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Molecular Distinction of Citrus *Phytophthora* Isolates in the Lower Rio Grande Valley of Texas

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ABSTRACT

***Phytophthora* infections causing foot rot, root rot, and gummosis in citrus are common in the Lower Rio Grande Valley (LRGV) of Texas. No previous studies have been conducted in Texas to characterize this fungus using molecular methods. We used the polymerase chain reaction (PCR) to characterize twenty two *Phytophthora* isolates from several root and soil samples. First round PCR with Ph2 and ITS4 primers resulted in a 700 base pair (bp) fragment. Primers Pn5B-Pn6 and Pc2B-Pc7 are highly specific for *P. nicotianae* Breda de Haan and *P. citrophthora* (R. E. Smith and E. H. Smith) Leonian, respectively. A highly sensitive nested PCR produced a 120bp fragment with primers Pn5B-Pn6 and no amplification with primers Pc2B-Pc7. This fragment was sequenced and a similarity search at GenBank showed 100% identity with *P. nicotianae*. These results reveal that *P. nicotianae* is the most prevalent species in LRGV citrus and it confirms the results of non-molecular identification completed previously.**

Additional Index Words: internal transcribed spacer, ribosomal DNA, polymerase chain reaction.

The Greek word *Phytophthora* means 'destroyer of plants.' This is a major plant pathogenic fungus with a wide range of hosts including agronomic and horticultural crops. Around 60 species have been reported that cause economic losses which account for billions of dollars world wide (Erwin and Ribeiro, 1996). This fungus can infect almost all parts of the citrus plant, causing root rot, foot rot and gummosis of the trunk, damping-off of seedlings, leaf blight, and brown rot of fruit. *P. nicotianae* Breda de Haan and *P. citrophthora* (R. E. Smith and E. H. Smith) Leonian are the most prevalent species (Menge and Nemec, 1997). *P. citrophthora* was also reported to be the causal agent of collar rot on pear trees in Greece, suggesting that *P. citrophthora* from citrus may pose a risk to pear (Elena and Paplomatas, 1999). Recently, *P. palmivora* (Butler) Butler was identified as the causal agent of citrus brown rot outbreaks in Florida (Graham et al., 1998).

Identification of *Phytophthora* to species level is crucial in developing quarantine methods, disease control, and disease resistance breeding. Traditional identification methods based on morphological characteristics using the microscope are tedious, require expertise, and sometimes may lead to wrong identification. Several molecular approaches such as isozyme analysis, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPDs), single-strand-conformation polymorphism of rDNA, and PCR of internal transcribed spacers (ITS) have been used in identifying some of the oomycetes (Foster et al., 1995; Ristaino et al., 1998; Kong et al., 2003; Cooke et al., 2000). Techniques like enzyme-linked immunosorbent assay are sensitive, but incapable of identifying *Phytophthora* down to the species level (Timmer et al., 1993).

Development of PCR-based genetic markers to obtain and sequence species-specific DNA fragments for *P. nicotianae* and *P. citrophthora* was reported (Ersek et al., 1994). The PCR technique requires very small amounts of target DNA; it is rapid, reliable, and applicable to different phytopathological research needs. The use of PCR primers based on nucleotide sequence of the ITS region of *Phytophthora* for identification of this fungus in citrus is well described (Ippolito et al., 2002). This system of ITS-based *Phytophthora* identification has more advantages compared to other methods since the ITS region of rDNA is well characterized, rDNA evolve in a neutral manner at a rate which often approximates to the rate of speciation, and it has proven successful in distinguishing fungal taxa (Bruns et al., 1991; Lee and Taylor, 1992). These ITS regions are highly variable and can be used to distinguish and relate closely related organisms such as different species in the same genus. Recently, a molecular phylogeny of *Phytophthora* and related oomycetes was constructed based on the ITS sequences of rDNA (Cooke et al., 2000). The objective of our research is to detect and characterize *Phytophthora* spp. infecting citrus in the LRGV with a PCR technique using primers designed by Ippolito et al. (2002) and to confirm the results with standard culture morphology.

MATERIALS AND METHODS

Fungal material. We used a baiting technique with grapefruit (*C. paradisi* Macf.) leaves to isolate the fungus from soil and root samples collected from citrus trees in McAllen, Mission, Weslaco, Catarina, and Houston. One milliliter of soil suspension and leaf discs were also transferred to selective agar media plates with a final concentration of antibiotics 10mg L^{-1} Pimaricin (Sigma-Aldrich, St. Louis, MO), 200 $mg.L^{-1}$ Vancomycin (Sigma-Aldrich), 100 $mg.L^{-1}$ PCNB (Sigma-Aldrich), and 50 $mg.L^{-1}$ Hymexazole (Sigma-Aldrich). These plates were incubated at room temperature in the dark for a week. The colonies showing morphological characteristics of *Phytophthora* were observed under a microscope. For DNA extraction, the fungus was grown at 24°C in a 10 mL water culture.

DNA extraction. DNA was extracted from several root samples using DNeasy plant mini kit (Qiagen Inc, Valencia, CA) according to manufacturer's instructions. DNA was also extracted from fungal mycelium using Master pure Yeast DNA purification kit (Epicentre, Madison, WI).

Polymerase chain reaction (PCR). PCR was performed in a total volume of 25µL containing 100ng DNA, 2mL 10X PCR buffer (Tris. HCl, KCl, $(NH_4)_2SO4$, 15mM $MgCl_2$; pH 8.7), 0.2 mM each of dNTPs, 2µM each of Ph2 and ITS4 primers, and 1U Hot star Taq DNA polymerase (Qiagen Inc.). The reaction was incubated in the DNA engine dyad Peltier thermal cycler (Bio-Rad laboratories, Hercules, CA). PCR reaction temperatures were 95°C for 15 min, followed by 35 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 1 min. A nested PCR was also conducted using 1µL of first round PCR product as a template with primers Pn5B-Pn6 and Pc2B-Pc7. The PCR conditions for the nested PCR were the same as the first round PCR, except the annealing temperature of 55°C. The PCR products were run on 1% agarose gels prepared in tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide and visualized under ultraviolet (UV) light.

Cloning and sequencing. The 120bp fragment amplified in the nested PCR with primers Pn5B-Pn6 was cut from the gel, purified using Qiaquick gel extraction kit (Qiagen Inc), cloned into pCR4-TOPO vector (Invitrogen Corporation, Carlsbad, CA), and sequenced (MWG-Biotech Inc, High Point, NC). The DNA sequence was compared to the GenBank database for homology.

RESULTS AND DISCUSSION

The results from the screening of *Phytophthora* isolates from citrus trees in McAllen, Mission, Weslaco, Catarina, and Houston are summarized in Table 1. First round PCR with Ph2 and ITS4 primers resulted in amplification of a 700bp fragment (Fig. 1A.). This fragment includes 5.8S rDNA gene and ITS2 region, common for 14 species of *Phytophthora* (Ippolito et al., 2002). The nested PCR with primers Pn5B-Pn6 amplified a 120bp fragment and primers Pc2B-Pc7 did not amplify any fragment (Fig. 1B.). Primers Pn5B-Pn6 and Pc2B-Pc7 are specific for *P. nicotianae* and *P. citrophthora*, respectively. These primers are highly specific as the target DNA is embedded within the first round PCR product (Ippolito et al., 2002). The sequence of the 120bp fragment is shown in Fig. 2. Similarity search for this nucleotide sequence with the GenBank database showed 100% identity with *P. nicotianae* ribosomal ITS2 (accession: Y08674). This PCR technique is convenient as the target DNA could be amplified with high specificity

Table 1. Identification of *Phytophthora* isolates from citrus in Texas by PCR.

Sample collection site	Collection dates	No. of isolates	Amplification with primers			Species identification
			a	b	c	
Mission	May 2005-Feb. 2006	6	+	+	-	*P. nicotianae*
McAllen	Jan. 2006	2	+	+	-	*P. nicotianae*
Weslaco	Sept. 2005-Oct. 2005	12	+	+	-	*P. nicotianae*
Catarina	March 2006	1	+	+	-	*P. nicotianae*
Houston	May 2006	1	+	+	-	*P. nicotianae*

PCR Primers designed by Ippolito et al., 2002. a: Ph2-ITS4, b: Pn5B-Pn6, and c: Pc2B-Pc7.

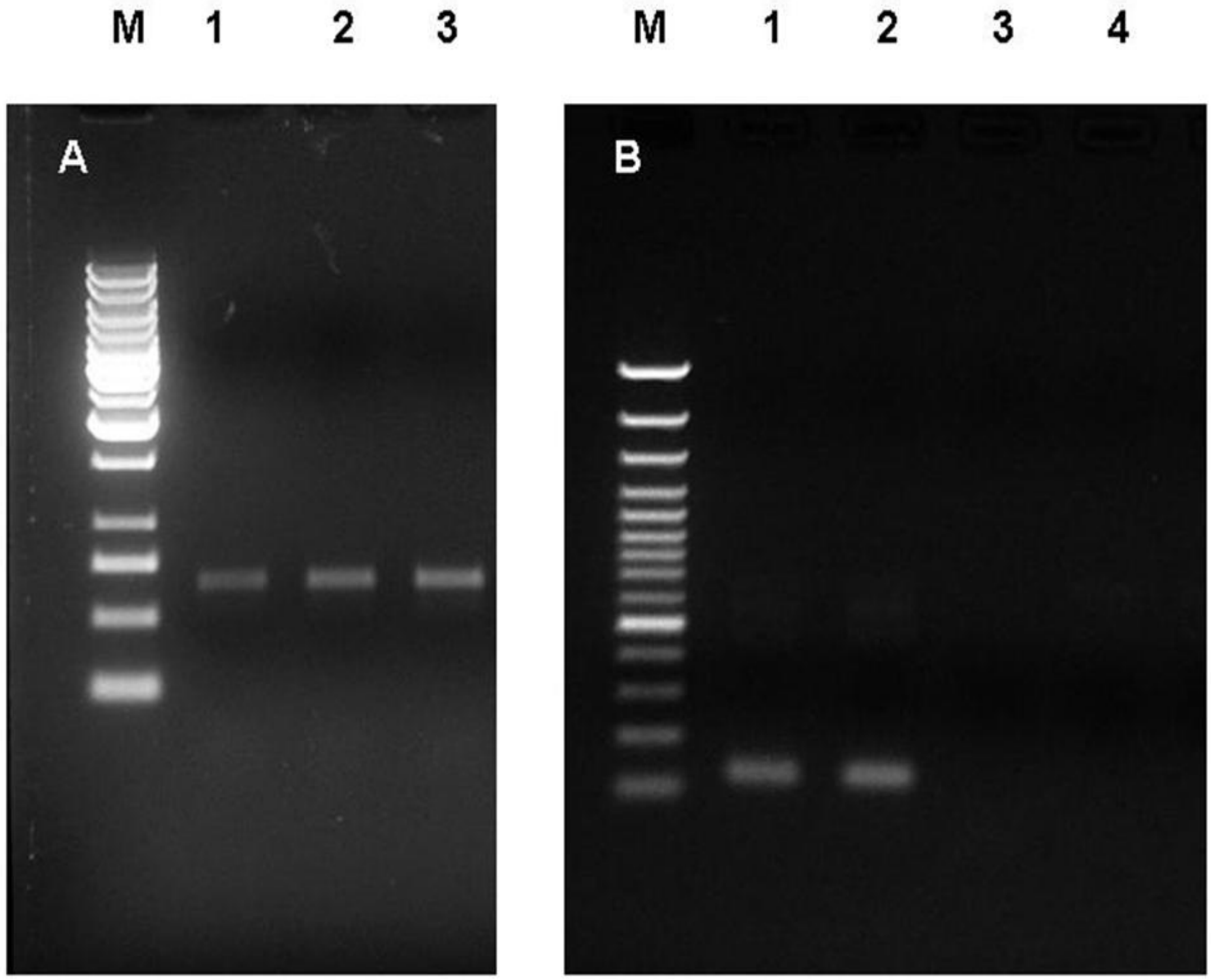

Fig. 1. Agarose gels showing amplification products of *Phytophthora* using specific PCR primers.
A: Lane 1-3 amplicons (700 bp) with primers Ph2-ITS4.
B: Lane 1-2 amplicons (120 bp) with primers Pn5B-Pn6 and Lane 3-4 amplicons with primers Pc2B-Pc7.
M: 100 bp DNA ladder plus.

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GAACAATGCAACTTATTGGACGTTTTTCCTGCTGTGGCGTGATGGACTGG
TGAACCATAGCTCGGTGGCTTGGCTTTTGAATTGGCTTTGCTGTTGCGAA
GTAGGGTGGCAGCTTCGGTT
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Fig. 2. The sequence of the 120bp fragment from ITS2 region of *Phytophthora nicotianae*

directly from the root samples. Results from *Phytophthora* assessed using selective medium are consistent with molecular analysis.

In California, *P. nicotianae* infection was reported to be predominant in summer and *P. citrophthora* in winter (Menge et al., 1988; Dirac et al., 2003). It was reported that a similar situation exists in southwestern and central parts of Arizona, where both fungi are important pathogens of citrus (Matheron et al., 1997). Temperature was attributed as the primary factor in the seasonality of infection and the abundance of a particular species. It was reported that the temperatures of 31°C and 26°C are optimum for the growth of *P. nicotianae* (Fawcett, 1936) and *P. citrophthora* (Dirac et al., 2003) respectively. Perhaps the high temperatures in Texas may be congenial for the growth of *P. nicotianae* compared to P. citrophthora. Moreover, it was previously reported that *P. nicotianae* is the predominant pathogen in Texas (Timmer, 1972, 1973) and Florida (Timmer et al., 1993).

In LRGV citrus, it is not uncommon to see trees dying with *Phytophthora* infection. Recently, a pest-disease complex, *Diaprepes-Phytophthora* was reported in the LRGV (Skaria and French, 2001). Here, several affected orange trees died quickly in 4-5 weeks showing symptoms of leaf wilt, yellowing, and defoliation. *D. abbreviatus* (L.) is a root weevil, native to the Caribbean region and was accidentally introduced into Florida in 1964 in a shipment of ornamental plants from Puerto Rico (Woodruff, 1968). It poses a serious threat to the citrus industry in that state and the management cost for the *Diaprepes-Phytophthora* complex was estimated to be as high as $500 to 600 per hectare (Muraro, 2000). The larvae feed on fibrous roots and the resulting root damage predisposes the root system to infection by *Phytophthora* spp. It was found that the management of *Diaprepes-Phytophthora* complex in a citrus orchard depends on which *Phytophthora* sp. is present and whether the soil and water conditions are conducive to the fungus (Graham et al., 2003, Rogers et al., 1996).

The potential threat posed by *Phytophthora* and *Diaprepes-Phytophthora* complex in LRGV citrus necessitates a simple and accurate method of *Phytophthora* detection. Based on the results of our experiments, *P. nicotianae* is the most prevalent species in LRGV citrus. The name, *P. nicotianae* was described in 1896 from Indonesia by van Breda de Hann and *P. parasitica* in 1913 from India by Dastur. Detailed comparisons by several researchers have shown that these two names were being applied to a single species. The nomenclature priority goes to the first description, and therefore, the name *P. nicotianae* has precedence over *P. parasitica*.

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Phytophthora species were isolated from rivers and streams in the southwestern United States by leaf baiting and identified by sequence analysis of internal transcribed spacer (ITS) ribosomal DNA (rDNA). The major waterways examined included the Rio Grande River, Gila River, Colorado River, and San Juan River.Â A second assay was used to estimate Phytophthora CFU in the Rio Grande River over three consecutive weeks as follows. Water was collected from the Rio Grande River and separated into four 1-liter aliquots, and each liter was incubated with five Salix species or Populus species leaves for 3 days.Â Six isolates recovered from the Rio Grande River generated low-quality chromatograms that did not align between the forward and reverse primers (Fig. 3A). The historic freeze that hit Texas pummeled Rio Grande Valley citrus growers. By Cayla Harris. Mani Skaria has been through this before. When the temperatures dropped below freezing earlier this month, it was impossible not to think back to the 1989 freeze that wiped out more than 20,000 acres of citrus. He had seen it first-hand, just a year after he moved to the Rio Grande Valley to work as a faculty member at the Texas A&M Citrus Center. A Washington Post article published that year was headlined â€œTEXAS CITRUS GROWERS FEAR CROP IS DESTROYED.â€Â â€œIt looks a little bleak here in the Valley right now,â€ said Dennis Holbrook, the owner of South Tex Organics based in Mission. â€œWeâ€™ve got all shades of dead brown. I guess thatâ€™s a way to put it.â€ Texas A&I University Citrus Center, Weslaco, TX 78596. The technical assistance of Mr. S. Villarreal is gratefully acknowledged. Accepted for publication 7 March 1977. Abstract. Timmer, L. w. 1977. Preventive and curative trunk treatments for control of Phytophthora foot rot of citrus. Phytopathology 67: 1149-1154. To evaluate the effectiveness of fungicidal trunk paints in young trees in Texas, captafol, CAC, CH, and copper salts of preventing infection by Phytophthoraparasitica,trunks of fatty and rosin acids at 40-60 mg/ml retained some activity.Â of citrus in the Lower Rio Grande Valley. J. Rio Grande. short-lived with no detectable activity 13 days after. Valley Hortic. Soc. 26:44-58. It is located in the Lower Rio Grande Valley of South Texas where subtropical and semi-arid climate prevail. Commercial citrus production of South Texas is almost entirely confined to Hidalgo, Cameron, Starr, and Willacy Counties with an approximate production area of 12,140 ha.Â Chaudhary, S. Identification and characterization of Phytophthora isolates from citrus orchards of South Texas. In Proceedings of the American Phytopathological Society Annual Meeting, At San Antonio, TX, USA, 31 January 2017. [Google Scholar]. Citrus tristeza virus (CTV) isolates collected from the Lower Rio Grande Valley in south Texas and east Texas were characterized using citrus indicators and molecular methods. The citrus indicators were Mexican lime (Citrus aurantifolia), sour orange (C. aurantium), sweet orange (C. sinensis) grafted to sour orange, Duncan grapefruit (C. Ã— paradisi), and Madam Vinous sweet orange, with some CTV isolates additionally indexed using the Texas commercial grapefruit cvs. Rio Red and Star Ruby, and Marrs and N-33 sweet orange. Severity ratings used 11 biotype groups or cumulative mean relative indic...Â All Texas CTV isolates produced vein clearing symptoms on inoculated Mexican lime plants.