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ORIGINAL ARTICLE

Towards a best practice methodology for the detection of *Phytophthora* species in soils

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Abstract

The genus *Phytophthora* contains species that are major pathogens worldwide, affecting a multitude of plant species across agriculture, horticulture, forestry, and natural ecosystems. Here, we concentrate on those species that are dispersed through soil and water, attacking the roots of the plants, causing them to rot and die. The intention of this study was to compare the soil baiting protocol developed by the Centre for Phytophthora Science and Management (CPSM) with two other baiting methods used in Australia. The aim was to demonstrate the effectiveness of each protocol for soil baiting *Phytophthora* species in different substrates. Three experiments were conducted: the first to test the sensitivity of each method to detect *Phytophthora cinnamomi*, the second to test the effect of substrate type (sand or loam), and the third to test the detection of species (*P. cinnamomi*, *P. multivora*, or *P. pseudocryptogea*). The specificity of different plant species baits was compared within and between the methods. Substrate type influenced isolation in all methods; however, the CPSM method was superior regardless of substrate, albeit slower than one of the other methods for one substrate. Comparing bait species between the three methods, *Quercus ilex* was the most attractive bait for *P. cinnamomi*, particularly in the CPSM method. The choice of protocol affected the isolation associated with each bait type. Overall, the multiple bait system used by CPSM was shown to provide the most sensitive and reliable detection of *Phytophthora* species from soil samples.

KEYWORDS

artificial inoculum, natural, pre-wetting, protocol, soil baiting, soil type

1 | INTRODUCTION

Diseases caused by *Phytophthora* are a threat both to global food security (Derevnina et al., 2016), and to the health, function, and biodiversity of native ecosystems (Hansen et al., 2012). Often transported via global plant trade, *Phytophthora* species are widespread in nurseries, forestry, ornamental plantings, and natural ecosystems worldwide (Burgess et al., 2017; Migliorini et al., 2015), and are naturally dispersed aerially and through soils and water (Erwin & Ribeiro, 1996), greatly assisted by anthropogenic means. There are more than 170 described species of *Phytophthora* (Scott et al., 2019) within 11 phylogenetic clades, and additionally many provisionally named species (Jung et al., 2017). It has been suggested that the growing number of species identifications might be due to recent introductions, improved methods of isolation, more intensive sampling, the advent of molecular tools to accurately differentiate

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between species, changing environmental conditions, or a combination of factors (Scott et al., 2019). Methods for the control of *Phytophthora* in horticulture and the ornamental trade include fungicidal treatments and strict hygiene to limit introduction and spread. Current broad-scale management plans for *Phytophthora*, in nurseries and in land management, focus on the restriction of the spread of the pathogen via strict hygiene, as eradication would be a lengthy, intense, and expensive process (Dunstan et al., 2010).

Baiting of soil/root samples is a simple indirect method of isolation that provides objective proof and a culture specimen for further phenotypic characterization (Cooke et al., 2007; Erwin & Ribeiro, 1996). This involves attracting motile zoospores released from the sporangia to a living bait. These spores are negatively geotropic and exhibit chemotaxis (Hardham, 2005); they swim upwards and towards baits. Baiting is a semiselective process, as other soil microbes lack swimming spores, so undisturbed containers are unlikely to have baits infected by other pathogens. Often, the bait plants exhibit highly specific symptoms or a lesion when infected (Cooke et al., 2007). The baits are then plated onto selective media for confirmation of presence of *Phytophthora*.

There appears to be a paucity of information for detecting *Phytophthora* in soils, particularly with respect to peak scientific bodies around the world, with mainly outdated methods in their information sheets/websites. For example, the European and Mediterranean Plant Protection Organization (EPPO) states that its database provides all pest-specific information for the region, and it is revised on a regular basis. However, the diagnostic protocols for regulated pests (PM7) for *P. cinnamomi* was prepared in 2003, based on references that are now 20–85 years old (EPPO, 2004). They recommend soil baiting techniques using avocado or pear fruits or leaves of *Rhododendron catawbiense*, and state they are equally effective. Although their datasheet acknowledges that *P. cinnamomi* is present in a considerable area of the EPPO region, the information presented is based on methods from the 1970s to 1990s. The CABI Invasive Species Compendium, with a lead partner US Department of Agriculture, is used by researchers, resource and environment managers in agriculture and forestry, and by policy makers. However, their datasheet on *P. cinnamomi* provides a link to the EPPO website for diagnosis (https://www.cabi.org/isc/datasheet/40957#todiagnosis). This is surprising given the substantial amount of on-going research worldwide. In Asia, the diagnostic manual for plant diseases in Vietnam (Burgess et al., 2008) recommends the insertion of the soil sample into apple, or to use a soil baiting method with petals or leaves as baits in cups (equal volumes of soil:water) with chilli and citrus leaves, rose petals, and seedlings of chilli, lupin, and soybean.

In Australia, Horticulture Innovation Australia (HIA), the not-for-profit research and development corporation for the horticulture industry, directs its growers to the Nursery and Garden Industry Australia (NGIA) publication that was produced by the Queensland Department of Agriculture and Fisheries (Nursery and Garden Industry Australia [NGIA], 2016). This acknowledges that there are a range of baits that can be used to detect pathogens, but focuses on lupin radicle baiting for *Phytophthora* in nurseries. Similarly, the Nursery Industry Accreditation Scheme Australia (Nursery Industry Accreditation Scheme and Australia [NIASA], 2013) suggests lupin baiting for the identification of problem areas by nursery managers. This is concerning, as they state it is "the preferred technique for nursery and growing media supplier on-site testing". It is imperative that advisory bodies update their information and techniques as new information is brought to light.

There are many papers describing baiting methods for *Phytophthora*; however, there have been few systematic studies undertaken to determine the best baiting protocol to use. An Australian study reported on marked variability in both baiting techniques and soil types (McDougall et al., 2002). However, there are a variety of baiting methods currently recommended by agencies around the world. Martin et al. (2012) reviewed detection progress and concluded that leaf baits were preferred over fruits, and while a wide variety of leaf baits are used, baits are selectively attractive to individual *Phytophthora* spp.

Many of the comparative methods studied do not include positive and negative controls, are conducted in only one season, and do not provide justification for choices, nor provide detailed methods. At this point, there is no standardization of media, timing, bait material, volumes/ratio, temperature, or container shape to use. It has long been understood that the use of multiple bait species results in more *Phytophthora* spp. to be isolated rather than just *P. cinnamomi* (Dance et al., 1975), yet many protocols and experiments use only one type of bait material.

The team at the Centre for Phytophthora Science and Management (CPSM) has developed a baiting protocol over the last 30 years that we believe maximizes the detection of *Phytophthora*. Our baiting method was shown to be a more reliable method to isolate *Phytophthora* than plating infected roots; investigating blackberry over four seasons, oak leaves baits (*Quercus ilex* and *Q. suber*) were more effective than direct plating of roots for the isolation of nine *Phytophthora* species (Aghighi et al., 2015). Importantly, the most pathogenic of the nine *Phytophthora* species recovered from blackberry, *P. bilorbang*, was only isolated by baiting rhizosphere samples from roots of naturally infected blackberry; it is a weak competitor and slow growing, and could not be isolated by direct plating (Aghighi et al., 2015).

In this study, we compared the CPSM soil baiting protocol to two methods commonly used in Australia and discuss the reasons for choices of major aspects of the protocol. One of the protocols that we include is that suggested by NIASA. NIASA provides the best management practice for Australian production nurseries and growing media manufacturers, and all NIASA accredited businesses operate in accordance with these practices. These experiments investigate sensitivity of each method, the effect of substrate type and inoculum source (young nonwoody, and woody roots), and provide a comparison of specificity of the different baits within two of the methods. The aim was to demonstrate the effectiveness of each protocol for soil baiting *Phytophthora* species in different substrates, and to discuss the reasons for this, leading towards the adoption of a more effective protocol.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Three experiments compared the sensitivity and specificity of the three baiting methods using different substrates, inoculum types and density, and *Phytophthora* species. Protocols for baiting were followed as described for Methods 1, 2, and 3. The substrates for each experiment were steam pasteurized at 65°C for 1 hr before inoculum added. All baiting containers were left open on the bench, between 22 and 25°C (room temperature, natural light) as supported by other researchers (Erwin & Ribeiro, 1996; Ferguson & Jeffers, 1999). A container provided a positive reading if the *Phytophthora* species was recovered from any of the baits.

2.1.1 | Baiting Method 1: CPSM baiting protocol

Approximately 200 g of substrate plus inoculum (1/3 of the container volume) was placed into 1 L plastic containers (11.5 × 16.5 × 7.5 mm deep, Genfac Plastics P/L) with 100 ml of distilled water and left overnight. An additional 500 ml distilled water was added to samples, and any floating debris was skimmed from the surface using sterile paper towel. If large root pieces came to the surface, sterilized paper towel was used to push them to one end of the container and hold them in place for the duration of the baiting. An assortment of five baits (leaves and petals) from susceptible host species was floated on the surface of the water. The type of bait used depended on seasonal availability; in these experiments leaves of *Q. ilex*, *Hibbertia scandens*, *Hedera canariensis*, *Pimelea ferruginea*, *Scholtzia involucrata*, *Rosa* sp., *Populus* sp., *Chameluacium uncinatum*, and *Eucalyptus sieberi* cotyledons and germinants of *Lupinus angustifolius* were used (prepared as described below for Method 3). Young, fully expanded leaves were collected immediately before use. - The number of baits (2–20) floated on the surface depended on the bait size to provide maximum surface coverage without overlapping of baits (Figure 1a).

Baits were checked daily, over 10 days, for infection symptoms, and lesions were excised and plated onto a *Phytophthora*-selective medium (NARH; Simamora et al., 2017). All culture plates were incubated at 22°C in the dark, checked daily for growth of *Phytophthora*, and isolates obtained were identified by microscopy based on cell and colony morphology. At day 10, containers without any baits with lesions had some symptomless tissue plated (one plate each container); however, no *Phytophthora* was isolated from these baits.

2.1.2 | Baiting Method 2

E. sieberi seeds were germinated in coarse vermiculite moistened with sterile water in the glasshouse. The cotyledons were harvested when seedlings were approximately 2 cm tall.

Approximately 300 g of substrate, plus inoculum, was mixed with an equal volume of distilled water in 1 L plastic containers (described above). *E. sieberi* cotyledons and *Pimelea* leaves (10–15 each) were floated as baits in each container (Figure 1b). Baiting containers were left open on the bench at 22–25°C and baits inspected on days 3 and 10 only. Lesions were excised and plated onto NARH, incubated and checked daily (as described in baiting Method 1, CPSM).

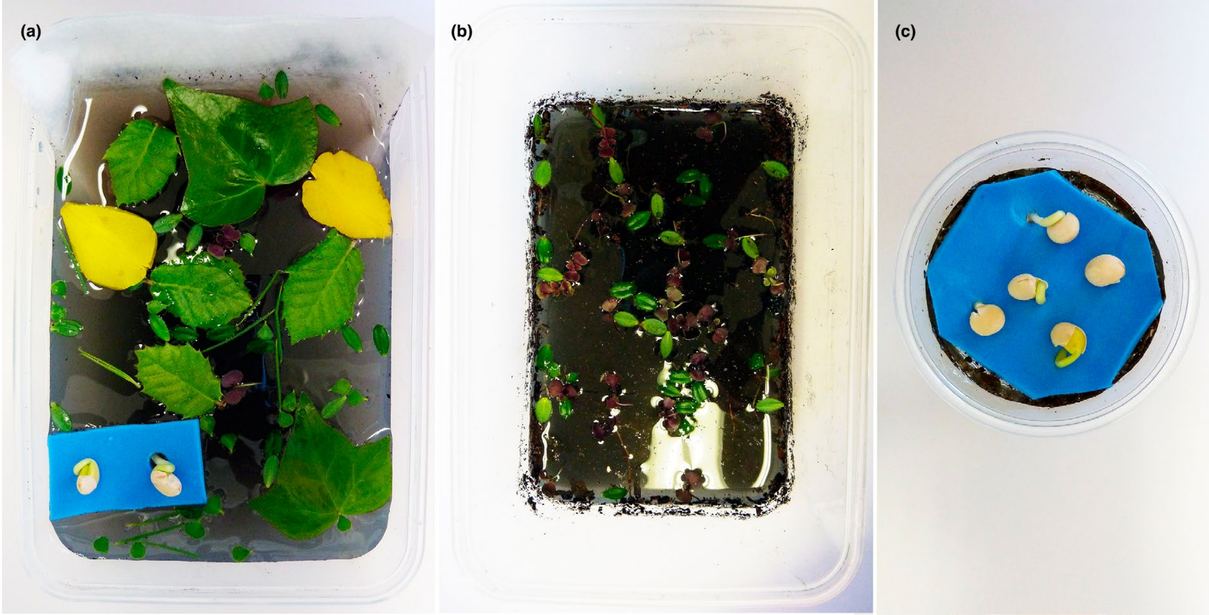


FIGURE 1 Baiting methods for the detection of *Phytophthora* in soil. (a) CPSM method (Phytophthora Science and Management, Murdoch University); (b) method using same container but with fewer bait types, and a lower water:soil ratio; (c) Nursery Industry Accreditation Scheme Australia (NIASA) recommended protocol using one bait type in a cup, with high water:soil ratio [Colour figure can be viewed at wileyonlinelibrary.com]

2.1.3 | Baiting Method 3: NIASA recommended protocol

L. angustifolius seeds were soaked in water overnight, and then held for 48 hr on moist sterile filter paper in plastic trays before use. Approximately 50 g of substrate plus inoculum was placed in a 250 ml polystyrene cup and filled almost to the top with distilled water. Five *L. angustifolius* seedlings, with 2 cm long roots, were used as baits by suspending them in a polystyrene float with their roots in water (Figure 1c). Cups were left open on the bench at 22–25°C for 7 days with baits inspected daily after 3 days. Discoloured lupin roots were removed, washed, surface sterilized (30 s in 50% ethanol, followed by two sterile water rinses), then dried between sheets of sterile blotting paper. Small segments were plated out onto NARH, incubated, and checked daily (as described above).

2.2 | *Phytophthora* isolates

Three *Phytophthora* species were used in the experiments. *P. cinnamomi* (isolate MP94-48; CPSM; Clade 7) was used in all experiments, and is considered moderately pathogenic to multiple hosts (Hüberli et al., 2001). *P. multivora* (isolate WAC13201; Clade 2) is a known pathogen of multiple hosts (Scott et al., 2009) and was used in Experiments 2 and 3. *P. pseudocryptogea* (isolate VHSC16118; Clade 8) was used in Experiment 3; this species has also been isolated from multiple hosts (Safaiefarahani et al., 2015). All these species have been recovered from *Banksia grandis*.

2.3 | Experiment 1: Sensitivity of *P. cinnamomi* detection

The sensitivity of each baiting method was examined using four different inoculum densities of *P. cinnamomi*. This experiment allows comparison of attractiveness of baits within and between methods.

Inoculum was prepared in 1 L flasks containing 400 ml vermiculite, 4 g millet, and 240 ml V8 broth (50 ml V8 juice [Campbell Grocery Products Ltd], 190 ml distilled water, 0.8 g $CaCO_3$, pH adjusted to 7). Flasks were autoclaved three times at 24 hr intervals (121°C for 20 min). *P. cinnamomi* isolate MP94-48 was grown on carrot agar (CA) plates (carrot extract 250 g/L; agar 15 g/L). After 1 week, the agar was cut into 0.5 cm cubes and used to inoculate the vermiculite. The flasks for the negative control were inoculated with a sterile plate of CA. The flasks were incubated at 25°C in the dark, and gently shaken each week. The flasks were fully colonized and ready for use after 4 weeks.

The three baiting methods were compared using river sand as the substrate. Four inoculum densities were used at 0.01, 0.1, 1, and 10 g/kg of substrate. There were 10 replicate bait containers and one negative control for each method, which was inoculated with 3 g of the sterile vermiculite/millet mix. Samples were baited and plated as per the three baiting protocols described above.

2.4 | Experiment 2: Detection from laboratory-produced root inoculum tested in two soil types

Specificity was further tested in two soil types (sand and loam) using a more natural inoculum source, young lupin roots infected with *P. cinnamomi* or *P. multivora*. There were 10 replicate bait containers for each method/*Phytophthora* species/soil type combination, and one negative control for each method/soil combination that was inoculated with uninoculated roots.

Seed of *L. angustifolius* 'Merrit' were germinated in moist paper towel for 12 hr. Germinants (c.30) were then aligned at the top of a fresh moist paper towel, which was rolled tightly so the shoots emerged at the top and the roots grew vertically down. To keep them moist, many rolls were packed upright in a container with some water, and incubated at 22–25°C. When the roots were approximately 5 cm long, a colonized agar plug with either *Phytophthora* species was placed adjacent to the root tip and the paper towel was rerolled and replaced in the water container. When the roots were approximately 10 cm in length, the lesion (discoloured portion of root) was harvested. All inoculated roots became infected, and Koch's postulates were confirmed by plating out material onto NARH medium and incubating for 3 days. Microscopic identification, comparing recovered isolates from control cultures, was used for positive identification of each species.

To the substrate, 5 cm of lupin root infected with either *Phytophthora* species was added to each container (with 2 cm for Method 3 containers). The substrate was rhizosphere soil collected in Western Australia from a typical coastal sand and a lateritic loamy sand from the Darling Scarp. The substrate was collected from areas where healthy plants existed. The soils were steam pasteurized at 65°C for 1 hr. Samples were baited and plated as per the three baiting methods provided above.

2.5 | Experiment 3: Detection from roots infected with different *Phytophthora* spp. and tested in two soil types

Roots of *B. grandis* grown in containers infested with *P. cinnamomi*, *P. multivora*, or *P. pseudocryptogea* were used to compare the effectiveness of the three methods in the two soil types.

The inoculum source for the baiting was 3-month-old *B. grandis* seedlings grown in free-draining containers containing sand with millet (*Panicum indicum*) inoculum (1% vol/vol of sand) prepared as in Experiment 1. The seedlings were flooded for 12 hr to allow sporangial production and zoospore release, and then left for the infection to develop naturally. The plants were watered daily to container capacity. After 3 months, the roots were harvested and gently washed to remove most of the sand. The root systems, inoculated with a single species, were chopped into approximately 1 cm sections, and thoroughly mixed. Several random subsamples were plated onto NARH, confirming successful infection of root pieces.

To the substrates (sand and loam as described in Experiment 2), infected *Banksia* root inoculum was added (5 g/kg of substrate). There

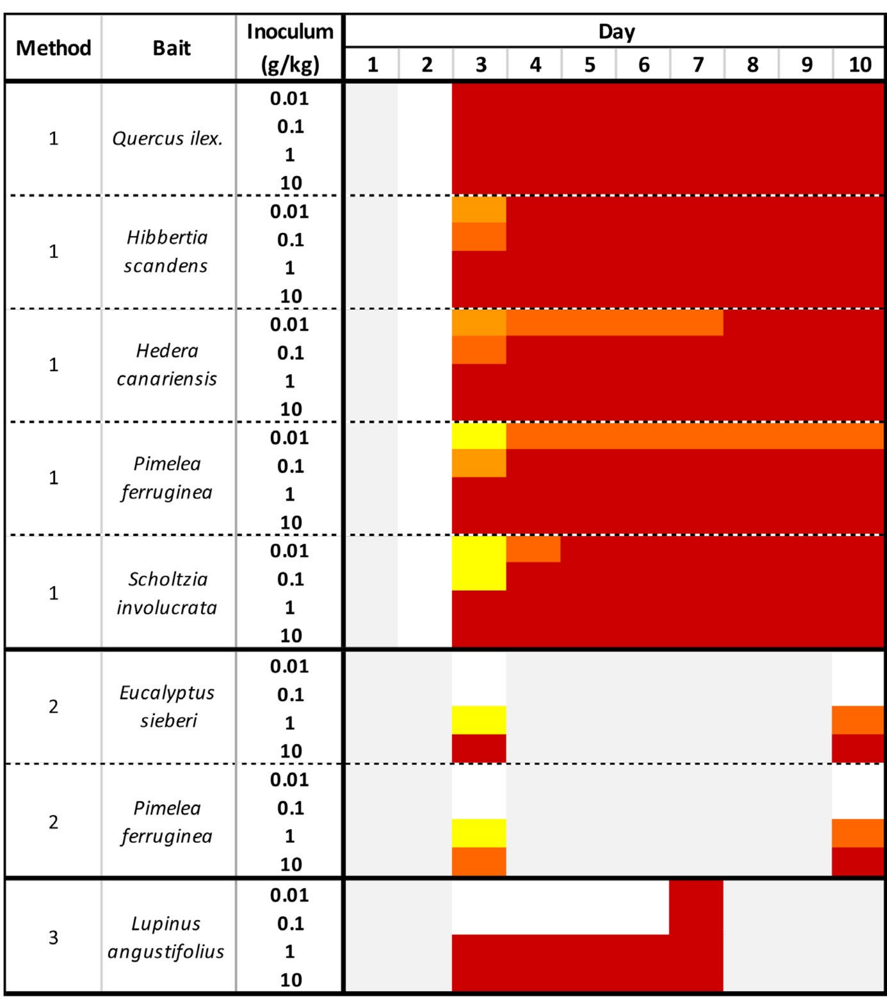
were seven replicates for each method × substrate × *Phytophthora* species combination, and one negative control for each method × soil combination that was inoculated with noninoculated roots obtained from *Banksia* grown in containers. Samples were baited and plated as per the three baiting protocols described above.

3 | RESULTS

By the end of each experiment, no lesions were observed on baits of the negative controls, nor was any *Phytophthora* recovered from portions of baits when plated out.

3.1 | Experiment 1: Sensitivity of *P. cinnamomi* detection

The most rapid and sensitive assay was Method 1, and the least sensitive was Method 2 (Figure 2). By day 3, Method 1 detected *P. cinnamomi* in all bait types at all inoculum densities. Method 3 produced the same result but only at the two highest inoculum densities (1 and 10 g/kg). Interestingly, at the two lower inoculum densities (0.01 and 0.1 g/kg) there was no isolation from lupins until day 7 when 100% of containers were positive. At day 10, Method 2 was not as sensitive as the other two methods and there were no positives at the two lower inoculum densities.



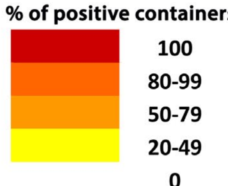


FIGURE 2 Experiment 1. Sensitivity: percentage of containers from which *Phytophthora cinnamomi* (isolate MP94-48) was recovered when comparing three soil baiting techniques. Sterile river sand (n = 10) was artificially inoculated with infected millet seed of increasing density. Grey shading indicates days when baits were not checked according to the different methods used. Method 1 (CPSM) used an overnight prewetting step, then a large water to soil ratio, with five bait types used in each rectangular container. Method 2 used a smaller water to soil ratio in the same container type, and a mixture of two baits only. Method 3 used a cup, with a high water to soil ratio and one bait type [Colour figure can be viewed at wileyonlinelibrary.com]

Method 1 revealed *Q. ilex* as the most attractive bait, closely followed by *H. scandens*, then *S. involucrata*. For this method, *P. ferruginea* was the least effective bait at the lowest inoculum densities. Method 2 also showed *P. ferruginea* to be the least attractive bait at day 3.

3.2 | Experiment 2: Detection from laboratory-produced inoculum tested in two soil types

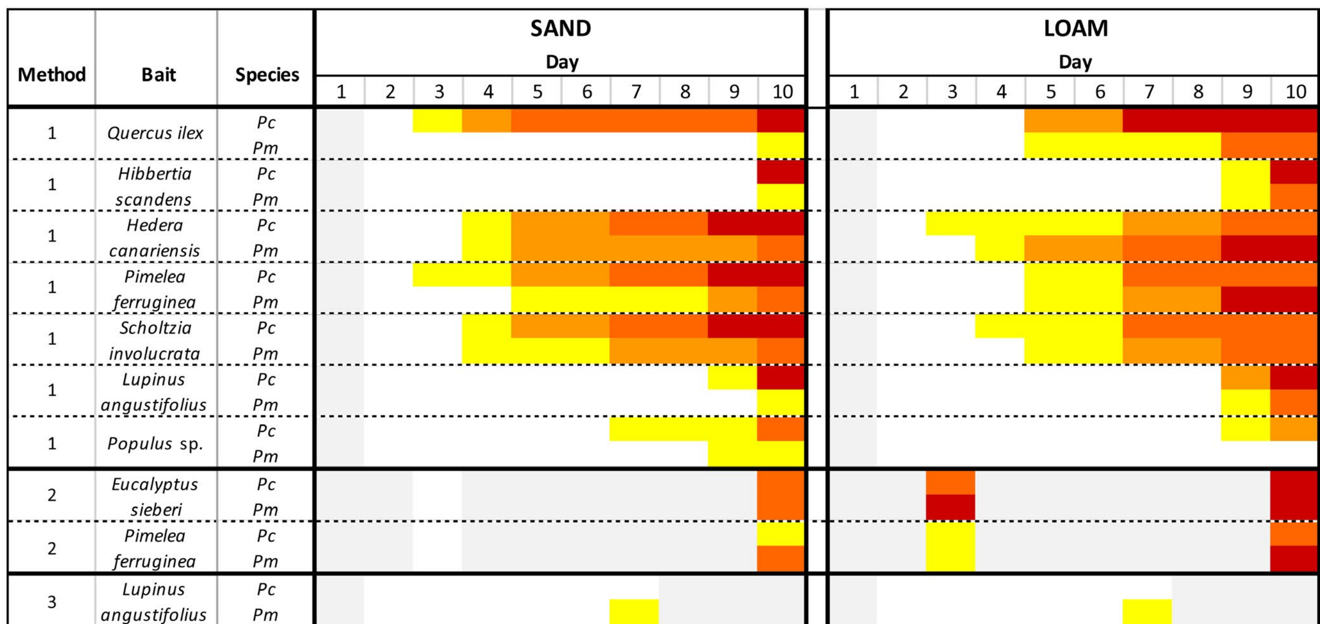
Overall, there was no difference in isolation between the substrates, nor between the species. Method 3 was out-performed by Methods 1 and 2. Method 1 provided the best results for both *Phytophthora* species, regardless of substrate, although it was slower in loam than Method 2 (Figure 3). For Method 1, *H. canariensis*, *P. ferruginea*, and *S. involucrata* gave similar results, for sand and loam, for both *Phytophthora* species, with at least 49% detection by day 5.

The fastest isolation for both species was Method 2, but only in loam at day 3, where both *Phytophthora* species were detected at >80% and <50% from *E. sieberi* and *P. ferruginea*, respectively. At day 3, there were no isolations from sand for method 2. At day 10, the isolation from sand was on average 20% lower than it was for loam.

By day 10, Method 2 showed a greater affinity for *P. multivora* than Method 1 in both substrates using both baits, but for *P. cinnamomi* Method 2 was generally 20% less sensitive (Figure 3). Method 3 performed poorly in the isolation of *P. multivora*, which was isolated from less than 50% of baits from both soil types on day 7.

In bait comparisons between methods, *P. ferruginea* returned the same percentage isolation by day 10 in loam, albeit faster in Method 2 (Figure 3). Method 1 showed a difference in specificity between sand and loam for the two species, where *P. cinnamomi* was isolated more frequently in sand, while more *P. multivora* was recovered in loam. In loam, both methods detected more *P. multivora* than *P. cinnamomi*. A comparison of the detection by *L. angustifolius* used by Method 1 and Method 3 showed Method 3 was faster to detect up to 50% *P. multivora* in both substrates compared to Method 1.

Q. ilex was useful for the isolation of *P. cinnamomi* as it was the most sensitive bait type for Method 1 (Figure 3). However, *P. multivora* showed a low affinity for this bait, particularly in sand. *H. scandens* and *L. angustifolius* were slow to display symptoms on baits but resulted in similar levels of isolation to the other baits at the end of the experiment. *Populus* sp. proved to be a poor bait for both these *Phytophthora* species.



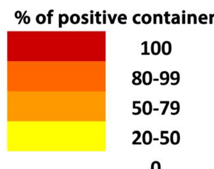


FIGURE 3 Experiment 2: Substrate, artificial inoculum: percentage of containers from which *Phytophthora cinnamomi* isolate MP94-48 (Pc) or *P. multivora* isolate WAC13201 (Pm) was recovered when comparing three soil baiting techniques. Sand or loam (*n* = 10) was artificially inoculated with infected *Lupinus angustifolius* roots. Grey shading indicates days when baits were not checked according to the different methods used. Method 1 (CPSM) used an overnight prewetting step, then a large water to soil ratio, with five bait types used in each rectangular container. Method 2 used a smaller water to soil ratio in the same container type, and a mixture of two baits only. Method 3 used a cup, with a high water to soil ratio and one bait type [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Experiment 3: Detection from roots infected with different *Phytophthora* spp. and tested in two soil types

Using *Banksia* roots as the inoculum source, there was no difference amongst the methods, and no difference between the species, and the rate of isolation was higher and faster than in Experiment 2 with the laboratory-inoculated roots of *L. angustifolius* (Figures 3 and 4). Most notable is the absence of detection of *P. cinnamomi* from many of the tests, entirely missing from Methods 2 and 3, and by four of the baits tested in Method 1 (sand and loam: *E. sieberi* and *P. ferruginea*; loam: *Rosa* sp. and *H. canariensis*), demonstrating the importance of using multiple baits. Again, Method 3 was the worst performer.

P. pseudocryptogea was consistently isolated quickly (2–3 days) by all three methods in all baits, with 100% isolation in all but *L. angustifolius* in Method 1 (Figure 4). *P. multivora* was also quickly detected but isolation was more variable than for *P. pseudocryptogea*; however, four of the baits in Method 1 and both baits in Method 2 had 100% isolation by day 3. Notably, *L. angustifolius* was the poorest bait type for *P. multivora* with isolation of <50% at best. *P. cinnamomi* was the most elusive pathogen under these conditions, with the pathogen being isolated from six sand baits and three loam baits using Method 1.

Under these experimental conditions, the best chance of isolating these three pathogens from both soil types is with *C. uncinatum* using Method 1 (Figure 4). A combination of *Rosa* sp. or *S. involucrata* and *C. uncinatum* should provide the best results using Method 1.

Comparing bait types between methods, *L. angustifolius* was a better bait when used in Method 3 compared to Method 1 for *P. multivora* and *P. pseudocryptogea* (Figure 4). *E. sieberi* provided better results when used in Method 2 than in Method 1, while recoveries from *Pimelea* were very similar between these two methods.

3.4 | Overall

A comparison of bait types between experiments demonstrates variability. For instance, *H. scandens* was a good bait in Experiment 1 (Figure 2), but poor in Experiment 2 (Figure 3). *H. canariensis*, *P. ferruginea*, and *E. sieberi* were good baits in Experiment 2 (Figure 3) but did not perform well in Experiment 3 (Figure 4). Another poor performer in Experiment 3 (Figure 4), *Q. ilex* was a good bait in Experiments 1 (Figure 2) and 2 (Figure 3).

4 | DISCUSSION

Method 1 was more sensitive for *P. cinnamomi* than the other two methods up to 7 days. It had the highest rates of isolation from the root-inoculated sand and loam substrates. From natural inoculum, this method was more sensitive and provided more consistent isolation of *P. cinnamomi*. Method 1 provided better detection of species from different clades, from both substrate types, regardless of inoculum type, if a mixture of baits was used.

Sensitivity in the testing procedure is paramount when detecting soil pathogens that can cause disease, even at low inoculum density, and are challenged by seasonal changes, physical and chemical properties of the soil, and microbial competition. *Q. ilex* used by Method 1 proved to be the most sensitive bait, with 100% isolation by day 3 at 0.1 g *P. cinnamomi*/kg substrate. Two other baits using Method 1 (*H. scandens* and *S. involucrata*) matched this by day 5, which outperformed the other two methods. Method 1 provided increased sensitivity and speed of isolation of *P. cinnamomi*. This might be attributed to the higher water:substrate ratio or the overnight prewetting followed by the flooding and placement of baits.

Although the importance of using more than one bait type was recognized in the 1970s, particularly when multiple *Phytophthora* species may be present in soil samples (Dance et al., 1975; Ferguson & Jeffers, 1999), this is not followed by many laboratories. The choice of baits has a large impact on the attractiveness to zoospores. Previous sampling surveys have demonstrated that different baits bias isolation (Smith et al., 2009), and Hüberli et al. (2013) showed that all species could not be isolated by a single plant bait. In the current study, where the experiments were run sequentially, thus falling into different seasons, the same bait type performed differently between experiments.

Method 1 deploys several species of bait leaves per container to maximize the chance of *Phytophthora* detection. It has been demonstrated that the current physiological activity of the bait plants affects detection of *Phytophthora* species, with summer leaves producing more variable results than the same plants leaves collected in autumn (Ferguson & Jeffers, 1999). The host species from which the bait tissue is derived also influences the efficiency of detection (O'Brien et al., 2009). Although the bait tissue is from susceptible host species, different host species have different levels of efficacy (Erwin & Ribeiro, 1996). Ferguson and Jeffers (1999) also demonstrated that *Phytophthora*-resistant species were colonized less than susceptible species, so it is important to use susceptible species. During detection of five species of *Phytophthora*, Ferguson and Jeffers (1999) found that while *Camellia* baits were good for detection of some species (including *P. cinnamomi*), *P. cactorum* was not isolated by 72 hr. CPSM chooses to use whole leaf baits rather than leaf discs, as the wounded cells around the perimeter are more easily colonized by *Pythium* and other contaminating organisms than intact baits (Ferguson & Jeffers, 1999). The multiple bait system used by Method 1 contributes to a more sensitive and reliable detection of *Phytophthora* species.

In Australia, it is promoted that *L. angustifolius* radicles "are used extensively in diagnostic laboratories because they detect many *Phytophthora* species" (Nursery and Garden Industry Australia [NGIA], 2016). However, the results of the current study demonstrated initial low sensitivity and later experiments showed poor isolation of *P. cinnamomi* and *P. multivora*. This observation is pertinent, given the importance of these two *Phytophthora* species as multihost pathogens worldwide. The early results were probably due to the use, at that time, of a cultivar of *L. angustifolius* susceptible to

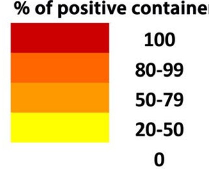


FIGURE 4 Experiment 3: Substrate, pot-infected root inoculum: percentage of containers from which *Phytophthora cinnamomi* isolate MP94-48 (Pc), *P. multivora* isolate WAC13201 (Pm), or *P. pseudocryptogea* isolate VHSC16118 (Pp) was recovered when comparing three soil baiting techniques. Sand or loam (n = 10) was artificially inoculated with infected *Banksia grandis* roots. Grey shading indicates days when baits were not checked according to the different methods used. Method 1 (CPSM) used an overnight prewetting step, then a large water to soil ratio, with five bait types used in each rectangular container. Method 2 used a smaller water to soil ratio in the same container type, and a mixture of two baits only. Method 3 used a cup, with a high water to soil ratio and one bait type [Colour figure can be viewed at wileyonlinelibrary.com]

P. cinnamomi. The new *L. angustifolius* cultivars in use have higher resistance to *P. cinnamomi* (unpublished data 2018, CPSM). Therefore, given the diversity of *Phytophthora* species present in Australia (Burgess et al., 2017), the NGIA method is not recommended, as it will undoubtedly yield false negative results. This is important, as nurseries worldwide are known to be a source of *Phytophthora* species spreading into managed and natural ecosystems (Jung et al., 2015; Parke et al., 2019). Consequently, it is essential that nurseries screening substrates for *Phytophthora* species use a wide variety of baits and baiting containers with a larger surface area than currently recommended.

Frequently, it is not possible to source uniform and suitable bait tissues from a single species on a year-round basis. Consequently, it is important to source baits from numerous plant species depending on the time of the year, and growth stage of the bait plants. Depending on location, different laboratories will have to source different baits-. Therefore, it should be expected that different diagnostic laboratories in different regions/countries will use different baits. Based on experience of developing the baiting protocol over 28 years, we have found it is important to use the leaves on either side of, and including, the youngest fully expanded leaves and avoid old and very young leaves and flowers. It is also

important to avoid plants that have been treated, or likely to have been treated, with fungicides (e.g., sourcing petals from florists). CPSM has a range of species maintained for use as bait plants including some in pots and others planted in the ground, which are regularly pruned, watered, and fertilized to stimulate leaf flush, and hence increase the year-round supply of suitable material. *Quercus* species, a genus not native to Australia, which we have found to be an excellent bait for many *Phytophthora* species, are maintained in pots and in the ground-. Planning can ensure that a variety of baits are always available.

The soil type can have a physical or chemical influence on the isolation of the pathogen. While Shew and Benson (1982) found no significant difference in *P. cinnamomi* propagules recovered from naturally infected clay versus sandy loam soils, baited clays were reported to be less inhibitory to *P. cinnamomi* detection than sands (Williams et al., 2009). In the current experiments, using laboratory-grown *P. cinnamomi*, isolation was faster by two bait types in sand compared to loam, and by two other bait types in loam compared to sand using Method 1. Using natural inoculum, *P. cinnamomi* was detected faster by five bait species (*Q. ilex*, *Rosa* sp., *H. canairensis*, *S. involucrata*, and *L. angustifolius*) in sand, while none of the bait types had faster or greater isolation in the loam soil. This could be due to the greater number of smaller silt and clay particles in the loam soil acting as a barrier to zoospore movement. Eden et al. (2000) showed that significantly more baits were infected from soils with a particle size range of 500–850 μm, suggesting that there was a physical blockage of zoospore movement in the soil, which may result in premature encystment. In substrates where the pore size was greater than the size of the zoospores there was little blockage of zoospores detected (Newhook et al., 1981), as found in sand in the current study.

Method 1 uses large shallow containers for baiting for multiple reasons. Detection of zoospores is more efficient in small volumes of soil (Eden et al., 2000), but due to the general low inoculum source in the soil, larger samples provide the best chance of detection. Therefore, a large surface area for the soil means that a shallower depth can be used for each sample. Furthermore, a larger surface area provides higher air exchange, enhancing the production of sporangia and zoospores. Method 1 uses a high water:substrate ratio (600 ml:200 g) and a large surface area. Although Method 3 uses 200 ml:50 g substrate, the small surface area reduces air exchange. Furthermore, Tsao (1983) suggested that the water-to-soil ratio should be high to assist in the dilution of chemicals that may be inhibitory to germination, hence the use of a 3:1 ratio in Method 1.

For dry soils, Method 1 engages a premoistening step overnight before the start of the assay, without any drying of samples before baiting. Oospores (and other survival structures) may be dormant in soil and the premoistening is thought to stimulate their germination. Air drying soils and then prewetting for several days increased detection of *P. citricola* (Ferguson & Jeffers, 1999) and *P. megasperma* (Stack & Millar, 1985). *P. nicotianae* was more detectable after drying and premoistening (Ioannou & Grogan, 1984). Conversely, for *P. cinnamomi*, premoistened soils did not result in a higher proportion of infections compared to soil samples that were not premoistened, and air drying did not give rise to infections, while there was 9% from nondried samples (Eden et al., 2000), and Meadows and Jeffers (2011) noted that *P. cinnamomi* isolation from dried and then remoistened soil samples was very rare. Other research groups/diagnostic facilities use longer periods for the premoistening step when soil samples are from dry areas, or the samples are dried after collection. For example, in the USA, dry soil samples are prewetted for 2–3 days prior to flooding and baiting for *Phytophthora* (USDA, 2010). In Iran, soil samples were dried and then moistened for a preincubation for 1–4 weeks before flooding to bait for *Phytophthora* (Mohammadi, 2013). The overnight premoistening step used by Method 1 has been employed to stimulate pathogen activity prior to flooding.

While Nursery and Garden Industry Australia (NGIA) (2016) advise that their technique has successfully recovered *P. cinnamomi* and 11 other *Phytophthora* species (not including *P. multivora* or *P. pseudocryptogea*), this technique had low sensitivity for *P. cinnamomi* in millet seed inoculum and infected root material in the current study. Using Method 1 we have, to date, isolated 54 species from soil samples in our laboratory.

Our recommendations for current best practice baiting methodology include

- Dry soils should be premoistened for at least 12 hr
- Use containers with larger surface areas, avoid using cups
- A soil-to-water ratio of 1:3 is recommended
- Ensure no organic matter is floating on the surface, as this allows true fungi to easily colonize baits and compete with *Phytophthora* species
- Use baits from as many different plant species as possible. Preference is for the leaves adjacent to the youngest fully expanded leaves or flower petals, ensuring they have not been treated with fungicides.
- Cover the surface area with baits to increase isolation success rates, as zoospores only travel 2.5–3.5 cm

The screening of substrates from nurseries for *Phytophthora* species needs urgent attention to reduce the number of false negatives. The promotion of an improved detection methodology will provide a more detailed understanding of *Phytophthora* distribution and potential invasion patterns on landscape scales, which is critical for effective conservation and management of ecosystems threatened by this pathogen.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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