Eur J Plant Pathol
DOI 10.1007/s10658-017-1248-z

A survey on tomato leaf grey spot in the two main production areas of Argentina led to the isolation of *Stemphylium lycopersici* representatives which were genetically diverse and differed in their virulence

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Accepted: 3 May 2017

Abstract Tomato gray leaf spot was first reported in Argentina in 1990. Since then, the disease has not only increased in endemic areas, but also disseminated in other tomato-growing areas. In a survey of plants with typical symptoms of Tomato grey leaf spot disease we isolated 27 *Stemphylium* representatives from the two main tomato-growing areas of Argentina. Cultural features such as sporulation, conidia morphometry among others revealed high variability between isolates, which was confirmed by Inter Simple Sequence Repeat (ISSR)-PCR technique. A molecular phylogenetic analysis comprising the Internal Transcribed Spacer (ITS) and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene partial sequences unambiguously identified all isolates as *Stemphylium lycopersici*. Based on disease severity on detached leaves, isolates were grouped in three categories (high, medium and low virulent). No correlation was found between phenotypic or genotypic characters and the geographical origin of the isolates.

Keywords *Stemphylium lycopersici* · Tomato gray leaf spot · Morphological variability · Genetic diversity · Virulence · Molecular phylogeny

Electronic supplementary material The online version of this article (doi:10.1007/s10658-017-1248-z) contains supplementary material, which is available to authorized users.

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Published online: 26 May 2017

Introduction

Tomato (*Solanum lycopersicum* L.) is a major crop worldwide. Approximately 4.6 million ha are cultivated annually yielding around 163 million tons (FAO 2016). Like other crops, it is negatively affected by various pests and diseases. Currently, the estimated number of tomato diseases is about 200 (Jones et al. 2014). Among the infectious diseases, more than 76 species of fungi have been described to be pathogenic on tomato (Farr and Rossman 2016).

Tomato gray leaf spot is a disease present in most tomato-growing areas around the world with warm temperatures and high relative humidity. The etiological agents of this disease are three species of the genus *Stemphylium* (teleomorph: *Pleospora*): *S. solani* G.F. Weber, *S. lycopersici* (Enjoji) W. Yamam. (syn. *S. floridanum* Hannon & G. F. Weber) and *S. botryosum* Wallr. f. sp. *lycopersici* Rotem, Y. Cohen, & I. Wahl (Jones et al. 2014). Under the conditions described, conidia of the fungus on the leaf surface develop a germination tube that penetrates the leaf mainly through stomata but also through the periclinal cell walls of the epidermis. Then, a vesicle develops inside the substomatal cavity, from where secondary hyphae appear and colonize the entire mesophyll. The first macroscopic lesions can be seen after 36 h of infection (Bentes and Matsuoka 2005). Symptoms consist in small brownish specks, which later became grayish slightly angular lesions that are surrounded by a yellow halo. As lesions mature, the center of developing lesions dried up and become brittle. In severe attacks, yellowing occurs along the entire leaf that present a high number of spots that might coalesce in large necrotic foliar areas (Blancard 2012; Jones et al. 2014).

Traditionally, *Stemphylium* species have been identified based on conidial morphology. However, such characters are not only variable but are also under the influence of environmental conditions (Leach and Aragaki 1970; Hawker 2016; Snyder and Hansen 1941; Neergaard 1945; Williams 1959; Joly 1962). Molecular biology provided researchers with neutral molecular markers that are unaffected by the environment. Because of this, today the molecular phylogenetic analysis is widely used to study relationships among species, which complements studies based on morphological features. Câmara et al. (2012) stated, by a multi-locus phylogenetic analysis of the internal transcribed spacer (ITS) and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene partial sequences, that the genus *Stemphylium* is a monophyletic clade in the *Pleosporaceae*. This was further confirmed by Inderbitzin et al. (2005, 2009) by means of a multilocus approach based on the nucleotide sequences of the ITS, *gpd*, elongation factor 1α (ef-1 α) and the noncoding region between the vacuolar membrane ATPase catalytic subunit A gene (*vmaA*) and a gene involved in vacuolar biogenesis (*vpsA)*.

Molecular methods also have been widely used to study and analyze genetic diversity among pathogens populations. Regarding *Stemphylium* species, genetic variability has been studied using Random Amplified Polymorphic DNA (RAPD) (Chaisrisook et al. 1995; Mehta 2001; Nasehi et al. 2014; Sy-Ndir et al. 2015), Enterobacterial Repetitive Intergenic Consensus (ERIC) (Mehta et al. 2002), Repetitive Extragenic Palindromes (REP) (Mehta et al. 2002) and Inter Simple Sequence Repeat (ISSR) (Nasehi et al. 2014; Al-Amri et al. 2016) fingerprints. Knowledge of the structure and dynamics of pathogens populations are essential to develop a strategy to manage the disease (McDonald and Linde 2002).

In Argentina, tomato gray leaf spot was first reported in the province of Corrientes in 1990 and, since then, the disease has also been found in the main tomato growing regions in the country. Until now, the disease has been associated with *S. solani* and *S. lycopersici*, though such studies relied solely on morphological features (Colombo et al. 2001; Ramallo et al. 2005; Colombo and Obregón 2008). Furthermore, no studies regarding the genetic variability and the population structure of *Stemphylium* species causing gray leaf spot in Argentina have been done. Therefore, the aim of this work was to confirm the identity of the causal agent of tomato gray leaf spot disease in plant materials collected from important tomato-growing regions of Argentina by means of conventional and molecular approaches and to characterize the etiological agent based on cultural, morphological, pathogenic as well as genetic features.

Materials and methods

Fungal samples

The work was carried out with twenty-seven *Stemphylium* isolates that belong to the culture collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Universidad Nacional de La Plata (UNLP).

Fungal isolates were obtained from tomato (*Solanum lycopersicum* L.) plants collected from the main tomato-growing areas in Argentina in 2010, 2011 and 2013 that presented typical symptoms of gray leaf spot (Table 1).

Cultural and morphological characterization

Cultural and morphological characteristics of the isolates were analyzed both in cultures grown on homemade and commercial potato dextrose agar (PDA). Homemade PDA was prepared by boiling 200 g of potatoes cv. Spunta in distilled water (1 l) for 1 h. Then, the filtered extract was mixed with 20 g of agar and 20 g of dextrose and it was finally boiled for an additional time (20 min). Commercial PDA was prepared according to the recommendations of the manufacturer Britania S.A. (Argentina). Each isolate was inoculated by placing a 7 mm plug from 7-day-old cultures at the center of a plastic Petri dish, which was incubated at 25 °C in continuous darkness for 7 days. The experiment was completely at random and the number of replicates was three per isolate per culture medium. Features such as growth rate, colony color, elevation, margin, zonation, culture medium pigmentation and sporulation were examined. Colony diameter was recorded as the mean of two colony diameters at right angles for each colony. Colors were designated according to the Munsell Colour Order System as implemented in the Virtual Colour Atlas v. 2.0.0720 web application (Virtual Colour Systems LTD 2013). Sporulation capacity was estimated by adding 5 ml of 0.01% Tween 20 to the culture, which was then scrapped with the help of a spatula. The suspension obtained was homogenized by pipetting and vortexing and then, a 100 μl aliquot was loaded in a modified Neubauer chamber, where spores were counted. Experiments were repeated at least three times and values were averaged. The number of conidia per square centimeter of fungal colony was estimated compared to the corresponding colony diameter. Conidia morphology was examined in lactophenol by light microscopy. Fifty mature conidia (±SD) were measured at ×100 magnification using a calibrated ocular micrometer. Both macroscopic and microscopic observations were photo-documented. Data were subjected to a one-way Analysis of Variance (ANOVA) and means were compared by the Least Significant Difference (LSD) test ($P = 0.05$) using InfoStat version 2015 l (Di Rienzo et al. 2015). In order to examine whether fungal growth was determined by the type of culture medium, a two-way nested ANOVA was carried out using the independent variables: culture medium and isolate.

Table 1 Origin of *Stemphylium* isolates

Isolate	Department	Tomato cultivar	Collection year
CIDEFI-200	Lavalle	Elpida	2011
CIDEFI-201	Lavalle	Elpida	2011
CIDEFI-202	Lavalle	Elpida	2011
CIDEFI-203	Lavalle	Elpida	2011
CIDEFI-204	Lavalle	Elpida	2011
CIDEFI-205	Lavalle	Elpida	2011
CIDEFI-206	Lavalle	Torry	2011
CIDEFI-207	Bella Vista	Elpida	2011
CIDEFI-208	Lavalle	Elpida	2011
CIDEFI-210	Bella Vista	Elpida	2010
CIDEFI-211	Lavalle	Elpida	2011
CIDEFI-212	La Plata	Elpida	2010
CIDEFI-213	Bella Vista	Elpida	2011
CIDEFI-214	Lavalle	Elpida	2011
CIDEFI-215	Bella Vista	Elpida	2011
CIDEFI-216	Bella Vista	Elpida	2010
CIDEFI-217	Lavalle	Torry	2011
CIDEFI-218	Bella Vista	Elpida	2011
CIDEFI-219	Lavalle	Torry	2011
CIDEFI-220	Bella Vista	Elpida	2011
CIDEFI-225	La Plata	Tomate Platense	2013
CIDEFI-226	La Plata	Tomate Platense	2013
CIDEFI-227	Lavalle	Elpida	2013
CIDEFI-228	Lavalle	Elpida	2013
CIDEFI-229	Lavalle	Elpida	2013
CIDEFI-230	La Plata	Elpida	2013
CIDEFI-231	La Plata	Elpida	2013

Virulence assessment

Virulence of *Stemphylium* isolates was evaluated in vitro on tomato cv. Elpida by means of a detached leaf assay. Briefly, detached leaflets from 45-days old tomato plants were placed with the adaxial side down on water-soaked filter paper inside a plastic Petri dish. Then, they were injured with a sterile tip on the abaxial side at three equidistant points where they were inoculated with a conidial suspension of 30 μl of a 10^3 conidia ml^{-1} suspension. The spore suspension was prepared as described before and filtered with sterilized cheesecloth. Since a few isolates failed to sporulate, the number of

colony forming units (CFU) was used as an estimate of the actual inoculum concentration. For these non sporulating isolates, 100 μl aliquots of serial dilutions from mycelial suspensions, prepared as described above, were plated on PDA and incubated for 48 h at 25 °C. After that time, the number of CFU was determined. Controls consisted in leaflets treated with a sterilized 0.01% Tween 20 solution. Petri dishes where sealed with Parafilm to prevent water loss and were incubated for a week at 25 °C. Symptoms were examined 7 days post inoculation (dpi) and the average lesion was determined from spot infections using the image analysis software for plant disease quantification Assess 2.0 (Lamari 2002). The experiment consisted of nine replicates of one leaflet per replicate inoculated with each isolate; the experiment was repeated twice. The inoculated fungi were re-isolated in order to fulfill Koch's postulates. Data were statistically analyzed by a one-way ANOVA and differences among treatment means were contrasted by the LSD test ($P = 0.05$), as implemented in InfoStat version 2015 l (Di Rienzo et al. 2015).

Molecular identification

Genomic DNA extraction

Total genomic DNA was extracted from axenic cultures using the CTAB method of Bornet and Branchard (2001). The quality and quantity of genomic DNA was evaluated by electrophoresis in a 0.7% agarose gel that was stained with ethidium bromide. Gels were visualized by means of UV light and images were captured with the software GeneSnap. Genomic DNA was quantified by comparing the bands of total DNA with those of a molecular marker of known concentration with the GeneTools image analyzer (SynGene, Cambridge, UK). Extracted DNA was stored at −70 °C until analysis.

PCR amplification and sequencing

Primers ITS4 and ITS5 (White et al. 1990; Table 2) were used to amplify the ITS. Primers GPDF and GPDR (Table 2), which were designed based on *gpd* sequences of *Stemphylium* spp. available in the GenBank (www.ncbi.nlm.nih.gov), were used to amplify a partial sequence of the *gpd* gene.

Both ITS and *gpd* PCRs were performed in a 15 μl reaction mixture containing 50 ng of template DNA, 0.3 μM of each forward and reverse primer, 1.5 μl 10X reaction buffer (500 mM KCl; 100 mM Tris-HCl, pH 9.0 a 25 °C; 1% Tween 20), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 unit of *Taq* polymerase (all Inbio Highway®, Buenos Aires, Argentina). To amplify the ITS fragment the thermocycler was programed as follows: 5 min at 94 °C followed by 33 cycles of 1 min at 94 °C, 45 s at 56 °C and 1 min at 72 °C, followed by a final extension step of 72 °C for 5 min. On the other hand, the fragment encoding the *gpd* was amplified by the following parameters: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C and a final extension that consisted in 5 min at 72 °C. Both amplification reactions were performed using a PTC-0150 MiniCycler (MJ. Research. Watertown, MA, USA). PCR products were resolved in 1% agarose gel electrophoresis stained with ethidium bromide. Gels were visualized by UV illumination, images were captured with GeneSnap and the DNA quantified with GeneTools image analyzer (SynGene). PCR products were purified by isopropanol precipitation and were sequenced at Macrogen (Seoul, Korea).

Table 2 List of primers used to perform all the PCR amplification described in this study

Primer	Sequence (5′-3′)
AA_5	GAG(AAG)5
AN	(CAA)5
BA_3	(AC)8CT
GA_5	TCA(GT)8
FA_5	TAC(GA)5
LA_5	CAG(AAC)5
ITS4	AAGCTTTCCTCCGCTTATTGATATGC
ITS5	GAATTCGGAAGTAAAAGTCGTAACAAGG
GPDF	GACATTGTCGCCGTGAAC
GPDR	ACTCGACGACGTAGTAGG

Sequence alignment and molecular phylogenetic analysis

The taxonomic position of the isolates was assessed by performing a molecular phylogenetic analysis. The analysis included seven representatives of five genera of the order *Pleosporales* (*Alternaria*, *Bipolaris*, *Cochliobolus*, *Pyrenophora* and *Setosphaeria*) that were chosen as outgroup and 23 representatives of the genus *Stemphylium* were included in the analysis.

Both ITS and *gpd* partial sequences were obtained from GenBank (www.ncbi.nlm.nih.gov; Table 3). Sequences were aligned with MEGA 5.10 (Tamura et al. 2011) using the default parameters of the ClustalW algorithm (gap opening penalty 15, gap extension penalty 6.66). The alignments were visually checked and manually optimized. Phylogenetic analysis was performed under both Maximum-parsimony (MP) and Maximum-likelihood (ML) criteria. Previously, the partition homogeneity test (PHT) (Farris et al. 1994) was performed in order to determine whether the two loci could be concatenated into a single dataset. PHT was run in PAUP* (Phylogenetic Analysis Using Parsimony) 4.0 b10 software (Swofford 2002) using the same parameters described below for MP analysis. MP based phylogenetic analysis was performed using PAUP* with the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1000 random sequence additions. Characters were treated as unweighed and gaps were treated as missing data. Due to the excessive computational time required to conduct a heuristic MP search, the number of saved trees was limited to 100 with scores of 1 or above for each random-addition-sequence replicate. Clade stability was assessed via 1000 bootstrap

Table 3 Additional strains used in the phylogenetic analysis

Specie	Strain	ITS[a]	*gpd*[a]
Alternaria alternata	EGS 34-016	AF071346	AF081400
Bipolaris australis	Turgeon 77,139	AF081448	AF081409
Cochliobolus sativus	Tinline A20	AF071329	AF081385
Pyrenophora japonica	DAOM 169286	AF071347	AF081369
Pyrenophora tritici-repentis	DAOM 208990	AF071348	AF081370
Setosphaeria minor	ATCC 62323	AF071341	AF081396
Setosphaeria rostrata	ATCC 32197	AF071342	AF081379
Stemphylium alfalfae	EGS 36-088	AY329171	AY316971
S. astragali	EGS 27-194.1	AF442777	AF443876
S. astragali	EGS 27-194.2	AF442779	AF443878
Pleospora tarda	EGS 04-118c	AF442782	AF443881
P. tarda	ATCC 26881	AF442781	AF443880
S. callistephi	NO 536	AF442783	AF443882
P. eturmiunum	EGS 29-099	AY329230	AY317034
P. gracilariae	EGS 37-073	AY329217	AY317021
S. gracilariae	EGS 37-073 extype	AF442784	AF443883
P. herbarum	EGS 30-181.1	AF442786	AF443885
S. lancipes	EGS 46-182	AF442787	AF443886
S. lycopersici	EGS 46-001	AF442790	AF443889
S. lycopersici	NO 425	AF442791	AF443890
P. paludiscirpi	EGS 31-016	AY329231	AY317035
P. sedicola	EGS 48-095	AY329232	AY317036
S. solani	EGS 42-027	AF442797	AF443896
S. solani	NO 545	AF442794	AF443893
P. tomatonis	EGS 29-089	AY329229	AY317033
S. trifolii	NO 615	AF442801	AF443900
S. trifolii	NO 553	AF442798	AF443897
P. triglochinicola	EGS 36-118	AF442802	AF443901
S. vesicarium	EGS 37-067	AF442803	AF443902
S. xanthosomatis	EGS 17-137	AF442804	AF443903

[a] GenBank accession number.

replications using the heuristic search options described above. As for the ML analysis, best-fit models of nucleotide substitution were assessed with jModelTest 2 (Darriba et al. 2012) software by using Akaike Information Criterion (AIC; Akaike 1974). Parameters of the chosen models were used in PhyML 3.1 software (Guindon and Gascuel 2003) to find the most-likelihood trees, whose branch support were estimated via 1000 bootstrap replicates.

Genetic diversity analysis

Diversity among all the isolates was analyzed by ISSR-PCR procedure (Bornet and Branchard 2001). Six micro-satellite primers were selected based on the number of polymorphic bands amplified and reproducibility of the reaction (AA_5, AN, BA_3, GA_5, FA_5 and LA_5, Table 2). PCR amplifications were carried out in a 25 μl volume containing 12 ng of template DNA, 1 μM of primer, 2.5 μl 10X reaction buffer (500 mM KCl; 100 mM Tris-HCl, pH 9.0 a 25 °C; 1% Tween 20), 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 units of *Taq* polymerase (all Inbio Highway®, Buenos Aires, Argentina). Amplifications were done using a PTC-0150 MiniCycler (MJ. Research. Watertown, MA, USA) thermocycler programmed as follows: an initial denaturation step at 94 °C for 7 min, followed by 33 cycles of 94 °C for 1 min, 48 °C (primers AA_5, AN and BA_3 and GA_5) or 53 °C (primers FA_5 and LA_5) for 75 s and 72 °C for 4 min, at the end all reactions had a final extension of 72 °C for 7 min. ISSR-PCR products were resolved in 1.5% agarose gels stained with ethidium bromide. Gels were run at 70 V, until the dye front gets close to the bottom of them. Gels were then exposed to UV illumination and images were captured with GeneSnap software (SynGene).

The ISSR-PCR banding patterns obtained from stable amplified bands were arranged into a binary data matrix, scoring 0 for the absence and 1 for the presence of band. A multivariate analysis was carried out using Past3 software (https://folk.uio.no/ohammer/past/). The Dice similarity index was used to create a similarity matrix from which a dendrogram was derived using the Unweighed Pair Group Method with Arithmetic Mean (UPGMA) algorithm. In order to measure the genetic variation within and among geographical distinct *Stemphylium* populations, an Analysis of Molecular Variance (AMOVA) was performed in Arlequin 3.5.2.2 (Excoffier et al. 2005).

Results

All the isolates obtained from diseased tomato tissue with typical symptoms of tomato gray leaf spot were initially identified as *Stemphylium lycopersici* by means of the ITS partial sequence. Therefore we decided to further characterize the isolates based on cultural, morphological as well as molecular features.

Cultural and morphological characterization

Cultural and morphological characters varied markedly between isolates, whether they were cultured on homemade or commercial PDA. Mean colony diameter ranged from 25.00 ± 1.00 mm (CIDEFI-212) to 79.33 ± 2.30 mm (CIDEFI-230) after 7 days of incubation at 25 °C. Isolates CIDEFI-200, CIDEFI-201, CIDEFI-210, CIDEFI-212 and CIDEFI-231 were among the slowest growing isolates (Fig. 1). Variations in colony diameter have also been found within the same isolate, as was the case of isolate CIDEFI-229 which reached a mean colony diameter of 70.33 mm on commercial PDA but grew only up to 25 mm when cultured on homemade PDA (Figs. 1 and 2). Differences between and within isolates have also been observed in other cultural characters such as colony color, texture, elevation, margin and the existence of zonation and its patterns. For instance, when isolate CIDEFI-203 was grown on homemade PDA it showed flat cottony colonies that were white, moderate olive green and brilliant yellow green in color and undulate margins that additionally presented a concentric zonation. Under the same conditions, the same isolate cultured on commercial PDA developed cottony colonies that were white, vivid yellow and pale orange yellow in color, with regular margins and the absence of zonation (Fig. 2).

None of the isolates sporulated on commercial PDA. However, on homemade PDA sporulation occurred though it varied markedly. On one extreme, isolates CIDEFI-201, CIDEFI-210, CIDEFI-212 and CIDEFI-231 did not sporulate. On the other extreme, isolate CIDEFI-218 produced 63,057 spores per square centimeter of aerial mycelia. The rest of the isolates differed in their capacity between these two extremes. It is important to mention that non sporulating isolates CIDEFI-201, CIDEFI-210, CIDEFI-212 and CIDEFI-231 shared cultural features such as the diffusion a vivid greenish yellow color that changed to a deep red one as the culture grew older. Cultural characteristics of all isolates

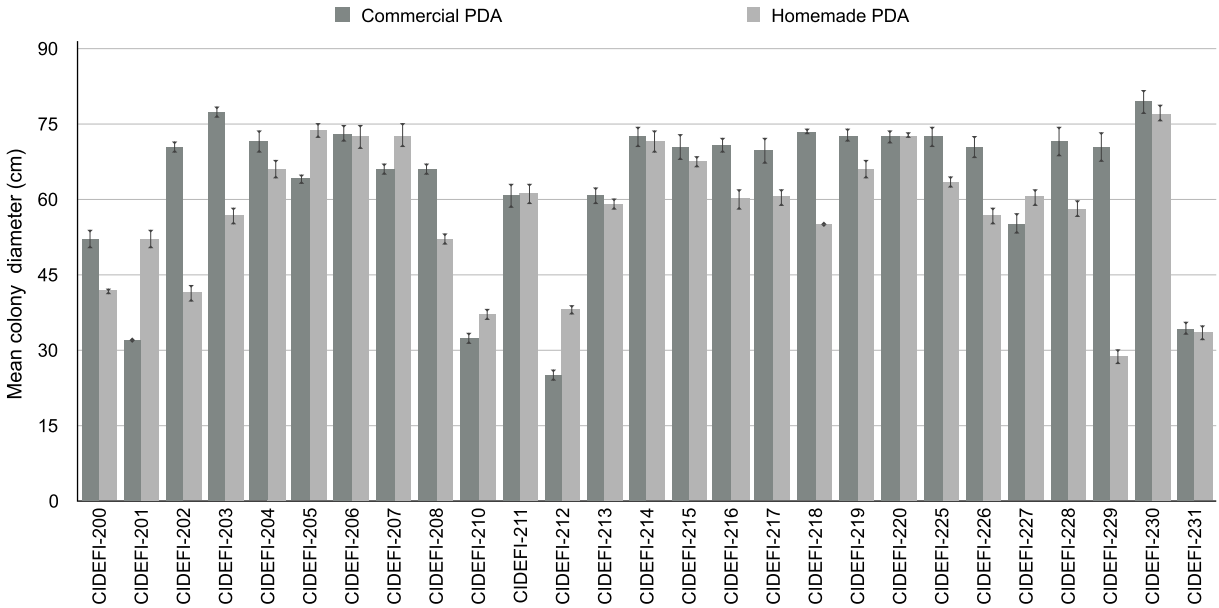

Fig 1 Mean colony diameter of *Stemphylium* isolates grown on homemade or commercial PDA medium after 7 day of incubation at 25 °C in continuous darkness. Values are means of three independent biological replicates and error bars represents the standard deviation

are described in Table 4 and pictures are exhibited in Electronic supplementary material 1 (ESM_1).

Conidiophores were light brown in color, septated and 3.6 μm wide with distinctly swollen apical cells that were 7.2 μm wide. Conidial shape was oblong, rounded or pointed at the apex, with a prominent dark brown scar at the rounded base. They were light brown and cell wall ornamentation was verruculose. Marked variations have also been observed in conidia dimensions and length to width (L:W) ratios, which varied from 2.2 (CIDEFI-230) to 3.1 (CIDEFI-206, CIDEFI-219). Morphological characteristics of conidia of all the isolates that sporulated in vitro are detailed in Table 5 and some examples are exhibited in Electronic supplementary material 2 (ESM_2).

Virulence

Whether inoculated as a spore or mycelia suspensions all isolates provoked disease on tomato cv. Elpida that developed typical symptoms of tomato gray leaf spot. Non sporulating isolates CIDEFI-201, CIDEFI-210, CIDEFI-212 and CIDEFI-231 were inoculated as a mycelial suspension of 4.10^3 CFU.ml^{-1},

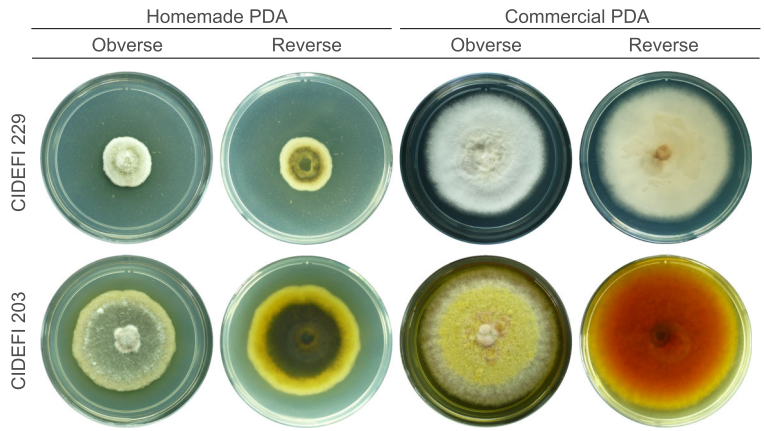

Fig 2 Variation in cultural characteristics of *Stemphylium* isolates grown on homemade or commercial PDA. Pictures were taken from 7-day old cultures grown on PDA at 25 °C in continuous darkness

Table 4 Cultural characteristics of *Stemphylium* isolates grown on homemade PDA and commercial PDA (between brackets)

Isolate	Colony character/Mycelial growth [a]								Media pigmentation [a, e]
	Diameter [d]	Obverse Colour [e]	Reverse Colour [e]	Texture	Elevation	Margin	Zonation	Conida per cm^2 [d]	
CIDEFI-200	41.66 [b] ± 0.58 [c] g	W (N 9) - MOG (7.5GY 4/4)	BYG (2.5GY 8/10) - MOG (5GY 3/4)	Cottony	Raised	Undulate	Concentric	2444 [b] ± 407 [c]	VGY (10Y 8/12)
	[52.00 ± 1.73] f	[W (N 9) - VY (5Y 8/12)]	[VRO (10R 5/14)]	[Cottony]	[Slightly raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-201	52.00 ± 1.73 g	PY (5Y 9/4) - PYP (7.5YR 9/2) - W (N 9)	VRO (10R 5/14) - B (N 9)	Cottony	Raised	Undulate	Absent	0	DR (7.5R 3/10) - VGY (10Y 8/12)
	[32.00 ± 0.00] c	[PY (2.5Y 9/4) - PYP (7.5YR 9/2) - W (N 9)]	[VRO (10R 5/14) - DRO (10R 4/12)]	[Velvety]	[Raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-202	41.33 ± 1.53 f	LYG (2,5GY 9/2) - MOG (5GY 4/4) - BGY (10Y 8/8)	LYG (2.5GY 9/6) - MOG (5GY 3/4)	Cottony	Raised	Undulate	Concentric	5796 ± 717 e	VGY (10Y 8/12)
	[70.33 ± 1.15] op	[W (N 9) - BY (5Y 8/8) - VY (2.5Y 8/12)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Undulate]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-203	56.67 ± 1.54 hi	BYG (2.5GY 8/8) - MOG (7.5GY 4/4) - W (N 9)	BYG (2.5GY 8/10) - MOG (5GY 3/4)	Cottony	Flat	Undulate	Concentric	8444 ± 458 fg	VGY (10Y 8/12)
	[77.33 ± 1.15] s	[W (N 9) - VY (5Y 8/12) - POY (7.5YR 8/4)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-204	66.00 ± 1.73 mn	BYG (2.5GY 8/8) - VPG (10GY 8/2) - W (N 9)	BGY (10Y 8/10) - MO (7.5Y 4/6)	Cottony	Raised	Undulate	Absent	2327 ± 338 c	VGY (10Y 8/12)
	[71.33 ± 2.08] opqr	[W (N 9) - LYG (5GY 8/4)]	[LYG (2.5GY 8/4) - MYG(2.5GY 7/4)]	[Cottony]	[Raised]	[Undulate]	[Absent]	[0]	[Absent]
CIDEFI-205	73.67 ± 1.53 r	LG (N 8)	W (N 9) - MOG (7.5GY 4/4)	Cottony	Flat	Entire	Absent	173 ± 75 a	Absent
	[64.00 ± 1.00] m	[W (N 9) - VY (5Y 8/12)]	[VGY (10Y 8/12) - LO (10Y 6/8)]	[Cottony]	[Slightly raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-206	72.33 ± 2.31 opqr	W (N 9)	LYG (5GY 9/4) - MOG (2.5GY 4/4)	Velvety	Flat	Undulate	Absent	1836 ± 442 bc	Absent
	[73.00 ± 1.73] pqr	[LYG (5GY 8/4) - PYG (5GY 8/2)]	[LYG (2.5GY 8/4) - MYG(2.5GY 7/4)]	[Cottony]	[Flat]	[Entire]	[Concentric]	[0]	[Absent]
CIDEFI-207	72,66 ± 2.51 pqr	BYG (2.5GY 8/8) - VPG (10GY8/2) - LY (2.5Y 8/6)	BYG (2.5GY 8/10) - MOG (5GY 3/4)	Cottony	Raised	Undulate	Radial	669 ± 133 a	VGY (10Y 8/12)
	[66.0 ± 1.00] mn	[W (N 9) - VY (5Y 8/12) - POY (7.5YR 8/4)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-208	52.00 ± 1.00 g	W (N 9) - LYG (5GY 8/4)	LYG (5GY 9/4) - MOG (2.5GY 4/4)	Cottony	Raised	Undulate	Concentric	959 ± 302 ab	Absent
	[66.0 ± 1.00] mn	[W (N 9) - BY (5Y 8/8) - BGY (10Y 8/19)]	[BYG (2.5GY 8/8) - LO (5Y 5/8)]	[Cottony]	[Raised]	[Undulate]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-210	37.00 ± 1.00 de	LY (5Y 9/6)	VRO (10R 5/14) - B (N 9)	Cottony	Flat	Undulate	Absent	0	DR (7.5R 3/10) - VGY (10Y 8/12)
	[32.33 ± 1.15] c	[W (N 9)]	[VRO (10R 5/14) - DRO (10R 4/12)]	[Velvety]	[Raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-211	61.00 ± 2.00 kl	BYG (2.5GY 8/8) - VPG (10GY 8/2) - W (N 9)	BYG (2.5GY 8/10) - MOG (5GY 3/4)	Cottony	Raised - Flat	Undulate	Radial	316 ± 219 a	VGY (10Y 8/12)

Table 4 (continued)

Isolate	Colony character/Mycelial growth [a]								Media pigmentation [a, e]
	Diameter [d]	Obverse Colour [e]	Reverse Colour [e]	Texture	Elevation	Margin	Zonation	Conida per cm^2 [d]	
	[60.66 ± 2.51] jk	[W (N 9) - POY (7.5YR 8/4)]	[BGY (10Y 8/10) - MO (7.5Y 2/4) - LOB (2.5Y 5/8)]	[Cottony]	[Raised]	[Undulate]	[Concentric]	[0]	[VGY (10Y 8/12)]
CIDEFI-212	38.00 ± 1.00 e	LY (5Y 9/6)	VRO (10R 5/14) - B (N 9)	Cottony	Slightly raised	Undulate	Absent	0	DR (7.5R 3/10) - VGY (10Y 8/12)
	[25.00 ± 1.00] a	[W (N 9)]	[VRO (10R 5/14) - DRO (10R 4/12)]	[Velvety]	[Raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-213	59.00 ± 1.00 ijk	GOG (7.5GY 3/2) - W (N 9)	LYG (5GY 9/4) - B (N 2)	Cottony	Raised	Undulate	Absent	46,796 ± 638 h	Absent
	[60.66 ± 1.54] jk	[W (N 9) - VY (5Y 8/12)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised - Flat]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-214	71.33 ± 2.09 opqr	BYG (2.5GY 8/8) - MOG (7.5GY 4/4) - W (N 9)	VGY (7.5Y 8/12) - DYB (10YR 3/6)	Cottony	Flat	Undulate	Absent	370 ± 80 a	VGY (10Y 8/12)
	[72.33 ± 2.08] opqr	[W (N 9) - BY (5Y 8/8) - VY (2.5Y 8/12)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-215	67.33 ± 1.14 no	BYG (2.5GY 8/8) - MOG (7.5GY 4/4) - W (N 9)	VGY (7.5Y 8/12) - DYB (10YR 3/6)	Cottony	Raised	Entire	Radial	371 ± 91 a	VGY (10Y 8/12)
	[70.33 ± 2.51] op	[W (N 9) - VY (2.5Y 8/12) - POY (7.5YR 8/4)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-216	60.00 ± 2.00 jk	W (N 9) - LYG (2.5GY 9/4) - POY (7.5YR 8/4) - GO (10Y 3/2)	LYG (5GY 9/4) - MOG (2.5GY 4/4)	Cottony	Raised	Undulate	Concentric	196 ± 196 a	Absent
	[70.66 ± 1.52] opq	[W (N 9) - BY (5Y 8/8) - VY (2.5Y 8/12) - POY (7.5YR 8/4)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-217	60.33 ± 1.53 jk	LYG (2.5GY 8/6) - VPG (10GY 8/2) - W (N 9)	PYG (2.5GY 9/2) - MYG (2.5GY 5/4)	Cottony	Raised	Undulate	Absent	4267 ± 1131 d	Absent
	[69.66 ± 2.51] no	[W (N 9) - LYG (5GY 8/4)]	[LYG (2.5GY 8/4) - MYG(2.5GY 7/4)]	[Cottony]	[Raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-218	55.00 ± 0.00 h	MYG (5GY 7/4) - VPG (10GY 8/2) - W (N 9)	PYG (2.5GY 9/2) - MYG (2.5GY 5/4)	Cottony	Flat	Undulate	Concentric	63,057 ± 1428 i	Absent
	[73.33 ± 0.58] qr	[LYG (5GY 8/4)]	[LYG (2.5GY 8/4) - MYG(2.5GY 7/4)]	[Cottony]	[Flat]	[Entire]	[Absent]	[0]	[Absent]
CIDEFI-219	66.00 ± 1.73 mn	BYG (2.5GY 8/8) - VPG (10GY 8/2) - W (N 9)	VGY (7.5Y 8/12) - DYB (10YR 3/6)	Cottony	Raised	Undulate	Absent	6549 ± 408 e	VGY (10Y 8/12)
	[72.66 ± 1.15] pqr	[W (N 9) - BY (5Y 8/8) - VY (2.5Y 8/12)]	[SGY (7.5Y 7/10)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-220	72.66 ± 0.58 pqr	BYG (2.5GY 8/8) - VPG (10GY 8/2) - W (N 9)	VGY (7.5Y 8/12) - DYB (10YR 3/6)	Cottony	Raised	Undulate	Radial	2857 ± 278 c	VGY (10Y 8/12)
	[72. 33 ± 1.15] opqr	[W (N 9) - BY (5Y 8/8) - VY (5Y 8/12)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-225	63.33 ± 1.15 lm	BYG (2.5GY 8/8) - VPG (10GY 8/2) - W (N 9)	BGY (10Y 8/10) - MO (7.5Y 4/6)	Cottony	Raised	Undulate	Radial	290 ± 100 a	VGY (10Y 8/12)
	[72.33 ± 2.08] opqr	[W (N 9) - LYG (5GY 8/4)]	[LYG (2.5GY 8/4) - MYG(2.5GY 7/4)]	[Cottony]	[Raised - Flat]	[Entire]	[Absent]	[0]	[Absent]

Table 4 (continued)

Isolate	Colony character/Mycelial growth [a]								Media pigmentation [a, e]
	Diameter [d]	Obverse Colour [e]	Reverse Colour [e]	Texture	Elevation	Margin	Zonation	Conida per cm^2 [d]	
CIDEFI-226	56.67 ± 1.53 hi	LYG (2.5GY 8/6) - VPG (10GY 8/2) - W (N 9)	PYG (2.5GY 9/2) - MYG (2.5GY 5/4)	Cottony	Raised	Undulate	Absent	807 ± 254 ab	Absent
	[70.33 ± 2.31] op	[W (N 9) - BY (5Y 8/8) - VY (2.5Y 8/12) - POY (7.5YR 8/4)]	[VY (5Y 8/12) - VRO (10R 5/14)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-227	60.33 ± 1.53 jk	LYG (2.5GY 8/6) - VPG (10GY 8/2) - W (N 9)	PYG (2.5GY 9/2) - MYG (2.5GY 5/4)	Cottony	Raised - Flat	Undulate	Absent	6736 ± 593 ef	Absent
	[55.00 ± 2.00] h	[W (N 9) - VY (2.5Y 8/12) - LO (10Y 5/4)]	[LYG (2.5GY 8/4) - MO (7.5Y 4/6) - DOY (7.5YR 6/12)]	[Cottony]	[Raised - Flat]	[Undulate]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-228	58.00 ± 1.73 ij	BYG (2.5GY 8/8) - BY (5Y 8/8) - W (N 9)	VGY (7.5Y 8/12) - DYB (10YR 3/6)	Cottony	Raised	Undulate	Absent	1892 ± 210 bc	VGY (10Y 8/12)
	[71.33 ± 2.89] opqr	[W (N 9) - VY (2.5Y 8/12) - POY (7.5YR 8/4)]	[VRO (10R 5/14) - DRO (10R 4/12)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]
CIDEFI-229	28.67 ± 1.54 b	MYG (5GY 7/4) - W (N 9)	PYG (2.5GY 9/2) - MYG (2.5GY 5/4)	Cottony	Raised	Undulate	Concentric	8607 ± 1721 g	Absent
	[70.33 ± 2.89] op	[W (N 9)]	[PYG (2.5GY 9/2)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[Absent]
CIDEFI-230	77.00 ± 1.73 s	BYG (2.5GY 8/8) - VPG (10GY 8/2) - MOG (2.5GY 4/4) - W (N 9)	BGY (10Y 8/10) - MO (7.5Y 4/6) - B (N 2)	Cottony	Raised	Undulate - Entire	Radial	397 ± 137 a	Absent
	[79.33 ± 2.30] s	[W (N 9) - BY (5Y 8/8) - VY (5Y 8/12) - POY (7.5YR 8/4)]	[VY (5Y 8/12)- DOY (7.5YR 6/12)]	[Cottony]	[Raised]	[Entire]	[Absent]	[0]	[VGY (10Y 8/12)]
CIDEFI-231	33.33 ± 1.5 c	PYP (7.5YR 9/2) - W (N 9)	VRO (10R 5/14) - B (N 9)	Cottony	Raised	Undulate	Absent	0	DR (7.5R 3/10) - VGY (10Y 8/12)
	[34.33 ± 1.15] cd	[W (N 9) - LY (5Y 9/6)]	[VRO (10R 5/14) - DRO (10R 4/12)]	[Cottony]	[Raised]	[Undulate]	[Absent]	[0]	[DR (7.5R 3/10) - VGY (10Y 8/12)]

[a] Values of three replicates taken from 7 day-old cultures grown on homemade/commercial PDA at 25 °C in darkness

[b] Mean

[c] Standard deviation

[d] Means from the same row followed by a letter in common are not significantly different according to LDS test at $P \leq 0.05$

[e] Colours according Munsell colour chart: B = Black, BGY = Brilliant greenish yellow, BY = Brilliant yellow, BYG = Brilliant yellow green, DOY = Deep orange yellow, DR = Deep red, DRO = Deep reddish orange, DYB = Deep yellowish brown, GO = Grayish olive, GOG = Grayish olive green, LG = Light grey, LO = Light olive,LOB = Light olive brown, LY = Light yellow, LYG = Light yellow green, MO = Moderate olive, MOG = Moderate olive green, MYG = Moderate yellow green, POY = Pale orange yellow, PY = Pale yellow, PYG = Pale yellow green, PYP = Pale yellowish pink, SGY = Strong greenish yellow, SYB = Strong yellowish brown, VGY = Vivid greenish yellow, VPG = Very pale green, VRO = Vivid reddish orange, VY = Vivid yellow, W = White

Table 5 Morphological characteristics of conidia of *Stemphylium* isolates

Isolate	Conidia [a, d, e] (μm)			
	Length (L)	Width (W)	Average L:W ratio	Transverse septa
CIDEFI-200	51.84 [b] ± 3.91 [c] i	17.47 ± 1.37 ij	2.99 ± 0.33 fghi	3(−4)
CIDEFI-201	Did not sporulate.			
CIDEFI-202	48.14 2.24 fgh	15.79 ± 1.20 bcde	3.06 ± 0.26 ij	(2-)3
CIDEFI-203	48.58 ± 4.66 h	16.90 ± 2.11 h	2.89 ± 0.23 efg	3(−4)
CIDEFI-204	51.22 ± 3.20 i	17.57 ± 1.57 j	2.93 ± 0.24 efgh	3
CIDEFI-205	45.60 ± 2.74 d	15.70 ± 1.30 bcd	2.92 ± 0.22 efg	(2-)3
CIDEFI-206	48.34 ± 4.09 gh	15.46 ± 1.20 ab	3.14 ± 0.34 j	(2-)3
CIDEFI-207	51.36 ± 4.63 i	16.94 ± 1.41 hi	3.05 ± 0.34 hij	3(−4)
CIDEFI-208	46.56 ± 3.32 def	16.03 ± 1.57 cdef	2.92 ± 0.26 efg	3(−4)
CIDEFI-210	Did not sporulate.			
CIDEFI-211	39.46 ± 2.75 c	15.74 ± 1.30 bcd	2.52 ± 0.26 c	(1-)2(−3)
CIDEFI-212	Did not sporulate.			
CIDEFI-213	47.23 ± 3.23 efgh	17.95 ± 1.63 jk	2.65 ± 0.32 d	3(−4)
CIDEFI-214	48.10 ± 4.11 fgh	19.25 ± 1.49 l	2.50 ± 0.17 c	3(−4)
CIDEFI-215	39.36 ± 3.03 c	16.32 ± 1.45 efg	2.43 ± 0.31 bc	(1-)2(−3)
CIDEFI-216	37.49 ± 5.98 b	15.46 ± 2.01 ab	2.45 ± 0.40 bc	3
CIDEFI-217	46.75 ± 5.04 defg	16.61 ± 1.52 gh	2.83 ± 0.36 e	3
CIDEFI-218	48.43 ± 5.83 h	16.18 ± 1.52 defg	3.01 ± 0.38 ghi	3
CIDEFI-219	48.67 ± 4.03 h	15.55 ± 1.39 bc	3.15 ± 0.37 j	3
CIDEFI-220	51.22 ± 4.67 i	18.14 ± 1.20 k	2.83 ± 0.28 e	3(−4)
CIDEFI-225	53.47 ± 5.06 j	18.43 ± 1.13 k	2.90 ± 0.25 efg	3(4)
CIDEFI-226	39.02 ± 3.46 bc	16.56 ± 1.00 fgh	2.36 ± 0.25 b	2(−3)
CIDEFI-227	45.98 ± 4.88 de	16.18 ± 1.06 defg	2.87 ± 0.47 ef	(2-)3
CIDEFI-228	39.79 ± 2.06 c	16.03 ± 1.23 cdef	2.50 ± 0.22 c	2(−3)
CIDEFI-229	46.75 ± 4.37 defg	17.47 ± 1.37 ij	2.68 ± 0.26 d	2(−3)
CIDEFI-230	33.02 ± 2.55 a	14.98 ± 1.33 a	2.21 ± 0.15 a	(1-)2
CIDEFI-231	Did not sporulate.			

[a] Values of three replicates taken from 7 day-old cultures grown on homemade/commercial PDA at 25 °C in darkness

[b] Mean

[c] Standard deviation

[d] Means from the same row followed by a letter in common are not significantly different according to LDS test at $P \leq 0.05$

[e] Conidial shape was oblong, rounded or pointed at the apex and with a prominent dark brown scar at the rounded base; color was light brown; and cell wall ornamentation was verruculose

1.10^3 CFU.ml^{-1}, 1.10^3 CFU.ml^{-1} and 7.10^3 CFU.ml^{-1}, respectively. Virulence assays confirmed that all isolates were pathogenic on detached tomato leaves. Symptoms developed 2 dpi and thereafter lesions expanded through the leaflet. Control leaflets treated with sterilized 0.01% Tween 20 solution remained healthy. We successfully re-isolated all the isolates from diseased leaflets fulfilling in this way Koch's postulates.

Isolates differed in the quantity of disease they provoke, which was shown by the level of severity of inoculated leaflets. Based on this, isolates can be roughly classified as highly virulent ones, like isolates CIDEFI-207, CIDEFI-215, CIDEFI-211, CIDEFI-228, CIDEFI-202, CIDEFI-216, CIDEFI-229, CIDEFI-220, CIDEFI-214, CIDEFI-230 and CIDEFI-226. The leaflet areas affected by these isolates ranged between

6.60 ± 1.05 cm^2 and 3.75 ± 1.11 cm^2. Isolates CIDEFI-208, CIDEFI-225 and CIDEFI-227 seemed to be medium virulence and affected a leaflet area between 2.31 ± 1.00 cm^2 and 1.77 ± 0.94 cm^2. Finally the less virulent isolates were CIDEFI-231, CIDEFI-203, CIDEFI-204, CIDEFI-219, CIDEFI-205, CIDEFI-218, CIDEFI-201, CIDEFI-200, CIDEFI-213, CIDEFI-210, CIDEFI-217, CIDEFI-212 and CIDEFI-206, which affected an area smaller than 1,07 ± 0,35 cm^2 (Table 6 and Fig. 3).

Table 6 Virulence of *Stemphylium* isolates towards tomato cv. Elpida evaluated by the detached leaf assay

Treatment	Affected leaf area (cm^2) [a]
Control	0.11 [b] ± 0.02 [c]
CIDEFI-200	0.56 ± 0.27 j
CIDEFI-201	0.64 ± 0.16 j
CIDEFI-202	5.54 ± 1.30 bcd
CIDEFI-203	1.03 ± 0.51 ij
CIDEFI-204	0.96 ± 0.30 j
CIDEFI-205	0.79 ± 0.24 j
CIDEFI-206	0.42 ± 0.13 j
CIDEFI-207	6.60 ± 1.05 a
CIDEFI-208	2.31 ± 1.00 h
CIDEFI-210	0.52 ± 0.17 j
CIDEFI-211	6.07 ± 1.03 ab
CIDEFI-212	0.50 ± 0.08 j
CIDEFI-213	0.55 ± 0.23 j
CIDEFI-214	4.44 ± 1.20 efg
CIDEFI-215	6.58 ± 1.38 a
CIDEFI-216	5.33 ± 1.30 bcd
CIDEFI-217	0.50 ± 0.09 j
CIDEFI-218	0.65 ± 0.21 j
CIDEFI-219	0.89 ± 0.35 j
CIDEFI-220	4.84 ± 0.97 def
CIDEFI-225	2.26 ± 1.06 h
CIDEFI-226	3.75 ± 1.11 g
CIDEFI-227	1.77 ± 0.94 hi
CIDEFI-228	5.71 ± 1.20 bc
CIDEFI-229	5.18 ± 1.56 cde
CIDEFI-230	4.30 ± 0.88 fg
CIDEFI-231	1.07 ± 0.35 ij

[a] Values of nine replicates taken 7 days post inoculation

[b] Mean

[c] Standard deviation

[d] Means from the same row followed by a letter in common are not significantly different according to LSD test at $P \leq 0.05$

Molecular identification

While the ITS sequence of all *Stemphylium* isolates was 579 bp long, the *gpd* partial sequence was 322 bp long. Regarding the latter, isolates presented *gpd* sequences that differed only in base number 70, having either a G or an A. This base is located in the third intron of the full-length *gpd* gene sequence (Locus tag: TW65_04473; Protein accession number KNG48731). This difference was used to divide isolates within two groups: Group-G and Group-A. The first was composed of isolates with a G: CIDEFI-201, CIDEFI-203, CIDEFI-214, CIDEFI-216, CIDEFI-218, CIDEFI-227 and CIDEFI-229. Group-A was integrated by isolates with an A in this position: CIDEFI-200, CIDEFI-202, CIDEFI-204, CIDEFI-205, CIDEFI-206, CIDEFI-207, CIDEFI-208, CIDEFI-210, CIDEFI-211, CIDEFI-212, CIDEFI-213, CIDEFI-215, CIDEFI-217, CIDEFI-219, CIDEFI-220, CIDEFI-225, CIDEFI-226, CIDEFI-228 and CIDEFI-230. All the ITS and *gpd* sequences were deposited in the DDBJ/EMBL/GenBank under the accession numbers presented in Table 7.

In order to make the analysis simpler but still informative, only an isolate of each of the two groups of organisms with the *gpd* was included in the phylogenetic analysis. CIDEFI-216 was selected as representative of Group-G and CIDEFI-217 was chosen of Group-A. Sequence alignment of the ITS and *gpd* sequences of the isolates and related taxa resulted in data sets of 521 and 294 bp long, respectively. The PHT of the combined ITS and *gpd* aligned sequences gave a *p*-value of 0.577000, thus both DNA sequences were concatenated into a single data set. ITS-*gpd* sequence data matrix contained a total of 815 characters, of which 504 were constant, 70 parsimony-uninformative and 241 parsimony-informative.

The most-parsimonious tree obtained from the ITS-*gpd* analysis had a tree length of 648 steps, a consistency index of 0.7577, a retention index of 0.7773 and a rescaled consistency index of 0.5890 (ESM_3). Regarding the ML approach, jModelTest selected HKY + I + G as the best-fit nucleotide substitution model from among 88 competing models for the ITS-*gpd* data (−ln L = 4055.5102; base freq: A = 0.2333, C = 0.2812, G = 0.2177, *T* = 0.2678; transition/transversion rates = 1.4080; gamma shape = 2.5790). When the selected molecular evolution model was incorporated into the phylogenetic analysis under ML criteria in

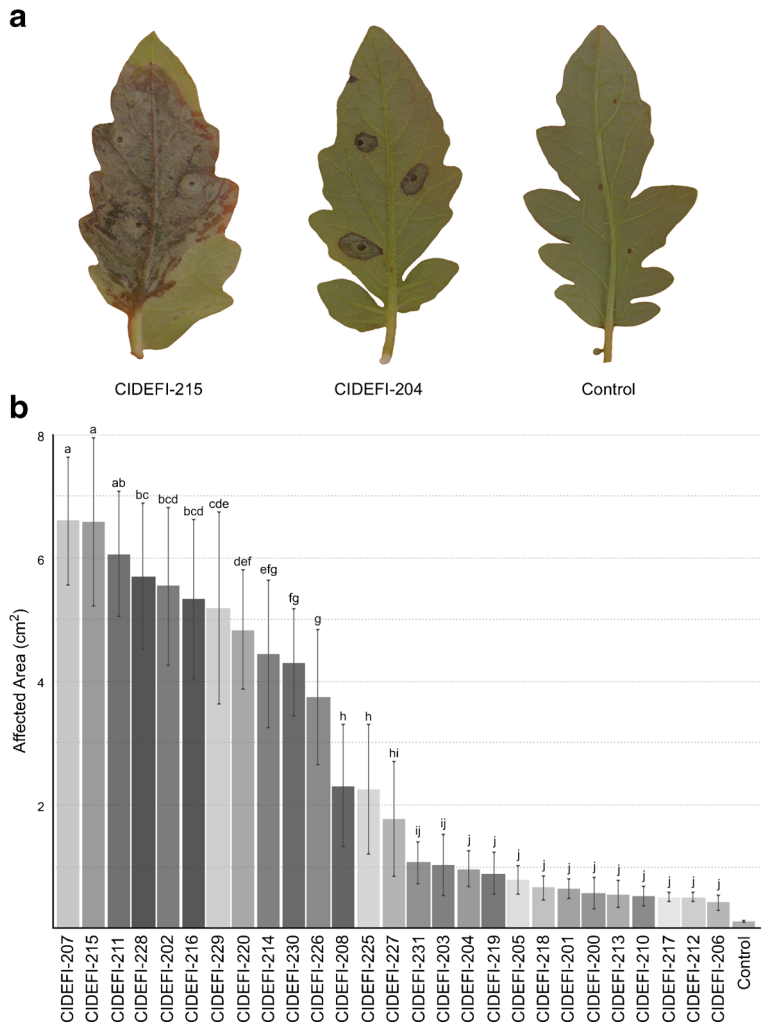

Fig 3 In vitro virulence of *Stemphylium* isolates against tomato cv. Elpida evaluated by the detached leaf assay. Symptoms (**a**) and necrotic area (**b**) of tomato detached leaflets 7 dpi with conidial/mycelial suspensions of *Stemphylium* isolates. Control leaflets were treated with a sterile 0.01% Triton X-100 solution. Values are means of nine independent biological replicates and error bars represents the standard deviation. Means followed by a letter in common are not significantly different according to LSD test at $P \leq 0.05$. The affected area was determined using the image analysis software for plant disease quantification Assess 2.0 (Lamari 2002)

PhyML, a single ITS-*gpd* tree was recovered (−ln L = −4113.82741; Fig. 4). Both approaches ML and MP resulted in a well-supported monophyletic *Stemphylium* clade. CIDEFI-216 and CIDEFI-217 isolates were placed in the same clade together with *S. lycopersici* and *S. xanthosomatis* with bootstrap values of 97% and 91% for the MP and ML approach, respectively. Inside these clades, isolates CIDEFI-216 was closely related to *S. xanthosomatis* and CIDEFI-217 to *S. lycopersici*.

Genetic diversity analysis

The 6 ISSR primers selected amplified 52 clear and reproducible bands that ranged from 250 bp to 2500 bp and were used to assess genetic diversity. Among them, 27 amplicons were recorded as polymorphic (52%). We built a dendrogram using the UPGMA algorithm and Dice coefficient based on the ISSR data. All the isolates of *Stemphylium* were clustered in two groups at a similarity coefficient of 0.83 (Fig. 5). At this level of similarity, it could be seen that CIDEFI-230 and CIDEFI-231 isolates, which had the same origin (Table 1), were separately clustered from the rest thought at a high level of similarity. At a higher similarity level of 0.88, the remaining isolates were subdivided in two groups. It is important to point out that there was no clear relationship between these clusters and the morphological characteristics or the geographical origin of the isolates. In fact, the AMOVA stated that 95.94% of the variation was the result of differences within geographically defined populations, while only 4.96% of the variation was attributed to differences between them.

Table 7 ITS and *gpd* GenBank accession numbers of isolates from this study

Isolates	ITS [a]	*gpd* [a]
CIDEFI-200 [A]	KF709429	KJ624421
CIDEFI-201 [G]	KJ624431	KJ624422
CIDEFI-202 [A]	KP026204	KP026203
CIDEFI-203 [G]	KP026205	KP026202
CIDEFI-204 [A]	KP026206	KP026201
CIDEFI-205 [A]	KP026207	KP026200
CIDEFI-206 [A]	KJ624432	KJ624423
CIDEFI-207 [A]	KJ624433	KJ624424
CIDEFI-208 [A]	KJ624434	KJ624425
CIDEFI-210 [A]	KJ624435	KJ624426
CIDEFI-211 [A]	KJ624436	KJ624428
CIDEFI-212 [A]	KJ624437	KP026199
CIDEFI-213 [A]	KJ624438	KJ624427
CIDEFI-214 [G]	KP026208	KP026198
CIDEFI-215 [A]	KP026209	KP026197
CIDEFI- 216 [G]	KJ624439	KJ624429
CIDEFI- 217 [A]	KP026210	KP026196
CIDEFI-218 [G]	KP026211	KP026195
CIDEFI-219 [A]	KJ624440	KJ624430
CIDEFI-220 [A]	KP026212	KP026194
CIDEFI-225 [A]	KJ624449	KP026189
CIDEFI-226 [A]	KJ624450	KP026188
CIDEFI-227 [G]	KJ624446	KP026183
CIDEFI-228 [A]	KJ624447	KP026186
CIDEFI-229 [G]	KJ624448	KP026187
CIDEFI-230 [A]	KJ624441	KP026185
CIDEFI-231 [A]	KJ624442	KP026184

[a] GenBank accession number

[G] Group-G: G in residue number 70

[A] Group-A: A in residue number 70

Discussion

The incidence of tomato gray leaf spot disease over the major tomato-growing regions of Argentina has increased considerably during the last three years. Although the disease is particularly important in Northern Argentina, it has recently been observed in southern Argentina as well as drier areas such as Mendoza. It appears that tomato gray leaf spot is spreading south, which might be related to changes in temperature and precipitation that most probably occurred due to global warming.

In Argentina, the etiological agents of tomato gray leaf spot were identified as two different species of *Stemphylium*. While Colombo et al. (2001) identified *S. solani* and *S. lycopersici* in diseased tomatoes growing in Corrientes province, Ramallo et al. (2005) identified *S. solani* in diseased greenhouse tomatoes growing in Tucumán. Both reports based their identification only on morphological characters. Even though diagnosis of *Stemphylium* species has been traditionally relied on morphological traits, the intrinsic variability of morphological characteristics within the genus raises some questions regarding the identification of the causative agent of the disease. Because of this we made a preliminary identification based solely on the ITS sequence, which confirmed that all of them belong to the genus *Stemphylium*.

Cultural characteristics as well as morphology of conidia have been used to define fungal species. Cultural characteristics of fungal isolates on PDA were typical of those described for members of the genus *Stemphylium* (Ellis 1971) though considerable levels of diversity were observed. In addition to this, we also found that certain characters of the isolates varied whether they were cultured on homemade or commercial PDA, which not only led to changes in their growth rate and pigmentation, but also in their sporulation capacity. While isolates exhibited a wide range of sporulation capacity on homemade PDA, no sporulation was observed on commercial PDA cultures. Thus, the differences observed between the two culture media used could be due to their chemical composition. It seems that sporulation is a demanding process that requires additional nutritional factors that are not provided in commercial PDA. Griffith et al. (2007) demonstrated that management of the potato crop used as source to elaborate the PDA medium plays a critical role in the quality of the resultant culture medium. Potatoes deficient in copper led to a reduction in pigmentation of various fungal cultures, and in some cases, the number of conidia also was affected. Malca and Ullstrup (1962) found that lactose was the best carbon source for the enhancement of sporulation in the *Pleosporales* fungus *Bipolaris zeicola* (previously named *Helminthosporium carbonum*) and *Exserohilum turcicum* (previously named *Helminthosporium turcicum*). More recently, Zhu et al. (2008) found that sporulation, unlike mycelial growth, in *Aschersonia aleyrodis* was affected by the content of lactose, vitamin B1, Fe^{2+} and

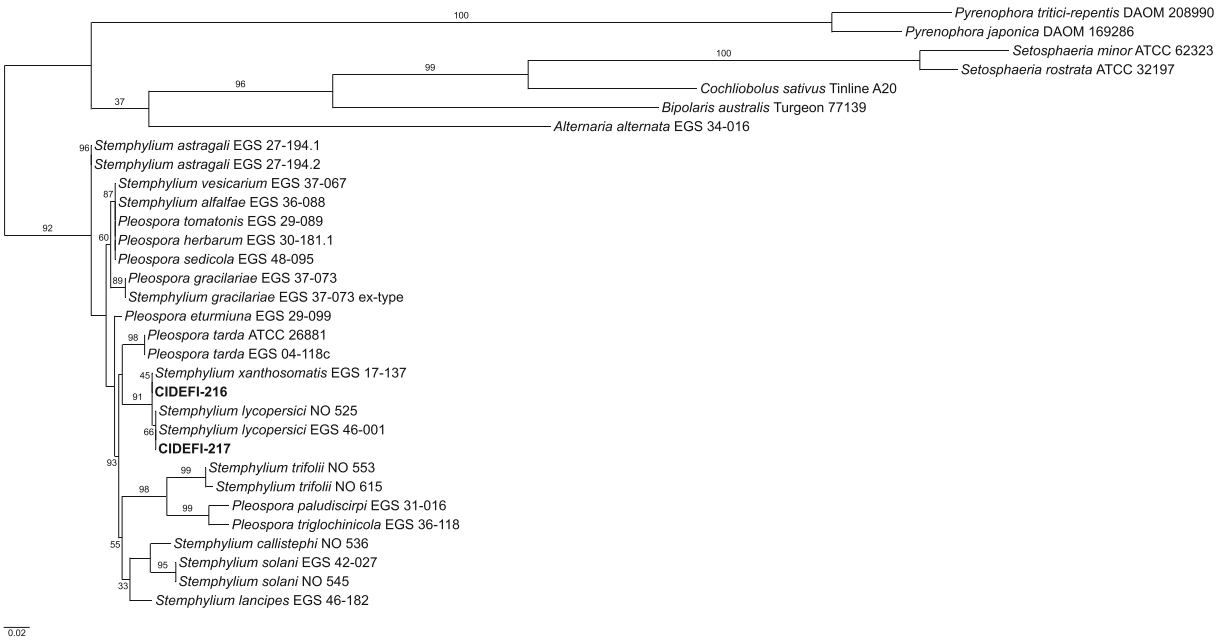

Fig 4 Maximum likelihood tree of *Stemphylium*/*Pleospora* inferred from the concatenated ITS-*gpd* data set. Sequences of seven representatives of five genera of the order *Pleosporales* (*Alternaria*, *Bipolaris*, *Cochliobolus, Pyrenophora* and *Setosphaeria*) were chosen as outgroups. Sequences generated in this study are in bold type letter. Numbers at the nodes represent bootstrap support values as a percentage of 1000 replicates. The scale bar represents the average number of nucleotide substitutions per site

tryptone of the culture medium. Both groups found that mycelial growth had different requirements. Therefore, it appears that sporulation of *Stemphylium* isolates is a demanding process that is variable among isolates suggesting this that they differ quite significantly in their metabolic capacity.

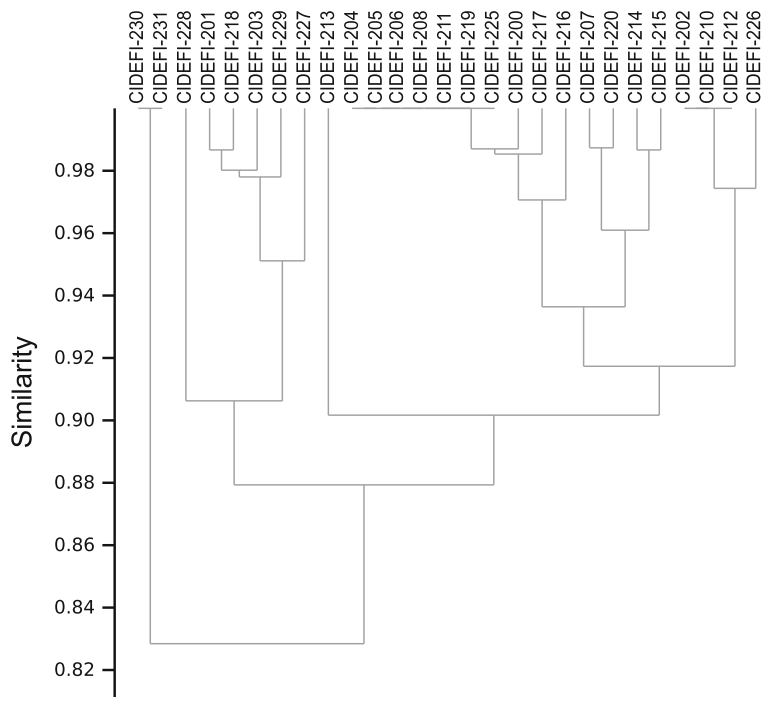

Fig 5 Dendrogram generated by UPGMA cluster analysis using the Dice similarity coefficient based on the ISSR fingerprint of *Stemphylium lycopersici* isolates

Although conidial shape, color and ornamentation were the same for the 27 isolates, some variation in their dimensions were observed, like the numbers of transverse septa and average L:W ratio, with the latter ranging from 2.2 to 3.1. Based on the earliest descriptions of spore morphology for *Stemphylium* species associated with gray leaf spot, we found that while some of our isolates fit closely to the *S. solani* phenotype, because of the shorter length, width and L:W ratio of the conidia, others presented morphological characteristics typical of *S. lycopersici,* since they produce bigger spores with L:W ratios equal or higher than 3 (Weber 1930; Hannon and Weber 1955; Ellis 1971; Ellis and Gibson 1975a, b). However, Kim et al. (2004), Kwon et al. (2007), Nishi et al. (2009), Tomioka and Sato (2011), Hong et al. (2012), Kurose et al. (2014), and Nasehi et al. (2015) described isolates of *S. lycopersici* with conidia with L:W ratios lower than 3. Although spore morphology has been traditionally used as a diagnostic tool to delimitate species of *Stemphylium*, this feature is under the influence of environmental factors. Leach and Aragaki (1970) showed that differences in temperature of culture incubation led to changes in conidia morphology of *S. lycopersici*. Furthermore, Tomioka et al. (1997); Tomioka and Sato 2011, Hong et al. (2012) and Nasehi et al. (2015) found that the dimension and L:W ratio of *S. lycopersici* conidia on leaf lesions were different from those grown on culture medium. In fact, previous reports showed that cultural as well as morphological characteristics are unreliable tools to be used to differentiate *S. lycopersici* from *S. solani* (Hong et al. 2012; Nasehi et al. 2015). It is evident that morphological characters should be supported with molecular data in order to precisely classify and determine the organism identity.

DNA markers are reliable neutral tools to evaluate genetic diversity and sequences of conserved genes to confirm the identity of fungi. The multi-locus phylogenetic analysis of the ITS-*gpd* partial sequences clustered all isolates in a clade together with *S. lycopersici* and *S. xanthosomatis* with highly significant bootstrap values both in MP and ML analysis, showing that they are distinct to *S. solani*. Only two *gpd* sequences were found within the 27 isolates. Therefore, we included in the phylogenetic analysis isolates CIDEFI-216 and CIDEFI-217 that represent both sequences that were clustered within the *S. xanthosomatis* and *S. lycopersici* sub-clades, respectively. Both *S. lycopersici* and *S. xanthosomatis* share morphological characteristics and had nearly identical ITS and *gpd* sequences. Although additional taxonomical studies are needed, several authors agreed that *S. xanthosomatis* may be a synonym of *S. lycopersici* with intra-specific variation (Câmara et al. 2002; Hong et al. 2012). Our results provide additional support to this hypothesis.

Virulence is one of the most important characteristic of pathogenic *Stemphylium* species. Virulence of *Stemphylium* isolates on tomato cv. Elpida detached leaves varied considerably, which was unrelated with the tomato cultivar from where isolates were collected and the geographical place of origin of the isolates. We found that an inoculum concentration of 10^3 conidia ml^{-1} was enough to provoke disease symptoms on detached tomatoes leaflets. Moreover, mycelial fragments at a concentration of the same order of magnitude were pathogenic on detached leaves, although apparently less virulent. Isolates CIDEFI-201, CIDEFI-210, CIDEFI-212 and CIDEFI-231, which were unable to sporulate on PDA were less virulent than spore producing isolates, since they affected a leaf area smaller than 1.07 ± 0.35 cm^2. Interestingly, these isolates shared nearly identical cultural features like a vivid greenish yellow and a deep red pigment that diffuse into the culture medium.

Satellite as well as micro-satellite DNA sequences within fungal genomes are useful tools to evaluate diversity. There are several different methods to evaluate diversity such as RAPD, ERIC, REP and ISSR-PCR. However, we decided to use ISSR-PCR due to its speed, reliability, simplicity, cost-effectiveness as well as the fact that it does not require previous knowledge of the genome sequence. Genetic variability based on the ISSR-PCR fingerprint distinguished 18 genotypes, among the 27 *S. lycopersici* isolates. Still, the level of similarity between accessions was high and there was no relation between the genetic clusters and the phenotypic characteristics, virulence, host identity and geographical origin of the isolates, except for the cluster formed by isolate CIDEFI-230 and CIDEFI-231. In view of these results, we found likely that the fungal pathogen has been introduced to the tomato-growing areas by few inoculum sources and it was subsequently spread by moving infected plant material from one place to another. Additionally, it is also evident that the fungus is also undergoing a process of genetic variation, as can be seen in the number of genotypes found. The latter aspect should be a cause for concern as it could be led to the

emergence of fungicide-resistant isolates or new races that are hazards for the existing resistant tomato cultivars.

This work included morphological as well as molecular characterization of pathogens isolated from tomato plants with typical symptoms of gray leaf spot, suggesting that *S. lycopersici* is the causal agent of this disease in the major tomato-growing areas of Argentina. The morphological, pathogenic and genetic variability exhibited by the 27 isolates studied suggest that the pathogen is under a rapid evolving process, which is of concern when developing phytosanitary programs. In order to perform an integral research framework of the tomato gray leaf spot pathosystem we have recently sequenced the genome of *S. lycopersici* (Franco et al. 2015). Since the tomato genome is also publicly available (Tomato Genome Consortium 2012), the availability of both genome sequences and additional experimental studies may lead to the development of more efficient strategies of control of the disease.

Acknowledgements This research was partially supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) of the Ministro de Ciencia, Tecnología e Innovación Productiva through the projects PICT 2012-2760 (Pedro Alberto Balatti) and PICT 2015-1620 (Mario Carlos Nazareno Saparrat).

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