Journal of Microbiological Methods 151 (2018) 90–98

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Detection of root-infecting fungi on cool-season turfgrasses using loop-mediated isothermal amplification and recombinase polymerase amplification

Brijesh B. Karakkat, Kurt Hockemeyer, Margot Franchett, Megan Olson, Cortney Mullenberg, Paul L. Koch*

Department of Plant Pathology, University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI, USA

ARTICLE INFO

Keywords:
Turfgrass
LAMP
RPA
Gaeumannomyces avenae
Ophiosphaerella korrae
Magnaporthiopsis poae

ABSTRACT

Root-infecting fungal pathogens such as *Gaeumannomyces avenae*, *Ophiosphaerella korrae*, and *Magnaporthiopsis poae* cause extensive damage to amenity turfgrasses in temperate climates. The diseases they cause are difficult to diagnose by visual symptoms or microscopic inspection, and traditional polymerase chain reaction-based assays require large financial investments in equipment such as thermal cyclers and highly trained staff. The primary objective of this research was to develop fast and accurate detection assays for the three pathogens listed above that did not require the use of thermal cycling equipment. Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) assays were developed for each pathogen based on known fungal cultures. The assays were tested on 27 samples received at the University of Wisconsin's Turfgrass Diagnostic Laboratory in 2016 and 2017 and both methods provided accurate diagnoses within about 30 min with minimal sample preparation. However, the RPA assays had lower levels of false positive contamination relative to the LAMP assays and are more likely to be effective in a field or diagnostic laboratory for improved turf root-pathogen detection.

1. Introduction

Root-infecting fungal pathogens of amenity turfgrasses cause several important diseases collectively referred to as patch diseases. Patch diseases are so named because of the similarity in their visual appearance; roughly circular patches of tan or brown turf 0.5 to 1 m in diameter. These patches are unsightly, can lead to extensive areas of dead turf, and can affect the playability of golf courses and athletic turf surfaces (Smiley et al., 2005). On golf courses and professionally managed athletic fields two or more fungicide applications targeting one or more patch fungi are often made annually to suppress disease symptoms (Latin, 2011). The most common patch diseases on cool-season turfgrasses are take-all patch caused by *Gaeuuammanomyces avenae,* necrotic ring spot caused by *Ophiosphaerella korrae*, and summer patch caused by *Magnaporthiopsis poae* (Clarke and Gould, 1993).

Take-all patch is primarily observed on creeping bentgrass (*Agrostis stolonifera*) but can also be observed on fine fescues (*Festuca* spp.) and bluegrasses (*Poa* spp.) (Nilsson and Smith, 1981). Necrotic ring spot symptoms are most commonly observed on Kentucky bluegrass (*P. pratensis*), annual bluegrass (*P. annua*), and fine fescues (Clarke and Gould, 1993). Summer patch symptoms are most commonly observed on annual bluegrass, Kentucky bluegrass, and fine fescues (Smiley et al., 2005), though reports have suggested creeping bentgrass can also serve as a host in certain conditions (Tredway, 2006). Microscopic inspection of roots afflicted with each disease reveals colonization of the root surface by dark brown runner hyphae and penetration into the root vasculature through simple or slightly lobed hyphopodia (Clarke and Gould, 1993; Kackley et al., 1990; Stowell and Gelernter, 2001; Walker, 1972).

Accurate diagnosis of take-all patch, necrotic ring spot, and summer patch are difficult despite their importance in turfgrass management. Microscopy can observe fungi colonizing the root surface but differentiating between the various fungal pathogens and between pathogens and non-pathogenic fungal species is arduous even for experienced diagnosticians (Kerns and Tredway, 2009; Singleton et al., 1992; Smiley and Craven Fowler, 1984; Smiley et al., 2005). Visual differences exist for each pathogen when grown in culture, but culturing pathogens from roots can take weeks and is often not fully indicative of the pathogen(s) present (Clarke and Gould, 1993). Multiple molecular detection methods have been developed in the past 30 years but most require

* Corresponding author.
E-mail address: plkoch@wisc.edu (P.L. Koch).

https://doi.org/10.1016/j.mimet.2018.06.011
Received 12 March 2018; Received in revised form 15 June 2018; Accepted 26 June 2018
Available online 28 June 2018

successful isolation and culturing of the pathogen (Raffle and Tsiang, 2009) and lack the portability to be conducted in a field setting (Abbott and Holland, 1975). Biochemical assays have been developed to detect each of these pathogens through immunoassay methods but most turfgrass managers and many diagnostic facilities do not have access to the specialized equipment such as plate readers that these assays require (Nameth et al., 1990). Moreover, past molecular assays to detect these pathogens have not been very sensitive or specific (Bateman et al., 1992; Zhao et al., 2012)

Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are relatively new alternatives to traditional PCR-based amplification technology (Craw and Balachandran, 2012). Both LAMP and RPA have prompt end-point detection, high sensitivity without the requirement of pure nucleic acid solution, rapid and efficient amplification of target DNA, simple naked-eye visualization of end-point results, and eliminate the requirement of a thermal cycler by performing the nucleic acid amplification at one temperature in a water bath or temperature block (Hoff, 2006). Therefore, LAMP and RPA represent a potential major step forward in advancement over existing methods in turf disease diagnosis.

The LAMP method was first developed in medical microbiology for the detection of pathogens in clinical diagnosis of human diseases (Mori and Notomi, 2009). In LAMP, the DNA amplification occurs by a strand-displacement *Bst* polymerase enzyme that uses six species-specific primers to create different amplified DNA products within 20 min at a constant temperature (Notomi et al., 2000). The different primers create a mixture of copies of stem-loop structures with multiple loops that are highly specific within the target sequence of DNA even if it is present alongside non-target DNA (Notomi et al., 2000). LAMP creates as many DNA copies as a traditional PCR but takes less time and amplifies without the need of thermal cycling (Notomi et al., 2000). In plant pathogen detection, LAMP has been used to detect *Fusarium* spp. that cause panama disease in banana (Li et al., 2013; Yasuhara-Bell et al., 2017), *Dickeya* spp. for potato soft rot (Yasuhara-Bell et al., 2017), and Pythium root rot (*P. aphanidermatum*) of tomatoes (Fukuta et al., 2013). The LAMP method is faster because only crude extract of infected plant tissues serves as the DNA template, rather than pure genomic DNA from culture (Temple and Johnson, 2011; Villari et al., 2017). The endpoint of the LAMP reactions are determined visually using DNA intercalating dyes such as EVA green (Lin et al., 2016) or measuring the accumulating magnesium pyrophosphate turbidity by metal indicators such as calcein (Norihiro et al., 2008).

The RPA method was initially developed by Piepenburg et al. (2006) for use in medical microbiology for the detection of human pathogens (Boyle et al., 2014; El Wahed et al., 2013). The DNA amplification process in RPA requires the same ingredients as PCR such as dNTPs and opposing oligonucleotide primer pairs. However, RPA works with four different enzymes for isothermal amplification, unlike traditional PCR that has only one DNA polymerase enzyme. The four RPA proteins are a recombinase, strand-displacing DNA polymerase I (Pol I), exonuclease IV (nfo), and single strand binding (SSB) proteins (Piepenburg, et al., 2006). During the start of the isothermal amplification, the primers form a complex with the recombinase that displaces the parental strands to attach to the cognate sites (Piepenburg et al., 2006). The SSBs stabilize the displaced parental DNA strand to keep them from reannealing while the Pol I adds nucleotides to synthesize the new strand. Through repetition on newly synthesized strands the Pol I creates an exponential increase in target DNA copies. No thermal cycling is required to cause the displacement and annealing as those steps are carried by different enzymes that require one constant temperature. Using a water bath or heating block, exponential amplification of DNA can be achieved with RPA in about 20 min. The probe-based technology has been used to create disposable detection kits such as TwistDX™ (Piepenburg, 2013) and the Agdia® AmplifyRP kit in the form of acceler8™ for plant pathogen detection. The acceler8™ works similar to a Taqman real-time PCR but uses antibodies to immunoreact to labeled probes to be read on a paper strip (Miles et al., 2015; Piepenburg, 2013). There is a control biotin probe to confirm the lateral flow of the reaction solution and a 6-carboxyfluorescein (FAM) probe that gets released only when amplification reaction occurs (Zhang et al., 2014).

The primary objectives of this study were to 1) develop LAMP and RPA detection assays for *G. avenae*, *O. korrae*, and *M. poae* on turfgrass hosts and 2) validate the methods developed for use by turfgrass diagnosticians on sample submissions from the field.

2. Materials and methods

2.1. Isolation and storage of reference isolates

Pure cultures of each fungus of interest were obtained and stored to serve as positive controls for assay development. In addition, other soil-inhabiting pathogens of turfgrass including *M. meyeri-festucae*, *G. graminis*, and *Pythium aphanidermatum* were collected to test the specificity of the assays. *Magnaporthiopsis poae* isolates M15 and M48 (Zhang et al., 2011) and isolates of the closely related *M. meyeri-festucae* (AG2 and FF isolates) (Luo et al., 2017) were received from Dr. Ning Zhang at Rutgers University. The *O. korrae* isolate was procured from American Type Culture Collection (Manassas, VA) as a frozen culture that was isolated from Kentucky bluegrass (*P. pratensis*) in Green Bay, WI (Tisserat et al., 1994). The *G. avenae* isolate was isolated by the authors and grown out on antibiotic-amended PDA plates (BD Difco, Sparks, MD) amended with 100 mg/l of tetracycline, chloramphenicol, and streptomycin sulfate from creeping bentgrass roots exhibiting take-all patch symptoms from Royal St. Patrick's Golf Course in Wrightstown, WI in June of 2016. *Gaeumannomyces graminis* (DR isolate) (Hernandez-R et al., 2016) was obtained from Dr. Maria Tomaso-Peterson at Mississippi State University and *P. aphanidermatum* (pom8 isolate) was obtained from Dr. Jim Kerns at North Carolina State University. All reference isolates were confirmed with 99% identity upon arrival following extraction of culture DNA, ITS PCR-amplification, Sanger sequencing, and querying the sequence results in BLASTn.

Storage of each isolate was conducted by transferring about 100 mg of fungal mycelium scraped with a sterile spatula from PDA cultures to sterile 2.0-ml nuclease-free microcentrifuge tubes with 0.2 N sodium hydroxide (NaOH) solution (about 2 µl per 1 mg of culture). To the tubes, 150 mg of 710 to 1180-µm sized acid washed glass beads were added and ground with sterile Kimble™ Kontes™ pellet pestle (Thermo Fisher, Waltham, MA) and vortexed five minutes until the suspension became turbid with culture extracts. The culture extract was diluted with PCR-grade nuclease-free sterile water (Fisher Scientific, Waltham, MA). Two and one µl of this extract were used for LAMP and RPA reactions, respectively.

2.2. LAMP assay development

The LAMP primers were designed for specific for each of the three fungal genes (Karakkat et al., 2018). To identify the right dilution of NaOH concentration in the crude extract for optimal LAMP reaction, two µl of crude fungal culture extract were added to various volumes of PCR-grade nuclease-free sterile water to create a dilution series of 1:20, 1:40, 1:80 and 1:160 of template. Once the right ratio of 1:40 was determined (data not shown), one µl of that dilution extract was added to all LAMP reactions.

A 10× primer mix tube was initially prepared for each of the three fungi. This 10× primer mix tube contained all six primers at concentrations of 16 µM FIP and BIP, 2 µM F3 and B3, 4 µM LoopF and LoopB in PCR-grade nuclease-free sterile water. LAMP reactions were carried out in 0.2-ml 8-Strip Standard PCR Tubes with individually attached clear flat caps (GeneMate UltraFlux®, VWR, Radnor, PA). A 1× reaction set up for one tube contained 1.25 µl of 10× primer mix for *M. poae* and *O. korrae* primer set and 0.62 µl for *G. avenae*, 6.25 µl of