Could DNA-Based Detection Technology Help Prevent Conifer Seed-Borne Pathogen Diseases?

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Abstract

Pathogens, often carried in seeds, can cause substantial economic losses to forest nurseries and put at risk the large investment in genetically selected seeds, as well as endanger reforestation efforts and, therefore, future forests. Mitigating plant health problems relies on rapid detection and identification of causal agents. Traditional detection protocols, however, rely on symptom manifestation, but many fungal pathogens exhibit a prolonged asymptomatic phase within their hosts. DNA-based detection assays based on the real-time polymerase chain reaction (qPCR) are among the most accurate, rapid, and cost-effective methods for detecting pathogens at the species level. The development of a DNA detection system for seed-borne pathogens would increase accuracy and speed in determining if seedlots are contaminated above an acceptable level and would help forest nurseries to make cost-effective management decisions. This paper was presented at the 2019 Joint Annual Meeting of the Forest Nursery Association of British Columbia and the Western Forest and Conservation Nursery Associations (Sidney, BC, September 30-October 2, 2019).

Introduction

North American forests must meet the public's socio-ecological and economic needs while simultaneously overcoming contemporary health challenges. To answer reforestation demands, British Columbia (B.C.) annually produces more than 200 million conifer seeds and seedlings with improved growing performance. However, adverse climate, pests, and diseases represent major threats to the sustainable supply of forest tree products. Seeds and seedlings are especially susceptible to diseases, which can be exacerbated by their environment, such as extreme temperatures, and water and mechanical stresses. As a consequence, seeds and seedlings also constitute a pathogen source representing an inconspicuous, yet significant, phytosanitary risk. For example, nursery trade of asymptomatically infected white pine (*Pinus strobus* L.) seedlings resulted in the introduction of the white pine blister rust (*Cronartium ribicola*) at the beginning of the 20th century in North America (Geils et al. 2010). Similarly, the trade of pine and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seeds and planting stock is of high risk, as these could contribute to global spread of the pathogen *Fusarium circinatum*, the fungal agent responsible for pine pitch canker (Cleary et al. 2019, Evira-Recuenco et al. 2015, Storer et al. 1998;). This aggressive fungus, recommended for quarantine regulation in Europe (Vettraino et al. 2018), is present in the United States and is a potential threat to Canadian forests.

In the current context of trade globalization, the risk of disseminating non-native and potentially invasive pathogens via seed exchange is increased (Burgess and Wingfied 2002, Cleary et al. 2019, Elmer 2001, Franić et al. 2019). Replacing genetically improved seed trees can take years, and the high cost of producing these seeds makes even small losses due to disease unacceptable. Seed and seedling diseases can cause substantial economic losses to nurseries and endanger reforestation efforts, and, therefore, future forests. With increasing seed losses reported across B.C. forestry nurseries, it is critical that we improve our capacity for identifying, detecting, and mitigating seed and seedling diseases to secure

the renewal of tomorrow's forests. Therefore, new approaches and tools are needed to prevent and mitigate seed and seedling losses that are emerging at critical phases within the forest renewal cycle.

Forest Seed Pathology: Battling the Unknown

A plant disease prevention program relying on pathogen detection requires, as an initial step, a priori knowledge of which organisms to target. Unfortunately, there are fundamental knowledge gaps in the current understanding of the microbial pathogens responsible for conifer seedling losses. Despite their historical and contemporary significance, the basic etiology and transmission processes of many important pre-emergent and post-emergent conifer seedling diseases remain poorly understood. Root rot and damping off of seedlings are among the most frequent diseases observed in tree nurseries, and are responsible for massive crop failure and economic losses. However, while root rot and damping off have been attributed to many common rhizosphere (root and soil) fungi (e.g., *Fusarium* and *Cylindrocarpon* spp.) and oomycetes (e.g., *Pythium* spp.), only a few species have been clearly associated with conifer diseases (Kope et al. 1996, Rossman et al. 2007).

Fungal species of the *Fusarium* genus are important causal agents of damping off and root rot in Douglas-fir seedlings. These species are known to be ubiquitous in most container and bareroot nurseries, occurring in soil, seeds, and roots and needles of asymptomatic and diseased Douglas-fir seedlings (Alexrood et al. 1995, Stewart et al. 2012). However, the biology of the diverse *Fusarium* species associated with Douglas-fir seeds and seedlings is still poorly explored. Particularly, the mode of infestation of Douglas-fir seeds remains uncertain. Two possibilities are that seed colonization occurs through systemic invasion from the mother plant vascular tissues to the embryo, or from seed-coat surface contamination from exterior cone parts. Historically, *F. oxysporum* was considered the most important cause of Fusarium root rot in Douglas-fir seedlings, but a direct relationship between this species and seedling mortality was never convincingly demonstrated. Moreover, specific strains of *F. oxysporum* are known to be benign to Douglas-fir seedlings and can even protect them from other virulent *Fusarium* species (Dumroese et al. 2012). Recent studies indicate that the highly virulent *F. commune*, a species closely related to, but distinct from, *F. oxysporum* is probably one of the major causes of disease in Douglas-fir seedlings (Kim et al. 2012, Stewart et al. 2012). That species, however, does not seem to be ubiquitous among conifers in forest nurseries, making it unlikely that it is the only problematic seed-borne *Fusarium* species in Douglas-fir. In a preliminary survey of 67 Douglas-fir seeds, we identified seven *Fusarium* species, including *F. proliferatum* (pathogenic on Douglas-fir seedlings) and *F. oxysporum*, but we never found *F. commune* on Douglas-fir or any of the four other conifer species included in the survey (figure 1).

Usually, a contamination level by *Fusaria* of greater than 5 percent within any conifer seedlot is considered to be significant for disease potential, and may consequently provoke pest-management actions (Peterson 2008). Systemic infestation of conifer seed, as well as surface contamination during seed development and management, by pathogenic *Fusarium* species makes testing for their presence a relevant step for managing it as a seed-borne organism (Peterson 2008). The presence of *Fusarium* on seedling roots in the absence of any disease symptoms is generally insufficient grounds for rejecting seedlings scheduled for outplanting. Potentially pathogenic fungi can, however, rapidly spread from seedling to seedling, as well as intensify within the roots of infected seedlings (Kope et al. 1996). When outplanted, systemically infected seedlings can have reduced performance and quickly succumb to planting shock and, if exposed to a subsequent heat or drought stress, will often die.

Management of Fusarium disease in forest nurseries could be greatly enhanced by accurate identification of the *Fusarium* species. Given their diversity and their functional variability, a clear identification of problematic species and establishment of causality between the presence of seed-borne species and seedling disease is required. Being able to differentiate pathogenic species from innocuous ones would enhance early-stage testing, thereby allowing rejection of seed lots contaminated with truly pathogenic species.

Opportunities for Development of a Pathogen-Detection System

A traditional approach to the complex problem of identifying and detecting fungal pathogens uses classical phytopathological concepts that rely on the combination of culture-based surveys and microscopy techniques. Although reliable, such approaches require high standards of knowledge in mycology and plant pathology, and are usually time-consuming, necessitating, in some cases, several weeks before being able to establish a proper diagnosis. So far, testing for conifer seed health in B.C. is carried out by the Plant Health Laboratory of the B.C. Ministry of Agriculture (Abbotsford, BC) using a culture-based method. The assay consists of plating and incubating subsamples of seeds on fungal-specific media and is successful in testing seedlots for the presence and rate of infection of three major seed-borne pathogens, including *Fusaria*. Identification of the cultures, however, relies only on morphological characters of spores, thus limiting the identification of *Fusarium* pathogens to the genus level only. Another limitation of cultural approaches is that they target fungi

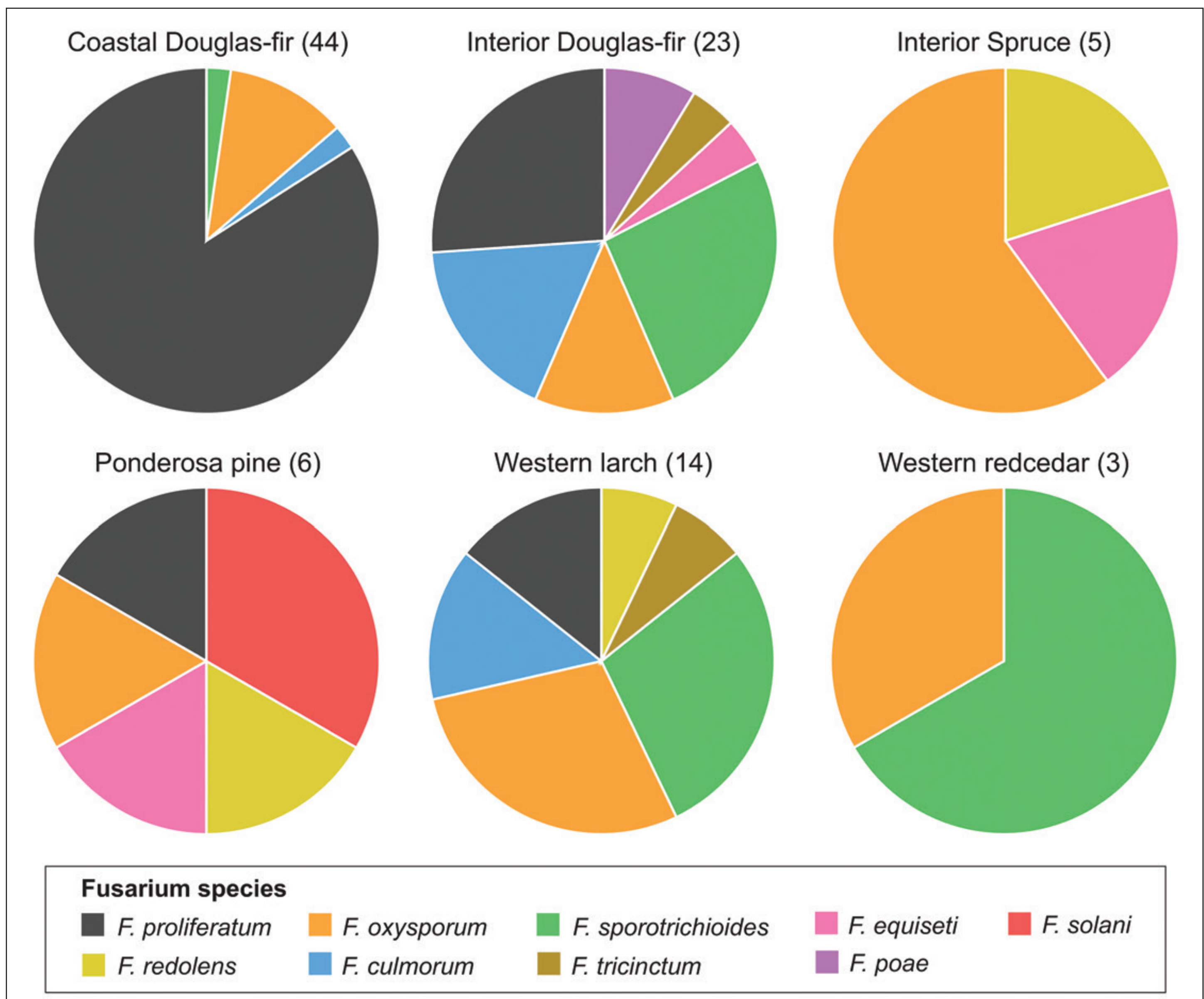

Figure 1. *Fusarium* species isolated from a preliminary survey of conifer seed-associated fungi in B.C. seedlots. *Fusarium* cultures were obtained by plating seeds on *Fusarium*-specific media at the Plant Health Laboratory of the B.C. Ministry of Agriculture (Abbotsford, BC) and cultures were identified using a DNA-barcoding approach. Numbers between brackets indicate the number of seeds tested for each conifer species.

that produce fruiting bodies or can be easily cultured on synthetic and semi-synthetic growth media. Many fungi (particularly endophytes; e.g., White and Cole 1986) do not sporulate in culture, making visual identification challenging. Many other fungi are notorious for being difficult or impossible to cultivate on culture media; good examples are the powdery mildew and smut fungi.

Over the last few decades, several new approaches have been developed for plant pathogen identification and detection. The use of monoclonal antibodies and enzyme-linked immunosorbent assays (ELISA) drastically increased the speed in which pathogen antigens could be detected in vivo. For example, this technique has been used for routine detection of the seed-borne fungal pathogen *Sirococcus conigenus* in spruce (*Picea* spp.) seedlots (Mitchell and Sutherland, 1986). However, ELISA assays have three major limitations: (1) immunological tests require the availability of an antibody that properly responds to a target pathogen; if it is not available, this requires extensive work to develop such an antibody; (2) the antibodies used to recognize proteins that are supposed to be unique to the targeted organism can sometimes cross-react with other species, resulting in false positives and therefore a lack of specificity (Kox et al. 2007, Luchi et al. 2020, Martinelli et al. 2015); and (3) antibody-based tests often lack sensitivity, which is frequently a problem when dealing with plant pathogens.

Innovations in genomics and molecular biology have provided a new toolbox that can address pathogen identification and detection challenges. The polymerase chain reaction (PCR) generates in a single in vitro reaction several million copies of "diagnostic" DNA region(s) located on the pathogen's genome. This method has the advantage of being sensitive, specific, and quick. PCR-based tests can be conducted by a broad range of users because they require less knowledge and expertise in mycology than classical culture-dependent approaches. In the context of plant-pathogen diagnostics, PCR-related methods can be used in two ways, i.e., for pathogen identification and for pathogen detection. The DNA-barcoding method uses a short, standardized DNA marker providing a high, interspecific variability (i.e., the sequence is different from those found in individuals from other species) and low intraspecific differences (i.e., the sequence is identical in individuals of the same species) that enables the identification of organisms at the species level (Hebert et al. 2003). Usually, the selected marker is present in several copies (e.g., about 100 tandem repeats per nucleus for the nuclear ribosomal internal transcribed spacer locus [ITS], formally selected as the universal DNA-barcode marker for fungi; Schoch et al. 2012) in the genome of the targeted organism, allowing a high sensitivity of the PCR amplification. DNA-barcoding has proved to be effective in identification and surveys of forest pathogens (Feau et al. 2009, 2011; Hidayati et al. 2014; Shestibratov et al. 2018).

The genetic variation within DNA barcodes has also been widely translated into taxon-specific rapid and sensitive detection assays using PCR and has been applied to forest pathogen detection (Vincelli and Tisserat 2008). Specifically, real-time PCR using TaqMan probes has become the gold standard in forest pathogen detection. The principle of this technology relies on a fluorescently labeled probe designed so that it hybridizes only to its target DNA sequence and releases a fluorescent signal (detected by the PCR machine) when the target site is amplified during PCR. TaqMan real-time PCR constitutes the most sensitive, specific, and rapid method available, and has been used to detect many forest pathogens in quantities as low as one single fungal spore (Bergeron et al. 2019, Feau et al. 2019, Lamarche et al. 2015).

Using real-time, PCR TaqMan technology has several benefits. The increase in genomic resources brought by next-generation sequencing makes it possible to mine entire genomes of plant pathogens to identify genes or genomic regions of higher discriminatory power than the conserved genes traditionally used to develop real-time PCR assays. Once identified, these unique genes can be translated into TaqMan probes of high specificity, reducing the risk of false positives (Feau et al. 2018). Another advantage of TaqMan-based detection is the possibility of combining (i.e., "multiplexing") several probes in the same PCR reaction. Probes can be labeled with different distinguishable fluorophores, which allows amplification and detection of two to four distinct sequences in one reaction tube. Probes targeting different taxonomy levels (e.g., *Fusarium proliferatum* species; *Fusarium* genus; Nectriaceae

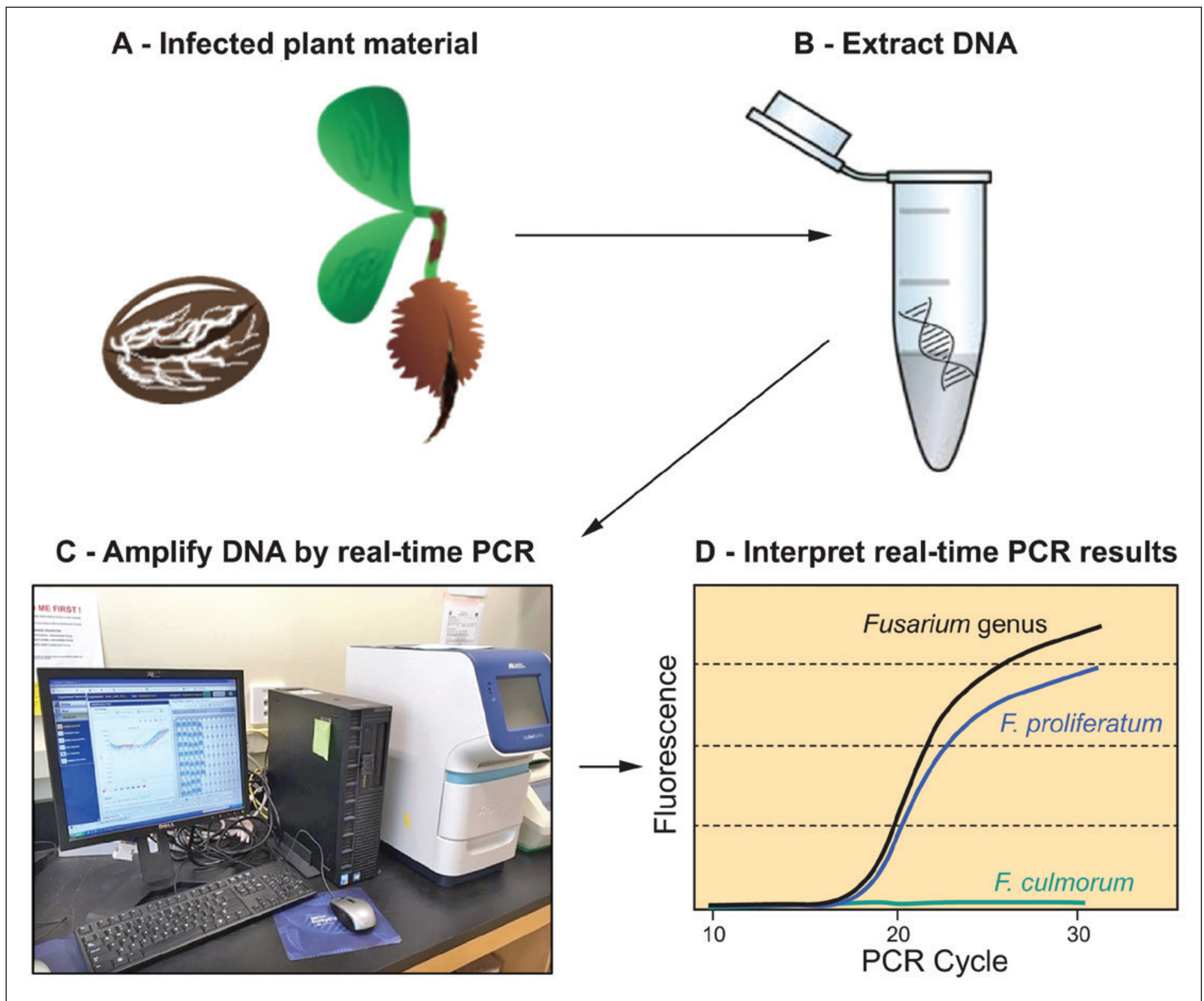

Figure 2. Pathogen detection theoretical workflow. (a) Infected material such as germinated and non-germinated conifer seeds is identified and collected, then (b) DNA is extracted from the infected material. (c) The extracted DNA is amplified by PCR in a real-time PCR machine. (d) TaqMan fluorescence curves produced in real-time PCR for three multiplexed detection assays tested on a DNA sample extracted from seeds. Each curve represents the accumulation of a PCR product (for a targeted gene) over the duration of the real-time PCR experiment. The test is positive for the *Fusarium* genus and *F. proliferatum* with an exponential accumulation of PCR products starting after 17 PCR cycles; for *F. culmorum*, no PCR product is accumulated, meaning that DNA of this species was not present in the tested sample. This illustrates the advantages of using multiplexed detection assays: three detection assays are combined in one reaction tube; two *Fusarium* species are targeted at the same time; two taxonomical levels are targeted (genus and species). (Photo by Nicolas Feau 2019)

family) can be combined together in a hierarchical way, providing vertical redundancy (figure 2). Another advantage of multiplexing is to increase redundancy by combining probes with different discriminatory powers. Confidence and reliability of the detection will increase by multiplexing probes of high sensitivity (by targeting a multicopy locus such as the ITS region) with species-specific genes providing a high-detection specificity (Feau et al. 2018, 2019). Multiplexing also allows querying different genome regions with different discriminative power. In the same reaction tube, sensitivity can be increased by targeting a multicopy gene, such as the ITS locus, while specificity of the detection can be achieved by targeting a species-unique gene.

Making a DNA-Based Detection System Operational for Seed-Borne Pathogens

A detection assay targeting seed-borne pathogens should be sensitive, specific, rapid, robust, inexpensive, and simple to implement and interpret (Maddox 1998, Walcott 2003). Depending on the probes designed, real-time, PCR TaqMan technology can provide a high degree of specificity, sensitivity, or both combined. The detection accuracy, however, will still depend on conditions that are inherent to the type of material tested (in this case, conifer seeds and the quality of the fungal DNA purified from these seeds). DNA quality is critical for the overall success of the detection test. Real-time PCR can suffer from the interference with inhibitory compounds found in seed extracts (Demeke and Jenkins 2010). Particularly, the yield and quality of the fungal DNA purified from conifer seeds can be limited by a high content of secondary metabolites. These compounds either impede DNA extraction or limit DNA polymerase activity (Bashalkhanov et al. 2008, Wilson et al. 1997), leading to false negative results. To overcome this problem, several protocols have been developed for plant and conifer tissues to separate pathogen DNA from inhibitory compounds and optimize PCR reaction conditions. For example, the combination of a DNA-enrichment procedure with quantitative PCR (qPCR) facilitated the sensitive detection of *Fusarium circinatum* from pine seeds (Ioos et al. 2009). Other solutions to this problem have been proposed for detection of *F. circinatum* (Dreaden et al. 2012), *Lophodermium seditiosum* (Bentele et al. 2014), and *Diplodia sapinea* (Decourcelle et al. 2015) in seeds. False-negative results caused by PCR inhibition can also be prevented by using a PCR internal control (e.g., a heterologous DNA template with priming sites identical to one of the primer pairs and probe used for the amplification) (Decourcelle et al. 2015, Ioos et al. 2005).

Poor PCR-based detection sensitivity also can result from low sampling intensity. Sample size and sampling methods used for other seed health tests are not necessarily appropriate for PCR-based tests as they might affect the quality and quantity of DNA extracted from seeds and, consequently, the result of the PCR test. One way to address this issue is to first determine the level of tolerance for the target pathogen in a seedlot at which the seedlot is considered to be significantly contaminated (Peterson 2008). Direct testing on seedlots can be done by extracting DNA and testing several individual seeds to determine if this threshold is reached. However, large numbers of seeds need to be tested to reduce the probability of having a Type I statistical error (accepting a seedlot with an actual greater level of contamination than the threshold) or a Type II statistical error (rejecting a seedlot with an actual smaller level of contamination than the threshold), making this approach economically unrealistic. Alternatively, indirect testing on sample units with a determined number of seeds can be carried out to determine if the contamination threshold is reached (Geng et al. 1983). Indirect testing minimizes the number of samples tested (by grouping seed samples in sample units) without affecting the efficiency of the DNA-based detection test. DNA is extracted from several sample units, each having a predetermined number of seeds, and the DNA extract of each sample is tested with the DNA-based detection assay. Several studies showed that fungal DNA can be efficiently extracted and detected from batches of 300 to 400 conifer seeds (Decourcelles et al. 2015, Dreaden et al. 2012). Geng et al. (1983) developed a statistical model providing the size and number of seed units needed as a function of an expected contamination threshold and the sensitivity of the test used (figure 3). For example, supposing that the tolerable disease rate threshold is 5 percent and that the sensitivity of the DNA-based test is 95 percent, we would need to test 6 sample units of 35 seeds each to be 99.99 percent confident that at least one test will result in a positive detection. With a sensitivity of 99 percent (which is more realistic for a DNA-based test), the number would drop to 5 sample units of 30 seeds each to reach the same confidence. Assuming an approximate cost of US$8 per PCR test (including the DNA extraction), testing one seedlot would cost less than US$50.

Conclusion and Perspectives

Traditional laboratory methods for tree pathogen diagnostics are accurate but slow and labor- intensive, requiring specialized personnel with mycological and plant pathology skills. Unfortunately, the availability of such trained staff to perform traditional techniques is in decline worldwide. DNA-based

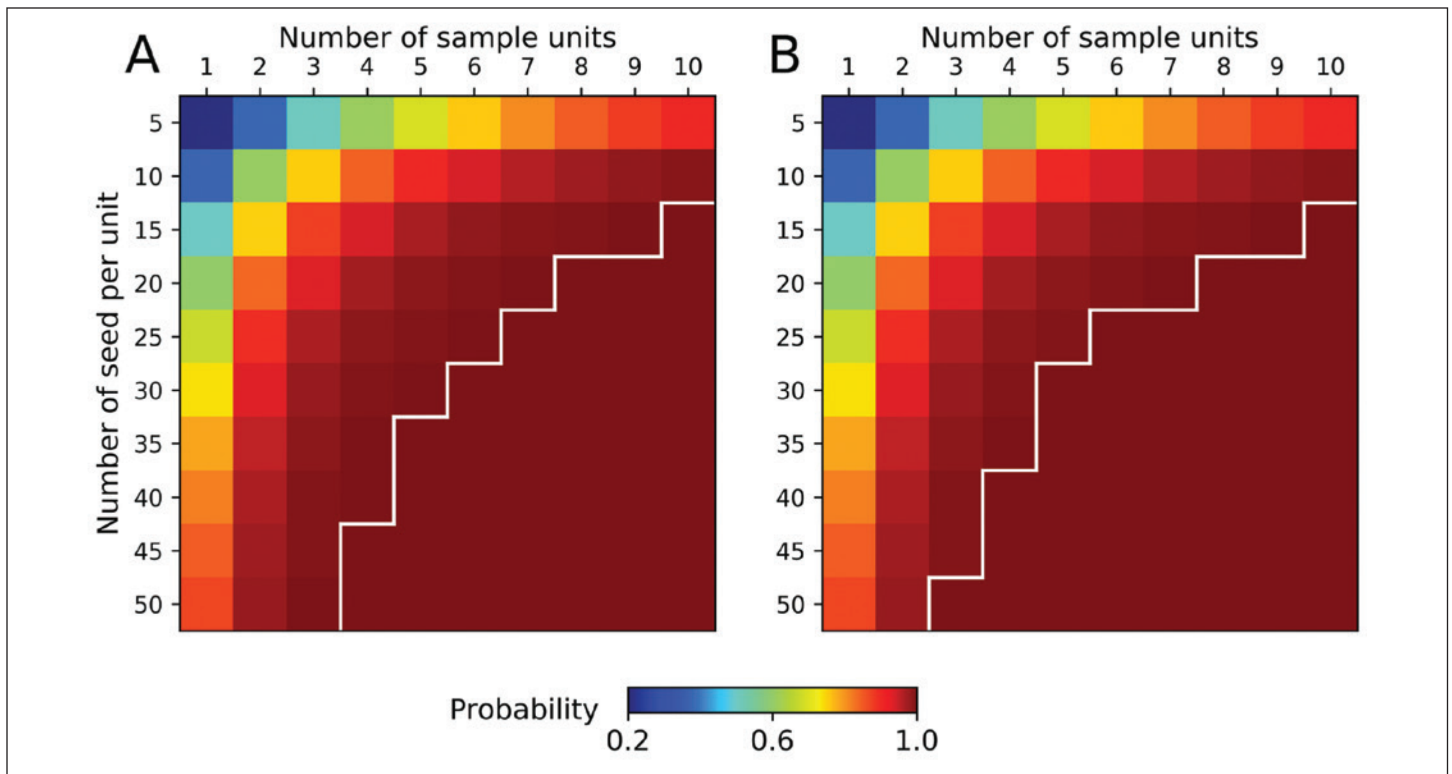

Figure 3. Probability of having at least one test resulting in a positive detection for different combinations of sample units and seeds per sample unit with an assay having a detection sensitivity of (a) 95 percent or (b) 99 percent. Combinations on the right of the white line have probabilities greater than or equal to 99.99 percent of resulting in at least one positive test. (Adapted from Geng et al. 1983)

technology has proven its utility in the rapid identification and detection of plant and forest tree pathogens. Major research progress has been made since the first development of DNA-based diagnostic tests to improve confidence in their results. Real-time PCR has become an established technique for the detection of known target pathogens due to its robustness and accessibility in high-throughput format. This accessibility and the popularity of this technology has driven down costs; real-time PCR is now a generic platform technology in plant diagnostic laboratories, usually exploited as a front-line diagnostic tool in plant health. We envision that this technology holds great potential for improving pathogen detection in conifer seeds, as it embodies many of the key characteristics including rapidity, specificity, sensitivity, and ease of implementation for routine testing on a diagnostic platform. With the implementation of PCR-based seed health testing in the seed and seedling industry, we can expect that this technology will eventually replace the seed detection assays currently employed, providing superior detection capabilities necessary for healthy seedling establishment.

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