Optimising methods for screening the susceptibility of host species populations to myrtle rust

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Executive summary

The highly invasive rust *Austropuccinia psidii* (myrtle rust) has an unusually large host range, with over 480 known hosts worldwide. The aim of this project is to carry out a pilot study to develop a fast, efficient, and cost-effective method that can screen plants for susceptibility to myrtle rust within any laboratory with a PC1 microorganism facility with MPI approval. Four methods were trialled, rooted cuttings, cut material in Ellepots, cut material in water agar (WA) in sealed jars and in WA in open falcon tubes. The cut material was inoculated within 24 hours of collection with the exception of the rooted cuttings. Rooted cuttings showed good results, however the method to set cuttings for this group of hosts needs to be investigated further to speed up the development of roots and new flush. Health of the cut material in Ellepots and WA in open falcon tubes deteriorated to a point where there was not enough healthy tissue to sustain myrtle rust infection in most cases, however some results were obtained from the former. The cut material set in WA in sealed jars showed promising results and warrants further investigation to optimise the health of the cuttings over time. Good infection rates were obtained using misting tents with humidity controllers. This means that these trials can be carried out in any PC1 microorganisms facility without the need for environmentally controlled chambers/rooms.

The problem

Understand variation in resistance among and within nursery varieties and test simple assay method using cuttings.

Client initiatives

Design a protocol to test variation in resistance to myrtle rust using cuttings and establish an experiment using nursery species/varieties to determine variation among species/varieties. Write a technical report of findings.

Key results

- The best results were seen with rooted cuttings, which maintained good general plant health. It takes time to root the cuttings and for new growth to re-establish and techniques will need to be optimised between species. Faster rooting and establishment of new growth will be achieved if cuttings are taken in spring.
- Good results were seen with cut material in WA sealed jars and general health remained at an acceptable level to score infection. Species with softer leaves will not maintain health as well as those with tougher leaves.
- Marginal results were seen with cut material in Ellepots due to the deterioration in health. Species with softer leaves will not be suitable for this method and many of those with tougher leaves could not be scored for infection at 21 days due to the lack of healthy tissues.
- No results could be obtained from the cut material in open falcon tubes. Within 10 days most cut material and died or dried out. This is not considered a suitable method to pursue.
- The use of misting tents with humidity controllers, means that these trials can be carried out in any PC1 microorganisms facility with the correct MPI approval without the need for environmentally controlled chambers/rooms.

Implications of results for the client

We have found that there are alternatives to using seedlings for susceptibility trials using cut material and rooted cuttings. Cut material and rooted cuttings also adds the benefit of working with clonal material and not having to rely on gathering seed which may not always be available for some species.

Further work

The WA in sealed jars method should be investigated further to see if cutting health can be optimised over the 21-day period. Further testing with larger numbers of replicates and species should be done and compared against results on rooted cuttings to better determine the viability of this method.

More work needs to be done on rooted cuttings of species that require screening, in particular native species. Time needs to be allocated so that engagement with iwi and hapū can take place. Given the impact this disease is having on some of our native Myrtaceae, including the impact on seed production, it is critical to pursue this method so screening of vulnerable populations can be done.

Optimising methods for screening the susceptibility of host species populations to myrtle rust

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Introduction

The highly invasive rust *Austropuccinia psidii* (myrtle rust) has an unusually large host range, with over 480 known hosts worldwide (J Soewarto, Giblin, & Carnegie, 2019). Susceptibility to myrtle rust varies among species and can also vary within a host species population (Berthon, Esperon-Rodriguez, Beaumont, Carnegie, & Leishman, 2018; Johansen, 2021; Junghans, Alfenas, & Maffia, 2003; Morin, Aveyard, Lidbetter, & Wilson, 2012; Pegg, Perry, Carnegie, Ireland, & Giblin, 2012; Sandhu & Park, 2013; Smith et al., 2020; Julia Soewarto et al., 2021; Tobias, Guest, Külheim, Hsieh, & Park, 2016; Yong et al., 2019; Zauza et al., 2010). For New Zealand, it is particularly important to understand if this variation exists within and among host species for numerous reasons including protecting our native species through seed banking more vulnerable genotypes, planting less susceptible genotypes, understanding susceptibility of exotic reservoir species, being prepared for incursions of different strains of myrtle rust, and to support industry to select resistant genotypes for propagation and production.

Since myrtle rust is an obligate biotroph, it is important to use plant material that is as close to its natural state as possible, whist still having a method where host material can easily be collected and efficiently inoculated and assessed for susceptibility. For this reason, we have chosen to trial fresh cut material and rooted cuttings. The standard method for experimental screening uses seedlings, and while the details are often not reported it requires a minimum of four months for seedlings to grow to suitable size for testing (Julia Soewarto et al., 2021; Zauza et al., 2010). There are also advantages and disadvantages when working with clonal cutting material versus sibling groups from seed when investigating the genetic basis of susceptibility. Furthermore, fruits are not always available for collection of seed. We inoculated cut material with new flush taken directly from the host plant (within 24 hours of taking the collection) using three methods and compared this to rooted cuttings.

The aim of this project was to carry out a pilot study to develop a fast, efficient, and cost-effective method, to screen host populations for susceptibility to myrtle rust which can be carried out in PC1 microorganisms facility which has MPI approvals without the need for environmentally controlled chambers/rooms. If successful, there are other applications that the method could be used for such as spore bulking and testing chemical control and biological control agents. At this pilot stage, we focussed on evaluating variation in susceptibility among species using exotic host species with a range of known susceptibilities. We were particularly interested in hosts commonly found in gardens around New Zealand.

Materials and methods

Host material:

Three host species were targeted as suitable host material, *Agonis flexuosa* (very susceptible), *Callistemon* sp. (variable susceptibility), and a *Syzygium* sp. (unknown susceptibility). *Agonis flexuosa* was collected from three mature trees planted in a Long Mile trial site at Scion. *Callistemon* sp. was collected from a hedge, approx. 50 m long. The *Syzygium* sp. was also collected from a hedge, around 10 m long, it is suspected this species was *S. paniculatum*, but it could not be confirmed as flowering was not observed.

Figure 1. Shows the setup of the four different cutting material methods.

1. *Rooted cuttings with new flush re-established:*

   Seventy-two cuttings of each species were propagated in Scion's nursery in Ellepots 4 cm (125cc) plugs with No. 8 bark mix from Daltons. Before the study, it was estimated that it would take 6-8 week for some root development and re-establishment of enough new flush to support infection. However, it was not until 14 weeks that the *Callistemon* sp. had enough cuttings with sufficient new growth to inoculate. At this time, a limited number of *Syzygium* sp. had a small amount on new flush, but leaves had not expanded. *Agonis flexuosa* had no new flush and could not be used for this method. Fourteen *Syzygium* sp. (10 treated and 4 controls) and 24 *Callistemon* sp. (20 treated and 4 control) were transferred to 6 by 8 cell trays in a randomised block layout. Controls were kept aside and added to the trays after inoculation.

2. *Cut material in Ellepots:*

   Each host species had cuttings taken (max length of 15 cm), with new flush and leaves. Cuttings were treated with rooting hormone (IBA + Talc powder). Fifty cuttings of each host were set in Ellepots 4 cm (125 cc) plugs with No. 8 bark mix from Daltons, in 6 by 8 cell trays in a randomised block layout, 12 controls of each host species were kept aside and added to the trays after inoculation. Host material was inoculated within 24 hours of collection.

3. *Cut material in WA in sealed jars:*

   Each host species had cuttings taken (max length of 15 cm), with new flush and leaves. Twenty-four of *Syzygium* sp. (20 treatment and four control), 26 of *Callistemon* sp. (22 treatment and four control) and 8 of *Agonis flexuosa* (six treatment and 2 control) were set into 15 mm depth of water agar in 1 L jars. Host material was inoculated within 24 hours of collection.

4. Cut material in WA in open falcon tubes:

Callistemon sp. and *Syzygium* sp. had cut material taken (max length of 15 cm), with new flush and leaves. *Agonis flexuosa* had no new flush present and was not used for this method. Twenty cuttings each of *Syzygium* sp. and *Callistemon* sp. (15 treatment and 5 control), were set in 50 ml flacon tubes with 40 ml of WA in 20 celled trays in a randomised block layout. Controls were kept aside and added to the trays after inoculation. Host material was inoculated within 24 hours of collection.

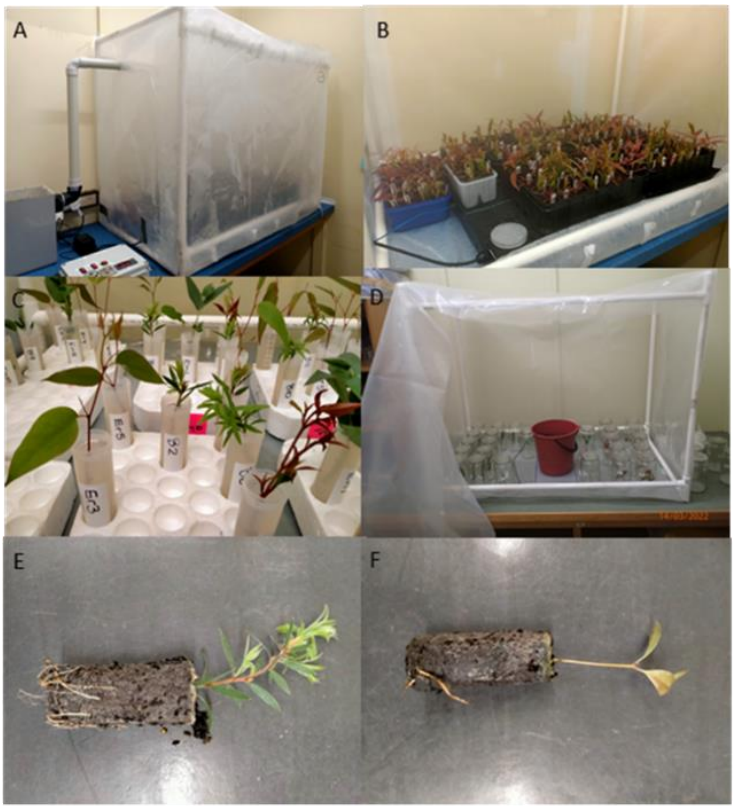


Figure 1. A. Tent set up with fogger. B. Cuttings in Ellepots. C. Cuttings in falcon tubes. D. Cuttings in WA in sealed jars. E. (*Callistemon* sp.) & F. (*Syzygium* sp.) set cuttings with new flush at 14 weeks.

Inoculation:

Inoculum was bulked on *Syzygium jambos* at Scion in January 2022 and was stored at -80 °C ready for use. The trays of rooted cuttings, cut material in Ellepots and in WA in open falcon tubes were placed in misting tents and inoculated using a fine mist spray of 1 x 10^5 urediniospores/ml and 0.05% Tween®, which was applied to ensure leaf and stem surfaces of the cuttings are coated but runoff of the spore suspension was avoided. Control plants were inoculated with a mixture of sterile water and 0.05% Tween®. Water agar plates were also sprayed with inoculum and assessed for spore germination after 24 hours. For the first 24 hours, cuttings were maintained at greater than 80% relative humidity, at approx. 18 °C, in darkness to simulate optimal conditions for germination. Thereafter, the humidity was maintained at 70 % or greater, at 20-24 °C, under continuous light (LED 50W/4000K), supplemented with normal daylight. Humidity was maintained by using a fogger box (containing distilled water and a fogger) with a fan which was piped into the

tent (Figure 1A). Humidity was controlled using a humidistat, and the fan ran while the fogger was running ensuring consistent humidity throughout the tent. Cuttings were monitored for progression of disease symptoms by visual assessment at days 10, 14, and 21 post inoculation. The cut material in WA in sealed jars were inoculated using the same method as above, but after the initial 24 hours, jars were sealed and maintained at a temperature of 20-24 °C, under light conditions described above.

Assessment:

Leaves and stems were assessed using the scale of Smith et al. (2020), (Table 1). Leaf and stem scores were added together (infection score) to give a susceptibility rating based around Smith et al. (2020), (Table 2).

Table 1. Leaf and stem visual assessment scale (Smith et al., 2020).

Scale	Leaf visual symptoms
L1	No symptoms or the presence of flecking evident
L2	Presence of a hypersensitive reaction with Fleck or necrosis
L3	Small pustules, <0.8mm diam., with one or two uredinia
L4	Medium pustules, 0.8-1.6mm diam., with about 12 uredinia
L5	Large pustules >1.6mm diam., with 20 or more uredinia on leaves, petioles and/or shoots
Scale	Stem visual symptoms
S1	No evidence of infection on stems
S2	Pustule present on stem but no lesion
S3	1-2 stem lesions, average length < 5 mm
S4	1-2 stem lesions, average length ≥ 5 mm
S5	> 2 but < 5 stem lesions, average length < 5 mm

Table 2. Susceptibility rating

Infection score (leaf + stem score)	Susceptible
1-3	No
4	Moderately
> 5	Yes

General health excluding any damage caused by myrtle rust infection was also scored. Unhealthy tissues included stems and/or leaves exhibiting necrosis, dieback, wilt, or dried green tissue deemed not to be caused by myrtle rust infection (Table 3).

Table 3. General health rating

Criteria excluding myrtle rust symptoms	Score
healthy	1
< 25% unhealthy	2
25-50% unhealthy	3
50-75% unhealthy	4
> 75% unhealthy	5

Results and discussion

Results:

Water agar plates that were sprayed with inoculum showed good germination rates of between 77 - 90% from three separate WA plates with a count of around 400 spores per plate.

Figure 2 shows typical symptoms and examples of leaf and stem scores and host health scores. The cut material in WA in open falcon tubes showed minimal infection after 10 days and most of the cutting material had dried and died over this time. For this reason, the results have not been shown as there was not enough heathy plant material to sustain infection after 10 days. Figures 3-5 shows infection scores (combined leaf and stem scores) over time for the other three methods. There was very little in the way of stem lesions and most of the infection scored could be attributed to leaf lesions. Figures 6-8 shows the health scores over time for these methods. Figure 9 shows the susceptibility of the different hosts for these methods over time.

Figure 2. A & B. *A. flexuosa* cuttings in Ellepots at 14 days (health 1) with L3, S1 and L4, S1 scores respectively. C. *Callistemon* sp. in Ellepots at day 10 (health 2) with L3, S1 score. D & E *Callistemon* sp. rooted cuttings (health 1) and in WA in sealed jars (health 2) respectively, at 21 days both with L5 and S1 score. F. *Syzygium* sp. control in WA sealed jars (health 1), at 21 days, L1, S1 score. G. *Callistemon*

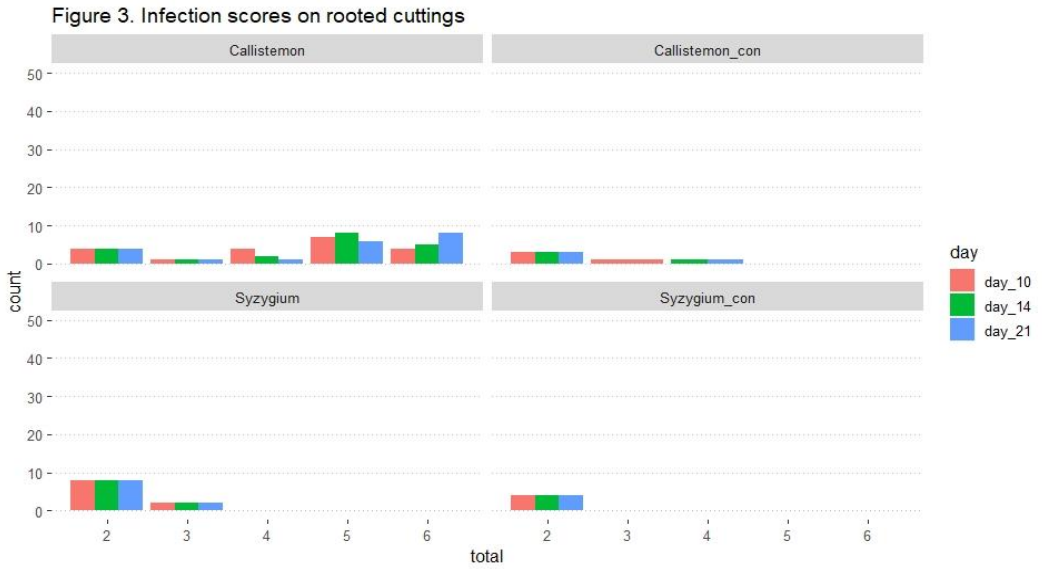


Figure 3. Number of replicates: *Callistemon* = 20; *Callistemon_con* = 4; *Syzygium* = 10; *Syzygium_con* = 4

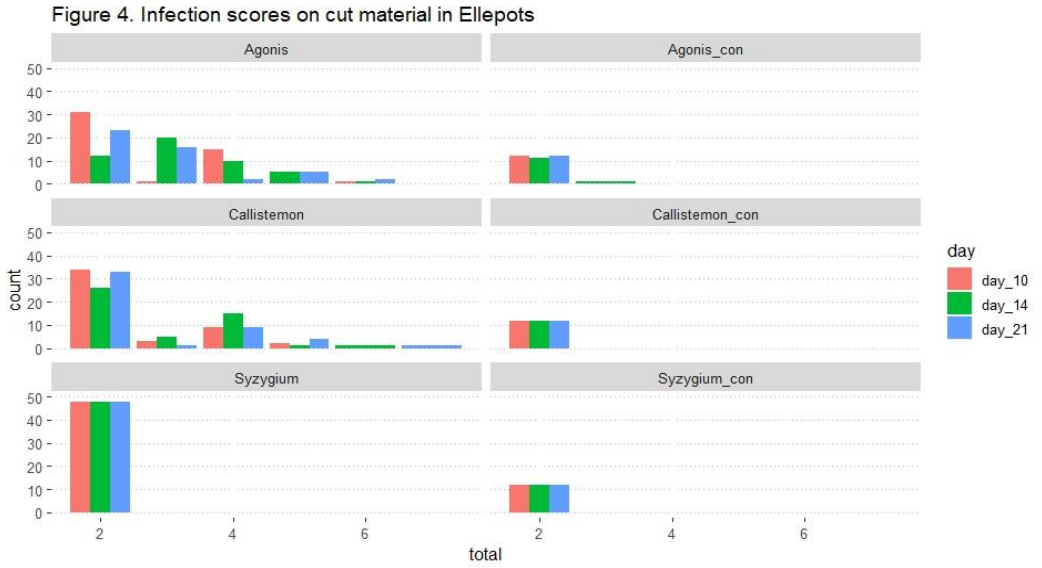


Figure 4. Total number of replicates: *Agonis* = 48; *Agonis_con = 12; Callistemon* = 48; *Callistemon_con* = 12; *Syzygium* = 48; *Syzygium_con* = 12

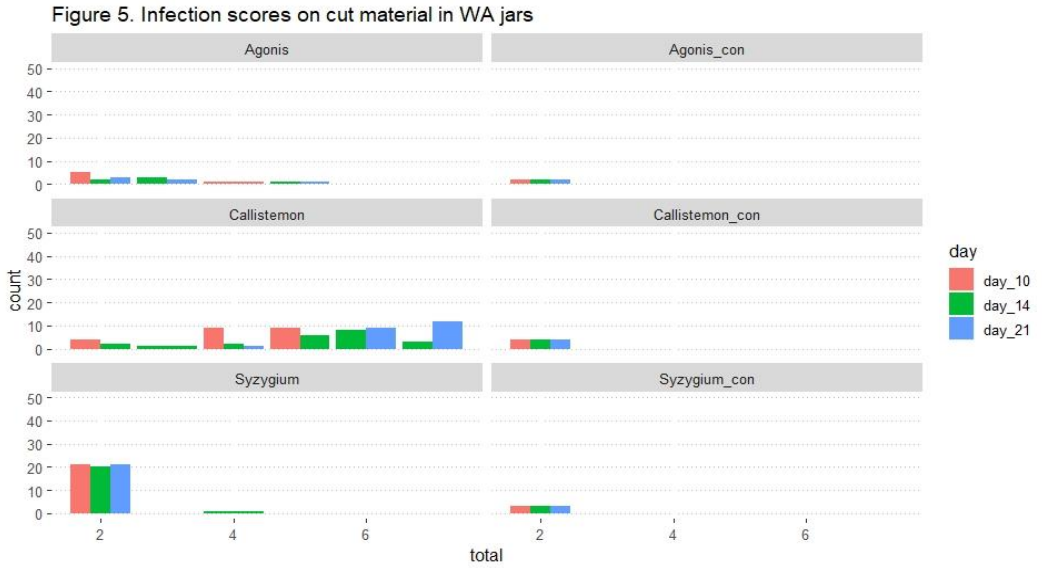


Figure 5. Total number of replicates: *Agonis* = 6; *Agonis_con* = 2; *Callistemon* = 22; *Callistemon_con* = 4; *Syzygium* = 20; *Syzygium_con* = 4

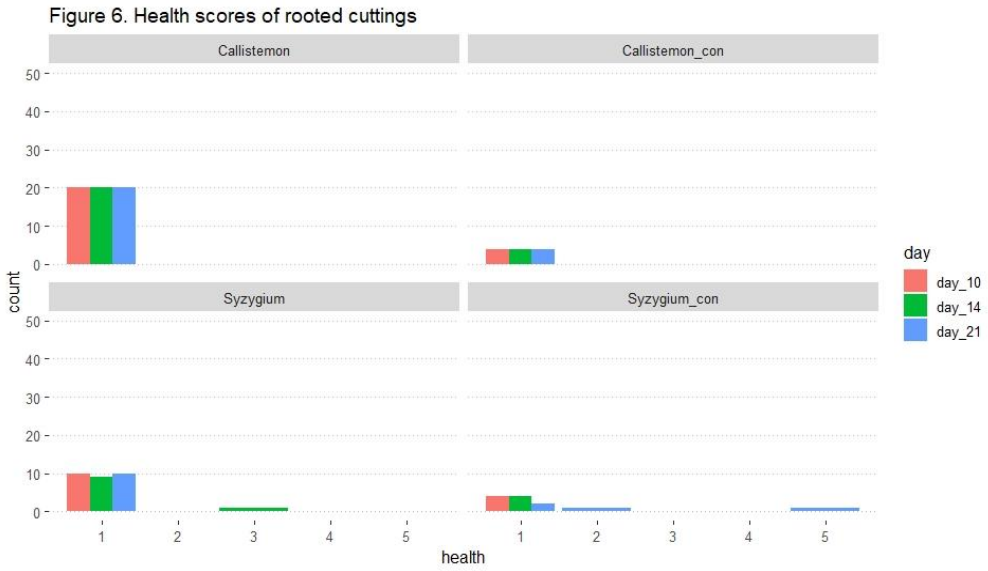


Figure 6. Total number of replicates: *Callistemon* = 20; *Callistemon_con* = 4; *Syzygium* = 10; *Syzygium*_con = 4

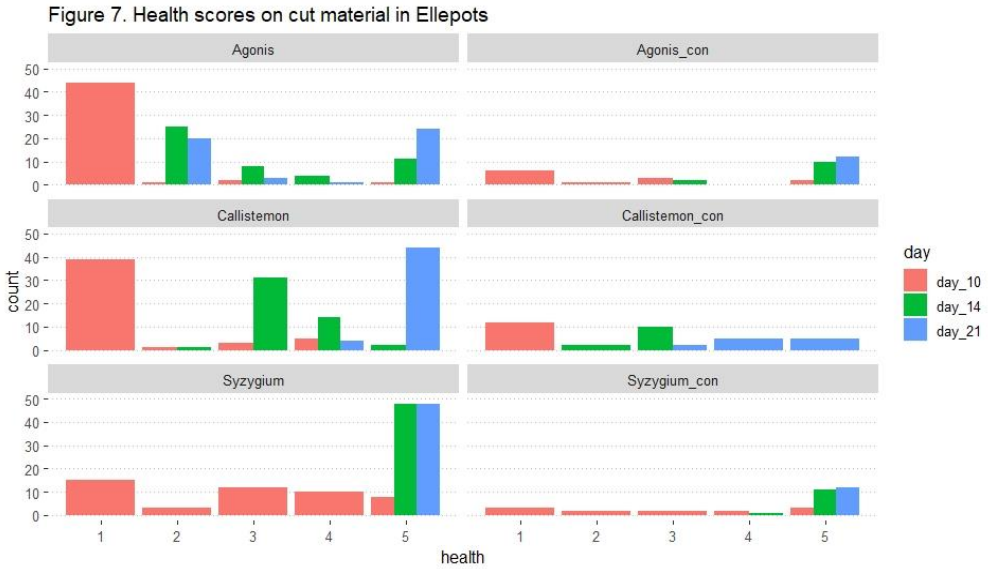


Figure 7. Total number of replicates: *Agonis* = 48; *Agonis_con* = *12; Callistemon* = 48; *Callistemon_con* = 12; *Syzygium* = 48; *Syzygium_con* = 12

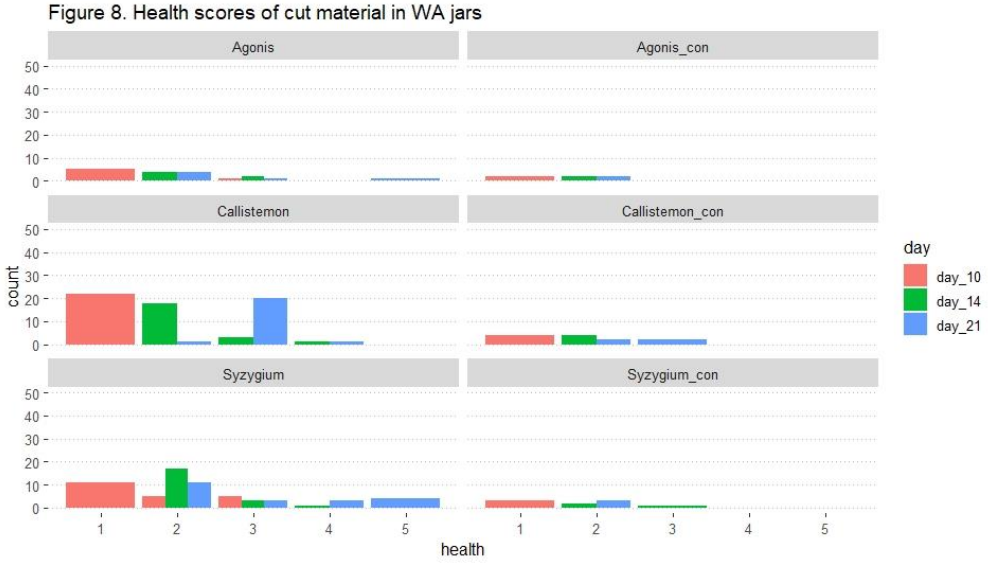
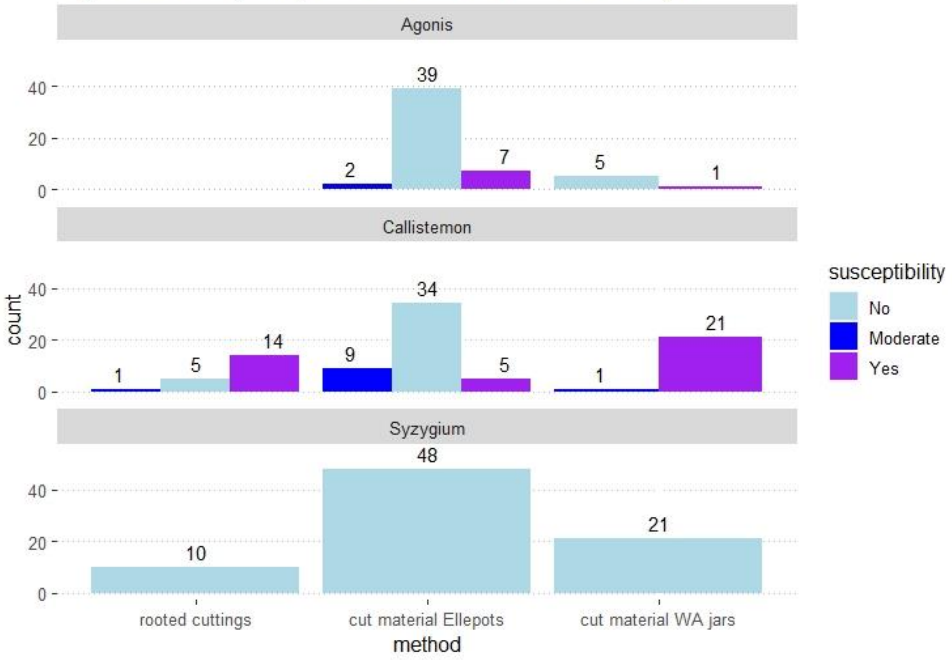


Figure 8. Total number of replicates: Agonis = 6; *Agonis_con* = 2; *Callistemon* = 22; *Callistemon_con* = 4; *Syzygium* = 20; *Syzygium_con* = 4

Figure 9. Susceptibility of hosts between methods at day 21



Rooted cuttings with new flush re-established:

Over half of the *Callistemon* sp. developed pustules after 10 days. Severity increased slightly on day 14 and again on day 21. A single *Callistemon* sp. control plant became infected. This is attributed to cross infection when the control plants were reintroduced to the randomised block layout with the inoculated cuttings. Health of this host was good over the assessment period. The number of *Callistemon* sp. individuals scored as susceptible increased over time and around three quarters of the cuttings were classed as susceptible after 21 days. Individuals that did not show symptoms at 10 days were also not showing symptoms after 21 days. A single cutting of *Syzygium* sp. showed a hypersensitive reaction, with no pustules forming. Otherwise, this species showed no signs or symptoms, and scored not susceptible. Most cuttings maintained overall health scores of 1 and 2 over the 21-day period.

Cut material in Ellepots:

Agonis flexuosa and *Callistemon* sp. had minimal visual symptoms after 10 days. There was no significant difference in severity over the 21 days in either of these hosts. General health of the material deteriorated over time and by day 21, half of *A. flexuosa* and almost all the *Callistemon* sp. cuttings had over 75 % dead tissues present. This level of decline left little living tissue for myrtle rust to progress infection and often symptomatic plant tissue was lost through leaf drop and alike. Less than a third of *A. flexuosa* and *Callistemon* sp. were classed as susceptible. The *Syzygium* sp. health deteriorated quickly and by day 14 they all had over 75 % dead tissues and were unable to sustain myrtle rust infection. No myrtle rust infection was noted on this host, and it scored not susceptible.

Cut material in WA in sealed jars:

Agonis flexuosa had a single individual with pustules at day 10, no pustules were observed on the other individuals throughout the 21-day assessment period. Susceptibility was significantly less than the cut material in Ellepots, but this could be attributed to the small number of replicates that were used in this method. All the *Callistemon* sp. individuals formed pustules after 14 days and were rated as susceptible with one of these classed as moderately susceptible. This was higher than either of the other methods for this host. The *Syzygium* had a single individual where pustules developed at day 14. By day 21 the leaf had dropped as no pustule development was recorded. This species was classed as not susceptible which was consistent with the other two methods. Health deteriorated in all species in this method, however by day 21 there were still enough material with healthy tissues to score infection for *A. flexuosa* and *Callistemon* sp., but only a few individuals had enough healthy tissues to score infection in the *Syzygium* sp. None of the controls showed infection.

Discussion:

The *Callistemon* sp. had more severe infection in the rooted cuttings than the other two methods, however susceptibility for this host was highest in the WA sealed jar method. This indicates that there may be a range of susceptibility within the hedge cuttings were sourced from. Due to lower sample sizes, it is possible some resistance was not picked up in the WA sealed jar method. The increased severity in the rooted cuttings indicates that myrtle rust is less aggressive on cut host tissue. This is not surprising since it is an obligate biotroph. Cut material set in Ellepots had many uninfected cuttings which could be attributed to the declining health of the host over the 21-day period. More work needs to be done on methods to keep the cut material in the best possible health for the duration of the infection period. *Agonis flexuosa* and *Callistemon* sp. both have quite tough leaves and showed better overall health than the *Syzygium* sp. which has much softer leaves; therefore, is less able to survive for long when cut. The WA sealed jar method had better health over the 21 days than the cut material in Ellepots for all hosts. It was unfortunate that we had no *A. flexuosa* to use in the rooted cutting method as this is a good benchmark for infection, however the cut material in Ellepots and WA sealed jars did behave in a similar way with only a few replicates being susceptible. As mentioned above, the deterioration in health of the cut material played a role in large number of uninfected individuals for the cut material in Ellepots. No infection was seen in the *Syzygium* sp. from any of the methods. This is an indication that this host might be resistant. However very few replicates were used in the rooted cutting method and the leaf material had not fully expanded, therefore not ideal and by day 21 all the individuals had died in the cut material in Ellepots and only a few individuals had enough healthy tissues to score infection in the WA in sealed jar method.

Cut material needs to have new growth for infection, when cuttings are set the new growth is removed and leaves are cut back to keep transpiration to a minimum. The fact that myrtle rust needs young material for infection complicates using cut material as a medium. However, the WA

sealed jars method should be investigated further as in the example of the *Callistemon* sp. excellent infection rates and progression of infection were achieved.

The rooted cutting method is another a good option. There is variation between species around the strike rate and time it takes for cuttings to re-establish new growth. In this case it took 14 weeks for us to have sufficient material for *Callistemon* sp., minimal material for *Syzygium* sp. and the third species (*A. flexuosa*) failed to strike. The host material was harvested at the end of summer, and we could have achieved greater success by harvesting and setting cuttings which are dormant (early spring). We would see much better and faster rooting without so much mortality and variability and they would be quicker to flush. Therefore, the initial period of 6-8 weeks could still be achievable, however any screening programme should allow some flexibility in timeline to allow for growth of new flush. It is important when comparing species that they are at a similar stage of flush, so they have similar inoculum loading and do not escape infection through lack of susceptible tissues. It should also be noted that there will be some species which will not strike cuttings within this timeframe or at all.

The misting tents with humidity control proved to be more than adequate set up to carry out these trials. This means laboratories with PC1 microorganisms facility with proper MPI approval can carry out these trials without the need for environmentally controlled chambers/rooms.

Recommendations and conclusions

The WA in sealed jars method should be investigated further to see if cutting health can be optimised over the 21-day period. Further testing with larger numbers of replicates and species should be done and compared against results on rooted cuttings to better determine the viability of this method.

More work needs to be done on rooted cuttings of species that require screening, in particular native species. Time needs to be allocated so engagement with iwi can take place. Given the impact this disease is having on some of our native Myrtaceae, including the impact on seed production, it is critical to pursue this method so screening of vulnerable populations can be done.

Time constraints limited our host selection but the full range of “lilly pilly” *Syzygium* species (*S. australe*, *S. paniculatum* and *S. smithii*) that are believed to vary in susceptibility and can provide a significant reservoir for the disease [12] and should be considered for future studies. Furthermore, any future testing should also allow for time to gain permissions for using our native and taonga species.

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