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ORIGINAL ARTICLE

Variability of *Fusarium* spp. isolates, causal agents of the soybean sudden death syndrome

Pablo R.P. de Melo Oliveira[a], Alexei Campos Dianese[b]*, Rodrigo Rocha Fragoso[b], André Freire Cruz[c] and Luiz Eduardo Bassay Blum[a]

[a]*Departamento de Fitopatologia, Universidade de Brasília, Campus Universitário Darcy Ribeiro, CEP 70910-900 Brasília, DF, Brazil;* [b]*Laboratório de Fitopatologia, Embrapa Cerrados, BR020, Km 18, CEP 73310-970 Planaltina, DF, Brazil;* [c]*Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Shimogamohangi-cho, Sakyo-ku, Kyoto 606-8522, Japan*

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Soybean (*Glycine max* L.) is the most widely cultivated crop in the world and an important commodity. Besides its main role in human nutrition, this grain is also used in animal feed and production of biofuels, among other purposes. Due to these factors, soybean became important in the global economy and is the most exported agricultural product from Brazil. Fungal diseases are among the limiting factors on soybean production; sudden death syndrome, caused by *Fusarium* spp., has been responsible for severe losses on this crop in Brazil. Four species of *Fusarium* can be considered causal pathogens: *F. brasiliense*, *F. crassistipitatum*, *F. tucumaniae*, and *F. virguliforme*. The *Fusarium* spp. isolates characterized in this work were collected in different soybean-producing regions in Brazil. The genetic variability of these isolates was determined through the random amplified polymorphic DNA (RAPD) technique. Disease severity was evaluated on moderately resistant and susceptible soybean cultivars in greenhouse trials. RAPD analysis demonstrated a great genetic diversity among the isolates and a clear tendency to split into two main species groups, *F. tucumaniae* and *F. brasiliense*, both prevalent in Brazil. The disease severity experiments, in which soybean plants were artificially inoculated, have shown that all isolates caused significant damage to the seedling root system. In fact, the genetic diversity of isolates does not correlate with disease severity, and also does not correlate with geographic distribution.

Keywords: *F. brasiliense*; *F. crassistipitatum*; *F. tucumaniae*; *F. virguliforme*; SDS

Introduction

Soybean (*Glycine max* (L.) Merrill), which has the North and Central regions of China as its center of origin, is the most extensively grown oilseed worldwide (Wilcox 2004). In the 2012/2013 season, USA, Brazil, and Argentina were responsible for 80% of the total 268 million tons produced globally (United States Department of Agriculture [USDA] 2014).

Among the various fungal diseases affecting soybean in Brazil, sudden death syndrome (SDS), caused by *Fusarium brasiliense*, *Fusarium tucumaniae*, and *Fusarium crassistipitatum* (Aoki et al. 2005, 2012), has caused severe losses, ranging from 20% to 80% (Freitas et al. 2004). It was first observed in São Gotardo, state of Minas Gerais, in 1981/1982 and is currently disseminated in all soybean-growing regions of the country, including the Cerrados, in the Midwest (Yorinori et al. 1993; Yorinori 1994; Embrapa Soja 2006). These pathogens are soil-borne, infecting the host's root system and causing significant reduction in nodulation and root mass (Hartman et al. 1997). Foliar symptoms include intervenal clorosis, leaf necrosis, and premature defoliation (Farias Neto et al. 2006).

Due to the polygenic nature of SDS resistance, the environmental influence on symptom intensity, and the absence of efficient chemical and cultural control methods, SDS became, undoubtedly, a major concern for soybean growers, technicians, and researchers (Fronza 2003). In 2013, Farias Neto et al. reported that soybean genotypes with moderate levels of

*Corresponding author. Email: alexei.dianese@embrapa.br

resistance were observed on field trials located in four different rural properties around Brazil's Federal District. Yet, these genotypes need to be tested in other growing regions.

Li et al. (2009) evaluated 123 isolates of *Fusarium virguliforme*, a species that causes SDS but does not occur in Brazil, in terms of aggressiveness/severity and found a wide range of variability. Scandiani et al. (2011) reported that *F. virguliforme* isolates were more aggressive than *F. tucumaniae*, when using two different soil infestation methods in greenhouse trials. There is no information about the SDS pathogens' population variability, and in terms of disease severity/aggressiveness in Brazil.

Random amplified polymorphic DNA (RAPD) based on polymerase chain reaction (PCR) is a very effective technique for genetic variability studies (Welsh & McClelland 1990; Williams et al. 1990). It involves amplification of random regions spread through the genome, using a series of short sequence primers that anneal to various sites in the genomic DNA (Fungaro 2000). A great number of fragment patterns can be created, generating a large amount of information about the genetic diversity among the individuals participating on the study (Ferreira & Grattapaglia 1996). Many studies have been carried out with RAPD in the genus *Fusarium* involving variability analysis, genetic mapping, isolate differentiation, population studies, among others (Bentley et al. 1995).

The present study aimed to evaluate the severity of *Fusarium* spp. isolates that cause SDS, collected from different soybean-growing regions in Brazil, and to analyze their genetic variability through RAPD.

Materials and methods

Pathogenicity test

Sixty isolates collected in various soybean-producing regions, from typical SDS root lesions, were morphologically characterized, according to the criteria described by Aoki et al. (2005), and kept in test tubes with potato dextrose agar (PDA) medium. Afterwards, the physiological variability of these *Fusarium* spp. isolates was evaluated in greenhouse trials (Table 1).

Three seeds of soybean cultivar Tuiuiú, susceptible to SDS, were sown into 140 ml plastic tubets containing autoclaved soil. After emergence, two seedlings were left per tubet. A total of 60 tubets were transferred to a greenhouse and irrigated daily to maintain soil moisture near field capacity level.

The inoculum was prepared according to the methodology described by Costa (1997). Petri dishes (90 mm diameter), containing six toothpicks of 15 mm in length, were sterilized. Then, PDA medium, with 20 mg/l of streptomycin, was poured into the plates leaving 3 mm of the toothpicks above the surface of the medium (as the medium hardened, the toothpicks were held in place using sterile tweezers). Afterwards, samples of the 60 *Fusarium* spp. isolates were taken from the test tubes, individually placed on the Petri dishes and incubated in a growth chamber for 13 days at 25°C (±2°C), 12-h photoperiod. Soybean plants were inoculated at the V2 stage (two sets of unfolded trifoliolate leaves) by introducing the *Fusarium* colonized toothpicks in the plant hypocotyl, between the soil and the cotyledons.

Fragments of seedling stems infected with the *Fusarium* isolates were collected 5 days after inoculation and taken to the plant pathology laboratory, where they were put in a solution containing 70% alcohol and 2% hypochlorite for 3 min. Later, these fragments were transferred to culture medium (PDA + 20 mg/l of streptomycin) for pathogen development. After 10 days of incubation, at 25°C (± 2°C), 12-h photoperiod, a new isolation was done with mycelial disks being transferred to new Petri dishes for 10 more days. This working collection was then transferred to tubes with the same selective medium.

Inoculum preparation

Samples of the 60 isolates were transferred to Petri dishes with selective medium (PDA + streptomycin) and incubated at 25°C, with 12-h photoperiod, for 7 days.

Sorghum seeds were used as carriers. About 2 kg of seeds were immersed in water for 24 h. Afterwards, 60 Erlenmeyer flasks of 100 ml received 30 g of sorghum seed and were sterilized for 25 min (121°C) on the day prior to inoculation. Finally, three disks of culture medium from each isolate were added to each flask in a sterile flow chamber. The flasks were placed in a growth chamber at 25°C (12-h photoperiod) during 7 days for fungal development.

After this period, the spore concentration in each flask was measured using a Neubauer chamber. In average, there were 7.38×10^5 conidia per 3 g of sorghum seed.

Greenhouse trial

Plastic tubets were filled with 100 ml of autoclaved soil; then, 3 g of sorghum seed inoculated with a *Fusarium* spp. isolate was deposited on the soil and covered with 20 ml of the same soil. Subsequently, three soybean seeds were sown in each tubet and also covered with 20 ml of soil (Figure 1). There were four tubets for each isolate. Two types of control, tubets with non-inoculated sterilized sorghum seeds and tubets with sterilized soil alone, were used. After

Table 1. Comparison of percentage DWL in soybean roots inoculated with *Fusarium* spp. isolates that cause sudden death syndrome (SDS), under greenhouse conditions (Embrapa Cerrados – Planaltina, DF, Brazil).

IS	DWL %	Location[a]	E[b]	IS	DWL %	Location[a]	E[b]
47	86.90 g	Passo Fundo/RS	*Fb*	54	66.60 b-g	Passo Fundo/RS	*Fb*
23	83.19 fg	Rio Verde/GO	*Ft*	43	66.54 b-g	São Gotardo/MG	*Fb*
37	82.97 fg	Passo Fundo/RS	*Ft*	19	66.54 b-g	São João D'Aliança/GO	*Ft*
53	82.79 fg	Luziânia/GO	*Fb*	17	66.14 b-g	Luziânia/GO	*Ft*
40	82.59 fg	Brasília/DF	*Fb*	33	65.82 b-g	Tupirama/TO	*F*[5]
11	82.05 fg	Ituverava/SP	*Fb*	38	65.22 b-g	Passo Fundo/RS	*Ft*
57	80.82 e-g	Cristalina/GO	*Fb*	9	65.17 b-g	Floresta/PR	*Fb*
21	80.69 d-g	Passo Fundo/RS	*Ft*	35	64.33 b-g	Formosa/GO	*Fb*
60	77.54 c-g	Cristalina/GO	*Fb*	15	64.20 b-g	Luziânia/GO	*Ft*
13	76.70 c-g	Cristalina/GO	*Ft*	30	64.18 b-g	Silvânia/GO	*Ft*
59	76.37 b-g	Cristalina/GO	*Fb*	34	63.57 b-g	Pedro Afonso/TO	*Fb*
7	76.10 b-g	Campo Mourão/PR	*Fb*	32	63.53 b-g	Silvânia/GO	*Ft*
20	74.62 b-g	Rio Verde/GO	*Ft*	29	63.44 b-g	Silvânia/GO	*Ft*
31	74.42 b-g	Silvânia/GO	*Ft*	56	62.12 b-g	Londrina/PR	*Fb*
4	73.58 b-g	Campo Mourão/PR	*Fb*	22	61.77 b-g	Rio Verde/GO	*Ft*
50	72.69 b-g	Formosa/GO	*Fb*	14	60.24 b-g	Cristalina/GO	*Fb*
2	71.82 b-g	Passo Fundo/RS	*Ft*	42	60.19 b-g	Brasília/DF	*Fb*
52	71.72 b-g	Ponta Grossa/PR	*Fb*	36	59.00 b-g	Formosa/GO	*Fb*
48	71.62 b-g	Chapadão do Sul/MS	*Fb*	6	58.54 b-g	Ponte Nova/MG	*Ft*
8	70.54 b-g	Brasília/DF	*Fb*	51	57.98 b-g	Ponta Grossa/PR	*Fb*
44	70.33 b-g	Planaltina/DF	*Ft*	41	57.39 b-g	Brasília/DF	*Fb*
5	70.22 b-g	Campo Mourão/PR	*Fb*	10	56.28 b-g	São Joaquim da Barra/SP	*Fb*
25	69.93 b-g	São João D'Aliança/GO	*Fb*	26	56.13 b-g	PAD/DF	*Fb*
49	69.31 b-g	Ponta Grossa/PR	*Fb*	18	54.46 b-g	Luziânia/GO	*Ft*
16	69.27 b-g	Luziânia/GO	*Ft*	28	53.08 b-f	Rio Verde/GO	*Ft*
55	69.25 b-g	Ponta Grossa/PR	*Fb*	58	50.74 b-e	Chapadão do Sul/MS	*Fb*
45	69.18 b-g	Planaltina/DF	*Ft*	27	50.72 b-d	Luziânia/GO	*Fb*
1	68.78 b-g	Chapadão do Sul/MS	*Fb*	3	48.76 bc	São Gotardo/MG	*Fb*
12	68.46 b-g	Cristalina/GO	*Ft*	46	46.42 b	Passo Fundo/RS	*F*
39	67.40 b-g	Planaltina/DF	*Fb*	C1	00.00 a		
24	67.08 b-g	Planaltina/DF	*Ft*	LSD[c]	30.08[c]		

Note: IS, isolates; DWL, percentage of dry weight loss; *Fb*, *Fusarium brasiliense*; *Ft*, *Fusarium tucumaniae*; *F*, *Fusarium* spp.
[a]City/state (RS, Rio Grande do Sul; GO, Goiás; DF, Distrito Federal; SP, São Paulo; PR, Paraná; MS, Mato Grosso do Sul; MG, Minas Gerais; TO, Tocantins) in Brazil where isolate was originally collected.
[b]Taxonomy of the species according to the morphological criteria of Aoki et al. (2005).
[c]Least significant difference by Tukey's test ($p \leq 5\%$).

germination, seedlings were thinned and only two plants per tubet were left.

Three soybean cultivars adapted to the Cerrado environmental conditions were used in the experiment: Milena (moderately resistant), Tuiuiú (susceptible), and Raimunda (susceptible). The resistance pattern was reported by Farias-Neto et al. (2013), according to field assays where the SDS severity assessment was based on foliar symptoms. Three experiments were conducted: Two with Milena and Tuiuiú, and one with Milena and Raimunda cultivars.

The experiment had a completely randomized block design and consisted of four replications per isolate and cultivar, each replicate with a tubet containing two plants.

Parameters evaluated and statistical analysis

Disease severity was determined 30 days after planting, by measuring the root dry weight. The roots were washed until all soil was removed and the above soil parts were cut off. Afterwards, the roots were placed in paper bags, dried for 9 days (60°C), and weighed.

Analysis of variance and mean values of percentage root dry weight loss (DWL) per isolate were calculated using Tukey's test (p = 5%) with the statistical software SigmaStat 2.0 from Jandel Corporation (1995).

Biological material and DNA extraction

Fifty-six SDS isolates collected from different soybean-producing regions of the country were used (Table 1). The cultivation was carried out as previously described. A *Fusarium solani* isolate taken from a different host (Embrapa Genetic Resources and Biotechnology) was used as a control.

The DNA extraction followed the cetyltrimethylammonium bromide method (CTAB) for fungus (Zolan & Pukkilla 1986). Sixty milligrams of

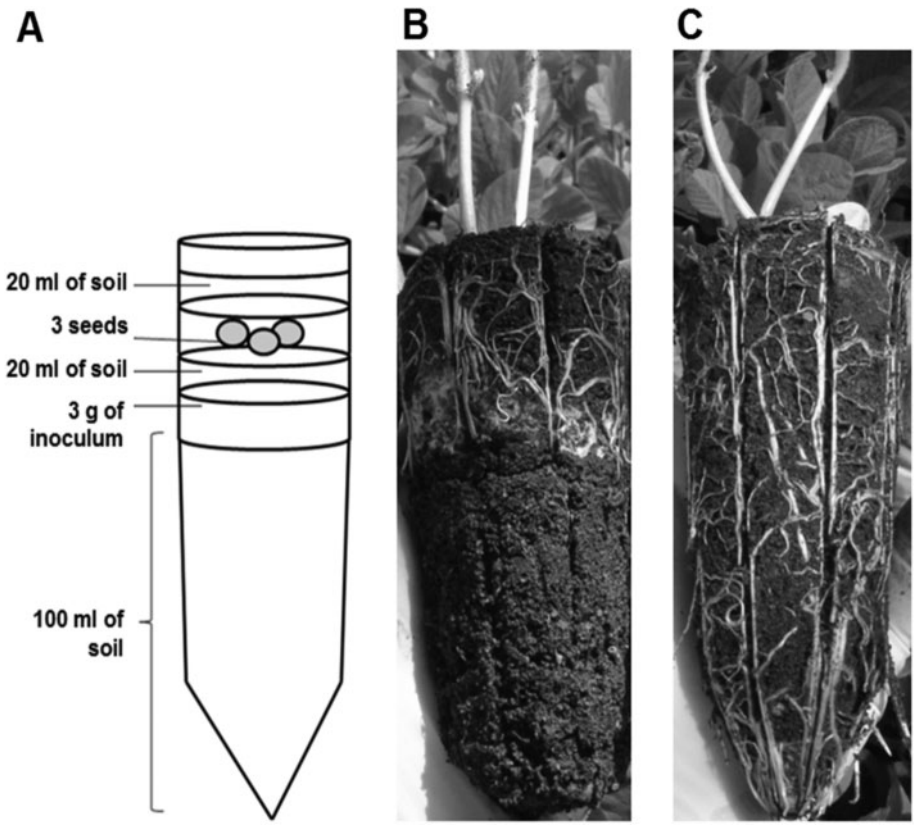

Figure 1. Representation of the inoculation method used to evaluate *Fusarium* spp. severity (A); root system of soybean seedlings inoculated with a *Fusarium* spp isolate (B); and non-inoculated (C).

mycelium per isolate were mixed to 450 µl of CTAB buffer. Afterwards, the protocol was followed as reported by Zolan and Pukkilla (1986). Finally, the pellets were dried and re-suspended in 100 µl of milli-Q water containing RNase at a final concentration of 40 µg/ml. Later, the samples were incubated at 37°C for 30 min, and the DNA was quantified using a spectrophotometer.

Amplification of DNA-RAPD and data analysis

PCR was done in a final volume of 13 µl, containing 12–15 ng of DNA as template, 1 µM of random primer, 1 U of DNA Taq Pol, 3 mM of $MgCl_{2,}$, 100 µM of dNTP, and 1X Buffer (Invitrogen). Thermal cycler (Model PTC-100 MJ Research, Inc.) was used with the following conditions: 94°C for 2 min, and then 40 cycles of 94°C for 15 s, 35°C for 30 s and 72°C for 90 s, with a final extension of 72°C for 6 min. The following universal primers from Invitrogen were used: OPD 7, OPD 4, OPB 9, OPB 7, OPB 17, OPF 14, OPE 20, OPE 11, OPD 8, OPF12, OPG 17, OPG 5, OPB 6, OPA 3, and OPR 8 (Silva et al. 2000). After the amplification, PCR products were analyzed by electrophoresis on 1% agarose gel in 1X TAE buffer. The gel was stained with ethidium bromide (0.5 µg/ml) for visualization of the fragments on a UV transilluminator and then photographed.

BioNumerics software was used to analyze the RAPD products. The primary function was to analyze a large number of information sets, which selected the data based on similarities and differences, summarizing and grouping the results to a conclusive manner.

The data were inserted as binary variables, i.e., the number 1 means the presence of fragment and 0 the absence. Thus, this program built a matrix, using the Jaccard similarity coefficient, which compares the number common fragments and total fragments involved, excluding the number of joint absences (a / a + b + c, where a means the quantity of positive agreed marks, b and c the amount of conflicted marks). These similarity matrixes were used to construct the dendrograms by unweighted pair group method using arithmetic average (UPGMA), which established genetic relationships among different isolates.

The genetic distance matrix was used to analyze the graphical dispersion in multidimensional scale using the method of principal coordinates with the software SAS and Statistica. The matching between the distance matrixes and the dendrogram was estimated with the co-phonetic correlation coefficient (r), according to Sokal and Rohlf (1962), using the computer program NTSYS pc 2.1 (Rohlf 2000).

Results and discussion

Severity of Fusarium *spp. isolates on soybean*

All experiments showed a significant difference between the 60 isolates and the non-inoculated controls, with the percentage of root DWL averaging 67.5% (Table 1). However, severity was not significantly different among the three cultivars used; consequently, all experiments were analyzed together, taking only into account the average percentage DWL.

Isolates 47, 23, 37, 53, 40, and 11 had the highest severity ratings, with losses ranging from 82.05% to 86.9% (Table 1). The isolates with the lowest DWL percentage, between 46.4% and 50.7%, and which differentiated significantly from the previous six isolates, were 58, 27, 3, and 46 (Table 1).

Scandiani et al. (2011), in a greenhouse experiment where the soil was also inoculated with infested sorghum seed, reported that *F. virguliforme* isolates were more aggressive than a group belonging to *F. tucumaniae*. In the present study, although significant differences were detected among the isolates, there was no interspecific separation. Therefore, isolates from *F. tucumaniae* and *F. brasiliense*, the two distinct species prevalent in Brazil, were present in both high and low severity groups (Table 1) (Aoki et al. 2005).

Also, there was no correlation between the location where the isolates were collected and level of severity (Table 1). Among the six isolates with the highest percentage root loss, two were collected in the state of Rio Grande do Sul (RS), in the southernmost region of Brazil, three were collected in states located in the Midwest (Goiás and the Federal District), and one in the state of São Paulo, which is located in the southeastern region of Brazil (Table 1). These results are similar to the ones obtained by Li et al. (2009), which reported that there was no association between the geographic origins of the isolates and their respective measures of severity. Moreover, the greenhouse results were distinct to those found by Farias-Neto et al. (2013), which reported that Cv. Milena was moderately resistant to SDS, based on evaluation of foliar symptoms. Foliar symptoms were rare in the greenhouse experiments, probably because of the inoculation method, resulting in greater and faster deterioration of the root system. Moreover, foliar symptoms in the field tend to appear late, when the soybean plants reach the R4 (full pod)/R5 (beginning of seed formation) stages (Almeida et al. 2005). In the present study, the greenhouse experiments had duration of only 30 days. Njiti et al. (1997) have stated that foliar symptoms are not a very efficient factor to evaluate soybean resistance to SDS, because, often, they are absent in infected plants observed at field level.

Genetic variability of Fusarium *spp. isolates causing SDS on soybean*

O'Donnell and Gray (1995) sequenced a region of approximately 600 bp amplified with the ITS5 and NL4 primers, and found for the first time that isolates of *F. solani* that caused SDS on soybean composed a genetically distinct group within this species. Achenbach et al. (1996) and Li et al. (2000), using RAPD markers, indicated that isolates causing SDS on soybean were genetically distinguished from *F. solani* f. sp. *phaseoli* that caused SDS on common bean, and could even form another *forma specialis*. Arruda et al. (2005) confirmed that SDS isolates were grouped in a different cluster from other *F. solani*, which were not related to the disease.

The present study aimed to better understand the genetic diversity of the soybean SDS pathogens occurring in Brazil using the RAPD technique. The polymorphism analysis of isolates was performed using 12 oligonucleotides of arbitrary sequence. The 12 selected primers (OPD 7, OPB 9, OPE 20, OPE 11, OPD 8, OPF12, OPG 17, OPG 5, OPB 6, OPA 3, and OPR 8) were analyzed and generated 1423 polymorphic fragments. The fragments amplified using the 12 primers ranged from 0.1 to 2.5 kb.

The analysis of fragment patterns generated by each primer led to the construction of a similarity matrix according to Jaccard's coefficient. The dendrogram was constructed using the UPGMA methodology (Figure 2), showing different levels of similarity (49.0–90.5%).

The circle in Figure 2 points out the separation of the two major clades of *F. brasiliense* and *F. tucumaniae* at 59% similarity. Five isolates morphologically characterized as *F. tucumaniae* appeared in a cluster with 29 *F. brasiliense* isolates (solid arrows in Figure 2). On a second cluster, four isolates previously characterized as *F. brasiliense* grouped together with 16 *F. tucumaniae* isolates (dotted arrows in Figure 2). Dubey and Suresh (2006) also observed differences between morphological characteristics and phylogenetic grouping among *Trichoderma* species when using RAPD. Corpas-Hervias et al. (2006) grouped three *Fusarium* species, which cause fusarium wilt of asparagus, in two genetically distinct clusters using RAPD. However, three isolates morphologically characterized as *F. solani* failed to be grouped in a single cluster, probably because of high intraspecific genetic diversity that was not identified with the RAPD primers used.

It was previously reported that *F. tucumaniae* presented high levels of genetic variability (Aoki et al.

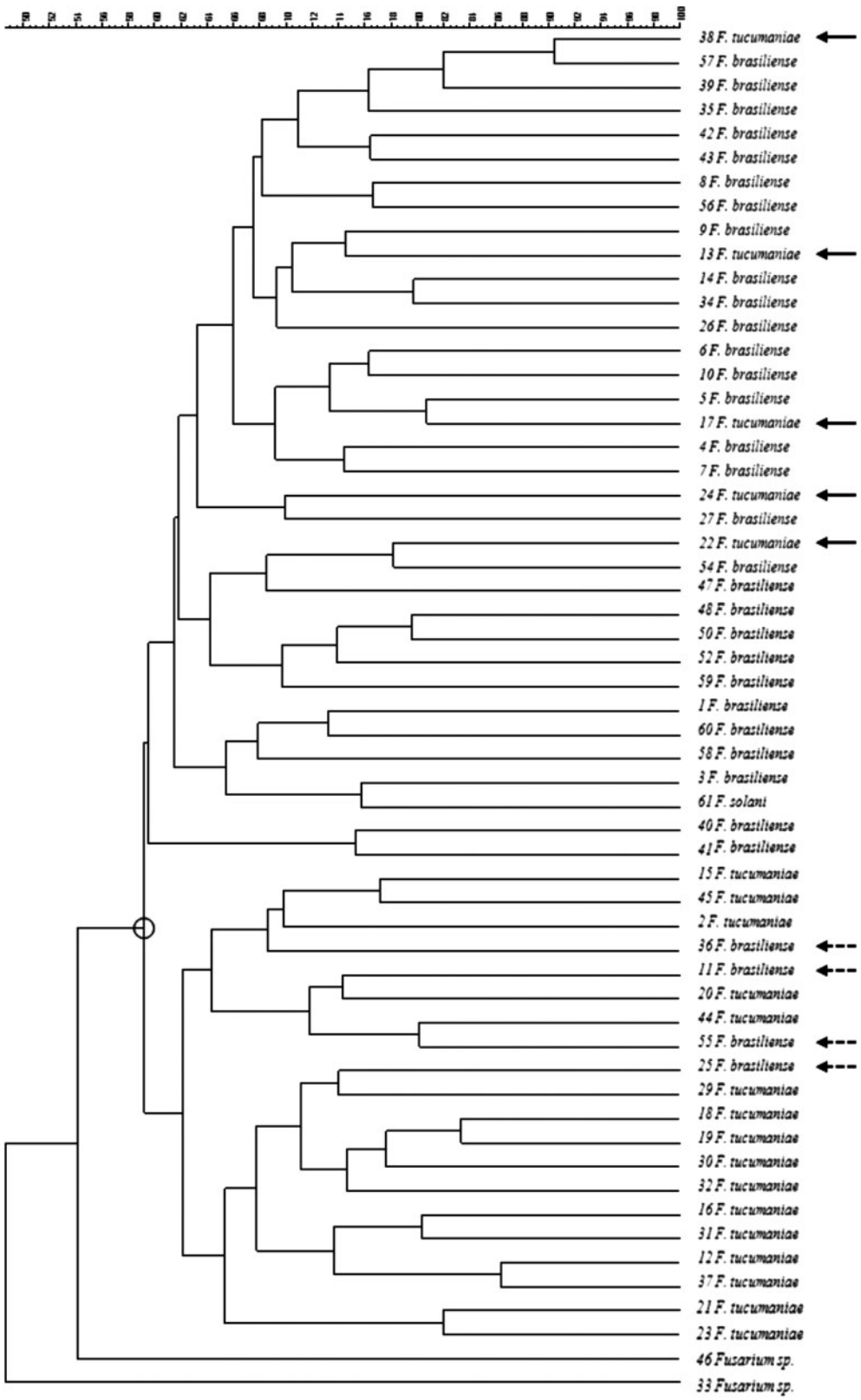

Figure 2. Dendrogram grouping of 57 *Fusarium* spp. isolates based on estimation of genetic distance using 1423 RAPD molecular markers. The UPGMA method was used as grouping criteria. The upper bar corresponds to the similarity percentage. The arrows point out differences between morpho-taxonomical characteristics and RAPD results.

2005), suggesting sexual recombination in the field (Covert et al. 2007), while some SDS species, like *F. virguliforme*, seem to be formed by a highly clonal population (O'Donnell et al. 2010). According to our results, *F. brasiliense* isolates showed genetic diversity as high as the one found in *F. tucumaniae* (Figure 2).

Considering the role of soybean in Brazilian economy and the losses due to SDS, these results present relevant information that will help to understand the population structure and genetic variability in the SDS–soybean interaction along the different soybean-growing regions of Brazil. This research will subsidize better integrated pest management (IPM) strategies for the control of this disease. Moreover, this is important background information for future breeding programs targeting resistance to SDS.

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