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High-Resolution Melting Analysis: a new molecular approach for the early detection of *Diplodia pinea* in Austrian pine

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ABSTRACT

The differentiation of *Diplodia pinea* from closely related species, such as *Diplodia scrobiculata* and *Diplodia seriata*, and its detection in plant tissue, represented a critical issue for a long time. Molecular screening tools have recently been developed to address this topic. In this study we applied one of the most sensitive and rapid diagnostic screening method so far developed, called High-Resolution Melting Analysis (HRMA), to detect *D. pinea* in Austrian pine (*Pinus nigra*). HRMA exploits differences in the melting behaviour of PCR products to rapidly identify DNA sequence variants without the need for cumbersome post-PCR methods. We developed a HRMA method to detect specific fungal sequences in the mitochondrial small subunit ribosome gene (mt SSU rDNA). The reliability of this technique was firstly assessed on DNA extracted from pure cultures of *D. pinea* and closely related species. Amplicon differences were screened by HRMA and the results confirmed by direct DNA sequencing. Subsequently, HRMA was tested on DNA from symptomatic and symptomless pine shoots, and the presence of the fungus was also confirmed by both conventional and molecular quantitative approaches. The HRMA allowed the distinction of *D. pinea* from closely related species, showing specific melting profiles for the each pathogen. This new molecular technique, here tested in a plant–fungus pathosystem for the first time, was very reliable in both symptomatic and symptomless shoots. HRMA is therefore a highly effective and accurate technique that permits the rapid screening of pathogens in the host.

Introduction

Diplodia pinea (Desm.) J. Kickx is a pathogenic fungus with a world-wide distribution that causes significant mortality and serious economic losses in pine plantations and nurseries (Stanosz *et al.* 2007; Swart & Wingfield 1991). The fungus is particularly injurious to Austrian pine (*Pinus nigra* Arn.) (Blodgett & Bonello 2003). In southern European forests it has a role in

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the decline of Austrian pine, which occurs mostly under conditions of adverse climatic or environmental factors (Maresi *et al.* 2002, 2007).

The most common symptoms of the disease are crown wilt, tip blight, cankers and a blue stain of the timber, while on seedlings the fungus causes damping off and collar rot (Swart & Wingfield 1991). *Diplodia pinea* can persist in asymptomatic pine tissue in latent phase for a long time, until unfavourable environmental factors predispose trees to fungal infections (Flowers *et al.* 2003; Stanosz *et al.* 2007). Studies examining the environmental conditions in nurseries and pine plantations have shown that stress factors, such as drought, enhance the susceptibility of the host, leading to an increase in the incidence and the severity of disease (Paoletti *et al.* 2001; Stanosz *et al.* 2001).

The taxonomy of *D. pinea* and species closely related to it had undergone significant revision in the past. Initially the fungus was most commonly named *Sphaeropsis sapinea*, which included four morphotypes (A, B, C and I) distinguished by their conidial and colony morphology, and by their aggressiveness to their host plants (de Wet *et al.* 2000, 2002; Smith & Stanosz 1995).

Some of these morphotypes were later elevated to species, with the A and C morphotypes together becoming *D. pinea*, and the B morphotype *Diplodia scrobiculata*. The I morphotype became *Diplodia seriata* (=“*Botryosphaeria*” *obtusa*) (Burgess *et al.* 2001). *Diplodia scrobiculata*, unlike *D. pinea*, is a relatively non-aggressive species that attacks the shoots of various conifers, including Austrian pine, causing canker lesions that are always however significantly smaller than those caused by *D. pinea* (Blodgett & Bonello 2003). *Diplodia seriata* is a common pathogen of many fruit crops, on which it causes cankers and dieback (Phillips 2002; Slippers & Wingfield 2007; Úrbez-Torres & Gubler 2009). It has been identified in more than 30 host plants, including a number of pine species (Punithalingam & Walker 1973).

It is difficult to detect and distinguish these closely related species in the Botryosphaeriaceae. However, molecular tools, together with morphological techniques, have enabled the phylogenetic relationships of these species to be revised (Crous *et al.* 2006; de Wet *et al.* 2003; Phillips *et al.* 2008). The molecular approach most commonly used to distinguish species within the Botryosphaeriaceae has been by comparing the DNA sequences from the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (Zhou & Stanosz 2001a). However, it is generally accepted now that in order to separate closely related or cryptic species it is necessary to compare sequences from multiple loci (Slippers *et al.* 2004; Phillips *et al.* 2008).

The choice of the target DNA region is an important consideration when designing molecular markers to detect and differentiate fungi (Khadempour *et al.* 2010). The ITS rDNA region is commonly employed for this purpose (White *et al.* 1990). However, in the case of *D. pinea* it is very difficult to use this region as a marker, since its sequence shows a very high level of homology to those of other closely related species such as *D. scrobiculata* and *D. seriata*. These species differ much more in the sequences of the mitochondrial small subunit ribosome gene (mt SSU rDNA) (Smith & Stanosz 2006; Zhou & Stanosz 2001b).

Sensitive techniques are needed so that a pathogen can be detected at an early stage of infection (Luchi *et al.* 2005a). For *D. pinea*, real-time quantitative PCR (qPCR) has been used for this purpose (Maresi *et al.* 2007). Recently, High-Resolution Melting Analysis (HRMA) has also been very successful in clinical studies (Erali *et al.* 2008; Mancini *et al.* 2010). This method is very sensitive in genotype scanning and rapidly identifies DNA sequence variants, without needing cumbersome post-PCR techniques (Wittwer *et al.* 2003). HRMA exploits the fact that PCR products with different sequences have distinct melting profiles. The signal change that signifies the transition from a double to a single strand is generated by fluorescent dyes that actively intercalate double stranded DNA with very low interference with the PCR reaction. The high-resolution melting profile of a PCR product produces a specific, sequence-related curve that rapidly distinguishes between sequences even when they differ by only one nucleotide. Thus, the HRMA of PCR products can detect single-point mutations and distinguish single-nucleotide polymorphisms (SNPs) (Wu *et al.* 2009). HRMA being very sensitive could conceivably serve to identify phytopathogenic fungi *in planta* even when they are present in very small amounts.

The aim of the present study was to devise a sensitive and specific HRMA assay for the molecular detection of *D. pinea* in Austrian pine shoots. To assess the reliability of the assay, the following samples were tested: i) DNA extracted from a pure culture of *D. pinea* and some other phylogenetically closely related fungi; ii) a DNA mixture of pine DNA and the DNA of each fungus at a known concentration, and iii) DNA from pine samples, to find out whether fungal DNA could be detected *in planta* in symptomatic and asymptomatic pine shoots.

Materials and methods

Pine sample preparation, isolation and real-time qPCR

The occurrence of *Diplodia pinea* on Austrian pine trees was investigated in two pinewoods in Tuscany, Italy. The first wood was located at Monte Morello (Florence; N 43°51′02.3″; altitude 580 m a.s.l.) where the fungus causes serious damage. This wood has previously been used by our team for other studies on the *D. pinea* – Austrian pine pathosystem (Feci *et al.* 2002). The second area was a young plantation of Austrian pine at Monte Senario (Florence; N 43°53′15.9″; E 11°19′55.1″; altitude 650 m a.s.l.) where tip blight is only rarely found.

Thirty pine samples were collected: 20 from the first site (ten symptomatic and ten asymptomatic shoots); and ten from the second site (five asymptomatic pine shoots and five samples of fascicles of healthy needles). One sample was collected per tree.

All samples were surface-sterilised with 70 % ethanol, 1.05 % NaOCl and Tween 80 (following Stanosz *et al.* 2001) and rinsed three times in sterile water. Each shoot and each needle fascicle were divided into two parts; the first part was used for isolation on potato dextrose agar (PDA), and the second for DNA extraction (Luchi *et al.* 2005b).

Fungal cultures from the plated fragments were identified on the basis of cultural and conidial characteristics. Colonies typical of *D. pinea* were incubated on a pine-needle agar medium to produce pycnidia *in vitro*, so that the identity of the colonies could be confirmed (Luchi *et al.* 2007). *D. pinea* in