Department for Environment Food & Rural Affairs

Phytophthora ramorum and Discrimination of non-target pathogens using detection dogs.

Final Report to Future Proofing Plant Health

Dr. Heather Dun- Forest Research

Luke Jones- Canine Assisted Pest Eradication (CAPE SPC)

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FUTURE PROOFING Plant Health

A Defra Network partnership delivering interdisciplinary plant health research to improve biosecurity and build capability

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Project Overview

This report details the second phase of an innovative project aimed at training a detection dog to specifically identify *Phytophthora ramorum* while discriminating it from other Phytophthora species and non-target organisms such as Pythium. This phase extended over a period of six months, following a successful proof-of-concept study. During the testing phase of the first study the trained dog demonstrated proficiency with a 100% successful first-time detection rate across ten tests, detecting *P. ramorum* sporangia in varied mediums such as water, soil, and plant material without being distracted by associated non-target scents (Green et al. 2023). This second phase builds on the success of the first phase to further train the same detection dog to distinguish *P. ramorum* from other non-target Phytophthora species. Both projects were a collaboration between Luke Jones of CAPE SPC and Forest Research pathologists Sarah Green and Heather Dun.

1. Objective

The primary objective of this phase was to refine the dog's detection abilities, emphasising the accurate discrimination of *P. ramorum* amidst a mixture of closely related pathogens and control scents. This phase aimed to confirm the dog's training under more challenging conditions and varied substrates to ensure reliability and effectiveness in real-world scenarios.

2. Methodology

2.1. Scent selection

The project's first stage involved gathering both target and non-target scents from Forest Research Roslin. These scents were preserved in Target Analyte Detection Devices (TADDs). The collection included:

- Target Scent: *P. ramorum* (isolate 16.13a, EU2 lineage collected from Glentrool and proven to be pathogenic in previous inoculation trials)
- Phytophthora Controls:
    - *P. cinnamomi* (isolate L8)
    - *P. pseudo-syringae* (isolate RG25)
    - *P. gonapodyides* (isolate IG41)
- Non-Phytophthora Control: Pythium (isolate SSS-L-10).
- Non-Target Controls: Carrot agar, filtered pond water, sterile distilled water, and blank TADDs

These scents were crucial for training the dog to recognise and discriminate the specific scent of *P. ramorum* from other similar pathogens and irrelevant scents. The alternative Phytophthora species were selected as they were ubiquitous species likely to be found in

the same environments as *P. ramorum* and thus discrimination between them would be important to prevent false positive detection of *P. ramorum*. Pythium was also included as it has previously been used in the proof-of-concept study (Green et al. 2023).

2.2. Scent preparation

Phytophthora ramorum scent

Phytophthora ramorum was grown on carrot agar plates at room temperature (15-20^{O}C) with supplemental lighting for 18 hours a day. The plates produced large numbers of sporangia after three weeks of growth. Sporangial suspension was produced by adding 2ml of filtered pond water onto the plates and then dislodging the caducous sporangia by gently scraping the plates with a sterile cell spreader. The resulting suspension from 10 plates was pooled and the concentration of sporangia present was calculated using a haemocytometer. For the first training runs a concentration of $3.2x10^5$ sporangia per ml was used, subsequently this was incrementally reduced to $6x10^4$ sporangia per ml. The sporangial suspension was then poured into a Target Analyte Detection Device (TADDs), a glass container with a special filter membrane to allow scent detection without contamination or spillage of material.

Alternative *Phytophthora* scent

Unlike *P. ramorum* none of control species produce caduceus sporangia (sporangia that easily break off the hyphae), so the preparation of the scent material was slightly different. All isolates were grown in the same conditions as the *P. ramorum*. To promote sporulation 10 $1cm^2$ cubes were cut from the edges of 3-week-old colonies, these cubes were then placed in petri dishes and flooded with filtered pond water, three plates of flooded cubes were made for each species. The pond water had been collected from a local pond and filtered with 1.2 um nitrocellulose filter membranes. The flooded plates were then incubated at room temperature with supplemental lighting for 18 hours a day for three days before inoculum was prepared. Examination under a microscope confirmed that sporangia had developed on the hyphae growing off the edges of the cubes in all species. These extended hyphae with sporangia were then cut off the cubes using a sterile scalpel and the agar cubes were removed from the plates. The water, detached hyphae and sporangia were then poured into the labelled TADDs.

Potted plant flow-through scent

For additional training a new scent derived from flow-through pond water from both inoculated soil and control (blank) soil. Rhododendron plants (cultivar “Wren”) in 1.5 l pots were purchased from a commercial nursery and first tested for the presence of *P. ramorum* in the soil. Each plant was flooded with 500ml of SDW three times with 20 minutes between each addition of water. The flow through was collected and filtered through 1.2 um nitrocellulose filter membranes. The membranes were then placed in 15ml of longmires buffer and shaken for 15 minutes. The Quigen blood and tissue extraction kit was then used to extract DNA from a 1.5 ml aliquot of buffer. The DNA was then tested

using a *P. ramorum* specific qPCR assay (Tomlinson et al., 2005). The Rhododendrons had no detectable P*. ramorum* DNA. One plant was then inoculated with 50ml of $5x10^5$ of *P. ramorum* sporangia per ml in filtered pond water on three points around the root ball (a control plant used SDW). Three days later each plant was flooded with 500ml of filtered pond water twice with 20 minutes between each addition of water. 100ml of flow through from each plant was then put in a TADD as the training scent. The rest of the flow though was then tested for *P. ramorum* DNA as detailed above. The control plant was negative and the inoculated plant was positive for *P. ramorum* DNA.

Figure 1: A Target Analyte Detection Devices (TADDs) used to safely store the scent material, there is a filter membrane in the black section to allow scent detection.

2.3. Training Regime

Initial Scent Recognition

The initial training phase focused on reinforcing the dog's ability to recognize *P. ramorum* in pond water, a new substrate introduced in this phase to simulate natural conditions, contrasting with the sterile distilled water used in the previous phase. This process was designed to both imprint the target scent in a new substrate and assess the dog's recognition of *P. ramorum* from phase 1. Over two training days and using non-target controls, the dog demonstrated it had retained the learning from phase 1 by accurately identifying *P. ramorum* in the new substrate.

Discrimination Training Phase

The discrimination training phase focuses on teaching the dog to reliably differentiate between the target scent (*P. ramorum*) and control scents introduced into the environment. This phase spanned six months and involves systematic exposure, positive reinforcement, and careful management to ensure balanced learning and progress.

1. Introduction of Control Scents into the Environment

Objective: To introduce control scents into the environment and monitor the dog's behaviour to ensure it can differentiate between the target scent and the non-target scents.

Gradual Introduction into the Environment: Control scents are introduced into the training environment. These scents are part of the training environment in various setups, and the dog's behaviour is monitored to see if it can differentiate between target and non-target scents. No cues are provided to the dog during this phase, it is allowed to explore the training environment freely.

Monitoring and Adjustment: Trainers observe the dog's responses, noting whether the dog ignores control scents and alerts only to *P. ramorum*. This phase helps assess the dog's ability to discriminate with direct exposure to the control scents.

2. Reinforcement Process

Objective: To reinforce correct behaviour when the dog identifies the target scent and ignores non-target scents introduced into the environment.

Positive Reinforcement: When the dog correctly identifies and alerts to the target scent (*P. ramorum*), it is rewarded with a toy, treats, verbal praise, or play. Early reinforcement is critical for building the dog's confidence and ensuring clear associations with the target scent.

Blank Scenarios (from the beginning): Blank scenarios, where no target scent is present, are used from the beginning. The dog is reinforced for not alerting in these scenarios, reinforcing that it should only alert when *P. ramorum* is present.

Variable Reinforcement: As the dog becomes more consistent in identifying the target scent, the frequency of rewards is adjusted to a variable schedule. This encourages the dog to stay focused without becoming dependent on constant rewards.

3. Managing Training Balance

Objective: To maintain a balance between training challenges, reinforcement, and managing the dog's progress to avoid frustration or stagnation.

Scent Refreshment: Every **4-6 weeks**, the TADDs containing both control and target scents are refreshed with new samples. This serves two purposes:

> **Concentration Reduction:** Over time, the concentration of the scents is reduced, training the dog to become more sensitive to traces of the target scent.
>
> **Maintaining a Healthy Active Scent:** Regular refreshment ensures the scent remains "active" and free from degradation, maintaining the quality of the scent samples and consistent dog responses.

Environmental Variation: As training progresses, the dog is exposed to different environments, including outdoor areas and spaces with distractions like other animals or people. This ensures the dog can generalise its training and perform in real-world conditions.

Managing Learning Plateaus: Over the course of 6 months, some dogs may hit learning plateaus, where progress slows. Trainers carefully monitor for this and make adjustments as needed, such as simplifying tasks or introducing new challenges to keep the dog engaged and progressing.

Risks and Challenges

Overexposure to Scents: Scent refreshment every 4-6 weeks helps prevent olfactory desensitisation. It ensures the scent remains active while gradually reducing concentration to challenge the dog’s sensitivity.

Learning Plateaus: Dogs may experience periods where progress slows. Trainers address this by adjusting the training pace or introducing new challenges to prevent stagnation and encourage continued development.

2.4. Testing Protocols

In March, extensive tests were conducted to evaluate the effectiveness of the training. All placements of target and non target scents were randomised using a number generator 1-12. The tests included:

- Test 1: Three trials of a blank searches without controls, to ensure no false positives.
- Test 2: Three trials of a blank searches with all controls present, to test for false alerts.
- Test 3: Three trials of target search with all controls, to simulate real-world conditions.

Each pot was cleaned and relocated in each trail to prevent pattern setting and scent marking by both dog and trainers. The dog was allowed one pass in each trial and failure to alert on a target scent was noted as a failure.

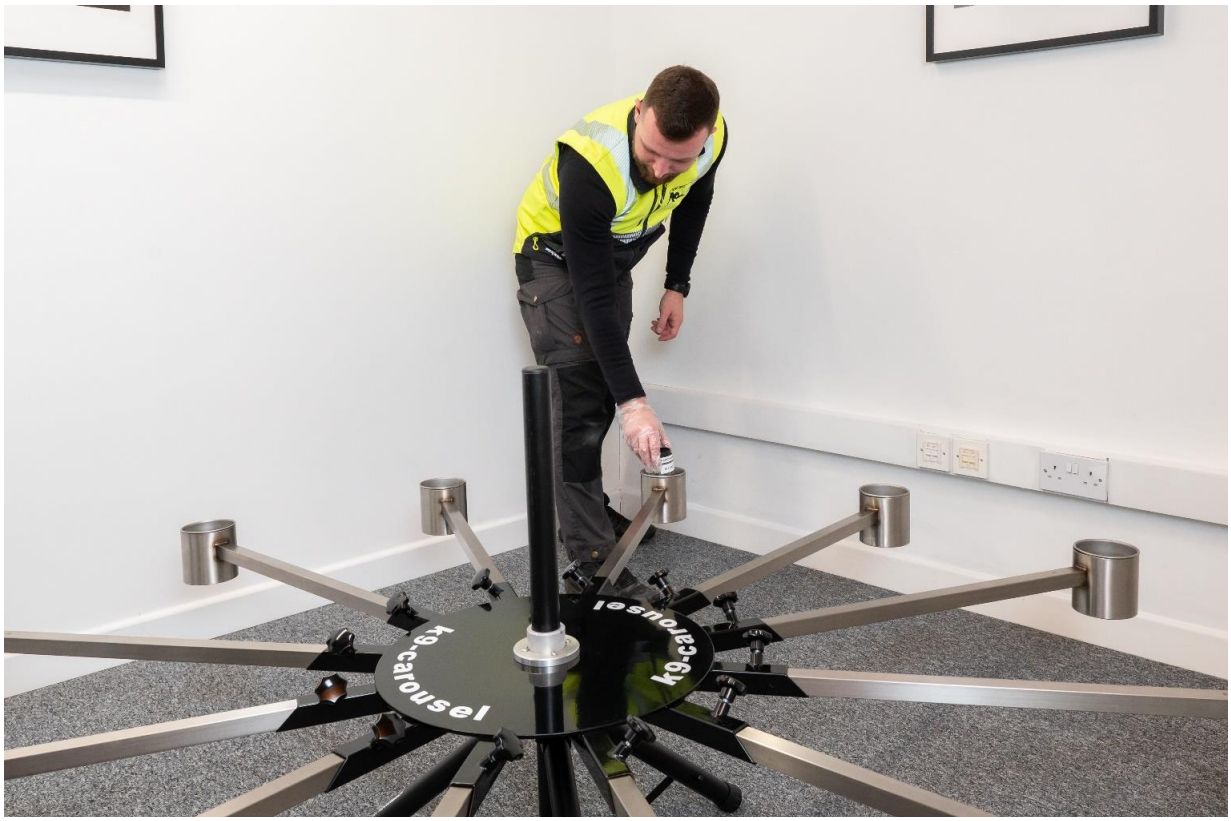

Figure 2: Training carousel being loaded with TADDs prior to training. The carousel can rotate between training runs to prevent the dog relying on special memory of where the target scent was previously located.

3. Results

The testing yielded the following success rates:

- Test 1 Blank search no controls: 100% success, confirming no false positives.
- Test 2 Blank search with all controls: 100% success, indicating no false alerts to control scents.
- Test 3 Target search *P. ramorum* and all controls: 89% success, with one failure noted during nine runs where the dog missed the target scent once. This was a critical learning point, indicating the need for further focus on early target detection.

Overall, these results demonstrate a high degree of accuracy in the dog's ability to discriminate *P. ramorum* from other scents, with an overall success rate of approximately 96% across all tests.

It is important to highlight that during testing, the dog was permitted only a single pass; however, in subsequent attempts, it successfully alerted to all target scents on each occasion.

3.1. Additional Training

Further training introduced in March focused on new scent derived from flow-through pond water from both inoculated soil and control (blank) soil. After an additional two weeks of training, the dog achieved a 89% success rate in discrimination trials, further validating the effectiveness of the training methodology.

Figure 3: Detection dog indicating target scent through characteristic stationary body positioning.

3.2. Live Training Demonstration

During a scent decontamination and replenishment session at Forest Research Roslin, a training demonstration was conducted for S. Green and H. Dun. Initially, the dog had difficulty identifying the target scent over several attempts. It was hypothesized that this might be due to a reduced concentration of sporangia/zoospores and the short exposure time of only 15 minutes in the TADD pots, which likely was insufficient for adequate scent release. Following the unsuccessful detection the carousel and all training aids were cleaned. The trial was repeated after the dog had a 15-minute break. Subsequently, the dog successfully identified the target scent in all trials. This demonstration proved highly informative for all involved, as it illustrated the factors influencing detection and provided insight into the dog's learning process.

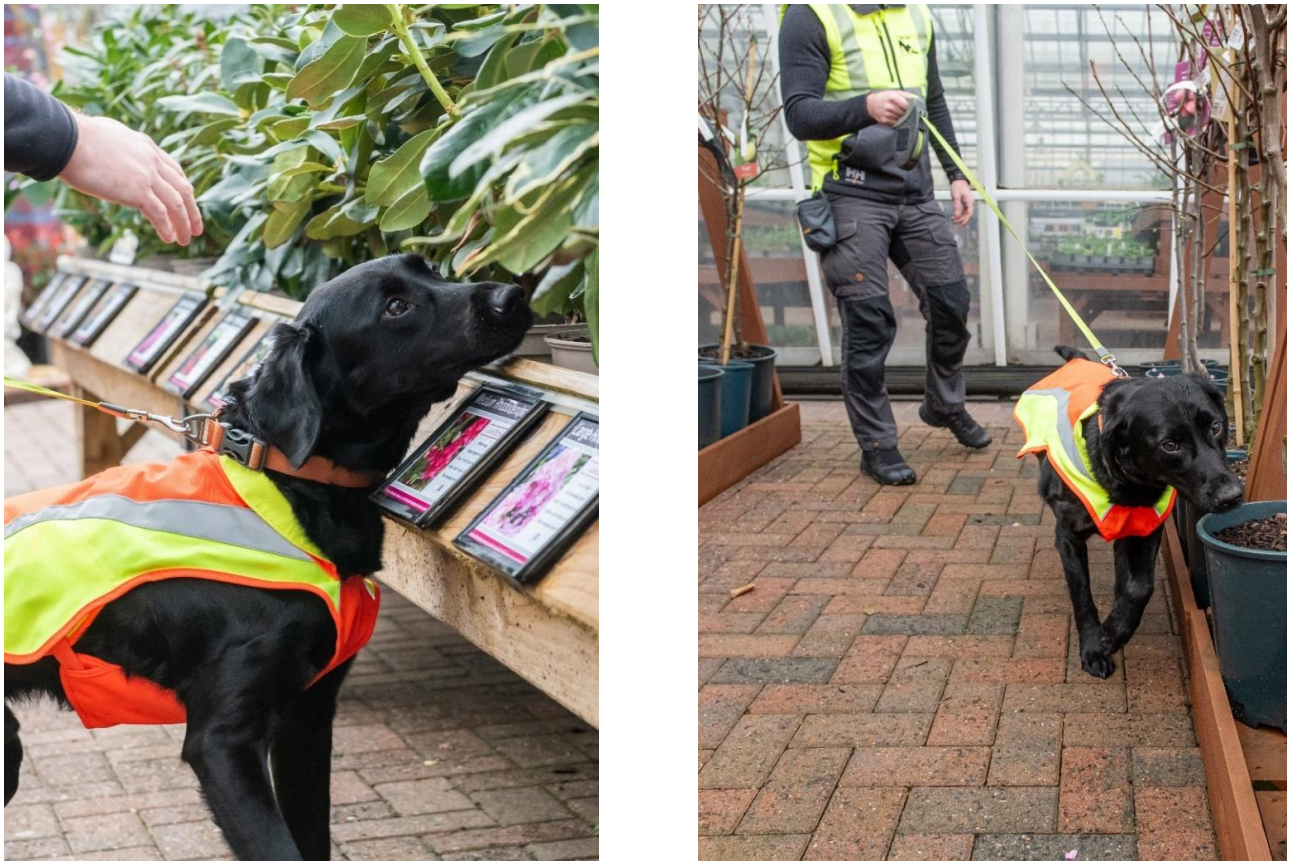

Figure 4: Dog searching rhododendron plants on benches (L) and large trees (R) during operational context training

3.3. Operational Context Training

To manage the risks associated with tree pathogens, 0.5mm pieces of Kong were used as a proxy scent in a working environment. This was carried out to simulate conditions without the direct use of pathogens. The detection of Kong was used at a garden center and nursery, specifically around conifer and potted tree areas, to simulate the possible use case of *P. ramorum* detection. This provided insights into operational strategies and visualisation of the potential structure for phase 3 of the project.

4. Conclusion and proposed next steps

The phase two project has significantly advanced the capabilities of detection dogs in identifying *P. ramorum* amidst complex scent environments. By demonstrating that the dog can discriminate between *P. ramorum* and the alternative ubiquitous species' likely to be found in the same environments this study provides robust evidence of the specific detection of *P. ramorum*. Continued training and refinement will be crucial as the project moves towards operational implementation in diverse and challenging real-world settings. The success of this phase not only reaffirms the feasibility of using detection dogs in managing plant pathogens but also highlights areas for enhancement in training protocols and scent handling techniques.

If funding is available for further work the next stage should include the following aspects:

- Expansion of Scent Training: Future training will include soil and plant materials to replicate the successes achieved in water-based environments.
- Deployment in Real-World Scenarios: Planning for the deployment of trained detection dogs in realistic settings to manage the spread of *P. ramorum* effectively. Potential settings include public gardens, plant nursery settings and potentially ports.
- Co-design with the SASA and APHA Plant Health Inspectorate: Collaboration with plant health inspectors to understand the logistics of day-to-day inspections and how detection dogs may be integrated into their current systems. Working together to establish a methodology for quick, accurate, and efficient surveying of plants in an operational setting using dog detection with the potential for deployment to assist routine inspections.

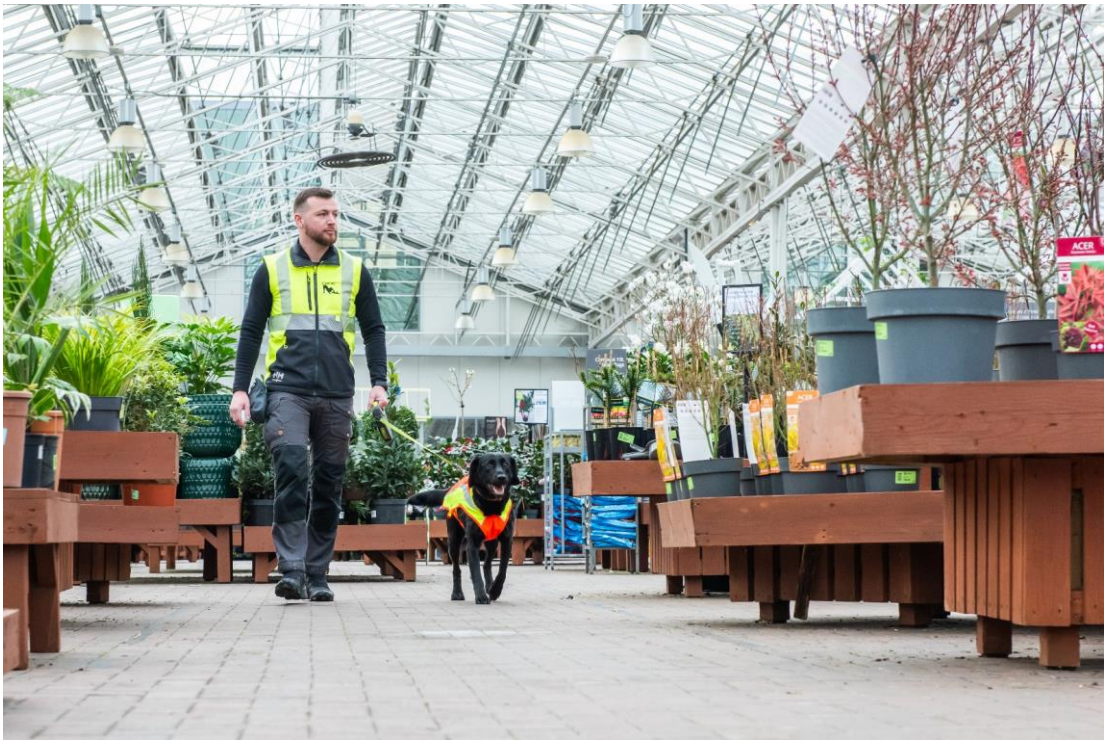

Figure 5: Operational context training was also carried out inside the garden center.

References

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