From Forest Nursery Notes Winter 2013

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Forest Pathology WILEY-BLACKWELL

For. Path. 42 (2012) 22–27 doi: 10.1111/j.1439-0329.2011.00716.x

PCR–DGGE method for *in planta* detection and identification of *Phytophthora* species

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Summary

We have developed a method to detect multiple species of *Phytophthora* directly in infected plant tissues. The method is based on the polymerase chain reaction and uses *Phytopthora*-specific primers and denaturing gradient gel electrophoresis (PCR–DGGE). The method distinguished most of the 16 *Phytophthora* species tested. Very closely related species might not, however, be identified using the method. The detection efficiency was high and successful in eight different plant tissues tested. The PCR–DGGE detection tool developed here will be a fast and inexpensive method suitable for pathogen surveys and research programmes.

1 Introduction

The genus *Phytophthora* is composed of over 90 species that include some of the world's most important plant pathogens. They cause economically significant impacts on crops and forests as well as on natural ecosystems throughout the world (Hansen et al. 2000; Brasier 2008; Hansen 2008). *Phytophthora* species have swimming zoospores and thick-walled resting spores, as well as generally wide host plant ranges. For example, *Phytophthora ramorum* Werres, De Cock & Man in 't Veld infects more than 100 plant species. It is also the causal agent of sudden oak death, which devastates oaks (*Quercus* spp.) and tanoaks (*Lithocarpus densiflorus* (Hook. & Arn.) Rehd.) among other trees, as well as increasing number of woody and herbaceous perennials over large areas in California and Oregon (Rizzo et al. 2002; Davidson et al. 2005; Prospero et al. 2009). Another example is *P. cinnamomi* Rands, which in southern Europe causes widespread decline of cork oaks (*Quercus suber* L.) (Brasier et al. 1993). It is also responsible for destructive impacts on whole ecosystems in Western Australia by threatening the Jarrah (*Eucalyptus marginata* Donn. ex Sm.) as well as the rich native flora (Weste 1994; Shearer et al. 2004). Species in the genus *Phytophthora* are also known to hybridize. The recently described hybrid species *P. alni* Brasier & Kirk. most likely originated in a European nursery and is now devastating alders in riparian ecosystems all over Europe (Gibbs et al. 2003; Brasier et al. 2004).

The predicted climate change will also promote the spread and establishment of pathogenic species in previously unaffected areas (Brasier 1996; Venette and Cohen 2006). Several important pathogens, including *P. alni*, *P. cinnamomi* and *P. ramorum*, enjoy passive dispersal through the horticultural trade and other transport networks (Hardham 2005; Brasier 2008).

To prevent the spread of *Phytophthora*, detection and identification tools have been developed with the aim to offer robust and sensitive properties. The classical methods are based on the isolation and culturing of the microbe to observe its morphology and growth. The process is time-consuming, and detection is often hampered because of difficulties in establishing mycelial cultures, perhaps because of the presence of antagonists or competitors, or inhibition by fungistatic compounds in the plant tissue (Malaczjuk 1983; Tsao 1983). Further difficulties in identification arise from the fact that morphological characters are indistinct, continuous between species or highly variable within a species. The difficulties in species identification have been addressed by using molecular approaches such as DNA sequencing or fingerprinting methods such as single strand conformation polymorphism (Kong et al. 2003).

For more efficient detection of *Phytophthora* spp. from infected plants, immunoassay-based test kits are commercially available, e.g., enzyme-linked immunosorbent assay (ELISA) and lateral flow immunochromatographic assay (Lane et al. 2007). These tests are not species specific but are intended for the initial screening of infected material. However, these immunodetection tests are known to yield false negatives (Bulluck et al. 2006; Kox et al. 2007).

DNA detection tests based on PCR amplification of templates obtained directly from plant material or environmental samples by species-specific primers have also been developed for several *Phytophthora* species (reviewed in O'brien et al. 2009). The amplification and detection of multiple species in a single reaction (i.e., multiplexing) is one of the aims of the new methods. Some level of multiplexing can be achieved by utilizing species-specific probes in PCR–ELISA analysis (Bailey et al. 2002).

For effective species separation, fingerprinting techniques, such as SSCP and RFLP (Kong et al. 2003; Drenth et al. 2006), are useful but produce rather complex patterns unsuitable for samples containing multiple species. Schena et al. (2008) designed a set of species-specific primers for detecting 15 *Phytophthora* species in single-round amplification from infected leaves or in a nested PCR of soil or water samples. Some cross reactions were observed in the study, i.e., primers designed for *P. cactorum* and *P. ilicis* Buddenh. & Young cross reacted with *P. idaei* Kennedy & Duncan and *P. nemorosa* Hansen & Reeser, respectively. Multiplex real-time PCR has been successful in detecting two (Ippolito et al. 2004) and four (Schena et al. 2006) *Phytophthora* species in a single reaction. Multiplexing in real-time PCR is restricted by the number of fluorescent probes, which is currently limited. Furthermore, the greatest challenge in using both conventional and real-time PCR for multiplexing is optimizing the conditions for a reaction containing several primers and probes.

Received: 22.11.2010; accepted: 24.2.2011; editor: L. Belbahri