Sensing Volatile Compounds

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Date: 24 Aug 2019

Publication No: BIO-T021

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Executive Summary

Plant diseases are responsible for major economic losses in the agricultural industry worldwide. Monitoring plant health and detecting pathogen infection early are essential to reduce disease spread and facilitate effective management practices. Plants emit many volatile organic compounds (VOCs) into their immediate surroundings that serve essential functions in growth, communication, defence, and survival. Studies show the VOCs released by plants change when the plant is infected with a disease compared to VOCs released under normal plant health conditions due to a change in its physiology. The present project has proposed the application of plant VOC profile monitoring for detecting diseases in plants. This is a new area of research which is showing promise for the rapid and accurate detection of pests and pathogens and infected plants that are not showing symptoms (cryptic infections). This project will assess potential VOC biomarkers associated with specific host-pathogen interactions using GCMS analysis. Additional metabolomic screening to confirm infection will be performed by NMR analyses of leaves and roots from *Agathis australis* plants. These results should highlight key differences between control and infected plants.

Analysis of VOCs produced by cultures of *Phytophthora agathidicida*, *P. kernoviae* and *P. pluvialis* showed that this method can be used to distinguish between these species. Differences in VOC profiles more effectively separated *P. agathidicida* from the other two species than *P. kernoviae* from *P. pluvialis*.

A static enclosure was created for the collection of VOC emissions from control and inoculated *Agathis australis* plants. Unfortunately, post-harvest testing revealed that the infection failed to take hold in the plants. Thus, VOC profiling and NMR analyses did not capture the biomarkers that we expected to find between control and infected plants.

Future work should be undertaken to examine VOCs profiles produced by other *Phytophthora* species before the technique can be widely applied. It was determined that the GC-MS analyses of VOCs from *Agathis australis* plants experiment would benefit from a dynamic enclosure that would allow clean air to be circulated around the plants and the ability to take samples over a longer period.

Introduction

Strengthening biosecurity through the detection of VOCs produced by pathogens and infected plants

Plant diseases are responsible for major economic losses in the agricultural industry worldwide. Monitoring plant health and detecting pathogen infection early are essential to reduce disease spread and facilitate effective management practices [1, 2]. While serological and PCR-based methods are the most available and effective to confirm disease diagnosis, they are not very reliable at the asymptomatic stage of infection. These techniques which need at least 1–2 days for sample collection, processing, and analysis are too expensive to perform throughout the growth cycle [1]. MPI identified E-noses as a technology capable of detecting even very faint odours from risk goods at a remote distance in their Biosecurity 2025 vision [3]. Better Border Biosecurity (B3) recognised innovative biosecurity solutions through the development of an insect odorant receptor array based biosensor for chemical detection and the identification of biogenic volatile organic compounds for improved border biosecurity [4-6]. In 2018, NZ government and industry Working Group Members put together the Biosecurity 2025 Strategic Direction 2 Tool Box for Tomorrow. The workplan cited the need for rapid identification methods to identify high-risk organisms and tools available to detect pests, pathogens and asymptomatic hosts [7]. Analysing the volatile metabolites profile released by plants could be used as a disease monitoring tool for early and rapid detection of plant diseases [2].

Plants emit many volatile organic compounds (VOCs) into their immediate surroundings that serve essential functions in growth, communication, defence, and survival [1, 8-10]. Studies show the VOCs released by plants change when the plant is infected with a disease compared to VOCs released under normal plant health conditions due to a change in its physiology [9, 11, 12]. The present project has proposed the application of plant VOC profile monitoring for detecting diseases in plants. This is a new area of research which is showing promise for the rapid and accurate detection of pests and pathogens and infected plants that are not showing symptoms (cryptic infections). During controlled laboratory experiments, GC-MS is the analytical instrument of choice for VOC profiling [13], because it uses a gas phase separation and detection system to provide comprehensive structural and chemical information on individual components of VOC mixtures.

Work over the past 2 years in Professor Woodward's lab in Aberdeen has demonstrated that VOC analyses can be used to detect fungal pathogens, such as *Ceratocystis platani* (cause of canker stain disease and mortality in *Platanus* spp.) and to distinguish between different species of *Phytophthora* [14]. The work carried out at Scion, along with the phytosanitary authorities in New Zealand, will add to these major breakthroughs by applying the method to detection of *Phytophthora* infections in tissues of both *Agathis* and *Pinus*, focused on *Phytophthora agathicida* and *P. pluvialis*, the main targets of the HTHF research programme. Also, VOCs produced by isolates of *P. kernoviae*, a species likely native to the Pacific basin and problematic in biosecurity elsewhere around the world, will also be examined.

Knowledge gained from the laboratory work will be applied to the development of new small, lightweight, and durable sensors that can be deployed in the field or on drones to detect the target pathogens in real time. These sensors could deliver results rapidly and effectively detect early infections using early induced volatiles as biomarkers. Detecting VOCs within or above the forest canopy has long been studied and a good understanding of plume behaviour and concentration dilution derived from modelling and field experiments is within the literature [15-20]. Air flow through a canopy can be described by sweep and ejection events. The wind rolls through the

canopy and eventually reaches the forest floor (a sweep - you hear the wind in the trees before you feel it). The air that is displaced and must leave the canopy, carries odour, gases, and particles up and out of the canopy (an ejection). This motion happens in all canopies whether it is short grass or tall trees. Wind speed does not have to be high for this momentum transfer to occur. The vertical transport of spores released from the soil and exiting a deep canopy with partial leaf coverage demonstrating particle transport from the forest floor during the sweep/ejection momentum transfer is also described in the literature [21]. Sensors have great potential for sustainable pest management since they analyse host responses and can decipher how plants respond to treatments and their health status [1].

For this project, laboratory experiments will be performed to identify specific VOC biomarkers by GCMS analysis. Additional metabolomic screening will be performed by NMR analyses of leaves and roots from *Agathis australis* plants. These results should highlight key differences between control and infected plants.

Key Milestones

Milestone 1 (December 2018):

- SPME GC-MS analysis of the headspace above microbial pathogens were grown in a liquid media (*Phytophthora* spp.).
- Report on results.

Milestone 2 (March 2019):

- Build “enclosures” to sample the VOCs emitted from branches of healthy and infected plants.
- The GC-MS analysis of enclosure samples.
- Liquid state metabolomic screening (i.e. NMR) of *Agathis australis* tissues.
- Report on results.

Methods

Pathogens

Cultures of *Phytophthora agathidicida*, *P. pluvialis* and *P. kernoviae* were obtained from SCION Pathology laboratory stocks. Three isolates of each species were sub-cultured to Elliott's medium [22] containing (g/L): sucrose 10, L-asparagine 1, KH_2PO_4 0.5, $MgSO_4$. $7H_2O$ 0.25, thiamine 0.001. Stock solution of trace elements (1 mL) was added; the stock solution contained, in 1 litre of water: $Na_2B_40_7.10H_20$, 88 mg, $CuSO_4.5H_20$, 393 mg, $Fe_2(SO_4)_3.6H_2O$, 910 mg, $MnCl_2.4H_20$, 72 mg; $Na_2MoO_4.2H_2O$, 50 mg, $ZnSO_4.7H_2O$, 4403 mg and EDTA, 5 g. Media were dispensed in appropriate volumes (see below) and autoclaved at 104 kPa for 20 minutes.

The isolates of *P. agathidicida*, *P. pluvialis* and *P. kernoviae* were subcultured to:

(1) Petri dishes containing 15-18 mL Elliott's medium gelled with 15 g/L agar.

(2) 7 mL Elliott's medium in 20 mL headspace vials.

(3) 75 mL Elliott's medium in 250 mL flat culture bottles

All cultures were incubated at 17.5 °C for 21-35 days.

Plant Materials

Two-year-old plants of *Agathis australis* family MW8L were raised in Dalton's pine-bark potting soil in standardised compost plastic pots (7 x 7 x 18 cm) in the *Agathis* glasshouse facility at SCION. Twenty plants were transferred to the laboratory for inoculation and treatment. Pots containing compost prepared at the same time as the pots with plants were also transferred to the laboratory for treatment.

Ten plants were inoculated with four 6 mm diameter plugs of *P. agathidicida* isolate 3118 mycelia grown for six weeks on Elliot's agar, one plug in each corner of the pot. Live mycelium was similarly placed in five pots containing compost but without *A. australis* plants. Five control plants and ten pots of compost without plants were each treated with four discs of Elliot's agar, as described above.

Following treatment, all plants and pots were flooded to within 10 mm of the surface of the compost and associated moss. All pots were covered with heavy-duty plastic bags 48 hours after treatment and the bags secured around the pot using rubber bands.

Five days after treatment, five inoculated plants were destructively harvested for NMR analyses of the root (all roots <2 mm diam.) and foliage tissues. All tissues were immediately frozen in liquid nitrogen before storing at -80°C until required.

Immediately before harvesting the remaining plants ten days after treatment, the percentage cover of moss and liverwort on the compost surface in each pot was estimated visually. Subsequently, the plastic bag enclosing the plants and pot was punctured with a needle 20 mm above the compost and approximately 30 mL of air withdrawn and transferred by syringe to a Tedlar sample bag. Plants were subsequently treated as described for harvest at day 5, and roots and foliage tissues frozen before analyses.

SPME GC-MS analyses of culture headspace volatiles

Volatile compounds were collected from the headspace of the 7 mL liquid cultures using a solid phase microextraction (SPME) technique. A three-phase Carboxen/PDMS 75 μm SPME fibre (Supelco Analytical) was exposed to the head space of the sample vials at room temperature for 45 min to accomplish VOC extraction. A Gerstel MPS autosampler (Gerstel GmbH, Germany) was used for automation of the procedure and for ensuring consistent SPME extraction conditions. Samples and controls were analysed on an Agilent model 7890B/7000A GC/MS triple quad instrument by automatic injection of the fibre which was held for 60s at 250°C inlet temperature. The helium carrier gas flow was held constant at 1.2 mL/min. The GC was fitted with a 30 m × 0.250 mm i.d. fused silica capillary column, with a 1.4 µm coating (DB-624, Agilent J&W). The GC oven was programmed from an initial temperature of 45 °C (held for 1 min) followed by a 12 °C per min increase to 150 °C. The oven temperature was then increased by 20°C per min to a final temperature of 250 °C, and held for 10 min. Full scan mass spectra were acquired from 35 to 450 *m*/*z*. The total time for each injection run was 30 min. Electron impact ionization at 70 eV was employed, with an interface temperature of 250 °C and a source temperature of 250 °C. Before analysis, the mass spectrometer was tuned according to the manufacturer's specifications. Full scan data were acquired and processed using Agilent MassHunter Workstation software (2016 version B.08.00).

Identification of the analytes was accomplished by NIST library comparison. All identifications are considered tentative unless confirmed with a reference standard.

NMR analyses of leaves and roots from *Agathis australis* plants

15-month-old seedlings underwent an inoculation experiment as set up by Steve Woodward. Root and leaf samples for chemical analysis were taken from five inoculated seedlings and collected at day 1 and six time points of the trial. The kauri leaves and roots were wrapped in tin foil and snapped frozen with liquid nitrogen and then were stored at -80 °C until required for analysis

The leaves and the roots were randomly subsampled and placed into a 5 mL cryogrinding vial and then ground under cryogenic conditions using a GenoGrinder operating at 1500rpm for 2 minutes.

The *Phytophthora agathidicida* mycelium were randomly subsampled and placed into a 5 mL cryogrinding vial and then ground under cryogenic conditions using a GenoGrinder operating at 1500rpm for 2 minutes.

The samples were then weighed to 0.100 g (+/- 15 mg). The frozen samples were warmed to room temperature, then a 1 mL aliquot of a pH 7.00 phosphate buffer (0.10 mol/L) with D_2O (10% v/v), an internal standard TSP (sodium salt of 3-(trimethylsilyl)-propionate acid-d4, 0.05 % w/v), and sodium azide (0.1 % w/v) to suppress microorganism activity was added. The samples were agitated in the GenoGrinder for 10 mins at 500 rpm, filtered through a 0.45 µm nylon membrane syringe filter and a 600 µL aliquot was then transferred to a 5 mm borosilicate NMR tubes for analysis.

One dimensional ^{1}H NMR spectra of the aqueous leaf samples were acquired on a Bruker Avance III 400 NMR fitted with a 5 mm Prodigy BBO cryoprobe (Bruker, Switzerland) operating at a ^{1}H frequency of 400.13 MHz. A standard Bruker "noesygppr1d" pulse sequence with water suppression achieved by the application of a 25 Hz presaturation field at a transmitter frequency offset (o1) of 1881.10 Hz was used. The internal probe temperature was set to 300K with a five-minute temperature stability delay. The spectral data were obtained in 65K data points, a relaxation

delay of 8 s and 64 scans. The spectra were then Fourier transformed, phased and baseline corrected, and all spectra were calibrated relative to TSP resonance at 0.00 ppm.

GC-MS analyses of VOCs from *Agathis australis* plants

Approximately 30 mL of air was transferred by syringe from inside the “enclosure” to a Tedlar sample bag. Tedlar bags were kept at 4 C until analysis. The analytical system consists of a Multi-Purpose Sampler (Gerstel GmbH, Germany), operated in large volume injection-mode and equipped with a 2.5 µL syringe and a temperature controlled cooled injection system (CIS) (Gerstel GmbH) used as interface, cold trap and injection system for the subsequent GC-MS analysis. Samples and controls were analysed on an Agilent model 7890B/7000A GC/MS triple quad instrument by automatic injection of a 10mL sample from each bag. The Multi-Purpose Sampler fills the syringe with 2.5 mL of the sample. With the split vent open and the CIS cooled down to -10 °C, the sample is injected into the glass insert filled with Tenax adsorbent. The split flow through the liner at these temperatures preferentially removes the solvent as a vapour while leaving the analytes of interest in the liner. This is repeated four times for a total sample volume of 10mL. After the solvent has been vented, the split is closed, and the CIS is ramped to at 12 °C per sec to 230 °C for splitless transfer of the analytes to the capillary column. The helium carrier gas flow was held constant at 1.2 mL/min. The GC was fitted with a 30 m × 0.250 mm i.d. fused silica capillary column, with a 1.4 µm coating (DB-624, Agilent J&W). The GC oven was programmed from an initial temperature of 35 °C (held for 2 min) followed by a 15 °C per min increase to 60 °C. The oven temperature was then increased by 20 °C per min to a final temperature of 220 °C, and held for 10 min. Full scan mass spectra were acquired from 35 to 450 *m*/*z*. The total time for each injection run was 30 min. Electron impact ionization at 70 eV was employed, with an interface temperature of 250 °C and a source temperature of 250 °C. Before analysis, the mass spectrometer was tuned according to the manufacturer’s specifications. Full scan data were acquired and processed using Agilent MassHunter Workstation software (2016 version B.08.00).

Analytes were identified as described above. VOC profiles from each plant were determined and further examined by principal component analysis, as described below.

Data Analyses

The mass percent of each component found by GC-MS was determined by the individual peak areas and the total area of all peaks found in the sample. The VOC profile of each microorganism was determined and further examined by principal component analysis to determine significant differences between groups. Phytophthora VOC data were normalised in a total ion chromatogram and evaluated by principal component analysis (PCA) with a Pareto scaling method using SIMCA 15.0.2 software (Sartorius Stedim Data Analytics AB, Sweden). Hierarchical cluster analysis (HCA) was also performed to test further if VOCs discriminated between the three *Phytophthora* species examined.

The resulting NMR spectra were used for untargeted secondary metabolite fingerprinting by bucketing the spectra from 0.00 to 10.00 ppm, with a bucket size of 0.04 ppm using AMIX software (Bruker, Germany). Bucketed data was evaluated using principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) with pareto scaling method using SIMCA 15 software (Sartorius Stedim Data Analytics AB, Sweden).

Results

SPME GC-MS analyses of culture headspace volatiles

As microbes grow and proliferate, they release a variety of volatile compounds that can be profiled and used for speciation. These VOCs were captured from the headspace above three strains of each Phytophthora species and analysed directly by GCMS. Figure 1 demonstrates peak differences found between *P. agathidicida*, *P. pluvialis* and *P. kernoviae*. Principal component analysis (PCA) was performed for a visual comparison of the three species and to determine discriminating peaks or biomarkers specific to each species. After assessing the media blank results, hexane, decane, and unknown four were excluded from further analysis. The media blank data were also excluded.

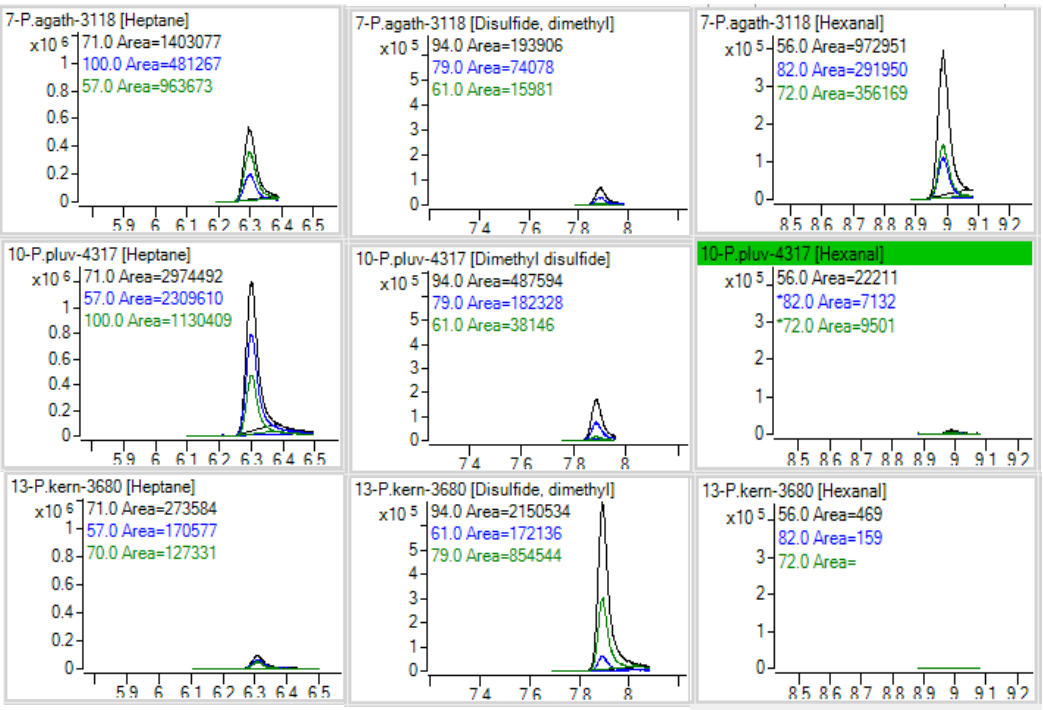

Figure 1: Peak comparison of extracted ion chromatograms for heptane, dimethyl sulphide and hexanal shown for *P. agathidicida*, *P. pluvialis* and *P. kernoviae.*

The PCA scatter plot is shown in Figure 2 (R2X(cum) = 0.867, Q2(cum) = 0.439) with PC 1 explaining 35.0% of the variance and PC 2 contributing 19.6%. The loadings plot (Figure 3) demonstrates that the VOC components separated along the PC 1 axis, based on changes in quantities, from heptane, 2-dodecane, pentane, dimethyl disulphide, hexane, N,N-diethyl- 1,4-benzenediamine and 2-methyl-3-thiolanone. The stronger drivers for the PC 2 component included heptane, dimethyl disulphide, acetoin and 2-dodecanone.

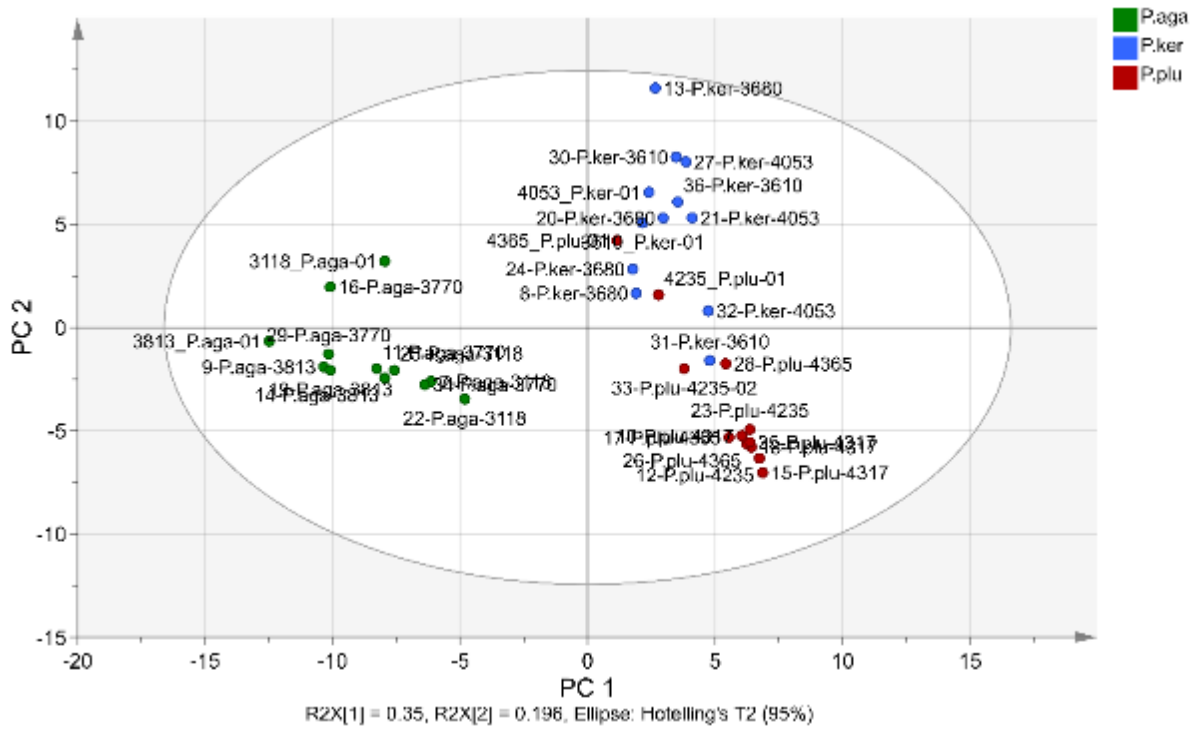

Figure 2: Score plot of PCA (PC 1 vs. PC 2) results obtained from VOC data from the *Phytophthora* isolates used. The Hotelling T2 ellipse is shown with 95% confidence interval.

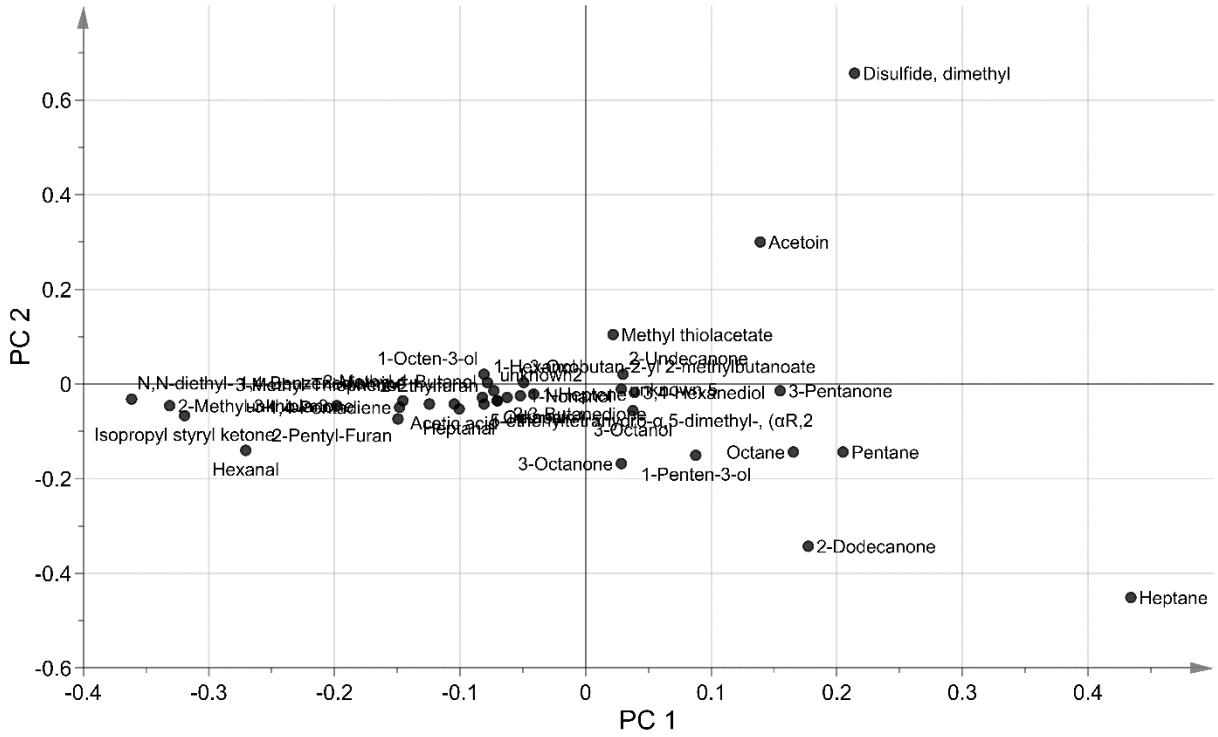

Figure 3: Loadings plot of PCA (PC 1 vs. PC 2) results obtained from VOC data from the *Phytophthora* isolates used.

Hierarchical cluster analysis (HCA) was also performed, and those results are shown in Figure 4. VOCs from *P. agathidicida*, *P. kernoviae* and *P. pluvialis* were separated based on HCA.

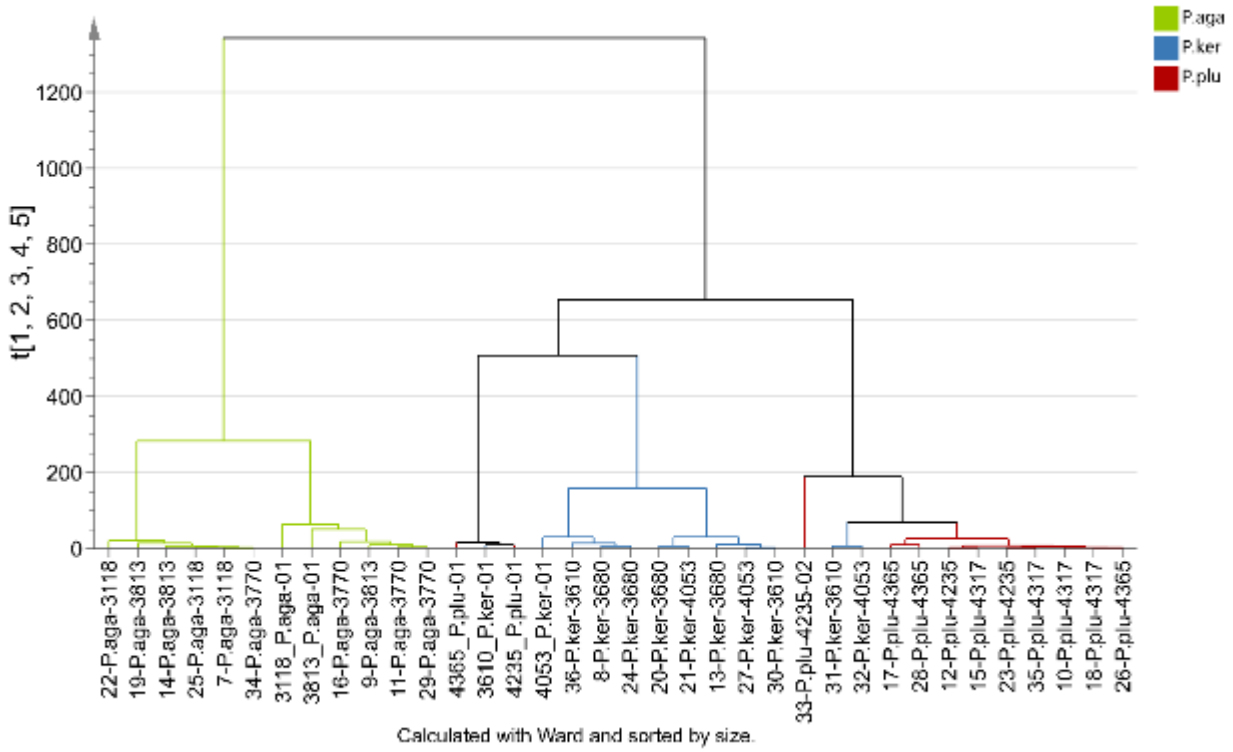

Figure 4: HCA analysis obtained from *Phytophthora* VOC data results.

NMR analyses of leaves and roots from *Agathis australis* plants

Neither leaves, or roots extracts showed any differences between Day 1 and Day 6 from the time of initial infection (Figure 5 & 6).

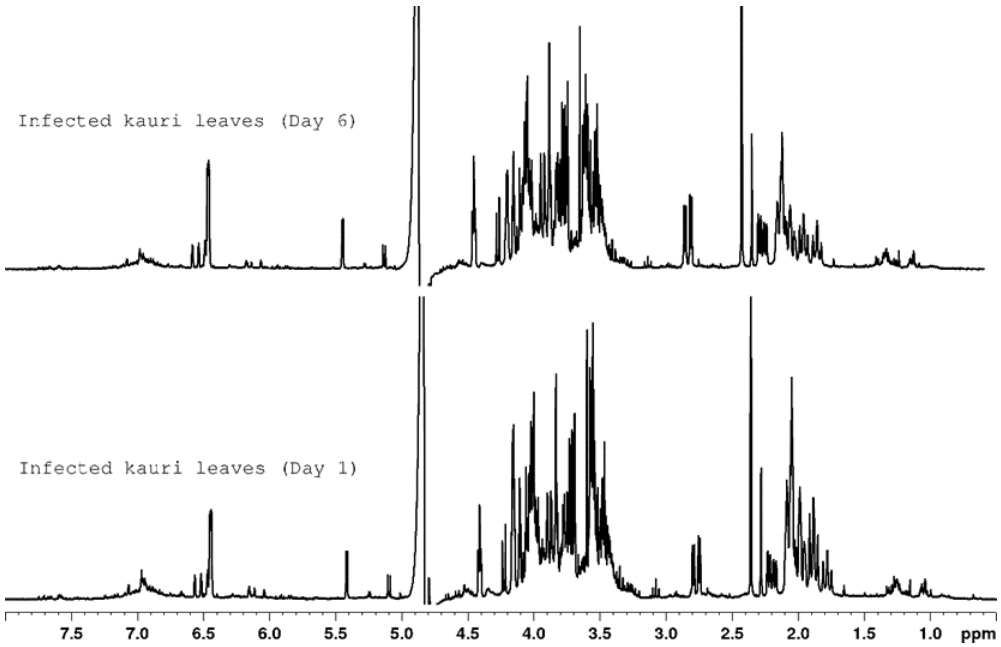

Figure 5: Day 1 & 6 ^{1}H NMR spectra of kauri leaves.

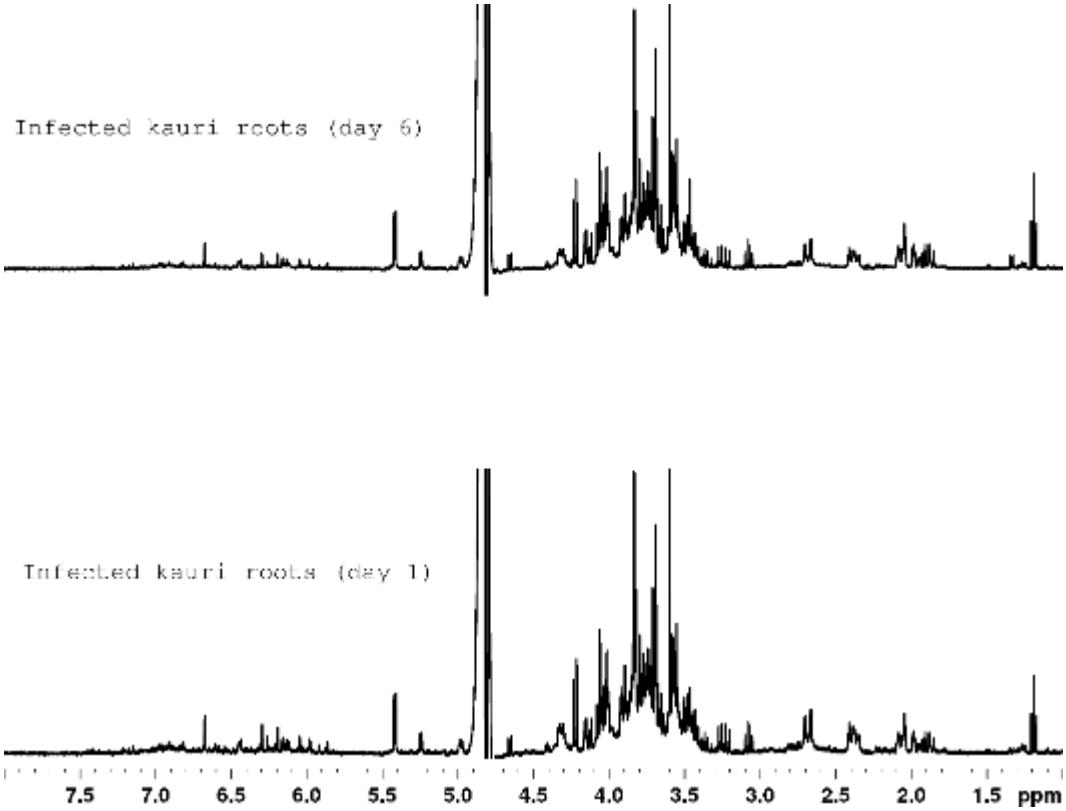

Figure 6: Day 1 & 6 ^{1}H NMR spectra of kauri roots.

GC-MS analyses of VOCs from *Agathis australis* plants

There are no standardized procedures or commercial enclosure systems available for quantitative measurements of biogenic VOC emissions. Thus, researchers must construct their own apparatus and follow methods unique to their laboratories. Vegetation enclosures can be static (no purge flow) or dynamic (flow-through). A static enclosure is the easiest (and cheapest) option. However, there are several drawbacks. Because air is not circulated, the CO_2 concentration is often not constant (due to photosynthetic uptake). Air temperatures can increase dramatically due to greenhouse heating. Elevated temperatures and non-realistic CO_2 concentrations create artificial conditions, which are not appropriate for measuring naturally occurring emission rates. Static enclosures cannot remain in place over long time periods. Hence, measurements made using these enclosures collect a very limited snapshot of the emissions. Overall, static techniques cannot yield realistic estimates of long-term emission rates or diurnal variability. However, headspace sampling of static enclosures can be valuable for chemical identification of biogenic VOC emissions and for developing and demonstrating analytical techniques [23].

A static enclosure was created for the collection of VOC emissions from control and inoculated *Agathis australis* plants. All pots of the two-year-old plants (inoculated and control) were covered with heavy-duty plastic bags 48 hours after treatment and the bags secured around the pot using rubber bands, as shown in Figure 7.

Figure 7: Static enclosures created for collection of VOC emissions from control and inoculated *Agathis australis* plants.

Analysis of the VOCs collected from the control, and inoculated plants revealed a few things. First, the samples should have been analysed immediately after their collection. Due to unfortunate circumstances, a broken part inside the instrument delayed sample analysis for 15 weeks. This delay could have resulted in the loss of some VOC compounds in the Tedlar bags. Several VOCs identified in the enclosure samples were determined to be from the bags used to cover the plants. These background compounds may have obscured biomarker VOCs from the analysis. It is also possible that these compounds could have negatively affected the plants and their normal respiration.

It's quite likely that a clear difference between the control plants and the inoculated plants was not realized because the plants were not affected by the pathogen. Adequate time was not given to prove that the inoculated pots resulted in infection of the *Agathis australis* plants before sampling. Following harvest, the root systems of each plant were surface sterilized (30 sec 70% ethanol then rinse in SDW), then 20 pieces of root segment were taken at random from across the root system. The root segments were plated on CRNH media, and the potting media was also baited with lupin radicals to confirm the viability of inoculum. After incubation, *P. agathidicida* was successfully recovered by baiting from the potting media. However, *P. agathidicida* did not grow from any of the root fragments plated onto CRNH, indicating that infection was not successful.

For the reasons stated above, it was determined that this experiment would benefit from a dynamic enclosure that would allow clean air to be circulated around the plants and the ability to take samples over a longer period.

Conclusions

- Analysis of VOCs produced by cultures of *Phytophthora agathidicida*, *P. kernoviae* and *P. pluvialis* can be used to distinguish between these species. Differences in VOCs profiles more effectively separated *P. agathidicida* from the other two species than *P. kernoviae* from *P. pluvialis*.
- Direct analysis of VOC from infected kauri was not possible from this experiment. Given the NMR and re-isolation tests, the likely reason was that the infection had not taken. Additionally, the method of collecting the VOC samples (syringe + Tedlar bags) proved difficult and a source of contamination in the GC-MS trace.

Future Work

- Future work should be undertaken to examine VOCs profiles produced by other *Phytophthora* species before the technique can be widely applied.
- It was determined that the GC-MS analyses of VOCs from *Agathis australis* plants experiment would benefit from a dynamic enclosure that would allow clean air to be circulated around the plants and the ability to take samples over a longer period.
- Healthy and infected *Pinus* species need to be tested.

Acknowledgements

The authors thank Colin Faluds, Catherine Banham, Gordon Teiman and Rita Tetenburg for their contribution to the growth of the kauri, inoculation, isolate preparation and culturing.

We thank Taoho Patuawa (Te Roroa) for obtaining permission to work with the kauri plants used in this project. The plants used in this experiment were grown from seed collected from and with whakapapa to kauri within Waipoua Forest, Northland in partnership with Te Roroa.

Thanks also to Rebecca McDougal for helpful discussions and support throughout the project.

References

1. Martinelli, F., et al., *Advanced methods of plant disease detection. A review.* Agronomy for Sustainable Development, 2015. **35**(1): p. 1-25.
2. Ray, M., et al., *Fungal disease detection in plants: Traditional assays, novel diagnostic techniques and biosensors.* Biosensors and Bioelectronics, 2017. **87**(Supplement C): p. 708-723.
3. MPI, *Biosecurity 2025 Protecting to Grow New Zealand*. 2016, Ministry for Primary Industries: Wellington. p. 1-58.
4. Cheema, J., *Olfactory receptor based Biosensor*. ongoing, University of Auckland.
5. Nixon, L., *Identification of biogenic volatile organic compounds for improved border biosecurity*. 2017, Lincoln University.
6. Teulon, D.A., *B3 Annual Report 2017/18*. 2018. p. 1-40.
7. Barnao, C., et al., *Biosecurity 2025 | Strategic Direction 2 Work Plan*. 2018. p. 1-16.
8. Baldwin, I.T., et al., *Volatile signaling in plant-plant interactions:" talking trees" in the genomics era.* science, 2006. **311**(5762): p. 812-815.
9. Scala, A., et al., *Green leaf volatiles: a plant's multifunctional weapon against herbivores and pathogens.* International journal of molecular sciences, 2013. **14**(9): p. 17781-17811.
10. Tholl, D., et al., *Practical approaches to plant volatile analysis.* The plant journal, 2006. **45**(4): p. 540-560.
11. Jansen, R., et al., *Detection of diseased plants by analysis of volatile organic compound emission.* Annual Review of Phytopathology, 2011. **49**: p. 157-174.
12. Sankaran, S., et al., *A review of advanced techniques for detecting plant diseases.* Computers and Electronics in Agriculture, 2010. **72**(1): p. 1-13.
13. Lytovchenko, A., et al., *Application of GC-MS for the detection of lipophilic compounds in diverse plant tissues.* Plant methods, 2009. **5**(1): p. 4.
14. Woodward, S. and R. Ebel, *VOC analyses for the detection of fungal pathogens; Unpublished*.
15. Larkin, N.K., et al., *Identification of necessary conditions for arctic transport of smoke from United States fires.* 2012.
16. Leclerc, M.Y., N. Meskhidze, and D. Finn, *Comparison between measured tracer fluxes and footprint model predictions over a homogeneous canopy of intermediate roughness.* Agricultural and Forest Meteorology, 2003. **117**(3-4): p. 145-158.
17. Murlis, J., M.A. Willis, and R.T. Cardé, *Spatial and temporal structures of pheromone plumes in fields and forests.* Physiological entomology, 2000. **25**(3): p. 211-222.
18. Strand, T., et al., *A simple model for simulation of insect pheromone dispersion within forest canopies.* Ecological Modelling, 2009. **220**(5): p. 640-656.
19. Thistle, H., et al., *Atmospheric dispersion from a point source in four southern pine thinning scenarios: basic relationships and case studies.* Transactions of the ASABE, 2011. **54**(4): p. 1219-1236.
20. Thistle, H.W., et al., *Surrogate pheromone plumes in three forest trunk spaces: composite statistics and case studies.* Forest Science, 2004. **50**(5): p. 610-625.
21. Finnigan, J., *Turbulence in plant canopies.* Annual review of fluid mechanics, 2000. **32**(1): p. 519-571.
22. Elliott, C.G., M.R. Hendrie, and B.A. Knights, *The Sterol Requirement of Phytophthora Cactorum.* Microbiology, 1966. **42**(3): p. 425-435.
23. Ortega, J. and D. Helmig, *Approaches for quantifying reactive and low-volatility biogenic organic compound emissions by vegetation enclosure techniques–Part A.* Chemosphere, 2008. **72**(3): p. 343-364.