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FULL PAPER

Phenotypic, molecular phylogenetic, and pathogenetic characterization of *Fusarium crassistipitatum* sp. nov., a novel soybean sudden death syndrome pathogen from Argentina and Brazil

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Abstract A novel soybean sudden death syndrome (SDS) pathogen from Argentina and Brazil is formally described herein as *Fusarium crassistipitatum* based on detailed phenotypic analyses of macro- and microscopic characters and phylogenetic analyses of multilocus DNA sequence data. *Fusarium crassistipitatum* can be distinguished from the other soybean SDS and bean (*Phaseolus*/*Vigna*) root rot pathogens (BRR) phenotypically by the production of yellowish colonies on PDA; and tall, stout, and mostly unbranched conidiophores with a thick-walled base, which form multiseptate conidia apically. Phylogenetic species recognition based on genealogical concordance of a six-gene dataset strongly supported the reciprocal monophyly of *F. crassistipitatum* with respect to the other SDS and BRR pathogens. Isolates of *F. crassistipitatum* were able to induce typical SDS foliar and root rot symptoms on soybean that were indistinguishable from those caused by three other SDS pathogens (i.e., *F. virguliforme*, *F. brasiliense*, and *F. tucumaniae*) on susceptible cultivars A-6445RG and N-4613RG in a pathogenicity experiment.

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Introduction

Sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Merr.) has become a constraint to the production of this crop in North and South America (Rupe and Hartman 1999). This economically devastating disease is characterized by foliar chlorosis and necrosis, root rot, vascular discoloration of stems and roots, defoliation, and death. Recent morphological and molecular phylogenetic analyses have shown three closely related species within clade 2 of the *Fusarium solani* species complex can induce soybean SDS (i.e., *F. virguliforme*, *F. tucumaniae*, and *F. brasiliense*), and that these fusaria are very closely related to two root rot pathogens of mung bean (*Vigna radiata* (L.) R. Wilczek) and snap and dry edible bean (*Phaseolus vulgaris* L.) (Aoki et al. 2003, 2005; O'Donnell et al. 2010). The two bean (*Phaseolus/Vigna*) root rot (BRR) fusaria, which were described as *F. phaseoli* and *F. cuneirostrum*, are referred to as BRR pathogens hereafter to distinguish them from the soybean SDS fusaria. A fourth soybean SDS pathogen, which was represented initially by a single isolate from Brazil, was originally reported as *F. cuneirostrum* (Aoki et al. 2005). However, subsequent phylogenetic analyses that included an isolate from Argentina revealed that it represented a genealogically exclusive lineage that was reported as *Fusarium* sp. (O'Donnell et al. 2010). Before the formal description of the SDS and BRR pathogens, they were typically reported as *F. solani* f. sp. *glycines* and *F. solani* f. sp. *phaseoli*, respectively, or as the Synder and Hansen species, *F. solani* (Mart.) Appel & Wollenweber (Nelson et al. 1983; Leslie and Summerell 2006).

Our working hypothesis is that the four soybean SDS and two BRR pathogens evolved from a common ancestor in the New World and the SDS fusaria independently moved onto soybean only after this crop was introduced to South America approximately 100 years ago. This hypothesis is based in part on all four soybean SDS fusaria having been found in pathogen surveys of Argentina whereas only one of the SDS pathogens, *F. virguliforme*, appears to be responsible for this disease within North America (Achenbach et al. 1996; Rupe et al. 2001; O'Donnell et al. 2010). In addition, all the close relatives of the soybean SDS and BRR pathogens appear to be endemic to South America (O'Donnell 2000). Interestingly, an extensive survey of soybean SDS in Argentina revealed that *F. tucumaniae* was the dominant soybean SDS pathogen, comprising 87% (163/187) of the isolates genotyped (O'Donnell et al. 2010). By comparison, *F. virguliforme* only accounted for 7% (17/187) of the pathogens in this survey. Moreover, *F. virguliforme* is the only SDS pathogen that has not been detected in Brazil, based on limited sampling (O'Donnell et al. 2010). In contrast to *F. virguliforme*, which appears to possess a highly clonal population structure (Achenbach et al. 1996; Li et al. 2000; Rupe et al. 2001; Aoki et al. 2003, 2005; O'Donnell et al. 2010), *F. tucumaniae* has been shown to possess a heterothallic or self-sterile reproductive mode based on laboratory (Covert et al. 2007) and field (Scandiani et al. 2010) studies. Koch's postulates have been completed for *F. virguliforme* (Roy et al. 1997) and *F. tucumaniae* (Scandiani et al. 2004).

Our recent survey of soybean SDS pathogen diversity in Argentina, using a novel multilocus genotyping assay, resulted in the discovery of eight additional isolates of the unnamed *Fusarium* sp., which included six isolates from the province of Salta and two from Tucumán (O'Donnell et al. 2010). In the present study, seven of these eight newly discovered isolates were combined with the two previously known isolates of this species (i.e., NRRL 31949 from Goiás, Brazil and NRRL 36877 from Santa Fe province, Argentina) to assess their phenotypic diversity, genealogical exclusivity employing phylogenetic species recognition (Taylor et al. 2000), and their ability to induce root rot and foliar symptoms on soybean, mung bean, and green bean in a pathogenicity experiment.

Materials and methods

Strains examined

Strains of *Fusarium* species included in the study, isolated from soybean (*Glycine max*), green bean (*Phaseolus vulgaris*), and mung bean (*Vigna radiata*) are listed in Table 1. Nine soybean SDS pathogens isolated from plants exhibiting typical symptoms of the disease at farmers' fields at Cristalina, Goiás State, Brazil, in 2000; at Zavalla, Santa Fe province, Argentina, in 2003; and at Las Cejas, Tucumán province and Las Lajitas, Salta province, Argentina in 2007, were studied and represent the novel soybean SDS pathogen described herein as *F. crassistipitatum*. Strain NRRL 31949 was reported originally as *F. cuneirostrum* in Aoki et al. (2005) primarily because its monophyly could not be assessed given that it was represented by a single strain. With the subsequent discovery of strain NRRL 36877 from Argentina, these two isolates were reported as *Fusarium* sp. (O'Donnell et al. 2010) because they formed a genealogically exclusive lineage. The nine strains of *F. crassistipitatum* were compared phenotypically with the three other soybean SDS (*F. tucumaniae* NRRL 34550, *F. virguliforme* NRRL 34551, *F. brasiliense* NRRL 31756) and the two BRR pathogens (*F. phaseoli* NRRL 31156, *F. cuneirostrum* NRRL 36024; Table 1). All strains included in the study are stored in liquid nitrogen vapors at −175°C in the Agriculture Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, USA, the NIAS Genebank Project-Microorganisms Section (MAFF), National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan, and the Culture Collection of Centro de Referencia de Micología (CCC), Fac. de Cs. Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina.

Examination of morphological characters and mycelial growth rates

Methods for the examination of morphological characters followed Aoki et al. (2003) and Aoki et al. (2005) to compare the various species within the SDS–BRR clade. *Fusarium* strains grown on potato dextrose agar (PDA; Difco, Detroit, MI, USA) and synthetic low nutrient agar (SNA; Nirenberg 1990) in 9-cm-diameter plastic Petri dishes were incubated at 20°C in complete darkness, under continuous fluorescent light (FL40S-W; Mitsubishi, Yokohama, Japan) or under daylight to examine morphological and cultural characteristics. Colony morphology, color, and odor were based on cultures grown on PDA. Colors cited are given according to Kornerup and Wanscher (1978). Average and standard deviation (SD) in the size of individual types of conidia were based on the measurement of 50 randomly selected conidia with a compound microscope, based on the number of septa and each cultural condition. Descriptive terms for microscopic morphology follow Nirenberg and O'Donnell (1998). A dried culture of the new species was deposited as the holotype in the herbarium of the U.S. National Fungus Collection (BPI), USDA/ARS, Beltsville, MD, USA. To examine mycelial growth rates at various temperatures, agar blocks approximately 5 × 5 mm were cut from the

Table 1 Strains included in the present study

Species	NRRL[a]	Geographic origin	Host (disease)	Source (year of isolation)	Equivalent[b]	Host inoculation
Fusarium crassistipitatum	31949	Brazil Goiás, Cristalina	*Glycine max* (SDS)	J.R. Menezes (2000)/ T. J. Yorinori, 01/00	MAFF 239052 = CCC 198-05	Tested
Fusarium crassistipitatum	36877[c]	Argentina Santa Fe, Zavalla	*Glycine max* (SDS)	M. Scandiani (2004)	MAFF 239757 = CCC 142-05	Tested
Fusarium crassistipitatum	43824	Argentina Tucumán, Las Cejas	*Glycine max* (SDS)	M. Scandiani/G. Salas (2007)	CCC 144-07	Tested
Fusarium crassistipitatum	43825	Argentina Tucumán, Las Cejas	*Glycine max* (SDS)	M. Scandiani/G. Salas (2007)	CCC 143-07	Tested
Fusarium crassistipitatum	46170	Argentina Salta, Las Lajitas	*Glycine max* (SDS)	M. Scandiani (2007)	CCC 169-07	Tested
Fusarium crassistipitatum	46171	Argentina Salta, Las Lajitas	*Glycine max* (SDS)	M. Scandiani (2007)	CCC 168-07	–
Fusarium crassistipitatum	46173	Argentina Salta, Las Lajitas	*Glycine max* (SDS)	M. Scandiani (2007)	CCC 174-07	Tested
Fusarium crassistipitatum	46174	Argentina, Salta, Las Lajitas	*Glycine max* (SDS)	M. Scandiani (2007)	CCC 175-07	–
Fusarium crassistipitatum	46175	Argentina Salta, Las Lajitas	*Glycine max* (SDS)	M. Scandiani (2007)	CCC 176-07	–
Fusarium tucumaniae	34550	Argentina Santa Fe, Pujato	*Glycine max* (SDS)	M. Scandiani (2001)	MAFF 239256 = CCC 128-02	Tested
Fusarium virguliforme	34551	Argentina Buenos Aires, San Pedro	*Glycine max* (SDS)	M. Scandiani (2002)	MAFF 239257 = CCC 101-03	Tested
Fusarium brasiliense	31756	Brazil Distrito Federal, Brasilia	*Glycine max* (SDS)	T. Nakajima (1992)/ T. J. Yorinori, SDS-1	MAFF 238550 = FRC S-1550 CCC 191-05	Tested
Fusarium phaseoli	31156	USA Michigan, Presque Isle	*Phaseolus vulgaris* (BRR)	Michigan State Univ., Hawks 2b	MAFF 239050 = CCC 190-05	Tested
Fusarium cuneirostrum	36024	Canada Ontario, Ridgetown	*Vigna radiata* (BRR)	T.R. Anderson, Mung 2 (1996)	MAFF 239492 = CCC 197-05	Tested

[a] NRRL, The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA/ARS, Peoria, IL, USA

[b] MAFF, Microorganisms Section of the NIAS Genebank, National Institute of Agrobiological Sciences, Tsukuba, Japan; CCC, Culture Collection of CEREMIC (Centro de Referencia de Micología), Fac. de Cs. Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina; FRC, *Fusarium* Research Center, Pennsylvania State University, University Park, PA, USA

[c] Ex-holotype strain

margins of 2-week-old cultures on SNA and placed in the center of 9-cm-diameter plastic Petri dishes containing PDA. These cultures were incubated in complete darkness at eight different temperatures at 5°C intervals from 5° to 40°C. Cultures were examined after 1 and 5 days under a dissecting microscope, and colony margins were marked with permanent ink on the reverse side of the Petri dishes. Radial mycelial growth rates were calculated as mean values per day by measuring the difference in colony size at 16 different positions around the colony during the 4-day incubation. Measurements of colony size were repeated at least twice and averaged.

Molecular biology

Mycelium was cultured in yeast-malt broth, harvested over a Büchner funnel, and freeze dried overnight. Total genomic DNA was extracted from approximately 50–100 mg dried, pulverized mycelium using a CTAB (cetyl trimethylammonium bromide; Sigma-Aldrich, St. Louis, MO, USA) protocol as previously described (O'Donnell et al. 2000). Five anonymous intergenic loci (i.e., 44, 51, 65, 83, and 96) and the entire nuclear ribosomal intergenic spacer (IGS) region of rDNA were separately amplified by the polymerase chain reaction and sequenced as described in Covert et al. (2007) and Aoki et al. (2005), respectively. Sequencing reactions were purified using ABI XTerminator (Applied Biosystems, Foster City, CA, USA) before running them on an automated ABI-Hitachi 3730 capillary sequencer (Tokyo, Japan).

DNA sequence alignment and phylogenetic analysis

Sequence chromatograms were edited with Sequencher version 4.9 (Gene Codes, Ann Arbor, MI, USA) and then were exported as NEXUS files. The nuclear IGS rDNA was aligned using the MAFFT program (http://align.bmr.kyushu-u-ac.jp/mafft/software) because of the number and complexity of length variable indels. Unweighted maximum parsimony (MP) analyses were conducted on the individual datasets using PAUP*4.0b10 (Swofford 2002) as previously described (Aoki et al. 2005). Maximum likelihood (ML) analyses were conducted with GARLI version 0.951 (Zwickl 2006) using the general-time-reversible model with a proportion of invariant sites and gamma-distributed rate heterogeneity (GTR + I + Γ). Nonparametric MP and ML bootstrapping followed previously described methods (O'Donnell et al. 2010).

Nucleotide sequence accession numbers

The DNA sequences generated in this study have been deposited in GenBank as JF920181–JF920284 and accessions cited in O'Donnell et al. (2010). The corrected ABI chromatograms and alignments have also been deposited in the web-accessible *Fusarium MLST* database (http://www.cbs.knaw.nl/fusarium; O'Donnell et al. 2010) at the Centraalbureau voor Schimmelcultures (CBS-KNAW) Fungal Biodiversity Center and the FUSARIUM-ID database (http://isolate.fusariumdb.org; Geiser et al. 2004; Park et al. 2011) at The Pennsylvania State University.

Pathogenicity tests on soybean, green bean, and mung bean plants

Eleven strains representing six different species were used in the pathogenicity experiments (Tables 1, 2, 3, 4, 5). Inoculum was prepared by growing each isolate on PDA for 1 week at 25°C in the dark and removing 6-mm-diameter plugs from the growing edge of the colony. Five plugs were added to a 500-ml flask containing 125 g sorghum grain that had been soaked in distilled water overnight, drained, and autoclaved for 60 min at 121°C on 2 consecutive days and allowed to cool (Hartman et al. 1997; Mueller et al. 2002). The inoculated grain was incubated at 25°C in the dark for 2 weeks and shaken daily. A field soil previously fumigated with methyl bromide (Roy et al. 1989; Melgar et al. 1994) was placed in plastic pots. A layer of infested sorghum seeds (3 g) was added to each pot and covered with 2 cm of soil (Scandiani et al. 2011). An initial experiment was conducted to assess the ability of six isolates of *F. crassistipitatum* to induce soybean SDS foliar and root symptoms on susceptible cultivars A-6445RG and N-4613RG, using the known soybean SDS pathogens *F. tucumaniae* NRRL 34550 and *F. virguliforme* NRRL 34551 as a positive control for pathogenicity (Table 2). In a separate experiment, the ability of *F. crassistipitatum* to induce various foliar symptoms (Table 3) and root rot symptoms (Table 4) on soybean cultivar NA-4313RG, green bean, and mung bean, and their impact on plant height and fresh weight (Table 5), was assessed using three soybean SDS and two BRR pathogens. In the latter experiment, infested oat grain was also used as the inoculum (Scherm and Yang 1996). Once inoculated, pots were placed on a greenhouse bench and grown under a natural photoperiod at 25° ± 3°C for 30 days. Soil was watered to saturation after planting and maintained at near field capacity throughout the study. The pots were arranged in a randomized complete block design. Uninoculated plants served as the negative control. Plants were assessed for incidence of typical SDS foliar symptoms (interveinal chlorosis and necrosis), chlorotic leaf mottling, leaf curling and rugosity, petiole retention, plant height, shoot and root weight, wilting, and root rot. At the end of the experiment, all plants within a replicate were rated for basal stem rot, and fresh shoot weight was determined after cutting the

Table 2 Soybean sudden death syndrome (SDS) foliar and root symptoms observed on two different cultivars of soybean grown in soil inoculated with *Fusarium crassistipitatum* or two SDS pathogens (% incidence and severity)

Species	NRRL	SDS foliar symptoms				SDS root symptoms			
		Soybean cultivar A-6445RG		Soybean cultivar N-4613RG		Soybean cultivar A-6445RG		Soybean cultivar N-4613RG	
		Incidence[a] (%)	Severity[b] (1–5)	Incidence[a] (%)	Severity[b] (1–5)	Incidence[c] (%)	Severity[d] (1–5)	Incidence[c] (%)	Severity[d] (1–5)
Fusarium crassistipitatum	31949	100.00	3.38	98.33	2.87	100.00	4.09	98.33	3.93
Fusarium crassistipitatum	36877	45.83	1.46	90.00	1.88	100.00	3.25	98.33	3.78
Fusarium crassistipitatum	43824	87.50	1.84	93.33	1.90	100.00	3.38	96.67	4.02
Fusarium crassistipitatum	43825	95.83	2.41	88.33	2.00	100.00	3.96	96.67	3.75
Fusarium crassistipitatum	46170	100.00	2.09	98.33	2.05	100.00	3.63	100.00	3.87
Fusarium crassistipitatum	46173	100.00	2.42	96.67	2.35	100.00	4.00	96.67	3.42
Fusarium tucumaniae	34550	8.33	1.08	36.67	1.42	100.00	3.08	100.00	4.03
Fusarium virguliforme	34551	91.67	3.17	100.00	3.65	100.00	3.92	100.00	3.58
Uninoculated negative control		0	1	0	1	0	1	0	1
LSD ($P \leq 0.05$)[e]		14.87	0.45	9.20	0.26	NS	0.41	NS[e]	0.31

NS not significant at $P \leq 0.05$

[a] Disease incidence (percentage of plants); plants with slight to typical foliar symptoms were scored

[b] Mean foliar severity ratings of 24 A-6445RG plants (three replications of four pots with two seeds per pot) and 60 N-4613RG plants (three replications of four pots with five seeds per pot) inoculated separately with one of the eight strains or the uninoculated negative control; foliar disease severity was based on a scale of 1–5 where 1 = no symptoms (0% foliage affected), 2 = slight symptom development with mottling and mosaic on leaves (1–20% foliage affected), 3 = moderate symptom development with interveinal chlorosis and necrosis on foliage (21–50% foliage affected), 4 = heavy symptom development with interveinal chlorosis and necrosis (51–80% foliage affected), and 5 = severe interveinal chlorosis and necrosis (81–100% foliage affected)

[c] Root disease incidence (percent of plants with root rot)

[d] Root severity was scored using the following scale: 1 = healthy roots and tap root, 2 = <25% of lateral roots and tap root necrotic, 3 = 25–50% of lateral roots and tap root necrotic, 4 = 51–90% of lateral roots and tap roots necrotic, and 5 = >90% of root system necrotic, plants dead; *Fusarium tucumaniae* NRRL 34550 and *F. virguliforme* NRRL 34551 were used as a positive control for pathogenicity

[e] Differences by Fisher's protected least significant difference (LSD) test

stems off at the soil line. In addition, the roots were removed from the soil and were washed under running water before they were rated for taproot necrosis and fresh weight. Data from the experiments were subjected to analysis of variance (ANOVA). Treatment means were compared using Fisher's protected least significant difference (LSD) at $P \leq 0.05$.

Results

Taxonomy

Our morphological and molecular phylogenetic analyses support the recognition of the soybean SDS isolates from Argentina and Brazil as a phenotypically and phylogenetically distinct species, which is formally described below. ***Fusarium crassistipitatum*** Scandiani, T. Aoki et O'Donnell, sp. nov.

MycoBank ID: MB 561257 Figs. 1–19

Coloniae in PDA radium per diem 1.3–1.9 mm ad 20°C obscuritate crescentes, albidae vel luteo-albae, pallide luteae vel aurantio-albae; in parte sporifera pustulae luteo-albae, dilute flavae, griseo-aurantiae; in luce fluorescente vel naturali pustulae similes; mycelium aerium parcum et in coloniis pionnotium simile, vel copiosum, laxum vel floccosum, album vel luteo-album; margo integra vel undulata; reversum hyalinum vel nonnumquam griseo-luteum, griseo-aurantiacum vel brunneo-aurantiacum; odor dulcis vel nonnumquam mucidus. Hyphae in SNA

Table 3 Different disease symptoms observed on soybean, green bean, and mung bean leaves grown in soil inoculated with *Fusarium crassistipitatum* or known SDS or bean root rot (BRR) pathogens

Species inoculated	NRRL	SDS foliar symptoms			Chlorotic mottling, leaf curling, leaf rugosity						Wilting					
		Soybean Trial[a]			Green bean Trial			Mung bean Trial			Green bean Trial			Mung bean Trial		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Fusarium crassistipitatum	31949	**93**[c]	**67**	**100**	**60**	**100**	**100**	0	0	0	0	0	0	**75**	**100**	**100**
Fusarium crassistipitatum	36877-(1)[b]	–[d]	**100**	–	–	**86**	–	–	0	–	–	0	–	–	**100**	–
	36877-(2)[b]	–	**100**	–	–	**85**	–	–	0	–	–	0	–	–	**100**	–
Fusarium tucumaniae	34550	**85**	31	**100**	**64**	**100**	0	0	0	**56**	0	0	0	25	0	12.5
Fusarium virguliforme	34551	**100**	**100**	**100**	**100**	**100**	**100**	19	0	0	0	0	0	19	**100**	**100**
Fusarium brasiliense	31756	**100**	**29**	**100**	**60**	**87**	**80**	12	0	6	0	0	7	37	0	**81**
Fusarium phaseoli	31156	0	0	0	7	50	0	12	0	0	0	0	0	0	0	0
Fusarium cuneirostrum	36024-(1)[b]	–	50	–	–	**75**	–	–	0	–	–	0	–	–	**87**	–
	36024-(2)[b]	–	29	–	–	**77**	–	–	0	–	–	0	–	–	**87**	–
Uninoculated negative control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Incidence is expressed as %. Inoculum in trials 1 and 2 was grown on sorghum seed; inoculum in trial 3 was grown on oat grain (see "Materials and methods")

[b] Two separately prepared inocula were used for *F. crassistipitatum* NRRL 36877 and *F. cuneirostrum* NRRL 36024 in Trial 2

[c] Symptom incidence >50% is indicated with bold numbers

[d] Not tested

1.5–6.5(–8) μm latae; chlamydosporae in hyphis et conidiis frequenter formantes, plerumque subglobosae, intercalares vel terminales, plerumque singulares, raro catenatae, hyalinae vel dilute flavidae, leves vel rugosae, 6.5–17.5 × 5–13.5 μm. Sclerotia absentia. Sporulatio plerumque praecox et copiosa; in PDA saepe subhyalina, plerumque flavescens. Sporodochia plerumque copiosa in SNA et PDA, parca in coloniis hypharum. Conidiophora aeria copiosa in SNA, raro in PDA, simplicia vel parce ramosa, plerumque crassa et erecta, ad 170 μm alta, 2–7.5 μm lata, basi plerumque clare pachyderma, apice monophialides integras formantia. Phialides aeriae simplices, subulatae vel subcylindricae, saepe collarette conspicuum ferentes. Conidia aeria biformia: (1) falcata vel nonnumquam curvatim cylindrica, (1–)3(–4)-septata, basi pediformia, a conidiis falcatis sporodochialibus saepe indistincta, praecipue in conidiophoris majoribus formata; (2) minuta, oblongo-ellipsoidea vel breviter clavata, nonnumquam reniformia, 0(–1)-septata, 4.5–8.5 × 2–3 μm, in conidiophoris usque 82 μm longis et 2–4 μm latis ad partes paucas coloniae formata. Conidiophora sporodochialia verticillata vel raro simplicia, monophialides ferentia. Phialides simplices, subulatae, ampulliformes vel subcylindricae, plerumque collarette conspicuum ferentes. Conidia sporodochialia biformia, a conidiis sporodochialibus in *Fusario cuneirostro* vel *Fusario brasilienso* saepe indistincta: (1) plerumque falcata, nonnumquam cylindrica vel modice curvata, medio latissima vel in dimidio superiore gradatim dilatata, deorsum modice angustata, cellula apicali plerumque cuneatim rostrata vel nonnumquam rotundata et cellula basilari clare pediformia vel rotundata, (1–)3–4(–6)-septata, in PDA et SNA formata; illa 3-septata 32–70 × 3.5–6.5 μm in SNA et 30.5–60 × 4–7 μm in PDA; illa 4-septata 43–69.5 × 4–6 μm in SNA et 41.5–63.5 × 4.5–6.5 μm in PDA; illa 5-septata 42.5–70.5 × 4.5–6.5 μm in SNA; (2) breviter clavata, nonnumquam ellipsoidea vel naviculiformia, recta vel leviter curvata, apice rotundata et basi truncata, (0–)1(–2)-septata, 15–31 × 4–6 μm, in PDA formata.

Holotypus: BPI 871490, cultura sicca (NRRL 36877 = MAFF 239757 = CCC 142-05 = M. Scandiani Zavalla 1) e radice *Glycines max* infecta, a M. Scandiani lecta in Zavalla, Santa Fe, Argentina, 2004, in Herbario BPI, USA deposita.

Colonies on PDA showing radial mycelial growth rates of 1.3–1.9 mm per day at 20°C in the dark. Colony color on PDA yellowish, white (1A1) to yellowish-white (4A2), pale yellow (4A3), or orange white (5A2); conidial pustules yellowish-white (4A2), pale yellow (4A3) to light yellow (4A4) or orange-grey (5B2) in the dark and under fluorescent light or daylight. Aerial mycelium sparse with pionnotal colony appearance, or developed abundantly, then loose to floccose, white (1A1) to yellowish-white (4A2). Colony margin entire to undulate. Reverse

Table 4 Different disease symptoms observed on soybean, green bean, and mung bean stems and roots grown in soil inoculated with *Fusarium crassistipitatum* or known SDS or BRR pathogens

Species inoculated	NRRL	Basal stem rot									Taproot rot								
		Soybean Trial[a]			Green bean Trial			Mung bean Trial			Soybean Trial			Green bean Trial			Mung bean Trial		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Fusarium crassistipitatum	31949	**93**[c]	33	**100**	**92**	**81**	**100**	**100**	**94**	**100**	**100**	**93**	**100**	**100**	**94**	**100**	**100**	**100**	**100**
Fusarium crassistipitatum	36877-(1)[b]	–[d]	50	–	–	**61**	–	–	**100**	–	–	**100**	–	–	**100**	–	–	**100**	–
	36877-(2)[b]	–	**61**	–	–	**64**	–	–	**100**	–	–	**100**	–	–	**100**	–	–	**100**	–
Fusarium tucumaniae	34550	38	15	33	**79**	27	**100**	**100**	19	**94**	**64**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Fusarium virguliforme	34551	**100**	**100**	**100**	**69**	**86**	**100**	**69**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Fusarium brasiliense	31756	**69**	29	**73**	**100**	31	**80**	**87**	44	**100**	**100**	**64**	**100**	**100**	**100**	**100**	**100**	**94**	**100**
Fusarium phaseoli	31156	0	0	0	29	**50**	**57**	31	25	37	15	0	38	**100**	**50**	**100**	**60**	0	37
Fusarium cuneirostrum	36024-(1)[b]	–	36	–	–	25	–	–	**75**	–	–	**79**	–	–	**100**	–	–	**100**	–
	36024-(2)[b]	–	36	–	–	**61**	–	–	**75**	–	–	**86**	–	–	**100**	–	–	**100**	–
Uninoculated negative control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Incidence is expressed as %; inoculum in trials 1 and 2 was grown on sorghum seed; inoculum in trial 3 was grown on oat grain (see "Materials and methods")

[b] Two separately prepared inocula were used for *F. crassistipitatum* NRRL 36877 and *F. cuneirostrum* NRRL 36024 in trial 2

[c] Symptom incidence >50% is indicated with bold numbers

[d] Not tested

Table 5 Plant height and fresh shoot and root weight of soybean, green bean, and mung bean plants inoculated with four soybean SDS and two BRR pathogens

Species inoculated	NRRL	Height[a]									Fresh shoot weight[a]									Fresh root weight[a]								
		Soybean Trial			Green bean Trial			Mung bean Trial			Soybean Trial			Green bean Trial			Mung bean Trial			Soybean Trial			Green bean Trial			Mung bean Trial		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Fusarium crassistipitatum	31949	69	60	**41**[c]	62	53	**34**	**44**	69	**43**	68	79	**27**	66	**39**	**29**	**19**	**23**	**8**	**46**	90	**20**	**38**	60	**35**	**7**	**7**	**6**
Fusarium crassistipitatum	36877-(1)[b]	–[d]	50	–	–	50	–	–	65	–	–	**45**	–	–	**24**	–	–	**22**	–	–	70	–	–	75	–	–	**11**	–
	36877-(2)[b]	–	54	–	–	54	–	–	64	–	–	**42**	–	–	**32**	–	–	**22**	–	–	65	–	–	73	–	–	**8**	–
Fusarium tucumaniae	34550	57	76	60	52	69	**49**	**46**	89	**40**	51	84	64	72	**44**	62	**34**	82	**15**	**42**	89	60	68	**44**	62	**13**	**14**	**4**
Fusarium virguliforme	34551	54	**34**	**29**	71	**41**	**34**	65	50	**24**	**44**	**27**	**15**	73	**38**	50	52	**10**	**1**	**38**	**31**	51	73	**38**	65	**30**	**7**	**1**
Fusarium brasiliense	31756	**46**	75	**36**	57	60	**44**	**48**	98	**24**	**22**	78	**35**	55	59	61	**37**	76	**10**	**11**	70	**41**	**47**	57	77	**17**	**27**	**1**
Fusarium phaseoli	31156	69	98	87	98	97	91	85	79	77	97	97	84	95	88	92	81	74	56	96	82	70	57	98	50	84	76	**31**
Fusarium cuneirostrum	36024-(1)[b]	–	75	–	–	54	–	–	65	–	–	74	–	–	**29**	–	–	**17**	–	–	62	–	–	**25**	–	–	**5**	–
	36024-(2)[b]	–	84	–	–	65	–	–	74	–	–	87	–	–	**34**	–	–	**16**	–	–	78	–	–	63	–	–	**9**	–
LSD ($P \leq 0.05$)[e]		7	8	10	12	15	9	10	12	7	10	15	31	15	20	13	10	26	18	19	19	44	15	23	21	18	18	10

[a] Plant height and fresh weight of shoot and root are expressed as a percentage of the height and weight of the corresponding uninoculated negative control plants; plants in Trials 1 and 2 were inoculated with sorghum grain infested with one of the six species; in Trial 3, infested oat grain was used as the inoculum

[b] Two separately prepared inocula were tested for *F. crassistipitatum* NRRL 36877 and *F. cuneirostrum* NRRL 36024 in Trial 2

[c] Plant height and fresh weight <50% are indicated with bold numbers

[d] Not tested

[e] Not significantly at the $P \leq 0.05$ using Fisher's protected least significant difference (LSD) test

Table 6 Summary of maximum parsimony phylogenetic analyses

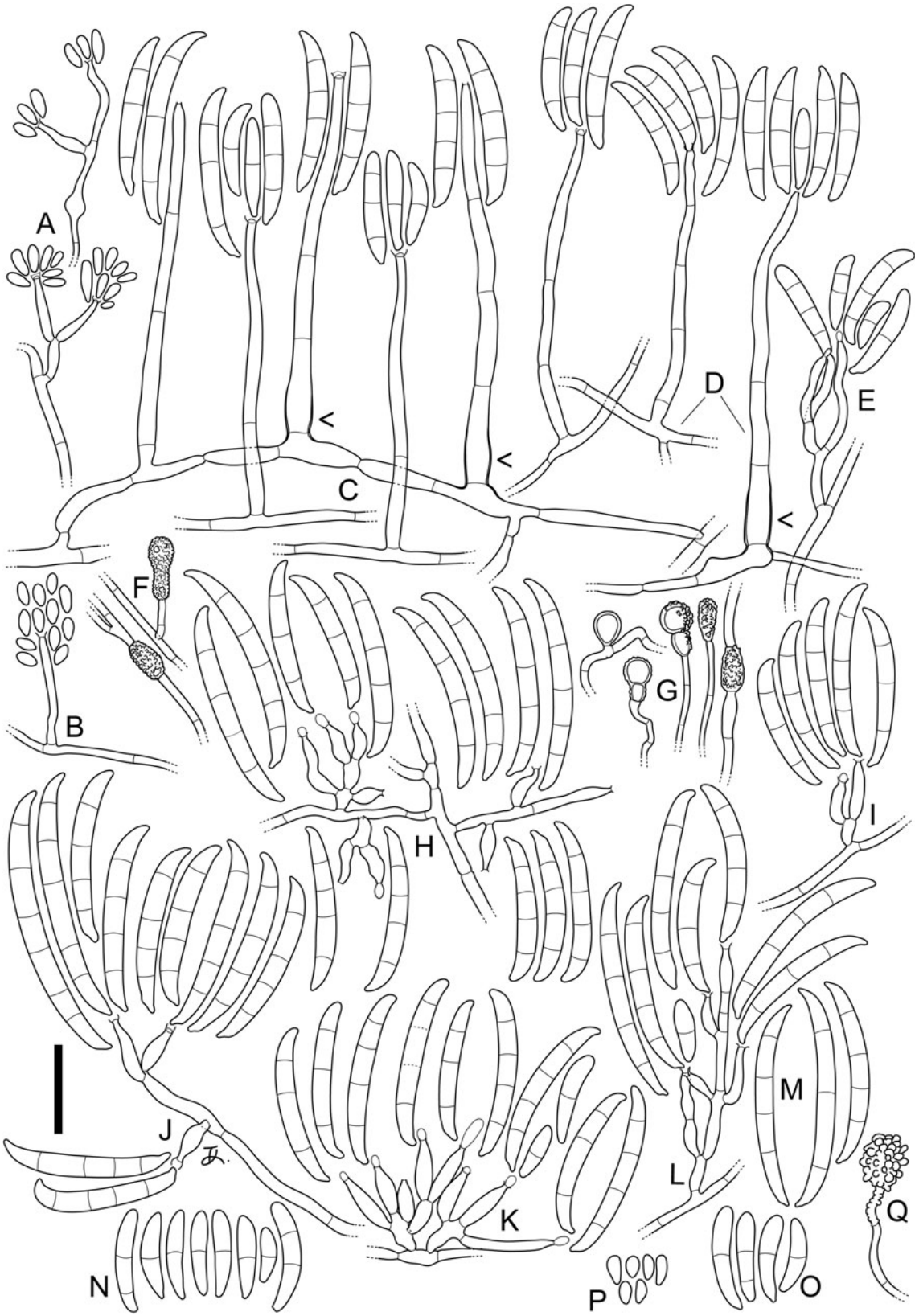
Locus[a]	Number of characters	Number of MPTs	MPT length	CI	RI	Syn	Aut	Bootstrap support (%): *Fusarium crassistipitatum*	No. of species supported as monophyletic
44	2,230	1	30	0.9	0.97	27	0	98	6
51	1,530	4	13	0.92	0.98	12	0	97	2
65	1,813	1	14	1.0	1.0	14	0	88	5
83	2,610	4	18	0.94	0.98	16	1	85	3
96	1,520	2	22	1.0	1.0	20	1	78	3
IGS rDNA	2,765	1	142	0.95	0.99	127	4	96	6

MPT most parsimonious tree, *CI* consistency index, *RI* retention index, *Syn* synapomorphy or parsimony informative character, *Aut* autapomorphy, uniquely derived character or parsimony uninformative character

[a] Five anonymous loci spanning intergenic regions designated 44, 51, 65, 83, and 96 were sequenced (Covert et al. 2007) together with the entire nuclear ribosomal intergenic (IGS) spacer region

pigmentation absent or sometimes greyish-yellow (4B3–4), greyish-orange (5B3–5) to brownish-orange (5C4). Exudate not observed. Odor sweet or sometimes moldy. Hyphae on SNA 1.5–6.5(–8) μm wide. Chlamydospores formed abundantly in hyphae and in conidia, mostly subglobose, intercalary or terminal, mostly single, rarely in chains, hyaline to pale-yellow, smooth to rough-walled, 6.5–17.5 × 5–13.5 μm. Sclerotia absent. Sporulation generally rapid and abundant; on PDA often light-colored, often with a yellowish pigmentation in the dark and under fluorescent light or daylight; sporodochia normally formed abundantly on SNA and PDA, but sparsely in some mycelial strains. Aerial conidiophores formed abundantly on SNA, infrequently on PDA, unbranched or sparsely branched, often stout and erect, up to 170 μm long, 2–7.5 μm wide, often thick-walled at the base, forming monophialides integrated in the apices. Aerial phialides simple, subulate to subcylindrical, often with a conspicuous collarette at the tip. Aerial conidia of two types; (1) falcate to sometimes curved cylindrical, (1–)3(–4)-septate, with a foot cell, morphologically indistinguishable from falcate sporodochial conidia, formed primarily on tall conidiophores; (2) minute, oblong-ellipsoidal to short clavate, occasionally reniform, 0(–1)-septate, 4.5–8.5 × 2–3 μm in total range, 6.2–6.3 × 2.5–2.6 μm on average (ex type: 4.5–8 × 2–3 μm in total range, 6.3 ± 0.8 × 2.5 ± 0.3 μm on average ± SD), formed in a small portion of the colony and on short and sometimes branched conidiophores up to 82 μm long, 2–4 μm wide. Sometimes an additional conidial type represented by 1–3-septate, straight or curved cylindrical conidia, with a rounded apex and with or without a foot cell, formed on tall or short conidiophores, representing an intermediate morphology of the first and the second types of aerial conidial structures. Sporodochial conidiophores branched verticillately, or rarely unbranched, forming apical monophialides. Sporodochial phialides simple, subulate, ampulliform to subcylindrical, often with a conspicuous collarette at the tip. Sporodochial conidia of two types, often morphologically indistinguishable from sporodochial conidia in *F. cuneirostrum* or *F. brasiliense*: (1) mostly falcate, sometimes cylindrical and gradually curved, widest at the midregion or widening gradually upward, with or without a rostrate apex resembling a wedge-shaped beak, sometimes with a rounded apex, gradually narrowing toward the base, often with a distinct, slightly protruding basal foot cell or sometimes rounded, (1–)3–4(–6)-septate, formed on PDA and SNA; 3-septate on SNA: 32–70 × 3.5–6.5 μm in total range, 45.3–49.9 × 5.0–5.3 μm on average (ex type: 32–56 × 4.5–6 μm in total range, 45.3 ± 5.4 × 5.1 ± 0.3 μm on average ± SD), on PDA: 30.5–60 × 4–7 μm in total range, 39.9–49.6 × 5.1–5.5 μm on average (ex type: 32.5–50 × 5–6 μm in total range, 39.9 ± 4.5 × 5.3 ± 0.3 μm on average ± SD); 4-septate on SNA: 43–69.5 × 4–6 μm in total range, 52.2–62.1 × 5.2–5.4 μm on average (ex type: 44.5–59 × 4.5–5.5 μm in total range, 52.2 ± 3.9 × 5.2 ± 0.21 μm on average ± SD), on PDA: 41.5–63.5 × 4.5–6.5 μm in total range, 49.1–55.8 × 5.2–5.5 μm on average (ex type: 42–57 × 4.5–6 μm in total range, 49.1 ± 2.9 × 5.2 ± 0.2 μm on average ± SD); 5-septate on SNA: 42.5–70.5 × 4.5–6.5 μm in total range, 51.8–60.0 × 5.4–5.5 μm on average (ex type: 42.5–70.5 × 4.5–6.5 μm in total range, 51.8 ± 5.9 × 5.5 ± 0.3 μm on average ± SD); (2) short-clavate to sometimes ellipsoidal or naviculate, straight or slightly curved, with a rounded apex and a truncate base, (0–)1(–2)-septate, 15–31 × 4–6 μm, formed on PDA.

Holotype: BPI 871490, a dried culture, isolated from a diseased root of soybean plant cv. A-6445RG exhibiting SDS symptoms, collected at Plot 5 of the Experimental Field "Jose V. Villarino," Facultad de Ciencias Agrarias,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◀ **Fig. 1** *Fusarium crassistipitatum* cultured on synthetic low nutrient agar (SNA) (*A–J*) and potato dextrose agar (PDA) (*K–Q*) in the dark (*A*, *C*, *G*, *J*, *L*, *O–Q* from NRRL 36877, ex holotype; *B*, *D–F*, *H*, *I*, *K*, *M*, *N* from NRRL 31949). *A*, *B*, *P* Aseptate, minute, short-clavate conidia formed on short aerial conidiophores. *C*, *D* Septate, falcate conidia with a wedge-shaped, pointed, or rounded apex and a foot cell formed on slender aerial conidiophores. *E* One- to 3-septate, straight or curved cylindrical conidia formed on short aerial conidiophores. *F*, *G*, *Q* Rough-walled, terminal or intercalary chlamydospores formed in hyphae. *H–M* Septate, falcate conidia formed on sporodochial conidiophores, mostly with a wedge-shaped pointed apex and a foot cell, but some possess rounded ends. *N*, *O* Straight or slightly curved, short-clavate to naviculate, 1–2-septate sporodochial conidia formed in culture on PDA. *Bar* 25 μm

Universidad Nacional de Rosario, Zavalla, Santa Fe Province, Argentina, 15 March 2004, M. Scandiani, deposited in the herbarium of BPI, USA.

Ex holotype culture: NRRL 36877 = MAFF 239757 = CCC 142-05

Etymology: Latin, *crassi-* (broad-) + *stipitatum* (stalked); based on the stout nature of the aerial conidiophores.

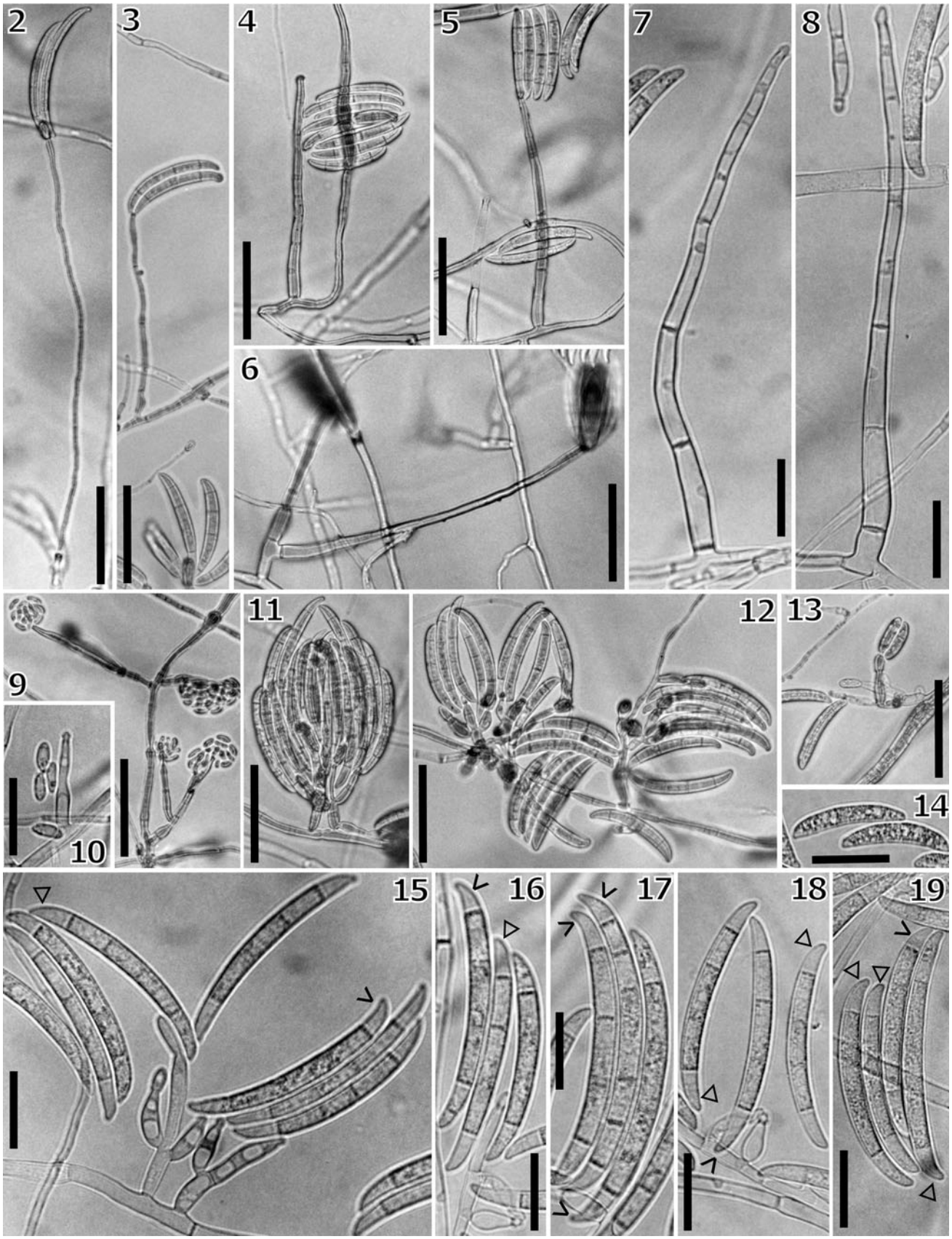
Isolates studied. NRRL 31949 = MAFF 239052 = CCC 198-05, *Glycine max*, Cristalina, Goiás, Brazil, J.T. Yorinori, 2000; NRRL 36877 = MAFF 239757 = CCC 142-05 = M. Scandiani, Zavalla 1, *G. max*, Zavalla, Santa Fe, Argentina, M.M. Scandiani, 2004; NRRL 43824 = CCC 144-07, *G. max*, Las Cejas, Tucumán, Argentina, M.M. Scandiani/G. Salas, 2007; NRRL 43825 = CCC 143-07, *G. max*, Las Cejas, Tucumán, Argentina, M.M. Scandiani/G. Salas, 2007; NRRL 46170 = CCC 169-07, *G. max*, Las Lajitas, Salta, Argentina, M.M. Scandiani, 2007; NRRL 46171 = CCC 168-07, *G. max*, Las Lajitas, Salta, Argentina, M.M. Scandiani, 2007; NRRL 46173 = CCC 174-07, *G. max*, Las Lajitas, Salta, Argentina, M.M. Scandiani, 2007; NRRL 46174 = CCC 175-07, *G. max*, Las Lajitas, Salta, Argentina, M.M. Scandiani, 2007; NRRL 46175 = CCC 176-07, *G. max*, Las Lajitas, Salta, Argentina, M.M. Scandiani, 2007.

Notes: *Fusarium crassistipitatum* typically produces sporodochial conidia with a rostrate apical cell and a slightly protruding basal foot cell. Because the apical cells of *F. crassistipitatum* conidia often taper and hook at the upper third or quarter of their length (Figs. 1H, J, L, M, 15–19), they look like a wedge-shaped beak. In this regard, *F. crassistipitatum* is morphologically similar to *F. cuneirostrum* (Aoki et al. 2005). Sometimes the multiseptate sporodochial conidia of *F. crassistipitatum* produce a rounded apical cell or an indistinct basal foot cell (Figs. 1H–L, 15–19), which is morphologically similar to *F. brasiliense* (Aoki et al. 2005). *Fusarium crassistipitatum*, however, possesses several unique morphological or phenotypic features. It is the only soybean SDS pathogen that produces a yellowish colony when cultured on PDA. Microscopically, the species produces diagnostic tall, erect conidiophores that are mostly unbranched and erect, and frequently thick-walled at the base (arrowheads in Figs. 1C, D, 4–8), with multiseptate conidia apically. When compared with the five other species within the SDS–BRR clade, *F. crassistipitatum* was the only species that produced stout aerial conidiophores. Strains of *F. crassistipitatum* also produce 1–3-septate, straight or curved cylindrical conidia, with a rounded apex and with or without a foot cell, on tall or short aerial conidiophores (Fig. 1E).

Fusarium crassistipitatum and the other species within the SDS–BRR are distinguished from other fusaria by the production of two distinct types of conidia on aerial and sporodochial conidiophores: these included (A-1) falcate, multiseptate aerial conidia with a foot cell formed mainly on tall conidiophores observed on SNA; and (A-2) minute, short-clavate to ellipsoidal aerial conidia formed on short conidiophores in a small portion of the colony on SNA and PDA. Two types of sporodochial conidia were also formed by the species: (S-1) falcate, multiseptate conidia with a foot cell formed on SNA and PDA; and (S-2) short-clavate to ellipsoidal or naviculate conidia on PDA. However, because the sporodochial conidial dimensions of *F. crassistipitatum* overlapped with other species within the SDS–BRR clade, especially *F. cuneirostrum* and *F. brasiliense* (Figs. 20, 21 for the S-1 type conidia), it lacks diagnostic value. Strains of *F. crassistipitatum* grow very slowly (1.3–1.9 mm per day at 20°C and 1.8–2.9 mm per day at 25°C), as commonly observed in the other soybean SDS and BRR pathogens (Fig. 22).

Molecular phylogenetic relationships among species

We sequenced five anonymous intergenic loci (see Table 6) and the entire IGS rDNA to investigate evolutionary relationships and species limits of the soybean SDS and BRR pathogens (Aoki et al. 2005; Covert et al. 2007; O'Donnell et al. 2010). Tree statistics and a summary of MP analyzes are presented in Table 6. Sequences of *F. virguliforme* were used to root the phylogenies based on more inclusive analyses (Aoki et al. 2005). MP analyses of the individual datasets provided strong bootstrap (BS) support for the monophyly of the two isolates of *F. crassistipitatum* sampled, including the ex-type strain NRRL 36877 (BS = 78–98%; Fig. 23). Evolutionary relationships of *F. crassistipitatum* were unresolved based on analyses of the locus 51, 65, and 96 partitions (Fig. 23); however, this species received modest support (BS = 75%) as a sister of *F. tucumaniae* from locus 44, moderate support (BS = 88%) as a sister of *F. phaseoli* + *F. tucumaniae* in the locus 83 phylogeny, and strong support (BS = 100%) as a sister group of *F. cuneirostrum* from analyses of the IGS rDNA. Phylogenetic relationships recovered from analyses of the combined six-gene dataset were concordant with the IGS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◀ **Figs. 2–19** Morphology of *Fusarium crassistipitatum* cultured in the dark (**Figs. 2–12**, **15–19** on SNA and **13**, **14** on PDA; **2–6**, **9**, **11–13** aerial view; **7**, **8**, **10**, **14–19** mounted in water). **2**, **3** Slender and narrow aerial conidiophores arising from hyphae on the agar surface, forming falcate septate conidia at the apex. **4–8** Stout and broad aerial conidiophores that are thick-walled at the base. **9**, **10** Minute conidia formed on short aerial conidiophores arising from hyphae on the agar surface. **11**, **12** Multi-septate sporodochial conidia and conidiophores formed on the agar surface. **13**, **14** Slightly curved, short-clavate 1-septate sporodochial conidia formed in culture on PDA. **15–19** Falcate sporodochial conidia formed on SNA; apical cells of some conidia are rostrate, resembling a wedge-shaped beak (*arrowheads*), but some conidia also possess rounded apical cells (*open triangles*). **2**, **4**, **7**, **11**, **15**, **16**, **18** from NRRL 36877, ex holotype; **3**, **5**, **6**, **8–10**, **12–14**, **17**, **19** from NRRL 31949. *Bars* **2–6**, **9**, **11–13** 50 μm; **7**, **8**, **10**, **14–19** 20 μm

rDNA phylogeny (data not shown), which can be explained by the fact that the IGS rDNA partition contained 58.8% of the synapomorphies (Table 6). Although evolutionary relationships were fully resolved by the IGS rDNA partition, none of the sister group relationships supported by this partition were supported by MP analyses of the five individual anonymous loci.

Based on the proven utility of locus 44 and the IGS rDNA for resolving the monophyly of species within the SDS–BRR clade (Table 6), these two regions were also sequenced in seven isolates of *F. crassistipitatum* from Argentina and the single isolate from Brazil. MP and ML analyses of the two-locus dataset strongly supported reciprocal monophyly of *F. crassistipitatum* with respect to the other species within the SDS–BRR clade. All eight isolates shared the same locus 44 and IGS rDNA allele, which suggests the isolates of *F. crassistipitatum* studied may represent a clone or clonal lineage (Fig. 24).

Pathogenicity tests on soybean, green bean, and mung bean plants

Our initial experiment established that the six isolates of *F. crassistipitatum* tested were able to induce typical SDS foliar and root rot symptoms on susceptible soybean cultivars A-6445RG and N-4613RG comparable to those induced by the known SDS pathogens *F. tucumaniae* NRRL 34550 and *F. virguliforme* NRRL 34551 (see Table 2). In a second set of experiments (see Tables 2, 3, 4; Figs. 25, 26), the two isolates of *F. crassistipitatum* tested (NRRL 31949 and NRRL 36877) induced typical SDS foliar symptoms, including chlorotic mottling and leaf curling on soybean, rugosity on green bean, and wilting on mung bean (see Table 3; Fig. 25b, h). The two isolates of

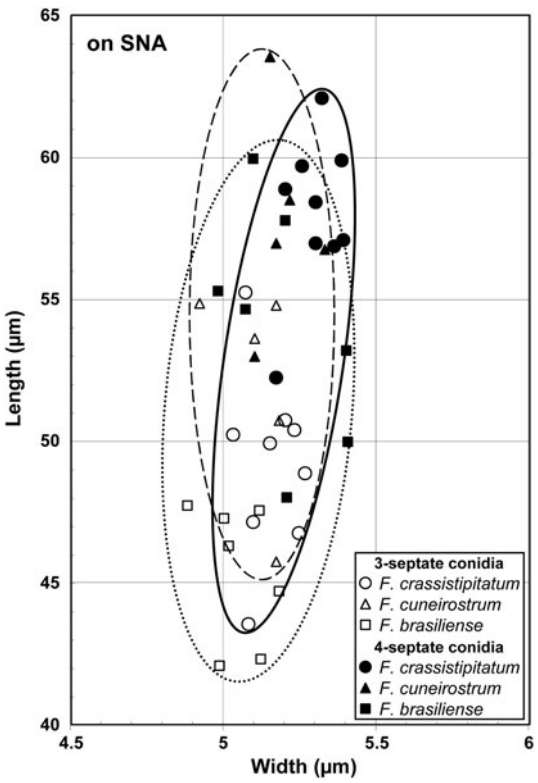


Fig. 20 Plots of mean values of length and width of 3- and 4-septate sporodochial conidia of *Fusarium crassistipitatum*, *F. cuneirostrum*, and *F. brasiliense* grown on SNA in the dark at 20°C

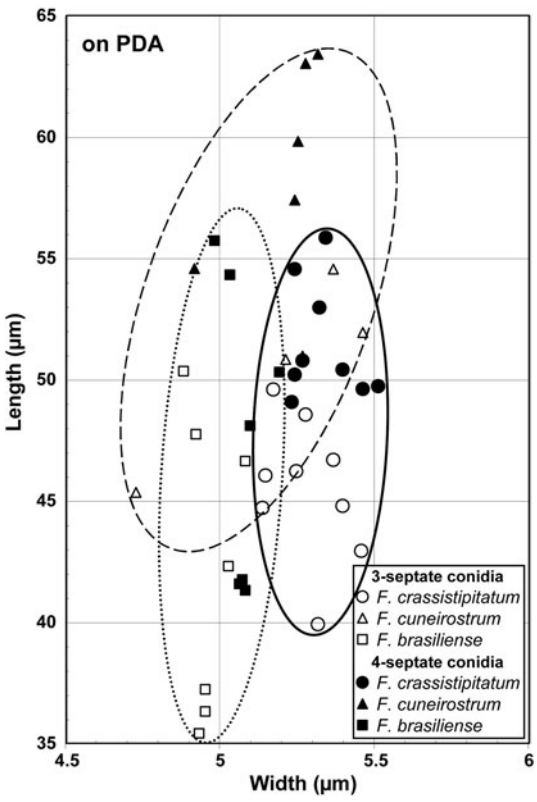


Fig. 21 Plots of mean values of length and width of 3- and 4-septate sporodochial conidia of *Fusarium crassistipitatum*, *F. cuneirostrum*, and *F. brasiliense* grown on PDA in the dark at 20°C

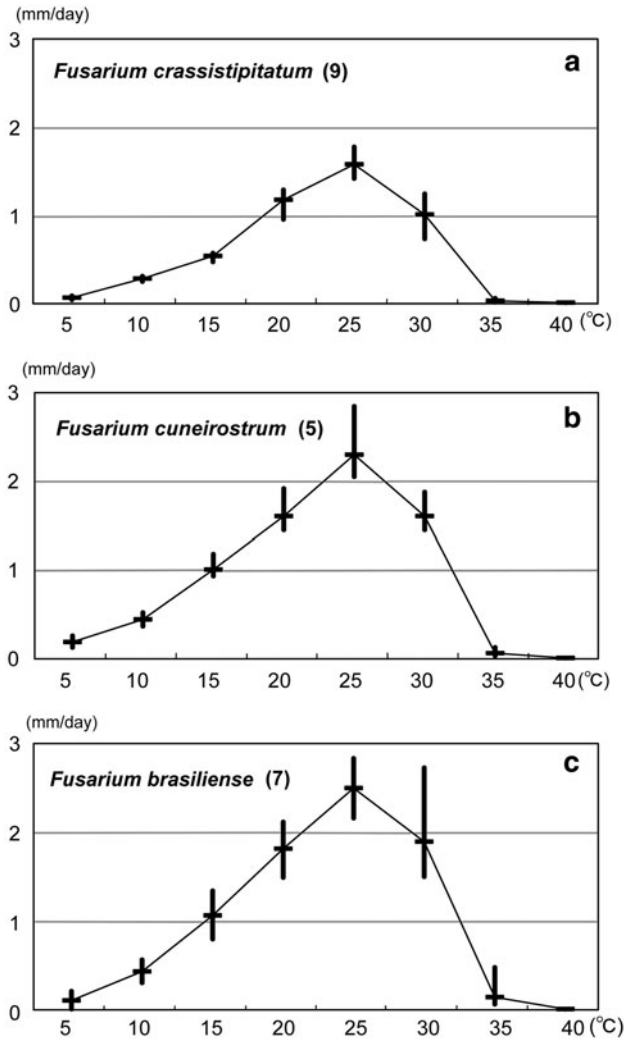


Fig. 22 Comparison of radial growth rates per day on PDA of *Fusarium crassistipitatum* (**a**), *F. cuneirostrum* (**b**), and *F. brasiliense* (**c**) at temperatures from 5° to 40°C. The *thick horizontal* and *vertical bars* indicate means and total ranges, respectively, of the strains of each species (number of strains examined given in *parentheses*)

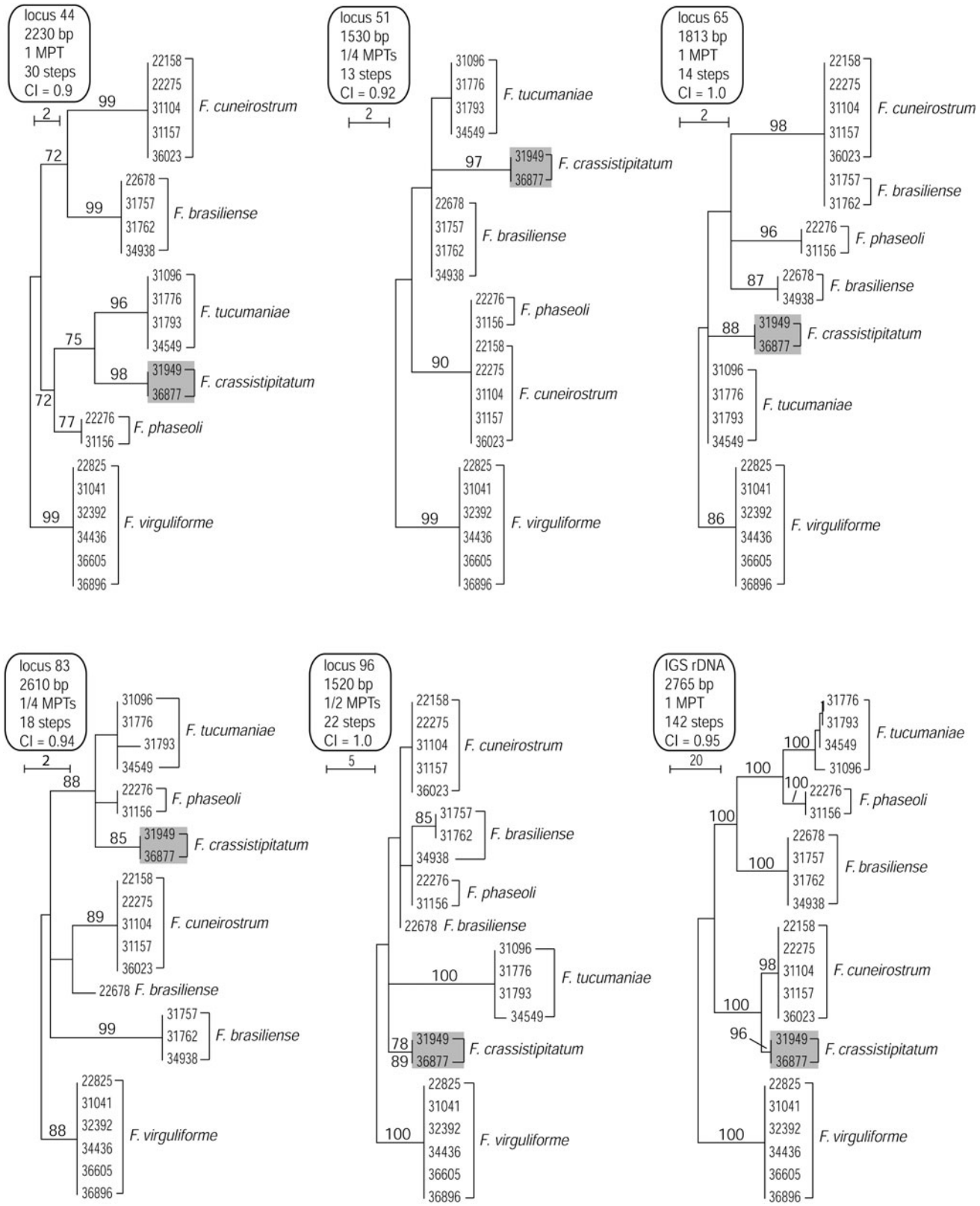
F. crassistipitatum were also able to induce basal stem rot and taproot rot on soybean, green bean, and mung bean (see Table 4; Fig. 26a). Similar foliar and stem and root rot symptoms were induced on these hosts by the three other soybean SDS pathogens (i.e., *F. tucumaniae* NRRL 34550 in Figs. 25e, k, 26c, *F. virguliforme* NRRL 34551 in Figs. 25c, i, 26d, and *F. brasiliense* NRRL 31756 in Figs. 25d, j, 26e), with the exception that *F. tucumaniae* NRRL 34550 only induced mild wilting on mung bean, and *F. brasiliense* NRRL 31756 only induced moderate wilting of mung bean in one of the three inoculation trials (see Tables 3, 4). By contrast, the BRR pathogen *F. phaseoli* induced mild taproot rot on soybean (Fig. 26f) but failed to induce foliar symptoms and basal stem rot on this host (Fig. 25f). Another BRR pathogen *F. cuneirostrum*, only

Fig. 23 Maximum parsimony (MP) phylograms inferred from five anonymous intergenic regions (i.e., loci 44, 51, 65, 83, and 96) and the nuclear ribosomal intergenic spacer (IGS rDNA) region for six species within the sudden death syndrome–bean root rot (SDS–BRR) clade. Note the two isolates of *Fusarium crassistipitatum* highlighted in *grey* are supported as reciprocally monophyletic by MP bootstrapping of all six loci. The *number above internodes* represents MP bootstrap support based on 1,000 pseudo-replicates of the data. Maximum likelihood (ML) bootstrap support is indicated *below internodes* only if it differed by ≥5% of the MP value. Each isolate is identified by the five-digit ARS Culture Collection accession number. The *scale bar* associated with each phylogram indicates number of mutations. *MPT*, most parsimonious tree; *CI*, consistency index; *RI*, retebtion index

induced mild foliar and basal stem rot in addition to moderate taproot rot on this host (Fig. 26g). However, the latter two pathogens did induce moderate foliar symptoms such as chlorotic mottling and leaf curling on green bean and taproot rot on green bean and/or mung bean (see Tables 3, 4; Figs. 25f, 26f, g). *Fusarium cuneirostrum* was the only BRR pathogen that induced wilting of mung bean (Table 3). In addition, the *F. crassistipitatum* isolates reduced height, fresh shoot weight, and fresh root weight of soybean, green bean, and mung bean, as did the strains of *F. tucumaniae*, *F. virguliforme*, and *F. brasiliense* used as a positive control for pathogenicity (Table 5).

Discussion

Fusarium crassistipitatum is morphologically very similar to other species within the SDS–BRR clade in that it frequently produces sporodochial conidia (Figs. 1H–M, 15–19) with a rostrate apical cell and a slightly protruding basal foot cell, as observed in *F. cuneirostrum* (Aoki et al. 2005), or sometimes with a rounded apical cell and an indistinct basal foot cell, as observed in *F. brasiliense* (Aoki et al. 2005). Therefore, *F. crassistipitatum* cannot be differentiated from these two species using conidial morphology alone (Aoki et al. 2005). *Fusarium crassistipitatum*, however, possesses diagnostic morphological or phenotypic features, including the production of tall and stout aerial conidiophores that frequently are thick-walled at the base (arrowheads in Figs. 1C, D; 4–8) and yellowish colonies when cultured on PDA. The five other species of the SDS–BRR clade also produce tall aerial conidiophores, but they are typically slender and relatively thin-walled (Aoki et al. 2003, 2005). Colony color of the other species within the SDS–BRR clade often turns greenish or bluish when they sporulate on PDA. By contrast, colonies of *F. crassistipitatum* remain yellowish even when they are cultured on PDA under fluorescent light or daylight and when they produce conidial pustules. Production of (1) falcate to curved cylindrical, multiseptate conidia with a foot cell formed on tall, erect conidiophores (Figs. 1C, D,



2–5) and (2) minute, short clavate 0(–1)-septate conidia on short conidiophores (Figs. 1A, B, P, 9, 10) appears to represent a synapomorphy that unites members of the SDS–BRR clade. A very slow growth rate on PDA is another common feature shared by the SDS–BRR clade pathogens. Although their slow growth presents some

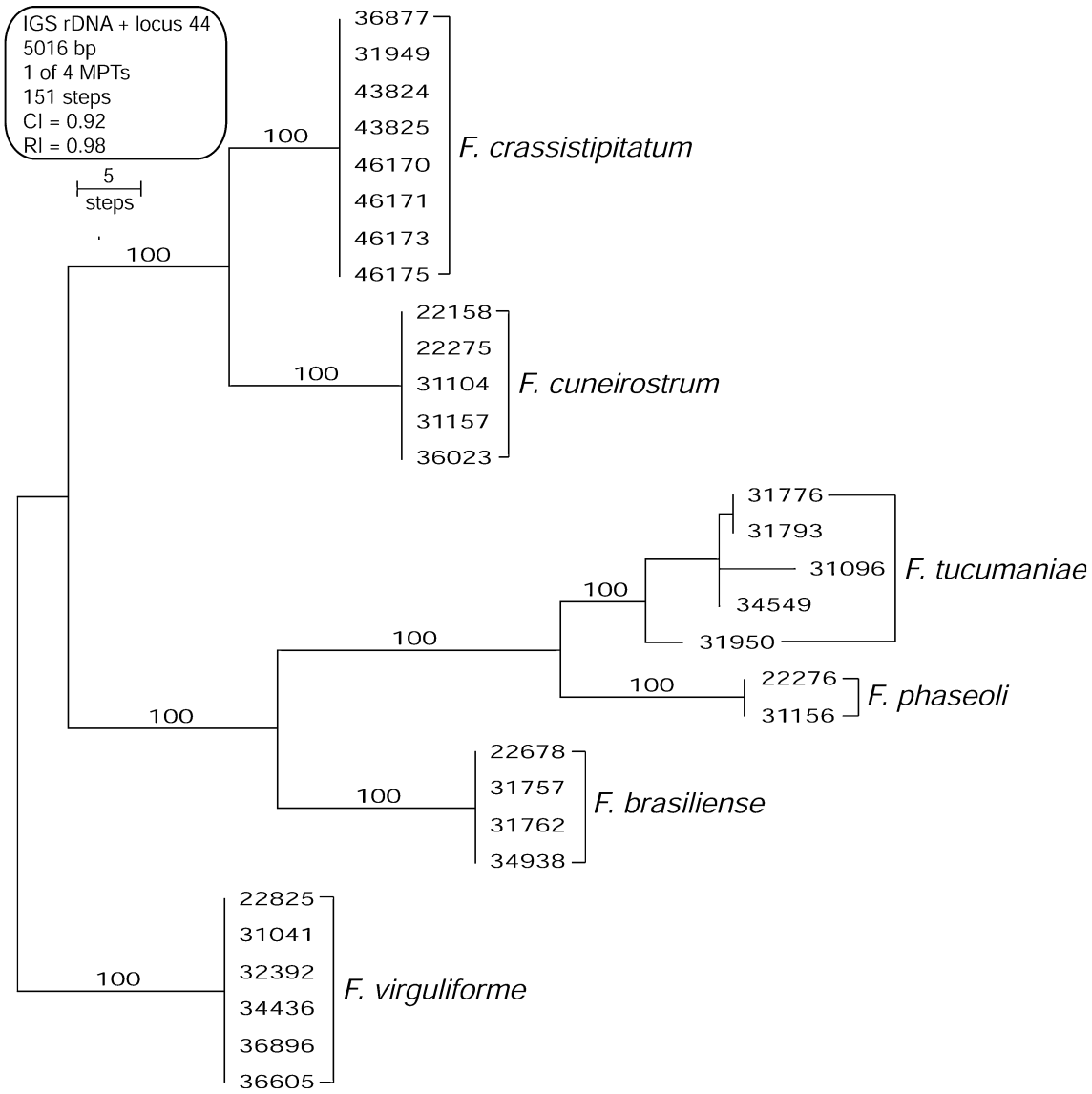


Fig. 24 One of four most parsimonious trees inferred from DNA sequence data from the nuclear ribosomal intergenic spacer region (IGS rDNA) and anonymous locus 44 for 30 isolates within the SDS–BRR clade. The 8 isolates of *Fusarium crassistipitatum* are strongly supported as genealogically exclusive by MP and ML bootstrapping. MP and ML bootstrap values were identical and are indicated *above internodes*. *MPT*, most parsimonious tree; *CI*, consistency index; *RI*, retention index

technical problems when isolating them from nature, it clearly distinguishes species within this clade from the common fast-growing members of the *Fusarium solani* species complex.

The MP and ML bootstrap analyses of nucleotide sequence data from portions of six loci, and the locus 44–IGS rDNA two-locus dataset, provided strong bootstrap support for the genealogical exclusivity of *F. crassistipitatum*. Therefore, this species fulfills the highly conservative criteria for species recognition under genealogical concordant phylogenetic species recognition (Taylor et al. 2000; Dettman et al. 2003). *Fusarium crassistipitatum* was originally discovered in the District of Goiás, Brazil as a single isolate (NRRL 31949) and was initially reported as a genetically divergent isolate of the BRR pathogen *F. cuneirostrum* (Aoki et al. 2005). However, through the discovery and analysis of the seven isolates from Argentina (O'Donnell et al. 2010), we were able to demonstrate herein that *F. crassistipitatum* is strongly supported as reciprocally monophyletic with respect to the five other species within the SDS–BRR clade.

If we assume the IGS rDNA phylogeny represents the best current hypothesis of evolutionary relationships within the SDS–BRR clade, then pathogenicity to *Phaseolus*/*Vigna*

Fig. 25 Foliar symptoms exhibited by soybean, green bean, and mung bean inoculated with four soybean SDS and one BBR pathogen. **a**–**j** Artificial inoculation experiment with strains of five *Fusarium* species using mung bean, dry (green) bean, and soybean. **a** Arrangement of eight plastic pots of each of the three hosts [*m1*–*m8*, mung bean; *g1*–*g8*, dry (green) bean; *s1*–*s8*, soybean]. Foliar appearance of mung bean, dry bean, and soybean plants observed after inoculation with *F. crassistipitatum* NRRL 31949 (**b**), *F. virguliforme* NRRL 34551 (**c**), *F. brasiliense* NRRL 31756 (**d**), *F. tucumaniae* NRRL 34550 (**e**), *F. phaseoli* NRRL 31156 (**f**), and uninoculated negative control (**g**). Foliar symptoms of soybean SDS inoculated with *F. crassistipitatum* NRRL 31949 (**h**), *F. virguliforme* NRRL 34551 (**i**), *F. brasiliense* NRRL 31756 (**j**), and *F. tucumaniae* NRRL 34550 (**k**)

Fig. 26 Root symptoms exhibited by soybean, green bean, and mung bean inoculated with four soybean SDS and one BRR pathogen. Root rot symptoms were caused by inoculation of *F. crassistipitatum* NRRL 31949 (**a**), NRRL 36877 (**b**), *F. tucumaniae* NRRL 34550 (**c**), *F. virguliforme* NRRL 34551 (**d**), *F. brasiliense* NRRL 31756 (**e**), *F. phaseoli* NRRL 31156 (**f**), *F. cuneirostrum* NRRL 36024 (**g**), and uninoculated negative control (**h**)

may have evolved convergently in *F. cuneirostrum* and *F. phaseoli* because they are strongly supported as sisters of the SDS pathogens *F. crassistipitatum* and *F. tucumaniae*, respectively, rather than forming an exclusive clade of BRR pathogens. However, it is important to note that the IGS rDNA phylogeny may not track with the species phylogeny within the SDS–BRR clade, because none of the sister group relationships supported by the IGS rDNA phylogeny was also supported by the five anonymous intergenic loci sampled, and because phylogenies inferred from this locus within the *F. oxysporum* species complex (O'Donnell et al. 2009) and the B trichothecene toxin-producing clade of *Fusarium* (O'Donnell, unpublished data) are highly discordant with those inferred from other loci. Therefore, more extensive molecular phylogenetic and phylogenomic analyses are needed to unravel the evolutionary history of species within the SDS–BRR clade. Fortunately, the whole genomes of several species within the SDS–BRR clade are being sequenced, so evolutionary relationships inferred from phylogenomic analyses should

soon be possible (Madan K. Bhattacharyya, personal communication)

Given the close relationship among the six species within the SDS–BRR clade, once the *MAT1-1* and *MAT1-2* idiomorphs have been identified within *F. tucumaniae*, it should be possible to design a highly conserved PCR assay to type isolates of each species for mating type to better understand their reproductive mode. In addition, sequence data from the *MAT* locus has also demonstrated utility for investigating species limits with *Fusarium* (O'Donnell et al. 2004). Development of an assay for *MAT* would be extraordinarily useful for screening large numbers of field isolates to assess whether heterothallic mating is possible in *F. virguliforme*, *F. crassistipitatum*, and *F. cuneirostrum*. Because of lack of nucleotide polymorphisms at the loci sequenced (i.e., 11 anonymous intergenic loci, plus *EF-1α* and IGS rDNA = 23.1 kb; O'Donnell, unpublished data), and data obtained using other molecular markers (Achenbach et al. 1996; Li et al. 2000; Rupe et al. 2001), the latter three species may be reproducing clonally on soybean and *Phaseolus*/*Vigna*, respectively.

Our recent SDS field survey in Argentina (O'Donnell et al. 2010) reported that *F. crassistipitatum* was the only SDS pathogen from Salta province ($N = 6$), and that it was also present in Tucumán ($N = 2$) and Santa Fe provinces ($N = 1$). Each of the six isolates of *F. crassistipitatum* inoculated on soybean induced foliar symptoms that ranged from slightly mosaic, mottling, and curling, to conspicuous interveinal chlorosis and leaf necrosis. In addition, all the isolates were able to induce moderate to heavy root rot symptoms. Differences in pathogenicity among the *F. crassistipitatum* isolates were observed, with NRRL 31949 *F. crassistipitatum* from Brazil identified as the most aggressive strain of this species studied. This isolate induced foliar disease similar in severity to *F. virguliforme* NRRL 34551. Given that at least four different fusaria can induce soybean SDS, further research is needed to determine what environmental conditions are conducive for optimal disease development under field conditions for each of the species.

With the completion of Koch's postulates for *F. crassistipitatum*, our study has increased the number of fusaria known to induce soybean SDS to four. *Fusarium virguliforme* was the first SDS pathogen for which Koch's postulates were completed; however, it was initially reported as *F. solani* (Roy et al. 1989; Rupe 1989), and subsequently as *F. solani* f. sp. *glycines* (Roy et al. 1997). More recently, these postulates have also been completed for *F. tucumaniae* (Scandiani et al. 2004) and *F. brasiliense* (Aoki et al. 2005). Thus, referring to these pathogens by the *forma specialis* designation, as noted previously (Aoki et al. 2005; Covert et al. 2007), obscures the fact that they comprise four morphologically and phylogenetically distinct species. Such knowledge is crucial to plant breeders' efforts directed at developing soybean cultivars with broad-based resistance to this disease and to agricultural scientists charged with preventing the SDS pathogens from being introduced into nonindigenous areas. The latter point is particularly relevant because, in contrast to South America where all four soybean SDS pathogens are responsible for this disease, *F. virguliforme* appears to be solely responsible for soybean SDS within North America (O'Donnell et al. 2010 and references therein). In contrast to *F. virguliforme* and *F. crassistipitatum*, which may be reproducing exclusively asexually on soybean, based on the lack of nucleotide polymorphism in the loci we sequenced, *F. tucumaniae* is the only soybean SDS pathogen that has been shown to reproduce sexually in the laboratory (Covert et al. 2007) and in the field (Scandiani et al. 2010). In theory, this sexually reproducing pathogen poses a much greater threat to soybean production because it is much more likely to overcome multilocus quantitative resistance in this host.

If our working hypothesis that soybean SDS evolution involved four independent host jumps of New World pathogens onto the Old World host *Glycine max* after it was introduced to South America roughly a century ago is correct, then it raises the following important questions: What hosts did the SDS pathogens evolve on, and do they currently harbor sexually reproducing populations of all four species in South America? This host–pathogen system is unique within *Fusarium* because it is the only one known that has involved multiple host jumps of native pathogens onto an exotic host (Slippers et al. 2005; Desprez-Loustau et al. 2007). Given that our pathogenicity experiments indicate the soybean SDS pathogens can also induce significant foliar and rot disease symptoms on green bean and mung bean, we hypothesize that their native hosts are likely Neotropical legumes of little or no economic importance. Even so, surveys to identify their indigenous hosts are essential for understanding the evolution of these pathogens, and these studies may also have significant practical value because one or more of the SDS pathogens might be reproducing sexually in close proximity to soybean production fields in South America.

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