Red Rhapsody', '2008-054', '3058-22-06', and 'NC95-19-1') were inoculated in each of two experiments (i.e. 10 and 17, in Table 5.2).

Table 5.1. Cg isolates used in the pathogenicity assays. All strawberry plants sampled were sourced from different runner farms.

Isolate no.	Host	Location of plants sampled and year[3]
NA1473	Strawberry[1]	Cooroy, Qld 1990[4]
N17360	Strawberry[1]	Wamuran, Qld 2008
N18931	Strawberry[1]	Bellmere, Qld 2009
N19211	Strawberry[1]	Stanthorpe, Qld 2010[4]
N19276	*Ozothamnus diosmifolius*[2]	Stanthorpe, Qld 2010[4]
NTW 121a/92	*Phyllanthus tenellus*[2]	Chevallum, Qld 1992[4]

[1] *Collected from crowns of symptomatic plant of Fragaria ×ananassa.*
[2] *Weed species with symptomatic lesions, from which Colletotrichum was recovered in laboratory isolations.*
[3] *Location of strawberry field from which plants were sampled.*
[4] *Runner production farm*

Disease resistance screening of strawberry genotypes for *Cg* isolate N19211 was conducted on 778 plants from 19 commercial cultivars currently or previously grown in Australia, as well as 4,046 plants from 258 breeding lines from the Australian Strawberry Breeding Program (ASBP). There were six to 40 replicate plants per genotype per experiment (Table 5.2). The cultivars Camarosa and Festival were used as susceptible controls, as they have previously been reported as highly susceptible to *Cg* (MacKenzie and Peres, 2007, MacKenzie et al., 2006, Peres and MacKenzie, 2012).

Within eight of the 22 experiments *Cg* resistance screening was also conducted on 1,594 seedlings from 34 families using isolate N19211 (Table 5.3). These plants were not clonally propagated and therefore were all genetically distinct. Seedling families were assessed in order to determine the most promising parent combinations/choices for *Cg* resistance. An additional 316 plants from 11 genotypes were included in the experiments and analysis as reference points for resistance.

5.2.2. Inoculation

Inoculum suspensions of six isolates of *Cg* (Table 5.1) were prepared at a spore concentration of 1×10^6 spores/mL according to the method of Neal et al. (2017). All plants were grown in 1:1 sterile peat and sand in 100 mm Spacesaver pots for six to 12 months prior to inoculation. Approximately 2 mL of inoculum was sprayed directly onto the crowns of each plant using a 500 mL hand/garden sprayer, to simulate field infection of crowns by *Cg* spores. Following inoculation individual plants were covered with a clear plastic bag for five days, creating a high humidity environment to encourage infection, and placed under shade cloth in an evaporatively cooled glasshouse set to reach a maximum of 30°C. The pots were arranged on a horticultural capillary mat on top of a 200 micron black plastic sheet, which covered a thermostatically-controlled electronic heat mat (Adloheat) maintained at 28°C and placed over a 25 mm Styrofoam sheet on the bench. Non-inoculated susceptible control plants of 'Festival' and 'Camarosa' were sprayed with sterile deionised water and subjected to the same conditions as the inoculated plants in the trial. Plant location within the glasshouse (experimental layout) and inoculation order was randomised for all experiments. Mortality events for individual plants were recorded on a weekly basis, starting at two weeks (14 days) post-inoculation and concluding at five to 11 weeks post-inoculation. Non-inoculated control plants were placed on a separate bench close to inoculated plants and not included in the analyses.

Table 5.2 Details of cultivars, number of plants per cultivar, number of breeding line genotypes evaluated, and total number of breeding line plants in clonal disease resistance screening experiments (including isolate x genotype).

Cultivar / breeding line	Experiment number and month and year conducted[1]																			Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
	Mar 2012	Apr 2012	Sep 2012	Apr 2013	Aug 2013	Nov 2013	May 2014	Sep 2014	Apr 2015	May 2015	Jul 2015	Oct 2015	Jan 2014	Feb 2016	Jun 2016	Sep 2016	Oct 2016	Apr 2017	Apr 2017	
Albion																12				12
Aussiegem																		12		12
Camarosa	8	8	7	10	12	40	6	10	10	6	12	10	10	10	10	10	6			185
Earlibrite												12								12
Earlisweet			17	10	12					6							6	12		63
Festival				10	12	6	6	10	10			12	10	12	12	9		12	12	133
Florida Radiance				10	12															22
Kabarla		6		10	12	6				6								6		46
Majestic		14																		14
Parisienne Belle						6														6
Parisienne Kiss						6												12		18
Phenomenal		20	17	10	12					6							6			71
Red Rhapsody				12	6					6		9					6			39
Redlands Crimson		20		10	12	23														65
Rubygem				10	12	6												12		40
Suncoast Delight						7														7
Sweet Charlie					8															8
Tioga		6																		6
Treasure			7	12																19
Total no. cultivar plants	**8**	**74**	**41**	**87**	**128**	**106**	**12**	**20**	**20**	**30**	**12**	**43**	**20**	**22**	**22**	**31**	**30**	**60**	**12**	**778**
No. breeding line genotypes	11	0	12	23	29	110	8	12	20	3	41	43	0	16	24	25	3	15	16	258
Total no. breeding line plants	71	0	127	207	346	684	48	217	344	18	480	446	0	192	267	254	18	177	168	4046
Grand total no. plants	**79**	**74**	**168**	**294**	**474**	**790**	**60**	**237**	**364**	**48**	**492**	**489**	**20**	**214**	**289**	**285**	**48**	**237**	**180**	**4824**

[1] 19 of 22 experiment conducted between 2012-2017 included clonal evaluations. See Table 3 for additional family assessments in three additional experiments. For practical reasons experiment number and year are not always sequential.

Table 5.3. Number of plants per family and per reference genotype evaluated in family disease resistance screening experiments using Cg isolate 19211. Genotypes are listed below the family totals.

Family/cultivar	Experiment							Total
	4	**5**	**7**	**9**	**20**	**21**	**22**	
	Apr 2013	Aug 2013	May 2014	Apr 2015	Jun 2013	Oct 2013	Sep 2016	
3058							8	8
3513		14		4				18
3519		21		9				30
3683		10		10				20
3685		26		19				45
3686		62		41				103
3689		5		3				8
3697		9		3				12
3701		15		14				29
3705		16		12				28
3715		3		3				6
3722		3		3				6
3726		7		6				13
3730		5		5				10
3733		5		3				8
3738		20		19				39
3740		8		2				10
3755						75		75
3756						48		48
3776					97			97
3782					67			67
3826					125			125
3827					89			89
3828					95			95
3830					171			171
3853					42			42
4110							73	73
4113							25	25
4118							237	237
4121							5	5
US 159-3499	8							8
US 292-3500	10		12					22
US 438-3501	10							10
US 70-3498			12					12
Total family plants	**28**	**229**	**24**	**156**	**686**	**123**	**348**	**1594**
Camarosa	10	27	12	10	6	10	10	85
Earlisweet	10		12					22
Festival	10		12		6	10		38
Florida Radiance	10		12					22
Kabarla	10		12					22
NC95-19-1	10		12			12		34
Phenomenal	10		12					22
Redlands Crimson	10		12					22
Rubygem	10		12					22
Sweet Charlie			8					8
Treasure	7		12					19
Total plants	**28**	**229**	**24**	**156**	**686**	**123**	**347**	**1593**

[1] *For practical reasons experiment number and year are not sequential.*

5.2.3. Statistical analysis

Genotype (cultivars and clonal breeding lines) and family data for isolate N19211 were analysed separately, as was the isolate pathogenicity data. A discrete time survival analysis based on Cox's proportional hazards model was performed on the mortality data (Neal et al., 2017, Southey et al., 2003). The data was coded with 0 for each week alive and then 1 when a mortality event occurred. For example a plant that was alive for four weeks post inoculation and then died in the fifth week would be coded as 0,0,0,0,1. Pedigree information which related the cultivars across the 22 experiments was included in the model for the genotype data so both additive and non-additive genetic effects could be predicted. Family data was analysed similarly but using a family model (with pedigree) rather than an individual model. Analyses were performed in ASReml-R using a binomial GLMM with complementary log log link function, and accounted for the different censoring times across experiments.

Hazard ratios were predicted for each genotype and family relative to the hazard of 'Camarosa'. The hazard gives the rate at which mortality events happen for each genotype/family. The hazard function may change over time but the proportional hazards model assumes the hazard for one genotype is a constant proportion of the hazard in the other genotypes. This proportion is called the hazard ratio (Duerden, 2014). Genotype hazard ratios greater than one suggest a higher rate of death (lower survival) than 'Camarosa'. Survival functions S(t), which is the probability a cultivar survives beyond time t, were also predicted for 'Camarosa' and the top five genotypes for disease resistance (Collett, 2003).

The isolate pathogenicity data was analysed using a discrete time survival analysis based on the proportional hazards model. The model included fixed effects for genotype, isolate, and genotype × isolate interaction.

5.3. Results

5.3.1. Isolate Pathogenicity

There was a significant (P<0.05) isolate effect and a significant (P<0.05) genotype effect but no significant interaction. Isolate N19276 had a significantly lower hazard (mortality) than the other isolates. The hazard ratios for each isolate with respect to isolate N19276 are shown in Figure 5.1. A hazard ratio of one suggests the same likelihood of mortality at any given time as N19276. One of the combinations ('Phenomenal' x isolate NA1473) showed no mortality over all plants. To avoid problems with predictions for this genotype and isolate, the predictions in Figure 5.1 are from the main effects model isolate + genotype.
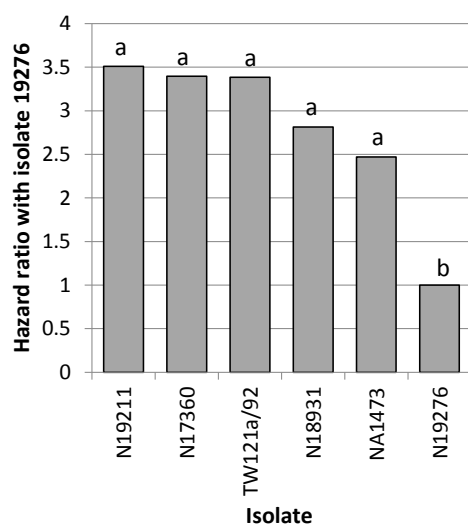

Figure 5.1 Hazard ratios relative to isolate N19276 for the six Cg isolates tested on cultivars 'Camarosa', 'Earlisweet', 'Kabarla', 'Phenomenal', 'Red Rhapsody', '2008-054', '3058-22-06', and 'NC95-19-1'. Higher values suggest higher pathogenicity. Hazard ratios with same subscript are not significantly different at the P = 0.05 level.

5.3.2. Genotype Screening

Significant additive genetic variance was detected in the genotype screening analysis. There was a small non-additive genetic variance component. Hazard ratios with the control 'Camarosa' are presented in Figure 5.2. These are based on the total genetic effects.

Narrow sense heritability for *Cg* resistance was relatively low at 0.23. Broad sense heritability is similar at 0.24, due to the limited non-additive genetic variance detected.

The five most resistant genotypes, from most resistant to least resistant, were: '3058-25M', 'US 438-3501(4)', '3058-22-04', 'US 438-3501(14)', '2014-243' (Figure 5.3). These genotypes had observed hazard ratios with 'Camarosa' of between 0.07 and 0.14, suggesting likelihoods of mortality at any given time of 7% and 14% of that observed for 'Camarosa'.

Parentage for the most resistant genotypes was strongly represented by progeny of open-pollinated 'US 438', resulting in the US 438-3501 population of which five of the ten most resistant genotypes were members. Also ranking well were offspring from '2013-001' (from open-pollinated 'NC95-19-1') crossed with 'Florida Radiance', resulting in the advanced breeding line '2014-243' and 12 of the top 25 genotypes for resistance.

The most resistant cultivars examined were 'Phenomenal', 'Earlisweet', 'Kabarla', and 'Florida Radiance'. The least resistant included 'Aussiegem', 'Treasure', 'Camarosa', 'Albion', 'Sweet Charlie' and 'Parisienne Belle'.
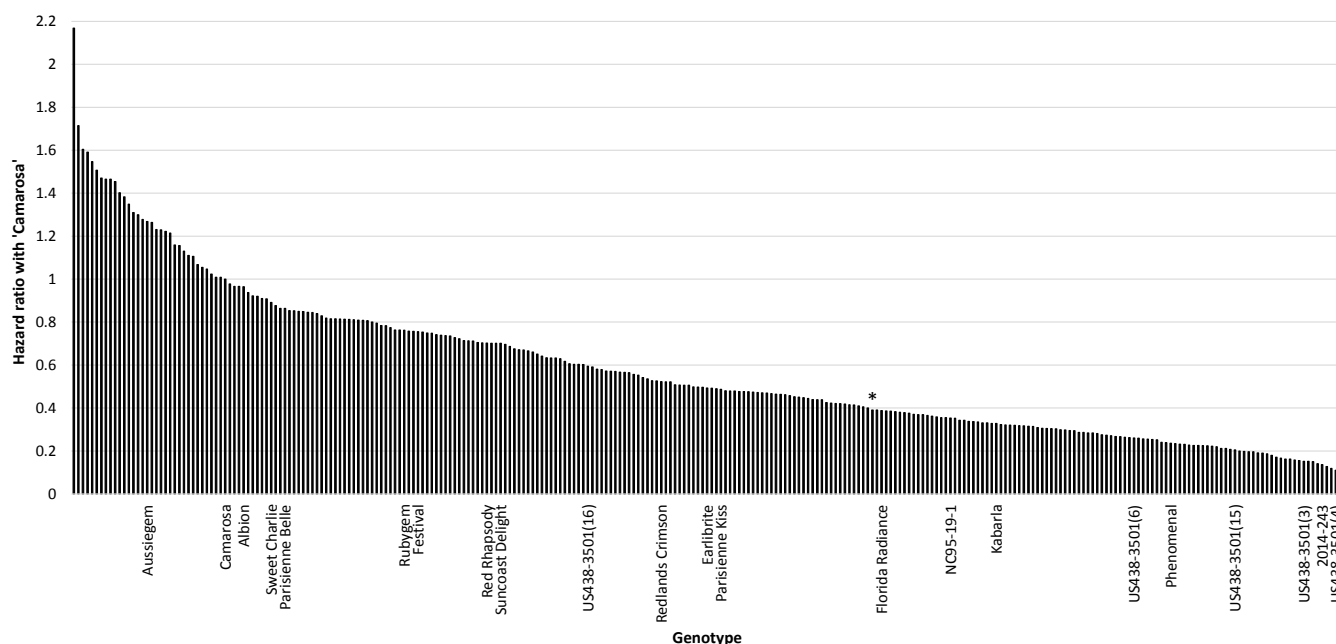

Figure 5.2 Hazard ratios with the control cultivar Camarosa for all genotypes assessed. All genotypes to the right of the asterisk have significantly lower hazard ratios than 'Camarosa'. For ease of reading, the only non-cultivar genotypes labelled on Fig. 2 are: cultivars, 'NC95-19-1' [a wild genotype with known resistance to Cg (Lewers et al., 2007)], elite breeding line '2014-243', and clones from the high performing family US 438-3051. The cultivars Treasure, Majestic, Tioga, Earlisweet and three members of family US 438-3501 are also not labelled for reasons of clarity. 'Treasure' is near 'Aussiegem', 'Majestic' is near 'Camarosa', 'Tioga' is near 'Florida Radiance', and 'Earlisweet' is near 'NC-95-19-1'. The three additional members of family US 438-3501 are in the cluster to the far right of the graph.
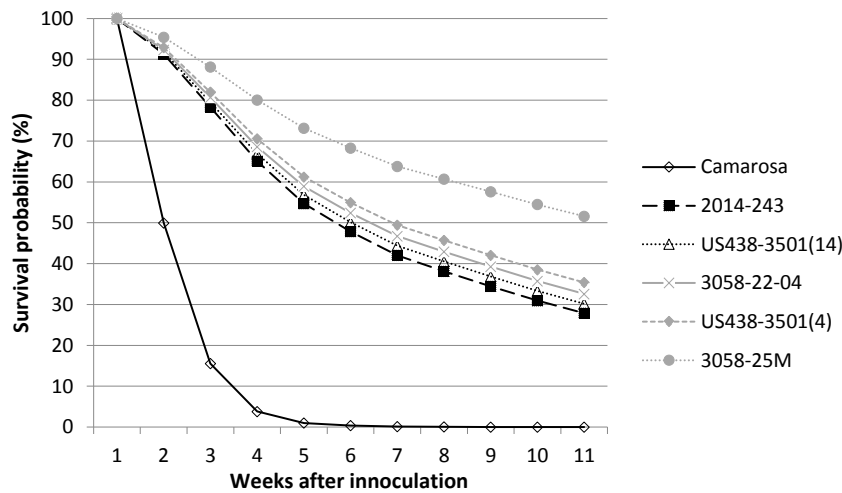
Figure 5.3. Survival function S(t), (i.e. the predicted probability a cultivar survives beyond time t), for the control cultivar Camarosa and the best five genotypes for disease resistance.

5.3.3. Family Screening

Hazard ratios of families with 'Camarosa' based on total genetic effects are presented in Figure 5.4. The most resistant family to *Cg* was 3726 ('3058-16' x '3058-16'), followed by 3738 ('Florida Radiance' x '3058-16') and 3058 (open pollinated 'NC95-19-1', of which genotype '3058-25M' is a member). Family US 438-3501 (open-pollinated 'US 438') ranked fourth.
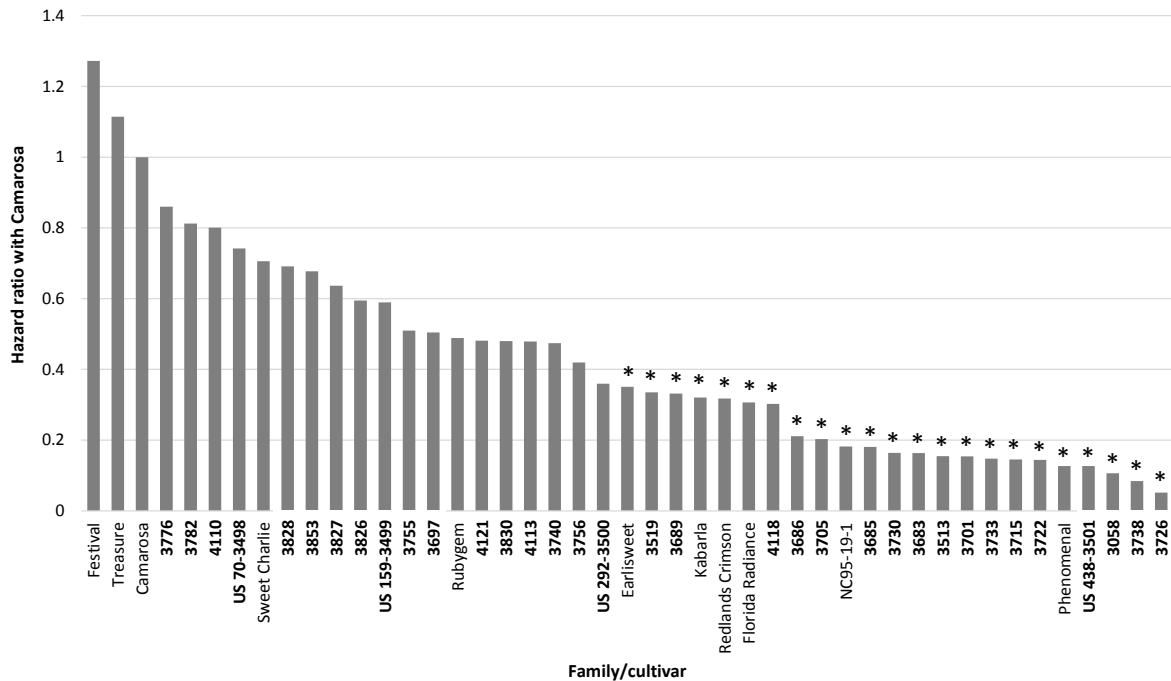

Figure 5.4 Hazard ratios with the control cultivar Camarosa for all families (bold font) and reference genotypes (regular font) assessed. Asterisked families and genotypes have significantly lower hazard ratios than 'Camarosa' (P<0.05).

5.4. Discussion

Results from the isolate pathogenicity experiments were similar to those from Neal et al. (2017). Isolate N19276 was collected from the non-strawberry host *Ozothamnus diosmifolius*, which may explain its lower pathogenicity to strawberry compared to the other isolates. This host is a native Australian Asteraceae cultivated for the cut flower industry (Ko et al., 1996). Similar to the findings here, MacKenzie et al. (2006), observed no genotype by isolate interaction from a series of experiments over three years across ten genotypes and 12 isolates sourced from Florida, USA. Australian isolates of *Cg* are also potentially less genetically diverse than those in the USA (Mackenzie et al., 2010). This lower diversity combined with the similar pathogenicity of all isolates other than N19276 suggest that the resistance screening results in this study from the single isolate N19211 are likely to be representative of a broader range of Australian isolates.

No cultivars to date have been reported to be immune to anthracnose crown rot (Mangandi et al., 2015), however North American studies have reported a range of differing responses. The cultivars Treasure, Festival, Sweet Charlie and Camarosa have previously been found to be susceptible to *Cg* crown rot (Chandler et al., 2000, Mangandi et al., 2015, Xiao et al., 2004). In this study 'Treasure', 'Sweet Charlie' and 'Camarosa' were similarly observed to have high susceptibility to Australian isolate N19211. Neal et al. (2017) found 'Festival' to be highly susceptible also, however with the inclusion of the additional data in this study it is now ranking as only moderately susceptible. By contrast, in a number of other studies 'Treasure' has been reported to show resistance to anthracnose crown rot (Chang, 2002, MacKenzie and Peres, 2007, MacKenzie et al., 2006). In the cases of Chang (2002) and MacKenzie and Peres (2007), however, the species of *Colletotrichum* was not specified.

'Phenomenal' ('Federator' x 'Pink's Prolific') was the most resistant named cultivar screened in our experiments. It was developed in 1906/07 in the Gympie region of Queensland, Australia (26.1834°S, 152.6657°E) by Charles A. Flay, and was the basis of the Queensland strawberry industry into the 1960s (Barnes et al., 2017, Darrow, 1966). In addition to showing high levels of resistance to *Cg* it also has resistance to *Macrophomina phaseolina* (Gomez et al., 2017). The assessed cultivars Redlands Crimson, Earlisweet, Kabarla, Aussiegem, Red Rhapsody and Parisienne Kiss have 'Phenomenal' in their ancestry. 'Earlisweet' and 'Kabarla' showed high resistance to *Cg*, 'Parisienne Kiss', 'Redlands Crimson' and 'Red Rhapsody' intermediate and 'Aussiegem' extremely low. Due to its high disease resistance 'Phenomenal' has recently been incorporated into the ASBP crossing program, and early data from a *Cg* inoculation field trial suggest it may be a promising parent.

In 2007 a wild population of *Fragaria virginiana* was reported to be resistant to American strains of *Cg* crown rots. This *F. virginiana* ssp. *grayana*, which originated from Monroe County, Mississippi was labelled 'NC95-19-1' in the USA germplasm (Lewers et al., 2007). While not possessing the traits necessary for agronomic success, 'NC95-19-1' has been incorporated into the ASBP as a parent to increase *Cg* resistance in the breeding population. This line is the female parent of the 3058 open-pollinated seedling population acquired from the United States Department of Agriculture National Clonal Germplasm Repository, Corvallis (Ore, USA). Its genotypic presence was evident in three out of the top five most resistant genotypes, namely clones '3058-22-04' and '3058-25M' and the breeding line '2014-243'.

Strawberry clones 'US 70', 'US 159', 'US 292' and 'US 438' have previously been reported as resistant to anthracnose crown rot incited by *C. fragariae*, and 'US 438' additionally to *C. acutatum* (Galletta et al., 1993). While these clones were not assessed for resistance to *C. gloeosporioides* in this study, open-pollinated offspring from 'US 70' (family US 70-3498) and 'US 438' (family US 438-3501) were assessed. A number of clones from both of these families showed moderate to high levels of resistance to *Cg*, suggesting that 'US 70' and 'US 438' may possess resistance to *Cg* in addition to the other *Colletotrichum* species reported. Despite this, family US 70-3498 was among the most susceptible overall of all families tested, suggesting that there may be segregation for a major gene effect in this family.

The absence of significant genotype x isolate effects, plus the spread of resistance levels within segregating families suggest polygenic resistance (Vanderplank, 1968) to Australian *Cg* isolates. For example, family 3058 (open-pollinated 'NC95-19-1'), included clones spanning the full spectrum from extremely high resistance to extremely low resistance. Despite relatively low heritability, breeding for increased resistance should be predictable. The recent addition of 'Phenomenal' into the ASBP breeding strategy may additionally incorporate different genetic sources of resistance to those already included from 'NC95-19-1' and 'US 438'. Being an historic Australian cultivar, 'Phenomenal' is likely to share different heritage and therefore genetic background to the North American 'US 438' and the wild Mississippi *F. virginiana* ssp. *grayana* 'NC95-19-1'.

While none of the breeding lines assessed here are agronomically suitable for commercial release, this study has allowed the identification of parents for improving varietal resistance to Australian *Cg* isolates by breeding programs. Resistance to crown rot caused by *Cg*, *C. fragariae* and *C. acutatum* have been found previously to be

highly correlated (MacKenzie et al., 2006, Peres and MacKenzie, 2012), and so the findings presented here may also be applicable to other countries and regions where other species of *Colletotrichum* are prevalent.

5.5. References

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6. Genetic variation in *Fusarium oxysporum* f. sp. *fragariae*

Michelle Paynter, Elizabeth Czislowski, Mark Herrington and Elizabeth Aitken

6.1. Introduction

Fusarium wilt of strawberry is caused by the fungal pathogen *Fusarium oxysporum* f. sp. *fragariae (Fof),* and is a major disease of strawberry worldwide. Soil-borne diseases including fusarium wilt have until recently been effectively controlled by pre-plant fumigation using methyl bromide/chloropicrin mixtures. With deregistration of methyl bromide for use in strawberry fruit production fields in 2005 however, fusarium wilt has become more prominent. Recently there have been high levels of plant death caused by fusarium wilt, mainly in Western Australia (Phillips and Golzar, 2008) but also in Southeast Queensland (Hutton and Gomez, 2010). In the production district of Western Australia, crown and root diseases of strawberry are considered a serious problem to strawberry production (Golzar et al., 2007). *Fof* has been reported as one of the most virulent pathogens causing crown and root diseases of strawberry (Fang et al., 2011), with the death of up to 50% of plants in the Perth district being attributed to *Fof* in the 2005 and 2006 season (Fang et al., 2010, Golzar et al., 2007, Phillips, 2008).

Fof is spread via infected planting material, contaminated implements, tools, pots and water (Burgess, 1981, Cooke et al., 2009). The spread and severity of disease is greater with the onset of hot wet weather (Hancock, 1999, Winks and Williams, 1965). *Fof* infects strawberry plants via the roots, moving into crown and vascular tissue (Figure 6.1) where it limits water and nutrient uptake. Plants will subsequently progressively wilt from the lower leaves to the top ones, eventually resulting in complete desiccation and plant death (Koike, 2009). This pathogen is difficult to eradicate and can remain viable in the soil as resting spores for many years (Smith and Snyder, 1975). To date, there is no cure for infected plants, and with increased outbreaks of this disease, genetic resistance is an economically viable method of control.

Figure 6.1 Strawberry crown showing vascular discolouration due to Fof infection.

F. oxysporum is sub-divided into *formae speciales* (form species) based on host specificity of isolates. The *forma specialis* of a *F. oxysporum* isolate has been typically assigned by pathogenicity tests and the ability of the isolate to cause disease symptoms. The pathogenicity of the pathogen is determined either by plant survival/death or by virulence, measured on a disease severity scale or index. Conventionally, the genetic diversity of *F. oxysporum* has been assessed by pathogenicity and by analysis of vegetative compatibility groups (VCGs), using nitrate non-utilizing (nit) mutants and pairing isolates with each other to verify VCG (Correll et al., 1987, Puhalla, 1985). Today, molecular techniques are commonly used to distinguish genetic variability among isolates and for phylogenetic analysis (Hyun and Park, 1996). A number of studies have shown the translation elongation factor 1-α (EF-1α) gene region to be useful for revealing genetic relationships among and within *formae speciales* of *F. oxysporum* (O'Donnell et al., 1998). Understanding the genetic diversity of Australian isolates is important for the breeding of strawberry cultivars resistant to fusarium wilt.

We were interested in characterizing variation and genetic diversity in Australian isolates of *F. oxysporum* harvested from strawberry plants to determine their virulence and genetic associations. As no research has been done with regard to the genetic variability of our Australian *Fof* strains, we tested 26 *F. oxysporum* isolates collected from regions in Australia representing locations of high intensity strawberry production. The majority of isolates came from regions within southeast Queensland where strawberry production is most intensive and from Western Australia where heavy infestations have been reported. Isolate virulence and genetic diversity were assessed by: virulence testing; analyses of VCGs; and sequence analysis of the translation elongation factor 1α (EF-1α) gene region.

6.2. Methods

Full details of the Methodology, Results and Discussion are described in our 2016 paper:

Paynter, M.L., Czislowski, E., Herrington, M.E., Aitken, E.A.B. 2016. Differences in pathogenicity, genetic variability and cultivar responses among isolates of *Fusarium oxysporum* from strawberry in Australia. Journal of the American Society for Horticultural Science, *141* 6: 645-652.

6.2.1. Pathogenicity tests

A sample of nine isolates were subjected to root dip inoculations (1 x 10^6 conidia/mL) on the susceptible cultivar Kabarla (Hutton and Gomez, 2006). A disease severity rating of 0 = healthy to 10 = dead was used to evaluate virulence (Figure 6.2). At 10 weeks post-inoculation diseased plants were sampled. *Fof* harvested from diseased crowns were used in a further virulence trial to verify pathogenicity. To confirm our results and fulfil Koch's postulates, five virulent isolates harvested from the crowns of plants from the pathogenicity test were chosen for virulence tests on the cultivar Kabarla. Isolate N17337 was chosen as a comparison.

Figure 6.2 External symptoms of Fof development in infected strawberry plant. Symptoms include stunting, lesions on petioles, necrotic spots, yellowing of leaves, wilting and plant death.

6.2.2. Vegetative compatibility group tests

Three biochemically different nitrate non-utilizing mutants (nit mutants) were obtained from potassium chlorate amended media. Sectors growing from restricted colonies, recognized by aerial mycelium (Figure 6.3a), were identified by phenotype when transferred onto minimal medium (MM) with one of three forms of a nitrogen source (NaNO2, NaNO3 and Hypoxanthine) in the media (Figure 6.3b). These were termed nit 1, nit 3 and Nit M respectively. All combinations of isolates were paired on MM to determine VCG. Samples paired were rated as belonging to the same VCG by a line of dense white aerial mycelium (heterokaryon formation) where the two isolates merge (Figure 6.3c).
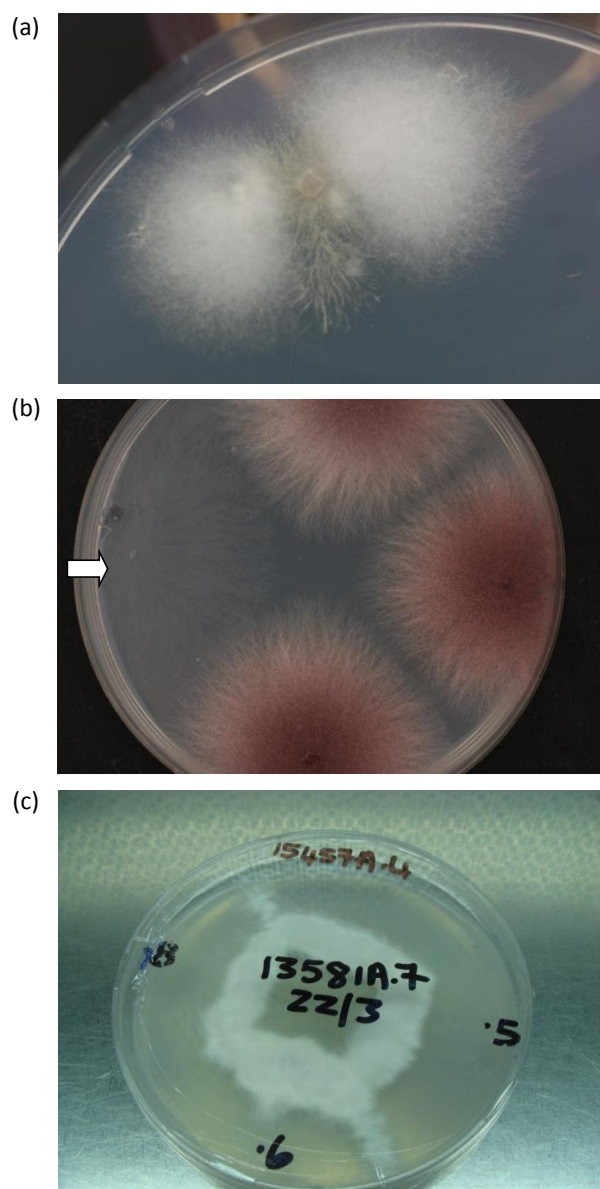
(a)

(b)

(c)

Figure 6.3. (a) Sectors of F. oxysporum *mycelia generated on MM media amended with potassium chlorate. Aerial mycelium produced from dense tightly packed sectors indicates a nit mutant. (b) Nit M (left, indicated by arrow) identified by sparse growth. (c) Heterokaryon formation formed by nit mutants belonging to the same VCG.Sequence and phylogenetic analysis*

6.2.3. DNA sequencing

DNA extractions of 26 *F. oxysporum* isolates were performed using a DNeasy Plant kit according to manufacturer's instructions (Qiagen, Victoria, Australia). The EF-1α gene region was amplified using forward and reverse primers EF-1 and EF-2 as described by O'Donnell et al. (1998) (Table 6.1). The EF-1α PCR products were sequenced in both directions using primers EF-3 (internal forward) and EF-22T (internal reverse) (Table 6.1). PCR product purification and sequencing were performed by Macrogen Inc. (Seoul, Korea). In order to understand the genetic relationships and evolutionary history of the *Fof* isolates, phylogenetic trees were constructed using the *F. oxysporum* EF-1α nucleotide sequences. Consensus sequences were produced using Geneious V7.1 (Biomatters Ltd, 2013) and aligned and edited using ClustalW in Geneious. Two sequences of EF-1α *Fof* downloaded from GenBank were included as comparisons; Maff744009 from Japan and KJ776745.1 from Turkey, and a sequence of EF-1α *F. oxysporum* f. sp. *verticillioides* (*Fov*) (KF466424.1) (GenBank) was used to root the data set.

Table 6.1 PCR and sequencing primers used to generate PCR products and for DNA sequencing.

Gene primer	Locus	Primer sequence (5' to 3')	Reference
EF-1	EF-1α	ATGGGTAAGGARGACAAGAC	O'Donnell et al., 1998
EF-2	EF-1α	GGARGTACCAGTSATCATGTT	O'Donnell et al., 1998
EF-3	EF-1α	GTAAGGAGGASAAGACTCACC	O'Donnell et al., 2008
EF-22T	EF-1α	AGGAACCCTTACCGAGCTC	O'Donnell et al., 1998

In order to understand the genetic relationships and evolutionary history of the *Fof* isolates phylogenetic trees were constructed using Geneious V7.1 (Biomatters Ltd, 2013). The EF-1α nucleotide sequences were rooted with *F. oxysporum* f. sp. *verticillioides* (KF466424.1) and inferred by the ML method based on the General Time Reversible model. Foz-BRIP39298 and N16004 were also included as outgroups. Branch lengths were measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6.

6.3.   Results and discussion

6.3.1. Pathogenicity tests

The rate and degree of symptom expression in plants varied among isolates (Table 6.2). It is apparent that there are significant differences in isolate virulence to strawberry. Plants inoculated with isolates N17337 and N13581 scored the highest disease severity ratings at 9.2 and 9.6 respectively. Isolates N13581, N17337 and N18462 were significantly more virulent than the lesser virulent isolates N15457, N15309 and N18582. There was no significant difference between the controls and N18421, N16004 and N18419. In our second test, confirming pathogenicity, there was no difference between the comparison isolate and re-isolate N17337a, showing the viability of the test.

Table 6.2. Disease severity visual ratings of F. oxysporum *isolates, taken at 8 weeks post-inoculation from virulence experiment. Ratings in right column represent those from re-isolation virulence tests.*

Isolate	Primary Disease Rating[z] (mean)(n=5)	Re-isolate code	Disease Rating[z] following re-isolation (mean)(n=6)	Pathogenicity rating[y]
n/a	n/a	**N17337**(comparison)	8.17_d	Pathogenic
N18421	0.6_a	**n/a**	n/a	Non-pathogenic
N16004	1.4_a	**n/a**	n/a	Non-pathogenic
N18419	1.4_a	**n/a**	n/a	Non-pathogenic
N18582	2.0_{ab}	**N18582a**	4.00_b	Pathogenic
N15457	3.4_b	**N15457a**	5.67_c	Pathogenic
N15309	7.2_c	**N15309a**	9.00_d	Pathogenic
N18462	7.8_{cd}	**n/a**	n/a	Pathogenic
N17337	9.2_d	**N17337a**	9.33_d	Pathogenic
N13581	9.6_d	**N13581a**	9.00_d	Pathogenic

Means with same subscript are not significantly different at P=0.05. LSD = 1.8
[z] Isolates were rated on a scale of 0-10, with 0=healthy and 10 = dead
[y] Isolates were considered pathogenic if plants had a mean disease severity rating >2

6.3.2. Vegetative compatibility group tests

Nit mutants were generated from all isolates. All mutants of an individual isolate were compatible with each other with the exception of six isolates which were designated as incompatible. Four isolates from the Sunshine Coast region were compatible with each other, showing a homogeneous population. These were grouped into VCGa. Similarly, all four isolates sampled from the Western Australia region belonged to the same VCG designated VCGb. The remaining 11 isolates, only compatible with one another, belonged to single-member VCGs.

6.3.3. Sequence and phylogenetic analysis

Twenty nine sequences were grouped into 10 lineages (Figure 6.4). There was no variation detected among the four EF-1a sequences in lineage (i), all of which were from Western Australia and comprised VCGb. Isolates from lineage (ii) were all closely associated and occurred with highest frequency, representing 34% of all isolates. Lineage (ii) included isolates from Queensland, New South Wales, and Japan. The lineages (iii) to (v) comprised isolates of heterokaryon self- incompatible (HSI) and single member (SM) VCGs, and originated from Queensland, South Australia, and Fof_KJ776745.1 sequence from Turkey. The outgroup Foz-BRIP39298 (*F. zingiberi*), was separate from the *Fof* isolates. The isolate N16004 was more distantly related to the other *Fof* isolates than Foz-BRIP39298.

Phylogenetic relationships among isolates resulted in several insights into the population profiled. Phylogenetic analysis of the EF-1α gene region clearly showed variation between isolates, and separated the isolates into three distinct clades and ten linages. Relationships within *Fof* were clearly defined by EF-1α analysis resolving interspecific and intraspecific genetic variation among the isolates. A close association was evident between VCG, EF-1α, and pathogenicity from the isolates profiled, and also between VCG and geographic origin.
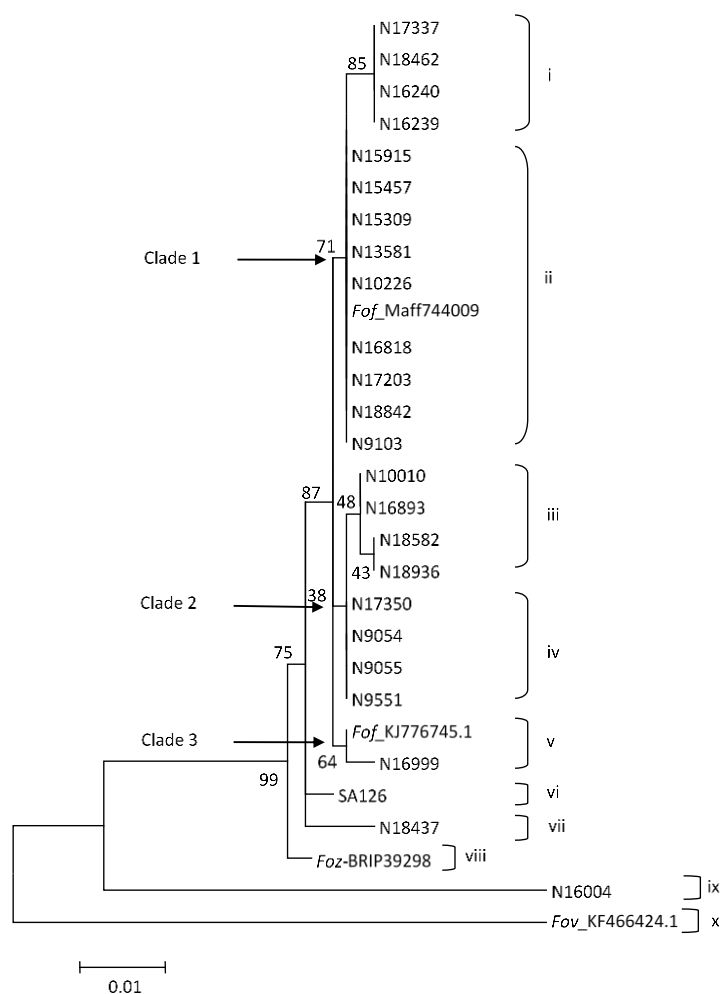

Figure 6.4. Phylogenetic tree of EF-1α sequences. Ten clonal lineages are identified by (i to x) and the three clades are indicated by arrows next to the corresponding interior branches. Numbers at the nodes represent bootstrap values from 1,000 replications.

6.4. Summary

Variation in the virulence of *Fof* strains is important when evaluating the resistance of plants to *Fof*. The results from the pathogenicity test showed a large variation in isolate virulence. The level of disease varied depending on isolate used, indicating heterogeneous populations within Australia. Genetic differences were identified through VCG and phylogenetic studies. Through characterization of nit mutants, two distinct VCGs were identified and corresponded to two of the 10 lineages identified by partial EF-1α.

6.5. Recommendations

As *Fof* is so varied in virulence, mutates easily (Burger et al., 2003), and is becoming a bigger threat to production in Australia, the continuation of pathogenicity testing of new pathotypes of *Fof* is recommended. It is also important to continue challenging cultivars and breeding lines for their resistance, so that the necessary information is available to make more informed decisions in selections for *Fof* resistance.

6.6. Refereed scientific publications

Paynter, M.L., Czislowski, E., Herrington, M.E., Aitken, E.A.B., 2016. Differences in pathogenicity, genetic variability and cultivar responses among isolates of *Fusarium oxysporum* from strawberry in Australia. *Journal of the American Society for Horticultural Science* **141**, 645-652.

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7. Cultivar responses to *Fusarium oxysporum* f. sp. *fragariae*

Michelle Paynter, Elizabeth Czislowski, Mark Herrington and Elizabeth Aitken

7.1. Introduction

Cultivar responses to *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) are important in resistance breeding and for the testing of pathogenic *Fof* strains. Knowledge of differences in the susceptibility of cultivars, and the presence of any cultivar x isolate interactions, allows breeders to select genotypes appropriate for use in transferring the resistance trait. It also provides growers with additional options for disease management strategies, i.e. using disease tolerant or resistant cultivars.

7.2. Methods

A subset of *Fof* isolates chosen from the pathogenicity test described in Chapter 6 were used in a glasshouse experiment to verify cultivar responses. Three virulent isolates from Queensland (N15309, N13581, and N18440) and one from Western Australia (N18462) were tested on eight cultivars: Camarosa, Earliblush, Festival, Fortuna, Kabarla, Redlands Joy, Rubygem and Sugarbaby. Nine plants (reps) per cultivar were inoculated in a randomized order (Figure 7.1). Six non-inoculated plants of each cultivar, dipped only in sterile water, were used as controls. A completely randomized design was used to place plants onto benches in a glasshouse. Disease severity ratings of 0 = healthy to 10 = dead were taken at weekly intervals post-inoculation.

Figure 7.1. Strawberry plants being root dip inoculated in Fof *conidia suspension at 1 x 10⁶ conidia/mL.*

Data were analysed in GenStat using ANOVA. Mixed models with smoothing splines were fitted to assess cultivar × isolate responses over time. Time intervals were equally spaced from 3 to 12 weeks post-inoculation and the intercepts were predicted at the midpoint of time (time 5).

For full details of methodology see manuscript:

Paynter, M.L., Czislowski, E., Herrington, M.E., Aitken, E.A.B. 2016. Differences in pathogenicity, genetic variability and cultivar responses among isolates of *Fusarium oxysporum* from strawberry in Australia. Journal of the American Society for Horticultural Science, *141* 6: 645-652.

7.3. Results and discussion

The assessed strawberry cultivars showed varying degrees of susceptibility to *Fof*, ranging from highly resistant to highly susceptible. Plants of the cultivar Fortuna showed moderate susceptibility across all isolates while cultivars Festival and Sugarbaby exhibited resistance (Table 7.1). The isolate N18462 from Western Australia was the most

aggressive isolate, resulting in higher disease severity ratings across most cultivars, with the exception of 'Festival' and 'Rubygem'. The cultivar Camarosa, which accounts for a high percentage of strawberries planted in Western Australia, was particularly susceptible to N18462.

Table 7.1 Means of visual rating for disease severity (where 0 = healthy plant, and 10 = plant dead), from eight strawberry cultivars inoculated with four Fof *isolates, taken at 12 weeks post-inoculation.*

Cultivar	Isolate			
	N13581	N15309	N18440	N18462
Camarosa	1.0_a	0.1_a	1.1_a	10.0_d
Earliblush	0.1_a	0.4_a	0.0_a	8.2_d
Festival	0.0_a	0.0_a	1.1_a	0.0_a
Fortuna	2.4_{abc}	3.9_{bc}	4.3_c	4.3_c
Kabarla	4.6_c	1.1_a	0.0_a	7.8_d
Redlands Joy	0.0_a	0.0_a	0.0_a	1.7_{ab}
DPI Rubygem	1.6_{ab}	1.1_a	0.0_a	0.0_a
QHI Sugarbaby	0.0_a	1.1_a	0.0_a	1.1_a

Means with same subscript are not significantly different at the P = 0.05 level. Standard error of differences: cultivar = 0.6, isolate = 0.4, cultivar x isolate = 1.3.

There was a significant (P<0.05) isolate effect, cultivar effect, and cultivar × isolate interaction by 7 weeks post-inoculation (i.e. cultivars performed differently in their disease response depending on which isolate was used). Fitting mixed models with smoothing splines showed significant cultivar × isolate interaction for intercepts, slopes and curvature of the splines (Figure 7.2).
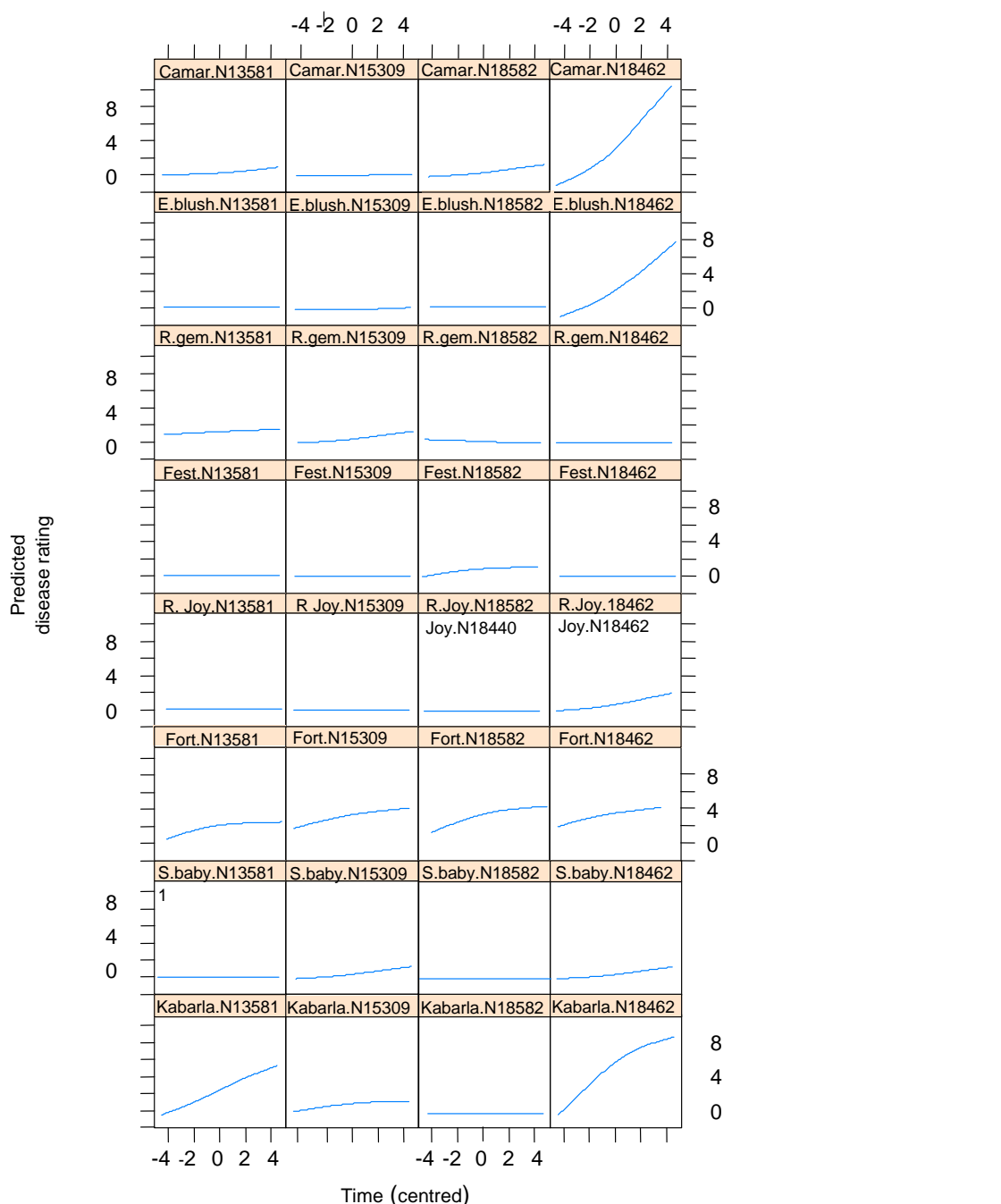
Figure 7.2 Analysis across times for cultivar × isolate response to Fof *using smoothing splines in ASReml. (Camar=Camarosa, E.blush=Earliblush, Fest=Festival, Fort= Fortuna, R. Joy=Redlands Joy, R.gem=Rubygem, S.baby=Sugarbaby).*

7.4.   Summary

The evaluation of strawberry cultivars showed varying degrees of susceptibility to *Fof* with some cultivar × isolate interaction. Cultivars 'Strawberry Festival', 'QHI Sugarbaby', and 'DPI Rubygem' had high levels of resistance across all isolates. Isolates from Western Australia were genetically distinct from those from Queensland and were more virulent to 'Camarosa', a major cultivar grown in Western Australia.

7.5.   Recommendations

Field management practices should ensure effective control across the whole production field and additionally avoid the spread of aggressive isolates e.g., 'N18462' to new localities.

7.6. Refereed scientific publications

Paynter, M.L., Czislowski, E., Herrington, M.E., Aitken, E.A.B., 2016. Differences in pathogenicity, genetic variability and cultivar responses among isolates of *Fusarium oxysporum* from strawberry in Australia. *Journal of the American Society for Horticultural Science* **141**, 645-652.

8. Screening for Fusarium wilt resistance

Michelle Paynter, Joanne De Faveri, Jodi Neal and Mark Herrington

8.1. Introduction

Due to increased disease outbreaks of the pathogen fusarium wilt of strawberry in Western Australia and Queensland, there is a need for the development of resistant cultivars. In order to develop new cultivars with increased *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) resistance, the strawberry breeding program screened genotypes from the subtropical breeding population to evaluate their potential for genetic gain.

This study identified and investigated two objectives relevant to the breeding of fusarium wilt resistance in strawberry. The first was to evaluate strawberry progeny for their susceptibility to *Fof*, and identify suitable parents for transferring the resistance trait. The second objective was to obtain estimates of genetic parameters (breeding values, variances and heritability) relevant to our population. In this study we predicted the potential of individual genotypes as a parent (through additive genetic effects), as well as the overall or total genetic effect of each genotype (by combining both additive and non-additive genetic effects), and provided estimates of both narrow- and broad-sense heritability.

8.2. Methods

To identify *Fof* resistance, three screening experiments were undertaken at the Maroochy Research Facility (MRF) in a secure glasshouse. Genotypes screened included selections from the subtropical breeding population and some cultivars. The screening process used plants clonally propagated by runners, aged approximately four months old with a small but well developed crown and a minimum of three to six mature leaves. The cultivars Kabarla and Festival were included in the screenings as controls. 'Kabarla' is known to be susceptible to *Fof* and 'Festival' resistant (Hutton and Gomez, 2006, Fang et al., 2012, Paynter, et al. 2016). See Appendix 1 for full methodology of the screening process. Two isolates of *Fof* were used as inoculum in the screening trials. Originally collected in Queensland, these were isolate N13581 harvested from 'Kabarla' in 2002 and N15309 harvested from 'Camarosa' in 2005. These isolates were initiated from storage in the MRF culture collection. Both isolates were identified as virulent, confirmed by pathogenicity tests performed at MRF, belong to the same vegetative compatibility group (VCG), and are genetically similar (Paynter, et al. 2016, Chapter 6). Isolates may lose their virulence due to cultural generation - caused by frequent sub-culturing on carbohydrate-rich media - and is common in *F. oxysporum* species (Leslie, et al. 2006). By using two isolates we safeguarded against loss of virulence.

Our previous work with *Fof* developed a strategy for increased efficiency of the inoculation process (Paynter and Herrington, 2016), addressing the need for an effective bioassay for the survival and development of *Fof* in the roots and crown. This involved inoculating rye grass seed to assist survival and proliferation of the pathogen (Smith, et al. 2008). The inoculation procedure used in our screenings combined the traditional root dip technique with the addition of sterilised ryegrass seed *Lolium perenne* L. cv. *Tetila*.

In order to prevent infection by other fungal pathogens, all plants used in the *Fof* screenings were subjected to a spray schedule of either the fungicide 'Switch' or 'Octave' for up to 4 weeks before inoculations and several times after. These fungicides are ineffective against *Fof*.

8.2.1. Experiment 1 – 2014 screening trial

In 2014, seventy eight genotypes and five cultivars were screened for their response to *Fof*. Plants were grown in 100mL pots containing steam-sterilised river sand and coir (1:1) with five plant replicates per genotype.

Isolates N13581 and N15309 were used in experiment 1 as inoculum. The isolates were plated onto 1/4 strength potato dextrose agar (PDA), and incubated in the dark at 27 °C for 2 weeks. Spores were collected from the plates by adding a small quantity of sterile deionised water and rubbing the agar surface with a glass spreader. The spore suspension was then filtered through four layers of cheesecloth. Conidial concentration was determined using a haemocytometer and adjusted to 1×10^6 conidia/mL using sterile water. Inoculum was used within three to five hours after preparation.

Prior to inoculation, five plants of each genotype/cultivar were removed from their pots, the crown and roots washed, drained, and placed into a plastic bag to keep moist and clean. Ten to twenty plants were immersed in the inoculum for 10 min in random order. Inoculum was replaced after every three to five immersions. Treated plants were placed back in their pots using potting medium previously described. Each pot was then randomly allocated onto one of five benches heated to 28 °C in a glasshouse with natural light and maintained to approx. 25 °C by a system of blowers and heaters. Disease development was monitored weekly and visual ratings taken after 14 weeks.

The severity of foliar *Fof* symptoms were assessed on a 0 to 10 visual rating scale:

0 = plant healthy, with erect growth and full vigour;
1 = plant generally healthy, with smaller canopy and moderate vigour;
3 = plant with slight wilt, with lower leaves affected;
5 = plant with moderate wilt, with the mature leaves collapse but young leaves still healthy;
7 = plant with severe wilt, with most of the plant collapsed and desiccated;
9 = plant with very severe wilt, with the entire plant collapsed and desiccated;
10 = plant dead.

Crowns were sampled for the recovery of *Fof* on plants of each genotype showing disease. The crowns were washed and surface sterilised in 0.5% sodium hypochlorite for 5 min, and rinsed three times in sterile water. They were then cut in cross sections and discoloured pieces plated onto 1/4 strength PDA and incubated at 27 °C in the dark. After 1 week, the plates were inspected for the presence of *Fof*.

Disease severity results at week 14 were analysed using REML (residual maximum likelihood) with a random table effect.

8.2.2. Experiment 2 - 2015 screening trial

Eleven genotypes were screened for their response to *Fof* in a 2015 experiment. The cultivars Kabarla and Festival were included in the screening and used as controls. Isolate N13581 was used as inoculum and prepared as described in Section 8.2.1. Disease scoring and analysis were also undertaken as per Section 8.2.1.

8.2.3. Experiment 3 – Genetic parameters

Full details of this experiment are described in our paper: Paynter, M.L., De Faveri, J., Herrington, M. E. (2014). Resistance to *Fusarium oxysporum* f. sp. *fragariae* and predicted breeding values in strawberry. *Journal of the American Society for Horticultural Science* **139**, 178-184.

In this experiment, we used progeny resulting from a partial incomplete diallel crossing to test for resistance to fusarium wilt, to obtain estimates of predicted breeding values and genetic parameters relevant to fusarium wilt resistance in our strawberry population. Two hundred and forty five progeny from 14 full-sib families were subjected to *Fof* (Table 8.1). Inoculation and assessment were undertaken as described in Section 8.2.1 above.

Analysis of disease response from severity ratings taken at 8, 10 and 14 weeks post inoculation were performed using a linear mixed model incorporating pedigree (inter-line relationships) on individual plant records. Pedigree information included up to four generations of ancestors. The model included terms for the random additive genetic effects for each of the genotypes (including parents), random family effects, residual non-additive genetic variance, replicate clone effects and table (location) effects. The analyses were performed using the ASReml-R package which provided residual maximum likelihood (REML) estimates of the variance components and Best Linear Unbiased Predictors (BLUPs) of the random effects in the mixed model.

Table 8.1 Crossing scheme used to produce progeny for Experiment 1. Indicated are family number, parents used, and number of strawberry progeny per family.

Family	Parent cultivars used in cross		Progeny (no./family)
	Female	**Male**	
2772	Festival	Festival	15
2773	Festival	Maroochy Jewel	26
2774	Festival	Kabarla	22
2775	Festival	Sugarbaby	27
2776	Maroochy Jewel	Maroochy Jewel	19
2777	Maroochy Jewel	Festival	10
2778	Maroochy Jewel	Kabarla	14
2779	Maroochy Jewel	Sugarbaby	23
2780	Kabarla	Kabarla	22
2781	Kabarla	Festival	19
2782	Kabarla	Maroochy Jewel	14
2783	Kabarla	Sugarbaby	22
2786	Sugarbaby	Sugarbaby	6
2787	Sugarbaby	Maroochy Jewel	6

8.3. Results

8.3.1. 2014 screening trial

There was a significant (P<0.001) genotype effect for disease severity at week 14. The majority of genotypes had low disease severity scores (i.e. high resistance) while several genotypes including 2011-101, 2009-030, 2011-208, and 2011-067 had high scores (i.e. low resistance, Figure 8.1).
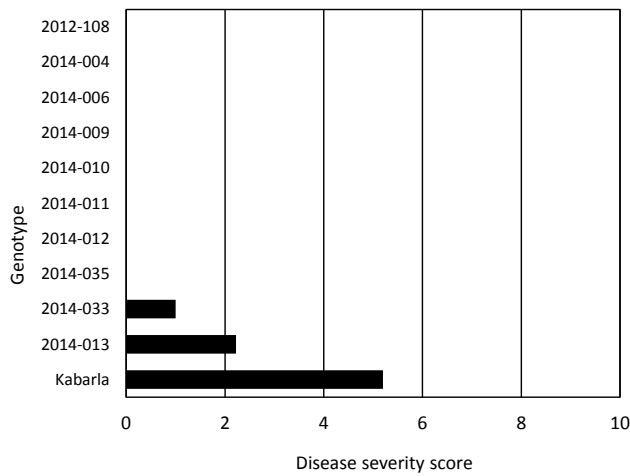
Figure 8.1 Predicted disease severity scores at week 14 for the 2014 screening experiment. Genotypes with values of zero are numerically then alphabetically sorted. Average standard error of difference = 1.5.

8.3.2. 2015 screening trial

In the 2015 screening trial, genotype 2014-035 showed wilting symptoms at 12 weeks but recovered 2 weeks later, suggesting that wilting may have been due to water shortage rather than *Fof*. One plant of 2014-033 suffered plant death, however the crown was very small at the time of planting and the plant died very soon post-inoculation. It is therefore likely that its death was not due to *Fof*.

There was a significant (P<0.001) genotype effect for disease severity. 'Kabarla' and 2014-013 had the highest disease severity scores (i.e. lowest resistance, Figure 8.2). All other genotypes with the exception of 2014-033 had predicted severity scores of zero.

Figure 8.2 Predicted disease severity scores at week 14 for the 2015 screening experiment. Genotypes with values of zero are numerically sorted. Average standard error of difference = 0.9.

8.3.3. Genetic parameters experiment

We observed varying degrees of susceptibility to *Fof* ranging from mildly resistant to highly susceptible among strawberry cultivars and progeny. Resistance appears to be under multigenic control.

Disease severity over time for all families is shown in Figure 8.3. Families with a low disease severity scores (i.e. showed good resistance) included 2772, 2775 and 2786, and were all from crosses involving 'Festival' and/or 'Sugarbaby'. Those with high scores (i.e. showed poor resistance) included families 2776, 2778, and 2782, derived from crosses involving 'Maroochy Jewel' and/or 'Kabarla'.

Figure 8.3 Mean disease severity over time for each family taken at weeks 4- 14 post inoculation (0 = healthy plant and 10 = dead plant). Dotted lines represent ±2 standard deviations from the mean.

Estimates of disease severity breeding values at week 14 post inoculation for the four parental genotypes ranged from 2 to 9.6 (Figure 8.4). Higher breeding values signified cultivars likely to pass on susceptibility to the next generation. Low values indicated cultivars likely to pass on good resistance. 'Maroochy Jewel' and 'Kabarla' were the most susceptible as parents while 'Festival' and 'Sugarbaby' were the most resistant.
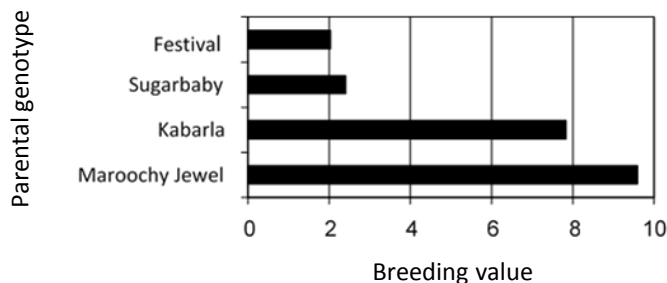

Figure 8.4 Predicted breeding values for severity of disease symptoms in strawberry caused by Fof *for parents, taken at week 14 post inoculation. Parent average standard error of differences = 0.78.*

Mean breeding values for disease severity for each of the 14 families encompassed a broad range, from 1.52 to 9.43 (average standard error of difference equal to 0.55) (Figure 8.5). Results were similar to those reported above, with low disease severity score observed for families 2775, 2786, and 2772, and high scores for families 2776, 2782, 2778, and 2780.
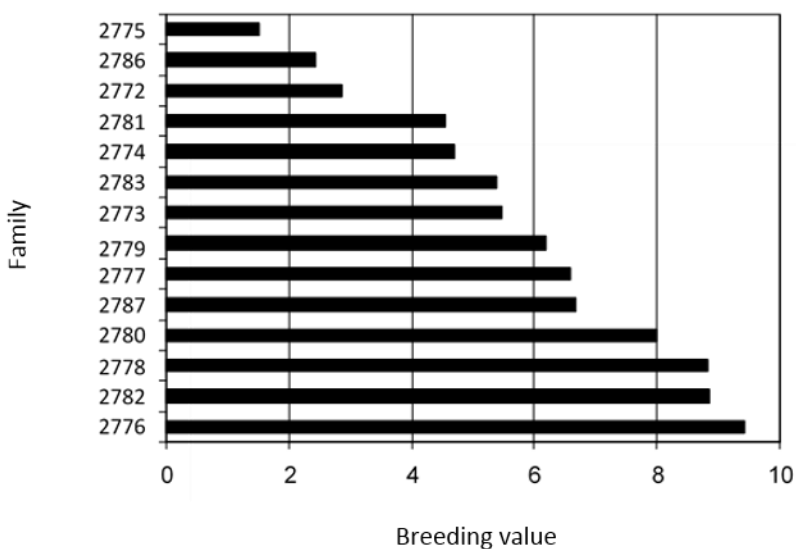

Figure 8.5 Predicted breeding values for severity of disease symptoms in strawberry caused by Fusarium oxysporum *f. sp.* fragariae *for each family taken at week 14 post inoculation. Family average standard error of differences = 0.55.*

Breeding values across the 245 individual genotypes ranged from 0.62 to 10.15 (average standard error of difference equal to 2.00). Among the most resistant 10% of progeny, the five best genotypes for resistance included: 2772-14, 2772-15, 2772-05, 2786-01 and 2775-30 (Figure 8.6). Genotype 2772-14 had the lowest disease severity breeding value at 0.62.
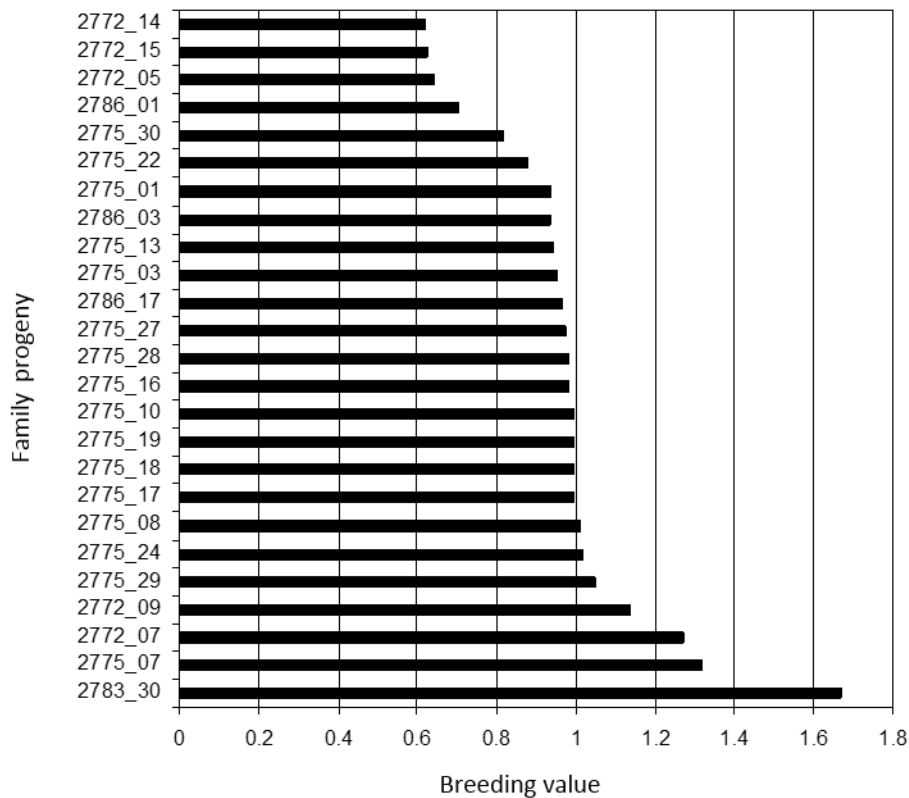
Figure 8.6 Predicted breeding values for severity of disease symptoms of the most resistant (lowest symptom expression) 10% of strawberry progeny to Fof *at 14 weeks.*

Narrow-sense heritability was estimated at 0.49 (±0.04) for the severity of disease, indicating that the observed phenotypic variation was moderately influenced by genetic factors. This suggests that resistance to *Fof* can be improved via phenotypic selection. Broad-sense heritability was estimated at 0.50 (±0.04).

The influence of time of assessment on breeding values was also investigated. There was a high correlation between breeding values among the assessment dates of 8, 10, and 14 weeks after inoculation (Figure 8.7).
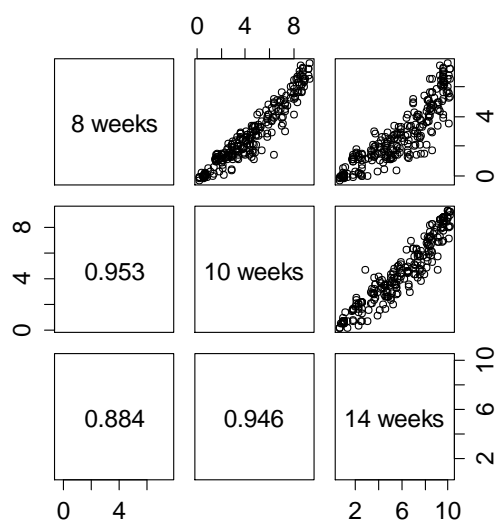

Figure 8.7 Relationship between disease severity breeding values at 8, 10 and 14 weeks following inoculation of strawberry progeny to Fusarium oxysporum *f. sp.* fragariae*. Above diagonal shows pair-wise plots, below diagonal gives corresponding correlation coefficients.*

8.4. Discussion

Overall, a large proportion of the genotypes screened in 2014 and 2015 displayed high levels of resistance to *Fof*. The recent cultivar releases 'Red Rhapsody' and 'Parisienne Kiss' from the Australian Strawberry Breeding Program both displayed disease severity scores of zero, suggesting that both have high resistance to *Fof*.

From our population, individuals in families 2772, 2775, and 2786 had the lowest breeding values for disease symptom severity, and are therefore likely to be the best parents for conferring disease resistance. There were many progeny that were predicted to have a better breeding value than any of the parents. For example, genotypes 2775-30, 2775-22, and 2775-13 possessed breeding values of 0.82, 0.88, and 0.95 respectively, whereas their parents 'Festival' and 'Sugarbaby' had breeding values of 2.04 and 2.42 respectively.

Several of the progeny most suitable as future parents were the result of self-pollination (e.g. 2772-14, 2772-15, and 2772-05 from 'Festival' and 2786-01 from 'Sugarbaby'). This suggests different loci for resistance may be present in the cultivars Festival and Sugarbaby, and that the number of progeny was not large enough to fully capture the range of recombination between the loci involved in these two parents. It is reasonable to expect that advantageous transgressive segregants will arise with recombination of additive genes among these parents. In support of this the moderate narrow-sense heritability (0.49) implies substantial additive gene action. Self-pollination to recover transgressive segregants, by concentrating alleles, may hold promise for improving the breeding values of parental lines. In this case improvements in breeding lines could be made using smaller populations.

This knowledge can be used to enable better predictions about progeny response to selection for the resistance trait and assist in the breeding of strawberry cultivars with increased resistance to fusarium wilt.

8.5. Refereed scientific publications

Paynter, M.L., De Faveri, J., Herrington, M.E., 2014. Resistance to *Fusarium oxysporum* f. sp. *fragariae* and Predicted Breeding Values in Strawberry. *Journal of the American Society for Horticultural Science* **139**, 178-184.

Paynter, M.L. and Herrington, M.E., 2016. Development of a glasshouse bioassay suitable for evaluating Fusarium wilt resistance in strawberry. *Acta Horticulturae* **1127**, 125-132.

8.6. References

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Leslie, J.F., Summerell, B.A., Bullock, S., 2006. The Fusarium laboratory manual, Vol. 2, No. 10, Blackwell Publishing Ltd, USA.

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Paynter, M.L. and Herrington, M.E., 2016. Development of a glasshouse bioassay suitable for evaluating Fusarium wilt resistance in strawberry. *Acta Horticulturae* **1127**, 125-132.

Smith, L.J., Smith, M.K., Tree, D., O'Keefe D., Galea. V.J., 2008. Development of a small-plant bioassay to assess banana grown from tissue culture for consistent infection by *Fusarium oxysporum* f. sp. *cubense*. *Australian Plant Pathology* **37**, 171-179.

9. A review and evaluation of methods to screen strawberry plants against charcoal rot

Apollo Gomez, Joanne De Faveri, Jodi Neal and Mark Herrington

9.1. Introduction

Approximately 10-12 years ago, detections of a crown rot disease called charcoal rot, caused by *Macrophomina phaseolina*, was reported causing significant losses in commercial farms in Queensland, Victoria and Western Australia (Golzar et al., 2007; Hutton et al., 2013). In 2006, Hutton et al. (2013) observed a commercial farm in Northern Victoria with more than 50% deaths due to charcoal rot. Similarly in Queensland in 2009, a Sunshine Coast producer also had more than 50% plant loss. In subsequent years, *M. phaseolina* has been regularly isolated from diseased plant samples submitted to the Department of Agriculture and Fisheries, Plant Pathology Group in Nambour, Queensland (Gomez, unpublished data). More recently, growers particularly in the Yarra Valley in Victoria and the Granite Belt district in Queensland, continue to experience significant plant losses due to charcoal rot in the 2017-18 season (Gomez, unpublished data; Mattner, personal communication). The disease has also been reported in other strawberry producing countries including Argentina (Baino et al., 2011), Chile (Sanchez et al., 2013), Israel (Zveibil and Freeman, 2005), Spain (Aviles et al., 2007), Turkey (Yildiz et al., 2010) and the United States (Mertely et al., 2005; Koike, 2008a). Sanchez et al. (2016) described *M. phaseolina* is an emerging and devastating pathogen of strawberry.

The fungus is a soil-borne pathogen that infects a wide host range among cultivated crops, such as sorghum, beans and maize (Dhingra & Sinclair, 1978), and also several weed species (Fuhlbohm et al., 2012). Researchers in Israel found that isolates from other hosts rotated with strawberry crops were pathogenic to strawberry, and emphasised the importance of avoiding rotations of crops that may host the pathogen (Zveibil et al., 2012). In Queensland, *M. phaseolina* has been isolated in a sorghum species used as a cover crop between strawberry seasons (Gomez, unpublished data).

Strawberry plants affected with charcoal rot show symptom progression of wilting of foliage, drying and death of older leaves with younger leaves often remaining alive, followed by complete plant collapse and subsequent plant death (Koike et al., 2013; *Figure 8.8*). When affected crowns are cut open longitudinally, dark brown necrotic areas in the internal cortex and vascular tissues are observed (Koike et al., 2013; *Figure 8.9*).

Figure 8.8 Strawberry plant collapse caused by charcoal rot.

Figure 8.9 Necrosis in internal cortex and vascular tissues of a strawberry plant crown.

M. phaseolina produces resting structures called microsclerotia, which are made up of branches of hyphae growing towards each other forming black, round to irregularly shaped structures held together by a melanin-like substance (Dhingra & Sinclair, 1978; Gangopadhyay and Wyllie, 1974). Microsclerotia allow the pathogen to survive in infected crop debris and in soil. *M. phaseolina* microsclerotia survived for at least 21 months in buried bean crop residues (Songa and Hillocks, 1998), and for up to three years in soil (Olaya and Abawi, 1996) and are considered highly resistant to unfavourable climatic conditions.

The disease is favoured by high soil temperatures (>27°C) (Strand, 2008). The average monthly maximum air temperatures in 2016 in strawberry producing areas in south-east Queensland (winter months) and Victoria (summer months) during strawberry production were 27 to 31°C and 33 to 42°C, respectively (Bureau of Meteorology website www.bom.gov.au). In addition, soil temperature under black-coloured plastic mulch at a depth of 5 cm is between 6.5 and 9.0°C higher than the air temperature (Hutton and Gomez, 2006).

Several reports on the increased incidence of charcoal rot worldwide has been attributed to the ineffectiveness of current soil fumigants, following the loss of methyl bromide in strawberry production (Mertely et al., 2005; Aviles et al., 2007; Koike, 2008b; Zveibil and Freeman, 2009). This is supported by previous research work done in Queensland by Hutton et al. (2013) that showed current soil fumigants, including chloropicrin and 1, 3-dichloropropene did not eradicate *M. phaseolina* in buried infected crowns.

With a wide host range, an ability to produce survival structures and withstand high soil temperatures, and the ineffectiveness of current soil fumigants, a management strategy of developing resistant cultivars has become increasingly important. Non-chemical approaches must be considered and developed, including the use of plants resistant to *M. phaseolina* (Aviles et al., 2009). The identification and development of resistant strawberry cultivars is considered the most cost effective and sustainable strategy for control of crown and root diseases of strawberry (Mackenzie et al., 2006). Maas (2012) highlighted the need to integrate and place greater emphasis on host-plant genetics and pathogen studies in the future to achieve healthy, disease-free plants and maximise strawberry production.

Breeding programs internationally now focus on host plant resistance and the importance of identifying the resistance of existing strawberry genotypes to soil borne diseases (Holmes et al., 2017). As part of the Australian National Strawberry Varietal Improvement Program, screening of current strawberry cultivars and breeding lines were conducted to identify potential resistance to *M. phaseolina*. In order to conduct screening tests, we first had to investigate inoculum production and inoculation methods that could potentially be used to screen both small scale (<50 plants) and larger scale inoculations (several hundred) of strawberry plants at a given time.

In this study, we report on developing a reliable and consistent method for *M. phaseolina* inoculum production and inoculation for future screening tests based on a review of previous methods reported, and assessing two different inoculum production and inoculation methods. In addition, experiments also investigated the susceptibility of strawberry cultivars Camarosa and Albion. A number of studies have observed that 'Camarosa' appears to be susceptible to *M. phaseolina* (Aviles et al., 2007; Baino et al., 2011; Koike, 2008a; Fang et al., 2011). For 'Albion', studies in California have found that it may also be susceptible to charcoal rot (Koike, 2008a; Koike,

2009). A study in Western Australia evaluated both cultivars and found 'Camarosa' was susceptible and 'Albion' was resistant when inoculated with *M. phaseolina* (Fang et al., 2012). The conflicting reports on 'Albion' warranted further testing to confirm the susceptibility of 'Albion' to an Australian isolate of *M. phaseolina*. The results of this study will aid the Australian National Strawberry Varietal Improvement Program in developing commercial breeding lines with resistance to *M. phaseolina*.

9.2. Review of *M. phaseolina* inoculum production and inoculation methods in strawberry

One common screening method for strawberry is the use of *M. phaseolina* colonised toothpicks. After the first detection of charcoal rot in Florida, Mertely et al. (2005) conducted a greenhouse trial to confirm pathogenicity by inserting a toothpick colonised with *M. phaseolina* into the crown of healthy strawberry plants. The toothpicks were sterilised three times, twice in deionised water and a third time in V8 juice. *M. phaseolina* isolates were grown on corn meal agar at 24°C. After four days, toothpicks were placed on the media and allowed to colonise for an additional five days. Six plants were inoculated by inserting a *M. phaseolina* colonised toothpick into the crown. Control plants were inoculated by sterile toothpicks without the pathogen. Wilting symptoms in one or more leaves were observed after three days. All inoculated plants collapsed or died within two weeks of inoculation, while plants inserted with non-inoculated toothpicks remained healthy.

A number of strawberry screening tests internationally adopted the methods of Mertely et al. (2005). Aviles et al. (2007) first reported *M. phaseolina* affecting strawberries in Spain. In their pathogenicity studies, toothpicks colonised by single-microsclerotia isolates were used to inoculate strawberry plants. After 58 days, mortality was between 67 and 100%. In California, Koike (2008a) was able to demonstrate plant mortality by four weeks after inoculations. In Argentina, Baino et al. (2011) observed signs of wilting at six days and 90% of the inoculated plants wilted within two weeks following inoculations. In previous research conducted in Queensland, Hutton et al. (2013) confirmed pathogenicity in strawberry plants using the toothpick method, but prepared *M. phaseolina* isolates on quarter-strength potato dextrose agar (PDA) amended with streptomycin sulphate, instead of corn meal agar.

In Israel, Zviebil and Freeman (2005) conducted pathogenicity tests by pouring *M. phaseolina* microsclerotia suspension into each pot of strawberry plants. The *M. phaseolina* inoculum was produced from single-microsclerotium isolates grown for three weeks on potato dextrose agar (PDA) plates and blended with 100 mL sterile distilled water. The suspension was filtered through eight layers of gauze and adjusted to a concentration of 1×10^5 sclerotia per mL. Each plant was grown in 1 L pots and inoculated by pouring 50 mL of the inoculum into the pot. Inoculated plants were incubated at 30°C with 12 hour day/night conditions. Diseased symptoms were observed 17 days after inoculation and plant mortality recorded six to ten weeks after inoculation. Non-inoculated plants remained healthy.

In Western Australia, Fang et al. (2011) conducted pathogenicity tests with *M. phaseolina* and compared the colonised toothpick inoculation method with plants grown in potting mix containing inoculated millet seed. Inoculum production and inoculation methods for the toothpick method were generally adopted as described by Mertely et al. (2005). *M. phaseolina*-colonised millet seed inoculum was prepared by soaking millet seed in a flask with deionised water, autoclaving at 121°C for 20 min on three consecutive days, inoculating the flask containing sterilised moist millet seed with *M. phaseolina* agar plugs, and incubating in darkness at 24°C for two weeks, shaking every two days to ensure uniform colonisation. Millet seed colonised by mycelia was mixed with sterile potting mix at a rate of 0.5%. The results showed significantly more severe disease on plants inoculated by the colonised toothpick than plants inoculated by the colonised millet seeds.

In Spain, Aviles et al. (2012) compared the colonised toothpick method and pouring microsclerotia suspension into plant pots. The microsclerotia suspension was produced by blending a 7-day PDA growing colony of *M. phaseolina* in 100 mL sterilised water and adjusting to 1.6×10^3 microsclerotia per mL. Plants were grown in 1 L pots of coir fibre substrate and 50 mL of suspension was poured in. Wilt and necrotic symptoms were observed after six days for the toothpick method and not until 27 days for the microsclerotia suspension tests.

Koike et al. (2013) prepared inoculum by combining a 3:1 mixture of fine washed sand and cornmeal placed in an Erlenmeyer flask with distilled water. The mixture was sealed with foil and autoclaved twice (24hr between autoclaving). Agar plugs with *M. phaseolina* growth were added and incubated at room temperature for three to four months. Flask contents with the microsclerotia-colonised sand and cornmeal mix were emptied out onto trays and dried at room temperature. Established strawberry transplants (runners) were uprooted, washed and roots trimmed to approximately 15-20 cm. The sand and cornmeal inoculum was sprinkled onto the roots, placed in nursery flats and covered with fresh peat moss. Fifteen plants were placed in each flat and maintained in a shade

house. Time to symptoms appearing and when plant death occurred were not reported and the authors found the inoculation conditions "very challenging".

In summary, the toothpick method involved colonising sterile toothpicks by placing them on the surface of *M. phaseolina* grown on corn meal agar or PDA media. Inoculum preparation took approximately 10-14 days. Toothpicks were directly inserted in each plant crown. Plant deaths may occur as quickly as two weeks after inoculation. The physical wounds caused by inserting toothpicks into the plant crowns did not appear to negatively affect plant health, as crowns inoculated with sterile toothpicks remained healthy. The microsclerotia suspension inoculum involved growing isolates for one to three weeks, blending the media and filtering the suspension. Strawberry plants were inoculated by pouring 50 mL of the suspension in the pot. First symptoms were observed by 17-27 days post-inoculation, and plant deaths occurred at 6-10 weeks. Preparation of the seed millet inoculum and cornmeal and sand mix inoculum were similar and much more involved, including repeated autoclaving and incubating that could take up to several weeks. Comparisons between the colonised toothpick and microsclerotia suspension showed faster onset of plant symptoms and mortality in the colonised toothpick method, although both appeared to have the same plant mortality levels. In contrast, when the colonised toothpick and seed millet inoculation methods were compared, plant mortality was significantly higher for the former. Based on the findings of this review, two experiments were conducted to evaluate the colonised toothpick and microsclerotia suspension methods, for Australian conditions and using a local isolate of *M. phaseolina* from strawberry.

9.3.   Methods

9.3.1. *M. phaseolina* isolate

M. phaseolina isolate N18924, obtained from the strawberry fungal isolate collection at Maroochy Research Facility, Nambour, Queensland, was used in two separate experiments. Isolate N18924 was originally recovered using methods of Hutton et al. (2013) from strawberry plants which had wilted and showed necrosis in the crowns, from a field at Glenview, Queensland. After one week, fungal growth was identified as *M. phaseolina* based on microsclerotia and colony morphology (Holliday and Punithalingen, 1970). Identification by DNA sequencing of the internal transcribed spacer (ITS) region confirmed the pathogen. The isolate was hyphal-tipped prior to storage on sterile deionised water (SDW).

9.3.2. Strawberry cultivars

Strawberry cultivars Camarosa and Albion were used for the two experiments. Transplants (runners) of 'Albion' and 'Camarosa' were purchased as certified commercial runners from the Toolangi Certified Strawberry Runner Grower's Co-operative Ltd., Victoria, Australia. All plants were grown in 1:1 sterile peat and sand in 100 mm Spacesaver® pots and maintained in a shade house until inoculated.

9.3.3. Experiment 1 – Toothpick inoculated method

9.3.3.1. Inoculum preparation

Inoculum preparation and inoculation method were adapted from Mertely et al. (2005) and Hutton et al. (2013). *M. phaseolina* isolate N18924 was initiated from storage by sub-culturing onto six Petri dishes (9 cm diameter). In each dish, 24 mL of potato dextrose agar (PDA) amended with streptomycin as described in Hutton et al. (2013) was poured to form a layer of agar 5 mm thick. A small piece of agar with *M. phaseolina* microsclerotia was placed in the middle of the plate and placed in a near-UV light incubator at 24°C. Bamboo toothpicks (Woolworth's brand) were autoclaved twice in deionised water and then once in V8 juice. In each plate, six toothpicks were placed on the surface of PDA media that had been incubated for four days. The plates were sealed with Parafilm and placed back in the near-UV light incubator for another seven days to allow *M. phaseolina* to colonise the toothpicks.

9.3.3.2. Inoculation method

Six plants each of 'Camarosa' and 'Albion' were inoculated by inserting one *M. phaseolina* colonised toothpick into the mid-crown area of each plant. Untreated control plants had an uninoculated toothpick sterilised twice in deionised water and once in V8 juice inserted into their crown. Treated and untreated control plants were placed in an evaporatively cooled glasshouse set to reach a maximum of 40°C, and with heated bench mats set at 30°C.

Time to mortality was recorded. Plants that showed wilting symptoms were tested to confirm presence of the pathogen. For this study, when *M. phaseolina* and/or a mix culture with *M. phaseolina* was present on the PDA plate, it was considered as plant death due to *M. phaseolina*. If the pathogen was not present, it is assumed plant died by other causes.

9.3.4. Experiment 2 – Microsclerotia suspension method

9.3.4.1. Inoculum preparation

Inoculum preparation and inoculation method was adopted from Zveibil and Freeman (2005) and Hutton et al. (2013), with some minor changes to the filtering of the suspension and concentration of microsclerotia used. Initiation of *M. phaseolina* isolate N18924 from storage was undertaken as described above. Twenty PDA plates were inoculated with a small piece of agar with *M. phaseolina* microsclerotia in the centre of the plates, sealed with Parafilm and placed in a near-UV light incubator at 24°C. After three weeks, PDA with microsclerotia embedded were blended in sterile deionised water for 45 seconds to produce a microsclerotia suspension. The inoculum suspension was filtered using two layers of cheesecloth. The volume of the inoculum suspension was adjusted with SDW to the total required volume (600 mL), determined by number of plants to be treated x 50 mL inoculum suspension per plant. Prior to determining the concentration, the inoculum suspension was mixed thoroughly and *M. phaseolina* microsclerotia were counted from a 1 mL sub-sample. This was performed three times to obtain an average concentration of 1.2×10^3 microsclerotia per mL.

9.3.4.2. Inoculation method

For this experiment, six plants each of 'Camarosa' and 'Albion' were inoculated by drenching the potting media with 50 mL of the inoculum suspension. For the untreated control plants, 50 mL of SDW was poured on the potting media. Plants were held in a glasshouse following inoculation, and mortality assessed and recorded as described previously. All plants that died during the experiment were tested to confirm the presence of the pathogen.

9.3.5. Data analysis

An analysis was performed based on the alive/dead plants on the final time point. This analysis used a Generalised Linear Model (GLM) regression analysis assuming a binomial distribution with logit link. Secondly, survival analysis based on the Cox proportional hazards model (Cox, 1972) was performed 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 were deaths and which were censored (plant still alive at the end of the trial). The analysis was used to predict the hazard function (the instantaneous risk of death at time t, conditional on survival to that time) for each cultivar. The hazard ratio between 'Camarosa' and a standard reference cultivar was investigated. In this study, 'Albion' was used as the reference cultivar, due to it being the leading cultivar used for temperate commercial strawberry production. A hazard ratio equal to or greater than one suggested equal or greater susceptibility than the reference cultivar. Hazard ratios less than one suggested lower susceptibility (greater resistance) than 'Albion'. Wald tests were performed to test the difference in hazard between cultivars. Control plants and plants that died of other causes were not included in the analysis.

9.4. Results

9.4.1. Experiment 1.

Symptoms of leaf yellowing and necrosis were observed on some inoculated plants two weeks after inoculation with isolate N18924. Wilt symptoms were first observed three weeks after inoculation, and by six weeks 100% of plants from both 'Camarosa' and 'Albion' had wilted. Laboratory isolations confirmed presence of *M. phaseolina*. Control plants treated with the sterile toothpicks did not wilt.

The survival functions for 'Albion' and 'Camarosa are shown in *Figure 8.10*. There was a no significant difference between the cultivars for plant mortality at the end of the experiment, when inoculated by the colonised toothpick method (P=0.92). In addition, the hazard ratio of 'Camarosa' (0.6) was not significantly different to that of the reference cultivar Albion (1), suggesting that the likelihood of mortality at any given time is the same for both cultivars when affected by *M. phaseolina* using this method of inoculation.
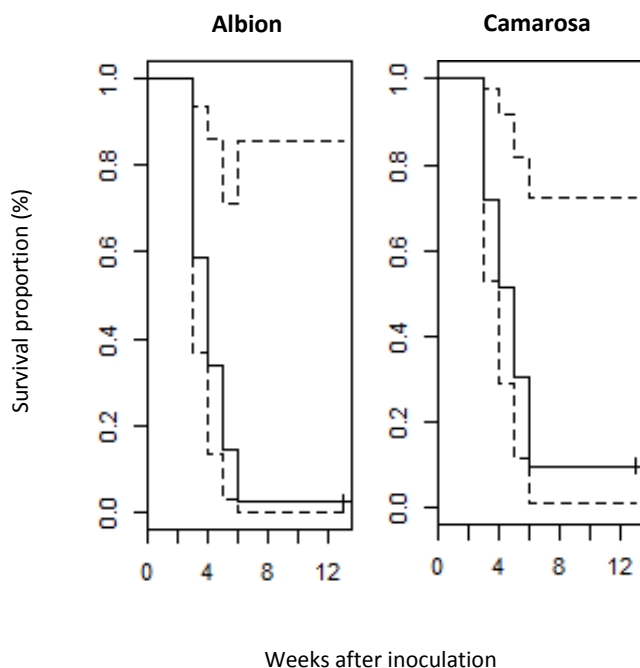
Figure 8.10 Survival functions (predicted survival percentage over time) for 'Albion' and 'Camarosa' for experiment 1.

9.4.2. Experiment 2

Leaf yellowing and necrosis started to appear four weeks after inoculation on some treated plants from both cultivars. Treated plants began to wilt six weeks following inoculation. At the end of the trial period (16 weeks after inoculation), 100% of 'Camarosa' plants and 67% of 'Albion' plants had wilted due to *M. phaseolina*. Plants drenched with SDW did not wilt.

The survival functions for both cultivars are shown in *Figure 8.11*. There was a no significant cultivar effect for mortality at the end of the experiment (P=0.86) when potting mix was drenched by *M. phaseolina* microsclerotia suspension. A significant difference was observed between the cultivars for hazard ratio (P=0.01). 'Camarosa' had a significantly higher hazard ratio (3.8) than reference cultivar 'Albion' (1), suggesting that 'Camarosa' may have a have a higher likelihood of mortality at any given time than 'Albion'.
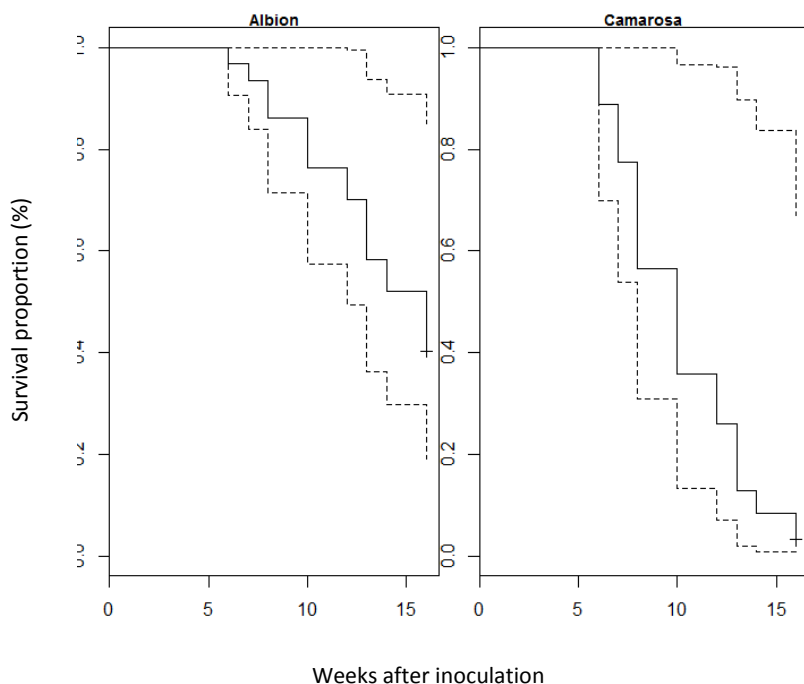
Figure 8.11 Survival functions (predicted survival percentage over time) for 'Albion' and 'Camarosa' for experiment 2.

9.5. Discussion

Results from the two separate experiments suggested that 'Camarosa' and 'Albion' are equally susceptible to charcoal rot in terms of plant mortality levels at the ends of the experiments. Our results for 'Camarosa' support previous studies that it is a susceptible cultivar to the pathogen. For 'Albion, however, our results differ with previous research done in Western Australia that found 'Albion' to be resistant to *M. phaseolina* (Fang et al., 2012). Our findings reflect, however, the experiences in open field production of 'Albion' in Queensland and Victoria, where growers have had significant losses due to charcoal rot (A. Gomez, unpublished data; S. Mattner, personal communication). In Western Australia, *M. phaseolina* has been isolated from fruit production fields, although *Fusarium oxysporum* is regarded as the major pathogen associated with crown rot diseases there (Fang et al., 2011).

Hazard ratio analysis showed no significant differences between 'Camarosa' and 'Albion' when inoculated by the colonised toothpick method. In contrast, 'Camarosa' had a higher likelihood of mortality than 'Albion' following inoculation using the microsclerotia suspension method. These results suggest that inoculation by microsclerotia suspension may be able to better distinguish differences in susceptibility between strawberry cultivars than the colonised toothpick method. Inserting colonised toothpicks into plant crowns is very invasive (Sanchez et al., 2016), and the microsclerotia suspension method more closely resembles natural infection processes in the field. We therefore recommend the microsclerotia suspension method for further screening of strawberry genotypes.

9.6. Conclusions

Based on the findings from this study, future screening of strawberry cultivars and breeding lines will be carried out by preparing and drenching potting media with a microsclerotia suspension of *M. phaseolina*. Although the colonised toothpick method resulted in faster development of symptoms, mortality levels were the same in the two separate experiments. Drenching pots with microsclerotia more closely replicates natural host-pathogen interactions, and may allow differences in cultivar response to be better observed. Future screening will continue to use 'Albion' as the reference cultivar for hazard ratios, due to its industry relevance as the leading temperate commercial cultivar.

9.7. References

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10. Response of strawberry genotypes inoculated with *Macrophomina phaseolina*

Apollo Gomez, Joanne De Faveri, Jodi Neal and Mark Herrington

10.1. Introduction

Charcoal rot, caused by the soil borne fungus *Macrophomina phaseolina*, is an important disease affecting strawberries. The strawberry industry in Australia is valued at $420M (Australian Horticulture Statistics Handbook Fruit 2014-15). Charcoal rot has been detected in Queensland, Victoria and Western Australia (Golzar et al., 2007; Hutton et al., 2013), and the three states account for 88% of the strawberry production in Australia (Australian Horticulture Statistics Handbook Fruit 2014-15). Hutton et al. (2013) reported commercial plant losses due to charcoal rot of more than 50% in Northern Victoria in 2006, and similar in a commercial farm in south-east Queensland.

There are no registered fungicides for strawberry production for control of charcoal rot. Growers rely on fumigating the soil, however current soil fumigants are only partially effective and do not eradicate the pathogen completely (Hutton et al., 2013). The management strategy of developing resistant cultivars has 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). Breeding programs internationally are now more focused on host plant resistance and the importance of identifying the resistance of existing strawberry genotypes to soil borne diseases (Holmes et al., 2017).

Our previous study (see Chapter 9) suggested that cultivars Camarosa and Albion were susceptible to *M. phaseolina*, an important step in order to establish a reference cultivar for future screening tests. Several host-pathogen studies have been conducted internationally to evaluate the response of other cultivars against *M. phaseolina*. There are cultivars that have been reported to show tolerance and/or resistance to *M. phaseolina*, including 'Seascape' (Koike, 2008), '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 (and susceptible) to *M. phaseolina*. Cultivars that have previously been reported to have resistance are either no longer grown in significant numbers in Australia or were not available for testing. There is limited information in Australia on the response of strawberry cultivars currently grown commercially to *M. phaseolina*.

This study evaluated the response of 41 strawberry genotypes, which included current and historical cultivars, and breeding lines, when inoculated with *M. phaseolina*. The breeding lines were developed from the Australian Strawberry Breeding Program (ASBP) for the sub-tropical and temperate areas of production in Australia.

10.2. Methods

10.2.1. Experimental design

A series of five glasshouse inoculation tests were conducted between 2015 and 2017. Disease resistance screening for strawberry genotypes for *M. phaseolina* isolate N18924 was conducted on 458 plants from twelve cultivars currently or previously grown in Australia (Table 10.1). An additional 469 plants from 41 ASBP breeding lines were also screened. Five to ten replicate plants were assessed per genotype per experiment. 'Albion' was used as the reference 'susceptible' genotype, as reported in Chapter 9.

10.2.2. Inoculation

As per the results of our previous work (Chapter 9), the microsclerotia suspension method was used for this study. For all experiments, *M. phaseolina* isolate N18924 was initiated from storage and a suspension of microsclerotia prepared. Two amendments in the inoculum production were applied for this study: i. blending time was increased from 45 seconds to 1 minute and the suspension was no longer filtered; and ii. the microsclerotia concentration

used for all experiments was increased to 1.4 x 10^3 microsclerotia per mL. Filtering the inoculum suspension took significant time and was inefficient, and more importantly, a substantial amount of microsclerotia were lost from being caught in the cheesecloth. Blending the suspension for an extra 15 seconds allowed more time to break down agar pieces. Transplants (runners) from all breeding lines and cultivars were produced from the ASBP collection at the Maroochy Research Facility, Nambour. All plants were grown in 1:1 sterile peat and sand in 100 mm Spacesaver® pots for six to 12 months prior to inoculation. Transplants of cultivars Albion and Camarosa were purchased as certified commercial runners from the Toolangi Certified Strawberry Runner Grower's Co-operative Ltd., Victoria, Australia. These plants were also grown as described above and maintained in the shade house until time of inoculation. For all experiments, a randomised block design was applied to the inoculation order of potted plants, as well as the physical arrangement of plants immediately after inoculation. The potted plants were inoculated by drenching the potting media with 50 mL of the microsclerotia suspension. Plants were placed in an evaporatively cooled glasshouse set to reach a maximum of 40°C. The pots were arranged on a bench that had a 25 mm Styrofoam sheet with a thermostatically-controlled electronic heat mat (Adloheat) maintained at 30°C and covered by a black plastic sheet and a horticultural capillary mat, as described by Neal et al. (2017). For the untreated control plants, 50 mL of sterile deionised water was poured on the potting media and subjected to the same conditions as the inoculated plants, but were kept physically separate from the inoculated plants. Plant mortality was recorded on a weekly basis. All plants that died during the experiment were tested to confirm the presence of the pathogen. For this study, when *M. phaseolina* and/or a mix culture with *M. phaseolina* was found to be present, it was considered as plant death due to *M. phaseolina*. If the pathogen was not present, it was assumed that the plant died of other causes.

10.2.3. Statistical analysis

All five experiments were analysed separately. Similar to Section 9.3.5, an analysis was performed on whether plants were alive or dead at the final time point in each experiment. A Generalised Linear Model (GLM) regression analysis assuming a binomial distribution with logit link was applied. Secondly, survival analysis based on the Cox proportional hazards model (Cox, 1972) was performed using the Survival package (Therneau, 2015) in R (R core team, 2015), as described in Section 9.3.5. Hazard ratios were determined with the reference cultivar 'Albion'. A hazard ratio greater than one suggested greater susceptibility than the reference cultivar, i.e. a higher likelihood of plant death at any given time compared with 'Albion'. Hazard ratios less than one suggested lower susceptibility (greater resistance) than 'Albion'. Wald tests were performed to determine significant differences in hazard between cultivars.

The hazard ratio analysis takes into account plant mortality and survival together for each genotype. In the case where there was no mortality or 100% mortality, comparisons become problematic because of inflated standard errors. If a genotype had no mortality, this was interpreted as suggesting resistance to *M. phaseolina*, and if there was 100% mortality, then the genotype was considered susceptible. Control plants and plants that died of causes other than *M. phaseolina* were not included in the analysis.

Table 10.1 List of genotypes (cultivars and breeding lines) and number of plants per genotype evaluated for each disease resistance screening experiment.

Genotype	Experiment Number					Total
	1	2	3	4	5	
Albion	20	20	20	20	20	172
Camarosa	20	20				112
Earlibrite		10				10
Earlisweet		5				5
Festival	20	10				30
Florida Radiance	20	10				30
Kabarla		9				9
Phenomenal		10				10
Red Rhapsody		10				10
Rubygem	20	10				30

Genotype	Experiment Number					Total
	1	2	3	4	5	
Suncoast Delight	20	10				30
Sweet Charlie		10				10
2005-027-001			10	10		20
2005-025-055			10	10		20
2005-069-063			10	10		20
2008-022-042			15	10		25
2006-221		6				6
2009-030		10				10
2010-113		6				6
2010-160		10				10
2011-174		10				10
2011-192		10			10	20
2011-216		9			10	19
2011-221		10				10
2011-237		10				10
2011-258		7				7
2012-017		10				10
2012-065		9			10	19
2012-108		10				10
2015-227			10			10
2015-228			10			10
2015-229			10			10
2015-230			10			10
2015-231			10			10
2015-232			10			10
2015-233			5			5
2015-234			10			10
2015-235			10			10
2015-236			10			10
2015-237			10			10
2015-238			10			10
2015-240			10			10
2015-241			10			10
2015-243			10			10
2015-244			10			10
2015-245			10			10
2015-246			10			10
2015-248			10			10
2015-249			10			10
2015-251			8			8
2015-252			6			6
2015-253			10			10
2015-254			10			10
Total number of plants	**120**	**257**	**296**	**60**	**50**	**927**

10.3. Results

10.3.1. Experiment 1

Laboratory isolations confirmed presence of *M. phaseolina* for plants that wilted. Control plants treated with the sterile toothpicks did not wilt due to *M. phaseolina*.

Analysis of plants dead and alive at the final experimental time point (16 weeks after inoculation, *Figure 10.1*), found a significant genotype effect (P=0.037). 'Suncoast Delight' had significantly lower plant mortality due to *M. phaseolina* than 'Albion', 'Camarosa' or 'Festival', and similar mortality to 'Florida Radiance' and 'Rubygem'. 'Rubygem 'also displayed significantly lower mortality than 'Albion'. Survival functions for all cultivars tested are shown in *Figure 10.2*.

Cultivars Rubygem and Suncoast Delight were observed to have significantly lower hazard ratios (P=0.01) than reference cultivar 'Albion' (*Figure 10.3*), suggesting that both cultivars may have a lower likelihood of mortality to charcoal rot at any given time than 'Albion'.

Figure 10.1 Predicted proportion dead for each cultivar for experiment 1 showing significant genotype effect (P=0.037) of plant dead/alive at final time point (week 16). Values with same subscript are not significantly different at the P = 0.05 level.
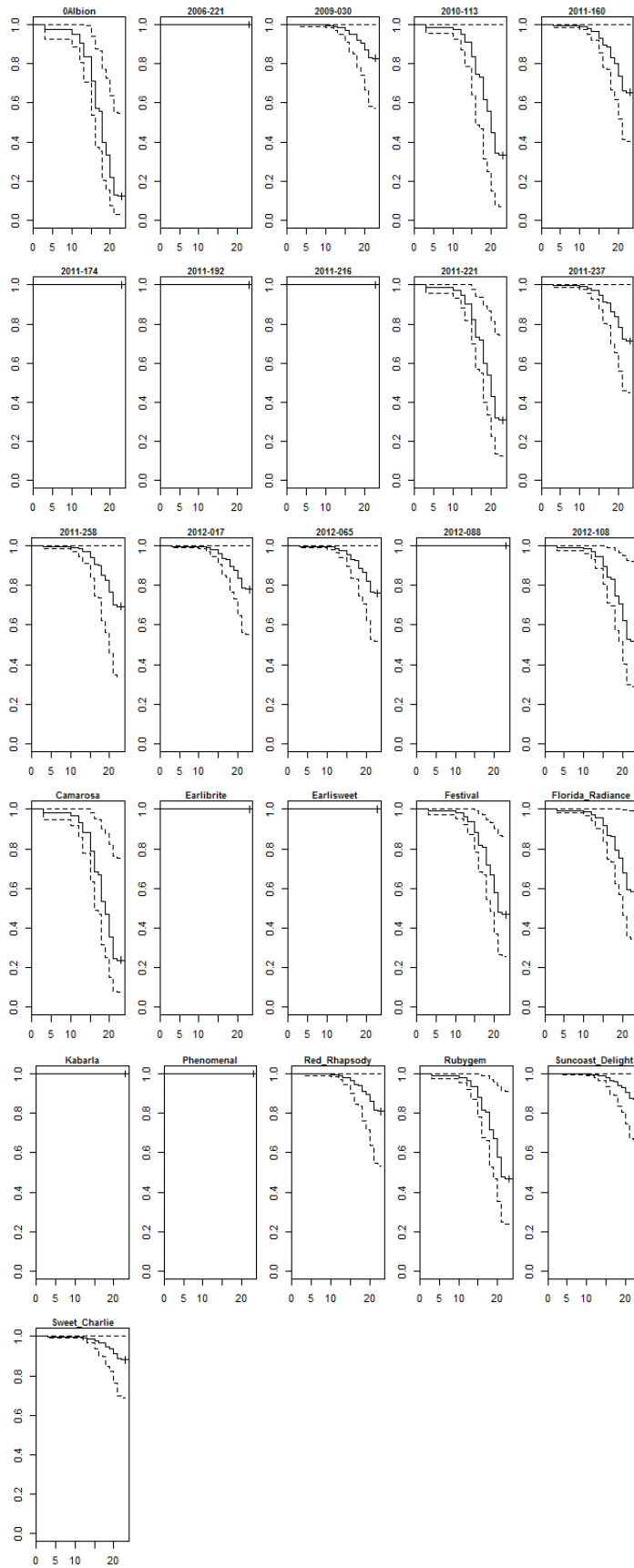
Figure 10.2 Survival functions (predicted survival proportion over time) for genotypes tested for experiment 1.
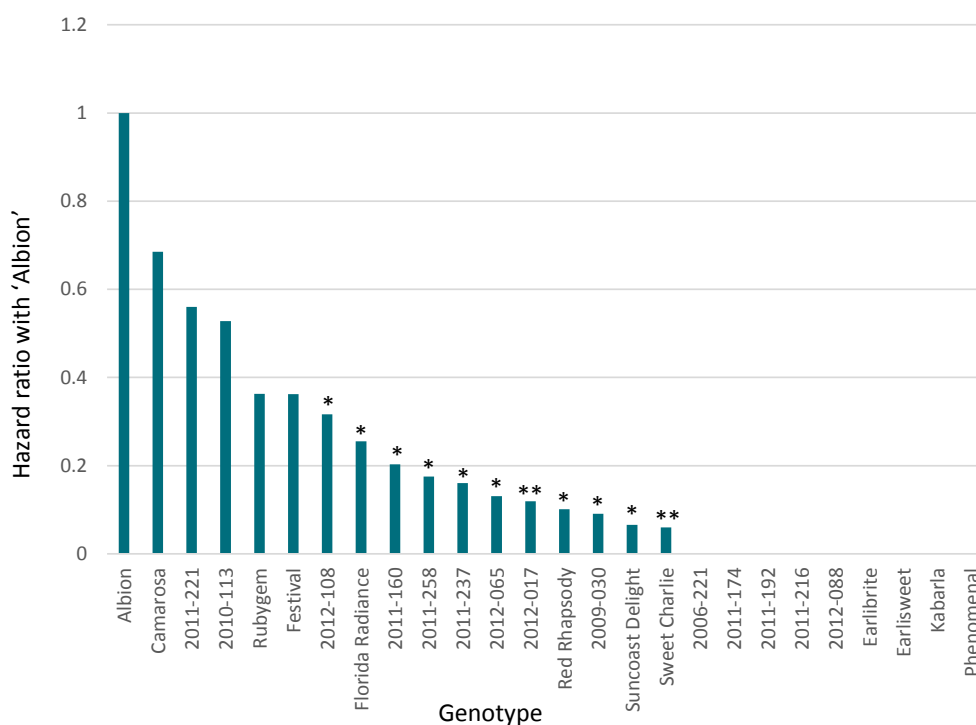

Figure 10.3 Hazard ratio with the reference cultivar Albion for all genotypes assessed in experiment 1. Asterisks denote significant differences from the reference cultivar Albion (P values: ** = 0.01; * = 0.05).

10.3.2. Experiment 2

Laboratory isolations confirmed presence of *M. phaseolina* for plants that wilted. Control plants treated with the sterile toothpicks did not wilt due to *M. phaseolina*.

There was no significant genotype effect (P=0.83) for plant mortality at the final experimental time point (22 weeks after inoculation). Ten genotypes had nil plant mortality due to *M. phaseolina* at the final time point

('Phenomenal', 'Kabarla', 'Earlibrite', 'Earlisweet', '2006-221', '2011-174', '2011-192', '2011-216' and '2012-088') and were excluded from the analysis. The survival functions for all genotypes are shown in *Figure 10.4*.

Significant genotype differences were, however, observed between hazard ratios. A number of genotypes were observed have significantly lower hazard than 'Albion' ('Florida Radiance', 'Red Rhapsody', 'Suncoast Delight', 'Sweet Charlie', '2009-030', '2011-160', '2011-237', '2012-017', '2012-065', '2012-108'; *Figure 10.5*), and hence a lower likelihood of mortality at any given time than 'Albion'.

Figure 10.4 Survival functions (predicted survival proportion over time) for genotypes (breeding lines) tested for experiment 2.

*Figure 10.5 Hazard ratio with the reference cultivar Albion for all genotypes assessed in experiment 2. Asterisks denote significant differences from the reference cultivar Albion (P values: ** = 0.01; * = 0.05).*

10.3.3. Experiment 3

Laboratory isolations confirmed presence of *M. phaseolina* for plants that wilted. Control plants treated with the sterile toothpicks did not wilt due to *M. phaseolina*.

Analysis of mortality at the final experimental time point (13 weeks after inoculations) showed a significant genotype effect (P=0.008; *Figure 10.6*). Genotypes '2015-246', '2015-251' and '2015-252' had nil plant mortality over the experiment. Genotypes '2009-065-063', '2015-227', '2015-231', '2015-235' and '2015-248' had significantly lower plant mortality at the end of the experiment than 'Albion'. In contrast, genotypes '2005-028-065', '2015-228' and '2015-229' had significantly higher plant mortality compared with 'Albion'. Survival functions for all genotypes tested are shown in *Figure 10.7*.

Hazard ratios for each genotype with respect to 'Albion' are shown in *Figure 10.8*. The analysis showed a significant difference between genotypes for hazard ratio. Genotypes that had nil plant mortality had hazard ratios of zero, suggesting resistance to N18924. Genotypes '2005-069-063', '2015-227', '2015-231', '2015-235' and '2015-248' were observed to have significantly (P<0.005) lower hazard, i.e. lower probability of death at any given time, than the reference cultivar Albion.
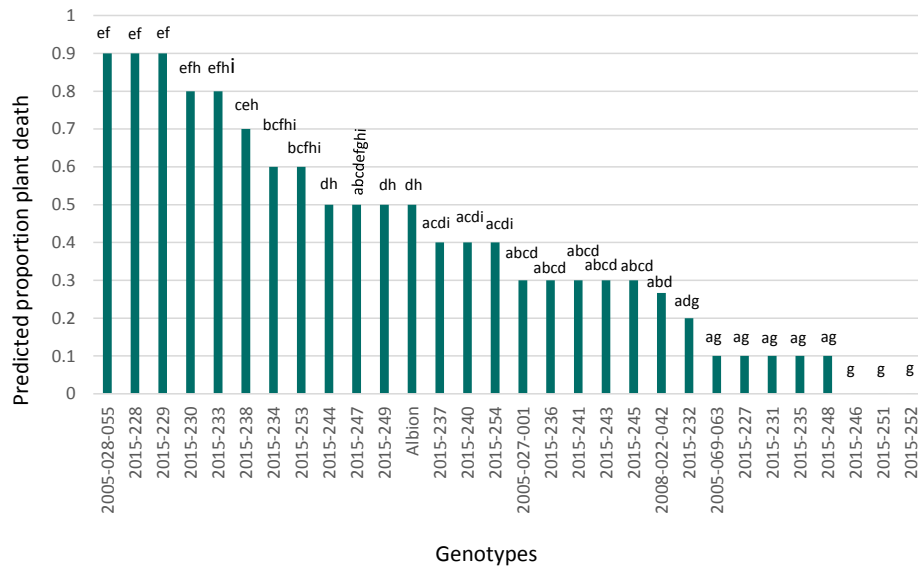
Figure 10.6 Predicted proportion of plant death at final time point (13 weeks after inoculations) for each cultivar for experiment 3. Values with same subscript are not significantly different at the P = 0.05 level.
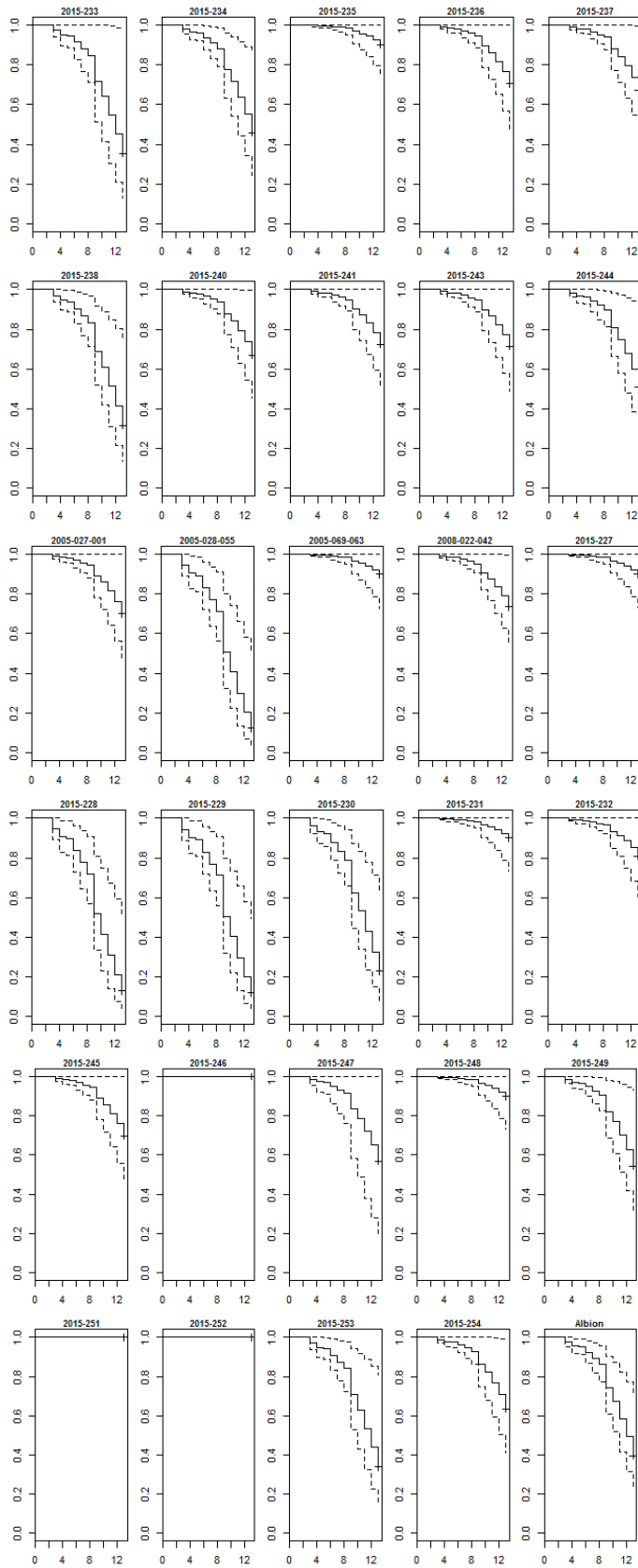
Figure 10.7 Survival functions (predicted survival proportion over time) for genotypes (breeding lines) tested for experiment 3.
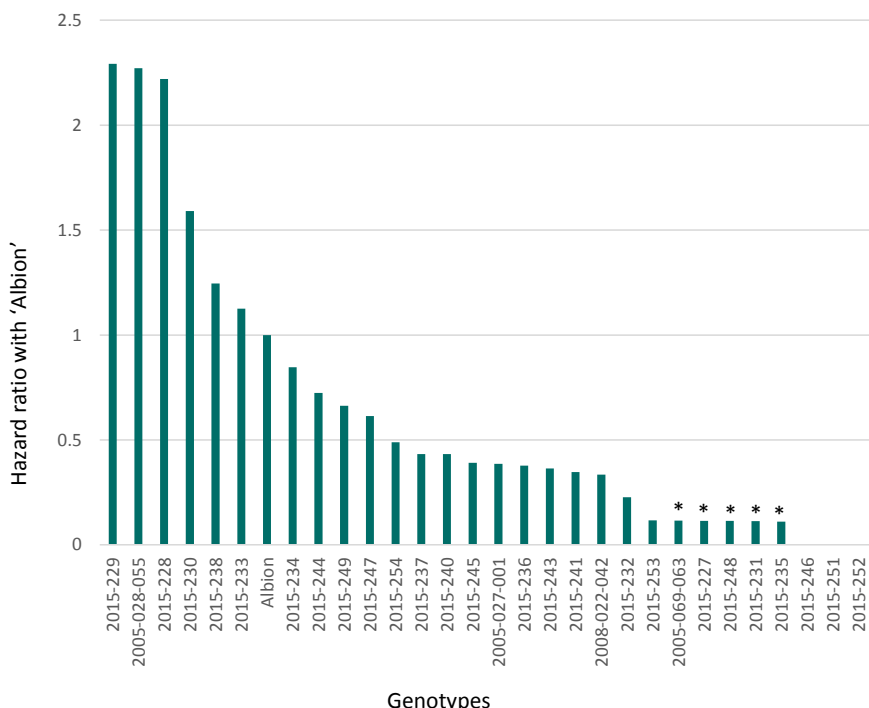
*Figure 10.8 Hazard ratios with the reference cultivar Albion for all genotypes assessed in experiment 1. Asterisks denote significant differences from the reference cultivar Albion (P values: ** = 0.01; * = 0.05).*

10.3.4. Experiment 4

Laboratory isolations confirmed presence of *M. phaseolina* for plants that wilted. Control plants treated with the sterile toothpicks did not wilt due to *M. phaseolina*.

Analysis of mortality at the final experimental time point (13 weeks after inoculation) for four breeding lines and 'Albion' showed no significant genotype effect (P=0.217). The survival functions for all genotypes are shown in *Figure 10.9*.

Hazard ratio analysis showed a significant difference between the genotype '2005-028-055' (hazard ration = 3.091) and Albion (*Figure 10.10*), with 2005-028-055' being significantly more likely to die at any given time. No genotypes were observed to have significantly lower hazard than 'Albion'.
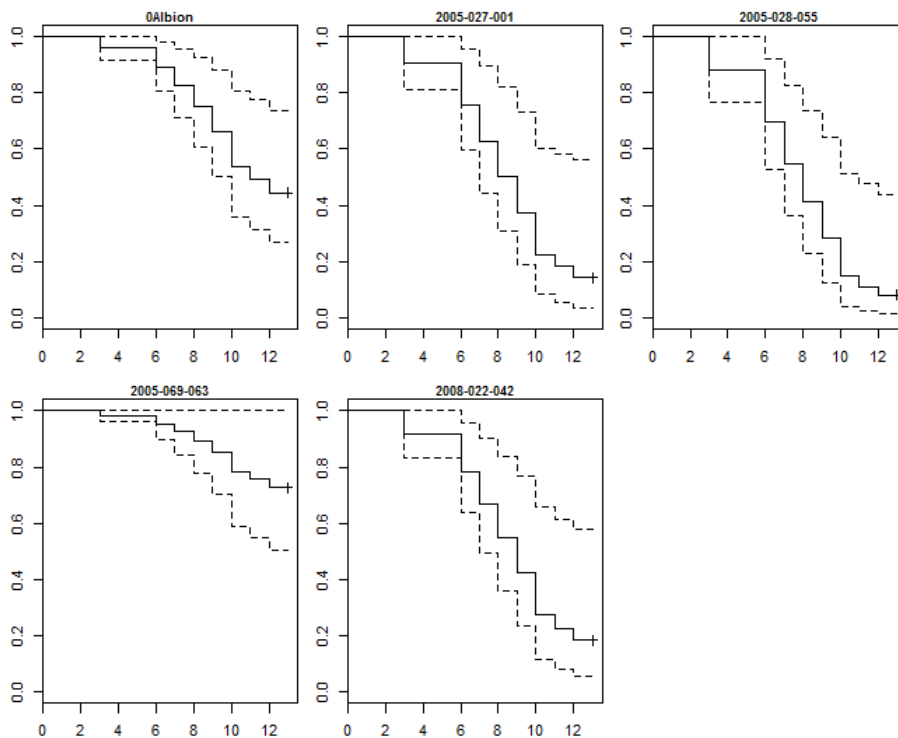
Figure 10.9 Survival functions (predicted survival proportion over time) for genotypes tested for experiment 4.
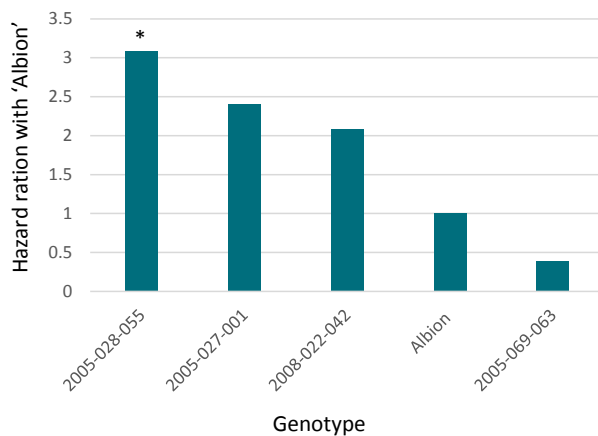

*Figure 10.10 Hazard ratio with the reference cultivar Albion for all genotypes assessed in experiment 4. Asterisks denote significant differences from the reference cultivar Albion (P values: ** = 0.01; * = 0.05).*

10.3.5. Experiment 5

Laboratory isolations confirmed presence of *M. phaseolina* for plants that wilted. Control plants treated with the sterile toothpicks did not wilt due to *M. phaseolina*.

Plant mortality analysis at the final experimental time point (13 weeks after inoculation) for three breeding lines and 'Albion' showed no significant genotype effect (P=0.806). Genotypes '2011-192' and '2011-216' had nil plant mortality. Interestingly, both genotypes were tested previously in experiment 2 and the outcome was the same. Survival functions for all genotypes are shown in *Figure 10.11*.

The hazard ratio analysis showed a significant difference between the genotype hazards (not presented). Genotypes '2011-192' and '2011-216' had nil plant mortality and therefore hazard ratios of zero, suggesting resistance to N18924. Genotype '2012-065' had significantly lower hazard (hazard ratio = 0.664; P=0.018) compared to reference cultivar Albion, which concurs with previous findings (from experiment 2) for this
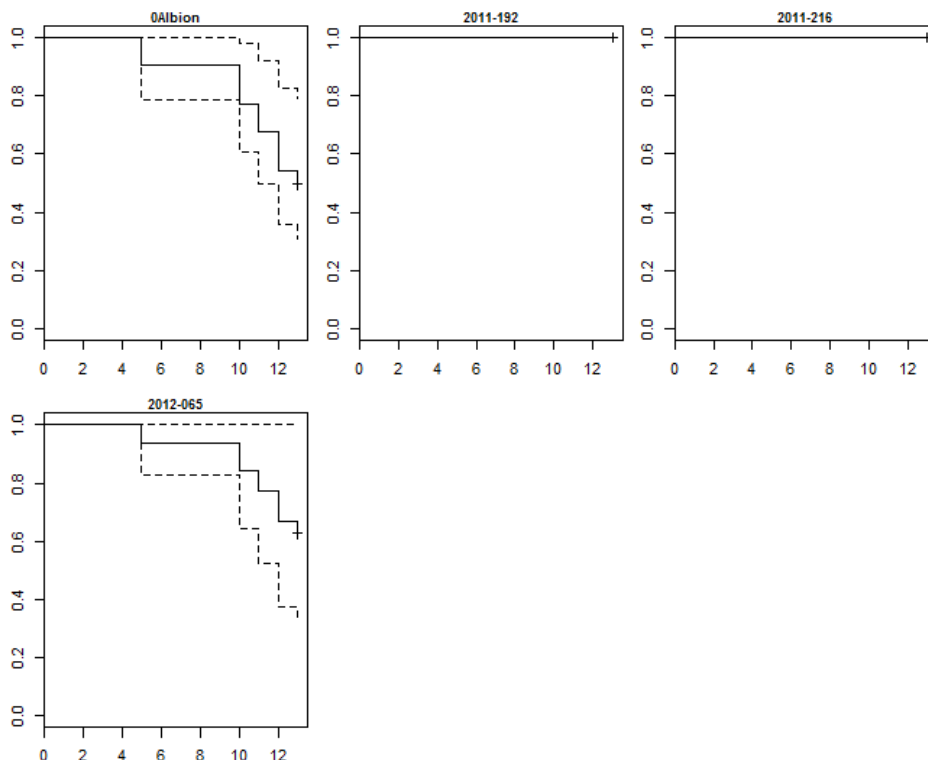
genotype.

Figure 10.11 Survival functions (predicted survival proportion over time) for genotypes tested for experiment 5.

10.4. Discussion

This study allowed the identification of differences in the susceptibility of different strawberry genotypes from five separate disease screening experiments using *M. phaseolina* isolate 18924. Based on plant mortality at the end time point and hazard ratio analyses, cultivars Phenomenal, Sweet Charlie, Suncoast Delight, Red Rhapsody, Kabarla, Earlibrite and Earlisweet appear to have greater resistance to charcoal rot than 'Albion'. Several breeding lines were also identified as showing resistance to N18924 and/or having greater resistance than 'Albion'. Genotypes '2011-192', '2011-216' and '2012-065' were tested twice in this study and produced the same outcomes each time.

Historical cultivars Phenomenal, Kabarla, Earlisweet and Earlibrite had nil plant deaths caused by *M. phaseolina* isolate N18924. These cultivars are no longer grown commercially in Australia, however are still available in the ASBP germplasm collection. ASBP cultivars 'Suncoast Delight' and 'Red Rhapsody' are currently grown commercially in Australia, with 'Suncoast Delight' being a parent of 'Red Rhapsody'. 'Sweet Charlie' also showed high resistance, and is a distant ancestor of both cultivars (M. Herrington, personal communication). In addition, 'Phenomenal' is in the ancestry of 'Sweet Charlie', 'Kabarla' and 'Earlisweet (M. Herrington, personal communication). This may suggest that resistance from 'Phenomenal' could have been passed on to later generations.

The genotypes identified here to have resistance to charcoal rot may be used in future crossing strategies to develop new elite breeding lines with resistance to the pathogen. This is the first study in Australia to report on strawberry genotypes with possible resistance to *M. phaseolina*. Future screening work should be conducted to confirm the results of this study and to further investigate host-pathogen studies by using different isolates of *M. phaseolina* from strawberry and non-strawberry hosts.

10.5. Conclusions

Pathogenicity studies are integral in understanding the resistances of commercial genotypes and to develop new *M. phaseolina* resistant genotypes (Sanchez et al., 2016). This study has demonstrated current strawberry cultivars grown in Australia with varying degrees of resistance when inoculated with *M. phaseolina*, including cultivars now in the germplasm collection and breeding lines currently being developed. These findings will assist in breeding efforts to develop *M. phaseolina* resistant cultivars in Australia to help minimise economic losses to charcoal rot.

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11. Evaluating pathogenicity of *Macrophomina phaseolina* isolates against strawberry

Apollo Gomez, Joanne De Faveri, Jodi Neal and Mark Herrington

11.1. Introduction

Previous host-pathogen studies with strawberry and *M. phaseolina* (i) allowed the development of standard procedures for inoculum preparation and plant inoculations (Chapter 9); (ii) confirmed the susceptibility of 'Albion', which became an integral part of subsequent and of future screening tests; and (Chapter 9); and (iii) identified possible genotypes with resistance and with greater resistance to reference cultivar Albion (Chapter 10). For consistency, these studies were based on the use of one *M. phaseolina* isolate previously reported as pathogenic to strawberry (Hutton et al., 2013).

Studies in Spain (Aviles et al., 2012) found that some cultivars may show different susceptibility to *M. phaseolina* depending on the isolate tested. The authors found 'Camarosa', a widely reported susceptible cultivar, responded differently when two *M. phaseolina* isolates were used. In addition, host-pathogen studies in Israel on cultivar Tamar found non-strawberry isolates of *M. phaseolina* were able infect strawberry plants (Zveibil et al., 2012). This study reported that host specialisation to strawberry was not evident, and stressed the importance of avoiding crop rotation of strawberry with the other crops tested.

Here, the pathogenicity of thirty *M. phaseolina* isolates was evaluated on the reference (susceptible) cultivar Albion. The isolates were from strawberry hosts, from non-strawberry hosts, and from non-strawberry hosts from cultivated strawberry fields. Investigation of host-pathogen interactions is integral to develop further understanding of the role non-strawberry isolates have on strawberry production.

11.2. Methods

11.2.1. Experimental design

A glasshouse inoculation test was conducted using thirty *M. phaseolina* isolates from a range of strawberry and non-strawberry hosts, collected in different years and locations (Table 11.1). Eight replicate plants were assessed per isolate in the experiment. In our previous studies (see Chapters 9 and 10), 'Albion' and *M. phaseolina* isolate N18924 were used as the reference cultivar and isolate respectively.

Table 11.1. Strawberry and non-strawberry isolates used in this experiment, and their collection year and location.

Isolate ID	Host Common Name	Year	Location	State
N13169	Strawberry	2001	Nambour	Queensland
N15922	Strawberry	2006	Elimbah	Queensland
N16892	Strawberry	2007	Wamuran	Queensland
N17093	Strawberry	2007	Wanneroo	Western Australia
N17336	Strawberry	2008	Elimbah	Queensland
N18307	Strawberry	2009	Wanneroo	Western Australia
N18924	Strawberry	2009	Glenview	Queensland
N18936	Strawberry	2009	Atherton	Queensland
N19332	Strawberry	2010	Bli Bli	Queensland
N20201	Strawberry	2010	Wanneroo	Western Australia
N22398	Strawberry	2013	Stanthorpe	Queensland
N22426	Strawberry	2013	Cobram	Victoria
N23002	Strawberry	2014	Wamuran	Queensland
N23063	Strawberry	2014	Chambers Flat	Queensland
N23392	Strawberry	2015	Pozieres	Queensland
N23452	Strawberry	2015	Glenview	Queensland
N23509	Strawberry	2015	Wamuran	Queensland
N23584A	Sorghum (cover crop)	2015	Wamuran	Queensland
N23584B	Sorghum (cover crop)	2015	Wamuran	Queensland
N23584C	Sorghum (cover crop)	2015	Wamuran	Queensland
BRIP55159	Chickpea	1995	Croppa Creek	Queensland
N22413	Chickpea	2007	Theodore	Queensland
BRIP53216	Mungbean	2010	Emerald	Queensland
BRIP55111	Mungbean	1994	Biloela	Queensland
BRIP29123	Peanut	2001	Dingo	Queensland
BRIP44996	Peanut	2004	Collington	Queensland
N22414	Sorghum	2013	Norwin	Queensland
N24125	Sorghum	2016	Emerald	Queensland
BRIP39285	Watermelon	2003	Walkamin	Queensland
BRIP42497	Watermelon	2003	Kununnura	Western Australia

11.2.2. *M. phaseolina* isolates

The majority of *M. phaseolina* isolates from strawberry were obtained from the strawberry fungal isolate collection at Maroochy Research Facility (MRF), Nambour, Queensland. Each isolate was originally recovered using the methods of Hutton et al. (2013) from symptomatic strawberry plants submitted by strawberry producers to MRF. Non-strawberry isolates BRIP53216, BRIP55111, BRIP29123, BRIP44996, BRIP55159, BRIP39285 and BRIP42497 were obtained from the Queensland Herbarium. Isolates N22413 and N22414 were obtained from the Leslie Research Centre, Toowoomba. Finally, isolate N24125 was obtained from University of Southern Queensland, Toowoomba. For reference isolate N18924, *M. phaseolina* identification by DNA sequencing of the internal transcribed spacer (ITS) region confirmed the pathogen. For all other isolates, fungal growth that developed was identified as *M. phaseolina* based on microsclerotia and colony morphology (Holliday and Punithalingen, 1970) only. All isolates used in the experiment were hyphal-tipped prior to storage on sterile deionised water.

11.2.3. Inoculation

As per the results of our previous work (see Chapters 9 and 10), the microsclerotia suspension method was used in this study. All *M. phaseolina* isolates were initiated from storage and a suspension of microsclerotia prepared as described in Chapter 10. Transplants (runners) from all breeding lines and cultivars were produced from the Australian Strawberry Breeding Program (ASBP) collection at the Maroochy Research Facility, Nambour. Runners of 'Albion' were purchased as certified commercial runners from the Toolangi Certified Strawberry Runner Grower's Co-operative Ltd., Victoria, Australia. The plants were grown in 1:1 sterile peat and sand in 100 mm Spacesaver® pots for six to 12 months prior to inoculation. A randomised block design was applied to the inoculation order of potted plants, as well as the physical arrangement of plants immediately after inoculation onto a heated glasshouse bench (as described in Chapter 10). For the untreated control plants, 50 mL of sterile deionised water was poured onto the potting media and subjected to the same conditions as the inoculated plants, but were kept physically separate from the inoculated plants. Plant mortality was recorded on a weekly basis. All plants that died during the experiment were tested to confirm the presence of the pathogen. For this study, when *M. phaseolina* and/or a mix culture with *M. phaseolina* was found to be present, it was considered as plant death due to *M. phaseolina*. If the pathogen was not present, it was assumed that the plant died of other causes.

11.2.4. Statistical analysis

A log rank test based analysis was performed on the survival functions of each isolate for 'Albion' on whether plants were alive or dead at the final time point. Secondly, survival analysis based on the Cox proportional hazards model was performed using the Survival package (Therneau, 2015) in R (R core team, 2015). Hazard ratios were determined relative to the reference isolate N18924 (used in previous studies). *M. phaseolina* isolates with hazard ratios greater than 1 have a higher hazard, and therefore greater pathogenicity to 'Albion', than isolate N18924. Control plants and plants that died of causes other than *M. phaseolina* were not included in the analysis

11.3.   Results

Laboratory isolations confirmed the presence of *M. phaseolina* for plants that wilted. Control plants treated with sterile deionised water did not wilt due to *M. phaseolina*.

With the exception of one isolate (N13169), 100% mortality was observed on 'Albion' plants inoculated with *M. phaseolina* isolates from strawberry, regardless of where the isolates came from. The isolates from sorghum grown as cover crops also caused 100% plant mortality at the final time point of the experiment (14 weeks after inoculation).

Significant differences in pathogenicity were observed between the isolates tested (P<0.001). The proportion of 'Albion' plants surviving over time for each isolate, as determined from survival functions, are shown in Figure 11.1.

Hazard ratios for each isolate with respect to reference isolate N18924 are shown in Figure 11.2. The analysis showed a significant difference in hazard ratios between *M. phaseolina* isolates. Isolate N17093, from strawberry in Western Australia, had a significantly higher hazard (P=0.006) than the reference isolate, i.e. a higher probability to cause charcoal rot than isolate N18924. Strawberry isolate N13169 showed a significantly lower hazard ratio than N18924 (P = 0.001). No other isolates from strawberry were significantly different to N18924. With the exception of isolates N23584A, N23584B and N23584C, all other *M. phaseolina* isolates from non-strawberry hosts displayed significantly lower hazards compared with N18924.
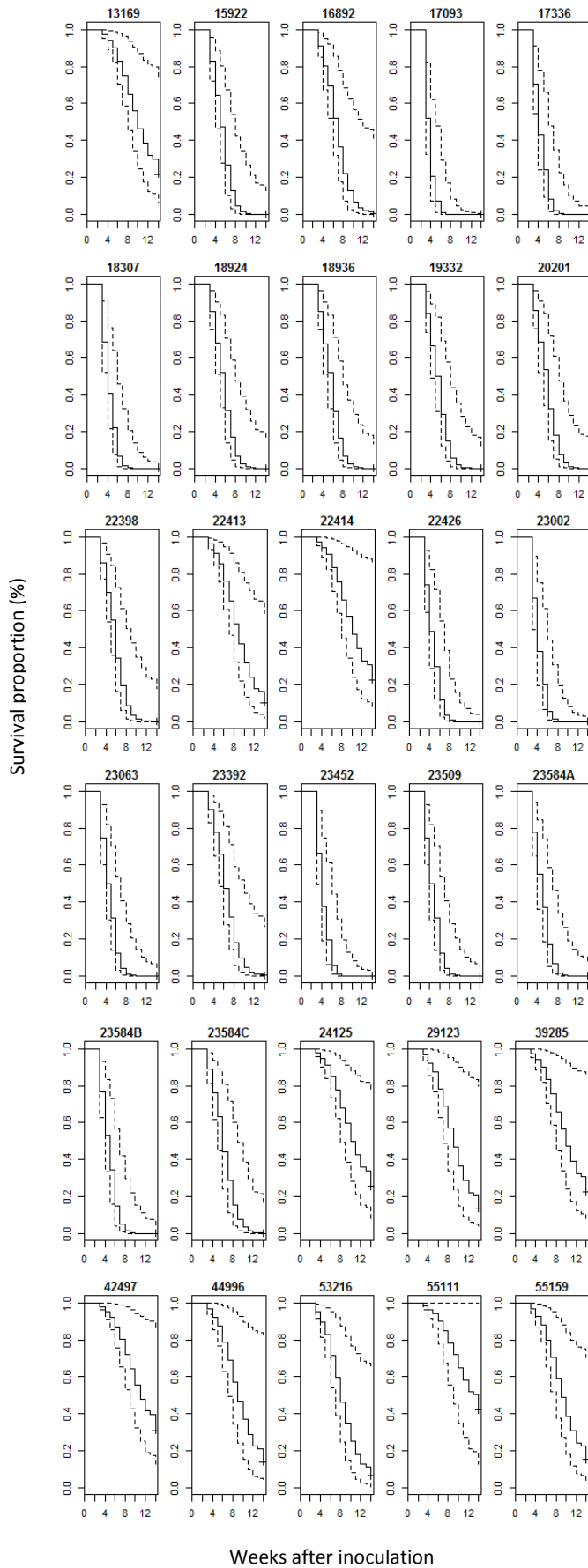
Figure 11.1. Survival functions (predicted survival proportions over time) for 'Albion' plants tested for each M. phaseolina isolate.
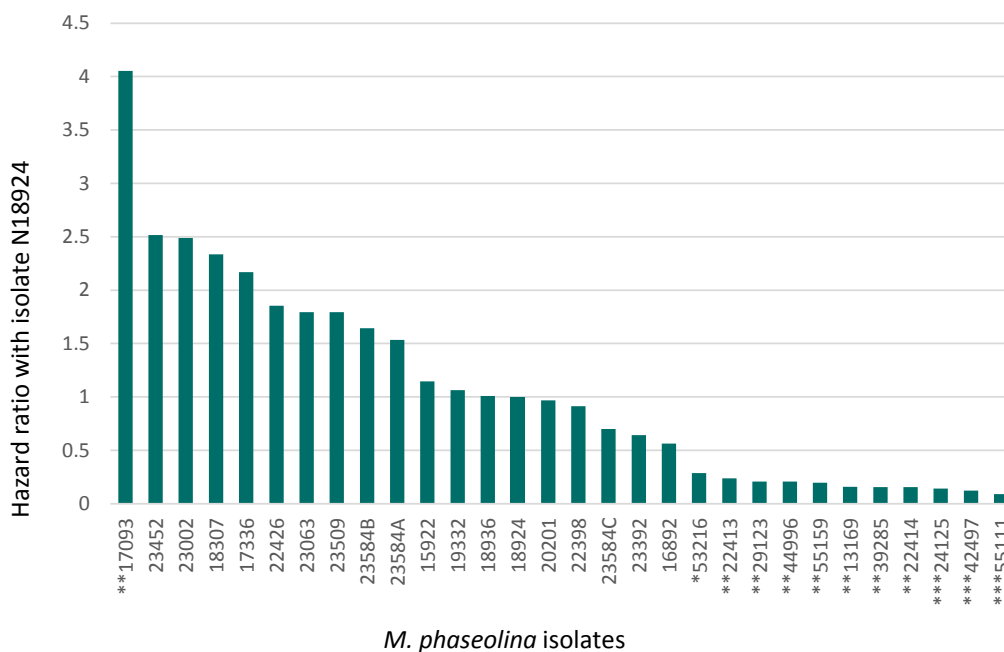
Figure 11.2. Hazard ratio with the reference isolate N18924 for all M. phaseolina isolates assessed. Asterisks denotes significant difference to reference cultivar. Asterisks denote significant differences from the reference cultivar Albion (P values: *** = 0.001; ** = 0.01; * = 0.05).

11.4. Discussion

Hazard ratio analysis between 17 *M. phaseolina* isolates collected from strawberry hosts showed only two isolates significantly different to reference isolate N18924 used in previous studies. Isolate N17093 had significantly higher pathogenicity than N18924, whereas isolate N13169 showed significantly lower pathogenicity. Symptoms on the plants were slower to express with isolate N13169 compared with other strawberry isolates. The age of this isolate, having been in storage for a number of years, may have been a factor in the lower pathogenicity observed.

Apart from isolate N17093, the Western Australian isolates were not significantly different to N18924. The isolate from Victoria was also not significantly different to N18924, suggesting that any of the strawberry isolates may be used for future screening.

Our study supports the findings of Zveibil et al. (2012), where *M. phaseolina* isolates collected from crops rotated with strawberry were able to infect strawberry plants. In our experiment, three isolates collected from sorghum in a commercial strawberry farm caused 100% plant mortality. All three isolates originated from the same location, but were from different plant samples i.e. N23584A from lesions on the base of a sorghum crown; N23584B from a root lesion, and N23584C from a symptomless root tissue. This is the first report in Australia where *M. phaseolina* isolated from a cover crop used in rotation with strawberry has been able to infect strawberry plants. Using alternate hosts of charcoal rot in strawberry production in Australia can increase the risk of charcoal rot infection, as cover crops are generally incorporated back in the soil and can potentially add more inoculum to the soil. Further work should be carried out to survey and monitor the pathogen on other alternate hosts, including weeds, in strawberry fields. Fuhlbohm et al. (2012) found several weeds in Queensland that are hosts of *M. phaseolina*.

All other alternate host isolates examined here were still able to infect strawberry, however displayed lower pathogenicity than N18924. Unlike the three sorghum isolates, the remaining alternative host isolates were not collected from strawberry farms.

11.5. Conclusions

Our study showed that strawberry plants may respond differently to various isolates of *M. phaseolina*. However, out of 17 strawberry isolates evaluated, only one showed significantly lower pathogenicity than the reference isolate. This distinction was more evident when isolates from strawberry and non-strawberry hosts were compared. The strawberry isolates typically recorded higher pathogenicity than non-strawberry isolates. For future screening, the isolates in the collection at MRF appear to be effective, potentially provided they have not been stored for a long time (>15 years).

The use of rotation crops and the presence of weeds that can host *M. phaseolina* may have large implications for the national strawberry industry, as presented by our results. In addition to previous studies (Chapters 9 and 10), efforts to develop strawberry cultivars with resistance to charcoal rot is crucial and should continue into the future.

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12. Container experiment for seed germination in-vitro

Michelle Paynter and Mark Herrington

12.1. Introduction

In recent years, low germination in soil of seed from crosses within the subtropical breeding node have prompted a shift to germination in tissue culture (TC). Traditionally the micropropagation of strawberry plants in-vitro has been in non-vented glass jars (Figure 12.1). Preliminary culture of strawberry seed into containers with breather holes (vented) (Figure 12.2) suggested that plants appeared larger and healthier, and plastic takeaway containers (Figure 12.3) with vents added appeared as effective, and more economical, than glass non-vented jars. Vents allow gas exchange while maintaining aseptic conditions. Plastic takeaway containers are a cheaper option than glass jars, and although not able to be reused, do not require washing up or storage. This experiment was undertaken to compare vented and non-vented tissue culture containers, to determine if they impact plant growth in-vitro. We also investigated if in-vitro container type affected acclimatization of the plantlets after being transferred to glasshouse conditions.

Figure 12.1. Glass jars typically used for seed germination in-vitro

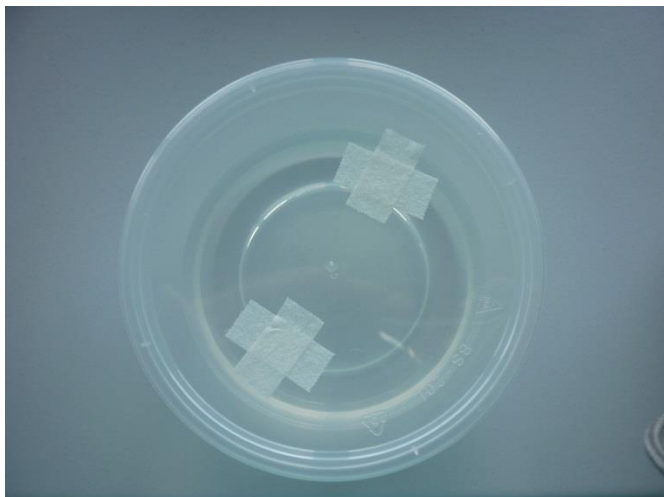*Figure 12.2. Vented polycarbonate 250 mL container with Microfiltration tape covering breather hole.*

Figure 12.3. Takeaway container with two holes covered with Microfiltration tape.

12.2. Methods

Three different containers were compared in this experiment: sterile plastic takeaway (TA) containers (500 mL) with vents added, TA containers (500 mL) without vents, and vented polycarbonate commercial tubs (250 mL). Vents were added to the TA containers using a 5 mm hole punch and the vent covered by adhesive Microfiltration tape.

Seed from the cross between 'Sugarbaby' and breeding line 2013-028 are known to germinate easily, and were therefore selected for use in this experiment. Seeds were placed into sterile 30 mL tubes and disinfested in 2% chlorine solution and two drops of Tween20. The tubes were then placed on a mechanical shaker for 20 minutes after which time the seeds were washed three times in sterile distilled water (Figure 12.4).

Figure 12.4. Sterilization of strawberry seed on a mechanical shaker.

All containers were prepared with quarter strength Murashige and Skoog SS media containing 1.11 g/L Murashige and Skoog, 20 g/L sugar and 4 g/L phytogel. The pH was adjusted to 6.0 prior to autoclaving. Twenty seed were placed into each of six vented TA containers (treatment one) and six non-vented TA containers (treatment two) using the template indicated in Figure 12.5. Ten seed were placed in each of 12 vented polycarbonate containers (treatment 3). Containers were placed in a randomized order in six blocks with four containers per block, on a shelf in a plant TC room. Each block included one container of treatment one, one of treatment two, and two containers of treatment three. Containers were exposed to a 12 hour light/dark cycle under LED lights. Temperature was maintained at 25 ˚C.

Light readings (µmol s^{-1} m^{-2} per µA) using a unit Li-cor LI 1400 datalogger were taken as close as possible to where the seed was positioned in the container, for each block and position.
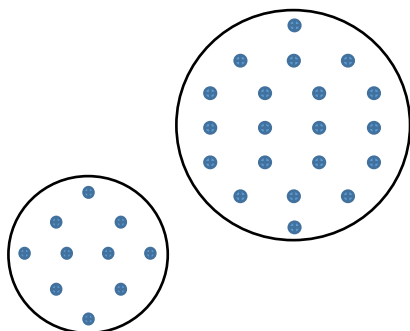

Figure 12.5. Seed placement for 250 mL and 500 mL containers.

Four plants that germinated on the same day from each treatment container were destructively sampled eight weeks after seed initiation. The plants were removed from the media and blotted gently with soft paper towel to remove any free surface media and moisture before being weighed. Fresh weight (TC plant fresh weight) was recorded after which plants were placed into a paper bag, labelled, and dried at 60 ˚C overnight. Plants were again weighed and recorded as 'TC plant dry weight'. The remaining plants were planted into 60 cell plug plant trays using a 50/50, peat /vermiculite (size 3) mix, and watered and fertilized as required. Six weeks after planting out, plants were removed from their cells and surface media gently removed. Each plant was weighed and recorded as 'end plant fresh weight'. Plants were then placed into a paper bag, labelled, and dried at 60 °C overnight. The plants were weighed and recorded as 'end plant dry weight'.

The data from the two vented polycarbonate containers in each block was combined. TC plant dry weight and end plant dry weight were each analysed using Analyses of Variance in Genstat. Light levels at each position were included as a covariate. For the analysis of end plant dry weight, covariates were included for 'end rows' (plants in cells at the ends of rows in the trays) and 'edge plants' (plants in the outside rows in each tray). Off-types, determined by unusual or excessive growth, were not included in the analyses.

12.3. Results and discussion

Seeds began to germinate approximately two weeks after initiation into culture, with a peak at week three of 34.2% of total seeds (Figure 12.6). Overall, 331 seed germinated out of a total of 360 (92%). One hundred and eleven seeds germinated in treatment one, 114 in treatment two, and 106 in treatment three. Four off-types were recorded.

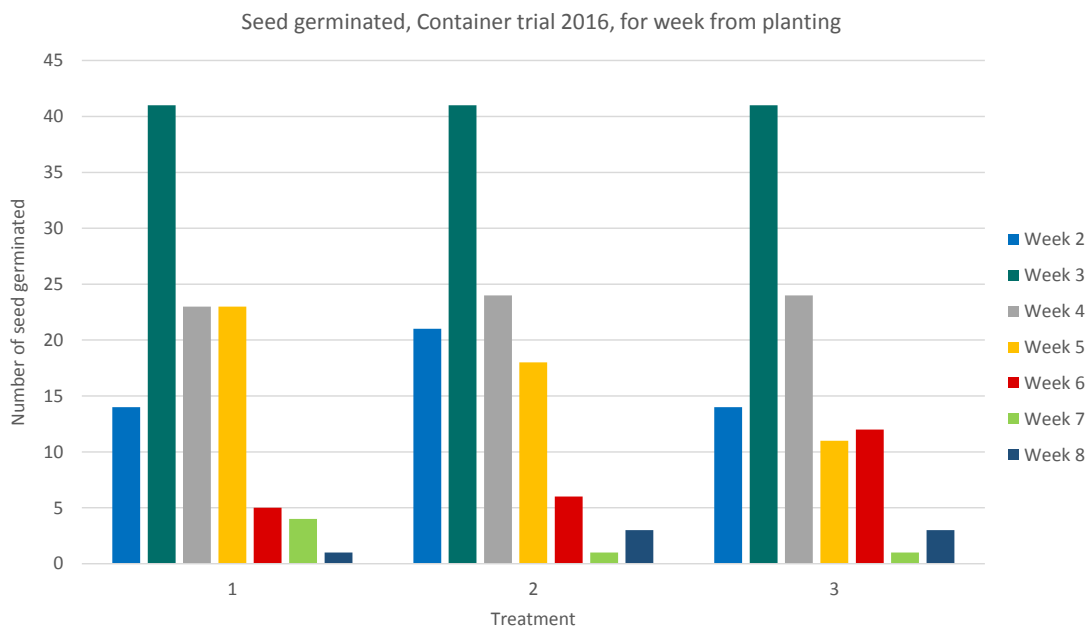Seed germinated, Container trial 2016, for week from planting

Figure 12.6. Number of seed germinating in TC at weeks 2 to 8 post seed initiation. Treatment 1 = TA with vents, treatment 2 = TA without vents, and treatment 3 = vented polycarbonate container.

No significant difference was observed for TC dry plant weight between treatments 1 and 3, although both were significantly different to treatment 2 (P = 0.02, Figure 12.7). There was a significant positive relationship between TC dry plant weight and light level (P=0.01).
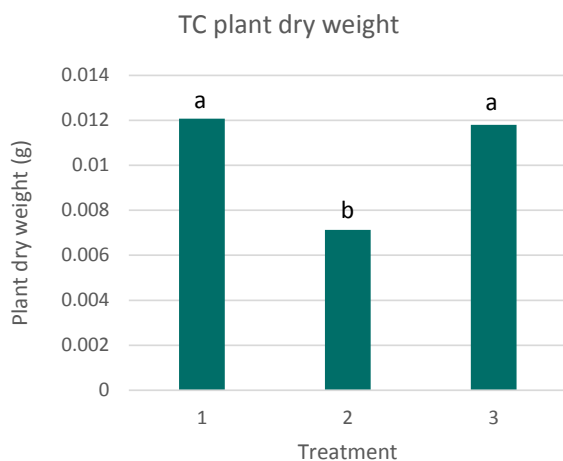

Figure 12.7. Treatment means for 'TC plant dry weight', taken eight weeks after seed initiation. Means with same subscript are not significantly different at the P = 0.05 level. Treatment 1 = TA with vents, treatment 2 = TA without vents, and treatment 3 = vented polycarbonate container.

The analysis of end dry plant weight showed no significant differences between treatments (P = 0.153). There was also no significant relationship between end dry plant weight and either light levels in TC (P > 0.05), the 'end row' covariate (P > 0.05) or the 'edge plant' covariate (P < 0.05).

12.4. Summary

The germination of seeds in tissue culture for the 'Sugarbaby' cross used in this experiment peaked at week three post initiation into TC for all treatments, with the majority of seed germinating by week six. This family of seed

proved very easy to germinate, with very few seed germinating after six weeks post seed initiation. Knowing the germination period or peak can be useful for the estimation of planting out dates. For harder to germinate seed more time may be needed for pre-seed treatments or in TC.

Seed initiated in containers with vents were observed to have larger growth rates in TC. Containers located closer to light sources in the culture room also had larger plants in TC. Treatment 3 containers had opaque lids which potentially lessened the amount of light reaching the seedlings, however there was no significant difference between either of the two container type with vents, whether opaque or clear-lidded (treatments 1 and 3).

Plant size in TC varied with container type, however there was no effect after the plants had been planted out in trays for six weeks. For the germination of strawberry seed in TC, all of the containers used in this experiment therefore appear to be similarly effective.

12.5. Recommendations

If the decision to use containers with vents is taken, container costs (incorporating single use or reusing), time to put holes into TA containers, and time investment required for repeated use (washing and storing, life expectancy) need to be considered. If plant growth in TC is an important requirement then using containers with vents is recommended. Size of container and transparency of lids may need further investigation.

13. Outputs

13.1. Commercialised varieties

Twelve strawberry varieties have been protected under Plant Breeder's Rights and positioned for commercialisation during this project. These include six varieties targeted for subtropical production: 'Red Rhapsody', 'Parisienne Kiss', 'Sundrench', 'Scarlet Rose-ASBP', 'Sunglow ASBP', 'Meadowsong', and 'Venus-ASBP'; two varieties targeted for temperate production: 'Summer Song', and 'Scarlet-silk'; and three varieties targeted for Mediterranean production: 'Rosalie-ASBP', 'Jubilee-ASBP', and 'Fanfare-ASBP'.

13.2. Peer reviewed scientific publications

Fredericks, C.H., Fanning, K.J., Gidley, M.J., Netzel, G., Zabaras, D., Herrington, M., Netzel, M., 2013. High-anthocyanin strawberries through cultivar selection. *Journal of the Science of Food and Agriculture* **93**, 846-852.

Herrington, M.E., Neal, J.M., Woolcock, L.L., 2017. 'Scarlet Rose-ASBP'. *Plant Varieties Journal* **30**, 119.

Herrington, M.E., Neal, J.M., Woolcock, L.L., 2017. 'Sunglow ASBP'. *Plant Varieties Journal* **30**, 120.

Herrington, M.E., 2016. Estimating production costs and profitability of strawberry genotypes in southeast Queensland using plant traits. *Acta Horticulturae* **1117**, 303-307.

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Herrington, M.E., 2015. 'Parisienne Kiss'. *Plant Varieties Journal* **28**, 92.

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Herrington, M.E., 2014. 'Red Rhapsody'. *Plant Varieties Journal* **27**, 107.

Herrington, M.E., Hardner, C., Wegener, M., Woolcock, L.L., 2013. Rain-damage on three strawberry cultivars grown in subtropical Queensland. *International Journal of Fruit Science* **13**, 52-59.

Neal, J.M., Ko, H.L., Gomez, A.O., De Faveri, J., Verbyla, A., Mayer, R., Herrington, M.E., 2017. Testing strawberry genotypes for resistance to *Colletotrichum gloeosporioides* in Queensland. *Acta Horticulturae* **1156**, 743-750.

Paynter, M.L., Herrington, M.E., 2016. Development of a glasshouse bioassay suitable for evaluating Fusarium wilt resistance in strawberry. *Acta Horticulturae* **1127**, 125-132.

Paynter, M., Czislowski, E., Herrington, E., Aitken, E.B., 2016. Differences in pathogenicity, genetic variability and cultivar responses among isolates of *Fusarium oxysporum* from strawberry in Australia. *Journal of the American Society for Horticultural Science* **141**, 645-652.

Paynter, M.L., De Faveri, J., Herrington, M.E., 2014. Resistance to *Fusarium oxysporum* f. sp. *fragariae* and predicted breeding values in strawberry. *Journal of the American Society for Horticultural Science* **139**, 178-184.

Whitaker, V.M., Osorio, L.F, Peres, N.A., Fan, Z., Herrington, M., Cecilia do Nascimento Nunes, M., Plotto, A., Sims, C.A., 2017. 'Florida Beauty' Strawberry. *HortScience* **52**, 1443-1447

Zeilmann, T., Netzel, M., Herrington, M., Rychlik, M., O'Hare, T. J., Fanning, K. J., 2014. New strawberry breeding lines – Enhanced phytochemical composition and bioaccessibility. *Journal of Nutrition and Intermediary Metabolism* **1**, 45.

13.3. Non peer reviewed publications

Barnes, A.J., Gomez, A., Neal, J.M., Herrington, M.E., 2017. Poster: The phenomenal 'Phenomenal': The cultivar developed in Queensland over a hundred years ago used in disease resistance research today. *American Society for Horticultural Science 2017 Annual Conference*. Poster Board # 366. Available at https://ashs.confex.com/ashs/2017/webprogramarchives/Presentation/Paper26832/The_Phenomenal_Phenomen

al_PDF.pdf

Herrington, M.E., Neal, J., Hardner, C., Wegener, M., Woolcock, L.L., Dieters, M.J., 2016. Poster: 'Breeding objective' for strawberry in subtropical Australia. *VIII International Strawberry Symposium*, 13-18 August 2016, Quebec City, Canada.

Herrington, M.E., Hardner, C., Wegener, M., Woolcock, L.L., Dieters, M.J., 2014. Poster: 'Breeding objective' for strawberry in subtropical southeast Queensland. *15th Australasian Plant Breeding Conference*, 26-29 October 2014, St Kilda, Victoria.

Herrington, M.E., Wegener, M., Hardner, C., Woolcock, L.L., Dieters, M.J., 2014. Poster: Estimating production costs and profitability of strawberry genotypes in southeast Queensland using plant traits. *15th Australasian Plant Breeding Conference*, 26-29 October 2014, St Kilda, Victoria.

13.4. Industry publications

Woolcock, L., Neal, J., Herrington, M., Barnes, A., 2017. The heritage, the nostalgia and the future: Breeding better berries for all. *Simply Red* **48**, 4-5.

Ko, L., Paynter, M., 2017. The importance of virus indexing of strawberry plants for breeding. *Simply Red* **47**, 1-3.

Neal, J., Herrington, M. and the rest of the National Strawberry Breeding Team, 2017. National Strawberry Breeding Program update. *Simply Red* **46**, 1-3.

Paynter, M., Neal, J., Herrington, M., 2017. Tissue culture in the National Strawberry Varietal Improvement Program. *Simply Red* **45**, 3.

Neal, J., Herrington, M., 2016. The economics of strawberry traits. *Simply Red* **44**, 2-3.

Gomez, A., Neal, J., Herrington, M., 2016. Identifying strawberry genotypes with resistance to charcoal rot. *Simply Red* **44**, 1.

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Neal J., 2015. Jodi Neal joins the National Strawberry Breeding team. *Simply Red* **39**, 8.

13.5. On-line survey

A short on-line survey was included in the December 2016 edition of Punnet-e News, with the aim to assess trends in the domestic strawberry substrate culture adoption to help positon future breeding directions. Twelve growers responded and of these 33% already used substrate. Of these 75% would maintain or increase their area over the next five years. Of the 66% who had not used substrate, 25% were unlikely to use it, 37% were likely and 37% were unsure. Perceived concerns included: economics i.e. whether extra expenses will result in increased profit, and whether substrate mitigates against big climatic extremes. Perceived benefits included: cost savings through reusable materials, water savings, no soil borne diseases, better work environment for staff, increased production and fruit quality, environmentally friendly, and marketing and sales advantages.

13.6. Presentations

Neal, J., Herrington, M., 2018. Breeding better berries: New Strawberry varieties driving industry growth. *Horticulture and Forestry Science Showcase*, 1 March 2018, Nambour, Queensland.

Neal, J., Herrington, M., 2018. Breeding better berries: Strawberry varieties for a profitable national industry. *BerryQuest International 2018*, 12 February 2018, Launceston, Tasmania.

Herrington, M., Neal, J., 2018. Breeding program update. *Queensland Strawberry Growers Association Annual General Meeting*, 6 February 2018, Glasshouse Mountains, Queensland.

Neal, J., Herrington, M., 2017. Rhapsody in red: New strawberry varieties driving industry growth. *Horticulture and Forestry Science Stakeholder Engagement Event*, 11 December 2017, Dutton Park, Queensland.

Neal, J., Herrington, M., 2017. Specialty strawberry selections. *Queensland Strawberry Growers Association Meeting*, 10 July 2017, Glasshouse Mountains, Queensland.

Neal, J., Herrington, M., 2017. Breeding program update for the sub-tropical production region. *Queensland Strawberry Growers Association Field Day*, 5 July 2017, Wamuran, Queensland.

Neal, J., Herrington, M., 2017. Strawberry breeding for the temperate production region. *Victorian Strawberry Forum*, 4 May 2017, Wandin, Victoria.

Herrington, M., 2017. National Strawberry Breeding Program, update for QSGA. *Queensland Strawberry Growers Association Meeting*, 7 February 2017, Glasshouse Mountains, Queensland.

Herrington, M., 2016. Strawberry breeding at Wandin as part of National Strawberry Breeding Program update. *Victorian Strawberry Growers Association Annual General Meeting*, 25 September 2016, Victoria.

Neal, J., Herrington, M., and the National Breeding Team, 2016. National Strawberry Breeding Program update. *Queensland Strawberry Growers Association Meeting*, 12 July 2016, Stanthorpe, Queensland.

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Herrington, M.E., Wegener, M., Hardner, C., Woolcock, L.L., Dieters, M., 2014. Estimating production costs and profitability of strawberry genotypes in southeast Queensland using plant traits. *XXIX International Horticultural Congress*, 17-22 August 2014, Brisbane, Queensland.

Herrington, M.E., Hardner, C., Wegener, M., Woolcock, L.L., Dieters, M.J., 2014. 'Breeding objective' for strawberry in subtropical southeast Queensland. *XXIX International Horticultural Congress*, 17-22 August 2014, Brisbane, Queensland.

Herrington, M.E., Wegener, M., Hardner, C., Woolcock, L.L., Dieters, M.J., 2013. Using plant traits to estimate production costs and profitability of strawberry genotypes in southeast Queensland. *2nd International Strawberry Congress*, 4-6 September 2013, Antwerp, Belgium.

13.7. Breeding program field days and site tours

Tour of Australian Strawberry Breeding Program elite selection trial at Red Jewel Experimental Farm, *Queensland Strawberry Growers Association Field Day*, 5 July 2017, Wamuran, Queensland.

Tour of breeding trials at Wandin Strawberry Research Centre, *Victorian Strawberry Forum*, 4 May 2017, Wandin, Victoria.

Temperate Reference group selection inspection of Wandin Strawberry Research Centre, 27 Feb 2017, Wandin, Victoria.

Tour of breeding trials at Wandin Strawberry Research Centre, *Victorian Strawberry Growers Association Field Day*, 25 November 2016, Wandin, Victoria.

13.8. Media coverage

Media release: Juicy news for strawberry lovers. *Queensland Government Department of Agriculture and Fisheries*, July 2017. Available at http://statements.qld.gov.au/Statement/2017/7/21/juicy-news-for-strawberry-lovers

A new Sunshine Coast-bred strawberry is selling out at nurseries across the country. *Seven News Sunshine Coast* (television), July 2017. Available at https://www.facebook.com/7NewsSC/videos/1540461016011327/

New Queensland varieties berry good news for strawberry lovers. *ABC Rural* (radio and website), July 2017. Available at www.abc.net.au/news/rural/2017-07-21/new-strawberry-varieties/8732138

Red Rhapsody hits Aussie shelves. *Produce Plus* (website), July 2017. Available at http://www.fruitnet.com/produceplus/article/172865/rhapsody-of-a-berry-hits-aussie-shelves

Coast farmers breed the ultimate strawberry. *Sunshine Coast Daily* (website), July 2017. Available at https://www.sunshinecoastdaily.com.au/news/coast-farmers-breed-the-ultimate-strawberry/3203200/

Juicy news for strawberry lovers. *Feature Magazine* (website), September 2017. Available at https://www.featuremagazine.com.au/single-post/2017/09/01/Juicy-News-for-Strawberry-Lovers

14. Outcomes

The specific goals in this project were to:

- Commercialise 2-6 (low chill - short day or day neutral) varieties with high consumer appeal and outstanding agronomic characteristics, which capture at least 25% of the total Queensland/NSW by 2018.
- This will contribute to the capture by the national breeding program of 22% of the total Australian market by 2018.

These outcomes have been achieved in the current project.

Twelve varieties have been commercialised or positioned for commercialisation under project BS12021, covering all three targeted production regions. These varieties have an estimated increased profitability for production over current international varieties, which in combination with an effective industry communication strategy, has resulted in excellent uptake of new Australian Strawberry Breeding Program (ASBP) varieties by industry.

In 2017, varieties from the ASBP captured 44% of the Queensland/NSW market, and 19% of the total Australian market. These plantings had a farm gate value of approximately $78 million and contributed ~2700 jobs in production alone. While 2018 runner sales of ASBP varieties are not yet available, indications from runner growers suggest that 2018 sales will be substantially higher than 2017 levels. In addition, several varieties produced by the subtropical breeding program are currently being trialled by strawberry producers in the Mediterranean and temperate regions, suggesting that there may be some overlap of varieties between regions.

Of the varieties released under this project, 'Red Rhapsody' is the oldest and therefore has the largest number of runners available for sale. This variety sold out in nurseries for the 2018 season. Various reports from industry have described 'Red Rhapsody' as a "game-changer", and a "grower's variety", with excellent and consistent production, an high proportion of premium quality fruit, and traits that have significantly reduced the time to pick and pack fruit, thereby reducing production costs. Flavour is also good and consistent, and supermarket chains have indicated a preference for 'Red Rhapsody' fruit. While adoption of the newer varieties is still in its early stages, runner sales are promising and reports suggest that at least several of these varieties are likely to have strong positions for domestic and international export markets.

15. Monitoring and evaluation

In the first three years the project's progress, direction and outputs were monitored through twice yearly meeting interaction, reporting to, and guidance under the Hort Innovation/Strawberries Australia National Strawberry Variety Improvement Steering Committee (NSVISC). This was in association with project milestones dates. At the inclusion of responsibility for the temperate region's breeding program under the joint national program, monitoring was undertaken though milestone achievements. From 2017 the project's progress, direction and outputs were monitored by Hort Innovation's Strawberry Variety Steering Committee (SVSC). This involved annual face to face meetings allowing interaction, reporting, and guidance. All milestones were achieved on time and within Hort Innovation's allocated budget.

An external review of the project was conducted in 2016 and was generally favourable. All recommendations from the review were incorporated where feasible under the current project. Others have been incorporated into the Chapter 16 recommendations below for future projects (e.g. incorporating DNA based selection methods into the breeding strategy).

16. Recommendations

16.1. Increase market size and value

Recent years have seen substantial increases in the number of strawberry runners planted nationally. For example, approximately 34 million runners were planted in the 2016 subtropical production season, which increased to over 50 million in 2017. Gross margin responses to the increased planting volumes (i.e. decreased prices because of increased fruit volume on the national market) indicate that a more disciplined production, quality maintenance and marketing strategy would be appropriate. Increasing the total market size by exporting fruit, making available higher value product (e.g. speciality fruit types), and national industry benchmarking would likely contribute to meeting a more disciplined production, quality maintenance and marketing strategy.

16.2. Focus for export

As suggested above, a strategic approach to the international export of fruit could greatly benefit the national industry. The breeding program should incorporate export-enhancing traits at appropriate levels, as specified from consultation with industry and international markets. Because Western Australia has an existing export focus, the breeding program should increase the focus of Mediterranean cultivar development in the near future into further developing higher export capacity product for that region.

In parallel with this, it is recommended that a nationally coordinated industry export management group be developed to coordinate production for specific export markets. Export needs to be deliberate, strategic and coordinated to maximise the national benefit - an ad hoc market driven approach is no longer sensible for long term benefit.

16.3. Focus for higher value product type

An additional approach for increasing market size and value is diversification of the strawberry sector. Unlike many other fruit and vegetable crops where multiple varieties with different traits are marketed, strawberries are sold as a single, generic product. In order to increase product diversification and value it is therefore recommended that the breeding program develop additional product types, e.g. different fruit colours and fruit flavours, thereby expanding the choices available to consumers. This can include varieties for different end use types, e.g. for salad, gifts, etc.

Additionally, a coordinated national marketing strategy should be implemented by industry to increase the virtual/experience/emotional value attached to strawberry and strawberry product types. Consumers are likely to pay more for the experience attached to a product than for the product itself. This will therefore encourage higher strawberry punnet values.

16.4. Develop variety benchmarking data

Currently, new varieties are released to industry with limited knowledge of requirements or optimums for nutrition, soil type, location, or other production factors. In order to cost-effectively and efficiently fill this knowledge gap, we recommend the development of a database of these production factors plus genotype performance (yield etc.) collected from the breeding program on-farm trials. The addition of data from runner producer trials, where possible, into such a database would allow statistical interrogation of all data to determine likely appropriate cultural practices for both new and old selections. Benchmarking would have the added benefit of improved commercial cultivation practises for specific varieties, increasing overall fruit quality and flavour, and thereby product value.

16.5. Increase focus on substrate culture

Nationally, strawberry production in substrate culture systems has increased dramatically, especially in Tasmania where it is also associated with protected cropping. Substrate culture gives benefits in the management of some soil borne diseases, access to labour, labour efficiency, and the potential for producing higher value product (minimises likelihood of soil residues on fruit etc.). Issues with substrate culture however include increased fruit fly

potential. Some diseases and pests (e.g. powdery mildew and mites) also potentially increase under protected cropping. The effect of cyclones or similar weather events on substrate culture and protected cropping has yet to be assessed, and the effective 'whole of life cycle' on environmental and economic terms has not yet been documented.

Cultivars capable of production in substrate culture should be determined, as well as documentation of plant characteristics suited to substrate culture. Breeding efforts should then be directed to providing new varieties specifically adapted to fruit production in substrate, and under protected cropping.

16.6.   Expand disease resistance focus

With expanding strawberry production in substrate culture and protected cropping nationally, powdery mildew is becoming increasingly problematic as a significant disease of strawberry plants and fruit. Work should be conducted to increase understanding and inclusion of powdery mildew resistance into the breeding program objectives. Screening and breeding for resistance to the crown wilt diseases *Macrophomina phaseolina*, *Colletotrichum gloeosporioides*, and *Fusarium oxysporum* f. sp. *Fragariae* should continue.

In addition, *Pestalotiopsis* sp. appears to be increasing in occurrence in commercial fields and breeding plots in the subtropical region. Defining the reaction of cultivars and germplasm to the *Pestalotiopsis* challenge should also be considered in the future.

16.7.   Incorporate DNA based selection into the breeding program

A number of technologies have potential for accelerating and increasing efficiencies in modern breeding programs. The USDA-SCRI funded RosBREED aims to incorporate advanced DNA-based methodologies into 22 US breeding programs focusing on eight rosaceous crops, including strawberry. Currently, DNA markers for "peach" and "sherry" flavours have been developed for strawberry. Markers such as these should be applied in the future to facilitate a more targeted breeding strategy for flavour.

Statistical genomics methods, such as genomic selection, use DNA markers spread across the genome to aid in the dissection of complex traits and predict the performance of selected crosses. In order to incorporate genome scale analyses, linkages should be developed with the RosBREED program to develop genetic markers for traits of interest, e.g. fruit soluble solids content (sugar content). This approach would allow genomic prediction and genome-wide association analyses of these traits within the breeding population, with a view to making improved selections and accelerating varietal development.

16.8.   Increase industry and consumer involvement

Industry and consumer engagement is critical to the development of superior varieties that successfully fulfil producer and consumer requirements, both now and under future market and environmental conditions. Formal reports of activities should be presented twice yearly to the Strawberry Variety Steering Committee (SVSC). Industry involvement should continue to be facilitated and expanded via the SVSC, local steering committees, on-farm trials of advanced selections, and seeking feedback at field days, meetings, and in general interactions with industry members.

16.9.   Investigate a permanent temperate trial site

The temperate region selection program is currently conducted on leased land in Wandin, Victoria, and present arrangements only allow for one more three-year lease period. This arrangement would be reassessed if a new project is contracted, however having long-term access to the Wandin research site would likely remain uncertain. With current staffing and lease arrangements it would be prudent to look toward having a more permanent temperate climate research site. In order to identify a suitable, more permanent location, we recommend commencing the comparison of genotype performance at Wandin with performance at other sites (e.g. Applethorpe), to determine correlations and therefore suitability.

16.10. Expand international seed exchange and linkages

For the ASBP to remain cutting edge, collaboration and information exchange with international strawberry breeding programs and scientists is essential. Established protocols used with the existing successful seed exchange agreement with the University of Florida Strawberry Breeding Program should be utilised to establish an additional international seed exchange agreement supporting the temperate and Mediterranean production regions. The existing agreement has been extremely beneficial for maintaining genetic variation within our subtropical breeding program. It has also resulted in the release of popular ASBP variety 'Rubygem' from Florida seed, and the recent release of Florida variety 'Florida Beauty' from ASBP seed.

Overseas deployment of cultivars for subtropical and temperate regions should be strengthened. Breeding team members should also attend and present at international conferences, such as the International Strawberry Symposium. This would provide a mechanism for international review of project activities and methods and ensure strong research collaborations are fostered and maintained.

17. Refereed scientific publications

Fredericks, C.H., Fanning, K.J., Gidley, M.J., Netzel, G., Zabaras, D., Herrington, M., Netzel, M., 2013. High-anthocyanin strawberries through cultivar selection. *Journal of the Science of Food and Agriculture* **93**, 846-852.

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Herrington, M.E., 2014. 'Red Rhapsody'. *Plant Varieties Journal* **27**, 107.

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18. Intellectual property, commercialisation and confidentiality

Twelve strawberry varieties have been protected under Plant Breeder's Rights and positioned for commercialisation during this project. These were: 'Red Rhapsody', 'Parisienne Kiss', 'Sundrench', 'Scarlet Rose-ASBP', 'Sunglow ASBP', 'Meadowsong', 'Venus-ASBP', 'Summer Song', 'Scarlet-silk', 'Rosalie-ASBP', 'Jubilee-ASBP', and 'Fanfare-ASBP'.

Acknowledgements

Appendix 1

Screening for Fusarium wilt resistance Methodology

The isolates were plated onto 1/4 strength potato dextrose agar (PDA) amended with 50 mg·L-1 streptomycin sulphate, and incubated in the dark at 24 °C for 2 weeks. Spores were collected from the plates following addition of sterile deionized water by rubbing the agar surface with a glass spreader. The spore suspension was then filtered through four layers of cheesecloth. The conidial concentration was determined using a haemocytometer and adjusted to 1×10^6 conidia/mL using sterile deionized water. After preparation, inoculum was used within 3 to 5 hours.

Experimental strawberry plants were propagated by runners and grown in pots containing steam-sterilized river sand and coir (1:1). The potted plants were then stored in a shade house, and watered and fertilized regularly as required until ready for inoculations.

To prepare sterilized ryegrass seed, the ryegrass seed was rinsed under running tap water, drained and soaked overnight in distilled water, after which the water was strained off and the seed rinsed several times with tap water until water was clear. The seed was then autoclaved at 121 °C for 20 min on three consecutive days.

Inoculation process: Ten replicate plants of each genotype/cultivar were removed from their pots and approximately 3 cm cut from the bottom of the root ball (soil and roots). The 3 cm section was put back into the pot and 20 mL of sterilized ryegrass seed spread on top. The crowns and remaining roots were washed, drained, and placed into plastic bags in a head-house to keep moist and clean. These were inoculated in a randomized order by immersing the remaining plant root ball in the inoculum (to 1 cm past the top of crown) for 10 minutes, then placed back on top of the ryegrass in their pot. Ten plants of cultivar Kabarla were immersed in sterile deionized water only to provide negative controls. Treated plants were placed back in their pots and refilled with steam-sterilized river sand and coir (1:1). Each pot was randomly allocated onto one of five benches heated to 28 °C in a glasshouse with natural light and maintained to approximately 25 °C. Disease development was monitored on the individual plants and visual severity ratings taken at weekly intervals post-inoculation. Severity of foliar symptoms was assessed on a 0 to 10 disease visual index (Hutton and Gomez, 2006).

The severity of foliar symptoms was assessed as follows:

0 = plant healthy, with erect growth and full vigour
1 = plant generally healthy, with smaller canopy and moderate vigour
3 = plant with a slight wilt, with lower leaves affected
5 = plant with a moderate wilt, with the mature leaves collapsed but young leaves still healthy
7 = plant with a severe wilt, with most of the plant collapsed and desiccated
9 = plant with a very severe wilt, with the entire plant collapsed and desiccated
10 = plant dead

The degree of resistance (x) to *Fof* was determined from the mean disease severity score by the following scale:

$x \le 2$ = resistant
$2 < x \le 4$ = moderately resistant
$4 < x \le 6$ = moderately susceptible
$6 < x \le 8$ = susceptible
$x > 8$ = very susceptible

Symptomatic plants across all experiments were sampled at 10 to 12 weeks post-inoculation to confirm the presence of *Fof*. The crowns were washed clean, surface sterilized in sodium hypochlorite and rinsed three times in sterile deionized water. Crowns were cut in cross section and discoloured pieces of crown plated onto 1/4 strength potato dextrose agar and incubated at 24 °C.

Appendix 2

Mixed model analysis of fusarium wilt resistance

To investigate the genetic effects influencing resistance to fusarium wilt, the disease severity scores for the screenings were analysed using a linear mixed as described:

$$y = X\tau + Z_g u_g + Z_o u_o + e$$

where y is the vector of observed disease ratings, τ is a vector of fixed effects (e.g., overall mean) with design matrix X, u_g is a vector of random total genetic effects with design matrix Z_g, u_o is a vector of other non-genetic random effects (e.g., replicate and table effects) with design matrix Z_o, and e is the vector of random residual effects.

All random effects in the model are assumed to be normally distributed with mean zero and the three random effect vectors (u_g, u_o, e) are assumed pair-wise independent. The variance models for the random non-genetic and residual effects are given by:

$$Var(u_o) = \sigma_o^2 I$$
$$Var(e) = \sigma_e^2 I$$

The vector of total genetic effects can be partitioned into three components namely additive, non-additive and family genetic effects. That is:

$$u_g = u_a + u_{na} + Z_f u_f$$

where u_a represents the vector of additive genetic effects with distribution $u_a \sim N(0, \sigma_a^2 A)$ where A is the known additive relationship matrix based on the pedigree information, u_{na} represents the non-additive or residual genetic effects with distribution $u_{na} \sim N(0, \sigma_{na}^2 I)$ and u_f represents the family genetic effects with distribution $u_f \sim N(0, \sigma_f^2 I)$.

Using the REML estimates of the variance components in the linear mixed model, the narrow-sense heritability (proportion of 'additive' genetic variance over the total variance) was estimated by:

$$h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_{na}^2 + \sigma_f^2 + \sigma_e^2)$$

Where σ_a^2 is the additive genetic variance of the individual genotypes, σ_{na}^2 is the non-additive genetic variance, σ_f^2 is the genetic variance between families, and σ_e^2 is the variance of the random residuals. Breeding values were predicted for each of the 245 progeny and 4 parents, obtained by the BLUPs. Broad-sense heritability was estimated as:

$$H^2 = (\sigma_a^2 + \sigma_{na}^2 + \sigma_f^2) / (\sigma_a^2 + \sigma_{na}^2 + \sigma_f^2 + \sigma_e^2)$$