Detection and Characterization of *Phytophthora* Species Infecting Tomato in Southern Italy by DNA-Based Methods

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Abstract

During summer and autumn 2002, in three regions of southern Italy, Apulia, Basilicata and Campania, where tomato is an economically important crop, unusual cool and wet weather conditions, very favorable to diseases caused by *Phytophthora* species occurred. Consequently, severe late blight, buckeye rot and/or stem and root rot symptoms on tomato were observed in several tomato growing areas of the above mentioned regions. In order to test the *Phytophthora* etiology of the tomato diseases occurring in southern Italy, symptomatic plants were examined by polymerase chain reaction (PCR) assays using universal fungal primers and primers specific for *Phytophthora* species. The primers used were directed to nuclear ribosomal DNA (rDNA) sequences. All symptomatic plants tested positive. The detected fungi were differentiated and characterized on the basis of primer specificity and extensive restriction fragment length polymorphism (RFLP) and sequence analyses of PCR-amplified rDNA. *P. infestans* was identified in tomatoes with late blight symptoms, whereas buckeye rot-affected tomato plants proved to be infected by *P. nicotianae*. No polymorphism among isolates of each *Phytophthora* species identified was observed by RFLP analysis. The diseases examined were known in southern Italy, but the pathogens were molecularly detected and characterized only from other geographic areas outside Italy. Thus, this is the first report from Italy on molecular detection and identification of *Phytophthora* species infecting tomato using nuclear rDNA markers.

INTRODUCTION

Phytophthora species are plant pathogenic organisms, members of the genus *Phytophthora* in the phylum Oomycota which cause significant damage to a wide range of economically important crops worldwide, including tomato (Agrios, 1997). Detection and identification of *Phytophthora* species have traditionally been based upon microscopic examination of morphological characters, isolation of the pathogen on selective media, and occasionally on physiological traits. However, lack of reliable morphological markers and a considerable morphological diversity occurring in some taxa may lead to inaccurate identification. Moreover, microscopic observations lack sensitivity and specificity. Isolation of *Phytophthora* species on culture media is difficult because these plant pathogenic organisms tend to be slow-growing on agar and the optimal method varies from species to species. In addition, once in culture, morphological characters typical of a given taxon are not consistently expressed (Duncan and Cooke, 2002).

During the last decade, considerable progress has been made in the application of DNA-based methods for detection, identification, and classification of *Phytophthora* species (Stammler and Seemüller, 1993; Stammler et al., 1993; Ristaino et al., 1998; Wangsomboondee and Restaino, 2002). In particular, specific and sensitive detection methods have been developed, mainly based on polymerase chain reaction (PCR) assay (Stammler and Seemüller, 1993; Bonants et al., 1997; Ristaino et al., 1998; Grote et al.,

2002; Ippolito et al., 2002). It also became possible to distinguish, characterize, and classify *Phytophthora* species on a phylogenetic basis, using restriction fragment length polymorphism (RFLP) and sequence analysis of PCR-amplified nuclear ribosomal DNA (rDNA) (Ristaino et al., 1998; Cooke and Duncan, 1997; Cooke et al., 2000a, 2000b; Cohen et al., 2003). In contrast to undefined genomic DNA fragments, the nuclear rDNA is a very attractive marker which provides valuable molecular information on fungi. It consists of highly repeated units, arranged in tandem, with each unit containing genes encoding the 18S, 5.8S and 28S rRNA respectively, interspeced with regions (internal transcribed spacer [ITS] regions, ITS1 and ITS2) which do not encode any functioning part of the ribosome. Sequence differences in the ITS regions are useful for distinguishing among *Phytophthora* species (Cooke et al., 2000b; Duncan and Cooke, 2002). Additionally, the high copy number of rDNA sequences present in the nuclear genome makes them ideal targets for detecting *Phytophthora* by increasing detection sensitivity in comparison with target sequences of single-copy genes.

During summer and autumn 2002, in three regions of southern Italy, Apulia, Basilicata and Campania, where tomato is a major crop, unusually cool, wet weather conditions, very favorable to *Phytophthora* disease development, occurred. Consequently, severe late blight, buckeye rot and/or stem and root rot symptoms on tomato were observed in several tomato growing areas of these regions. In order to test the *Phytophthora* etiology of the tomato diseases occurring in southern Italy, symptomatic plants were examined for occurrence of *Phytophthora* species by PCR amplification. The detected fungi were differentiated and characterized on the basis of primer specificity and extensive RFLP and sequence analyses of PCR-amplified rDNA.

MATERIALS AND METHODS

Plant Samples and *Phytophthora* Reference Isolates

Sampling of diseased and nonsymptomatic plants was carried out from May 2002 until September 2002. A total of 53 tomato (*Lycopersicon esculentum* L.) plants showing typical symptoms of late blight disease was sampled in commercial fields of tomato located in the Vulture plain area of Basilicata (20 plants), in Apulia near Foggia (15 plants) and Campania near Battipaglia (18 plants). Twelve tomato plants showing mainly buckeye rot symptoms were sampled in the Metapontino plain (Basilicata). Several nonsymptomatic field-collected and healthy greenhouse-grown plants of tomato were used as healthy controls. DNA samples from the reference isolates of *P. infestans* and *P. nicotianae*, whose molecular identification had already been determined (Camele et al., 2004), were included for comparison.

DNA Isolation, Primers and PCR Amplification

For DNA isolation from diseased and healthy plants, stem, leaf and fruit tissues were used. Stem, leaf, and fruit lesions were excised as aseptically as possible, cut into small pieces with scissors, and ground to a fine powder (1 g) in liquid nitrogen with a sterile mortar and pestle. Healthy tissues were processed similarly. Tissue powder was extracted according to the DNA isolation protocol of Doyle and Doyle (1990). Some plant samples were also extracted using the DNeasy Plant Mini Kit (Qiagen, Milano, Italy) according to the manufacturer's instructions.

All primers employed for PCR amplification were directed to nuclear rDNA sequences. The universal primer pair ITS6/ITS4 (White et al., 1990; Cooke et al., 2000a) amplifies a ribosomal fragment that extends from the 3'-end of the 18S rRNA gene to the 5'-end of the 28 rDNA, which includes the ITS1 and ITS2 regions and the 5.8 rDNA, from many eukaryotes. Also, the following species-specific primer pairs were used: ITS5/PINF (Trout et al., 1997) that specifically amplifies DNA of *P. infestants*, DC3/DC8 (Boersma et al., 2000) and Pn5B/Pn6 (Ippolito et al., 2002). Both of these primers are specific for *P. nicotianae*.

Amplifications were performed with an automated thermal cycler (Hybaid,

Middlesex, United Kingdom) in a 50-µl reaction volume containing 125 µM of the four dNTPs, 0.5 µM of each primer, 1 U of DyNAzyme EXT DNA polymerase, 1x polymerase buffer (both Finnzymes, Espoo, Finland), 1-3 µl of template DNA (20-50 ng), and water. The reaction mixture was subjected to 35 cycles at the following conditions: 30 s denaturation at 95°C (120 s for the first cycle), 30 s annealing at 55°C (65°C with primers DC3/DC8), and 60 s extension at 72°C (10 min for the final cycle). In nested PCR assays, initial amplification ('first round') was carried out with primers ITS6/ITS4. The products obtained were then re-amplified with the species-specific primers mentioned above. For amplification with nested primers, either 1 µl of undiluted PCR products or 3 µl of 1:50 dilution obtained in the initial amplification were used as template in the same reaction mixture as in the first round. Five microliters of PCR products were analyzed by electrophoresis in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) in presence of 0.5 µg/ml ethidium bromide. DNA bands were visualized using a UV transilluminator.

RFLP and Sequence Analyses of rDNA

Ten microliters of PCR products obtained with the primer pair ITS6/ITS4 were separately digested with *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II and *Hae*III restriction endonucleases following the manufacturer's instructions (MBI Fermentas, Vilnius, Lithuania). Seven to twelve microliters of the digests were used to resolve the restriction fragments on vertical 5% polyacrylamide gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8). After electrophoresis, the DNA was visualized with ethidium bromide as described above. Molecular weights were determined using the 1-kb DNA ladder (BRL Life Technologies, USA).

For sequencing, the ITS6/ITS4 PCR products (approximately 5 µg) were electrophoresed in 1.5% agarose gel. Fragments with sizes corresponding to the expected amplified sequences were excised from the gel and eluted using the QIAquick gel extraction kit (Qiagen). Sequencing of both strands was performed by a commercial service (Bio Molecular Research and Biotechnology Centre, University of Padua, Padua, Italy). Primers for sequencing PCR products were the same as for PCR amplification. The obtained sequences were then used as query sequences in a BLAST 2.0 search (Altschul et al., 1997). Alignments of sequences determined in this study and others retrieved from the GenBank database were performed by using CLUSTAL, version 5, using DNASTAR's LaserGene software (DNASTAR).

RESULTS AND DISCUSSION

With PCR amplification using the primer pair ITS6/ITS4, the target DNA was amplified from all samples collected from diseased tomato plants (data not shown). The target DNA was also amplified from the *P. infestants* and *P. nicotianae* reference isolates. However, the primers ITS6/ITS4 did not amplify DNA from samples of asymptomatic field-collected and healthy greenhouse-grown tomato plants. Nested PCR assays employing species-specific primer pairs in the second round were also used to differentiate the tomato-infecting fungi. When the amplification products obtained with primers ITS6/ITS4 were re-amplified in nested PCR assays with primer pair ITS5/PINF, an amplification product of the expected size was obtained only from samples taken from late blight-affected tomatoes and the *P. infestants* reference isolate. In contrast, the primer pairs DC3/DC8 and Pn5B/Pn6 initiated amplification in template DNA from tomato plants showing mainly buckeye rot and/or stem and root rot symptoms as well as from the *P. nicotianae* reference isolate (Fig. 1). Species-specific primer pairs did not amplify DNA from samples of asymptomatic and healthy tomato plants.

Following separate digestion with *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II and *Hae*III restriction endonucleases, all ITS6/ITS4 amplicons from late blight-diseased tomato plants and the *P. infestants* reference isolate showed the same restriction profiles for each of the enzymes. Those from tomatoes with buckeye rot proved to be indistinguishable from the *P. nicotianae* reference isolate (Fig. 2 and data not shown).

The ITS6/ITS4 amplicons from two isolates from late blight-affected tomato (Ly.es.LB1 and Ly.es.LB2) and one from tomato with buckeye rot symptoms (Ly.es.BR1) were used for rDNA sequence analysis. Isolates Ly.es.LB1 and Ly.es.LB2 proved to be identical showing 100% ITS1, 5.8S and ITS2 sequence similarity. They had a sequence similarity of 100 and 99.5% with *P. infestants* isolates from Korea (GenBank accession no. AF228084) and The Netherlands (GenBank accession no. AF266779), respectively. The Ly.es.BR1 isolate proved to be nearly identical to a Chinese *P. nicotianae* isolate (GenBank accession no. AY202128) sharing 99.9% ITS1, 5.8S and ITS2 sequence similarity. Ly.es.LB1 and Ly.es.LB2 isolates each had a total ITS1, 5.8S and ITS2 region sequence length of 795 nucleotides whereas that of Ly.es.BR1 isolate was 803 nucleotides long. For all sequences determined, expected sizes based on analysis of putative restriction sites were in agreement with fragment sizes obtained by enzymatic RFLP analysis of ITS6/ITS4 amplicons.

The diseases studied were known previously in southern Italy, but isolates of the pathogens were molecularly detected and characterized only from other geographic areas outside Italy. This is the first report from Italy on molecular detection and identification of *Phytophthora* species infecting tomato using nuclear rDNA markers, and the work further extends knowledge on rDNA RFLP profiles of *P. infestants* and *P. nicotianae*. To our knowledge, RFLP analysis of nuclear rDNA from *Phytophthora* species had previously been determined using only a few restriction endonucleases.

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Figures

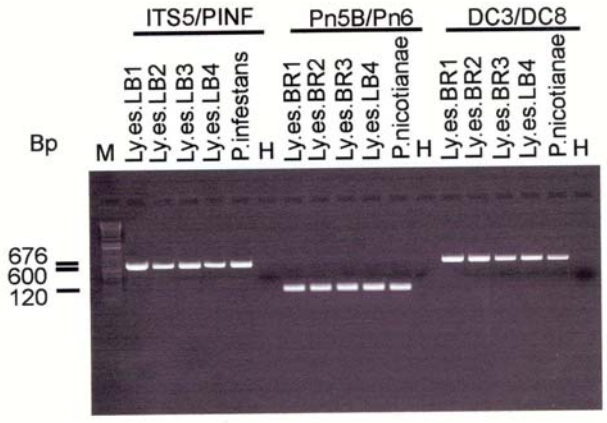

Fig. 1. PCR amplification of *Phytophthora* ribosomal DNA using species-specific primer pair. M, 1-kb DNA ladder (BRL Life Technologies); Ly.es.LB1 to Ly.es.LB4, late blight-affected tomato plants; P. infestans, reference isolate of *P. infestans*; H, healthy tomato; Ly.es.BR1 to Ly.es.BR4, tomato samples with buckeye rot symptoms; P. nicotianae, reference isolate of *P. nicotianae*.

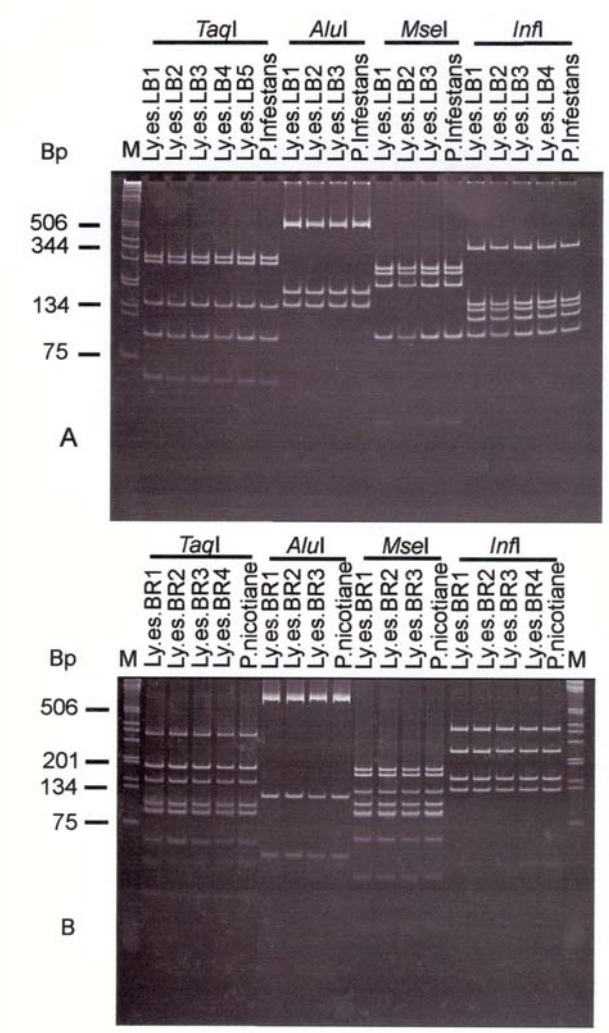

Fig. 2. Restriction profiles of ITS6/ITS4 amplicons obtained from *Phytophthora* reference isolates and tomato plants showing (A) late blight and (B) buckeye rot symptoms, respectively. See Fig. 1 for abbreviations.