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|---|---|
| **Project title:** | **Characterising broad-acting resistance to bacterial canker of cherry and elucidating tissue-specific mechanisms of immunity** |
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| **Project leader:** | Dr Richard Harrison, NIAB |
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| **Previous report:** | N/A |
| **Key staff:** | Dr Glyn Barret, University of Reading |
| **Location of project:** | NIAB, Cambridge |
| **Industry Representative:** | Harriet Duncalfe, Berry Gardens |
| **Date project commenced:** | 1st Oct 2020 |

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# AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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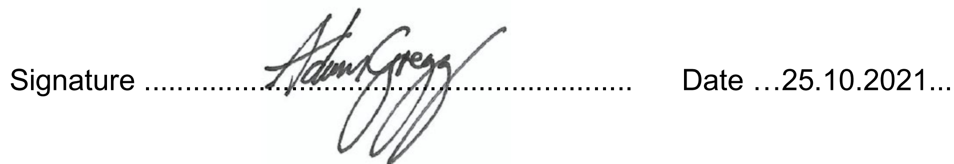

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# GROWER SUMMARY

## Headline

This project seeks to characterise the genetics of resistance towards bacterial canker in cherry (*P. avium*). Initially, phenotyping experiments will be carried out on different wild and sweet cherry tissues. This will be followed by genotyping studies. This project will lead to the development of molecular markers, furthering progress towards identification novel sources of resistance and improved breeding strategies.

## Background

The cherry industry in the UK has seen a significant resurgence in recent years from producing ca. 1,000 ton in 2012 to >6,000 tons in 2019 (British Summer Fruits, 2019). High intensity orchards have helped to achieve this output but has also increased bacterial canker susceptibility through increased local abundance and transmission potential.

*P. syringae* disease is distributed widely throughout the world and the bacteria commonly exists as an epiphyte and is ubiquitous in the natural world, including rainwater, rivers, and soil (Morris *et al*., 2013). It is an aerobic Gram-negative rod shaped γ-proteobacteria (1.5 µm long and 0.7 – 1.2 µm width), cells are motile exhibiting polar flagellum (Arnold & Preston, 2018). The *P. syringae* species complex is subdivided into >60 pathovars depending on which plant species it infects (Gutiérrez-Barranquero *et al*., 2019). Diseases caused by *P. syringae* pathogenicity include annual crops such as tomato (causing bacterial spot), and beans (causing halo blight), as well as woody plants and trees including on horse chestnut (causing bleeding canker) and kiwifruit (causing canker) (Lamichhane *et al*., 2014, Bartoli *et al.,* 2015).

The three main *P. syringae* clades responsible for canker in cherry are *P. syringae* pv. *syringae* (Pss), *P. syringae* pv. *morsprunorum* race 1 (Psm R1), and *P. syringae* pv. *morsprunorum* race 2 (Psm R2) these are located in phylogroup 2, phylogroup 3 and phylogroup 1, respectively (Hulin *et al*., 2018, Taylor *et al*., 1989).

*P. syringae* is able to infect in any season and cause lesions on all aerial plant tissues (leaves, fruit and blossom) and in autumn/winter seasons can infect dormant woody tissues through scars caused by mechanical and freeze/thaw damage. Symptoms include dead, sunken, water-soaked bark often accompanied with and gummosis as well as leaf shotholes.

Treatments such as copper-based biocides cause adverse environmental effects and not completely effective due to increasing copper resistance (McLeod et al., 2017) and crop spraying has adverse ecological effects. Biological pathogen control attained through genetic

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resistance is a key part for the global transition to sustainable agriculture (Sonmez & Mamay, 2018).

*P. syringae* pathogenicity is complicated due to the biochemical complexities derived from the multitude of virulence factors working in concert. In terms of defining host specificity the most important factor is the pathogen's effector/toxin repertoire. Other virulence factors include hormones (or hormone-like compounds), cell wall degrading enzymes, and exopolysaccharides (Xin, *et al.,* 2018). Plants have evolved a means for detecting these virulence factors and activating host defences.

Plant recognition receptors and NB-LRRs encoded by R genes, when triggered, activate downstream signalling enacting defence responses (Bucherl *et al*., 2017, McHale *et al*., 2006). A robust understanding of *P. syringae* effectors, effector interplay and corresponding resistance genes (R genes), may lead to the development of resistant cherry cultivars, greatly benefiting the cherry breeding strategies.

Biological control measures achieved through genetic understanding are much more financially–and ecologically favourable than the current chemical methods (Leung et al., 2020). Therefore, the biochemical mechanisms and underlying genetics that determine the interaction between host recognition receptors and pathogen effectors are key to understanding resistance. Genomic, effector-informed, research has the potential to provide development of biological control through enabling accelerated breeding strategies and other potential control measures (Lenaerts et al., 2019).

## Summary

- *P. syringae* is the causal agent of bacterial canker. This bacterium is ubiquitous with the environment and the species complex is vast with over 60 pathovars affecting many economically important crop species.
- *P. syringae* affects all areal plant tissues and can infect anytime throughout the year.
- Copper based treatments are becoming less effective due to increased resistance. Genetic solutions towards pathogen control are much more favourable and are key to the future transition to sustainable agriculture.
- *P. syringae* pathogenicity is complex and a range of factors affect host-pathogen interaction.
- The understanding of R gene-effector relationships is key to understanding genetic resistance towards *P. syringae* pathovars, and genomic research can facilitate

accelerated breeding strategies and has the potential to achieve continuous biological control.

## Financial Benefits

This is a 4-year project, no financial benefits can be reported at this stage in the project. However, this project will build knowledge towards genetically informed breeding strategies to avoid future bacterial canker tree loss.

## Action Points

At this stage of the project no action points can be recommended.

# SCIENCE SECTION

## Introduction

Previous research has shown that some commercial cherry cultivars exhibit broad-acting partial resistance towards all pathogens. This resistance is likely to be quantitative involving a range of traits, meaning a continuous distribution between resistance and susceptible phenotypes in a progeny resulting from the segregation of alleles with variable effects at several loci. In contrast, wild cherry has been shown to exhibit strong resistance which may result from only a few major genes. Therefore, the understanding of genes which control this strong resistance is key to establishing robust disease-resistant commercial cherry varieties.

Initial steps of this project have been made towards resistance phenotyping which involved development and optimization of pathology assays to understand extent and timings of disease/immune response. Assays currently in development include measurement of reactive oxygen species, ion leakage, leaf & shoot pathology, and biophoton emission on cherry leaf material and cell suspensions.

Future resistance characterization will include effector/bacterial localization experiments using fluorescent tags, tracking of fluorescently tagged bacteria, and the study of immune gene expression through RNA sequencing experiments.

The final work package of this project will involve genotyping of the cherry material. Genotyping data will be generated from the established multiparental population at EMR, Kent. The cherry population is structured/linked – often linked by one parent allowing for analysis of many recombination's within different full-sib families. Also, parent trees are often grandparents of others, these will have enough contribution of resistance QTL to enable mapping. High depth Illumina sequencing of parental founders and shallow (DArT or skim)

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sequencing of progeny to enable identification of candidate loci leading to improved genomic prediction, breeding and selection of existing material.

This work builds towards the development of molecular markers for use in breeding resistant cultivars. Compared to traditional breeding techniques, genomic approaches allow for higher accuracy and a more informed approach to breeding. Also, if genomic techniques are combined with speed breeding the generational cycle and speed of implementation can further accelerated.

This first year of the project has been used to develop and optimise phenotyping assays to understand immune/disease response between various *P. avium* cultivar and *P. syringae* strain combinations (Table 1). Assays were first tested using *Nicotiana benthamiana* during *P. avium* dormancy. *N. benthamiana* was used as a control alongside *P. avium* lea once material was available.

**Table 1.** Pathology assays which have been tested this year and are in development as well as their role in differentiating phenotype using different cultivar-strain combinations.

| Assay | Target |
|---|---|
| Shoot dip inoculation | Woody resistance phenotype |
| Leaf disc infection | Dynamics of infection/ resistance response without infiltration |
| Infiltration assay for hypersensitive response | Identification of effector-triggered immunity in leaves |
| Ion Leakage assay | Cumulative ETI response |
| ROS- Luminol Assay | Dynamics of PAMP & effector triggered oxidative burst |
| Callose Assay | PAMP- triggered immunity |
| ROS-DAB Assay | ROS snapshot at different timepoints |

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## Materials and methods

**Plant Material –** 1–2-week-old Cherry leaf material was collected from small trees grown in 5L pots located outdoors at Park Farm, Cambridge. The cultivar collection was an assortment of wild and cultivated varieties which have previously been shown have differential resistance/susceptibility towards *P. syringae*. 1–2-week-old leaves used. Shoot material was collected from the EMR cultivar collection during the winter while the trees were dormant.

*N. benthamiana* plants were grown from seed in a growth chamber at 24°C, 60% humidity, and 16-hour photoperiod for 4-5 weeks before use.

**Bacterial Material -** *P. syringae* stock plates were grown from glycerol stocks on Kings B agar at 28°C. Fresh stock plates were made every Friday. Prior to each experiment, overnight cultures were made by subculturing 1-2 colonies from stock plates into 10mL lysogeny broth incubated at 28°C at 180RPM.

**(1) Shoot Inoculation -** Five 12cm shoots per strain were sterilised in 70% ethanol. Bacteria was grown overnight in 10mL LB at 20°C. Bacteria was the spun down at 300RPM for 10 mins. The supernatant was removed, and the bacteria was resuspended in 10mL 10mM $MgCl_2$. The bacterial concentration was adjusted to 0.2 $OD^{600}$ and diluted 1in10 to give a final concentration of $2x10^7$CFU/mL and total volume of 50mL. Bacterial suspension was put into a sterile jar, the top of the shoots was cut and dipped in the bacterial suspension for 5 minutes. $MgCl_2$ was used as a negative control. Following inoculation, the top of the shoots were wrapped in parafilm, the bottom of the shoots were cut, placed in universals filled with 10mL sterile dH20 (randomised design) and incubated at 16°C for 6 weeks.

**(2) Ion Conductivity -** Bacterial suspensions were made and diluted to $2x10^8$CFU/mL. Bacterial suspensions were syringe infiltrated into leaves and 7.5mm leaf discs were cut out with a cork borer before the inoculum dried. Two leaf discs per well were floated in 2mL sterile ultrapure water and were incubated for 30 mins at 20°C. Following incubation, water was removed and replaced with 2mL of fresh ultrapure water. 100µl was sampled every 4 hours and ion conductivity was measured using a LAQUAtwin conductivity meter EC-11.

**(3) ROS-Luminol Assay -** One day before ROS assay performed 0.8mm leaf discs punched out using a cark borer and halved using a razor. Each leaf half was floated adaxial side up in an individual well of a 96-well plate contained 150 µl sterile dH20 and incubated for 20-24 hours at 22°C. The elicitation solution was prepared containing bacteria ($2x10^8$CFU/mL), luminol (17mg/mL, 2 µl/mL) and horse radish peroxidase (500x, 2 µl/mL). Incubated dH2O was removed from each well and replaced with 100 µl elicitation solution and placed in a luminometer. Luminescence was measured for either 90 mins or 16hours.

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**(4) ROS-DAB staining -** Leaves were syringe infiltration with a bacterial suspension of $6x10^8$CFU/mL and incubated at 22°C under continuous light for 1-2hours ($MgCl_2$ was infiltrated as a negative control). While inoculated leaves were incubating, DAB staining solution was prepared. Following incubation, leaf discs were punched out from the inoculated leaves and placed into a 12-well plate with 2mL DAB staining solution. 12-well plate was vacuum infiltrated for 5 mins (-0.1MPa) and incubated at 22C, 100RPM for 4 hours. Leaf discs were then decolourised overnight in a solution of ethanol: acetic acid: glycerol (3:1:1). Stained leaf discs were imaged using a stereo microscope.

**(5) Leaf infiltration -** leaves were infiltrated with bacterial suspension from the abaxial surface using a blunt-ended syringe. Leaves placed water agar and incubated at 22°C, 16-hour photoperiod. Leaves were assessed at chosen time points using a Bio-Rad ChemiDoc MP imaging system (Universal Hood III), and images were acquired on excitation by a light source in the green visible spectrum, reading emission of emitted in the red visible spectrum (filter 605/50).

**(6) Leaf Disc Vacuum Infiltration –** 1 cm Leaf discs were punched out using a cork borer. Leaves were submerged in a bacterial suspension and vacuum infiltrated for 10 mins (-0.1MPa). Infiltrated leaf discs were then placed on water agar and incubated at 22°C, 16-hour photoperiod. Red light imaging was performed as above.

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## Results

### (1) Shoot Inoculation

The results of this experiment were compromised due to fungal growth which was observed at the inoculation sites following the 6-week incubation, therefore this experiment needs to be repeated in the coming winter. However, the results from this run (Figure 1 & 2) suggest that Cobtree, Arger Fern E, Howley wood, Cherryhill Copse A, Groton B and Burghley Wood all appear to have high susceptibility to PsmR2, with tested strains showing lesion lengths of >2cm. PsmR1-5244 infection of Burghley wood showed the largest average lesion size (4.16 cm). Snarkhurst, Marlow common, and FD1-57-4/122 appeared to have high tolerance towards all Ps strains tested with no lesions exceeding 1cm in length. *P. incisa* also showed good resistance towards all strains generally, Psm R1-5300 being to only strain to display significant average lesions length on this cultivar (1.96cm).

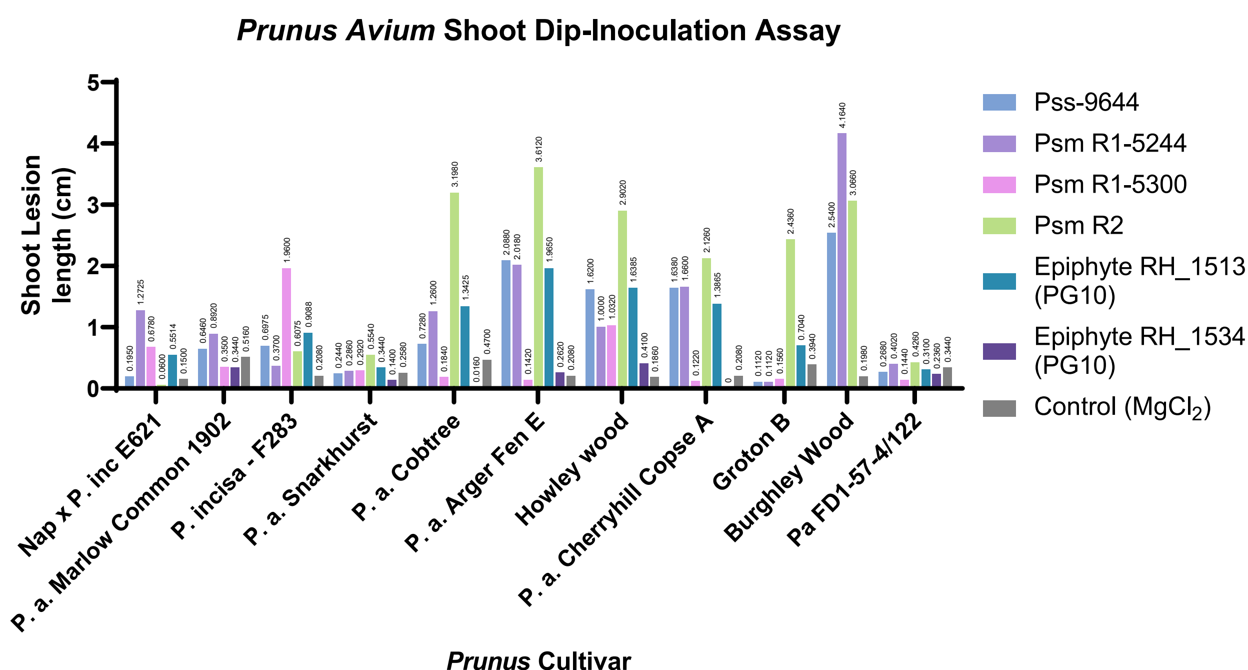


**Figure 1. Shoot Inoculation summary data.** Cut shoots (N=5) dipped for 5mins in $2x10^7$CFU/mL *P. syringae* and incubated for 6 weeks at 16°C. Lesion length measured with callipers following incubation period. Increased lesion length indicates susceptibility (N=5).

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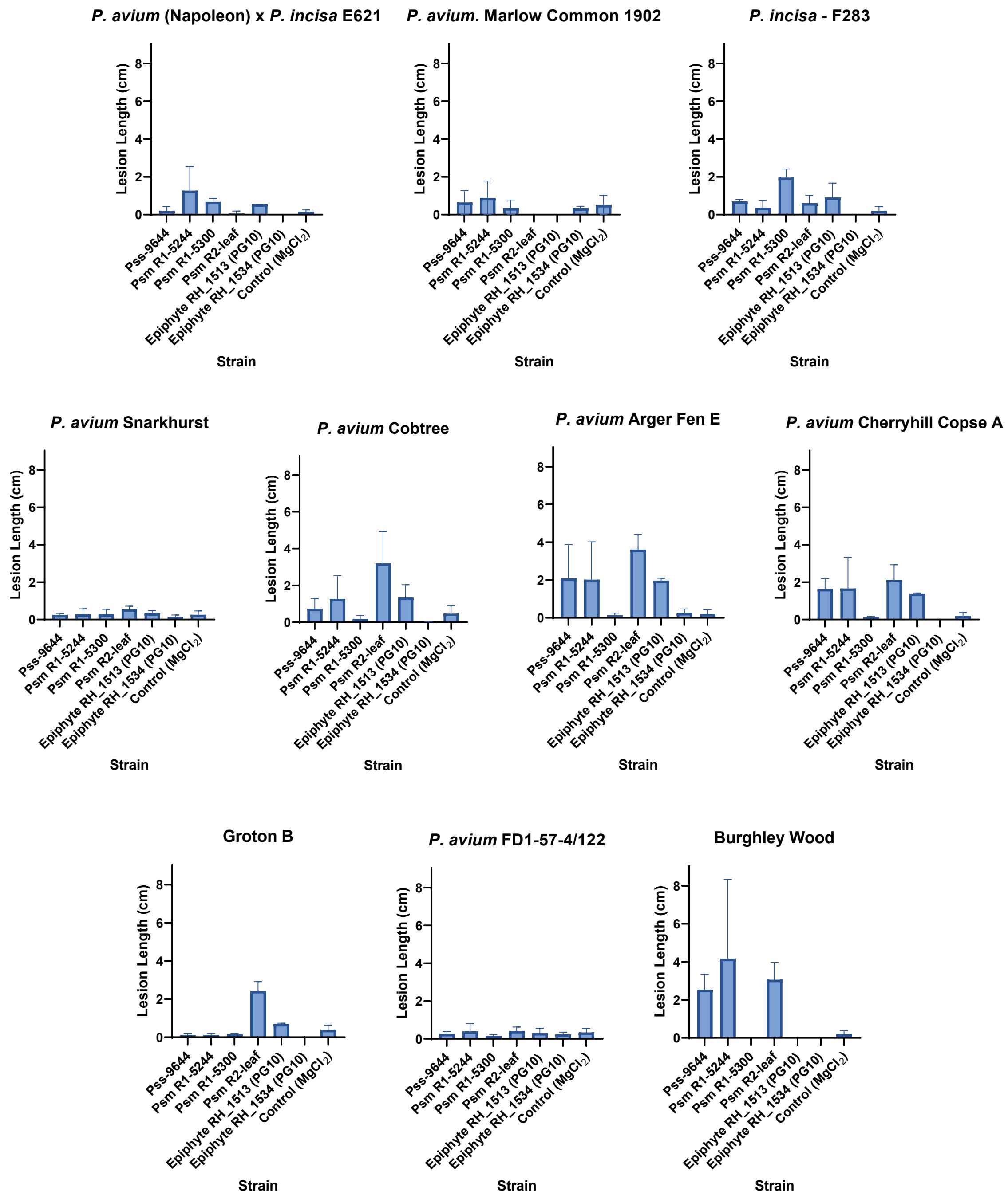


**Figure 2. Individual shoot inoculation data.** Same data shown in Figure 1. Shown per cultivar with error bars. Increased lesion length indicates increased susceptibility (N=5 per cultivar).

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### (2) Ion Conductivity

Results shown are pilot data of the initial assay test using *N. benthamiana* (Figure 3). Ion leakage is a cumulative measure of cell death due to hypersensitive response or pathogen induced necrosis. Results show good differentiation between strains. Pss-9644 shows greatest pathogen response and tissue breakdown (194.22 μs/cm) followed by Psm R2 (131.22 μs/cm), while the epiphyte and control appeared to have no effect on ion leakage over 24 hours (41.89 and 41.11 μs/cm respectively). This assay did not work when tried on *P. avium*, all strains tested did not differ from the control and stayed at background levels for the duration of the 24-hour experiment (data not shown).

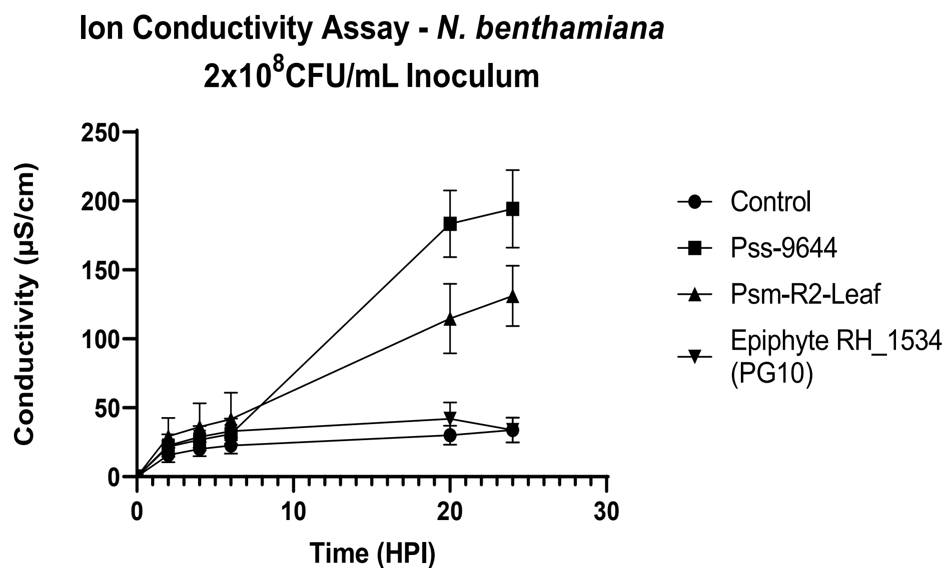


**Figure 3. *N. benthamiana* Ion conductivity assay.** Leaves were syringe infiltrated with *P. syringae* strains and floated in sterile ultra-pure $dH_2O$. Ion conductivity was measured at various timepoints by aliquoting 100 μL of sample $dH_2O$ into a LAQUAtwin conductivity meter EC-11 (N=9 er strain).

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### (3) ROS-Luminol Assay

The ROS-luminol assay is a chemiluminescent assay showing the dynamics of pathogen induced oxidative burst following infection. Both virulent and avirulent bacteria elicit PTI ROS peaks however only avirulent bacteria should show the second ETI ROS peak (Trujillo, 2016). PTI peaks are short, high intensity and occur shortly after infection while ETI peaks are much broader and occur 2-16 hours post infection. Initial results were promising when using *N. benthamiana* leaf tissue (Figure 4 & 4a). Initial tests were a 1.5hr time course experiment and the results revealed clear PTI peaks at ~20 minutes post infection. Test 1 (Fig 4) shows similar responses between all strains tested. Test 2 (Fig 4a) shows much more differentiation between strains. The epiphytic strains showed a much-reduced response compared to the virulent stains tested.

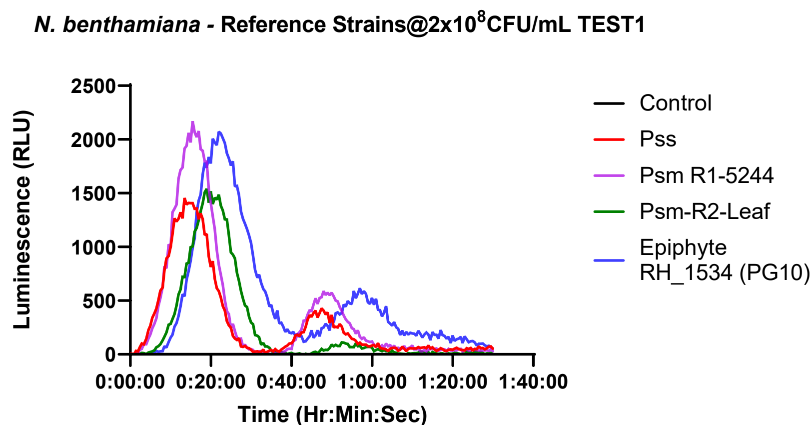


**Figure 4. ROS-Luminol assay**. Initial assay development test using N. benthamiana and P. syringae reference strains (N=5). ½ 0.8mm leaf discs floated in 2x10$^8$CFU/mL *P. syringae* along with the $H_2O_2$ reactants luminol and HRP. Dynamics of oxidative burst in response to bacterial elicitors measured over 90 minutes.

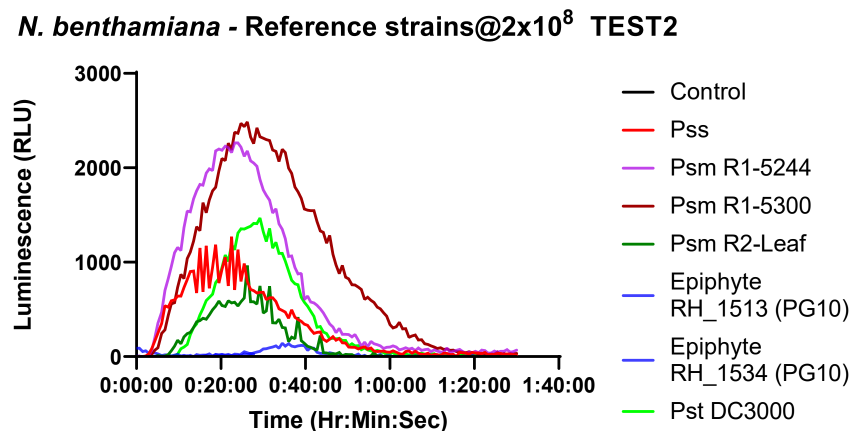


**Figure 4a. ROS-Luminol assay**. Second development test using *N. benthamiana* and *P. syringae* reference strains (N=5).

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Figures 5 shows the first test using *P. avium* leaf tissue and is an exact repeat of the method used in the previous two runs. No measurable response can be seen in the *P. avium* samples.

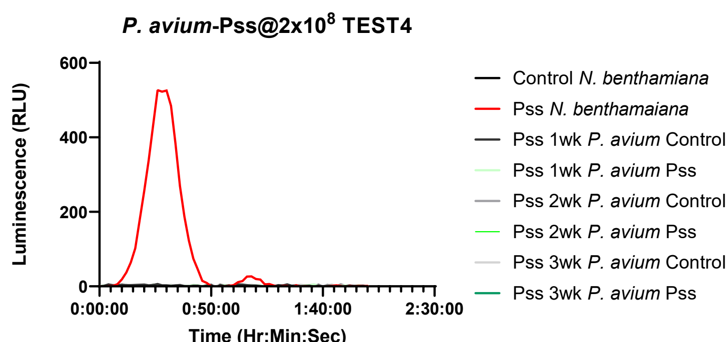


**Figure 5. ROS-Luminol Assay using *P. avium*.** First test including *P. avium* (N=5) *N. benthamiana* included as a positive control.

The time course was increased from 1.5hrs to 16 hrs (Figure 5a and 5b), as it was thought that *P. avium* may respond later than *N. benthamiana*. Also, this increase run time would show both PTI and ETI responses. Pathogen loading was also increase to $6x10^8$ CFU/mL. Figure 5b shows results after further adjustment to the method: vacuum infiltration, increased leaf material/decreased disc size, and the addition of a surfactant. Figure 5c shows the added step of microneedle rolling of leaves to increase pathogen entry into test tissues. *N. benthamiana* indicated an age dependant response (Figure 5c), young leaves show a strong PTI response and a much smaller ETI response while older leaves had an inverse trend, showing a weaker PTI and stronger ETI. No response could be measured in *P. avium* tissues (Fig 5 & 5a-c). Further experimentation is required to develop a dynamic ROS assay.

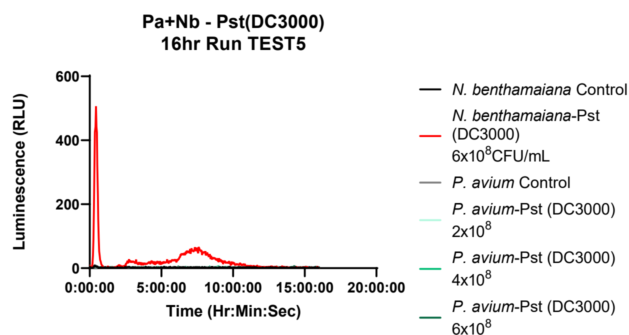


**Figure 5a. ROS-Luminol Assay using *P. avium*.** Second test including *P. avium* (N=5). *N. benthamiana* included as a positive control and the method modified to increase testing time from 90 mins to 16hours to test whether *P. avium* showed a delayed PTI response.

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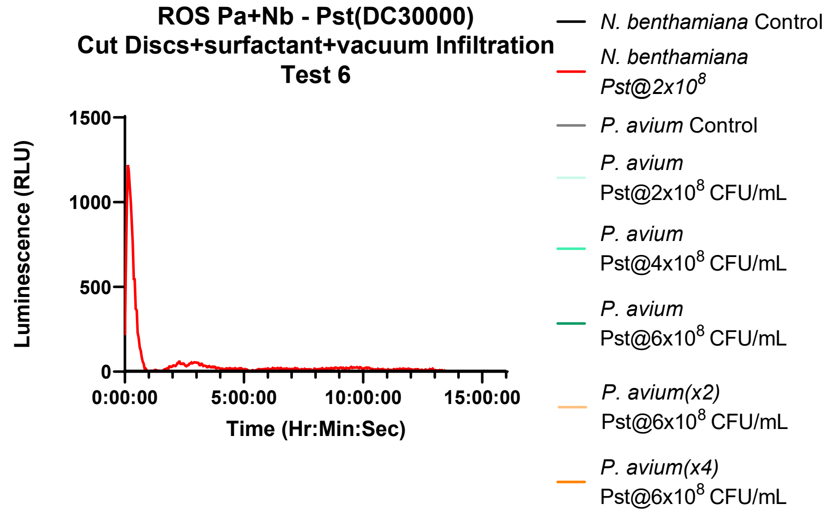


**Figure 5b. ROS-Luminol Assay using *P. avium*.** Third test including *P. avium* (N=5). N. benthamiana included as a positive control and the method was modified to increase testing time to 16hours and discs were cut into 8ths (2, 4 & 8 1/8$^{th}$ leaf discs tested per well). Leaf material was also vacuum infiltrated to saturate leaf material with elicitation solution, and surfactant (Silwet) was added to further facilitate bacterial entry to the leaf.

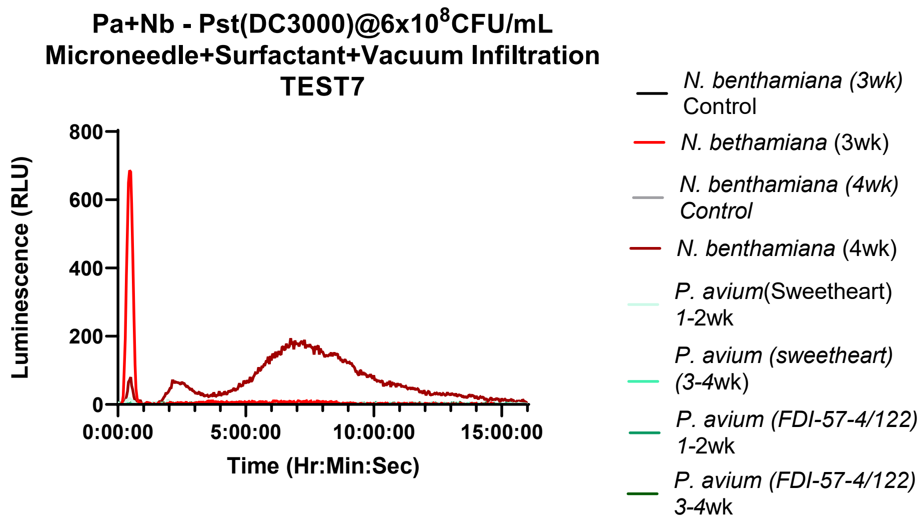


**Figure 5c. ROS-Luminol Assay using *P. avium*.** Third test including *P. avium* (N=5). N. benthamiana included as a positive control. Different aged leaf material was tested to see if leaf age was the issue in previous tests. The method was also modified by increasing testing time to 16hours, and perforating leaf material using a 0.5mm microneedle roller. Perforated leaf material was then vacuum infiltrated, and a surfactant (Silwet) added to elicitation solution.

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#### 4) ROS-DAB Assay

As an alternative to the luminol based ROS assay, DAB staining was performed. DAB staining is a histochemical method for detection of superoxide anions and hydrogen peroxide showing snapshot data of the oxidative burst in response to pathogen colonisation. The data shown is pilot data from the initial validation experiment. Tissues were stained 2 hours post infection. Clear differentiation between strains can be seen. The difference between pathogenic strains and control discs confirmed that a ROS response was being elicited. Increased staining was seen in the Pss-9644 samples (1), which was the most virulent strain tested. R1_5300-plum (2) was the second most stained samples which has been shown to be less virulent on cherry. The epiphyte (3) and control (4) discs appeared comparable with little staining seen.

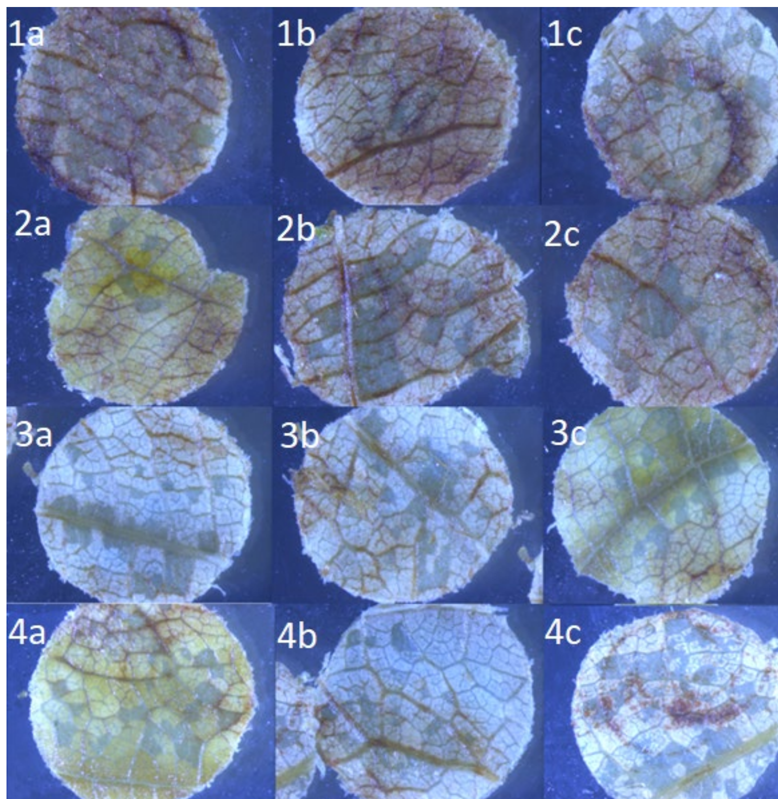


**Figure 6. ROS-DAB Assay 2 hours post infiltration.** P. avium (Sweetheart) leaves syringe infiltrated (2hr incubation), stained with DAB (4hr incubation) and decolorised overnight (N=3). (1) Pss-9644, (2) Psm R1-5300, (3) Epiphyte RH_1534, (4) Control ($MgCl_2$). Increased $H_2O_2$ observed in Pss-9644 and Psm R1-5300 samples. RH_1534 & control appear similar indicated little $H_2O_2$ generation except for damage caused through infiltration.

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### (4) Leaf Infiltration

To quantify hypersensitive response/disease symptoms between cultivars red-light imaging was tested. This experiment only had a single timepoint at 40 hours post infection. Background reads were variable between leaves tested, however clear differences can be seen between controls and bacterial strains. Three *P. avium* cultivars were tested in this pilot run: Groton B, Sweetheart, and Van (N=3). Pss-9644, R1-5244 and R1-5300-plum consistently had higher intensity values compared to the epiphyte and control. Groton B showed greatest susceptibility towards R1-5244 while Van showed greatest susceptibility towards the plum strain R1-5300. This was a pilot experiment; additional repeats are required to achieve confidence in the trends seen. Initial results were promising showing red-light imaging to be a viable method for quantification of disease/immune response in *P. avium* leaf material.

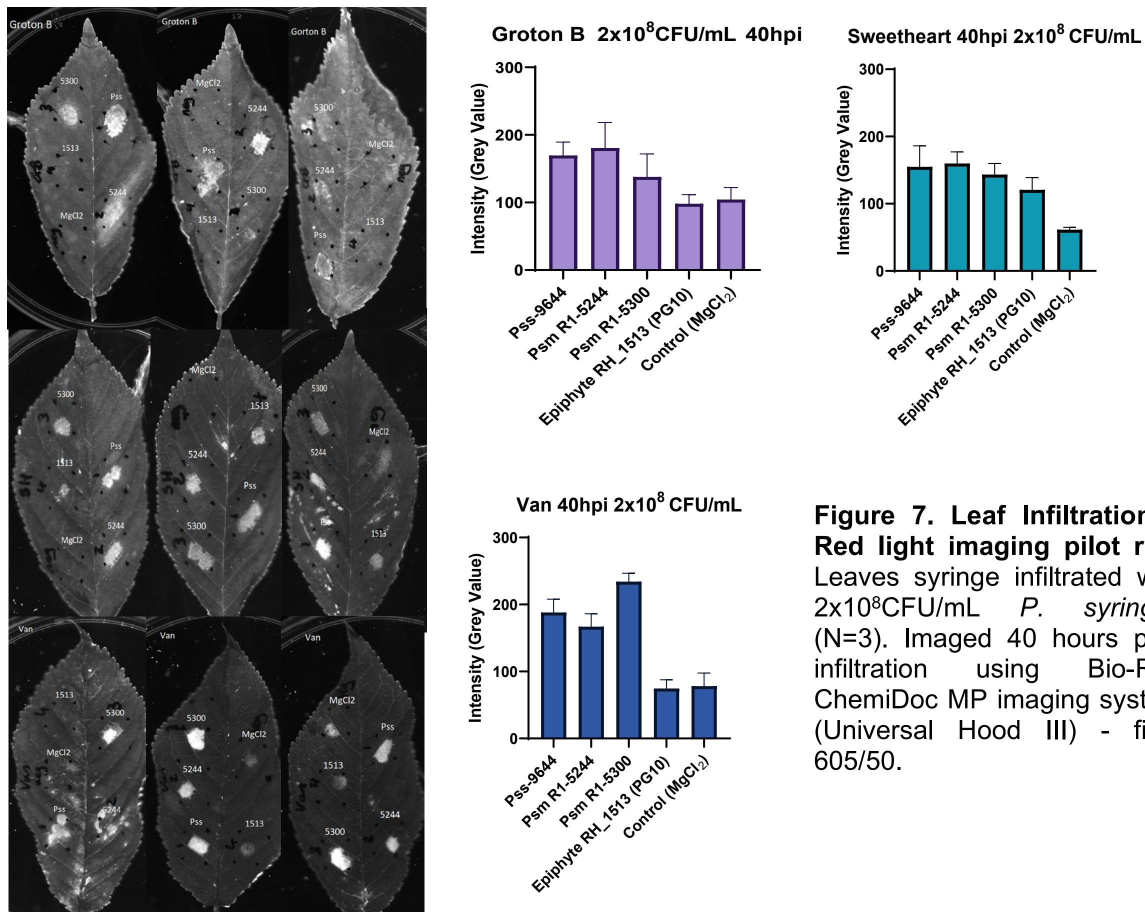


**Figure 7. Leaf Infiltration – Red light imaging pilot run.** Leaves syringe infiltrated with 2x10$^8$CFU/mL *P. syringae* (N=3). Imaged 40 hours post infiltration using Bio-Rad ChemiDoc MP imaging system (Universal Hood III) - filter 605/50.

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To determine disease timings a red-light imaging experiment was set up with numerous timepoints (Figure 8 & 9). *P. avium* (Napoleon) leaves were samples and images were taken every 4 hours. Two inoculation concentrations were tested, $10^6$ (Figure 8) and $10^7$ (Figure 8a). Pss-9644 and Psm R1-5244 of consistently showed increased values compared to the other strains tested. The most virulent strains, 9644 and 5244 averaged grey values of ~30 at $10^6$ and ~38 at $10^7$ 40 hours post infiltration. Variability was seen between repeat infiltration sites but clear differences between strains can be seen.

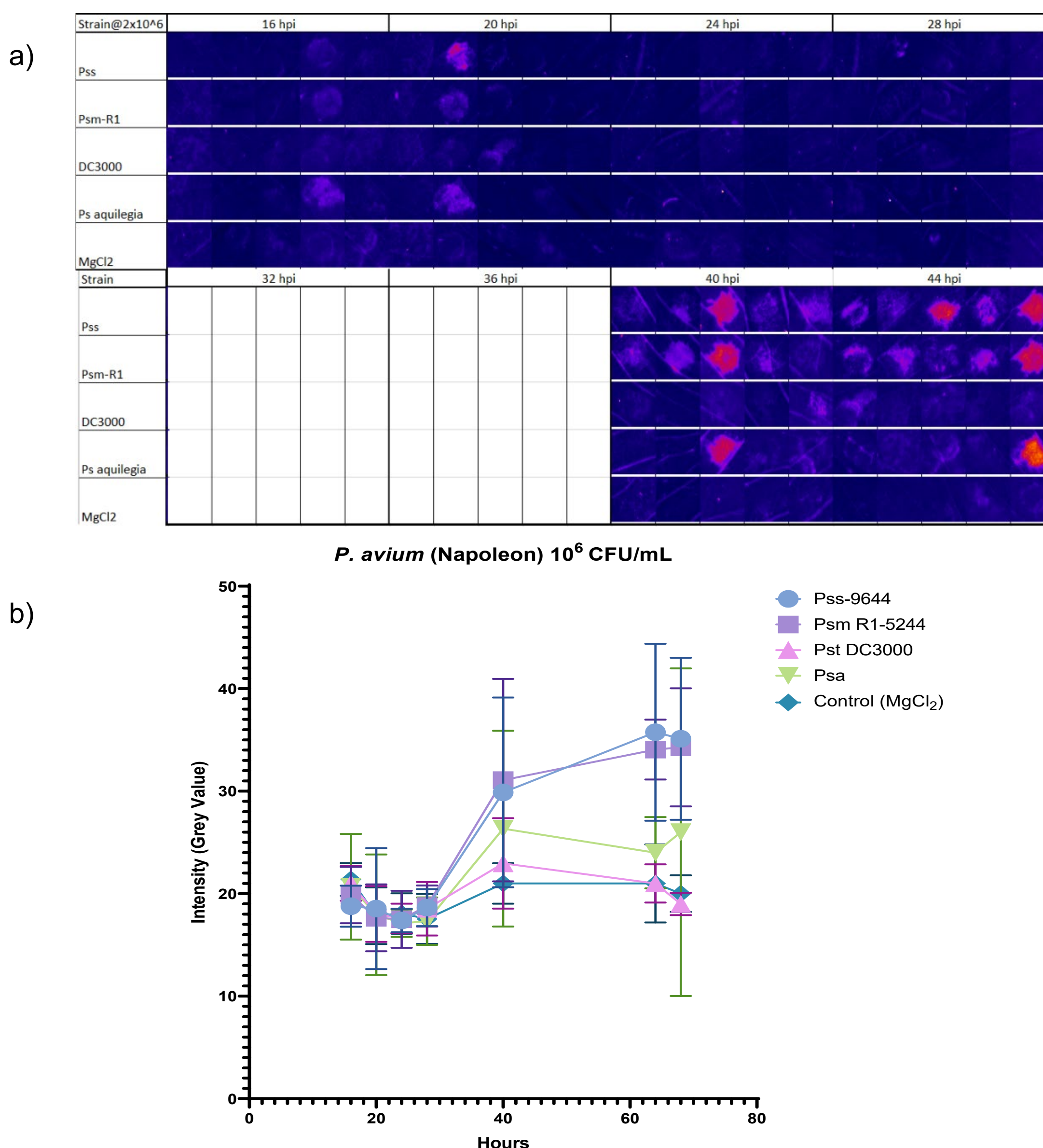


**Figure 8. a) Example of red-light imaged leaf infiltration (16 – 44 hpi) sites b) Grey values of re-light images calculated using ImageJ**. Leaves infiltrated with $10^6$ CFU/mL *P. syringae* (N=5), and images taken every 4 hours Bio-Rad ChemiDoc MP imaging system (Universal Hood III) - filter 605/50.

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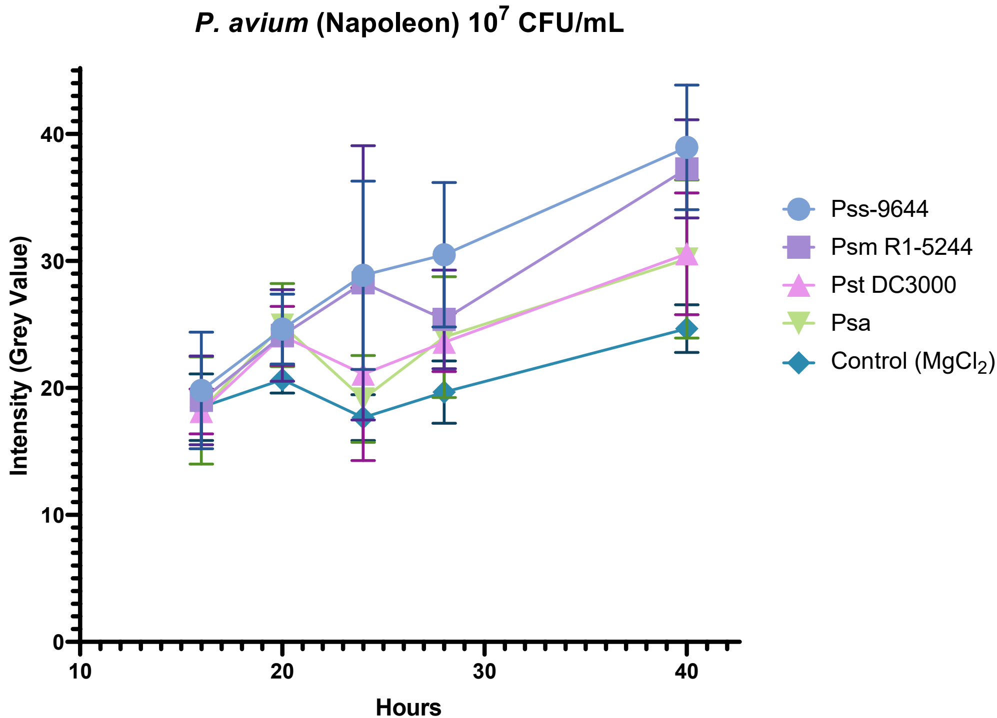


**Figure 9 Leaf infiltration assay.** Leaves were syringe infiltrated with $2x10^7$ CFU/mL *P. syringae* (N=5). Leaves were imaged every 4 hours Bio-Rad ChemiDoc MP imaging system (Universal Hood III) - filter 605/50 to monitor disease progression. Red-light intensity was quantified using ImageJ.

## Discussion

This first year has been used to test and develop phenotyping assays. Much progress and knowledge has been gained but further development and optimisation is required. Red light imaging, cut shoot and DAB assays proved the most promising so far. These will be repeated in much greater depth during the next growing season. ROS and ion conductivity tests require further development for use with *P. avium*.

Leaf infiltration is the traditional standard in plant pathology testing. The extent of disease is normally gauged through symptom scoring. The red-light imaging method was an attempt to quantify leaf response to pathogens for numerical comparison between cultivars and to accurately determine disease timings or immune response. Much effort has been towards increasing reproducibility of this method (results not shown) and the current iteration of the method with enough repeats will yield interesting and useful phenotypic data between cultivars. Further experimentation using many cultivar strain combinations is planned for the next growing season.

Shoot assays are a tried and tested method and has been shown to generate reliable data. Due to the availability of shoot material this experiment could only be performed once, and

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due to the fungal contamination, true insights could not be gained from this first attempt. However, additional sterilisation steps will be taken this winter to reduce fungal growth and a much broader and reliable data set will be generated.

The ROS-DAB assay showed success and will be carried out in greater depth with many strain cultivar combinations once leaf material becomes available next spring. This method does not show temporal data, only showing a snapshot of oxidative response but with rigorous experimental design this method can reveal useful insights into cultivar disease phenotype.

The failure of the ROS-luminol and ion conductivity assays, when using *P. avium* leaves, has shown that additional steps or modifications are required. The next attempts to get these assay working has been through the creation of *P. avium* leaf cell suspensions (Figure 10). It is hoped that assaying leaf suspensions will overcome the physical/structural difficulties of working with *P. avium* leaves. Leaf cell suspensions have the highest surface area to volume ratio possible and will also guarantee much greater standardisation between tests - allowing exact cell densities, growth conditions and health between test samples, while also removing seasonal and weather variation between repeated tests. Specific cellular ROS fluorescence and luminescence assay kits are commercially available, and these will be tested as an alternative to the luminol assay used previously. As an added benefit, the *P. avium* leaf cell suspensions can be maintained over the winter months overcoming the relatively short leafing period of *P. avium* trees.

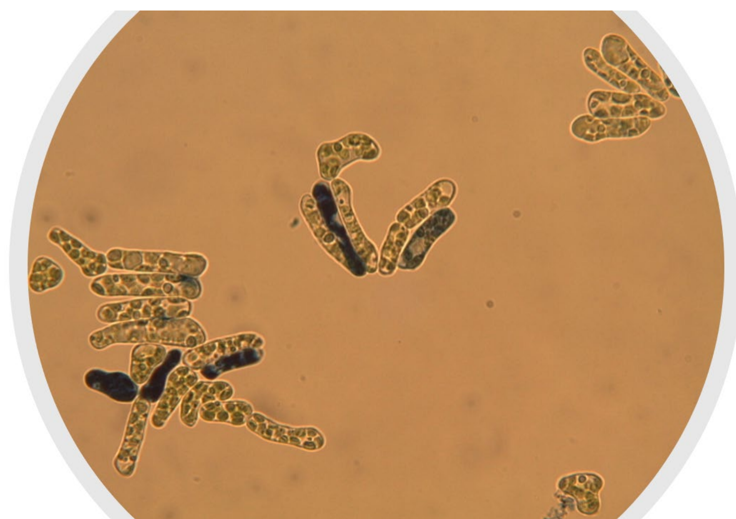

**Figure 10. *P. avium* cell suspension** (x400) 2 weeks post leaf digestion. Cells stained with Evan's blue viability stain and imaged using a light microscope.

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## Conclusions

- The work undertaken up till now has been to develop a suite of assays suitable for phenotyping *P. avium* cultivars using various *P. syringae* strains or mutants.
- This year's work has highlighted the difficulties of plant pathology in tree species, but many insights have been gained.
- Shoot pathology tested will be repeated this winter with additional sterilisation steps.
- Leaf infiltration coupled with red-light imaging has shown promise, these experiments will be carried on in much greater detail next growing season.
- The ROS and ion conductivity assays did not work as planed but will be performed using cell suspension in the future to overcome the difficulties of working with *P. avium* leaf material.
    - The ROS-DAB assay is working well and will be further explored next spring, additional image post processing and analysis steps will be worked on to allow quantification of stained regions for a more accurate comparison between samples.
- A method for creation of cell suspensions will be finalised.
    - Cell suspensions will be used as test samples in ROS assays and allow for additional assays development such as a coculture cell death/bacterial growth assay and other microscopy-based testing.

## Knowledge and Technology Transfer

- CTP Autumn Event 25th November 2020
- AHDB Tree Fruit technical Day presentation 26th February 2021
- CTP Summer Event July 6th – 7th 2021

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