A Survey on Occurrence of *Cladosporium fulvum* Identifies Race 0 and Race 2 in Tomato-Growing Areas of Argentina

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Abstract

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The presence of *Cladosporium fulvum* (syn. *Passalora fulva*), causal agent of tomato leaf mold, was confirmed in the two main greenhouse-production areas for tomato in Argentina. Using both morphological characters and internal transcribed spacer sequencing, we confirmed the presence of physiological races of this pathogen. A diagnostic multiplex polymerase chain reaction (PCR) was also developed, using primers derived from *C. fulvum* avirulence (*Avr*) genes. In all, 20 isolates of *Cladosporium* spp. were obtained as monospore cultures and 12 were identified as *C. fulvum.* By this method, we showed that, of these 12 isolates, 5 were race 0 (carrying functional *Avr2, Avr4, Avr4E*, and *Avr9* genes) and 7 were race 2 (lacking the *Avr2* gene). Race identity was confirmed by testing their virulence on a set of tomato differentials carrying different *Cf* resistance genes. All *Avr* genes could be amplified in single or multiplex PCR using DNA isolated from in vitro grown monospore cultures but only three *Avr* could be amplified when genomic DNA was isolated from *C. fulvum*-infected necrotic leaf tissue.

Tomato (*Solanum lycopersicum* L.) can be heavily infected by leaf mold (Cooke 1883), a disease caused by *Cladosporium fulvum* (Braun et al. 2003) (syn. *Passalora fulva*). This nonobligate biotrophic fungus infects tomato plants under conditions of high relative humidity and temperatures around 20°C (Thomma et al. 2005). Conidia settle on the lower side of the leaf, germinate, penetrate through open stomata, and colonize the intercellular space (De Wit 1977; Lazarovits and Higgins 1976). Within a week, on the upper side of the leaves, pale green or yellowish diffuse spots appear and gradually grow and become brownish. On the lower side, the fungus starts to sporulate (Blancard 1992; Thomma et al. 2005) and, in a few weeks, the spots become necrotic.

Leaf mold disease complies with the typical gene-for-gene relationship (De Wit 1992; Flor 1971). In the intercellular space of tomato leaves, the fungus secretes effector proteins that function as virulence factors on tomato plants lacking matching *C. fulvum* (*Cf*) resistance genes but function as avirulence (*Avr*) factors on tomato plants carrying the matching *Cf* genes (Dixon et al. 1996, 1998; Jones et al. 1994; Parniske et al. 1997; Takken et al. 1998; Thomas et al. 1997). Of the *Avr* genes of *C. fulvum*, four had been cloned and sequenced at the start of this study: *Avr2* (Luderer et al. 2002), *Avr4* (Joosten et al. 1994), *Avr4E* (Westerink et al. 2004), and *Avr9* (Van Kan et al. 1991). Four additional extracellular proteins (Ecp) have been identified that are also considered to be virulence factors and are recognized by matching *Cf-Ecp* genes (Bolton et al. 2008; Joosten et al. 1994; Laugé et al. 2000; Van den Ackerveken et al. 1993; Van Esse et al. 2006).

In the past, races of *C. fulvum* were identified using a set of tomato differentials carrying different *Cf* resistance genes. (Bailey 1947; Kooistra 1964). Recent studies showed that new races of *C. fulvum* often appeared within a short period of time, especially after the introduction of a single *Cf* gene in new tomato cultivars (Iida et al. 2010). In Japan, only cultivars carrying the *Cf-2* resistance gene have been cultivated for many years. However, since 2006, new varieties of tomato carrying the *Cf-9* gene were released. Soon, the new race 2.9 of *C. fulvum* appeared (Iida et al. 2010), which could overcome both the *Cf-2* and the *Cf-9* gene (Enya et al. 2009; Satou et al. 2005; Yamada and Abiko 2002).

In Argentina, leaf mold is a rather new disease, mostly affecting tomato produced in greenhouses in the provinces Corrientes and Buenos Aires. All commercial hybrids that have been grown in these provinces are susceptible to the disease (Rollan et al. 2013). Not much is known about physiological specialization of *C. fulvum* population in Argentina. Recently, Rollan and colleagues identified two races of *C. fulvum* (Rollan et al. 2013)

Molecular biology has provided new tools that can be used for diagnostic purposes; they are accurate and not highly influenced by the environment. Such tools include intersimple sequence repeat markers that are used to study diversity among and within pathogen populations (Stenglein and Balatti 2006) and specific markers that allow the identification of species or races (Theerakulpisut et al. 2008).

Recently, Stergiopoulos and colleagues (2007) developed a set of primers based on the sequences of cloned *Avr* genes of *C. fulvum*. They were used to study the allelic variation of *Avr* genes in a collection of isolates of *C. fulvum*. Yan et al. (2007) developed a real-time polymerase chain reaction (PCR) assay aimed at detecting *C. fulvum* on tomato leaves with primers derived from three fungus-specific sequences. In this study, using morphological as well as molecular tools, we isolated and identified *C. fulvum* from the two main greenhouse production areas for tomato in Argentina. In addition, we developed a diagnostic multiplex

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PCR to identify races of the fungus using primers derived from four known *C. fulvum Avr* sequences.

Materials and Methods

Isolation, subculture, and storage of monospore isolates from diseased tomato leaves. From six different locations in the two main tomato production areas of Argentina, located in the provinces of Corrientes and Buenos Aires, diseased tomato leaf samples showing typical tomato leaf mold symptoms, presumably caused by *C. fulvum*, were collected. The samples were obtained from tomato (*S. lycopersicum* L) cultivars 'Elpida' (Enza Zaden), 'Tarija', 'Potosí' (BHN Research), 'Arcos' (Wisdom Seeds), 'Compak', 'Keitor' (Syngenta Seeds S.A.), 'Cherry Colly', and 'Colibrí' (Clausen) (Rollan et al. 2013) (Table 1).

Conidia were collected from sporulating lesions and were transferred with a needle to petri plates filled with water agar (Biopack) medium. Germinated conidia produced mycelium that was transferred and grown on 2% potato dextrose agar (PDA; Britania). Cultures of fungal isolates were maintained on PDA at 4°C. Isolates were deposited in the culture collection of the Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Pcia de Buenos Aires, Argentina.

Determination of virulence spectrum of monospore isolates on a differential set of tomato cultivars carrying different *Cf* genes. The virulence spectrum of the monospore isolates was determined by inoculating them on a set of differentials of 'Money Maker' (MM) tomato carrying no (*Cf-0*) or different resistance genes (*Cf-2, Cf-4, Cf-5*, or *Cf-9*). Seed were placed in trays containing sterilized perlite-soil mix substrate and were cultivated in greenhouses under controlled conditions of growth at 28 ± 2°C. Plantlets were transplanted to 2-liter plastic pots containing the same soil medium. Plants were inoculated with a conidial suspension of 1×10^5 conidia ml^{-1} essentially as described before (Rollan et al. 2013). Twenty days after inoculation, susceptible genotypes developed pale green to yellow spots on the abaxial leaf surface and pale brown to olivaceous brown sporulation on the adaxial side.

PCR amplification of internal transcribed spacer. Genomic DNA of fungi was isolated using the cetyltrimethylammonium bromide (Sigma-Aldrich) method (Galván et al. 2003) and quantified by comparing with a λ *Hin*dIII marker (Invitrogen) after electrophoresis on 0.7% agarose gels that were stained with ethidium bromide.

Genomic DNA was mixed with primers internal transcribed spacer (ITS)-4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990) to amplify the ITS region. Reactions contained 1× amplification buffer (Inbio Highway), 1.5 mM $MgCl_2$ (Inbio Highway), 0.15 μM each primer, 200 μM each deoxynucleoside triphosphate (Inbio Highway), 25 ng of DNA template, and 1 U of *Taq* DNA polymerase (Inbio Highway) in a 15-μl volume. Reactions were performed in a PTC-1152 Mini Cycler (MJ Research) programmed as follows: an initial step at 94°C for 4 min; followed by 33 cycles of a denaturing step at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min; with a final extension at 72°C for 5 min. Control experiments lacked template DNA. Amplified fragments were visualized and compared with a 100- to 1,000-bp marker (Inbio Highway) on 1% agarose gels stained with ethidium bromide and documented by means of an image analyzer (Gene Genus analyzer; Syngene).

PCR amplification of *Avr2*, *Avr4*, *Avr4E*, and *Avr9* genes. The primers used in the amplification reactions were essentially those described by Stergiopoulos et al. (2007). We slightly modified them because the universal MP13 sequence was eliminated (AVR2f: 5′-CATCAGCA TATCCTCTTCCATCC-3′, AVR2r: 5′-CAGTACGTTCAAAAGCA GATAAGG-3′, AVR4f: 5′-ACGGTAGGTCTGTACACGAGCC-3′, AVR4r: 5′-ACCGAACTGGGTCATGGAATG-3′, AVR4Ef: 5′-GCC CGGTATATCGCTGTGC-3′, AVR4Er: 5′-CGGAACCCCTGGCT GAGA-3′, AVR9f: 5′-AATACAACCTTGAAACAGCTAGG-3′, and AVR9r: 5′-GGACTCTACGGGGCTTGG-3′) (Table 2). The PCR mixture contained 1× amplification buffer (Inbio Highway), 1.5 mM $MgCl_2$ (Inbio Highway), 0.2 mM dNTPs (Inbio Highway, Argentina), 0.4 μM forward and reverse primer, 1 U of T-Plus *Taq* DNA polymerase (Inbio Highway), and 30 ng of genomic template DNA. PCR was performed in a 15-μl volume with a thermocycler (Model Multigene gradient; Labnet International, Inc.) programmed as follows: an initial denaturing step at 94°C for 5 min; then, 40 cycles of 30 s at 94°C; an annealing step of 1 min at 63, 65, 53.5, or 62.5°C for *Avr2, Avr4, Avr4E*, and *Avr9*, respectively; and 1 min of extension at 72°C. There was a final extension of 7 min at 72°C. Amplified PCR products were separated by electrophoresis in 1% agarose gels that were stained with ethidium bromide. A 100- to 1,000-bp DNA ladder marker (Inbio Highway) was used to estimate the size of the amplicons. Gels were observed and documented in a GeneGenius analyzer (Syngene) and the bands were processed by means of GeneSnaps, GeneTools, and GeneDirectory software (Syngene).

Multiplex PCR amplification of *Avr* genes. Multiplex PCR was performed in a 15-μl volume with a thermocycler programmed as previously described, except for the annealing temperature that was changed to 58.4°C. Reactions were performed with genomic DNA isolated either from monospore cultures (40 to 60 ng) or necrotic diseased leaf tissue (100 ng), 1.5 mM $MgCl_2$ (Inbio Highway), 0.20 μM each pair of primers (corresponding to *Avr*2, *Avr4, Avr4E*, and *Avr9*), 0.2 mM dNTPs (Inbio Highway), and 1 U of T-Plus DNA polymerase (Inbio Highway) in 1× reaction buffer (Inbio Highway). PCR products were resolved by electrophoresis in 1% ultrapure agarose (Invitrogen) gels that were stained with ethidium bromide and exposed to UV light with a transilluminator. Gels were documented in a GeneGenius analyzer (Syngene) and the bands were processed by means of GeneSnaps, GeneTools and GeneDirectory software (Syngene).

The amplified bands generated in multiplex PCR were eluted from the gels as described by Sambrook et al. (1989), precipitated, and sequenced by Macrogen Inc.

Sequencing of ITS and *Avr* genes. The amplified DNA fragments were precipitated by adding 10% of a 3-M NaAc solution and one volume of isopropyl alcohol. The DNA was sequenced by the dideoxy termination method (Sanger et al. 1977) using the BigDye Terminator Cycle Sequencing Ready Reaction kit and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems, Macrogen). The sequences of the ITS and the *Avr* genes were deposited in the National Center for Biotechnology Information (NCBI) GenBank.

Table 1. Place of isolation and identity of the isolates used in this study

Species, isolate, cultivar[a]	Site of isolation[b]
Cladosporium cladosporioides	
CFOT7 (this article), Tarija	Los Hornos
AAS17 (this article), Arcos	Arana
AAS2 (this article), Arcos	Arana
HD10 (this article), ND	Los Hornos
CFOPC (this article), Potosi	Olmos
CFP14 (this article), ND	Abasto
AAS16 (this article), Arcos	Arana
C. sphaerospermum	
HD8 (this article), ND	Los Hornos
C. fulvum	
ALH (this article), Elpida	Los Hornos
ELH (this article), Elpida	Los Hornos
EMP (this article), Elpida	Los Hornos
CoA (this article), Colibrí	Arana
CH6 (this article), Elpida	Corrientes
EOP (this article), Elpida	Olmos
ColA (this article), Colibrí	Arana
CK813 (this article), Keitor	Corrientes
ELS (this article), Elpida	Etcheverry
ComA (this article), Compak	Arana
EAV (this article), Elpida	Arana
CHEAV (this article), Cherry-Colly	Arana
Ensifer fredii	
HH103 (Cleyet-Marel 1987), Soybean	Vietnam
Humicolopsis cephalosporioides	
EU (Allegrucci et al. 2009), Nothofagus	Tierra del Fuego

[a] ND = not determined.

[b] Los Hornos Pcia de Buenos Aires, Arana Pcia de Buenos Aires, Olmos Pcia de Buenos Aires, Abasto Pcia de Buenos Aires, Corrientes = Pcia de Corrientes, Etcheverry Pcia de Buenos Aires, and Tierra del Fuego = forest soil.

Results

Isolation and identification of *C. fulvum* as the causal agent of the disease. Twenty monosporic cultures were obtained from diseased leaves and analyzed. Eight isolates developed 40-mm colonies in 15 days on 2% PDA. Among them, one produced small spherical conidia (ball like, 3 to 4 μm) and the other seven small ovoid conidia (measuring about 3 to 7 by 2 to 4 μm). They were identified as *C. sphaerospermum* and *C. cladosporioides*, respectively (Bensch et al. 2010; Crous et al. 2006, 2007, 2009; Schubert et al. 2007; Zalar et al. 2007). The 12 remaining isolates grew at a slower rate and developed 40-mm colonies in 30 days. They produced one-celled, large, pale green, ovoid conidia in accordance with previous reports (Crous 2009; Rollan et al. 2013) and were identified as *C. fulvum* (Table 1). The morphological identification was confirmed by analysis of the ITS region sequences.

All 12 isolates of *C. fulvum* were inoculated on a differential set of tomato cultivars. Isolates ELH, EOP, ELS, ComA, and EAV infected only MM-*Cf*-0 tomato, which indicated that they should be designated race 0, because they contain functional *Avr*2, *Avr*4, *Avr*4E and *Avr*9 genes (Boukema 1981). The remaining seven isolates of *C. fulvum* infected MM-*Cf*-0 and MM-*Cf*-2 tomato, which indicated that they lack a functional *Avr2* gene and should be designated race 2 (data not shown).

These results were confirmed by PCR with primers derived from *C. fulvum Avr* genes and genomic DNA from *C. fulvum* as template. DNA fragments of 570, 806, 640, and 710 bp were amplified (Fig. 1), each representing the expected size of *Avr*2, *Avr*4, *Avr*4E, and *Avr*9, respectively. These bands were sequenced and a BLAST analysis indicated that they corresponded to *Avr*2, *Avr*4, *Avr*4E, and *Avr*9.

Multiplex PCR as a diagnostic tool. Determining the virulence spectrum of *C. fulvum* on a set of differentials containing different *Cf* genes is time consuming and expensive. Therefore, we developed a molecular diagnostic tool aimed at allowing researchers to identify the causal agents. Because the annealing temperature of the reactions designed to amplify *Avr*2, *Avr*4, *Avr*4E, and *Avr*9 (Stergiopoulos et al. 2007) varied within a narrow temperature range, it was postulated that it would be possible to amplify all *Avr* genes in one reaction. The method would be more powerful if the multiplex assay could be performed directly on diseased tissue, avoiding fungal isolation. PCR containing genomic DNA of *C. fulvum* race "0" (isolate ELH) and all the primers homologous to *Avr*2, *Avr*4, *Avr*4E, and *Avr*9 were run within a temperature range of 52 to 65°C. All four *Avr* genes were successfully amplified at an annealing temperature of 58°C (Fig. 2A). Such results were obtained if the reactions contained 40 to 60 ng of template DNA from *C. fulvum* and 20 ng of each primer.

In order to confirm that the PCR are specific, reactions containing template DNA of *C. fulvum* mixed with increasing concentrations of *C. cladosporioides* DNA (20, 30, and 40 ng) or DNA isolated from unrelated fungi or bacteria were performed. *Avr* genes were amplified only in reactions containing template DNA from *C. fulvum* (Fig. 2B and C).

The sizes of the fragments amplified with the multiplex PCR were identical to those obtained in individual PCR and to those described by Stergiopoulos et al. (2007) and Peteira et al. (2011). The identity of the *Avr* genes was further confirmed by analyzing the sequence of the amplicons, which showed that they were homologous to *Avr*2, *Avr*4, *Avr*4E, and *Avr*9.

To develop the diagnostic tool further, the multiplex PCR was attempted on DNA extracted from infected tissue of tomato leaves inoculated with race 0 of *C. fulvum*. In four amplification reactions aimed at amplifying each *Avr* gene using template DNA from infected leaf tissue, we successfully amplified the *Avr*2, *Avr*4, *Avr*4E, and *Avr9* genes (Fig. 3A). However, when genomic DNA was extracted from infected leaf tissue, the multiplex reaction only amplified *Avr*2, *Avr*4, and *Avr4E*. *Avr*9 could only be amplified in reactions containing the *Avr9*-specific pair of primers (Fig. 3B). Also, in this case, the amplified bands were sequenced and all appeared to represent correct *Avr* gene sequences.

As described above, among all the *C. fulvum* isolates analyzed, only two different races were found. However, the *Avr* gene sequences were polymorphic (Fig. 4; Supplementary Fig. S2). *Avr4* was polymorphic at the 5′ end compared with other isolates from Argentina but also compared with those sequences available at the NCBI database that corresponded to *C. fulvum* representatives isolated in Europe. Regarding *Avr4E*, the sequences isolated from Argentina were the same but they were polymorphic compared with those available at the NCBI database that correspond to an isolate from another country (Fig. 4; Supplementary Fig. S2).

Discussion

C. fulvum occurs worldwide and causes large economic losses in new tomato-growing areas. This might be due to the fact that, in these new areas, the populations of the causal agent are not known and tomato cultivars lack *Cf* resistance genes. Therefore, whenever the environmental conditions are optimal, outbreaks of the disease may occur (Butler and Jones 1949). Tomato leaf mold is a new disease that affects greenhouse-grown tomato in Argentina. Therefore, in order to start to develop strategies to control the disease, we isolated 20 monosporic cultures from plants with typical symptoms of leaf mold from the two main production areas.

Twelve isolates were identified as *C. fulvum* using morphological characteristics as well as ITS (Schoch et al. 2012) and *Avr* sequences (Stergiopoulos et al. 2007). The four *Avr* genes could be amplified using primers homologous to cloned *Avr* genes of *C. fulvum* (Stergiopoulos et al. 2007) and enabled us to discriminate between races. We found that five isolates of *C. fulvum* were race 0 and seven isolates were race 2, as previously reported by Rollan et al. (2013). These findings suggest that tomato hybrids used in these two main areas in the province of Corrientes and Buenos Aires lack *Cf* resistance genes (except *Cf-2*). Therefore, in the future, hybrids carrying at least two *Cf* resistance genes out of the five known (*Cf-2*, *Cf-4*, *Cf-4E Cf-5*, and *Cf-9*) should be used to manager the disease, provided that races 0 and 2 are the only ones present in Argentina.

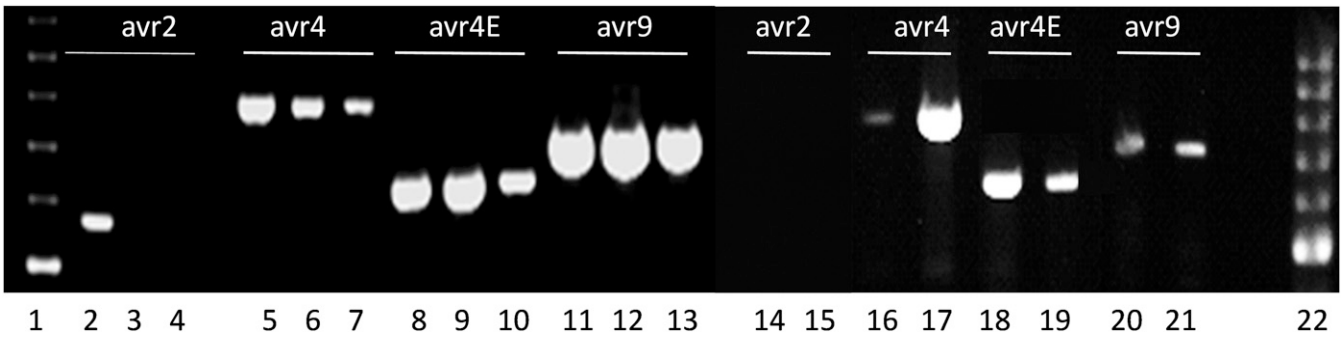

Fig. 1. Amplification of avirulence genes *Avr2* (570 bp), *Avr4* (806 bp), *Avr4E* (640 bp), and *Avr9* (710 bp) in polymerase chain reaction containing genomic DNA isolated from different isolates of *Cladosporium fulvum* collected from tomato hybrid Elpida. Lanes 1 and 22: 100- to 1,000-bp marker; lanes 2, 5, 8, and 11: isolate ELH; lanes 3, 6, 9, and 12: isolate EMP; lanes 4, 7, 10, and 13: isolate ALH; lanes 14, 16, 18, and 20: isolate CoA; lanes 15, 17, 19 and 21: isolate CH6.

The tomato–*C. fulvum* interaction complies with the gene-for-gene hypothesis proposed by Flor (1956). As with other pathosystems, a classical way of identifying the virulence spectrum of isolates of *C. fulvum* is to perform virulence assays on a set of differential tomato cultivars carrying different *Cf* genes. Such experiments are laborious and time consuming. Several molecular studies described the identification, cloning, and characterization of both *Avr* and *Cf* genes, which provided researchers with tools for management of the disease (Dixon et al. 1996, 1998; Jones et al. 1994; Joosten and De Wit 1999; Stergiopoulos et al. 2007; Yan et al. 2008). Therefore, we developed a diagnostic tool for identification of *C. fulvum* as well as identifying the race.

We successfully amplified *Avr* genes *Avr2*, *Avr4*, *Avr4E*, and *Avr9* in single PCR; all of the reactions included the temperature of the annealing step within a narrow window, which suggested that amplifications might be performed in a single multiplex PCR. Such a reaction amplified all four *Avr* genes from *C. fulvum* template DNA but not from bacteria (*Ensifer fredii*) or fungi, including *Humicolopsis cephalosporioides* or *Cladosporium* spp. Also, the presence of contaminating DNA did not outcompete *C. fulvum* DNA, confirming that the reaction is specific: the DNA of saprophytes or opportunistic pathogens that might be accompanying *C. fulvum* in lesions does not interfere with the reaction.

The multiplex PCR was specific and particularly accurate when template DNA was isolated from cultures of *C. fulvum* grown in vitro. However, when the reaction was run with genomic DNA isolated from infected leaf tissue, only *Avr2, Avr4*, and *Avr4E* were amplified. We do not have an explanation for the failure to amplify *Avr9* under the conditions described, because the gene was amplified in reactions containing only *Avr9* primers. One possibility is that, because the *Avr9* sequence is short and located in a repeat-rich region, the corresponding primers might anneal less efficiently. Therefore, both single and multiple PCR effectively detected the presence or absence of *Avr* genes. However, multiplex PCR can only be successfully applied to identify the virulence spectrum of a race, when changes in virulence are correlated with the loss of an *Avr* gene, as can sometimes be the case for *C. fulvum*. Thus far, overcoming resistance genes such as *Cf-9* has always been correlated with loss of the *Avr9* gene, while overcoming *Cf-2* and *Cf-4E* has often, though not

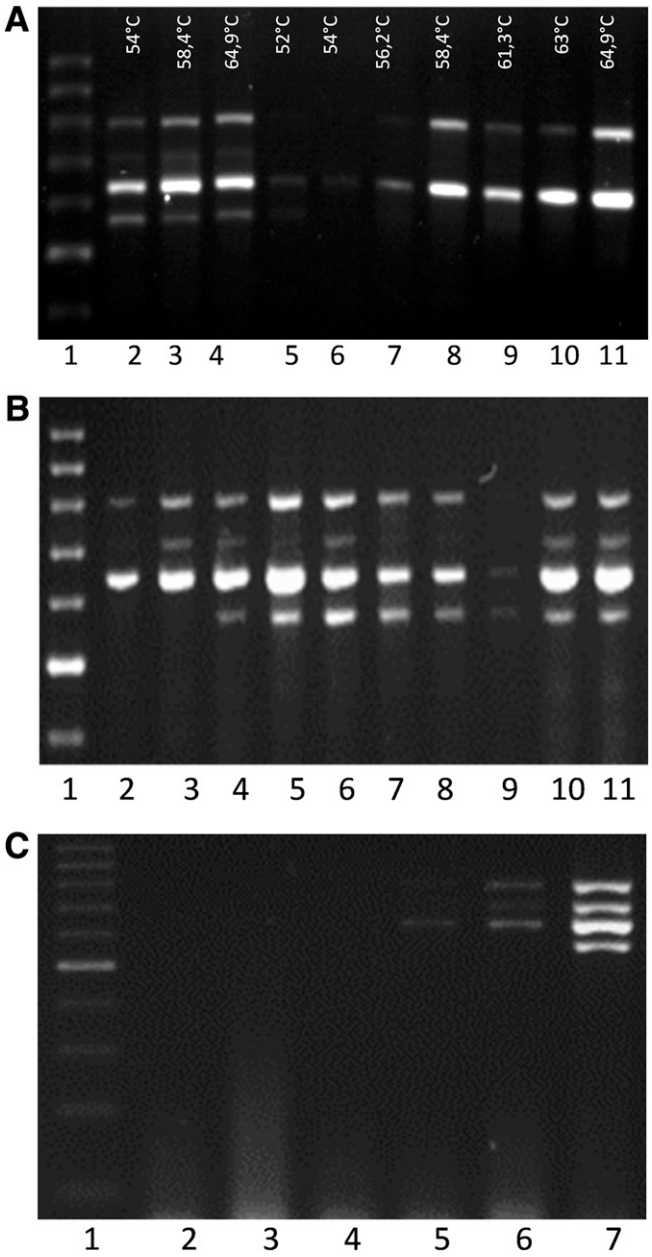

Fig. 2. A, Multiplex polymerase chain reaction (PCR) of *Avr2* (570 bp), *Avr4* (806 bp), *Avr4E* (640 bp), and *Avr9* (710 bp) using different amounts of primers and annealing temperatures. Genomic DNA (40 ng) was used for isolate ELH. Lane 1, 100- to 1,000-bp marker; lanes 2 to 4, 20 ng of each primer; lanes 5 to 11, 12 ng of each primer. **B,** Multiplex PCR of *Avr2* (570 bp), *Avr4* (806 bp), *Avr4E* (640 bp), and *Avr9* (710 bp) in the presence of increasing amounts of template DNA from *Cladosporium fulvum* and competitive DNA from other organisms. Lane 1, 100- to 1,000-bp marker; lanes 2 to 4 reactions with 40 ng of genomic DNA of ALH, EMP, and ELH, respectively; lanes 5 to 8, reactions with 60, 45, 30, and 15 ng of genomic DNA of isolate ELH, respectively; lanes 9 to 11, reactions with 40 ng of genomic DNA of isolate ELH mixed with 40, 30, and 20 ng of genomic DNA of *C. cladosporioides*, respectively. **C,** Multiplex PCR of *Avr2* (570 bp), *Avr4* (806 bp), *Avr4E* (640 bp), and *Avr9* (710 bp). Lane 1, 100- to 1,000-bp marker; lanes 2 to 4, reactions with 40 ng of template DNA from *Ensifer fredii* HH103, *Humicolopsis cephalosporioides*, and *C. cladosporioides*, respectively; lanes 5 to 7, reactions with 40 ng of template DNA from isolates ALH, EMP, and ELH, respectively.

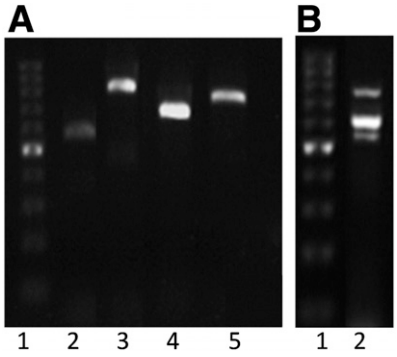

Fig. 3. A, Amplification of *Avr*2 (570 bp), *Avr4* (806 bp), *Avr4E* (640 bp), and *Avr9* (710 bp) in reactions containing genomic DNA isolated from necrotic leaf tissue infected by ELH. Lane 1, 100- to 1,000-bp marker; lane 2, *Avr2*; lane 3, *Avr4,* Lane 4, *Avr4E*; lane 5, *Avr9*. **B,** Lane 1, 100- to 1,000-bp marker; lane 2, multiplex polymerase chain reaction using 100 ng of genomic DNA isolated from necrotic leaf tissue infected by ELH.

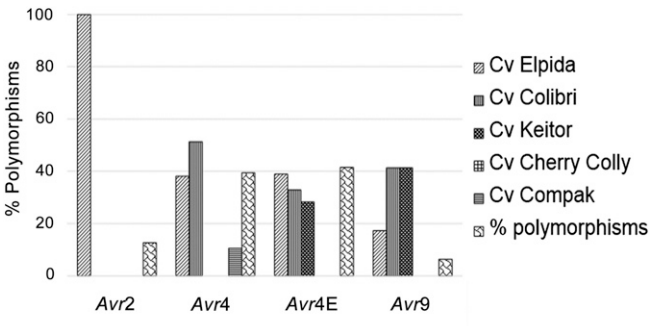

Fig. 4. Relative quantity of polymorphisms found in each *Avr* sequence as related to the tomato hybrid of isolation and number polymorphisms accumulated in each *Avr*.

always, been correlated with loss of the corresponding *Avr2* and *Avr4E* genes, respectively (Luderer et al. 2002; Van den Ackerveken et al. 1992; Westerink et al. 2004). Such a change is not based on loss of *Avr* gene but on nucleotide polymorphisms in the *Avr* genes leading to amino acid changes. In the Avr protein, the amplified bands need to be sequenced. This has been the case with races overcoming both the *Cf-4* (Joosten et al. 1994, 1997) and *Cf-2* and *Cf-4E* genes (Luderer et al. 2002; Westerink et al. 2004).

Multiplex PCR combined with sequencing of the multiplied bands can be an alternative for testing the virulence spectrum of races on a differential set of tomato lines carrying different *Cf* genes. However, new polymorphisms in *Avr* genes that have not been reported before may evolve that could overcome matching *Cf* genes. In such instances, the multiplex PCR will not be sufficient. The *Avr* genes will need to be tested for ability to cause a hypersensitive response (HR) when coexpressed in plants carrying the complementary *Cf* gene (van der Hoorn et al. 2000).

Although the areas sampled were distant, only two races of *C. fulvum* were found. This likely reflects the fact that few *Cf* genes are present in the hybrid cultivars that are used in Argentina. The analysis of the sequences of *Avr*2, *Avr4*, *Avr4E*, and *Avr9* in race 0 showed that they were almost identical to published sequences, except for a few polymorphisms that likely do not affect the biological activity of Avr proteins, because HR reactions occurred in cultivars carrying the corresponding *Cf* gene. The virulence spectrum of a given race of *C. fulvum* might be determined by polymorphisms in *Avr* genes, including nucleotide substitutions, frame-shift mutations. and the complete loss of an *Avr* gene. This means that, after having obtained the amplicon of an *Avr* gene, it still needs to be sequenced to see whether it may contain nucleotide polymorphisms that result in an Avr protein that can escape recognition by a matching *Cf* resistance gene. Moreover, the sequence of an *Avr* gene might help to identify the origin of a particular isolate within production areas and might allow researchers to follow its evolutionary history. Stergiopoulos and colleagues (2007) studied the allelic variation of effectors within isolates of *C. fulvum* and found that *Avr* genes seem to be under continuous selection pressure imposed by *Cf* genes present in tomato cultivars grown in production areas. In addition, neutral mutational shifts can occur that do not affect the biological activity of the encoded proteins. The presence of polymorphisms within the *Avr* gene sequences suggested that races might be under an evolutionary process, which might be important considering the reduced number of isolates analyzed.

In conclusion, two races of *C. fulvum*, race 0 and race 2, were identified within 12 isolates of *C. fulvum* obtained from the two most important greenhouse-grown tomato production areas in Argentina. The multiplex PCR method described successfully amplified four *Avr* genes from a mixture of genomic fungal DNA and also from genomic DNA isolated from infected leaf tissue although, in this case, two reactions are needed. Such methodology, much quicker than a virulence assay, is an important complementary diagnostic tool for identification. Introduction of *Cf-4*, *Cf-5*, and *Cf-9* resistance genes in the new tomato hybrids can protect them against the prevailing race 0 and 2 of *C. fulvum*. These results suggest that combining two or even three *Cf* genes into one hybrid should reduce the impact of the disease (Hörger et al. 2012).

Table 2. List of primers homologous to the 5′ and 3′ end of *Avr*2, *Avr4*, *Avr4E*, and *Avr9*

Primer	Sequences
AVR2f	5′-CATCAGCATATCCTCTTCCATCC-3′
AVR2r	5′-CAGTACGTTCAAAAGCAGATAAGG-3′
AVR4f	5′-ACGGTAGGTCTGTACACGAGCC-3′
AVR4r	5′-ACCGAACTGGGTCATGGAATG-3′
AVR4Ef	5′-GCCCGGTATATCGCTGTGC-3′
AVR4Er	5′-CGGAACCCCTGGCTGAGA-3′
AVR9f	5′-AATACAACCTTGAAACAGCTAGG-3′
AVR9r	5′-GGACTCTACGGGGCTTGG-3′

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