National Myrtle Rust Transition to Management (T2M) Program

Final Report

Genetic basis of pathogenicity in *Uredo rangelii*

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Summary:

Myrtle rust was first time detected in Australia on the central coast of NSW in April 2010, from *Agonis flexuosa*, *Callistemon viminalis* and *Syncarpia glomulifera* plants. Based on the tonsured urediniospores, initially this pathogen was described as *Uredo rangelii* but later on with the detection of teliospores and studying the sequence of the rDNA ITS region, it was considered to be a strain of *Puccinia psidii* sensu lato, a guava rust causing fungal pathogen that was first detected in Brazil in 1884. To differentiate it from guava rust caused by *P. psidii* sensu stricto, investigating agencies named the disease Myrtle rust based on the name *Myrtus communis* the original host of *U. rangelii*. When efforts to eradicate myrtle rust in Australia proved unsuccessful, a "National Myrtle Rust Transition to Management (T2M) Program" was initiated, under which the University of Sydney was assigned the research project "Genetic basis of pathogenicity in *Uredo rangelii*". The main aim of the project was to establish a "National Myrtle Rust Screening Facility" at the Plant Breeding Institute (PBI), to deliver advisory services to the industry.

Project milestones included the collection and preservation of single pustule increased isolates of *P. psidii*, the establishment of protocols for germplasm screening, development of a scale to characterise host response, large scale phenotyping of *Eucalyptus* and non-*Eucalyptus* species, identification of potential differentials for surveying pathogenicity of *P. psidii*, and the development of microsatellite markers to study the genetic variability among the Australian isolates of *P. psidii*.

More than 20 rust isolates representing different geographical locations of Australia were increased from single pustules and preserved in liquid nitrogen at PBI. An isolate with accession number 115012, collected originally from *A. flexuosa*, was used as standard culture for DNA extraction and for all germplasm testing. Inoculum of isolate 115012 (PBI rust culture no. 622) was also provided to the Department of Primary Industries, NSW for *P. psidii* genome sequencing.

Myrtle rust was found to infect only young foliage, and *Syzygium jambos* was used as the susceptible control and for increasing rust inoculum. Successful infections were achieved with urediniospore inoculations followed by incubation at 20°C plus >95% RH for 24 hrs and at a

post incubation temperature of 22 ± 2°C. Thousands of myrtaceous plants from 39 *Eucalyptus* species, 10 hybrids of *Eucalyptus* and 110 non-*Eucalyptus* species were screened for myrtle rust resistance. Out of 39 *Eucalyptus* species tested, 23 showed a varied response against *P. psidii* whereas *Eucalyptus cladocalyx* was found to be highly resistant. Similarly, out of 10 hybrids of *Eucalyptus* tested, seven showed the presence of resistant to susceptible plants. A majority of the non-*Eucalyptus* species was susceptible. Whereas some Callistemons, Leptospermums and Melaleucas showed a level of resistance against *P. psidii*, all guava cultivars tested were highly resistant. From the seed lots tested for resistance, individual genotypes of *E. globulus* and *E. grandis* showing resistant to susceptible infection types were identified as potential differentials for myrtle rust pathogenicity survey. Further work is required to establish a full set of differentials. Testing of more than 5,000 plants from other 158 seed lots of *Eucalyptus* species is under progress at PBI.

Genome sequencing using standard culture 622 suggested a genome size of ~ 142 Mb, and 22,819 simple sequence repeat (SSR) motifs were identified across the *P. psidii* genome. Sequence analysis revealed that A/T mononucleotide and AT/TA dinucleotide stretches were the most abundant motifs, whereas CG/GC were the least common and occurred across the genome only five times. Using software OLIGO® 7, a set of 240 SSRs was selected for primer synthesis. A diverse range of 17 isolates of *P. psidii* including 14 from Australia (NSW, QLD and ACT Canberra), two from Brazil and one from Hawaii was selected to study genetic diversity in the pathogen. Out of 240 SSRs developed, 178 amplified DNA from the different isolates and 110 were found to be polymorphic. Polymorphic markers differentiated the two Brazilian isolates from each other and that from all the Australian isolates and one from Hawaii. Further work is in progress to find the exact sizes of these polymorphic markers. Genotyping using SSRs revealed that all the Australian isolates were similar to the one from Hawaii and that there is no genetic diversity among the Australian isolates of *P. psidii*. This supports the hypothesis that only one genotype/ strain of *P. psidii* was introduced into Australia.

The "National Myrtle Rust Screening Facility" at PBI proved to be very successful, with a huge demand for germplasm screening. Continuation of the program is highly recommended. Facilities at PBI can be very helpful in myrtle rust training and already one PhD student has started working on this rust at the facility.

Aim of the Project:

The key aim of the project was to establish the "National Myrtle Rust Screening Facility" at the Plant Breeding Institute, in order to deliver germplasm screening and advisory services to industry. Under the Federal Government's funding through Plant Health Australia, till 30th June 2013 all the rust screening and advisory services were provided free of charge and in future the facility will adopt the "Australian Cereal Rust Control Program" Fee for Service model, and subject to demand, will run on a user pays system.

Milestones:

Under this program, different milestones were set to understand the host resistance and susceptibility to the pathogen and to study genetic variability among Australian isolates of *P. psidii*.

- ✓ Collection and preservation of single pustule increased isolates of *P. psidii* representing different geographical areas of Australia.
- ✓ Establishment of protocols for successful infection required for germplasm screening.
- ✓ Development of a rust infection scale to characterise host response for the ease of the industry.
- ✓ Large scale phenotyping of *Eucalyptus* species to reveal the presence of rust resistance.
- ✓ Large scale phenotyping of non-*Eucalyptus* species as a pre breeding approach for resistance against *P. psidii*.
- ✓ Identification of *Eucalyptus* plants as potential differentials for the pathogenicity survey of *P. psidii* in Australia and worldwide.
- ✓ Development of polymorphic microsatellite markers to study the genetic variability among *P. psidii* isolates in Australia.

Introduction:

Rust disease on myrtaceous plants is caused by the fungus *Puccinia psidii* Winter, which is native to South America where it was first described from Brazil on guava (*Psidium guajava* L.) in 1884 (Winter 1884); hence the common name "guava rust". In the same country during 1944, *P. psidii* was first recorded on non-native eucalypts (*Corymbia citriodora* (Hook) Hill & Johnson syn: *Eucalyptus citriodora* Hook) (Joffily 1944), leading to the often used vernacular name of Eucalyptus rust.

According to the Australian National Botanical Gardens (http://www.anbg.gov.au/aust-veg/australian-flora-statistics.html), there are 70 genera containing 1,646 species of Myrtaceae in Australia. In the family Myrtaceae, *Eucalyptus* is the biggest genus with ~ 850 species, followed by *Melaleuca* with 176 species. Due to the presence of nearly half of the world's Myrtaceae species in Australia, and the wide host range of *P. psidii*, plant pathologists always considered *P. psidii* a significant biosecurity threat to Australia and New Zealand (Glen *et al*. 2007; Grgurinovic *et al*. 2006; Langrell *et al*. 2008; Navaratnam 1986; Office of the Chief Plant Protection Officer 2007; Ridley *et al*. 2000; Mireku and Simpson 2002; Tommerup *et al*. 2003). For many years, guava/eucalyptus rust has been considered as one of the most serious exotic plant disease threats to Australia (Glen *et al*. 2007, Tommerup *et al*. 2003) because many Australian *Eucalyptus* and other myrtaceous species proved highly susceptible to this rust when tested in South America (Alfenas *et al*. 2004; Ferreira 1983; Tessmann *et al*. 2001).

It was the 22nd of April 2010 when the exotic rust of Myrtaceae was detected for the first time in Australia, from *Agonis flexuosa*, *Callistemon viminalis* and *Syncarpia glomulifera* plants grown at a property on the central coast of NSW (Carnegie *et al*. 2010). Based on the morphological description of tonsured urediniospores, similar to earlier descriptions of a fungal species in the *P. psidii* sensu lato complex given by Simpson *et al*. (2006), this pathogen was described as *Uredo rangelii*, though its DNA sequence of the rDNA ITS region was similar to that of *P. psidii* (Carnegie *et al*. 2010). Originally, *U. rangelii* was described from the host plant *Myrtus communis* and named after a Brazilian plant pathologist Eugenio Rangel renowned for having a profound interest in Myrtaceae rust causing pathogens (Simpson *et al*. 2006). Based on the host name *M. communis*, the disease was named "myrtle rust" to differentiate it from "guava rust" caused by *P. psidii* sensu stricto (Department of Agriculture,

Fisheries and Forestry 2010; Carnegie *et al*. 2010). Later on in NSW, the discovery of teliospores matching those of *P. psidii* sensu stricto indicated that myrtle rust in Australia was caused by a strain of *P. psidii* sensu lato but with tonsured urediniospores (Carnegie and Lidbetter 2012).

Myrtle rust can infect actively growing young leaves, twigs, flower buds and young fruits of many plants belonging to the family Myrtaceae (Coutinho *et al*. 1998; Tommerup *et al*. 2003). It can also infect non-myrtaceous plants, as *Heteropyxis natalensis* classified as Heteropyxidaceae was found to be highly susceptible (Alfenas *et al*. 2005). Rust caused by *P. psidii* is the most destructive disease of *Eucalyptus* (Graça *et al*. 2011; Junghans *et al*. 2003) and can even lead to the death of young plants (Alfenas *et al*. 2009). In Central and South America including Brazil, the Caribbean Islands, Florida and Hawaii, *P. psidii* is very damaging and has been reported from 129 species in 33 genera of Myrtaceae and its host range is also expanding in Australia where it has already been recorded on 107 host species (Carnegie and Lidbetter 2012). In different studies, the evaluation of Australian species of Myrtaceae showed a varied response against *P. psidii* and a range of resistant to highly susceptible species including eucalypts were identified (Morin *et al*. 2012; Zauza *et al*. 2010). Using molecular markers, a major locus (*Ppr1*) contributing resistance against *P. psidii* was mapped in *E. grandis* (Junghans *et al*. 2003) and was positioned on the reference genetic map for *Eucalyptus* (Mamani *et al*. 2010).

Environmental conditions required for *P. psidii* spore germination and successful infection were determined in different studies (de Piza and Ribeiro 1988; Ruiz *et al*. 1989). Generally, infection occurs at a temperature range of 15–25°C in the presence of high humidity or leaf wetness for 8 hours in darkness, and sporulation can be observed within 10–12 days after inoculation (Alfenas *et al*. 2003; Marlatt & Kimbrough 1979; Rayachhetry *et al*. 2001). While testing key forestry species in Australia, an extended latent period (inoculation to sporulation) of four to five weeks was observed under winter conditions (Carnegie and Lidbetter 2012). More than 76% of hardwood and softwood plantation areas in Australia are in the climatic potential range of *P. psidii* and can be at risk of this rust causing pathogen (Kriticos *et al*. 2013).

The life-cycle of the guava/eucalyptus rust complex is still unclear and there are contradictions as to whether the rust is autoecious or heteroecious. Figueiredo (2001) considered *P. psidii* to be autoecious and reported that basidiospore infections on host *Syzygium jambos* led to the production of aeciospores that were morphologically similar to urediniospores but didn't observe any production of pycniospores. Ramsfield *et al.* (2010) also claimed that the *P. psidii* complex is autoecious and that it does not require an alternate host species to complete the lifecycle. Simpson *et al.* (2006) questioned these reports, regarded the *P. psidii* complex as heteroecious with an unknown alternate host producing aeciospores. Sexual recombination that occurs through cross-fertilisation of spermagonia is a major source of genetic variation in rust fungi, although other processes such as mutation and somatic hybridisation between isolates can also contribute to genetic diversity (Park and Wellings 2012). Over the decades, national surveys of pathogenic variability in the rust pathogens that infect cereal crops in Australia have shown that in the absence of an alternate host and subsequent sexual recombination, genetic diversity is generated by periodic exotic incursions, single-step mutations and somatic hybridisation except in the case of *P. hordei* Otth. only, sexual hybridisation (Park 2008; Park *et al.* 1995; Park and Wellings 2012; Wellings and McIntosh 1990).

Currently, it is believed that there is only one genotype/strain of *P. psidii* is present in Australia. Different strains of guava/eucalyptus rust may have the ability to impact host species differently (Tommerup *et al.*, 2003; Alfenas personal comm.). In cereal rust pathogens, the development of new strains is very common, and each is characterised using a set of differential lines or cultivars with known genes of resistance. High levels of pathogenic variability in wheat stripe rust causing pathogen *P. striiformis* Westend. f. sp. *tritici* has resulted in many resistance genes in wheat being rendered ineffective (Wellings 2007) and in wild grasses (Park and Wellings 1992; Wellings 2011). A new race of *P. graminis* f. sp. *tritici* (*Pgt*) "Ug99" was detected in 1999 in Uganda, has overcome the genes for stem rust resistance present in many of the world's wheat varieties (Boshoff *et al.* 2000).

Although information on variability obtained from pathogenicity on differential genotypes is important in the genetic control of rusts, it is of limited use in assessing genetic variation in these pathogens. Both biochemical and molecular markers have been applied to evaluate genetic diversity among various plant pathogens (McDermott and McDonald 1993).

Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated DNA sequences composed of 1–6 base pair arrays that are highly polymorphic and evenly distributed in abundance across genomes. SSRs are co-dominant, generate maximum genetic information, and are inherited according to Mendelian laws (Liu *et al.* 1999). SSRs are robust PCR-based markers and are usually associated with a high frequency of length polymorphism (Weber 1990; Tóth *et al*. 2000). Due to their informative power, high throughput and PCR reproducibility, SSRs are the preferred choice of markers for a variety of studies including discrimination, kinship, population genetics and mapping (Jarne and Lagoda 1996).

To date, SSRs have been developed and applied to study different rust pathogens. SSRs developed specifically for the crown rust pathogen *P. coronata* f. sp. *avenae* were highly polymorphic among 35 isolates, with an allelic diversity of two to 16 alleles per locus (Dambroski and Carson 2008). Similarly, SSR markers developed from a urediniospore derived expressed sequence tag (EST) resource were used to study genetic diversity among the Australian and New Zealand isolates of *P. coronata* f. sp. *lolli,* causing crown rust on rye grass (Dracatos *et al.* 2009). In another study, 118 isolates of *P. triticina* collected from the Middle East and Central Asia were genotyped using 23 SSRs (Kolmer *et al.* 2011). All the Middle Eastern isolates differed from the Central Asian isolates, suggesting a lack of pathogen migration between the two regions. In another study that compared North American and South American isolates of *P. triticina* using SSRs, a high degree of similarity was found, suggesting that it was introduced to America from a common origin (Ordoñez *et al.* 2010). SSRs have also been developed and used to genotype *Pgt* isolates. Keiper *et al*. (2006) used 110 SSRs to genotype 10 pathogenically diverse isolates of *Pgt* and demonstrated that some of these SSRs were also useful in revealing polymorphism among isolates of the oat stem rust pathogen *P. graminis f. sp. avenae*. Recently, the *Pgt* pathotypes TTKSF, TTKSP and PTKST, all believed to belong to a clonal lineage typified by pathotype TTKSK ("Ug99") and selected South African isolates of *Pgt*, were genotyped using SSR markers. The four "Ug99" pathotypes shared only 31% similarity with other South African pathotypes and it was concluded that pathotypes TTKSP and PTKST arose in South Africa as a result of exotic introduction (Visser *et al.* 2011). More recently Karaoglu *et al*. (2013) have developed a set of novel SSR markers for *Pgt*, which showed an average PIC value of 0.71. These markers are currently being used to study the genetic diversity among global isolates of stem rust causing pathogen *P. graminis*.

Zhong *et al*. (2008) developed a set of 15 polymorphic microsatellite markers present in the genome of *P. psidii,* which revealed 71 alleles among 22 *P. psidii* isolates including 18 from Brazil and four from Florida. The primers for these SSRs were designed by sequencing clones from a genomic DNA library enriched only for a dinucleotide SSR motif of (AG), and previous studies have shown that such markers often are not highly polymorphic (Bailey, 2013). Studying the genetic variability among the Australian isolates of *P. psidii* is very important as addressing these knowledge gaps will improve our understanding of how variation may evolve in the population of myrtle rust in Australia. Genetic variability in the pathogens influences their evolutionary potential and eventually diverse strains can have implications for the durability of genetic resistance present in the host species (McDonald and Linde 2002).

Materials and Methods:

Germplasm:

There was a huge demand for testing of *Eucalyptus* and non-eucalypt species against myrtle rust. Young plants or seed lots of different species (Tables: 3–6) were provided for testing by the following clients.

Public institutes:

1. Australian National University, Canberra, ACT
2. University of Tasmania, Hobart, TAS
3. Ornamental Eucalypts Development Program, the University of Adelaide, SA
4. Department of Primary Industries, Forest Science Centre, NSW
5. Department of Primary Industries, Parks, Water and the Environment, TAS
6. Forest Science and Industry Development, Department of Agriculture and Food WA
7. Department of Environment and Conservation, WA
8. The Australian Botanic Garden, Mount Annan, NSW
9. The Royal Tasmanian Botanical Gardens, TAS
10. The Royal Botanical Gardens, Cranbourne, VIC

Private businesses:

1. Bangalow Wholesale Nursery, Brooklet, NSW
2. New Flora, Plant Breeding Institute, Cobbitty, NSW
3. Ozbreed Pty. Ltd., NSW
4. Unique Plants Pty. Ltd., QLD
5. Wafex Flowers, Melbourne, VIC
6. Yuruga Nursery Pty. Ltd., QLD

Potential international clients:

Comvita Pty. Ltd. and SCION (New Zealand Forest Research Institute Ltd) from New Zealand Limited also showed interest in getting their myrtaceous germplasm tested at PBI.

Raising seedlings:

Seed lots requiring stratification were treated with 50% bleach (White King: Sodium Hypochlorite 42g/L) for 5 minutes, washed with tap water, dried on the filter paper and were sown/spread in Petri-dishes containing 1% water agar solution or in the zip locked sandwich plastic bags containing sterilised soil mix (fine bark and coarse sand (50%) and vermiculite (50%)). Soil mix in the bags was moistened using distilled water. Petri plates and bags containing seed were kept in a refrigerator at 5ºC for 2 weeks followed by another 2 weeks in a room kept near the window at room temperature under natural day/night conditions. Germinating seeds were transplanted and seedlings were raised in sterlised soil mix beds using microclimate producing sowing trays (Fig. 1). Transplanting from petri plates proved very laborious and time consuming compared to the spreading of seed containing soil mix from the sandwich bags.

Seed lots not requiring stratification were sown directly in the microclimate producing sowing trays. Sowing trays were covered with transparent covers to create microenvironment required for better germination. Covers were removed after 2 weeks of sowing to avoid any damping of the germinating seedlings under humid conditions.

Fig. 1 Raising seedlings from different seed lots of *Eucalyptus* species in the greenhouse

Rust inoculum:

Isolates of *P. psidii* (previously *U. rangelii*) were collected from different geographical locations of Australia. Each rust sample was used to generate a single pustule isolate, which was preserved in liquid nitrogen at the PBI. During rust surveys (2011–2013), 34 samples of myrtle rust were received from different locations across NSW, QLD and the ACT. For DNA extraction, three additional international samples of dead (in ethanol) urediniospores of *P. psidii* were sourced, including two from Brazil and one from Hawaii (Table 1). Samples of infected leaves and twigs were collected in paper bags along with information on date of collection, host and location. In the survey, different species of *Agonis*, *Astromyrtus*, *Backhousia*, *Chamelaucium*, *Eucalyptus*, *Melaleuca*, *Metrosideros*, *Rhodamnia*, *Rhodomyrtus* and *Syzygium* were found to be infected with myrtle rust. Each myrtle rust sample was assigned with a unique accession number.

A standard culture (PBI collection rust culture no. 622) of single pustule increased isolate (Au_3) with accession number 115012 was used for all the germplasm testing. This isolate was collected in 2011 from *A. flexuosa* plant grown in a street of Leonay, NSW. Single pustules increased urediniospores from the same culture were provided to the Department of Primary Industries (DPI), NSW for *P. psidii* genome sequencing. A diverse range of isolates including

three from overseas was selected for studying the genetic diversity among the Australian isolates of *P. psidii* (Table 1).

Table 1 Details of *Puccinia psidii* isolates used in the molecular study

Isolate ID	Acc. No.	Original host	Location	Year of Collection
Au_1	115001	*Syzygium jambos*	Lismore, NSW	2011
Au_2	115010	*Rhodamnia rubescens*	Onley S. F., NSW	2011
Au_3[STD]	115012	*Agonis flexuosa*	Leonay, NSW	2011
Au_4	125004	*Eucalyptus pilularis*	Newry, NSW	2012
Au_5	125005	*Rhodamnia rubescens*	Lansdowne S. F., NSW	2012
Au_6	125008	*Syzygium sp.*	CSIRO, Canberra	2012
Au_7	125009	*Melaleuca quinquenervia*	Manly vale, NSW	2012
Au_8	125013	*Metrosideros excelsa*	PBI SP Collection	2012
Au_9	125014	*Chamelaucium uncinatum*	Toowoomba, QLD	2012
Au_10	125015	*Astromyrtus Sp.*	Toowoomba, QLD	2012
Au_11	125016	*Agonis flexuosa*	Toowoomba, QLD	2012
Au_12	125017	*Syzygium sp.*	Warrawee, NSW	2012
Au_13	135001	*Rhodamnia maideniana*	Mooball, NSW	2013
Au_14	135002	*Rhodamnia rubescens*	Nightcap N. P., NSW	2013
Bz_15	135005	*Eucalyptus grandis*	Vicosa, Brazil	2013
Bz_16	135006	*Psidium guajava*	Vicosa, Brazil	2013
Hw_17	135007	*Syzygium jambos*	Hawaii	2013

[STD:] Standard rust culture used for germplasm screening and *Puccinia psidii* genome sequencing

Protocols were standardised for the inoculation and post inoculation requirements for successful infection of *P. psidii* on its susceptible host the rose apple, *S. jambos*, previously reported as highly susceptible to myrtle rust (Carnegie and Lidbetter 2012; Morin *et al*. 2012; Pegg *et al*. 2012). This host was used for the single pustule rust increase and for increasing inoculum of standard rust culture 622. Young *S. jambos* plants with young and actively growing leaves were used as controls in all the testing and for rust increases.

Inoculation:

In the rust survey, each sample of leaves and or twigs infected with myrtle rust was cut into small pieces and immersed in light mineral oil (Univar Solvent L naphtha 100, Univar Australia Pty Ltd) and sprayed over adaxial and abaxial leaf surfaces of a *S. jambos* plants using an aerosol hydrocarbon propellant pressure pack (Fig. 2). For larger inoculations, an airbrush attached to a motorized compressor was used to spray the urediniospore suspension on test plants in the inoculation room (Fig. 4). The chamber (Fig. 3) door was kept closed for 5 minutes to allow urediniospores to settle on the leaves completely. Spray nozzle fittings were stored in 70% ethanol and rinsed thoroughly with tap water before each inoculation to prevent cross contamination. In addition, the inoculation chamber was washed thoroughly with pressurised tap water following each inoculation.

Fig. 2 Hydrocarbon pressure pack

Fig. 3 Inoculation chamber

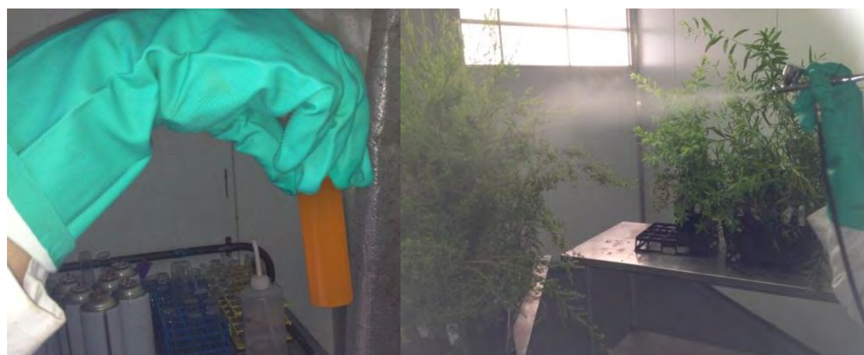

Fig. 4 Inoculation with urediniospores suspended in oil (2mg/ml) using air brush

Incubation:

Myrtle rust-inoculated plants were incubated for 24 hrs under plastic hoods in a dark room maintained at 20°C. Mist was created inside the hoods by an ultrasonic humidifier. Hoods were sealed by filling the trays with de-chlorinated water (tap water stored in open drums for a week). The humidifier was run at 15 minute intervals to create >95% RH in the hoods. After incubation, plants were moved to naturally lit microclimate rooms maintained at 22 ± 2°C (Fig. 5). Within 7 days post inoculation (dpi), infection was visible on the young leaves of *S. jambos* (Fig. 6). Infection was very slow on the adaxial leaf surface compared to the lower side or abaxial leaf surface. Within 14 dpi, pustules were fully developed and had grown through to the adaxial surface of leaves and heavy sporulation was observed on the lower side of the leaves (Fig. 7). A single pustule was excised carefully and used to inoculate a new *S. jambos* plants as described earlier.

Fig. 5 Post-incubation: plants kept in a microclimate room running at 22 ±2°C

Fig. 6 Seven day old infection on abaxial and adaxial leaf surface *S. jambos* leaves

Urediniospore suspensions:

Suspensions of 20 mg freshly collected urediniospores per 10 ml of light mineral oil and 0.05% of Tween 20 were used to inoculate fresh leaf growth of *S. jambos* plants. Plants were incubated as described earlier and infection was compared 14 dpi on the abaxial leaf surfaces. Coverage was much better with oil suspension, which resulted in more pustules per unit of leaf area (Fig. 7) compared to the leaf inoculated with Tween 20 suspension (Fig. 8).

Fig. 7 Oil suspension inoculated

Fig. 8 Tween 20 suspension inoculated

Collection and storage of rust inoculum:

At 14 dpi, urediniospores resulting from the single pustule inoculation were tapped and collected on glassine paper (Fig. 9). In case of bulk increase, rust spores were collected using a motorized cyclone collector. Rust was collected twice a week from the infected leaves until 4 weeks only after inoculation. In some cases, infections older than 5 weeks started to produce white spores, thought to be a result of infection by an unidentified hyper-parasitic fungus (Fig. 11). Urediniospores collected in Petri dishes were sieved to remove any trash, and were desiccated for 2 weeks over silica gel beads in an air tight container (Fig. 10). During the process of each desiccation, at least once a week, the silica beads were replaced with oven dried beads. Approximately 50 mg of dried rust spores were sealed in aluminium pouches and stored in the liquid nitrogen for future use. Rust packets taken out of liquid nitrogen were given a 4 minute heat shock in a water bath at 40ºC to acclimatise the urediniospores before using for inoculation.

Fig. 9 Collection of rust spores

Fig. 10 Desiccation of rust spores

Fig. 11: L–R: Rust spores turning white in old infections and a pustule under microscope

Rust scale:

A greenhouse scale was developed to measure the rust response of the different species tested against *P. psidii*. The scale as described in Table 2 is based on different infection types (ITs) produced by a range of highly resistant to highly susceptible genotypes. Combination of scale was used to describe mixed or different ITs as shown in Figures 12 and 13. This rust scale will be very helpful for the industry, especially for private businesses to be able to categorise and promote their plants accordingly.

Table 2 Greenhouse scale developed for scoring host response against *Puccinia psidii*

Infection Type	Scale	Host Response
No visible sign of infection	0	Highly resistant (HR)
Mild hypersensitivity/flecks/dark flecks/necrosis	;/;+/N/1	Resistant (R)
Restricted pustule/dark gray surrounding/chlorosis/necrosis	1+ to 2+/CN	Moderately Resistant (MR)
Small to medium sized pustules low in frequency and may be with some chlorosis present	3 to 3C	Moderately Susceptible (MS)
Fully developed pustules on leaves and medium to high in frequency	3+	Susceptible (S)
Abundance of fully developed pustules on leaves, twigs and buds	4	Very susceptible (VS)

; = Light brown flecking, ;+ = Dark & bigger sized flecks, C = Chlorosis, N = Necrosis

Fig. 12 Different ITs observed in *Callistemon viminalis* varieties; L–R: HR (0), R (;), R (;+N), R–MR (1+C), MR (22+C), MS–S (33+C) and S (3+)

Fig. 13 Different ITs observed in *Eucalyptus globulus* ; L–R: HR (0), R (1-C), MR (22+C), S (3+) and VS (4)

Development of microsatellite markers:

Extraction of genomic DNA from urediniospores:

DNA of standard culture number 622 and other isolates of *P. psdii* selected for studying genetic diversity was extracted from the established single pustule cultures. Freshly collected urediniospores were desiccated over silica for 12 hrs. A sample of 25–30 mg of urediniospores of each rust isolate was put in labelled Lysing Matrix C tubes (Impact resistant tubes with 1.0

mm silica spheres, Mp Biomedical, Ohio, USA). One ml of 2x CTAB extraction buffer [(CTAB 2% (w/v), 20 mM EDTA (pH 8.0), 1.4 M NaCl, Polyvinylpyrrolidone (PVP; 40000 MW) 1% (w/v), 100 mM Tris-HCl (pH 8.0) and dH2O] was added to each sample, mixed well by inversion and tubes were submerged in ice for 2 min. Tubes were then shaken for 15 s on a FastPrep® Cell Distrupter (Qbiogene, USA) at speed 6, returned to ice for 3 min and shaken again for 20 s at the same speed. Tubes were kept in a pre-warmed water bath at 65˚C for 30 min and inverted every 10 min, after which they were removed, mixed well by inversion and the solution in each tube/sample was divided (~ 500 µl in each tube) into two new 1.5 ml Eppendorf tubes to generate duplicate extractions. DNA extraction was carried in a fume hood by adding ~ 250 µl of cold phenol, followed by ~ 250 µl of cold chloroform: isoamyl alcohol, to each tube. Samples were mixed gently by inverting (~ 100 times) the tubes until a thick emulsion formed. Tubes were centrifuged at 13,000 rpm for 15 min and the supernatant was transferred into sterile 1.5 ml Eppendorf tubes. The process of phenol and chloroform: isoamyl alcohol extraction was repeated. About 50 µl of 3 M NaOAc and ~ 500 µl of cold isopropanol were added to each tube and tubes were then stored at -20˚C. The following day, the tubes were centrifuged at 13,000 rpm for 30 min and the DNA pellet thus formed was drained carefully. The pellets were washed with 500 µl of ethanol, centrifuged at 13,000 rpm for 15 min, drained carefully and allowed to air dry. The dried pellet was re-suspended in 100 µl double distilled autoclaved water (ddH_2O) and stored overnight at 4˚C. The following day, 5 µl of Rnase-A (10 µg/µl) was added to each tube and incubated at 37˚C for 2 hrs. All DNA samples were quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop® Technologies) and diluted to working dilution of 10 ng/µl using ddH_2O.

Genome Sequencing:

P. psidii genome sequencing was performed by BGI Genomics using Illumina HiSeq 2000 at 30 x Coverage. Sequence data was downloaded from CDTS available at http://cdts.genomics.org.cn/ where ~ 142 Mb sequence size was generated with some redundancies. The genome sequence contained 57,500 scaffolds with a size range of 1,000 to 30,000 bp each.

SSRs analysis for abundance:

From the *P. psidii* genome sequence data, SSRs were identified using a PYTHON based program as described by Karaoglu *et al*. (2005).

Primers design and synthesis:

Two hundred and forty SSR sequences containing dinucleotide to hexanucleotide repeat motifs were selected for primer design using OLIGO® Version 7 software (Molecular Biology Insights, Inc., USA) according to the methods described by Karaoglu *et al*. (2013). Primers were synthesized and supplied by Sigma Aldrich Australia Pty Ltd.

Screening of SSRs:

PCR amplification and electrophoresis:

PCR was performed using 15 µl of reaction containing 2.0 µl of genomic DNA (10 ng/µl), 1.5 µl of dNTPs (0.2 mM), 1.5 µl of 10x PCR buffer (NH4 Reaction buffer, Bioline), 0.9 µl of 50 mM $MgCl_2$ (Bioline), 0.9 µl of each forward and reverse primer (2 mM), 0.15 µl (5 u/µl) of *Taq* DNA (Bioline, Australia) and 7.15 µl of ddH_2O. The PCR amplification profile comprised an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 55–56°C (locus specific), 30 s extension at 72°C and a final extension step of 7 min at 72°C. Reactions were performed in a 96-well DNA thermocycler (Eppendorf Mastercycler, Germany). PCR products were resolved on 3% agarose (Agarose, Molecular Grade, Bioline) gels at 110 V electrophoresis for 3 hrs. For staining, 1.0 µl of GelRed™ (Biotium) was added per 100 ml of gel solution. One hundered bp HyperLadder™ IV (Bioline) was used as ladder. The separated bands were visualised under an ultra violet light unit fitted with a GelDoc-IT UVP Camera.

Results and Discussion:

Germplasm screening:

Under the National Myrtle Rust Screening Facility program at PBI, seed lots or young plants belonging to 39 *Eucalyptus* species (Table 3), 10 hybrids of *Eucalyptus* (Table 4) and 110 non-*Eucalyptus* species (Table 5) were screened for myrtle rust response. *Eucalyptus* species tested against myrtle rust showed varied responses as different ITs were observed within the same lot. Out of the 39 *Eucalyptus* species tested, seven showed very poor germination and there were less than 10 plants available for testing and results were hence questionable. One species, *E. cladocalyx*, was found to be highly resistant as it did not develop any sign of infection against *P. psidii*. The remaining *Eucalyptus* species were found to be MR–S (4 species), MR–VS (3), R–S (3), R–VS (13), S–VS (4) and another four were VS (Table 3). For example, in case of *E. pilularis*, a range of rust response (R, MR, S and VS) was observed among different plants (Fig. 14) and resistant to highly susceptible plants were observed in the tested seed lot of *E. crebra* as well (Fig. 15). The presence of resistance against *P. psidii* in *Eucalyptus* species has been reported (Morin *et al*. 2012; Zauza *et al*. 2010) and a single locus contributing resistance against *P. psidii* was mapped in *E. grandis* (Junghans *et al*. 2003; Mamani *et al*. 2010). Many native species of *Eucalyptus* like *E. baueriana*, *E. burgessiana*, *E. camphora*, *E. cloeziana*, *E. deanei*, *E. elata*, *E. globoidea* and *E. tereticornis* were susceptible to myrtle rust (Fig. 16), with important implications especially in situations such as post-fire regeneration.

Plants raised from seed lots of the two *Eucalyptus* hybrids *E. conveniens* x *E. tetragona* and *E. websteriana* x *E. crucis* were moderately susceptible to susceptible against *P. psidii*, whereas all other hybrids produced resistant to susceptible individuals except for one hybrid (*Corybmia calophylla* x *Corybmia ficifolia*) for which results were not clear due to few plants (Table 4). All the *Eucalyptus* hybrid seed lots were sourced from open pollinations and it was not possible to establish the inheritance of resistance based on the number of plants tested.

Fig. 14 Different ITs observed in *Eucalyptus pilularis* ; L–R: R, MR, S and VS

Fig. 15 Different ITs observed in *Eucalyptus crebra* ; L–R: R, MR and VS

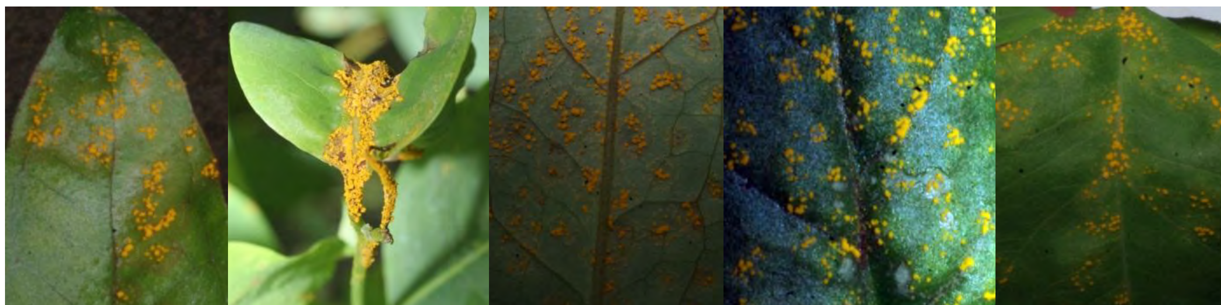

Fig. 16 L–R: Susceptible species; *Eucalyptus baueriana*, *Eucalyptus cloeziana, Eucalyptus elata, Eucalyptus globoidea* and *Eucalyptus tereticornis*

Table 3 Response of *Eucalyptus* species tested against *Puccinia psidii* in the greenhouse

Sr. No.	Genus	species	Host response*
1	*Eucalyptus*	*agglomerata*	R–S
2	*Eucalyptus*	*argophloia*	?
3	*Eucalyptus*	*baileyana*	R–S
4	*Eucalyptus*	*baueriana*	S–VS
5	*Eucalyptus*	*bosistoana*	?
6	*Eucalyptus*	*brunnea*	?
7	*Eucalyptus*	*burgessiana*	S–VS
8	*Eucalyptus*	*caleyi*	?
9	*Eucalyptus*	*camaldulensis*	R–VS
10	*Eucalyptus*	*camphora*	VS
11	*Eucalyptus*	*cinerea*	MR–S
12	*Eucalyptus*	*cladocalyx*	HR
13	*Eucalyptus*	*cloeziana*	S–VS
14	*Eucalyptus*	*cornuta*	R–VS
15	*Eucalyptus*	*crebra*	R–VS
16	*Eucalyptus*	*dalrympleana*	?
17	*Eucalyptus*	*deanei*	S–VS
18	*Eucalyptus*	*dunii*	MR–S
19	*Eucalyptus*	*elata*	VS
20	*Eucalyptus*	*fastigata*	MR–VS
21	*Eucalyptus* (*Corymbia*)	*ficifolia*	R–VS
22	*Eucalyptus*	*forrestiana*	R–VS
23	*Eucalyptus*	*gillii*	R–VS
24	*Eucalyptus*	*globoidea*	VS
25	*Eucalyptus*	*globulus*	R–VS
26	*Eucalyptus*	*grandis*	R–VS
27	*Eucalyptus*	*guilfoyleii*	MR–S
28	*Eucalyptus*	*jacksonii*	MR–S
29	*Eucalyptus*	*largiflorens*	?
30	*Eucalyptus*	*lehmannii*	R–VS
31	*Eucalyptus*	*megacarpa*	R–VS
32	*Eucalyptus*	*melliodora*	?
33	*Eucalyptus*	*microcorys*	MR–VS
34	*Eucalyptus*	*moluccana*	MR–VS
35	*Eucalyptus*	*occidentalis*	R–VS
36	*Eucalyptus*	*pilularis*	R–VS
37	*Eucalyptus*	*tereticornis*	VS
38	*Eucalyptus*	*torquata*	R–VS
39	*Eucalyptus*	*woodwardii*	R–VS

* = Host responses as described in Table 2, ? = Not clear

Table 4 Response of *Eucalyptus* hybrids tested against *Puccinia psidii* in the greenhouse

ID	**Parent A**	**Parent B**	Host response*
H1	*Corybmia calophylla*	*Corymbia ficifolia*	?
H2	*Eucalyptus conveniens*	*Eucalyptus tetragona*	MS–S
H3	*Eucalyptus pyriformis*	*Eucalyptus macrocarpa*	R–S
H4	*Eucalyptus pyriformis*	*Eucalyptus macrocarpa*	R–S
H5	*Eucalyptus pyriformis*	*Eucalyptus macrocarpa*	R–S
H6	*Eucalyptus websteriana*	*Eucalyptus orbifolia*	R–S
H7	*Eucalyptus websteriana*	*Eucalyptus crucis*	MS–S
H8	*Eucalyptus youngiana*	*Eucalyptus macrocarpa*	MR–VS
H9	*Eucalyptus youngiana*	*Eucalyptus macrocarpa*	R–VS
H10	*Eucalyptus youngiana*	*Eucalyptus macrocarpa*	R–VS

* = Host responses as described in Table 2, ? = Not clear

Most of the non-*Eucalyptus* species tested were susceptible to highly susceptible against myrtle rust, whereas all the guava (*P. guajava*) varieties screened were highly resistant (Table 5). In the report, commercial names of all the cultivars, hybrids and selections are coded (ID; Table 5) due to intellectual property rights on the material. Out of 110 lots including both released and unreleased cultivars, hybrids and selections from similar or different plant species, 67 were totally susceptible (40:S, 22: S–VS, 5:VS) and four *C. viminalis* lots showed MS–S type of response. In two of the *Callistemon* lots, there were only few plants with fresh growth and their responses to myrtle rust were not clear. Plants from the remaining 40 lots showed varied responses to myrtle rust (Table 5), for example, the callistemons (Fig. 17). Species from genera like *Austromyrtus*, *Chamelaucium*, *Kunzea*, *Metrosideros* and *Syzygium* were totally susceptible to myrtle rust (Table 5) and in some cases infections were also recorded on flowers as well (Fig. 18).

Fig. 17 Different ITs observed in *Callistemon viminalis*; L–R: R, MR, MS, S and VS

Table 5 Response of Non-Eucalypts tested against *Puccinia psidii* in the greenhouse

Sr. No.	ID	Botanical Name	Host Response*
1	A_s_1	*Acmena smithii*	MR–MS
2	A_s_2	*Acmena smithii*	?
3	Aust_?_1	*Austromyrtus sp.*	VS
4	C_c_1	*Callistemon citrinus*	S
5	C_c_2	*Callistemon citrinus*	S
6	C_c_3	*Callistemon citrinus*	S
7	C_c_4	*Callistemon citrinus*	?
8	C_h1	*Callistemon Hybrid*	S
9	C_h2	*Callistemon Hybrid*	MS
10	C_h3	*Callistemon Hybrid*	S
11	C_h4	*Callistemon Hybrid*	MS–S
12	C_h5	*Callistemon Hybrid*	MS
13	C_h6	*Callistemon Hybrid*	S
14	C_?_1	*Callistemon sp.*	S
15	C_?_2	*Callistemon sp.*	?
16	C_v_1	*Callistemon viminalis*	MR–MS
17	C_v_2	*Callistemon viminalis*	MS–S
18	C_v_3	*Callistemon viminalis*	S
19	C_v_4	*Callistemon viminalis*	HR
20	C_v_5	*Callistemon viminalis*	HR–R
21	C_v_6	*Callistemon viminalis*	S
22	C_v_7	*Callistemon viminalis*	S
23	C_v_8	*Callistemon viminalis*	MS–S
24	C_v_9	*Callistemon viminalis*	R–MR
25	C_v_10	*Callistemon viminalis*	MR–MS
26	C_v_11	*Callistemon viminalis*	HR–R
27	C_v_12	*Callistemon viminalis*	MS–S
28	C_v_13	*Callistemon viminalis*	S
29	C_v_14	*Callistemon viminalis*	S
30	C_v_15	*Callistemon viminalis*	S
31	C_v_16	*Callistemon viminalis*	S
32	C_v_17	*Callistemon viminalis*	S
33	C_v_18	*Callistemon viminalis*	S
34	C_v_19	*Callistemon viminalis*	S
35	C_v_20	*Callistemon viminalis*	S
36	Cham_?_1	*Chamelaucium sp.*	S
37	Cham_?_2	*Chamelaucium sp.*	S
38	Cham_?_3	*Chamelaucium sp.*	S
39	Cham_?_4	*Chamelaucium sp.*	S
40	Cham_?_5	*Chamelaucium sp.*	S
41	Cham_?_6	*Chamelaucium sp.*	VS

42	Cham_?_7	*Chamelaucium sp.*	VS
43	C_f_1	*Corymbia ficifolia*	R–VS
44	K_a_1	*Kunzea ambigua*	S
45	K_a_2	*Kunzea ambigua*	S
46	K_a_3	*Kunzea ambigua*	S
47	L_glau_1	*Leptospermum glaucescens*	S–VS
48	L_glau_2	*Leptospermum glaucescens*	S–VS
49	L_grandi_1	*Leptospermum grandiflorum*	S–VS
50	L_grandi_2	*Leptospermum grandiflorum*	S
51	L_grandi_3	*Leptospermum grandiflorum*	S–VS
52	L_h1	*Leptospermum hybrid*	R–S
53	L_h2	*Leptospermum hybrid*	R–MR
54	L_h3	*Leptospermum hybrid*	R–S
55	L_h4	*Leptospermum hybrid*	S
56	L_h5	*Leptospermum hybrid*	MR–S
57	L_h6	*Leptospermum hybrid*	S
58	L_lani_1	*Leptospermum lanigerum*	S–VS
59	L_lani_2	*Leptospermum lanigerum*	S–VS
60	L_lani_3	*Leptospermum lanigerum*	S–VS
61	L_lani_4	*Leptospermum lanigerum*	S–VS
62	L_niti_1	*Leptospermum nitidum*	R–S
63	L_niti_2	*Leptospermum nitidum*	R–S
64	L_ripa_1	*Leptospermum riparium*	S–VS
65	L_ripa_2	*Leptospermum riparium*	R–VS
66	L_rup_1	*Leptospermum rupestre*	S–VS
67	L_rup_2	*Leptospermum rupestre*	S–VS
68	L_sco_1	*Leptospermum scoparium*	S–VS
69	L_sco_2	*Leptospermum scoparium*	*S*
70	L_sco_3	*Leptospermum scoparium*	S–VS
71	L_sco_4	*Leptospermum scoparium*	S
72	L_sco_5	*Leptospermum scoparium*	R–VS
73	L_sco_6	*Leptospermum scoparium*	S–VS
74	Lo_conf_1	*Lophostemon confertus*	HR
75	M_a_1	*Melaleuca alternifolia*	R–S
76	M_e_1	*Melaleuca ericifolia*	S–VS
77	M_e_2	*Melaleuca ericifolia*	R–VS
78	M_g_1	*Melaleuca gibbosa*	S–VS
79	M_g_2	*Melaleuca gibbosa*	S–VS
80	M_g_3	*Melaleuca gibbosa*	S–VS
81	M_p_1	*Melaleuca pallida*	S–VS
82	M_p_2	*Melaleuca pallida*	S–VS
83	M_p_3	*Melaleuca pallida*	VS

84	M_q_1	*Melaleuca quinquenervia*	R–VS
85	M_s_1	*Melaleuca squarrosa*	*S*
86	M_s_2	*Melaleuca squarrosa*	R–S
87	M_s_3	*Melaleuca squarrosa*	S–VS
88	M_s_4	*Melaleuca squarrosa*	R–S
89	M_v_1	*Melaleuca virens*	S–VS
90	M_v_2	*Melaleuca virens*	R–VS
91	Met_c_1	*Metrosideros collina*	S
92	Met_c_2	*Metrosideros collina*	S
93	Met_e_1	*Metrosideros excels*	S
94	Met_e_2	*Metrosideros excelsa*	S
95	Met_t_1	*Metrosideros tomentosa*	S
96	P_g_1	*Psidium guajava*	HR
97	P_g_2	*Psidium guajava*	HR
98	P_g_3	*Psidium guajava*	HR
99	P_g_4	*Psidium guajava*	HR
100	P_g_5	*Psidium guajava*	HR
101	P_g_6	*Psidium guajava*	HR
102	Syz_j_1	*Syzigium jambos*	VS
103	Syz_p_1	*Syzigium pinnacle*	S
104	Syz_l_1	*Syzygium luehmanii*	S–VS
105	Syz_w_h1	*Syzygium wilsonii/S. luehmannii*	S
106	Syz_w_h2	*Syzygium wilsonii/S. luehmannii*	S
107	Syz_w_h3	*Syzygium wilsonii/S. luehmannii*	S
108	T_l_1	*Tristaniopsis laurina*	HR
109	W_?_1	*Waterhausea sp.*	HR?
110	W_f_1	*Waterhausea floribunda*	MS–S

* = Host responses as described in Table 2, ? = Not clear

Most of the *Leptospermum* species tested, including *glaucescens*, *grandiflorum*, *lanigerum*, *riparium*, *rupestre* and *scoparium*, were susceptible to myrtle rust. One exception were a seed lots from *L. riparium* (L_ripa_2) and L. *scoparium* (L_sco_5), which showed the presence of resistant plants (Table 5). Hybrids of *Leptospermum* and *L. nitidum* showed a range of resistant to susceptible response against myrtle rust in the greenhouse (Fig. 19). In the case of *Acmena smithii*, and *Waterhausea floribunda*, infection was observed on some very young and actively growing leaves (Table 5). In addition to all the guava cultivars tested, the non-*Eucalyptus* species *Lophostemon confertus* and *Tristaniopsis laurina* were highly resistant against *P. psidii* (Fig. 20). In some plant species (*A. smithii*, *L. confertus*, *T. laurina* and *W. floribunda*) it was very hard to get uniform new growth for testing. Though results were repeated in many cases,

it was not possible to retest all the plants. Test results are based on the number of plants tested and in case of non-*Eucalyptus* species turned highly resistant against *P. psidii*, it is advisable to retest the bigger lots of these particular species.

Fig. 18 Myrtle rust infected; A: *Chamelaucium sp*., B: *Chamelaucium sp*. flowers, C; *Kunzea ambigua*, D: *Metrosideros excelsa*, E: *Syzygium luehmanii* and F: *Leptospermum sp.* flower

Fig. 19 L–R: *Leptospermum nitidum* (R), *L. nitidum* (S) and *L. lanigerum* (VS)

All the seed lots of the tea tree species *Melaleuca gibbosa* and *M. pallida* were highly susceptible to *P. psidii* but seedlings raised from the seed lots of *M. quinquenervia* and *M. alternifolia* produced resistant to susceptible responses (Fig. 21). One seed lot of each *M. ericifolia* and *M. virens* was found to be susceptible whereas a second lot of each of these two species produced resistant to susceptible plants. Of the remaining four lots of *M. squarrosa* tested for response to *P. psidii*, two were susceptible and two showed a response that was variable (Table 5).

Fig. 20 Resistant *Lophostemon confertus* and *Tristaniopsis laurina*

Fig. 21 Different ITs observed in *Melaleuca alternifolia*; L–R: R, R, MR, S and VS

Testing of other 158 seed lots of *Eucalyptus* species (Table 6) is in progress at PBI. These seed lots were sourced from Tasmania, Victoria and Western Australia, and are being tested as a preemptive measure for myrtle rust management, especially in the states of TAS and WA where this pathogen is not reported yet.

Table 6 Myrtle rust testing of *Eucalyptus* species in progress at the Plant Breeding Institute

Sr. No	***Eucalyptus* species**	**Source**	**Location**
1	*Eucalyptus acies*	FPC WA	Albany
2	*Eucalyptus amygdalina*	UTAS	Beulah 135C
3	*Eucalyptus amygdalina*	UTAS	Retreat 217A
4	*Eucalyptus amygdalina*	UTAS	Kingston
5	*Eucalyptus amygdalina*	UTAS	Moulting Lagoon Game Reserve
6	*Eucalyptus annulata*	FPC WA	Albany
7	*Eucalyptus aquilina*	FPC WA	Esperance
8	*Eucalyptus archeri*	UTAS	Ben Lomond
9	*Eucalyptus archeri*	UTAS	Mt Saddleback
10	*Eucalyptus archeri*	UTAS	Projection Bluff
11	*Eucalyptus aspersa*	FPC WA	Perth Hills
12	*Eucalyptus barberi*	UTAS	Butlers Ridge Nature Reserve
13	*Eucalyptus barberi*	UTAS	Douglas Apsley National Park
14	*Eucalyptus barberi*	UTAS	Southern population
15	*Eucalyptus brandiana*	FPC WA	Albany
16	*Eucalyptus brookeriana*	UTAS	Salmon River 109H
17	*Eucalyptus brookeriana*	UTAS	Brookeriana Forest Reserve
18	*Eucalyptus calcicola* subsp. *calcicola*	FPC WA	Blackwood
19	*Eucalyptus calcicola* subsp. *unita*	FPC WA	Albany
20	*Eucalyptus calycogona*	FPC WA	Albany
21	*Eucalyptus cephalocarpa*	RBG VIC	Cranbourne
22	*Eucalyptus cerasiformis*	FPC WA	Esperance
23	*Eucalyptus cernua*	FPC WA	Albany
24	*Eucalyptus clivicola*	FPC WA	Albany
25	*Eucalyptus clivicola*	FPC WA	Albany
26	*Eucalyptus coccifera*	UTAS	Mt Field National Park
27	*Eucalyptus coccifera*	UTAS	Mt Wellington
28	*Eucalyptus conferruminata*	FPC WA	Albany
29	*Eucalyptus cordata*	UTAS	Corbett's Hill

30	*Eucalyptus cordata*	UTAS	Coombe's Hill
31	*Eucalyptus cordata*	UTAS	Bluestone Tier
32	*Eucalyptus cordata*	UTAS	Snug Plains
33	*Eucalyptus creta*	FPC WA	Esperance
34	*Eucalyptus dalrympleana*	UTAS	Wentworth 009A
35	*Eucalyptus dalrympleana*	UTAS	Clumner 171X
36	*Eucalyptus dalrympleana*	UTAS	Roses Tier 131F
37	*Eucalyptus delegatensis*	UTAS	Brady's 062E
38	*Eucalyptus delegatensis*	UTAS	Mt Foster 059A
39	*Eucalyptus delegatensis*	UTAS	Kara 004B
40	*Eucalyptus depauperata*	FPC WA	Esperance
41	*Eucalyptus desmondensis*	FPC WA	Albany
42	*Eucalyptus dolichorhyncha*	FPC WA	Esperance
43	*Eucalyptus erectifolia*	FPC WA	Albany
44	*Eucalyptus falcata*	FPC WA	Albany
45	*Eucalyptus flocktoniae*	FPC WA	Albany
46	*Eucalyptus foliosa*	FPC WA	Esperance
47	*Eucalyptus frenchiana*	FPC WA	Esperance
48	*Eucalyptus globulus*	UTAS	Blue Gum Hill
49	*Eucalyptus globulus*	UTAS	St Helens
50	*Eucalyptus globulus*	UTAS	King Island
51	*Eucalyptus globulus*	UTAS	Domain
52	*Eucalyptus goniantha subsp. goniantha*	FPC WA	Albany
53	*Eucalyptus goniocalyx*	RBG VIC	Cranbourne
54	*Eucalyptus gunnii* subsp. *divaricata*	UTAS	Todds Corner
55	*Eucalyptus gunnii* subsp. *gunnii*	UTAS	Snug Plains
56	*Eucalyptus gunnii* subsp. *gunnii*	UTAS	Central Plateau
57	*Eucalyptus gunnii* subsp. *gunnii*	UTAS	Lake St Clair
58	*Eucalyptus halophila*	FPC WA	Esperance
59	*Eucalyptus hebetifolia*	FPC WA	Albany
60	*Eucalyptus incrassata*	FPC WA	Albany

61	*Eucalyptus incrassata*	FPC WA	Albany
62	*Eucalyptus johnstonii*	UTAS	Repulse 036B
63	*Eucalyptus johnstonii*	UTAS	Mt Wellington
64	*Eucalyptus latens*	FPC WA	Perth Hills
65	*Eucalyptus lehmannii*	FPC WA	Albany
66	*Eucalyptus lehmannii* subsp. *parallela*	FPC WA	Albany
67	*Eucalyptus leptocalyx*	FPC WA	Albany
68	*Eucalyptus ligulata* subsp. *ligulata*	FPC WA	Esperance
69	*Eucalyptus ligulata* subsp. *stirlingica*	FPC WA	Albany
70	*Eucalyptus littorea*	FPC WA	Esperance
71	*Eucalyptus marginata*	FPC WA	Swan Coastal
72	*Eucalyptus medialis*	FPC WA	Albany
73	*Eucalyptus megacarpa*	FPC WA	Albany
74	*Eucalyptus megacornuta*	FPC WA	Albany
75	*Eucalyptus melanophitra*	FPC WA	Albany
76	*Eucalyptus misella*	FPC WA	Esperance
77	*Eucalyptus morrisbyi*	UTAS	South Arm.
78	*Eucalyptus morrisbyi*	UTAS	Risdon Hill
79	*Eucalyptus nebulosa*	UTAS	Serpentine Ridge
80	*Eucalyptus newbeyii*	FPC WA	Albany
81	*Eucalyptus nitida*	UTAS	Temma 011C
82	*Eucalyptus nitida*	UTAS	Sumac 004D
83	*Eucalyptus nitida*	UTAS	Melaleuca airstrip
84	*Eucalyptus obliqua*	UTAS	Togari 003C
85	*Eucalyptus obliqua*	UTAS	Kara 007D
86	*Eucalyptus obliqua*	UTAS	Gladstone 223B
87	*Eucalyptus obliqua*	UTAS	Franklin 023E
88	*Eucalyptus oleosa* subsp. *oleosa*	FPC WA	Esperance
89	*Eucalyptus ovata*	UTAS	Grove
90	*Eucalyptus ovata*	UTAS	Peggs Beach
91	*Eucalyptus ovata*	UTAS	Nunamara

92	*Eucalyptus ovata*	RBG VIC	Cranbourne
93	*Eucalyptus pachyloma*	FPC WA	Albany
94	*Eucalyptus pauciflora*	UTAS	Story 016B
95	*Eucalyptus pauciflora*	UTAS	Ross
96	*Eucalyptus pauciflora*	UTAS	Dungrove
97	*Eucalyptus pauciflora* subsp. *pauciflora*	RBG VIC	Cranbourne
98	*Eucalyptus perinniana*	UTAS	Strickland
99	*Eucalyptus perinniana*	UTAS	Hungry Flats
100	*Eucalyptus petrensis*	FPC WA	Swan Coastal
101	*Eucalyptus phenax*	FPC WA	Albany
102	*Eucalyptus pileata*	FPC WA	Albany
103	*Eucalyptus pleurocarpa*	FPC WA	Albany
104	*Eucalyptus praetermissa*	FPC WA	Albany
105	*Eucalyptus preissiana* subsp. *lobata*	FPC WA	Esperance
106	*Eucalyptus proxima*	FPC WA	Albany
107	*Eucalyptus pryoriana*	RBG VIC	Cranbourne
108	*Eucalyptus pulchella*	UTAS	Coles Bay Road
109	*Eucalyptus pulchella*	UTAS	Garden Island Creek, Scars Road
110	*Eucalyptus pulchella*	UTAS	Garden Island Creek, Channel Hwy
111	*Eucalyptus pulchella*	UTAS	Glenlusk
112	*Eucalyptus purpurata*	FPC WA	Albany
113	*Eucalyptus radiata*	RBG VIC	Cranbourne
114	*Eucalyptus radiata* subsp. *radiata*	UTAS	Lemonthyme
115	*Eucalyptus redunca*	FPC WA	Albany
116	*Eucalyptus regnans*	UTAS	Styx 035C
117	*Eucalyptus regnans*	UTAS	Cascade 157C
118	*Eucalyptus regnans*	UTAS	Oldina 027G
119	*Eucalyptus regnans*	UTAS	Snug Tiers Nature Recreation Area
120	*Eucalyptus retusa*	FPC WA	Albany
121	*Eucalyptus rigens*	FPC WA	Esperance
122	*Eucalyptus risdonii*	UTAS	Government Hills, north side

123	*Eucalyptus risdonii*	UTAS	Meehan Range
124	*Eucalyptus rodwayi*	UTAS	M6 road East of Mt. St John
125	*Eucalyptus rodwayii*	UTAS	Maggs 135U
126	*Eucalyptus rodwayii*	UTAS	Bradys 017C/023E, Wentworth
127	*Eucalyptus rubida*	UTAS	Rossarden Crown Land
128	*Eucalyptus rubida*	UTAS	Derwent Valley
129	*Eucalyptus semiglobosa*	FPC WA	Esperance
130	*Eucalyptus sieberi*	UTAS	Urana 020D
131	*Eucalyptus sieberi*	UTAS	Beaumaris
132	*Eucalyptus sinuosa*	FPC WA	Albany
133	*Eucalyptus* sp. (M.E. French 1579)	FPC WA	Esperance
134	*Eucalyptus spathulata*	FPC WA	Albany
135	*Eucalyptus stoatei*	FPC WA	Esperance
136	*Eucalyptus subcrenulata*	UTAS	Plenty 003D
137	*Eucalyptus subcrenulata*	UTAS	Hartz Mountains National Park
138	*Eucalyptus subcrenulata*	UTAS	Mt Field
139	*Eucalyptus subcrenulata*	UTAS	Crystall Hill
140	*Eucalyptus suggrandis*	FPC WA	Albany
141	*Eucalyptus surgens*	FPC WA	Esperance
142	*Eucalyptus talyuberlup*	FPC WA	Albany
143	*Eucalyptus tenuiramis*	UTAS	Freycinet National Park
144	*Eucalyptus tenuiramis*	UTAS	Huon Road
145	*Eucalyptus tenuiramis*	UTAS	Bothwell Tip
146	*Eucalyptus tenuiramis*	UTAS	Lovely Banks
147	*Eucalyptus tetraptera*	FPC WA	Albany
148	*Eucalyptus uncinata*	FPC WA	Albany
149	*Eucalyptus urnigera*	UTAS	Mt Wellington
150	*Eucalyptus urnigera*	UTAS	Lake Echo
151	*Eucalyptus vernicosa*	UTAS	Hartz Mt
152	*Eucalyptus vernicosa*	UTAS	Moonlight Ridge
153	*Eucalyptus vesiculosa*	FPC WA	Albany

154	*Eucalyptus viminalis*	UTAS	UTAS
155	*Eucalyptus viminalis*	UTAS	UTAS
156	*Eucalyptus viminalis*	UTAS	UTAS
157	*Eucalyptus viminalis*	UTAS	UTAS
158	*Eucalyptus websteriana* subsp. *norsemanica*	FPC WA	Esperance

Development of SSRs:

To study the genetic diversity among the Australian isolates of *P. psidii*, a new set of highly polymorphic SSR markers were developed from genome sequence information. As part of this process, a survey of the nature and abundance of SSRs was evaluated across the sequenced genome. Based on genome sequencing of *P. psidii* standard culture (622) of single pustule increased isolate (115012), originally collected from *A. flexuosa* in Leonay, NSW, the genome size was estimated at ~ 142 Mb.

SSR Abundance:

A total of 22,819 SSR motifs were identified across the *P. psidii* genome. Most of these (13,028) consisted of mononucleotide repeat motifs, followed by 7,031 dinucleotide, 2,198 trinucleotide, 251 tetranucleotide, 147 pentanucleotide and 164 hexa nucleotide repeat motifs (Fig. 22). In a similar study, Karaoglu *et al*. (2013) found that mononucleotide repeats were the most frequent in the *Pgt* genome.

Relative abundance and most frequent SSR repeats:

The relative abundance of SSRs was calculated as the number of SSRs identified per Mb of sequence analysed (Karaoglu *et al*. 2013) and was compared with the relative abundance of SSRs in several cereal rust pathogens for which genome sequence information is available. SSRs are very densely distributed in the *P. psidii* genome compared to the wheat stripe rust causing pathogen *P. striiformis f.sp. tritici*, and sparse in comparison to *Pgt* and *P. hordei* (Table 7). The calculated relative abundances of tetranucleotide, pentanucleotide and

hexanucleotide repeats was much lower than that of the dinucleotides and trinucleotides, whereas the mononucleotide repeats showed the highest levels of relative abundance (Table 7).

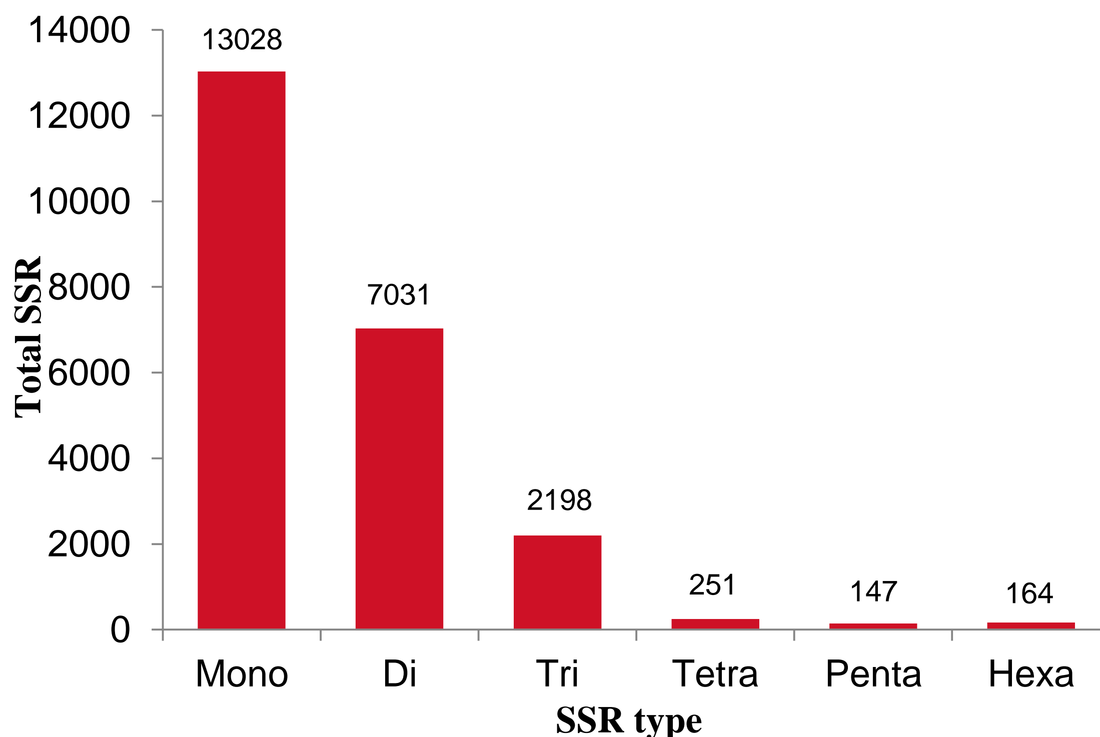

Fig. 22 Number of different types of SSRs present across the *Puccinia psidii* genome

Table 7 Relative abundance of SSRs in *Puccinia psidii* in comparison to three other cereal rust causing pathogens

Repeat Type	***P. psidii***	***P. striiformis* f. sp. *tritici***	***P. graminis* f. sp. *tritici***	***P. hordei***
Mono	91	-	255.7	149.7
Di	49.5	11.51	76.2	74.9
Tri	17.72	6.3	47.3	33.6
Tetra	1.76	2.8	3.9	5.1
Penta	1.03	0.7	3.4	2.7
Hexa	1.15	0.5	1.1	3
Total	160.7	22.08	387.6	269.1

Sequence data was also analysed for the most frequent SSR repeat motifs. Sequence analysis revealed that A/T mononucleotide stretches were more abundant compared to the C/G stretches, and that the longest stretch of motif A was with 6,511 repeats followed by 6,332 repeat units of motif T, 95 repeat units of motif C and 90 repeat units of motif G. After the mononucleotide repeat motifs, the AT/TA dinucleotide stretches were the most abundant motifs occurring 6,055 times followed by CT/TC 315 times, AG/GA 293 times, AC/CA 191 times, GT/TG 172 times and the least occurring CG/GC for only 5 times (Table 8). The lowest occurrence of CG/GC motifs is a general trend in fungi containing larger genome sizes. Trinucleotide and hexanucleotide repeat motifs contained C and G nucleotides in addition to the A and T nucleotides whereas tetranucleotide and pentanucleotide stretches were the combinations of A and T nucleotides only (Table 8).

Table 8 Most frequent SSR repeats present in the *Puccinia psidii* genome

Repeat Type					
Mono	**Di**	**Tri**	**Tetra**	**Penta**	**Hexa**
A (6511)	AT/TA (6055)	AAT/ATA/TAA (614)	AAAT (21)	AAATT (9)	AAATCA (12)
T (6332)	CT/TC (315)	TTA/TAT/ATT (585)	TAAA (14)	AAAAT (7)	AATTTG (7)
C (95)	AG/GA (293)	AGT/ATG/GAT/GTA/TAG/TGA (344)	TTTA (14)	ATTAA (6)	TGATTT (7)
G (90)	AC/CA (191)	ACT/ATC/CAT/CTA/TAC/TCA (282)	AATA (13)	ATTTT (6)	TTGATT (7)
	GT/TG (172)		ATTT (12)		
	CG/GC (5)				

Longest repeat motif:

The longest repeat motif identified was the trinucleotide ATG with a stretch of repeat occurring 89 times, followed by a stretch of TTG with 81 repeat units. In mononucleotides, there were three stretches of each A and T repeat motifs containing 26 repeat units of each. The longest dinucleotide repeat motif was GA occurring 21 times in a stretch of this repeat. In tetra, penta and hexanucleotide repeats the longest repeat motifs were the TCTT with 11 repeat units, ATTAG with 40 repeat units and ATATAA with 22 repeat units respectively (Table 9).

Table 9 Details of longest repeat motifs found in the *Puccinia psidii* genome

Repeat Type					
Mono	**Di**	**Tri**	**Tetra**	**Penta**	**Hexa**
A^{26}	GA^{21}	ATG^{89}	TCTT^{11}	ATTAG^{40}	ATATAA^{22}
A^{26}	TC^{18}	TTG^{81}	ATGG^{10}	AGTAG^{12}	AAATTA^{11}
A^{26}	TC^{15}	TAT^{26}	ATTT^{10}	AATTT^{11}	CTGACA^{10}
T^{26}	TA^{15}	TTG^{24}	CATC^{9}	TGATC^{9}	ATCAAA^{10}
T^{26}	GA^{14}	CAA^{21}	TTGA^{9}	TGATG^{8}	TCACTA^{9}
T^{26}	AT^{14}	CAT^{21}	GATG^{9}	TTTGA^{8}	TTAAAA^{9}
T^{26}	AT^{14}	TAT^{20}	ATTT^{9}		ATTAAA^{9}
	AT^{14}	ATT^{20}	GAAG^{9}		
			TTTC^{9}		

Genetic diversity among Australian isolates of P. psidii:

A set of 240 SSRs was selected for primer synthesis using software OLIGO® 7. Out of the 240 primers designed, 10 were developed from dinucleotide repeat motifs, 86 from trinucleotides and 48 each from tetra, penta and hexanucleotides (Table 10). A diverse range of 17 isolates of *P. psidii* including 14 from Australia (NSW, QLD and ACT Canberra), two from Brazil and one from Hawaii (Table 1) was selected to assess genetic diversity in the pathogen with these 240 markers. More than 74% of the markers amplified DNA from the different isolates at an annealing temperature of 56ºC in the PCRs performed. Out of the 178 amplifying SSRs, 110 showed polymorphism between the Australian and Brazilian isolates of *P. psidii* (Table 10). For example, the marker Pp5_32 amplified similar bands for the Australian isolates (Au_1 to

Au_14) and an isolate from Hawaii (Hw_17), and different bands for both the Brazilian isolates (Bz_15 and Bz_16) as shown below (Fig. 23). Polymorphic SSRs clearly separated the two Brazilian isolates (Bz_15 and Bz_16) from each other and from all Australian isolates, plus the one from Hawaii (Hw_17). Using these markers, all Australian isolates were found to be similar to the one from Hawaii.

Forward primers for each polymorphic locus were 5′ labeled by incorporating the fluorophore dye 6FAM and fluorescently labeled PCR products will be used for fragment analysis using an ABI3730XL (Macrogen Inc. Geumchun-gu Seoul,Korea) capillary analyser. Sequence data of the primers is not provided in the report because the results are due for publication in a refereed journal.

Table 10 Detail of 240 primers designed using OLIGO® Version 7 and used in the genotyping

Repeat	No. of primers designed	Amplified at TM 56ºC	Total Polymorphic	Percent polymorphic	No. of alleles
Di	10	7	6	83.33%	2–3
Tri	86	57	36	63.15%	2–3
Tetra	48	37	21	58.33%	3
Penta	48	38	22	58.82%	3
Hexa	48	39	25	74.07%	3–4
Total	240	178	110	74.16%	

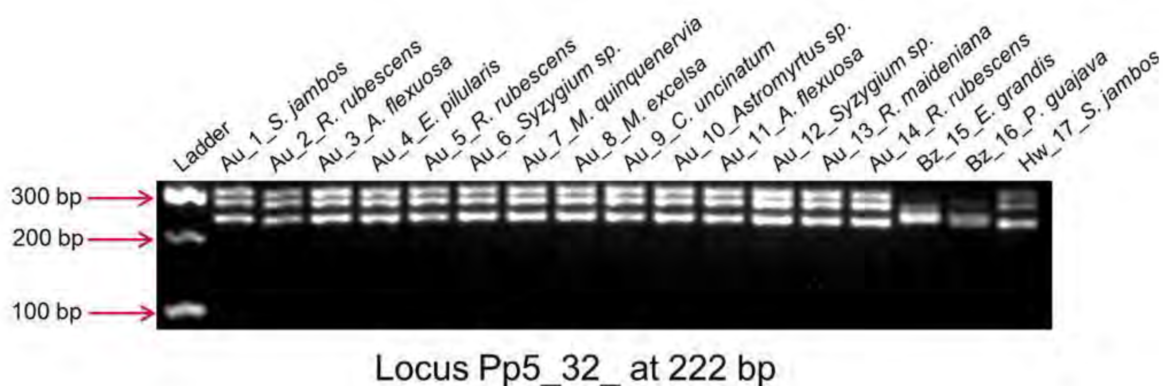

Fig. 23 Polymorphic locus Pp5_32 showing three different allele types as one allele for isolates Au_1 to Au_14 and Hw_17, second allele for Bz_15 and third allele for Bz_16

Conclusions:

All milestones of the project were met successfully. The research outcomes have increased our understanding about susceptibility and resistance among the myrtaceous host plants tested and about the myrtle rust causing pathogen *P. psidii*, under Australian conditions. Myrtle rust isolates collected from different geographical locations of Australia were increased from single pustules and preserved in liquid nitrogen at PBI. Standard culture (622) of single pustule increased isolate (Au_3) was used in DNA extraction for genome sequencing and for all the germplasm testing. Rust culture 622 was also provided to the DPI, NSW for *P. psidii* genome sequencing.

Rose apple *S. jambos* was used as susceptible control and is highly suitable for increasing the rust inoculum. Myrtle rust can infect only young and actively growing foliage. Within two weeks after inoculation, rust infections were established successfully with inoculations of urediniospores suspended in mineral oil (2mg/ml) followed by incubation at 20°C plus >95% RH for 24 hrs and at a post incubation temperature of 22 ± 2°C in the microclimate rooms.

Most of the *Eucalyptus* species tested showed a varied response of resistance to susceptibility against *P. psidii*, whereas majority of the non- *Eucalyptus* species were susceptible. The local strain of *P. psidii* is not virulent on *P. guajava*, as all the guava cultivars tested were highly resistant. Resistant to susceptible plants of *E. globulus* and *E. grandis* that were identified have potential as differentials for assessing pathogenic diversity in the myrtle rust pathogen. Further work is required to establish a full set of differentials including different *Eucalyptus* species and methods of their propagation for the continuous supply of true to type plants. Testing of another 158 lots of *Eucalyptus* species is under progress at PBI.

Out of 240 SSRs developed from *P. psidii* genome sequencing, 178 amplified the PCR products and further 110 showed polymorphism among the Australian and Brazilian isolates of *P. psidii*. Polymorphic markers differentiated the two Brazilian isolates from each other and from all Australian isolates and one from Hawaii. Polymorphic SSRs revealed that all the Australian isolates were found similar to the one from Hawaii, and that there is no genetic diversity among the Australian isolates of *P. psidii* examined, suggesting that a single genotype

of the pathogen was introduced. Molecular work is in progress to find the exact sizes of these polymorphic markers.

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Inclusions:

Figures: 23

Tables: 10

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