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

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Abstract

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). C. fulvum 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
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 Coronientes 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), 'Compák', 'Keitor' (Syngenta Seeds S.A.), 'Cherry Colly', and 'Colibri' (Clausen) (Rollán 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 on 0.7% agarose gels that were stained with ethidium bromide for 4 min; followed by 33 cycles of a denaturing step at 94°C for 1 min and a 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 GeneSners, 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 MgCl₂ (Inbio Highway), 0.20 μM each pair of primers (corresponding to Avr2, Avr4, Avr4E, and Avr9), 0.2 mM dNTPs (Inbio Highway), and 1 μl 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 GeneSners, 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

<table>
<thead>
<tr>
<th>Species, isolate, cultivar</th>
<th>Site of isolation</th>
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<tbody>
<tr>
<td>Cladosporium cladosporioides</td>
<td></td>
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<tr>
<td>CFOT17 (this article), Tarija</td>
<td>Los Hornos</td>
</tr>
<tr>
<td>AAS17 (this article), Arcos</td>
<td>Arana</td>
</tr>
<tr>
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<td>Arana</td>
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<tr>
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<tr>
<td>Eusph. f. e.</td>
<td></td>
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<tr>
<td>HH103 (Cleyet-Mareil 1987), Soybean</td>
<td>Vietnam</td>
</tr>
</tbody>
</table>

\* ND = not determined.

\* ND = not determined.

\* ND = not determined.

\* ND = not determined.
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 Avr2, Avr4, Avr4E and Avr9 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 Avr2, Avr4, Avr4E, and Avr9, respectively. These bands were sequenced and a BLAST analysis indicated that they corresponded to Avr2, Avr4, Avr4E, and Avr9.

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 Avr2, Avr4, Avr4E, and Avr9 (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 Avr2, Avr4, Avr4E, and Avr9 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).

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 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 Avr2, Avr4, Avr4E, and Avr9.

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 Avr2, Avr4, Avr4E, and Avr9 genes (Fig. 3A). However, when genomic DNA was extracted from infected leaf tissue, the multiplex reaction only amplified Avr2, Avr4, and Avr4E. Avr9 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.
The tomato–\textit{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 \textit{C. fulvum} is to perform virulence assays on a set of differential tomato cultivars carrying different \textit{Cf} genes. Such experiments are laborious and time consuming. Several molecular studies described the identification, cloning, and characterization of both \textit{Avr} and \textit{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 \textit{C. fulvum} as well as identifying the race.

We successfully amplified \textit{Avr} genes \textit{Avr2}, \textit{Avr4}, \textit{Avr4E}, and \textit{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 \textit{Avr} genes from \textit{C. fulvum} template DNA but not from bacteria (\textit{Ensifer fredii}) or fungi, including \textit{Humicolopsis cephalosporioides} or \textit{Cladosporium} spp. Also, the presence of contaminating DNA did not outcompete \textit{C. fulvum} DNA, confirming that the reaction is specific: the DNA of saprophytes or opportunistic pathogens that might be accompanying \textit{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 \textit{C. fulvum} grown in vitro. However, when the reaction was run with genomic DNA isolated from infected leaf tissue, only \textit{Avr2}, \textit{Avr4}, and \textit{Avr4E} were amplified. We do not have an explanation for the failure to amplify \textit{Avr9} under the conditions described, because the gene was amplified in reactions containing only \textit{Avr9} primers. One possibility is that, because the \textit{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 \textit{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 \textit{Avr} gene, as can sometimes be the case for \textit{C. fulvum}. Thus far, overcoming resistance genes such as \textit{Cf-9} has always been correlated with loss of the \textit{Avr9} gene, while overcoming \textit{Cf-2} and \textit{Cf-4E} has often, though not

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{A, Multiplex polymerase chain reaction (PCR) of \textit{Avr2} (570 bp), \textit{Avr4} (806 bp), \textit{Avr4E} (640 bp), and \textit{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 \textit{Avr2} (570 bp), \textit{Avr4} (806 bp), \textit{Avr4E} (640 bp), and \textit{Avr9} (710 bp) in the presence of increasing amounts of template DNA from \textit{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 \textit{C. cladosporioides}, respectively. C, Multiplex PCR of \textit{Avr2} (570 bp), \textit{Avr4} (806 bp), \textit{Avr4E} (640 bp), and \textit{Avr9} (710 bp). Lane 1, 100- to 1,000-bp marker; lanes 2 to 4, reactions with 40 ng of template DNA from \textit{Ensifer fredii} HH103, \textit{Humicolopsis cephalosporioides}, and \textit{C. cladosporioides}, respectively; lanes 5 to 7, reactions with 40 ng of template DNA from isolates ALH, EMP, and ELH, respectively.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Fig. 3. A, Amplification of \textit{Avr2} (570 bp), \textit{Avr4} (806 bp), \textit{Avr4E} (640 bp), and \textit{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, \textit{Avr2}; lane 3, \textit{Avr4}; lane 4, \textit{Avr4E}; lane 5, \textit{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.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Fig. 4. Relative quantity of polymorphisms found in each \textit{Avr} sequence as related to the tomato hybrid of isolation and number polymorphisms accumulated in each \textit{Avr}.}
\end{figure}
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 amplified 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 Avr2, 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 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).

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