Investigation note

Fusarium oxysporum, causal agent of stevia wilt in Veracruz, Mexico

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Abstract

The wilting of stevia plants (*Stevia rebaudiana* Bertoni) is one of the most important phytosanitary problems in the production area of Veracruz. The objective of this study was to identify the causal agent of wilt symptoms in stevia plants in a plantation located in Martinez of the Torre, in the state of Veracruz, Mexico. In 2015, Stevia plants were observed and collected with symptoms of root rot, basal stem necrosis and wilting. From the samples, *Fusarium* fungal colonies were continuously obtained. For the identification of the fungus, a morphological characterization and sequence analysis of the region of the transcribed internal spacer (ITS) and of the partial sequence of the gene of elongation factor 1-alpha (EF1-α) were performed. In addition, the pathogenicity of a representative isolate of *Fusarium* in stevia plants was verified. The results of the morphological characterization, analysis of ITS and EF1-α sequences, as well as the pathogenicity tests, determined that *Fusarium oxysporum* is the causal agent of the wilt symptoms of stevia plants in Martinez of the Torre, Veracruz.

Keywords: *Fusarium oxysporum*, *Stevia rebaudiana*, etiology, fungi.

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Stevia (*Stevia rebaudiana* Bertoni) is native to the semi-arid Paraguayan northwest, its leaves are extracted glycoside compounds, approximately 300 times sweeter than sugar cane. Antibiotic and anti-fungal properties are also attributed to stevia (Arturo *et al*., 2009). Some of the effects produced by stevia ingestion in humans are: hypotensive and hypoglycemic effect, as well as presenting anti-inflammatory and antimicrobial action (Marcavillaca, 2016).

Among the main stevia producing countries are Japan, China, Korea, Taiwan, Thailand, Indonesia, Laos, Malaysia and the Philippines; these countries represent 95% of world production (Herrera-Cedano *et al*., 2012). In America, it is grown mainly in Paraguay, Brazil, Argentina, Colombia, Perú and Mexico. The total cultivated area in Mexico is 58 ha, distributed in the states of Sinaloa, Nayarit, Jalisco, Colima, Michoacan, Guerrero, Oaxaca and Chiapas (SIAP, 2015).

There are several phytopathogenic fungi that have been reported to induce diseases in stevia plants, where the susceptibility to them increases with excessive levels of water (Álvarez, 2005), such is the case of fungi such as *Alternaria alternata*, *Alternaria steviae*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Septoria steviae*, *Uromyces* sp., *Verticillium dahliae* (Farr and Rossman, 2017), *Rhizoctonia* sp., *Colletotrichum* sp., *Pestalotia* sp. *Ascochyta* sp., *Phomopsis* sp., *Curvularia* sp., *Drechslera* sp., *Cercosporella* sp., *Thielaviopsis* sp. (Arturo *et al*., 2009), *Macrophomina phaseolina* and *Fusarium semitectum* (Hilal and Baiuomy, 2000). With respect to bacterial diseases, only *Pseudomonas cichorii* has been registered causing leaf spot (Strayer *et al*., 2012). Whereas, *Tomato spotted wilt* virus (Chatzivassiliou *et al*., 2007) and *Cucumber mosaic* virus (Chatzivassiliou *et al*., 2016) are the registered viruses inducing diseases in stevia.

Stevia wilt has been previously reported in Brazil (Mendes *et al*., 1998), Egypt (Hilal and Baiuomy, 2000), Venezuela (Arturo *et al*., 2009; Salazar *et al*., 2015) and India (Hegde and Chavan, 2009). This disease has been associated mainly with *Fusarium oxysporum*, although other species such as *F. solani* (Hegde and Chavan, 2009) and *F. semitectum* (Hilal and Baiuomy, 2000) may also be associated.

The objective of this study was to identify the causal agent of the symptoms of root rot, basal stem necrosis, and wilting of stevia plants in Martinez of the Torre, Veracruz, by combining morphological characterization, sequence analysis of the region ribosomal DNA of the transcribed internal spacer (ITS) and the partial sequence of the gene elongation factor 1-alpha (EF1-α), in addition to pathogenicity tests.

During 2015, directed samplings were conducted in a stevia plantation located in Martínez of the Torre, Veracruz, Mexico. Twenty plants with symptoms of root rot, basal stem necrosis, foliar chlorosis and wilting were collected. The incidence of the disease in the field was estimated at 25%.

For isolation, the roots of the plant samples were washed with water to remove soil debris and cut into 5 mm segments from the root rot and basal necrosis of the stem. Subsequently, the segments were disinfested with 1% sodium hypochlorite for 2 min, washed three times with sterile distilled water and dried on sterile absorbent paper. Five disinfested segments were seeded from each plant

collected in Petri dishes with potato dextrose agar (PDA) culture medium. The boxes were incubated at a temperature of 25 °C and in continuous darkness. The developed fungal colonies were transferred to boxes with fresh PDA medium. The fungal isolates were purified by the monosporic culture technique and stored at -80 °C in cryogenic tubes containing 15% glycerol.

From the fungal isolates obtained, temporary preparations were made in lactophenol and in glycerin, in order to observe and characterize the main reproductive structures in light microscopy. The identification at the gender level was carried out with the taxonomic keys of Barnett and Hunter (2006), while the identification at the species level was carried out using the descriptions reported by Leslie and Summerell (2006).

Pathogenicity tests were performed on 10 stevia plants, using a representative isolate of the fungus. The fungal isolate was increased in Petri dishes containing PDA culture medium. From the conidia grown in culture medium, a conidia suspension adjusted to a concentration of 1 x 10^6 spores ml^{-1} was obtained. The roots of 10 plants were immersed in the spore suspension for 30 min, then the plants were planted in unicel cups containing sterile soil and placed in a greenhouse at 25 °C. Ten plants whose roots were immersed only in sterile distilled water, served as control. The plants were observed every 24 h and the advance of the symptoms was recorded. The complete pathogenicity test was performed twice.

The extraction of genomic DNA from a representative isolate of the morphologically identified fungus was carried out by macerating 100 mg of mycelium obtained from a colony with 7 days of growth. Subsequently, the protocol indicated in the Plant DNeasy Mini Kit extraction kit (Qiagen®, EE. UU) was followed. The DNA quality was verified by electrophoresis in 1% agarose gel with running buffer TBE 0.5 X with the use of 5 µl of DNA and carried to 90 volts. The gel was analyzed in a Gel-Doc mod 2000 transilluminator (Biorad®, EE. UU.).

For the PCR amplification, the primers ITS5/ITS4 (White *et al*., 1990) and EF2/EF1 (O'Donnell *et al*., 1998) were used. The reaction mixture was prepared in a final volume of 25 µl, 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of each primer, 1U DNA polymerase (Promega®, EE. UU) and 100 ng of DNA. The PCR was carried out in a C1000 thermal cycler (Biorad®, EE. UU), with an initial denaturation of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final extension of 72 °C for 10 min. The alignment temperature was 55 and 54 °C for ITS5/ITS4 and EF1/EF2, respectively. The amplified products were verified by electrophoresis in 1% agarose gel with running buffer TBE 0.5 X with the use of 5 µl of the PCR product, to carry electrophoresis at 80 volts. The gel was analyzed on a Gel-Doc mod 2000 transilluminator (Biorad®, EE. UU).

The fragments amplified with the ITS5/ITS4 and EF1/EF2 primers were purified by the DNA clean & concentrator protocol (Zymo Research®, EE. UU). In a 1.5 ml microcentrifuge tube, 5 volumes of the DNA Binding buffer were added and mixed by inversion. The mixture was transferred on a Zymo-Spin column in a 2 ml collection tube. It was centrifuged for 30 s at 8 000 rpm, and the supernatant discarded. The 200 µl of DNA Wash buffer was added into the column and centrifuged 30 s for 8 000 rpm. The supernatant was discarded and changed to a new 2 ml tube. The 60 µl

Elution DNA buffer was added directly to the column and incubated for 1 min. The column was transferred to a new 1.5 ml tube and the DNA was diluted. The purified DNA fragments were sent to be sequenced to the company Macrogen (dna.macrogen.com). The sequences obtained were compared in the NCBI database (www.ncbi.nlm.nih.gov) with the BLASTn tool.

From the samples of infected plant tissue that were planted in Petri dishes with PDA culture medium, 92% of colonies belonging to the genus *Fusarium* were obtained.

The fungus obtained developed well in PDA culture medium forming macroconidia, microconidia and chlamydospores. The colony developed in PDA medium was cream-colored with slight violet pigmentation and cottony growth. The microconidia were hyaline, from oval to ellipsoidal, of 5.1-9.8 × 2.3-2.9 μm. The macroconidia were hyaline, 20.2-30.2 × 2.5-4.7 μm, elongated and curved, with the apical cell slightly acute, the basal cell with foot shape and presenting 3 to 5 septa. Individual and pairwise chlamydospores were observed, distributed terminally and interspersed in the hyphae. All the characteristics coincided with those reported by Leslie and Summerell (2006) for the species *Fusarium oxysporum*.

Eight days after the inoculation (ddi), it was observed that the inoculated isolate of *Fusarium oxysporum* caused root rot, basal necrosis of the stem and wilting in all the plants inoculated with the spore suspension. Twelve ddi, the inoculated plants died due to complete obstruction of the vascular bundles. While, the stevia plants that were used as control, remained asymptomatic and free of the disease. From the infected tissues of the symptomatic plants, *Fusarium* colonies were re-isolated that presented the same morphological characteristics of the isolates obtained from the stevia plants in culture fields.

The results of the pathogenicity tests of this study coincide with the symptoms registered in stevia plants by Salazar *et al*. (2015): however, the development times of symptoms and death of plants were different, even though the concentration of spores used for the inoculation was the same. This could be due to various factors such as greenhouse conditions or virulence of the isolate.

The analysis with the BLASTn tool of the ITS sequence obtained in this study showed 99% identity with the sequence of *F. oxysporum* deposited in GenBank with accession number KJ439169. Whereas, the sequence EF1-α showed 100% identity with various EF1-α sequences of *F. oxysporum* (GenBank accession numbers KY508354, KU507197, KU507192, KU507184 and KU507180). The above confirmed the results of the morphological identification carried out in this study.

In general, the results of this research coincided with those reported by Arturo *et al*. (2009) and Salazar *et al*. (2015), who determined through morphological identification and pathogenicity tests, that *Fusarium* sp. and *F. oxysporum* are the cause of the wilt symptom in stevia plants produced in Maracay and in Aragua, Venezuela, respectively. On the other hand, Hilal and Baiuomy (2000) registered *F. oxysporum* causing wilting of stevia plants in Egypt, however, their study lacks evidence of pathogenicity and molecular analysis. Other *Fusarium* species that have been reported to cause root rot of stevia are *F. solani* in Egypt (Hilal and Baiuomy, 2000) and India (Hegde and Chavan, 2009), in addition to *F. semitectum* in Egypt (Hilal and Baiuomy, 2000).

With respect to the control of stevia wilt, Hilal and Baiuomy (2000) indicated that the chemical treatment based on methyl thiophanate and the biological treatment based on *Trichoderma harzianum*, present an effective control of the disease. However, there is little specific information on wilting management strategies of stevia plants, so it is suggested to conduct studies aimed at evaluating various control strategies, in order to obtain integrated management tools to minimize the damage caused for this disease in stevia growing areas in Mexico.

Conclusions

The combination of results of the morphological characterization, analysis of ITS and EF1-α sequences, as well as pathogenicity tests, confirmed that *Fusarium oxysporum* is the causal agent of the symptoms of root rot, basal stem necrosis, leaf chlorosis and wilting of stevia plants in the town of Martinez of the Torre, in the state of Veracruz, Mexico.

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