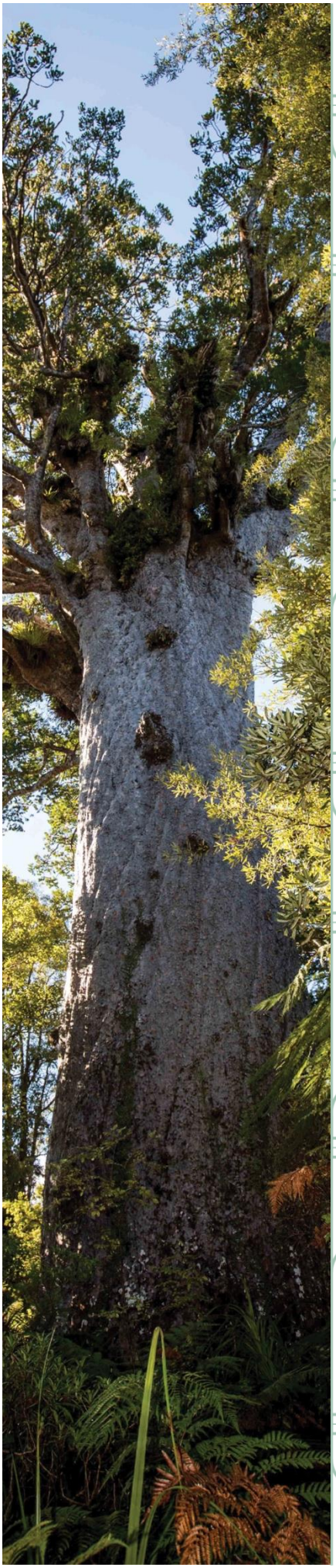

TIAKINA KAURI
KAURI PROTECTION

Approved soil baiting method for *Phytophthora agathidicida*

Tiakina Kauri

2023

Version 1.2 (01/05/2023)

Biosecurity New Zealand
Ministry for Primary Industries
Manatū Ahu Matua

Tiakina Kauri - approved soil baiting method for *Phytophthora agathidicida*

Purpose and background

The method below describes the operating procedure for isolating *Phytophthora agathidicida* (PA) from fresh or stored soils, which was standardised and approved by Tiakina Kauri. Baits obtained using this method may be used to identify PA using molecular or laboratory culture tests, including the loop-mediated isothermal amplification (LAMP) or the standard soil bioassay and morphological assessment.

The approved methods (Version 1.1) were agreed by attendees at a 16 Jan 2023 workshop to standardise the soil baiting method (Appendix A). Some components of the methods require additional evidence to determine if further standardisation is necessary, and changes will be made as part of continuous improvement as evidence becomes available (these are detailed in Appendix A). The next review of the approved method is due in 2024.

	Name
Drafted by:	Lindsay Bulman (Technical Advisory Group lead)
Reviewed by:	Karyn Froud (Biosecurity Research) Lauren Waller (Tiakina Kauri)
Workshop attendees:	Yue Chin Chew (Auckland Regional Council) Darryl Herron (Scion) Lee Hill (BioSense Ltd.) Wellcome Ho (MPI's Plant Health and Environment Lab) Ian Horner (Plant and Food) Sarah Killick (Auckland Regional Council) Pam Taylor (Scion) Kat Wardhaugh (Scion) Richard Winkworth (Massey University/Ampersand Ltd.)

	Name	Role	Signature/date
Approved by:			

Note: *Phytophthora agathidicida* is an unwanted organism and must not be spread or propagated without the permission of a chief technical officer. This precludes any laboratories that are not CTO approved from soil baiting for *P. agathidicida*.

Detailed methods

The full baiting procedure requires at least ten days. Day one: air dry soils, day four: moist incubation (or later if soils are very wet and require more time), day six: lupin set up, day eight: flooding and baiting, day 10: harvest.

General handling of samples

1. Care is needed to ensure there is no cross contamination between samples
    a. All materials and containers used throughout the baiting process must be new or autoclaved.
    b. Surfaces should be kept clean and sanitised regularly.
    c. Maintain spatial separation between samples
    d. Extreme care should be taken to avoid flicking or splashing from one container to another, particularly if pouring from one container to another.
    e. Sterilise tools in ethanol, or a DNA-denaturing product if a DNA-based test is planned, and by hot flaming between samples.

f. Gloves should be changed between batches[1] or when wet or contaminated

2. DO NOT carry out DNA extraction or PCR steps in the same work area as soil processing and isolate culturing
3. When baiting for a DNA-based test it is good practice to include at least 2 negative controls (sterile soil) to a random location within each large batch of samples to detect cross contamination. The negative controls should be added from the soil drying step using sterile soil (e.g. autoclaved) and processed in the same way as the test samples (e.g. wetting, flooding, adding baits, etc.).

Air dry soils

1. Label the drying container with the soil ID reference.
2. Add a paper towel to line the container (optional, but helps the soil dry more quickly).
3. Add sufficient soil to the container to ensure it is approximately 1/3 filled.
4. Include root material, humus and decomposed organic material, but exclude rocks and leaf litter if present.
5. Break up any soil clumps using a new or autoclaved stirrer (replace between each container). Ensure that the clumps don't explode and cast soil into other soil samples or the bench surface.
6. Dry in a well aerated lab at an ideal temperature range of 19-22℃, but not below 10℃ or above 25℃.
7. Air dry soil for a minimum of 3 days and continue drying until the entire soil sample is dry.

Moist incubation

1. Gently tip the dried soil into the container for moist incubation, or if using the same container, gently remove and discard the paper towel from beneath the soil. Ensure there is NO cross-contamination of soil.
2. Using a water spray bottle, spray the soil to wet it. Add water, little by little, so as not to over-wet the samples. Keep spraying and stirring with a clean stirrer until the soil is completely moistened but not saturated. It should have a shine to it and excess water should not be seen pooling anywhere.
3. Place a lid on the container for 10 to 20 minutes.
4. Clean the bench surface of between samples. If a DNA-based test is planned (e.g. LAMP), use a DNA-denaturing product. After 10 to 20 minutes check the soil to make sure that there are no dry areas and spray in more water as needed.
5. Leave sealed with a cover to incubate for 3 to 4 days between 19-22℃ with natural or artificial light for at least 8 hours per day.

Lupin set up[2]

1. Each sample requires 5 lupin ('NZ Blue') radicles. To ensure that, use 15 seeds.
2. Using a new or autoclaved stirrer very gently mix the seeds with vermiculite or germinate seeds between moist paper towels. Ensure all seeds are covered. Add water. Too much or too little water and the seeds won't germinate. There shouldn't be any water visible at the bottom of the container. Cover loosely allowing a little space for air movement.
3. Store the seed mix in the dark and ensure the mixture doesn't dry out.
4. Germinate until radicle is approx. 1 cm long (2 -3 days).

Flooding and Baiting

1. Pour RO (reverse osmosis), distilled or deionized water down the side of the container, filling to at least 2 cm above the soil level.
2. Float 5 mature cedar (*Cedrus deodara*) needles on the water surface. Cedar bait should have vesicle end submerged to break the surface tension of the water.
3. Select lupins with a radicle of ~1cm.
4. Place 5 lupins on the water surface if using a 1000ml container, or 3 lupins if using a 500ml container (a new polystyrene float may be used, if the lupins are long, lay them on the float with ~ 1cm of the radicle submerged in the water). Parafilm stretched over the container surface can be used as an alternative to polystyrene floats to suspend the lupins. The radicle must not touch the soil, allowing for room for 2 days' growth.
5. Do not move, disturb, or fully cover the container once it has been flooded.

[1] Batches are samples grouped by location. It is expected that management agencies, mana whenua or land owners will define batches to a general geographical area at a level that meets their management needs, i.e. catchment or forest.

[2] Lupins are not required for LAMP test baiting.

6. Bait for a minimum of 2 days, and a maximum of 3 days, between 19-22℃ with natural or artificial light for at least 8 hours per day.

Harvest

1. Remove all baits from the container using forceps, sterilised in alcohol, or a DNA denaturing product when baits will be tested using a molecular test, and flamed between each soil sample. (Note: Solution used for sterilisation should be changed between batches of soil).
2. For cutting samples, use a cutting board lined with a new, double layered tissue paper for each sample. Clean the cutting board surface with a denaturing product between batches.
3. Using sterile tools, cut the portion of each lupin radicle that has been in the water. For culturing:
    a. Rinse baits in distilled or RO water to remove organic/soil particles.
    b. Surface sterilise the baits for 30-60 seconds in a 50-70% ethanol bath, rinse once in a sterile deionized water bath for 30 seconds, blot dry on a labelled clean paper towel.
    c. Using sterile tools plate entire baits onto two plates of PARPH media.
    d. Label each plate, using the soil ID reference.
    e. Wrap and/or bag plates and incubate at 19-22^{0}C in the dark. Inspect every 2-3 days for 5 days.
    f. Sub-culture onto V8 or carrot agar for subsequent identification
4. For DNA tests:
    a. Remove baits, place individual baits into separate Eppendorf tubes, label the lid and down the side of the tube, always label from the lid end towards the bottom of the tube and freeze at -20^{0}C.

Appendix 1

Specifications

Step	Variable	Standard	Comment
Airdrying soil	Soil storage temperature	19-22^{0}C	Exact temperature is deemed unimportant so long as soil dries and it is not above 25^{0}C. (10-25^{0}C max/min long term storage lower end of range). Recommend that maximum, minimum and optimal temperature range is determined.
	Duration	Minimum 3 days. Continue drying until the entire soil sample is dry.	Number of days will depend on the saturation of the soil.
Lupin germination preparation		Use 'NZ Blue' lupin variety. Germinate until radicle is approx. 1 cm long.	Germination method will not affect test result. Lupin and cedar needles were selected because of year round availability and efficacy.
Moist incubation	Temperature	19-22^{0}C	Maximum temperature 23^{0}C. Recommend that maximum, minimum, and optimal temperature range is determined. Efficacy may be reduced at low temperatures.
	Baiting vessel	500-1000ml round or rectangular container	Recommend that the assumption there is no difference in efficacy between 500ml and 1000ml containers be tested.
	Lighting	Minimum requirement is natural or artificial light for 8 hours during daytime hours	
	Duration	3-4 days	
	Wetting	Spray with Di or RO water to thoroughly moisten soil throughout (not just surface).	
	Exposure	Lid closed tight	
Flooding and baiting	Volume of soil	Minimum 150m soil depth (e.g., aim to fill a 1000ml container to 1/3) with a well homogenised soil sample.	Recommend that the assumption there is no difference in result through using different volumes of soil be tested.
	Lupin	3 radicles in a 500ml vessel and 5 radicles in a 1000ml vessel floated on surface with radicles submerged.	Recommend that optimal lupin radicle numbers and cedar vs lupin efficacy be tested.
	Cedar	5 mature *Cedrus deodara* needles floated on surface.	
	Duration	Minimum 2 days, maximum 3 days.	Baits are colonised very rapidly. No more than 3 days.
	Float	Ensure that baits remain at the surface.	
	Incubation	19-22^{0}C in light (min daylight hours)	

Harvest and culturing	Harvest	Remove baits, rinse, soak in 70% ethanol for 30-60 sec, rinse once, blot dry, place on PARPH for culturing. For DNA tests: Remove baits, place radicles and cedar needles into separate Eppendorf tubes and freeze at -20^{0}C.	
	Incubation	19-22^{0}C in dark	
	Duration	5 days	
	Inspection	Every 2-3 days up to 5 days	
	Sub-culturing	Onto agar (V8, or thin carrot). 7-10 days.	