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A five-minute DNA extraction method for expedited detection of *Phytophthora ramorum* following prescreening using *Phytophthora* spp. lateral flow devices

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ABSTRACT

In a direct comparison with established methods for *Phytophthora ramorum* detection (isolation followed by morphological identification, or conventional DNA extraction followed by TaqMan real-time PCR) a rapid, simplified detection method in which membranes of lateral flow devices (LFDs) are added directly to TaqMan real-time PCR reactions was used to test 202 plant samples collected by plant health inspectors in the field. *P. ramorum* prevalence within the 202 samples was approximately 40% according to routine testing by isolation or TaqMan real-time PCR. The diagnostic sensitivity and specificity of the rapid detection method were 96.3% and 91.2%, respectively. This method can be used in conjunction with *Phytophthora* spp. lateral flow devices to reduce the number of samples requiring testing using more laborious conventional methods. The effect of combining prescreening for *Phytophthora* spp. with *P. ramorum*-specific tests is discussed in terms of the positive and negative predictive values of species-specific detection when testing samples collected in different inspection scenarios.

1. Introduction

Phytophthora ramorum is the causal agent of die back and leaf blight of a wide range of ornamental plants (principally rhododendron) in the UK and throughout Europe (Werres et al., 2001) and is also the cause of extensive oak mortality ('sudden oak death') on the west coast of North America (Rizzo et al., 2002). EU-wide emergency measures were implemented in 2002 (Anonymous, 2002), and in the UK there is an ongoing programme of surveillance for the presence of this pathogen by Defra's Plant Health and Seeds Inspectorate (PHSI), who have the authority to enforce eradication and containment measures including the destruction of infested material. A number of methods have been developed for the nucleic acid-based detection of *P. ramorum*, including several based on real-time PCR (Bilodeau et al., 2007; Hayden et al., 2004; Hayden et al., 2006; Hughes et al., 2006; Schena et al., 2006; Tomlinson et al., 2005; Tooley et al., 2006) These methods have been found to have high specificity and sensitivity, detecting less than 12 fg *P. ramorum* DNA (Hayden et al., 2004), and can be used for testing both cultured pathogen and infected plant material. The majority of assays reported to date have been used in conjunction with DNA extraction methods based on spin columns or processing of magnetic beads (Anonymous, 2006; Bilodeau et al., 2007; Hughes et al., 2006; Kox et al., 2007; Tooley et al., 2006;) or using organic solvents such as phenol and chloroform (Anonymous, 2006; Hayden et al., 2004; Schena et al., 2006). These methods generally result in high quality DNA extracts, but they are also time-consuming even when automated for high-throughput use.

In England and Wales, samples of plant material taken by plant health inspectors are sent to the Food and Environment Research Agency (Fera) for *P. ramorum* testing. The diagnostic method used in the laboratory depends primarily on the host plant: the majority of rhododendron samples are tested directly by TaqMan real-time PCR (Hughes et al., 2006), while other hosts are tested by plating of plant material on semi-selective media followed by morphological examination. In accordance with the EPPO diagnostic protocol for *P. ramorum* (Anonymous, 2006), positive identification of the pathogen is possible on the basis of an unambiguous result for either real-time PCR or morphological examination. In practice, only samples of the most common host in the UK (rhododendron) are tested by real-time PCR, and any ambiguous real-time PCR results are confirmed by isolation and morphological examination. Further to this, partial sequencing of the ITS region of the rRNA gene is carried out to confirm the identity of the pathogen in samples from new outbreak sites and in previously unrecorded hosts.

Phytophthora spp. LFDs are used by some plant health inspectors for screening samples in the field (Lane et al., 2007). The use of these devices has been found to be a suitable prescreening method (Kox et al., 2007; Lane et al., 2007) due to the high diagnostic sensitivity of this method compared to methods which identify *P. ramorum* at the species level (cultural and/or PCR-based methods). Prescreening reduces the number of samples sent to the laboratory for testing, resulting in a considerable cost saving, but several thousand samples

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are still sent to the laboratory every year. Sending samples to the laboratory for testing has a number of disadvantages including the movement of potentially infectious material away from outbreak sites; a requirement for stringently observed quarantine procedures at the testing laboratory; and the possibility of discrepant results due to uneven distribution of the pathogen or degradation of samples in transit.

Fera have developed a method for extraction of nucleic acid from plant material using LFDs (Danks and Boonham, 2007). A section of the LFD nitrocellulose membrane can be added directly to a DNA amplification reaction, such as real-time PCR, without any additional processing. LFDs run at inspection sites could be sent to the laboratory for testing by TaqMan real-time PCR. This approach would obviate the need to send plant material to the laboratory and has the advantage of expediting real-time PCR testing, since conventional DNA extraction is not required. In order to evaluate the potential utility of this approach, 202 samples sent to the laboratory at Fera for routine testing for *P. ramorum* were also tested by DNA extraction using the LFD method followed by real-time PCR. The results were compared to those obtained by routine testing using established methods.

2. Materials and methods

2.1. Samples

Samples of plant material with suspected symptoms of *P. ramorum* were collected by the PHSI as part of ongoing surveillance for *P. ramorum*. 198 out of 202 samples were leaf material, as recorded by the diagnostician who received the sample (the remaining samples were recorded as stem/shoot, leaf/twig/branch, or leaf litter). Samples were dispatched from the field in sealed plastic bags containing a small piece of damp tissue. On receipt in the laboratory, the material was examined for the presence of typical symptoms, and sub-samples were taken from the leading edge of any identified lesions. Sub-sampled material was washed briefly in distilled water to remove any debris from the surface. Samples were predominantly rhododendron (141 samples) but also included *Pieris* (14 samples), *Viburnum* (7 samples), *Magnolia* (7 samples) and *Camellia* (6 samples).

2.2. Routine laboratory testing

Samples were tested either by plating on semi-selective media followed by morphological assessment of any growth, or by DNA extraction directly from the plant material using a magnetic bead-based extraction method followed by TaqMan real-time PCR. This is in accordance with the EPPO diagnostic protocol (Anonymous, 2006), in which a sample can be identified as positive on the basis of an unambiguous result obtained by either real-time PCR or morphological examination. The majority of rhododendron samples (113 out of 141 samples) were initially tested directly by TaqMan. The remaining rhododendron samples were tested by culturing if the sample originated from a previously unrecorded outbreak site or if there was considered to be insufficient material to allow subsequent culturing if the TaqMan result was ambiguous. All non-rhododendron hosts were tested by culturing only. Following assessment of symptoms, excised pieces of tissue were tested immediately by either conventional DNA extraction followed by TaqMan real-time PCR or culturing on semi-selective media. Duplicate samples were stored at 4 °C, prior to testing by LFD followed by TaqMan real-time PCR.

For detection by culturing, pieces of tissue were plated out on P_5ARP[H] semi-selective media (Jeffers and Martin, 1986). The plates were examined microscopically after 6 days for the presence of *P. ramorum* growth (Werres et al., 2001).

Alternatively, DNA was extracted from the material using a KingFisher ML platform (Thermo Scientific, Waltham, MA). Briefly, samples (typically 200–500 mg) were homogenized in 10 volumes of Buffer C1 from the NucleoSpin Plant kit (Machery Nagel, Düren, Germany), incubated at 65 °C for 30 min and centrifuged for 2 minutes at 6000×*g*. DNA was extracted from the clarified lysates by adding 1 ml PB Binding Buffer (Qiagen, Hilden, Germany) and 75 µl Magnesil paramagnetic particles (PMPs) (Promega, Madison, WI) and processing the samples using a KingFisher ML to wash the PMPs three times in 70% ethanol and elute the DNA in 200 µl molecular grade water.

DNA extracts were tested by TaqMan real-time PCR for *P. ramorum* and plant cytochrome oxidase (COX) using primers and probes described by Hughes et al. (2006), as shown in Table 1. Real-time PCR was carried out on an ABI 7900HT (Applied Biosystems, Foster City, CA) using TaqMan Core Reagents (Applied Biosystems) as described by Hughes et al. (2006), except that the *P. ramorum* and COX reactions were carried out in separate wells. Samples for which the COX TaqMan Ct value >28 or the *P. ramorum* Ct value >36 were retested by culturing, the result of which was taken as the final result.

2.3. LFD DNA extraction and TaqMan real-time PCR

Phytophthora spp. LFDs were obtained from Forsite Diagnostics Ltd (York, UK). Samples were placed in bottles containing 5 ml LFD Buffer C and 5 ball bearings (5 mm diameter) and shaken or vortexed for 2 to 3 min. Approximately 60 µl of Buffer C from the bottle was run on an LFD and the result recorded after 5 min. Positive results are indicated by the formation of 2 lines on the device; negative results are indicated by a single control line. Devices were left at room temperature for several hours or overnight before testing by TaqMan real-time PCR. For real-time PCR testing, the devices were dismantled and sections (approximately 1.5 mm by 2 mm) were cut from the membrane and added directly to TaqMan real-time PCR reactions. Sections were generally taken from the centre of the membrane, although it is not necessary to sample from any particular region of the membrane (Danks and Boonham, 2007). Membranes were tested for *P. ramorum* and plant DNA (cytochrome oxidase) using the *P. ramorum* primers and probe used by Tomlinson et al. (2005), shown in Table 1, and the COX primers and probe described above. A base substitution was introduced into the forward primer Pram-114Fc used for routine testing and described by Hughes et al. (2006) in order to increase discrimination between *P. ramorum* and the closely related pathogen *P. lateralis* when testing highly concentrated DNA extracted from cultures. As a result of this mismatch, the Ct values obtained using this primer are higher than recorded for the perfect match primer Pram-114F. The LFD extraction method results in the addition of smaller amounts of DNA to the real-time PCR reaction, so the perfect match primer Pram-114F was used when testing LFD membranes (Tomlinson et al., 2005).

Real-time PCR reactions were set up in 96-well plates using TaqMan Core Reagents (Applied Biosystems) consisting of 1×Buffer A and 0.025 U/µl AmpliTaq Gold, plus 0.2 mM each dNTP, 5.5 mM $MgCl_2$, 300 nM forward primer, 300 nM reverse primer, and 100 nM probe. The final volume of each reaction was 25 µl, and all reactions were carried out in duplicate. DNA extracted using conventional procedures was used as a positive control, and negative controls containing nuclease-free water instead of DNA were included in every run. Real-time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems) using cycling conditions of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and results were analyzed using default threshold settings.

3. Results

3.1. Results of routine laboratory testing

Results were obtained for all 202 samples tested using either TaqMan real-time PCR or morphological examination. Twenty four samples gave real-time PCR results which were considered to be ambiguous and were subsequently retested by isolation. Out of 202