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SHORT COMMUNICATION / *COMUNICAÇÃO*

First report of *Diaporthe phaseolorum* var. *caulivora* infecting soybean plants in Brazil

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ABSTRACT

In March 2006, diseased soybean plants (*Glycine max*) were found in the municipalities of Passo Fundo and Coxilha, state of Rio Grande do Sul, Brazil, showing withered, brown leaves and light to reddish-brown discoloration and necrosis (canker) of the lower half of the stem. Cultural characteristics in potato dextrose agar showed identical patterns to those described for *Diaporthe phaseolorum* var. *caulivora* (*Dpc*). The rDNA-ITS sequence data from the Brazilian strain CH 40/06 (GenBank accession number EU622854) were compared to homologous sequences from the NCBI GenBank database, and a high similarity with *Dpc* strains was verified. A phylogenetic reconstruction was performed to compare EU622854 with other related sequences and grouped with *Dpc*. This group was highly supported by the bootstrap test (99%). Morphology of the Brazilian specimen was also similar to that described in the literature for *Dpc*, but the ascospores were slightly narrower than the American original material of *Dpc*. In field surveys performed in the 2006/07 crop season, *Dpc* was also found in five additional municipalities in RS.
Keywords: stem canker, *Phomopsis phaseoli* f. sp. *caulivora, Glycine max.*

RESUMO

Primeiro relato de *Diaporthe phaseolorum* var. *caulivora* infectando plantas de soja no Brasil

Em março de 2006, plantas de soja (*Glycine max*) doentes foram encontradas em Passo Fundo e em Coxilha, Rio Grande do Sul, Brasil. O quadro sintomatológico caracterizava-se por plantas com folhas secas e de coloração marrom; necrose (cancro) na metade inferior da haste, de coloração marrom-clara a marrom-avermelhada, envolvendo a mesma, seguindo-se murcha e morte da planta. Características culturais em batata dextrose ágar mostraram padrões idênticos àqueles publicados para *Diaporthe phaseolorum* var. *caulivora* (*Dpc*). Os dados de seqüenciamento de DNA do isolado brasileiro CH 40/06 (número de acesso GenBank EU622854) foram comparados às seqüências homólogas de NCBI GenBank, e alta similaridade foi verificada com outros isolados de *Dpc*. Árvore filogenética foi elaborada a fim de comparar EU622854 com outras seqüências de *Dpc*, cujo agrupamento foi altamente confirmado pelo teste bootstrap (99%). Características morfológicas foram similares às descritas para *Dpc* na literatura, embora os ascosporos do espécime brasileiro fossem mais estreitos que o original americano. Na safra 2006/07, *Dpc* foi também encontrado em outros cinco municípios do RS.
Palavras-chave: cancro da haste, *Phomopsis phaseoli* f. sp *caulivora, Glycine max.*

Soybean (*Glycine max*) is affected worldwide by the *Diaporthe/Phomopsis* complex, which is composed of three varieties of *Diaporthe phaseolorum*, anamorph *Phomopsis phaseoli: D. phaseolorum* var. *meridionalis* F.A. Fernández, and *D. phaseolorum* var. *caulivora* Athow and Caldwell, both causal agents of soybean stem canker, and *D. phaseolorum* var. *sojae* (Lehman) Wehm., causal agent of pod and stem blight; and by *Phomopsis longicolla* Hobbs (teleomorph unknown), primary agent of seed decay (Pioli *et al*., 2003).

Soybean stem canker, caused by *D. phaseolorum* var. *meridionalis* (*Dpm*) was found for the first time in Brazil in 1989 and, in the next crop season, it was observed in almost all the Brazilian soybean-producing areas, leading to yield losses approaching 100% in some fields (Yorinori *et al*., 1989; Yorinori, 1996). The use of resistant soybean cultivars, released seven to eight years after the first detection of the disease, led to an effective control of this disease in Brazil. Nowadays, every soybean cultivar has to be tested for resistance to soybean canker caused by *Dpm* in order to be registered and released for cultivation by the Serviço Nacional de Proteção de Cultivares (National Service to Protect Cultivars), a government agency within the Brazilian Ministry for Agriculture, Livestock and Food Supply – MAPA.

In 1999, *Diaporthe phaseolorum* var. *caulivora* (*Dpc*) was found for the first time in South America occurring in the main soybean-producing region of Argentina (Pioli *et*

al., 2001). More recently (March 2006) diseased soybean plants with symptoms of stem canker were found in Passo Fundo and Coxilha municipalities, state of Rio Grande do Sul (RS), Brazil, in cultivars previously known as resistant to *Dpm* canker. The symptoms were withered brown leaves associated with light to reddish-brown discoloration and necrosis (canker) of the lower half of the stem, especially around the nodes (Figures 1-3).

Portions of the discolored cortical stem tissue were plated in potato dextrose agar (PDA) amended with streptomicine sulphate, and a *Phomopsis*-like white-grey to brownish colony developed. A strain isolated from cultivar BRS 154 from Passo Fundo RS was deposited in the Coleção Micológica de Referência (Mycological Reference Collection) at the University of Brasília, accession number UB 20868 (CH 40/06 Embrapa Soy culture collection), and chosen for further molecular, pathogenic and morphological studies.

For DNA extraction, a pellet containing mycelium obtained by single-ascospore culturing in PDA was crushed on liquid nitrogen and treated with CTAB extraction buffer (50mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 2% hexadecyltrimethyl-ammonium bromide-CTAB), followed by phenol/chloroform purification, precipitated with ethanol and stored at –80ºC (Almeida *et al*., 2003). DNA concentration was determined through spectrophotometer analysis and the DNA sample was stored at –20ºC. The nuclear rDNA region, including internal transcribed spacer 1 (ITS-1), 5.8S rRNA gene, and internal transcribed spacer 2 (ITS-2), was amplified with the primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.,* 1990) in an Eppendorf Mastercycler gradient thermocycler. Amplification reactions were performed in 50 µL-volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 0.5 µM each primer (ITS5 and ITS4), 10 ng of genomic DNA and 2.5 U Taq DNA polymerase. Temperature and time parameters for PCR amplification were 94ºC for 3 min for the first cycle and 1 min for the remaining cycles, 55ºC for 1 min, and 72ºC for 2 min for 35 cycles, followed by a final extension at 72°C for 7 min. Amplified products were analyzed by electrophoresis in 1.3% agarose gel and visualized after staining with ethidium bromide. It was observed that the band patterns were identical to those produced by *Dpc* isolates.

The band produced by CH 40/06 was excised from the gel and the amplicon was purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen®). Sequencing was performed by the chain-termination method using the ABI Big Dye Terminator Cycle sequencing kit v. 2.0 (Applied Biosystems®) on an ABI PRISM model 3100 DNA sequencer. The DNA sequence data of ITS-1, 5.8S ribosomal RNA gene, and ITS-2, from the strain CH 40/06 were deposited in GenBank (accession number EU622854). The sequence EU622854 was compared to homologous sequences from the NCBI GenBank database using BLAST (Altschul *et al.*, 1997), and a high similarity (99-100%) was verified with *Dpc* strains.

A phylogram was constructed in order to compare the ITS sequences of CH 40/06 strain with other homologous sequences from NCBI GenBank database (Fig. 4). A multiple sequence alignment was performed using Clustal W (Thompson *et al.,* 1994) and a neighbor-joining phylogenetic tree was constructed from Kimura 2-parameter pairwise distances using MEGA 4 (Tamura *et al.,* 2007). The consistency of phylogenetic resolution was supported by a bootstrap analysis using 1,000 replicates. Indels and gaps were treated as missing data.

Biometric characteristics were measured for the strain CH 40/06, cultivated in PDA medium at 25ºC, in the dark. The colony growth was fast (90 mm diameter in five days), and semi-immersed black stromata (1.0–3.0 mm diameter) developed around 20 days later. Black, globose perithecia arranged in caespitose groups were formed after 40 days in stromata and were 210–320 x 290–410 µm at the base. Perithecial beaks were 280–320 µm long, 80–110 µm wide at the base and 50–60 µm wide at the apice (Fig. 5). The eight-spored asci (5–6 x 32–40 µm) were elongated clavate with thin, evanescent walls. Ascospores (2–2.5 x 8–10 µm) were hyaline, ellipsoidal, two-celled, biguttulate at each cell (Fig. 6). The morphology of the Brazilian strain was equivalent to that reported by Athow & Caldwell (1954) for *Dpc*, with a slight difference in ascospore width, which is narrower in the Brazilian material. This difference was also reported by Pioli *et al*. (2001) for the Argentinian strain of *Dpc* (ascospores: 2.5–2.7 x 7.7–8.9 µm).

In Petri dishes, strain CH 40/06 was paired on PDA with a strain for *Dpm*, CH8 (GenBank accession number FJ357154), which has been used since 1989 by Embrapa Soy as the reference strain for testing soybean genotypes for resistance to stem canker. After five days, barrage reactions were evident as black lines along the zone of mycelial contact between expanding colonies (vegetative incompatibility reaction, according to Smit *et al*., 1997), but not between the two CH 40/06 colonies (Fig. 7).

Pathogenicity tests were performed in greenhouse conditions (temperatures around 25ºC) using the toothpick method described by Yorinori (1996), in soybean plants at V1 growth stage (completely unrolled leaf at the unifoliolate node, according to Fehr *et al*., 1971), with isolates CH8 and CH 40/06 and the soybean cultivars BR 23 (susceptible to *Dpm*) and BRS 154 (original source of the CH 40/06). Fifteen days later, it was observed that CH 40/06 did not cause disease symptoms in cv. BR 23, but it caused internal stem discoloration without leading to plant death of cv. BRS 154. Plant death was observed when BR 23 was inoculated with *Dpm*, but no symptoms resulted from inoculation of BRS 154, leading to the conclusion that CH 40/06 was different from *Dpm*. *Dpc* was reisolated on PDA from BRS 154 stem portions.

In this study, the combination of molecular tests and

FIG. 1 - Soybean field affected by stem canker caused by *Diaporthe phaseolorum* var. *caulivora*. Ciríaco, RS, 2008.

FIG. 2 - Soybean plant with stem canker caused by *D. phaseolorum* var. *caulivora*, showing brown discoloration in the stem (arrowed) and withered leaves above de canker. Passo Fundo, RS, 2008.

FIG. 3 - Soybean stem showing a canker caused by *D. phaseolorum* var. *caulivora*. Passo Fundo, RS, 2007.

FIG. 5 - Protruding beaks of *D. phaseolorum* var. *caulivora* perithecia on a stem of a naturally infected soybean plant (arrowed).

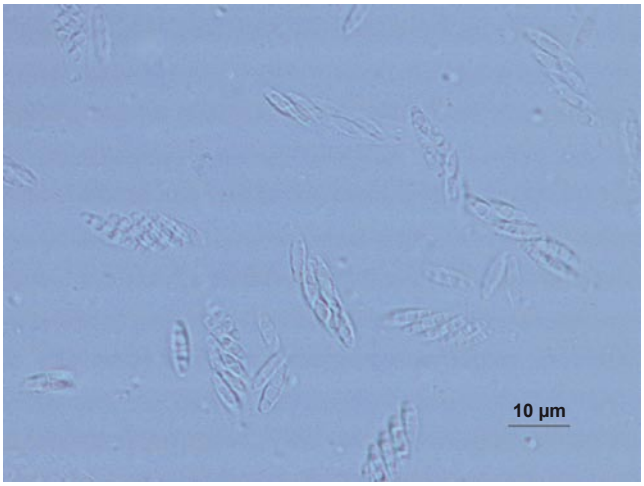

FIG. 6 - Asci and ascospores of *D. phaseolorum* var. *caulivora* from stems of soybean cv. BRS 154, RS, Brazil, 2006.

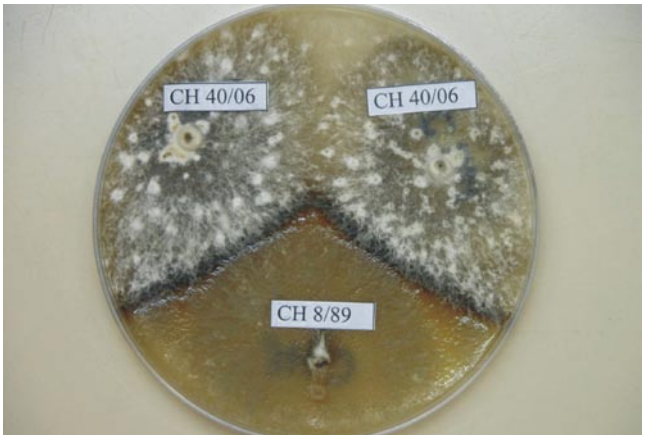

FIG. 7 - Determination of vegetative compatibility between colonies of *D. phaseolorum* var. *caulivora* (CH 40/06) and *D. phaseolorum* var. *meridionalis* (CH 8/89). Same strains merge along the line of contact; a black barrage reaction line is formed along the zone of contact of incompatible strains.

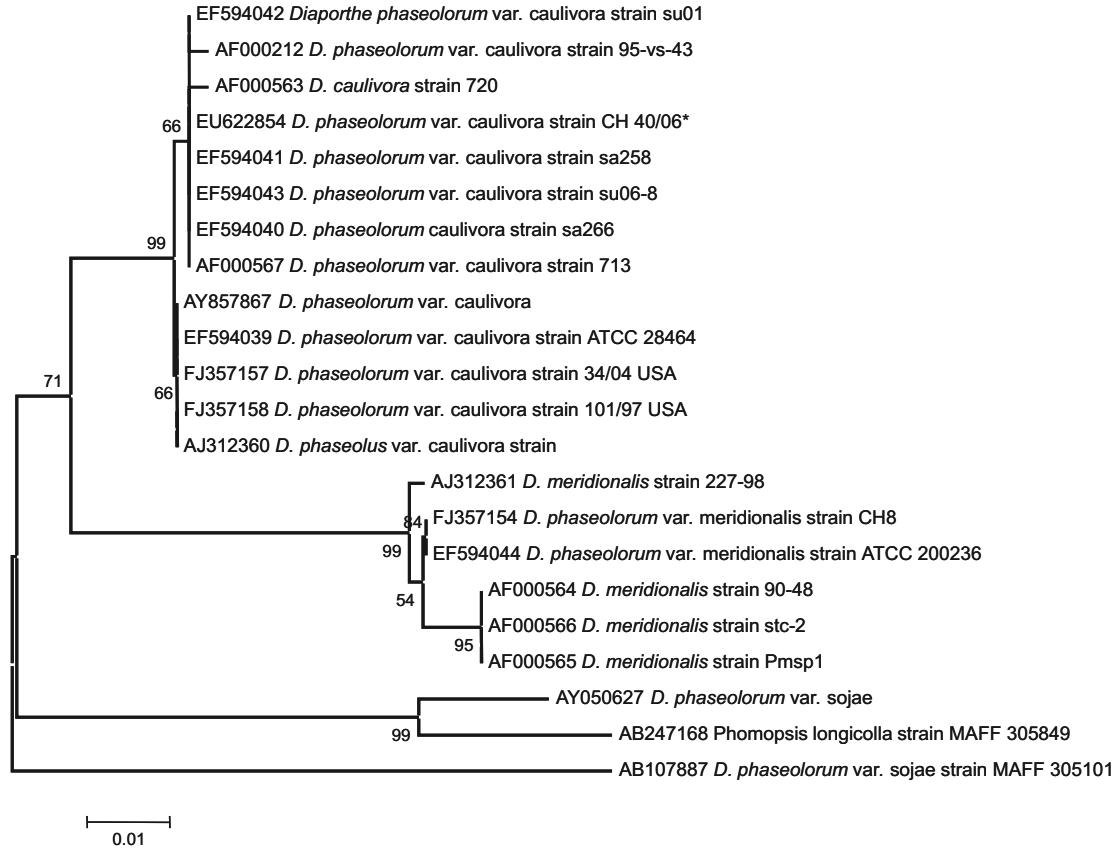

FIG. 4 - Unrooted phylogenetic tree based on rDNA ITS-1 and ITS-2 sequences showing the relationships among *D. phaseolorum* var. *caulivora* strains and other species. The distance between two strains is the sum of the branch lengths between them. Asterisk (*) indicates Brazilian *D. phaseolorum* var. *caulivora* strain from this study.

cultural aspects showed that isolate CH 40/06 clearly belongs to *Dpc*. According to Pioli *et al*. (2002), clustered perithecia, smaller asci and ascospores, and the development of fluffy mycelia with age are the main morphological characteristics that distinguish *Dpc* from *Dpm*. Nevertheless, molecular techniques allow for a more precise identification. Since the 1990's, molecular techniques have been used as a tool for identification and characterization of *D. phaseolorum* strains, additionally to morphologic characteristics and pathogenicity and vegetative compatibility tests (Fernández & Hanlin, 1996; Zhang *et al*., 1997; 1998; Pioli *et al*., 2001; 2002; 2003; Vechiato *et al*., 2003).

Some of the previous works on *D. phaseolorum* (Fernández & Hanlin, 1996; Pioli *et al*., 2001; 2003), have recorded asci and ascospore dimensions that were somewhat different from those given in Athow and Caldwell (1954). The same was observed for the Brazilian isolate (CH 40/06), which otherwise had the combination of characteristics typical for *Dpc*. The biometric differences between ascospores of the Brazilian *Dpc* strain and the original *Dpc*, described by Athow and Caldwell in 1954, were also observed by Pioli *et al*. (2003) on Argentinian records of *Dpc*. It also suggests that the probable origin of the Brazilian isolate is soybean seed material brought from Argentina.

Field surveys, performed by officials of the Department of Agriculture – MAPA in Rio Grande do Sul during the 2006/2007 crop season, yielded soybean plants bearing stem cankers. The fungus involved in the disease was isolated and identified by the phylogenetics described herein. The results evidenced that *Dpc* was present in five additional municipalities of Rio Grande do Sul: Marau, Pontão, Quatro Irmãos, Lagoa Vermelha, and Ibirubá.

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