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Microsatellite markers reveal low breeding system efficacy and pollen contamination can limit production of full-sib avocado progeny

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ABSTRACT

Phytophthora cinnamomi causes a severe root rot in avocado, *Persea americana*. Breeding tolerant rootstocks is thought to be the most promising method for phytophthora root rot disease control but breeding avocado is challenging. The avocado flowering syndrome (synchronous protogynous dichogamy), combined with high flowering and low fruit set, render controlled pollination exceedingly difficult. Juxtaposing complementary flowering types of elite parent cultivars (cultivars that produce progeny with tolerance to phytophthora root rot) was performed in an effort to increase the number of full-sib progeny for elite maternal parents and, hypothetically, the number of phytophthora root rot tolerant progeny. Although high outcrossing rates were achieved (estimated ~93%), the majority of progeny had a non-elite paternal parent (56% of progeny were offtypes) implying maternal trees were pollinated by non-elite distant trees. Among progeny that could be confidently genotyped, a high number of cross types were detected (33). Contrary to our hypothesis, a significant portion of the progeny were the result of crosses between like, and not complementary, flowering types. The spatial distribution of productive trees and grafts helped to explain these data, as productive grafts were directly adjacent to grafts of the same flowering type more often than that of the complementary flowering type. Selfed progeny were significantly less tolerant to phytophthora root rot than outcrossed progeny. Progeny resulting from crosses between an elite maternal parent and non-elite pollen donor (offtypes) were less tolerant than full-sib progeny resulting from crosses between elite parents. Maternal effects may interfere with identifying truly disease tolerant selections. Thus, to reduce maternal effects and non-elite pollen donor contamination, removal of seedling cotyledons before screening for disease tolerance and better isolation of elite parent trees and windbreaks may improve breeding efficacy. This study also demonstrates the usefulness of microsatellite markers in parentage analysis where a high proportion of the putative parents are closely related.

1. Introduction

The invasive pathogen *Phytophthora cinnamomi* causes a lethal disease of avocado, *Persea americana* Mill., phytophthora root rot (PRR) (Menge and Ploetz, 2003; Pegg et al., 2002). As an oomycete with several persistent spore types (Erwin and Ribeiro, 1996; Judelson and Blanco, 2005; Kassaby et al., 1977; Krober, 1980) and a broad host range, *P. cinnamomi* can often be considered a permanent resident once established (Zentmyer and Mircetich, 1966).

There are few effective control measures for PRR and integrated strategies are usually needed to manage the disease (Coffey, 1987). Breeding tolerant rootstocks is among the most promising components of disease management (Bijzet and Sippel, 2001; Menge et al., 1992; Menge, 2001; Violi et al., 2006). Avocado shares no evolutionary history with *P. cinnamomi*. Thus, there is little natural resistance upon which to select and most individuals are highly vulnerable to PRR. For this reason, modern plant breeding with the use of molecular tools constitutes a promising approach.

The physiological basis for PRR tolerance in avocado is unknown. Conflicting reports of varietal differences in tolerance exist (see list of PRR tolerant cultivars and racial background in Menge et al., 1992). However, previous work in Florida on the subgenus *Persea* provides some evidence that progeny with *P. americana* var. *americana* and *P. americana* var. *americana* × *P. americana* var. *guatemalensis* pedigrees had significantly greater

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Table 1
(a) Avocado accessions that were established as grafts in 2004 in paired combinations on trees at TREC-Homestead.

Elite cultivar	Variety[a]	Accession[b]	Flowering type[c]	Number of productive grafts (2006)
Catalina	W	17248	A	6
Family	W	2895	B	3
Girrardin	W	23343	A	3
Hiawassee	GW	26063	A	2
Maxima	W	23271	B	2
Monroe	GW	19852	B	4
Pollock	W	19846	B	2
Simmonds	W	670	A	5

(b) Rootstocks and other cultivars at TREC-Homestead.

Cultivar	Flowering type[c]
Booth-7	A
Booth-8	B
Lula	A
Peterson	A

[a] Varieties are: W, which are pure West Indian race, *P. americana* var. *americana*, or GW, which are hybrids between the Guatemalan race, *P. americana* var. guatemalensis, and West Indian race.
[b] All accessions were from the USDA-ARS clonal germplasm repository in Miami (MIA numbers are shown for each accession).
[c] Flowering phenotypes are: A, where flowers are first female receptive in the morning and later perfect or functional as a male that afternoon; or B, where flowers are first female in the afternoon and then perfect or male functional the following morning.

tolerance to PRR than those with other backgrounds (Ploetz et al., 2002). PRR tolerance from these cultivars in the USDA-ARS Miami germplasm collection was estimated to have a broad-sense heritability of 0.45 (Ploetz et al., 2002). Based on these findings, current efforts to select for PRR tolerant individuals have focused on producing progeny from these "elite parents" and with the ultimate goal of identifying molecular markers associated with PRR tolerance.

Avocado has a flowering syndrome called synchronous protogynous dichogamy (Kubitzki and Kurz, 1984) involving two flowering types typically referred to as "A" and "B" (Stout, 1923). Under "optimal" environmental conditions, each flowering type becomes functionally female or male at alternate times of the day (Sedgley, 1977; Sedgley and Annells, 1981; Sedgley and Grant, 1983). This pollination syndrome theoretically promotes outcrossing. However, controlled pollinations are usually not effective. In a given season, up to one million flowers are produced per tree (Monselise and Goldschmidt, 1982) but only a very small number of the flowers set fruit [(0.02–0.07%) (Inoue and Takahashi, 1990; Cameron et al., 1952)]. Additionally, self-pollination is possible (Davenport et al., 1994). Thus, placing trees of complementary flowering type close together (juxtaposing) may increase outcrossing between target parents.

The main assumptions tested for this study were that full-sib progeny from putatively elite parents will have greater tolerance to PRR and that juxtaposing cultivars with complementary flowering phenotypes will increase production of full-sib progeny collected from elite maternal parents. Cultivars with complementary flowering types were grafted onto individual trees. The objective of this study was to assess the efficacy of this design by determining: (1) outcrossing rates among progeny and if the majority of the pollen donors are among the elite parents; (2) if the majority of progeny resulted from parents with complementary flowering types; (3) the parentage of PRR tolerant progeny; and (4) if outcrossed progeny are more PRR tolerant than progeny from self-pollination.

2. Materials and methods

2.1. Study site and selection of elite parents

The study sites are located at the USDA-ARS Miami and at the University of Florida Tropical Research and Education Center in Homestead, Florida (TREC). In the winter of 2004, pairs of elite cultivars with compatible flowering types (type A and B) were grafted to hatracked trees (i.e., the original scion was cut and replaced with elite parent cultivars) within the avocado collection at TREC (Table 1). In addition to the elite parent cultivars listed in Table 1, 'Booth-7', 'Booth-8', 'Lula' and 'Peterson' cultivars are contained within the collection and interspersed among grafted trees.

All fruit was collected from grafted trees between August and November 2006. There were several cases of rootstock or original scion intrusion (i.e. a grafted branch failed and the rootstock, or original scion, grew in its place). Intrusion was evident based on morphological characteristics in ten cases. Following the parentage analysis, 24 additional progeny were found to be the product of rootstock, or former scion, intrusion. These seedlings were excluded from the PRR analyses where the associated questions only concerned elite maternal parents. These data were included in the analyses associated with mating system efficacy as they likely had an impact on seedling parentage. Within three years, 27 of the 58 elite parent grafts produced fruit. Fruit was processed and planted as described below.

2.2. Production and inoculation of seedlings

Seeds were planted in 1 L pots in a greenhouse at the USDA-ARS facility in Miami, Florida. Substrate consisted of steam-sanitized potting mix (Nursery Mix; Atlas Peat & Soil, Inc., Boynton Beach, FL) composed of 50% pine bark, 10% sand and 40% coir pith.

In February 2007, 4-month old progeny were transplanted into new 1 L pots and inoculated with *P. cinnamomi*. Inoculum was prepared by inserting PARPH agar plugs colonized by *P. cinnamomi* into flasks containing 20 mL autoclaved (45 min) millet seed and allowing the sealed flasks to incubate at room temperature for two weeks. Inoculated millet seed was then homogenized and mixed with potting substrate in a large cement mixer (0.1% by volume) (Ploetz et al., 2002). Ocular estimates of percent root necrosis were taken in May 2007 and the presence of *P. cinnamomi* was determined as described in Ploetz et al., 2002. Two seedlings died weeks prior to collection of root necrosis data and were excluded from the analysis.

2.3. Laboratory analyses

Prior to inoculation, leaf samples were collected from each 2–3-month old seedling for extraction of DNA ($N = 246$). In addition,

Table 2
Cross types identified for progeny collected from "elite" maternal parents (superior tolerance to phytophthora root rot). Elite parents are those found to be relatively tolerant to PRR. Offtypes are defined as progeny resulting from a cross which appeared to involve a non-elite pollen donor. One hundred and twenty offtypes were detected.

Maternal parent	Pollen donor													
	Booth-8	Catalina	Family	Girrardin	Hiawassee	Lula	Maxima	Monroe	Peterson	Pollock	Simmonds	No. unknown donor	No. known donor	Total
Catalina		3		27			1	2	3	1	2	33	39	72
Family	2	1	2	2						2		8	9	17
Girrardin				2			4		2		1	3	9	12
Hiawassee					1	1						9	2	11
Maxima				14				2		2	2	11	20	31
Monore												4	0	4
Pollock				1	1	1			2	1	5	12	11	23
Simmonds	1		1						3	8	5	25	18	43
Total	3	4	3	46	2	2	5	4	10	14	15	105	108	213

leaves from all elite parent cultivars and other cultivars at TREC were collected (Table 1b). Non-elite parent cultivars on the TREC station were collected as they were possible pollen donors.

Twelve microsatellite markers were used in the study (Table 2). Markers were selected from over 100 markers available for avocado based on consistency of amplification and their ability to discriminate among parents used in the study. All markers were only partially informative due to the close relatedness of elite parents; putative parents shared at least one allele with another putative parent.

DNA extraction was performed as in Schnell et al. (2003). PCR protocols followed those described in Borrone et al. (2007), for the primers described therein. For the primers developed by Sharon et al. (1997), PCR was performed as in Schnell et al. (2003). Protocols within this study only differed in that PCR amplifications were performed using 10–20 μL reactions containing 0.50–0.25 ng μL^{-1} genomic DNA.

Capillary electrophoresis was performed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) and PCR products were prepared, processed and resulting electropherograms analyzed as described in Schnell et al. (2003).

2.4. Parentage analyses

Parentage analysis on progeny of maternal cultivars was performed using CERVUS ver. 3.0.3 (Kalinowski et al., 2007; Marshall et al., 1998; Slate et al., 2000). Default settings were used, except that we assumed sampling of 60% of possible paternal parents and 100,000 iterations were run for the paternity simulation. Twelve possible parents were genotyped and included in the analyses, these included the eight elite parent cultivars and other non-elite cultivars at TREC (Table 1). Other potential pollen donors (not included in the analysis) were >100 m from the study trees; numerous farms and yards containing *P. americana* exist in the vicinity of the study site but the opportunity for gene flow to our study trees was thought possible but unlikely to substantially contribute.

Pollen donors were only assigned to progeny if maternal parent, putative pollen donor and progeny together had a combined positive log-likelihood score (trio LOD). All other progeny were either not assigned pollen donors (nine were excluded because too few loci could be confidently scored) or were categorized as "offtypes." Offtypes were defined as having a non-elite parent (either identified as genotyped non-elite cultivars that were at TREC or cultivars not sampled in this study per the negative combined LOD scores). For the purposes of this study, selfs refer to progeny that were the result of autogamy, geitonogamy or pollen exchange between two grafts of the same cultivar (i.e. crosses among clones). Elite parents are known to be closely related (Schnell et al., 2003), resulting in low trio LOD for some progeny despite a lack of mismatches among progeny and known maternal parental loci. Fortunately, a high proportion of the progeny contained rare alleles (frequency <8%) that increased the confidence with which progeny could be categorized as offtypes (see results section titled *"Heterozygosity, null and rare alleles"*).

2.5. Statistical analyses

All analyses were performed in JMP 5.1.1 [(JMP, 1989–2002) Version 5.1.1 SAS Institute Inc., Cary, NC]. Percent root rot necrosis data were strongly skewed. The Box-Cox algorithm was used to attain a normal distribution:

$$\frac{(\text{Percent root necrosis}^2 - 1)}{165.0751}.$$

One-way ANOVAs were then performed using transformed data to detect maternal and pollen donor effects on progeny disease tolerance. Post-hoc tests were performed using Tukey-HSD. A standard *t*-test was performed to determine if outcrossed progeny were more PRR tolerant then selfed progeny.

2.6. Spatial considerations

Many grafts within the study failed to produce fruit. Also, the position of productive grafts in relation to potential pollen donors of like and complementary flowering types varied. Only trees and grafts that had the ability to contribute pollen to the elite maternal parent grafts (i.e. grafts and trees that produced fruit and/or flowers) were included in the spatial analysis. To address the potential impact of the spatial distribution of flowering types on progeny, a Wilcoxon signed rank-sum test was used to determine if the position of like and complementary flowering type grafts was the same for productive grafts on the same tree and for productive distant trees. The number of fruit produced per graft was included in the analysis to estimate the potential contribution of each graft as a pollen donor and to determine the relative impact of neighboring pollen donors on the population.

Data relating to number and distance of trees of a given flower type from grafts were treated as an ordinal (distance classes) rather than continuous (absolute distance). Distance classes were used to account for variability in the contribution of pollen from a given tree due to pollen load or tree size. The inclusion of ordinal data paired with difficulties meeting the assumptions for parametric tests, rendered the non-parametric, Wilcoxon rank-sum test more appropriate (Sokal and Rohlf, 1995).

We asked if a given fruit (collected from an "A" or "B" type graft) was positioned next to the same and complementary flowering type grafts an equal number of times for: (1) productive grafts on

the same tree; (2) for Trees 1 row away (between ~3 and 8 m from grafted branch) and (3) for Trees 2 rows away (between ~8 and 20 m from grafted branch)?

3. Results

3.1. Heterozygosity, null and rare alleles

The mean number of alleles per locus was higher for progeny (7.67) than it was for the maternal parent population (5.25). Mean expected heterozygosity of all markers was 0.677 for parents and 0.602 for progeny populations.

In several cases, mismatches that were detected in the parentage analysis, among known parents and offspring were attributed to the occurrence of null alleles. Out of the progeny genotyped, null alleles were detected in just 14 cases and the mean observed genotyping error rate was 0.056. Despite "allele dropout", the average amount of missing data was low, 1.4 loci/sample. Null alleles have previously been reported for the six loci involved (Ashworth et al., 2004; Borrone et al., 2007; Sharon et al., 1997).

Alleles that had a frequency in the population of less than 8% for a given locus were defined as rare. Seventy percent of these rare alleles were not detected in the elite parent population. In ~84% of cases where such alleles were detected in the elite parent population, they occurred in only one cultivar. Thus, rare alleles contributed significantly to the confidence with which parents were assigned to progeny. Specifically, the frequency of rare alleles that did not occur in the elite parent population was significantly higher for progeny that could not confidently be assigned known parents (i.e. negative LOD scores) relative to those with known parents (ANOVA: $F = 15.61$, d.f. = 3, $P < 0.0001$, $N = 246$). Among progeny with negative LOD scores, the average number of non-elite parent rare alleles per seedling was 1.45, whereas in progeny categorized with positive LOD scores and with confidence greater than 80%, none of these alleles were detected.

3.2. Parentage analysis

Progeny with an elite maternal parent were the result of at least 33 unique cross types (Table 2). Twenty-four seedlings were determined not to have an elite maternal parent and are not included in the table. These included five for which a maternal parent appeared to be the rootstock, and 19 where the maternal parent was either 'Booth-8', 'Lula' or 'Peterson' (the former scions). However, 56% of the progeny from elite parent grafts were not the result of crosses between two elite parents and pollen donors other than those found at TREC seemed to be contributing substantially (Table 2). Among the full-sib progeny (parents were both elite cultivars), the three most common cross types were 'Simmonds' × 'Pollock' (8), 'Catalina' × 'Girrardin' (27) and 'Maxima' × 'Girrardin' (14).

3.3. Mating system efficacy

The majority of seedling progeny genotyped were not a result of exchange between parents of complementary flowering types. Including progeny with a non-elite maternal parent, only 46% of the progeny could confidently (positive combined trio LOD scores) be assigned pollen donors (113 out of 246 seedlings). Selfing among parent cultivars was low. Specifically, for progeny giving positive combined trio LOD scores, only 14.29% were the result of selfing or pollen exchange between trees of the same cultivar (35 out of 113 progeny for which both pollen donor cultivars were confidently assigned).

In addition, a chi-square test was performed to determine if seedling progeny were more likely the result of maternal parent (either type A or type B) receiving pollen from a paternal parent of the same versus a complementary flowering type. The test indicated that there were significant differences in the number of progeny resulting from parents with complementary flowering types versus those resulting from parents with the same flowering type ($\chi^2 = 24.33$, d.f. = 1, $P < 0.0001$, $N = 112$). Significantly, more type A maternal parents produced seedling progeny resulting from pollination from a type A pollen donor (75%) than from a type B pollen donor (25%). In contrast, more type B maternal parents produced seedling progeny resulting from pollination with a type A pollen donor (73%). Overall, 58% of seedling progeny were the product of parents with like flowering type behavior.

3.4. Distribution of productive grafts on individual parent trees

Only seven of the 29 trees grafted with complementary flowering type cultivars supported fruit production in both the type A and type B grafts in 2006 (see Table 1 for number of grafts per cultivar and cultivar flowering type). Five other trees contained only one productive type A fruiting graft, four trees had only one productive type B fruiting graft and four trees had two main branches of the A flowering type due to rootstock intrusion. The remaining grafted trees died or both grafts failed to produce fruit.

Type A and B grafts produced 133 and 123 fruit, respectively. For a given tree, disproportionate amounts of fruit from type A grafts and B grafts were adjacent to productive grafts of the same flowering type indicating that the potential for pollen exchange among the same types appears greater than that of complementary types for this distance class. Fruit from type A grafts were located next to other productive type A grafts/trees more often than were fruit from type B grafts ($\chi^2 = 25.791$, d.f. = 1, $P < 0.0001$, $N = 256$). Likewise, fruit from type B flowering grafts were located next to other productive type B grafts/branches more often than were fruit from type A grafts ($\chi^2 = 17.293$, d.f. = 1, $P < 0.0001$, $N = 256$).

3.5. Relative distribution of type A and B flowering type trees to productive grafted branches

Fruit collected from grafts of A and B flowering types were directly adjacent (i.e. trees within 8 m of grafts) to type A flowering type trees equally frequently ($\chi^2 = 0.648$, d.f. = 1, $P = 0.421$, $N = 256$). Alternatively, fruit collected from grafts of A and B flowering types were not directly adjacent to type B flowering type trees equally frequently ($\chi^2 = 5.962$, d.f. = 1, $P = 0.015$, $N = 256$). Here, fruit from type A flowering grafts were directly adjacent to type B flowering trees more often than fruit from type B grafts. Significant differences in the number of type A trees within 8–20 m of fruit from type A and B grafts were also detected. Fruit collected from type B grafts were surrounded by more type A trees in the 8–20 m distance class relative to fruit collected from type A grafts ($\chi^2 = 19.108$, d.f. = 1, $P < 0.0001$, $N = 256$) whereas no differences were detected for the proximity of either graft type to type B trees ($\chi^2 = 3.806$, d.f. = 1, $P = 0.051$, $N = 256$).

3.6. Phytophthora root rot tolerance among progeny

The majority of seedlings were highly susceptible to PRR (Fig. 1). Percent root necrosis was detectably higher for seedling progeny that resulted from self-pollination (Fig. 2; t-test: t-ratio = 2.11, d.f. = 18.06, $P = 0.049$). No significant differences in PRR susceptibility among the three most common cross types ('Simmonds' × 'Pollock', 'Catalina' × 'Girrardin' and 'Maxima' × 'Girrardin') were detected (ANOVA: $F = 2.74$, d.f. = 2, $P = 0.074$). Further comparisons among full-sib families were not performed as the remainder of full-sib families contained few progeny.

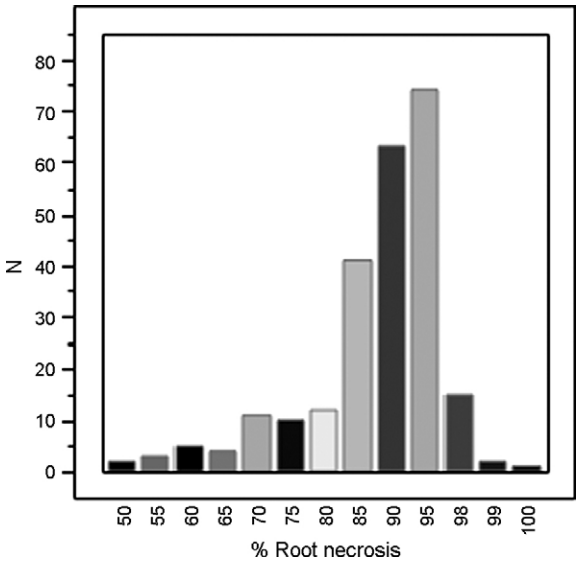

Fig. 1. Frequency distribution for percent root necrosis. N = the number of progeny within each root necrosis class. N = 246.

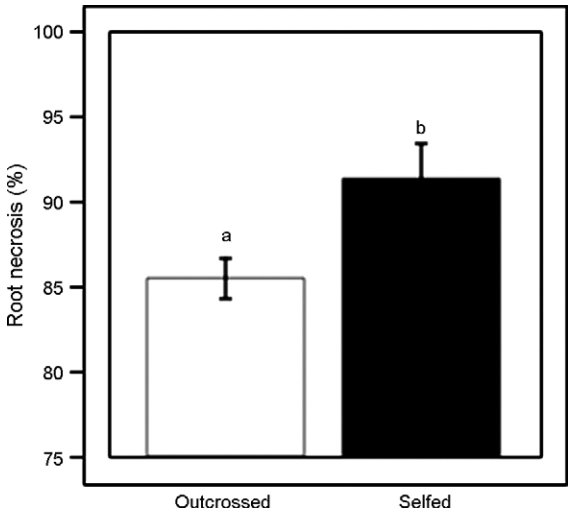

Fig. 2. Percent root necrosis for selfed and outcrossed progeny. Means for percent root necrosis caused by *Phytophthora cinnamomi* ±1 S.E. Means (compared with *t*-tests) not followed by the same letter are significantly different at $P \leq 0.05$. N = 106.

Although there were no detectable differences for root rot tolerance among most common cross types, maternal parents were important to predicting root rot tolerance (ANOVA: F = 3.488, d.f. = 7, P = 0.002). Post-hoc comparisons revealed that 'Catalina' was a superior maternal parent relative to 'Simmonds', 'Hiawassee' and 'Family' (Fig. 3). Conversely, no pollen donor effects could be detected among elite paternal cultivars. However, there was a significant difference in percent root necrosis between progeny pollinated by 'Girrardin' and the "offtypes" (ANOVA: F = 2.621, d.f. = 6, P = 0.018 and see Fig. 3).

4. Discussion

The goal of this breeding effort was to increase the production of full-sib progeny resulting from crosses between elite cultivars (i.e. cultivars known to produce relatively PRR tolerant progeny). Although outcrossing rates for progeny from elite maternal parents were high (~93%), pollen donors could only be identified for less than half of the progeny genotyped. Considering that all potential pollen donors within the grove were genotyped, and the high proportion of rare alleles among the progeny that were not detected among the elite parents, it appears that cultivars outside the grove were effectively pollinating elite parent cultivars.

The high contribution of pollen donors from outside of the grove was unexpected, as Kobayashi et al. (2000) found that the average outcrossing rate decreased sharply for several pollinators between 1 and 5 rows of 'Hass' trees in Southern California. In contrast, Degani et al. (1997) found that in Israel a significant correlation between distance to pollen donor and outcrossing was dependent upon the cultivar and only became apparent over distances greater than 20 m. For a pure 'Hass' grove in California, Vrecenar-Gadus and Ellstrand (1985) detected outcrossing rates

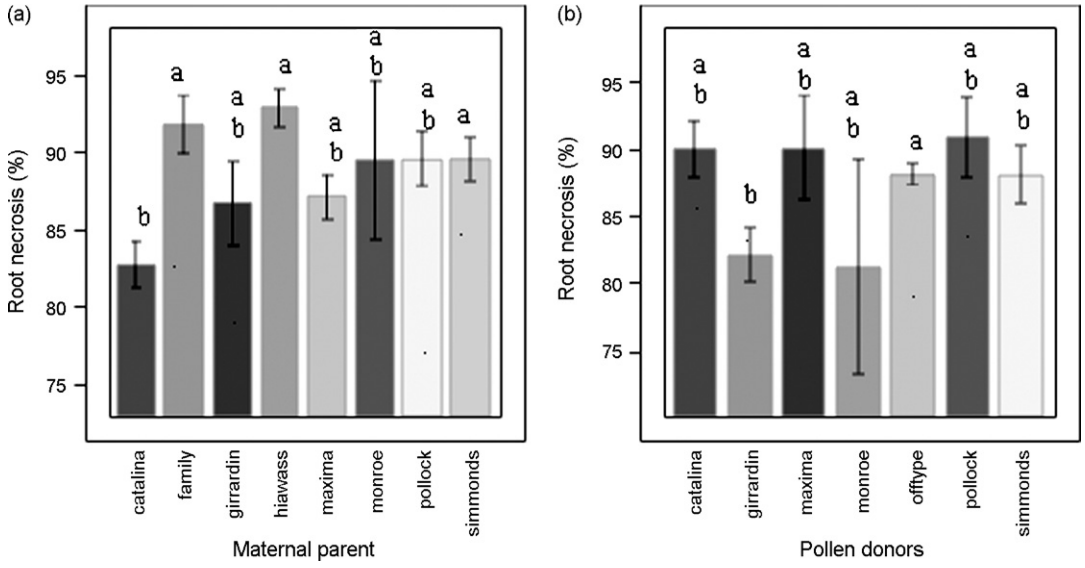

Fig. 3. Maternal (a) and paternal (b) effects on Phytophthora root rot tolerance. Named cultivars consist of elite parents that have produced progeny with superior tolerance to Phytophthora root rot. Offtypes are non-elite pollen donors. Hiawass = 'Hiawassee'. Means for percent root necrosis caused by *Phytophthora cinnamomi* ±1 S.E. Means (compared with Tukey–Kramer HSD) not followed by the same letter are significantly different at $P \leq 0.05$. (a) N = 211 and (b) N = 206. Two cultivars ('Hiawassee' and 'Family') were eliminated from the analysis of paternal effects because they only contained two and three scores, respectively.

above 42% indicating that distant pollen donors (~80 m from 'Hass' grove) can indeed contribute significantly to fruit production. Similarly, Borrone et al. (2008) proposed that pollen donors that were between 100 and 300 m away from study trees parented nearly a quarter of the seedling population.

Interestingly, the negative effect of non-elite pollen donors on PRR tolerance among progeny was detectable. For 56% of the progeny resulting from a cross between elite maternal parents and an "offtype" pollen donor, PRR tolerance was less than that of progeny for which the pollen donor was the elite cultivar 'Girrardin'. Thus, pollen contamination from non-target cultivars represents a considerable obstacle to improving upon PRR tolerance.

In South Africa, breeders have addressed the problems associated with pollen contamination by isolating groves composed of cultivars with desirable traits far from non-target pollen sources. Buffer zones composed of dense vegetation surrounding breeding blocks may also help to reduce the influx of contaminating pollen. Further, high-density plantings of target cultivars is generally recommended over dually grafting trees with target cultivars as graft failure rates for dually grafted trees are high (Stout and Savage, 1925; Stout, 1933).

In this study, for the portion of progeny that could confidently be assigned two parents, crosses between like flowering types occurred more often than crosses between complementary flowering types. Examination of the spatial distribution of flowering types helped to explain these data. Productive type A and B flowering branches were found adjacent to like flowering type branches on the same tree more often than complementary flowering type branches. Also, within 8–20 m, maternal grafts were next to complementary flowering types more often than like flowering types.

The greater proportion of hybrid progeny resulting from crosses between different cultivars of like flowering type strongly conflict with the longstanding idea that inter-planting cultivars of complementary flowering type is required to achieve a high outcrossing rate (Markle and Bender, 1992; Stout, 1923). Instead, our results imply that high outcrossing rates can be achieved among different cultivars of the same flowering type. However, almost half of the pollen donors were of unknown flowering type and the proportion of progeny resulting from complementary flowering type crosses could obviously change significantly if these pollen donors could be identified.

Few other studies have examined outcrossing in groves composed of relatively equal numbers of type A and B pollen donors. In groves consisting of both type A and B pollen donors, hybrid 'Gwen' progeny were predominantly (~83%) the result of crosses between complementary flowering types (Sulaiman et al., 2004). Other results obtained in our lab for a commercial orchard consisting of interplanted 'Simmonds' (flowering type A) and 'Tonnage' (flowering type B) cultivars were similar for 'Tonnage' progeny but equivocal for 'Simmonds' progeny (Borrone et al., 2008). Eighty-eight percent of 'Tonnage' hybrid progeny appeared to have resulted from crosses with 'Simmonds'. However, nearly half of the 'Simmonds' hybrid progeny were pollinated by an unknown donor (Borrone et al., 2008). In contrast, studies that demonstrate the selfing rates can be high (Davenport et al., 1994; Davenport et al., 2006) support the idea that crosses between like flowering types are sometimes significant.

Spatial relationships alone may not explain parentage results within this study. In a study involving nine cultivars, no barriers to pollen tube growth or ovule penetration between like flowering cultivars were detected (Sedgley, 1979). In the absence of such barriers, crosses between complementary flowering types would be favored due to higher pollen availability of the complementary flowering type coinciding with stigma receptivity. Temperature extremes could influence the pollen availability as temperature can alter flowering behavior by delaying or omitting the female flowering stage (Sedgley, 1977; Sedgley and Grant, 1983; Sedgley and Annells, 1981). West Indian cultivars grown in Florida generally have overlapping flowering seasons (Davenport, 1982). Also, flowering types are not equally susceptible to temperature extremes and atypical intervarietal differences may emerge in unusual years (Sedgley, 1977; Sedgley and Grant, 1983). The result of which could conceivably be a reduction in the availability of target pollen donors during some years and overlap of functionally male and female flowers of the same flowering type (Lesley and Bringhurst, 1951).

To compound the difficulties of trying to control avocado crosses, avocado is alternate bearing and the production of floral shoots may vary annually (Monselise and Goldschmidt, 1982). Salazar-Garcia et al. (1998) found that the high yield of an "on" year was followed by the production of fewer floral shoots. Consequently, annual variation in flower production could reduce the production of full-sib progeny.

Promoting outcrossing has been suggested important to preventing inbreeding depression. Selective abscission of flowers and fruitlets favoring outcrossed progeny has been reported (Degani et al., 1986; Degani et al., 1989; Degani et al., 1997) and was attributed to embryo "hybrid vigor". Selfed progeny were significantly less tolerant to PRR relative to outcrossed progeny. However, due to high outcrossing rates any comparison of heterozygosity estimates between selfed and outcrossed progeny in our study would be based on a very small number of selfed progeny. Therefore, we were not able to test for heterosis and cannot attribute the relatively poor tolerance of selfs to inbreeding depression.

Modest differences were detected among progeny parented by different elite cultivars. There was also evidence of differential maternal and paternal effects on PRR tolerance detected in this study. Several studies lend support to the idea that maternal effects could be problematic to identifying progeny with superior tolerance to PRR (e.g. Magrath and Mithen, 1993). Maternal effects on isoflavonoid concentrations in soybean embryos have been documented in several studies (Chiari et al., 2006; Dhaubhadel et al., 2003) and have strong implications for Phytophthora-caused disease tolerance. Specifically, isoflavonoids are both important as precursors to phytoalexins (Hammerschmidt, 1999) and as powerful chemoattractants to *Phytophthora* spp. zoospores (Morris et al., 1998).

The duration of maternal effects on disease tolerance in seedlings may only persist as long as the developing plant is dependent on resources supplied by the cotyledons. In avocado, cotyledons can contribute to seedling growth for nearly a year (Eggers and Halma, 1937). Thus, initial screening efforts may only work to eliminate the most susceptible individuals and long-term efforts are required to select for tolerant individuals. Some breeding programs have begun to remove the cotyledons prior to PRR screeining (Greg Douhan, Stephan Köhne and John Menge, personal communications). Such efforts may reduce the vigor of the developing seedling but also reduce maternal effects on PRR tolerance.

Seedling survival and root necrosis following inoculation with *P. cinnamomi* represents only one test of tolerance to the pathogen. Further, tests of root attractiveness to *P. cinnamomi* zoospores, repeated inoculations, and field trials spanning over many years are among some of the screening steps necessary to categorize a given selection as truly PRR tolerant (Menge, 2001). Nonetheless, the results reported herein support findings reported in previous work (Ploetz et al., 2002) and represents an important initial screening effort. Specifically, PRR tolerance among elite cultivars appears to be heritable because tolerance in progeny resulting

from crosses involving non-target pollen donors was detectably less than those resulting from crosses with elite pollen donors.

5. Conclusion

The evaluation of the breeding approach described herein has broad-scale applications to other pathosystems involving the many ecologically important and the economically valuable species that also produce large numbers of open-pollinated flowers and largely have dichogamous flowering behavior (Kubitzki and Kurz, 1984). Further, our work demonstrates the efficacy with which microsatellite markers can be used in breeding trees with complex pollination syndromes and in parentage analysis where a significant number of the parents are closely related.

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