*Tropical Plant Pathology*, vol. 37(2):142-145, 2012
Copyright by the Brazilian Phytopathological Society. Printed in Brazil
www.sbfito.com.br

**SHORT COMMUNICATION / *COMUNICAÇÃO***

# Detection of isometric, dsRNA-containing viral particles in *Colletotrichum gloeosporioides* isolated from cashew tree

**Lívio C. de Figueirêdo[1], Girlene S. de Figueirêdo[1], Ágata C.H. Giancoli[1], Francisco A.O. Tanaka[2], Leonor A.O. da Silva[1], Elliot W. Kitajima[2], Spartaco A. Filho[1] & João Lúcio Azevedo[2]**

[1]Universidade Federal do Amazonas, Multidisciplinary Support Center, Graduate Program in Biotechnology. Av. General Octávio Jordão Ramos 3000, Mini-Campus Universitário, 69077-000, Manaus, AM, Brasil; [2]Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Universidade de São Paulo, ESALQ, 13418-900, Piracicaba, SP, Brazil

Author for correspondence: Lívio C. de Figueirêdo, e-mail: liviocf@gmail.com

**ABSTRACT**

Fungi are disease-causing agents in plants and affect crops of economic importance. One control method is to induce resistance in the host by using biological control with hypovirulent phytopathogenic fungi. Here, we report the detection of a mycovirus in a strain of *Colletotrichum gloeosporioides* causing anthracnose of cashew tree. The strain *C. gloeosporioides* URM 4903 was isolated from a cashew tree (*Anacardium occidentale*) in Igarassu, PE, Brazil. After nucleic acid extraction and electrophoresis, the band corresponding to a possible double-stranded RNA (dsRNA) was purified by cellulose column chromatography. Nine extrachromosomal bands were obtained. Enzymatic digestion with DNAse I and Nuclease S1 had no effect on these bands, indicating their dsRNA nature. Transmission electron microscopic examination of extracts from this strain showed the presence of isometric particles (30–35 nm in diameter). These data strongly suggest the infection of this *C. gloeosporioides* strain by a dsRNA mycovirus. Once the hypovirulence of this strain is confirmed, the strain may be used for the biological control of cashew anthracnose.
**Key words:** *Anacardium occidentale*, anthracnose, biological control, hypovirulence, mycovirus, phytopathogenic fungi.

Fungi are the most important disease-causing agents in plants with serious economic impact. Chemical methods to control phytopathogenic fungi can affect human health and have environmental consequences. In an attempt to minimize dependence on chemical methods, alternative means of pathogen control have been investigated (Yu et al., 2010). One alternative is to use hypovirulent phytopathogenic fungal strains that can biologically control phytopathogenic fungi of the same or related species. Fungal hypovirulence is usually caused by mycoviral infection. Hypovirulent fungi cause milder infection and may induce hypovirulence in other pathogenic strains via viral transfer (Pearson et al., 2009).

All currently known mycoviruses have isometric, double-stranded RNA (dsRNA)-containing particles, and are classified into three families: *Chrysoviridae*, *Endornaviridae*, *Partitiviridae* and *Totiviridae*. Particle size ranges from 25 to 50 nm in diameter, except for endornaviruses which do not produce virions (Chu et al., 2002; Ghabrial & Suzuki, 2009).

In this communication, we report the presence of isometric dsRNA viral particles in a strain of *Colletotrichum gloeosporioides* (Penz.) Sacc., the causal agent of anthracnose in cashew tree.

*Colletotrichum gloeosporioides* URM 4903 was isolated from anthracnose lesions on a cashew tree (*Anacardium occidentale* L.) in the town of Igarassu, Pernambuco, Brazil (7°50’3.74”S and 34°54’22.87”O), and deposited in the mycology collection (URM) at Federal University of Pernambuco, Recife, PE. This strain was chosen after preliminary tests revealed poor pathogenicity in detached cashew leaves and the presence of extrachromosomal bands in agarose gel electrophoresis (unpublished data).

PDA (20% potato extract, 2% dextrose, 1.5% agar) and PD (20% potato extract, 2% dextrose) media were used to grow this *C. gloeosporioides* strain. The strain, when needed, was incubated at 28°C for 7 days before use.

Mycelia for nucleic acid extraction were obtained after inoculating PD medium with conidia of the *C. gloeosporioides* strain. After 3 days of continuous shaking, mycelia were filtered and ground to a fine powder with liquid nitrogen. Total nucleic acids were extracted as described by Raeder & Broda (1985) and analyzed by agarose gel electrophoresis at 3 $Vcm^{-1}$ and ethidium bromide staining (0.5 $\mu g\ mL^{-1}$).

Bands corresponding to the total fungal nucleic acids were obtained after agarose gel electrophoresis (Figure 1A). They were purified by cellulose column chromatography according to the method of Morris & Dodds (1979) with modifications. The cellulose column was mounted on a disposable 10 mL syringe, with 2.5 g of microgranular cellulose evenly distributed. The column was buffered with

successive washings with STE buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M NaEDTA, pH 7.0) and 15% ethanol. After sample application, the column was washed with STE-15% ethanol to remove DNA and single-stranded RNA. The putative dsRNA that remained in the column was eluted with STE without ethanol and precipitated by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol (-20°C) for approximately 12 h. The precipitate was collected by centrifugation at 10,000 *g* for 15 min. The supernatant was discarded, and the precipitate was washed with 70% ethanol, dried in a laminar flow cabinet and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The several stages of chromatography were visualized after agarose gel electrophoresis.

After chromatography, the purified material was digested with DNAse I and nuclease S1. Nuclease S1 (35 U/mg) digestion was performed at 37°C for 30 min. DNAse I (20 mg/mL in 30 mM $MgCl_2$) digestion was performed at 37°C for 20 min, followed by incubation at 65°C for 15 min to inactivate the enzyme. Digestion results were analyzed by 1% agarose gel electrophoresis as previously described.

We attempted to visualize the putative mycovirus in the *C. gloeosporioides* strain by examining negatively stained mycelial extracts by transmission electron microscopy. Hyphae and spores of the fungus grown in PDA medium were carefully removed with a sterile needle, transferred to microtubes containing small spherical glass beads, and homogenized in 0.2 mL 0.05 M cacodylate buffer (pH 7.2). A small drop of this material was placed on a parafilm sheet, and Formvar/carbon-coated 300 mesh grids were floated on it for 15 min. The grids were removed, and excess liquid was removed with a piece of filter paper. The grids were washed with drops of distilled water and floated on a droplet of 1% aqueous uranyl acetate for 5 min. After removal of excess liquid, the grids were dried and examined in a Zeiss EM 900 transmission electron microscope with digital image recording. Although in low concentrations, isometric particles (30-35 nm in diameter) were consistently found in the fungal extracts (Figure 1B). They were essentially similar to other mycoviruses in the *Chrysoviridae*, *Partitiviridae* and *Totiviridae* families, whose diameters range from 25 to 50 nm and the genomes are comprised of dsRNA molecules.

The presence of dsRNA in fungi can be demonstrated by several processes after total nucleic acid extraction and purification. The *C. gloeosporioides* strain isolated from cashew tree showed extrachromosomal bands after total nucleic acid electrophoresis (Figure 1A). After cellulose column chromatography we observed nine bands, all of which were resistant to DNAse I and Nuclease SI digestion, indicating that they consisted of dsRNA (Figure 1C).

In most cases, these dsRNAs are associated with hypovirulence of phytopathogenic and entomopathogenic fungi and thus may provide an alternative method for biological control (Anagnostakis and Day, 1979; Dalzoto et al., 2006). In some fungi, the presence of dsRNA is related

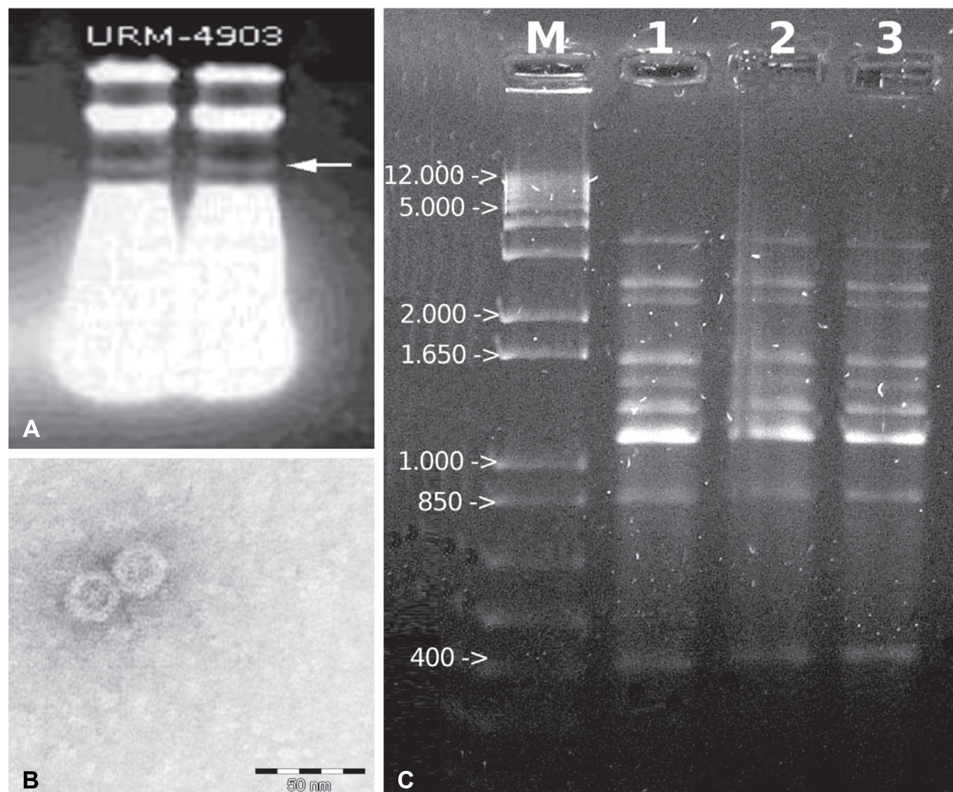


**FIGURE 1 - A.** Agarose gel electrophoresis profile of total nucleic acids extracted from *Colletotrichum gloeosporioides* URM 4903 isolated from cashew tree (*Anacardium occidentale*). The arrow indicates the dsRNA bands; **B.** Isometric particles, ca. 30-35 nm in diameter, in a negatively stained hyphal extract of strain URM 4903; **C.** Agarose gel electrophoresis profile of nucleic acids obtained by cellulose column chromatography and enzymatic digestion. M: 1 kb DNA ladder (in base pairs); Lanes: 1, untreated control sample; 2 and 3, samples digested by DNAse I and Nuclease S1, respectively.

to several phenotypic alterations (Day et al., 1977; Zhou & Boland, 1997, 1999; Liu et al., 2003a; Suzuki et al., 2003; Kwon et al., 2009). Viral infection reduces the levels of α and β subunits of protein G, changing the transduction signal of this protein in the host (Dawe et al., 2004). Therefore, transcription factors regulated by protein G are downregulated, including CpST12, which is necessary for fungal virulence and expression of genes that respond to hypovirulence (Deng et al., 2007). Hypovirulent strains of phytopathogenic fungi have been reported in several species such as *Fusarium oxysporum* (Kilic & Griffin, 1998), *Rhizoctonia solani* (Liu et al., 2003a; Cardinale et al., 2006) *Sclerotinia sclerotiorum* (Li et al., 2003), *Helicobasidium mompa* (Osaki et al., 2006), *Botrytis cinerea* (Wu et al., 2007), and *Fusarium graminearum* (Kwon et al., 2009). The best examples have been described in *Cryphonectria parasitica*, the causal agent of chestnut blight, a devastating disease of the American chestnut tree (*Castanea dentata* [Marsh] Borkh) (Anagnostakis & Day, 1979; Rae et al., 1989; Liu et al., 2003b; Lin et al., 2007; Turchetti et al., 2008).

To the best of our knowledge, dsRNA has been reported only once in *Colletotrichum lindemuthianum* (Rawlinston et al., 1975) and *C. gloeosporioides*, which cause different anthracnose diseases on *Stylosanthes* (Dale et al., 1988). This study is the first to report a possible dsRNA virus in *C. gloeosporioides* causing anthracnose of cashew tree. Once the hypovirulence of *C. gloeosporioides* URM 4903 has been proven, we may test its utility as an alternative biological control agent for cashew tree anthracnose.

## ACKNOWLEDGEMENTS

We thank the staff of the Microorganism Genetics Laboratory of the Federal University of Amazonas (UFAM), who allowed us to use their facilities and instruments. We also thank the Brazilian Research Council (CNPq) for supporting grants to Lívio C. de Figueirêdo and the Postgraduate Program in Biotechnology of UFAM for supporting this project.

## REFERENCES

Anagnostakis SL, Day PR (1979) Hypovirulence conversion *in Endothia parasitica*. Phytopathology 69:1226-1229.

Cardinale F, Ferraris L, Valentino D, Tamietti G (2006) Induction of systemic resistance by a hypovirulent *Rhizoctonia solani* isolate in tomato. Physiological and Molecular Plant Pathology 69:160-171.

Chu Y, Jeon J, Yea S, Kim Y, Yun S, Lee Y, Kim K (2002) Double-stranded RNA mycovirus from *Fusarium graminearum*. Applied and Environmental Microbiology 68:2529-2534.

Dale JL, Manners JM, Irwin JAG (1988) *Colletotrichum gloeosporioides* isolates causing different anthracnose diseases on Stylosanthes in Australia carry distinct double-stranded RNAs. Transactions of the British Mycological Society 91:671-676.

Dalzoto PR, Glienke-Blanco C, Kava-Cordeiro V, Ribeiro JZ, Kitajima EW, Azevedo JL (2006) Horizontal transfer and hypovirulence associated with double-stranded RNA in *Beauveria bassiana*. Mycological Research 11:1475-1481.

Day PR, Dodds JA, Elliston JE, Jaynes RA, Anagnostakis SL (1977) Double-stranded RNA in *Endothia parasitica*. Phytopathology 67:1393-1396.

Dawe AL, Segers GC, Allen TD, McMains CC, Nuss DL (2004) Microarray analysis of *Cryphonectria parasitica* Gα- and Gβγ-signalling pathways reveals extensive modulation by hypovirus infection. Microbiology 150: 4033-4043.

Deng F, Allen TA, Nuss DL (2007) Ste12 transcription factor homologue CpST12 is down-regulated by hypovirus infection and required for virulence and female fertility of the chestnut blight fungus *Cryphonectria parasitica*. Eukaryotic Cell 6:235-244.

Ghabrial SA, Suzuki N (2009) Virus of plant pathogenic fungi. Annual Review of Phytopathology 47:353-384.

Kilic O, Griffin G (1998) Effect of dsRNA-containing and dsRNA-free hypovirulent isolates of *Fusarium oxysporum* on severity of Fusarium seedling disease of soybean in naturally infested. Plant and Soil 201:125-135.

Kwon S-J, Cho S-Y, Lee K-M, Yu J, Son M, Kim K-H (2009) Proteomic analysis of fungal host factors differentially expressed by *Fusarium graminearum* infected with *Fusarium graminearum* virus-DK21. Virus Research 144:96-106.

Li GQ, Huang HC, Laroche A, Acharya SN (2003) Occurrence and characterization of hypovirulence in the tan sclerotial isolate S10 of *Sclerotinia sclerotiorum*. Mycological Research 107:1350-1360.

Lin H, Lan X, Liao H, Parsley TB, Nuss DL, Chen B (2007) Genome sequence, full-length infectious cDNA clone, and mapping of viral double-stranded RNA accumulation determinant of hypovirus CHV1-EP721. Journal of Virology 81:1813-1820.

Liu C, Lakshman DK, Tavantzis SM (2003a) Quinic acid induces hypovirulence and expression of a hypovirulence-associated double-stranded RNA in *Rhizoctonia solani*. Current Genetics 43:103-111.

Liu Y-C, Linder-Basso D, Hillman BI, Kaneko S, Milgroom MG (2003b) Evidence for interspecies transmission of viruses in natural populations of filamentous fungi in the genus *Cryphonectria*. Molecular Ecology 12:1619-1628.

Morris T, Dodds J (1979) Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69:854-858.

Osaki H, Nakamura H, Sasaki A, Matsumoto N (2006) An endornavirus from a hypovirulent strain of the violet root rot fungus, *Helicobasidium mompa*. Virus Research 118:143-149

Pearson MN, Beever RE, Boine B, Arthur K (2009) Mycovirus of filamentous fungi and their relevance to plant pathology. Molecular Plant Pathology 10:11.

Rae B, Hillman B, Tartaglia J, Nuss D (1989) Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. EMBO Journal 8:657-663.

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology 1:17-20.

Rawlinston CJ, Carpenter JM, Muthyalu G (1975) Double-stranded RNA virus in *Colletotrichum lindemuthianum*. Transactions of the British Mycological Society 65:305-330.

Suzuki N, Maruyama K, Moriyama M, Nuss DL (2003) Hypovirus papain-like protease p29 functions in trans to enhance viral double-stranded RNA accumulation and vertical transmission. Journal of Virology 77:11697-11707.

Turchetti T, Ferretti F, Maresi G (2008) Natural spread of *Cryphonectria parasítica* and persistence of hypovirulence in three italian coppiced chestnut stands. Forest Pathology 38:227-243.

Wu MD, Zhang L, Li GO, Jiang DH, Hou MS, Huang HC (2007) Hypovirulence and double-stranded RNA in *Botrytis cinerea*. Phytopathology 97:1590-1599.

Yu X, Li B, Fu Y, Jiang D, Ghabrial SA, Li G, Peng Y, Xie J, Cheng J, Huang J, Yi X (2010) A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. Proceedings of the National Academy of Sciences, USA 107:8387-8392.

Zhou T, Boland GJ (1997) Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa*. Phytopathology 87:147-156.

Zhou T, Boland GJ (1999) Mycelial growth and production of oxalic acid by virulent and hypovirulent isolates of *Sclerotinia sclerotiorum.* Canadian Journal of Plant Pathology 21:93-99.

*TPP 365 - Received 5 August 2011 - Accepted 18 March 2012*
*Section Editor: F. Murilo Zerbini*