Best Practices for Plant Disease Diagnostic Laboratories

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1. Introduction

Best practices in plant disease diagnostics aims to ensure laboratories avoid false positive and false negative results of a diagnostic test. These practices can also help in the identification of the right disease-causing organism and not the secondary contaminating organisms. The Combination of best practice and best judgement can guide diagnosticians to accurate results by asking these questions:

- Is the tissue sample adequate and sufficient to diagnose disease and detect the causal agent?
- Does the diagnostic test under assessment accurately distinguish between a healthy and disease specimen?
- Is a test valid?
- Is the test fit-for-purpose?
- Can the application of this valid, accurate diagnostic test be replicated for similar specimens?

Best practices in plant disease diagnostics starts with sample collection and continues until the communication of the results with the client. It may even extend to recommendations of management measures.

A decision on the cause of plant abnormality should only be made by connecting the dots of symptom and sign confirmed on a sample by acceptable tests.

2. Steps in best practices

2.1. Sample collection and shipping

Labs should have instructions detailing these procedures

2.1.1. Instructions should include details on how much material, what type of material, and what plant parts to collect.

2.1.1.1. Specimens should be collected at all levels of symptom expression (mild to heavy) if possible.

2.1.1.2. Sample tissue should be taken from the junction of diseased and healthy tissue.

2.1.1.3. Care should be taken to prevent contamination of the sample from the environment and cross contamination between other samples.

2.1.1.4. Instructions should also be provided on how to store the sample before shipment such as refrigerated or not, plastic or paper bags, sealed or not.

2.1.2. Sample shipping instructions should include:

2.1.2.1. Sample shipping instructions should include:

2.1.2.1.1. Where to send the sample.

2.1.2.1.2. What information should be included with the with the sample.

2.1.2.1.3. How to bag the sample; e.g. put in a Ziptop plastic bag to prevent drying of sample, pressing of the sample between paper and cardboard.

2.1.2.1.4. When and how to send the sample?

2.1.2.1.4.1.1. Samples should be sent on the first or second day of the week to ensure delivery to the diagnostic laboratory before the end of the week. If the sample is collected later in the week the sample should be stored to best preserve the suspected pathogen type. Perishable specimens such as succulent leaves or herbaceous stems should be shipped using expedited or overnight mail especially during the summer months

2.2. Sample storage

2.2.1.The lab should have a dedicated space for sample storage that is separate from the spaces where test assays and media are stored and from the area used for sample triage and tissue plating. Samples should be kept separate from one another to prevent cross-contamination of the samples. This may be accomplished by using a physical barrier (e.g. bags, boxes, etc) or space. Samples should be stored in the lab according to the needs of each sample. Samples suspected of virus, bacterial, or insect disease causing agents should be refrigerated. Sample suspected of fungal disease causing agents should be kept at room temperature and if possible pressed between paper and cardboard for preservation. Moisture control should be a priority for all samples to prevent their decay and destruction. Potted plants and large trees may be kept at room temperature. Whenever possible samples should be kept according to the time they were received by the lab.

2.3. Sample triage

2.3.1. The sample should have its unique identification number, reception date, physical condition, and relevant initial observations recorded. Photographs of the sample may also be taken.

2.3.1.1. Initial observations:

2.3.1.1.1. Stippling- the plant sample should be checked under a stereo microscope for the presence of small insects (two spotted spider/broad mites, thrips, aphids, mealy bug, scales etc.).

2.3.1.1.2. Virus like symptoms (mottling, distortion, mosaic, etc)- the plant should be tested for virus. Testing for virus should be completed immediately.

2.3.1.1.3. Suspected environmental stress, nutrient imbalance or chemical injury- If whole plant sample is available a bioassay should be performed. If only a portion of the plant was submitted the client should be contacted about sending a whole plant for bioassay testing. If a plant specimen is still intact the plant may be planted and kept under observation to check if the new growth is symptom-free.

2.3.1.1.4. Suspected fungal infection- the sample should be checked for the presence or sign of conidia, spore, fruiting body or intact organism. If these structures are not readily available, incubation of subsample containing healthy and diseased area should be done in a moist chamber.

2.3.1.1.5. Suspected bacterial infection- the sample should be checked for the presence or sign of a bacterial infection. This should include checking for bacterial streaming in tissue collected from the water-soaked, halo area around a leaf spot or blighted tissue area. If bacterial streaming is detected, a small tissue sample should be homogenized and streaked out on nutrient agar media to derive single colonies. Single colonies should be further tested on selective or semi-selective differentiation media specific for a genus of interest.

2.4. Microscopy

2.4.1.Equipment

2.4.1.1. Microscope should be cleaned and serviced annually. If using measurements for diagnostics, a calibrated microscope reticle has to be used. Calibrations on camera software (Leica, Olympus etc.) is also recommended.

2.4.2.Fungi and Oomycetes

2.4.2.1. Culturing out from host tissue material

2.4.2.1.1.1. Direct observation under stereoscope

2.4.2.1.1.2. Tape mount – Clear tape is used on surface of host material to grab easy to dislodge structures. Tape is placed on a slide containing a drop of water or glycerin.

2.4.2.1.1.3. Slide mount

2.4.2.1.1.3.1. Remove fungal structures using flame sterilized tweezers or dissection needle and place on a glass slide containing water, glycerin, or 85% lactic acid, cover with a glass cover slip. Tap cover slip gently to remove air bubbles and break up structures. Placing

the slide on a slide warmer set to 50°C for a few minutes can help in clearing structures and improving visibility of important structures.

2.4.2.1.1.3.2. To increase visibility and contrast, stain structures with an appropriate stain. Cotton blue or lactophenol blue are recommended for hyaline fungi. Staining can negatively impact identification if too much stain is used or it obscures natural pigmentation.

2.4.2.1.1.3.3. Photos should be taken with a camera attached to the microscope and store on the sample electronic folder.

2.4.2.1.1.4. Water Float/Baiting: Dilute the soil sample appropriately and surface sterilize baits before placing on the surface of suspension

2.4.2.1.1.5. Moist chamber

2.4.2.1.1.5.1. If no fungal structures are observed, a subsample should be placed in a moist chamber. The subsample should contain both healthy and symptomatic tissue. The moist chamber should consist of a labeled sealed container and lightly moistened paper towel. The sample should be checked after 24 hours for growth. If no growth is observed the moist chamber maybe continued, with the sample being checked daily for growth.

2.4.2.2. Diagnostic measurements

2.4.2.2.1. Measurements should be taken and compared with reference materials to confirm that the size is consistent.

2.4.2.2.2. The best resouces to use to when confirming an identification are the original descriptions, illustrations and images of the type fungi. These literature references can be found on Mycobank and the ARS databases. What compendia/articles are the “gold standard”?-APS compendia, photos available online from other diagnosticians or extension specialist

2.4.2.2.2.1. Compendia and other sources of information are best used as screening aids.

2.4.3.Bacteria

2.4.3.1. Extraction from host material

2.4.3.1.1. Check for bacterial streaming (see detail 2.3v , in case of doing this with stem, take the stem piece very close to the ground/crown area). Avoid getting tissue contaminated with soil bacteria.

2.4.3.1.2. For leaf lesions, cut through the water soaked lesions that are new

2.4.3.2. Biochemical tests such as gram staining, KOH test, ability to grow at different oxygen levels, ability to utilize different nutrient sources, production of fluorescence, oxidase activity detection, etc.

2.4.4.Nematodes

2.4.4.1. Extraction from host material (source: EPPO PM 7/119 Nematode Extraction https://onlinelibrary.wiley.com/doi/full/10.1111/epp.12077)

2.4.4.1.1. Vermiform extraction from plant tissue

2.4.4.1.1.1. Plant tissue suspension in water method

2.4.4.1.1.1.1. Roots, tubers and bulbs should be washed free of soil. Seeds, foliage and wood chips do not need to be washed. Add tissue to petri dish or erlenmeyer and fill with sterile water. Tear apart tissue using an appropriate tool such as scalpel, blade or knife. Observe nematodes on compound scope.

2.4.4.1.2. Baermann funnel/Oostenbrink dish method

2.4.4.1.2.1. Roots, tubers, bulbs, foliage, and wood should be chopped into approximately 1cm pieces. Seed may be left whole or cut. Plant issue is placed on top of cheese cloth/ filter paper in sieve. Water is added to the funnel/ dish until covering plant material. Sample preparation should be collected and counted after 24 to 72 hrs.

2.4.4.1.3. Vermiform Extraction from soil: take rhizosphere soil of a suspected plant and process/extract sooner. Soil should be stored at 5-8°C

2.4.4.1.3.1. Baermann funnel/Oostenbrink dish method

2.4.4.1.3.1.1. Create a slurry of soil and water. Rinse slurry through a series of three sieves (#20, #60, and #325) with water. After water running through the bottom sieve (#325) is clear, collect the remaining material from the bottom sieve and add it to the top of the cheese cloth/ filter paper in sieve. Water is added to the funnel/dish until covering the material. Sample preparation should be collected after 24 to 72 hrs.

2.4.4.1.3.2. Cyst nematode extraction from soil

2.4.4.1.3.2.1. The soil sample should be representative of the field from which it was taken and should from the depth where the nematodes are expected to be found.

2.4.4.1.3.2.2. For dry soil methods: soil should be thoroughly dried before extraction with large clumps broken up and the sample thoroughly mixed.

2.4.4.1.3.2.3. Baunacke method

2.4.4.1.3.2.3.1. Rinse the dried soil through a #60 sieve. You can place a larger sized sieve (ex. #20) on top of the #60 sieve to remove larger debris. Once the water running from the bottom sieve is clear, the material in the sieve should be transferred to a beaker with water. Mix the suspension and allow to settle. The cysts can be removed from the top of the water using a dissecting scope. Alternatively, the mixture could be poured into a petri dish or a square gridded petri dishes with water for easier observation. Cysts can sink and be missed if they're in a taller beaker.

2.5. Culturing

2.5.1.Equipment

2.5.1.1. Incubator

2.5.1.1.1. Temperature monitoring should be performed to make sure incubator is working as specified.

2.5.1.1.2. Light (light and dark hour) should be optimum to enhance production of reproductive structures. Incubate plates under near UV (black) light if needed

2.5.1.2. Autoclave-regular autoclave tests should be performed to document that the autoclave is functioning properly.

2.5.1.2.1. Depending on your media, select dry/liquid cycle and optimum exposure time and repetition as needed

2.5.1.2.2. Safety: When opening autoclave after cycle operator must make sure the pressure has been released, open the door slowly and wearing autoclave gloves. Media bottles should be loosely capped.

2.5.1.2.3. Antibiotics and other chemicals should be added to media after it has cooled to 50°C.

2.5.1.3. Biological Safety Hood – the hood should be certified annually

2.5.1.3.1. Sterilize the hood by turning on UV light for 30 minutes or wiping with 70% ethanol before and after each use

2.5.1.4. Balance – the balance should be regularly checked with a calibrated weight to ensure accuracy.

2.6. ELISA

2.6.1.Equipment and reagents

2.6.1.1. Pipettes – pipettes should be calibrated annually

2.6.1.1.1. Pipettes should meet the volume range used on the protocol

2.6.1.2. Coated ELISA plate

2.6.1.2.1. Known positive and negative/buffer controls should be included in each plate. The controls should be positioned on a plate to avoid cross-contamination of the test samples during the process of ELISA.

2.6.1.2.2. Healthy plant control extracts should be included on each plate, especially if using a new host material. Inclusion of the healthy plant extract will allow operator to eliminate background noise created by the antigen present in different host tissue.

2.6.1.3. Plate reader: If you choose to use plate reader, make sure it is calibrated and data can be processed by appropriate software. Make sure to save the data on each sample's electronic file.

2.6.1.3.1. Calculation guidance and "threshold" setting

2.6.1.4. Take a photograph of the developed ELISA plate. This is important especially if you do not use a plate reader to record data.

2.6.2.Virus, Fungi, Bacteria, and Oomycetes

2.6.2.1. Extraction from host material

2.6.2.1.1. Plant tissue should be selected based on kit instructions.

2.6.2.1.2. If using non-standard plant tissue for the first time, results of non-standard tissue should be compared with standard plant tissue results from the same sample. If comparable, then verification of the non-standard tissue has been completed for that host/tissue type.

2.7. Lateral Flow Device (LFD)

2.7.1.Equipment Needed

2.7.1.1. Pipettes –should be calibrated annually

2.7.1.1.1. Pipettes should meet the volume range used on the protocol

2.7.2. Virus and other pathogen groups

2.7.2.1. Extraction from suspected host material should include exact suggested amount of tissue. Maceration of tissue should be done with different tools based on the tissue type such as succulent, woody, leaf, stem. Positive control and healthy sample should be included to rule out false positive and negative.

2.7.2.2. Read the technical note included with the specific test to be cognizant of potential cross reactions / false positive.

2.7.2.3. Make sure the correct dilution specified in the manufacturer instructions is achieved.

2.8. Bioassay

2.8.1. Fungal pathogen: Inoculation of plants should be made with spore suspension originating only from fresh colonies followed by incubating at a disease conducive environment (optimum temperature, high relative humidity). Adding a few drops of surfactant (Tween-20, Silwet L-77, Soltrol 170) with spore suspension ensures spores can stick on the host surface. If woody stems are inoculated with fungal plug, it should be placed underneath the bark followed by wrapping with parafilm to prevent drying up.

2.8.2. Bacterial pathogen: Plants that are used for confirming soil contaminated with a certain type of bacterium should be infection free, as such starting with a tissue culture plant that is highly sensitive to the disease. For example, use of tissue cultured ginger seedling for bioassay of soil contaminated with *Ralstonia solanacearum*.

2.8.3. Virus: Indicator plants such as Nicotiana (tobacco) and Chenopodium (lambsquarter) can be used if symptom on a host is suspected but symptom is not typical or strong enough. Extraction of virus particles should be done in buffers having pH ~7.0. Using abrasive material such Celite and charcoal or carborundum may be necessary for effective inoculation (https://doi.org/10.1111/j.1744-7348.1945.tb06240.x).

2.8.3.1. Indicator plants should be grown under low light and high humidity to keep them 'softer' that

3. Communicating diagnostic results with clientele

Conclusive results should be sent to the sample submitter as well as the actual client where the sample originated through most secured channel. In many cases, clientele may expect recommendation for remedial measures. In that case, recommendations should be made by an expert who has the authority to do so and accompany a disclaimer if any specific products are recommended. What detailed results will be included in the communications should be dependent on expectation of the clientele. Research sample results should include enough information on the causal agents and tests performed so that client can draw some inference on the source of the problem and can take precaution in the future to prevent similar problem. If samples are from growers who are more interested in receiving a timely recommendation, detailed results and description of tests are not necessary.