Use, limitations, and interpretation of bench leachate baiting tests on nursery stock produced following the *Phytophthora* BMPs

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Version: 1 October 2019

This document discusses sampling and other considerations related to using the bench leachate baiting method (http://phytosphere.com/BMPsnursery/test3_4bench.htm) to detect *Phytophthora* in batches of container nursery stock. In particular, we discuss how interpretation of negative test results (no *Phytophthora* detected) differs between plants produced through careful and consistent implementation of the best management practices (BMPs) for excluding *Phytophthora* from nursery stock (http://phytosphere.com/BMPsnursery/Index.htm) and plants produced without following these BMPs.

Phytophthora root rot symptoms in nursery plants

For many pests and pathogens that affect leaves and stems, visual inspections can be effective for identifying affected plants or groups of plants, so careful inspections can be used to reject affected plants. Unfortunately, visual inspections are largely ineffective for detecting Phytophthora root rots (Osterbauer et al. 2014). Infection occurs long before visible symptoms develop aboveground, so most diseased plants may not show visible symptoms. For example, Standish et al. (1982) showed that in nursery-grown *Juniperus* spp., foliar symptoms only became apparent when over 50% of the root systems were decayed by *P. cinnamomi*. We have observed that many Californian drought-tolerant native species (e.g., toyon, *Heteromeles arbutifolia*) growing under nursery conditions do not show obvious top symptoms even when nearly all their roots are decayed. This is related to the fact that plants are irrigated frequently in nurseries, usually with overhead irrigation. Drought tolerant plants even with only a few (or in some cases no) viable roots can remain green for an extended period under the wet, humid, and often partially-shaded conditions found in nurseries.

Top symptoms can eventually develop in *Phytophthora*-infected plants, especially in hot weather, but most symptoms produced on above-ground portions of plants are not unique to Phytophthora root rot. Symptoms can include dieback (including recent dead plants), off-color, stunting, and low vigor or reduced growth. The same symptoms may be associated with water stress due to inadequate irrigation or other factors. Some *Phytophthora*-infected plants develop stem cankers at the base. Although this is a more reliable indicator of *Phytophthora* infection, such cankers typically develop only when disease is very advanced and do not develop in all *Phytophthora*-infected hosts.

The initial symptom of Phytophthora root rot is decay of fine roots. Decay may or may not extend into larger woody roots. Careful root inspection can identify some potentially *Phytophthora*-infected plants at early stages of disease, but examining roots is difficult, time-consuming, and may be destructive. At early disease stages, many healthy-appearing roots will be present, and it may be necessary to pull apart or wash out the entire rootball to find symptomatic roots. Furthermore, dislodging soil and roots while removing plants from pots can inadvertently spread contamination unless plants are handled carefully according to phytosanitary protocols, making this an impractical method for inspecting large numbers of plants.

In many woody species, healthy and diseased roots may not show clear differences in color or appearance. It can also be difficult to distinguish between root turnover caused by abiotic issues (such as excessive heat or episodic drought stress) and roots that have died due to *Phytophthora* infections. Hence, even careful, thorough visual root inspections will likely not identify all likely *Phytophthora*-infected plants. Because symptoms are not definitive, roots suspected to have *Phytophthora* infections

need to be subjected to further testing (e.g., culturing, DNA-based tests) to confirm the presence of *Phytophthora*.

General limitations of testing for detecting diseased nursery plants

If inspection alone is inadequate, why don't we simply test nursery stock to determine if it is infected with *Phytophthora*? Various assays can be used to detect and identify *Phytophthora* species associated with nursery plants. These tests vary in difficulty, cost, sensitivity, and suitability for particular applications. However, caution is warranted because all testing methods can yield false negative results, i.e., *Phytophthora* is not detected when it is present. False negative results may arise for many reasons. All testing methodologies (e.g., immunoassay [ELISA], culturing, baiting, DNA-based methods) have limits in sensitivity and are subject to factors that can interfere with the tests. The quality, quantity, size, and condition of the sample, as well as the sampler's skill and experience level, can affect whether a pathogen is detected in a sample. False negative results may be obtained due to any of the following reasons:

- sample size is too small to capture detectable levels of the pathogen for the test being used;
- the amount of infection in the sampled plants is below detectible levels, e.g., because plants were recently infected;
- sampled plant(s) or roots are not infected, even though other nearby roots or plants are infected;
- infected roots are decayed by secondary organisms that interfere with detection of *Phytophthora*;
- *Phytophthora* species present does not infect baits being used or does not grow well on media used;
- the diagnostic test is run incorrectly or under conditions that reduce its efficacy.

Leachate baiting method sensitivity and limitations

Like other testing methods, the bench leachate baiting test (http://phytosphere.com/BMPsnursery/test3_4bench.htm) has inherent limits in sensitivity and the test is best used for certain applications. Currently, this test is the most efficient test available for detecting sporulating *Phytophthora* in a set of infected nursery plants. It tests the entire root systems of all plants in the test array for the presence of *Phytophthora* by detecting zoospores released from sporangia present in the root system during repeated irrigations. The test is nondestructive and does not expose other plants to inoculum beyond what may occur during normal hand watering. The test does not generate false positives if bait spots are confirmed by culturing. Culturing of bait lesions can identify which *Phytophthora* species are present.

The bench leachate test relies on the presence of *Phytophthora* sporangia on infected root systems. Sporangia can form quickly on infected roots (within 1-3 days), but are also relatively short-lived. Hence, conditions in the week prior to the test that influence sporulation can affect the sensitivity of the test. These include:

1. **Moisture regime before the test**. Sporulation occurs most readily when soil is moist, so if containers have been allowed to dry out excessively before the test, test sensitivity will be reduced, i.e., a false negative result may be obtained.
2. **Temperature regime before the test.** Moderate average soil temperatures (about 64-75 F) in the week before the test appear to be optimal for detecting a number of *Phytophthora* species. For some *Phytophthora* species that we have tested under controlled conditions, detection efficiency is significantly reduced if the previous week average soil temperatures are below 64 F. High water temperatures limit the length of time that zoospores are able to swim, so tests conducted under high temperature conditions can also have reduced sensitivity. If tests are conducted outside of the optimum temperature regime, at least some *Phytophthora* species can still be detected, but false negative results will become more common.

In addition, test results can be affected by the length of time that a plant has been infected.

1. If a plant has been infected only recently, e.g., by spores splashing from an adjacent or nearby container, the infection may not have had time to spread to enough roots to generate detectable amounts of inoculum. In this situation, an infected plant will generate a false negative result.
2. On the other end of the spectrum, plants that have been infected for an extended period may also generate low amounts of inoculum if few roots capable of supporting active sporulation are left. *Phytophthora* sporulates most readily on newly-infected roots. As the roots are killed, they are invaded by secondary microorganisms including *Pythium* species, fungi, and bacteria. The leachate test may still detect *Phytophthora* in this situation if the species produces oospores or chlamydospores that can germinate to produce sporangia or zoospores, but test sensitivity will be reduced. If the *Phytophthora* species present does not produce oospores or chlamydospores (e.g., *P. cambivora*), false negative results may be more likely when testing plants in which most or all of the roots have been dead for an extended period.

Beyond these factors, the sensitivity of the leachate test is limited by the overall concentration of zoospores collected in the zoospore collection vessel. If the zoospore concentration in the leachate is excessively diluted by leachate from plants producing few or no zoospores, *Phytophthora* will not be detected. Based on many controlled tests using known numbers of infected and noninfected plants, the test is likely to detect *Phytophthora* when the infection rate in the test array is at least 2.5% to 5% and other factors noted above are not limiting. This detection limit is also affected by the strength of the inoculum source. If the infected plant(s) in the array release low amounts of inoculum due to one or more of the factors discussed above, a false negative may result even if the infection rate is 5% or higher.

To maximize the detection efficiency of the test, we try to ensure that those factors that can be controlled are as close to optimal conditions as possible. Typically, the moisture requirements are met by normal irrigation practices, but if the watering schedule is reduced (e.g., if plants are dormant), we advise that that plants be irrigated during the last several days before testing. The optimum temperature range can be attained with no additional effort if testing is done during a period of appropriate weather. If ambient temperatures are cooler than optimal, plants can be moved to a warmer environment such as a greenhouse for at least 3 days before testing. Due to scheduling, it may be necessary to conduct some testing under suboptimal temperatures. When this occurs, it is important to realize that the false negative rate is likely to increase and interpret results with this in mind.

Testing should also be conducted when plants are well-established within a given container size. This provides a higher root density; a higher density of infected roots will produce more inoculum. Therefore, testing should occur before, rather than after, plants are moved into larger containers. Also, while small plants can be tested, it should be recognized that tests on plants with small root systems in large containers are likely to be inefficient due to the low density of potentially infected roots.

Finally, test efficiency is optimized by minimizing the number of plants in a test that are not infected (see http://phytosphere.com/BMPsnursery/test2_4benchleachsize.htm for a full discussion). Although it is not possible to visually detect which plants may be infected with *Phytophthora*, we can increase the odds of including infected plants in the test by selecting plants that may show even subtle symptoms (such as being smaller than average) or have other risk factors (more exposure to potential sources of contamination). This is referred to as biasing the sample to include infected plants (if they are present). Furthermore, limiting the number of plants in each test avoids over-dilution of inoculum, especially in groups of plants that do not show any apparent symptoms to guide selection (i.e., sampling bias would not be strong). This is done by running multiple separate tests with fewer plants rather than a single test with a high number of plants.

When test conditions are optimized to the degree possible and we are able to successfully bias the sample to include infected plants in the test array, the success rate (ability to detect *Phytophthora* when present in the tested plants) is still affected by the infection rate in the batch being tested. If the batch infection rate is 1% , selection bias has to be very strong to have a detection success rate greater than 50%. In contrast, if the batch infection rate is 5%, even a random (unbiased) test of 40 plants has more than a 50% chance of detecting *Phytophthora* (Figure 4, http://phytosphere.com/BMPsnursery/test2_4benchleachsize.htm).

Furthermore, we have also found that although the standard green pear bait used in the leachate test can detect a wide variety of *Phytophthora* species, some species do not infect pears readily and will be detected at reduced efficiency even under optimal test conditions. If the *Phytophthora* species present does not readily infect pears, detection efficiency may be much lower than expected and may be nearly zero for *Phytophthora* species that rarely infect pear baits.

Although a single positive result is definitive that *Phytophthora* is present in the tested array, a single negative result is not conclusive. If tests are conducted under conditions that permit at least reasonable levels of detection efficiency, greater confidence can be obtained by successive tests of the same material. For instance, if the detection efficiency of each test was 50% (1 in 2 chance of a false negative) the statistical chance of two false negatives from two different tests of the same material is 25% (1 in 4).

The process of conducting the leachate test maximizes opportunities for zoospore release. Even if the number of zoospores leached from containers during the test is too low to be detected, the test conditions may increase opportunities for zoospores to infect other roots within the container. A second test, conducted at least a week after the first, could have a greater likelihood of detection if sporangia are produced on newly infected roots. Hence, repeated negative test results from the same set of plants provide better confidence than a single negative test. It should be noted that although the prolonged irrigation period during the test may promote additional root infections in plants that are already infected, they have no effect on a healthy plant. If *Phytophthora* is not present, tested plants simply get a generous irrigation that does not adversely affect plant health.

Application of the bench leachate method

Plants produced following Nursery *Phytophthora* BMPs

A comprehensive set of best management practices for producing nursery stock free of *Phytophthora* (nursery *Phytophthora* BMPs) has been developed. These are available from http://calphytos.org and in a version with more explanatory text at http://phytosphere.com/BMPsnursery/Index. These BMPs are incorporated into the "Nursery Evaluation Form for Systems Approach to Clean Nursery Production". This is an online spreadsheet form used by nurseries to document their compliance with the nursery *Phytophthora* BMPs in conjunction with review and inspection by a qualified evaluator. When implemented fully and consistently, the nursery *Phytophthora* BMPs effectively eliminate the possibility of *Phytophthora* contamination in nursery stock.

However, not every nursery is consistently in full compliance with the nursery *Phytophthora* BMPs. If critical departures from the BMPs occur, *Phytophthora* infections may occur. Such contamination events are more likely to occur in nurseries that also maintain plant material that is not BMP-compliant because such material can serve as a nearby source of contamination. Under the BMPs, nurseries conduct their own internal testing of nursery stock to monitor for these types of deviations, with bench leachate baiting as the preferred method for general monitoring. If an issue is detected, the nursery can take steps to identify the source(s) of the contamination, eliminate infected material, and reestablish clean production in affected areas.

If a nursery is doing a good job at implementing the BMPs and has a thorough internal testing program, there is a very low chance that BMP-compliant plant material will be infected. The objective of

predelivery testing by a client is to provide a final check that the plant material is free of detectable *Phytophthora*. The prospect of predelivery testing provides an added incentive to the nursery to be vigilant about BMP compliance and internal testing, because a *Phytophthora* detection near the delivery date is a highly undesirable outcome. Furthermore, because predelivery tests should represent at minimum a second test of the stock, predelivery testing increases the confidence level of negative results.

The practices noted above — including the use of sampling bias, limiting test size, testing when root density is high, and optimizing other testing parameters — help ensure that the predelivery tests are as sensitive as possible. No single negative result from a predelivery test is definitive, but if enough tests are conducted, significant contamination associated with departures from the BMPs are likely be detected. Negative predelivery test results can also be given greater weight if the nursery's own testing of the material also shows no *Phytophthora* detections. The nursery's documented adherence to the nursery *Phytophthora* BMPs, when combined with valid negative (no detection) test results, provides a high level of assurance that plants are free of *Phytophthora* to the maximum extent attainable. When used with BMP-compliant stock, testing helps increase confidence that stock is free of detectable *Phytophthora*, but testing plays a secondary confirming role rather than being the only basis for assessing plant health.

Non-BMP compliant nurseries

Bench leachate testing can be used to survey for *Phytophthora* in conventional nurseries that do not comply with the nursery *Phytophthora* BMPs. In many cases, such nurseries have relatively high *Phytophthora* infection levels, so detections are commonly made if testing is conducted under conditions that allow for good sensitivity. One issue that can reduce test sensitively in conventionally-produced nursery stock is the application of chemicals that suppress the activity of *Phytophthora* and other water molds (Oomycetes). The use of these chemicals, mostly registered systemic fungicides that are labeled for use against *Phytophthora*, is not permitted under the *Phytophthora* BMPs precisely because they can interfere with the detection of *Phytophthora* in testing procedures such as baiting (Shishkoff 2014). Because these chemicals do not eliminate infections or eradicate *Phytophthora* from infected plants or infested soil, fungicide-treated infected plants may remain undetected. This can ultimately result in greater spread of *Phytophthora* within the nursery.

Given the limitations of testing described above, it is not possible to use testing to reliably identify individuals or groups of non-infected plants from a generally contaminated nursery. In such nurseries, all plants are assumed to have a similar risk profile, and consequently similar infection rates or at least similar levels of exposure to contamination. Cross-contamination is likely to go on more or less continuously, so it is possible negative test results might be obtained from plants that are contaminated but not yet producing detectable levels of inoculum.

Non-BMP compliant plants within a BMP-compliant nursery

If a plant batch in a BMP-compliant nursery has tested positive for *Phytophthora*, it represents a situation that is analogous to that in a non-compliant nursery. By definition, a *Phytophthora* detection indicates that some significant departure from the BMPs occurred. Technically, the affected part of the nursery, which could be restricted to a single bench, is no longer BMP-compliant if *Phytophthora* is present in the stock. The *Phytophthora* BMPs outline a process for dealing with this situation if it develops. First, the entire batch is assumed to be infected at some unknown level because the plants in the batch have a shared risk profile. Adjacent lots that are not separated by barriers or adequate distances (as specified in the BMPs) are also at risk of cross-contamination and are quarantined for additional testing.

Additional testing of the affected batch and adjacent batches is used to obtain a better estimate of the infection rate within the contaminated stock and the distribution of contamination within and between benches. Other nursery factors, such as the distance between lots and benches, any past movement of the stock, irrigation patterns, and cultural practices (such as pruning) should be taken into account to help delineate which plants are at higher risk. Because of the high potential for spread of inoculum via splash

in tightly-packed plant arrays and the fact that newly-contaminated plants may not be detectable in testing, a single test cannot provide definitive information on the extent of the infestation. Multiple tests are needed, including repeated tests of material that has a higher risk of contamination based on detections. A combination of good nursery records, presence of gaps or barriers that minimize the risk of spread via splash, and data-driven testing are needed to delineate and eradicate the inadvertent *Phytophthora* contamination within the nursery.

A related situation exists in nurseries that have both BMP-compliant and noncompliant production within the nursery. The most common reason for noncompliance is use of non-heat-treated potting media, a critical departure from the *Phytophthora* BMPs. Other cultural BMPs may be followed, but the noncompliant plants have to be considered likely to be contaminated, and therefore potential sources of inoculum. Hence, noncompliant production needs to be physically separated from BMP-compliant plants as discussed in the BMPs.

If some *Phytophthora* BMPs are followed, noncompliant nursery stock may have somewhat lower levels of *Phytophthora* infection than stock produced under nursery practices that employ few or no BMPs, depending on the sources of contamination that exist in the nursery. If the contamination levels are low or nonuniform within noncompliant plants, it may be difficult to document whether plants in a batch are infected. A single negative leachate test result does not provide a high degree of confidence that plants are not infected because it is being used as the sole means to determine if the plants are free of *Phytophthora*.

To obtain a reasonable level of confidence, each batch should be tested in its entirety in relatively small test arrays that allow for high test sensitivity and at least two rounds of testing should occur. If all tests conducted under these parameters are negative (no detections):

- *Phytophthora* may not be present in the tested plants; or
- *Phytophthora* may be present but inoculum production is below detectable levels; or
- *Phytophthora* may be present but is a species that does not readily infect pear baits.

In the absence of information that all inputs have been pathogen-free through compliance with the BMPs, we have less confidence that the stock is actually free of *Phytophthora* even after extensive testing. This contrasts with the situation in fully BMP-compliant stock described above, in which BMP compliance provides the primary evidence that the plants are likely to be free of *Phytophthora*.

Conversely, if *Phytophthora* is detected within a batch, the entire batch needs to be considered to be contaminated at some level, under the assumption that all plants in the batch have the same risk profile. In most cases, only a percentage of the plants in a batch that tests positive will actually be infected. However, given the limitations of testing, it is difficult and usually uneconomical to conduct enough testing to determine which plants within a contaminated batch are free of *Phytophthora* with a high degree of confidence.

Conclusions

Testing for the presence of *Phytophthora* is an integral part of producing clean plant material under the nursery *Phytophthora* BMPs. Bench leachate testing is the best technique identified to date to conduct this testing. However, like all test procedures, bench leachate testing has limitations and test results need to be interpreted within the context in which the testing occurs. Greater confidence in negative (no detection) test results from predelivery tests is possible if the nursery can clearly document good BMP compliance, which includes their own internal testing of the same plant material. In a BMP-compliant nursery, the documented process of clean production provides most of the confidence that the stock is free of *Phytophthora*. Testing plays a supporting role to provide confirmation of the clean production process or, alternatively, to uncover problems that may have developed.

In contrast, in a noncompliant nursery or portions of a BMP-compliant nursery that have fallen out of compliance, we do not have a clean process to provide evidence that *Phytophthora* has been excluded from the stock. In this context, testing provides the only evidence that the material is free of *Phytophthora*. Because the detection rate from a single bench leachate test is much less that 100% when the infection rate in the tested batch is low, negative (no detection) test results from any single test of noncompliant stock cannot be viewed as definitive. To attain a reasonable level of confidence that noncompliant stock is at least largely free of *Phytophthora*, testing needs to be considerably more intensive, including repeated testing of the same material over time. Such testing can be used to estimate the contamination level within noncompliant stock, but the procedures are not designed to allow for reliable separation between infected and noninfected plants within a batch that is known to be contaminated with *Phytophthora*.

References

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