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Epidemiology of Fusarium Agave Wilt in *Agave tequilana* Weber var. azul

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Abstract

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Fusarium oxysporum is reported as the principal causal agent limiting production of *Agave tequilana* Weber var. azul, but frequent isolation of *F. solani*, and symptoms typical of *F. solani* as a pathogen like severe reddish coloured root rot and loss of soil anchorage are frequently associated with diseased agaves. Inoculations of agave plantlets with *F. solani* induced typical agave root rot symptoms in greenhouse trials. The incidence of both pathogens was determined molecularly with specific primers in the ITS2 sequence. Dispersion patterns of agave wilt, determined in plantations of different age, indicated a tendency to produce aggregated patterns over time as the disease spread from the initial symptomatic plant to adjacent plants. Although both fungi were isolated from agave diseased plants, and in spite of the higher percentage of detection and root rot symptoms, it is concluded that *F. solani* may have a greater impact in agave wilt.

Keywords: Pathozone; soil-borne; plant pathogens; dispersion pattern

Agave tequilana Weber var. azul is the only raw material authorised by the Official Mexican Standard (NOM-006-SCFI-2005; www.crt.org.mx/images/Documentos/NOM-006-SCFI-2005.pdf) to produce the alcoholic beverage tequila. This beverage is important to the Mexican economy. In 2015, 248.3 million litres of tequila were produced and 74% was exported. To obtain this production, the Tequila Regulatory Council (CRT) reported the use of 859.2 thousand tons of agave heads (stems) (CRT 2016; www.crt.org.mx/EstadisticasCRTweb/). The agave tequilero takes from six to eight years to mature sufficiently for processing. Agave wilt is the most important disease that affects the survival of agave until it is sufficiently mature for harvest (CRT 2010). This disease has mainly been associated with *Fusarium oxysporum* (Luna-Hernández 1996; Aceves-Rodríguez 2002; Ortiz *et al*. 2011), however the disease symptoms which include severe reddish-brown necrosis in roots, crown and the lower part of the stem, and root rot (Luna-Hernández 1996; Uvalle *et al.* 2010; Avila-Miranda 2011), are unusual for a vascular wilt pathogen like *F. oxysporum* (Beckman 1987, 1989; Pegg 1989; Agrios 2005; Leslie & Summerell 2006).

In previous work, *F. oxysporum* isolates from stems of plants with agave wilt symptoms were classified into two groups according to their genetic diversity

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(determined by the DNA marker BOX-PCR) and vegetative compatibility groups (VCG). The compatible group included isolates with similar BOX-PCR fingerprints and the same VCG; meanwhile, the incompatible group included isolates with high diversity in their BOX–PCR fingerprints and the VCG could not be identified (Avila-Miranda *et al*. 2012). Only the isolates of VCG of *F. oxysporum* could reproduce symptoms of vascular wilt in inoculated agave plantlets (Avila-Miranda *et al*. 2010). On the other hand, *Fusarium solani* was frequently isolated from reddish rotted crown and stem tissue from agave plants with wilt symptoms (Avila-Miranda *et al*. 2010, 2012; Flores *et al.* 2010), but its pathogenicity was not tested. *F. solani* is generally associated with a strong necrotrophic process in roots, crowns, and stems in both monocot and dicot species (Graham *et al*. 1985; Leslie & Summerell 2006; Koike 2011; Correia *et al*. 2013; Farr & Rossman 2015).

In this work, genetic diversity of *Fusarium* isolates from the stem or crown tissue of agave plants with agave wilt symptoms was determined, and specific primers for identification or discrimination of both pathogens in plants were designed. The distribution pattern of diseased plants with agave wilt in commercial agave fields was determined; the pathogenicity of *F. solani* on agave plants was evaluated. These analyses provide a better understanding of the specific contribution of *F. oxysporum* and *F. solani* to the agave wilt disease.

MATERIAL AND METHODS

Pathogenicity testing of Fusarium solani. A pathogenicity test was performed using the FsG strain, morphologically identified as *F. solani* according to Booth (1971) and Leslie and Summerell (2006). Isolate was obtained from one field agave plant with severe symptoms of reddish rot on the stem. The fungus was grown on potato dextrose agar (PDA) for two weeks and then a conidial suspension was formulated and adjusted to 1.6×10^4 conidia/ml. Agave plants reproduced *in vitro* with 3 months of *ex vitro* growth were transplanted to sterile plastic pots containing a sterilised mix of peat moss, vermiculite, and sandy soil mixture (1 : 1 : 8 v/v/v) and acclimated to greenhouse conditions (28 ± 2°C), with watering and fertilisation every week. *F. solani* was inoculated two weeks later into the substrate introducing 3 ml of the conidial suspension; control plants were not inoculated. Severity of internal root rot was determined 240 days post-inoculation in 30% of the primary roots of each plant, in relation to their total length, slicing them using a scalpel, and their examination under a stereoscopic microscope. Induction of necrosis by FsG was confirmed by plating pieces of roots 1 cm in length, from areas with healthy-diseased tissue. The growth of *F. solani* was recorded and contrasted with the non-inoculated treatment.

***Diversity on ITS1-5.8S-ITS2 of F. solani and F. oxysporum* isolates.** Ten *F. solani* isolates (FsA, FsC, FsF, FsG, FsH, FsK, FsO, FsP, FsQ, and C7), in addition to *F. oxysporum* isolates, including six of the same VCG (FoxC, FoxD, FoxR, Fox24, Fox27, and Fox29) and nine non-vegetative compatibles (FoxA, FoxB, FoxCh, FoxE, FoxF, FoxQ, Fox23, Fox30, and Fox34) were plated on sterile cellophane disks overlaid on Petri dishes with PDA and grown for 10 days at 27°C. Recovered mycelia were used for total DNA extraction according to the supplier's instructions (ZR Plant/Seed MiniPrep Kit, Zymoresearch®).

Amplifications of the ITS1-5.8S-ITS2 region of these isolates were achieved using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*. 1990). The PCR reaction of 50 µl contained 0.04 U/µl Taq polymerase Amplicasa® (BioTecMol), 10× PCR buffer, 1.5 mM/l $MgCl_2$, 0.2 mM of each dNTPs (Bio-Rad), 0.8 µM of each specific primer, and 30 ng/µl of DNA template. The amplifications were performed with initial DNA denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 7 minutes. PCR products were purified (Gel/PCR DNA Fragments Extraction Kit, IBI Scientific) and sequenced. The obtained sequences were compared in the GenBank (http://www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST). Differential regions identified with the BioEdit sequence alignment editor (Hall 1999) in each group of strains were used for the design of specific primers by the Primer-BLAST program, and tested in pairs with ITS1 or ITS4 primers. Optimisation of PCR amplifications was performed using a temperature gradient.

Detection and frequency of F. oxysporum and F. solani in agave fields. Four commercial agave fields, with plants over 3 years old and a high incidence of agave wilt, were sampled. In each field, four groups of three or four contiguous diseased plants

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Figure 1. Patchy distribution of diseased plants frequently observed in commercial agave fields with epidemics of agave wilt. All these plants had reddish rot symptoms on the crown

were selected, and were considered as the half of a patchy distribution point, where on one edge of the patch there was a plant with the older infection, presumably infected by the primary source of inoculum from the soil; meanwhile, the contiguous plants were considered infected by a secondary dispersion of the pathogen, product of the contact of healthy roots with the diseased ones (Figure 1). Plants were removed from the soil including the principal roots (minimally of 20 cm in length) and sectioned to get the internal tissue from the stems and tissue from the border between healthy and reddish necrotic areas in the crowns. Tissues were sterilised by immersion in a sodium hypochlorite, alcohol, and distilled water (1 : 1 : 8) solution for 1.5 min, and then rinsed three times in sterile distilled water, and finally they were plated on PDA media. Isolates were identified to the genus based on microbiological and morphological criteria (Leslie & Summerell 2006). The incidence of one or both *Fusarium* species in plants was determined using the designed primers.

Determination of dispersion patterns of agave wilt. The dispersion pattern of agave wilt disease was evaluated in 2013 in sixteen commercial fields sown in 2008, 2009, 2010, and 2011, selecting groups of four fields for plants from 5 to 2 years old, respectively. The incidence of agave wilt was recorded quantifying stunted plants with dry areas in the tips of the leaves at a different height level, corresponding to level 3 in severity according to the scale reported by Avila-Miranda *et al.* (2010). Four hundred agave plants were evaluated in each field, distributed in a lattice pattern of 20 rows of plants separated 3 m from each other, by 20 plants in the row, separated 1 m from each other, in a total area of 1200 m^2. Join-count statistics was used to analyse the spatial association of diseased plants. A standardised version of join-count BW was executed by the SAS program in the file a:\chapter16*ex1.sas* (Gumpertz 1997; Madden *et al.* 2007) according to the formula:

$$\text{Standardised join-count} = \frac{BW - E(BW) + 0.5}{s(BW)} > \Phi^{-1}(0.01) = -2.326$$

where: BW – number of diseased-healthy joins in adjacent quadrants within rows, under free sampling, and expectation and variance of join-count BW were: E(BW) = (1/2)E(*r*) and Var(BW) = (1/4)Var(*r*) (Gumpertz 1997). The value of 0.5 is used as a correction for continuity as counts are discrete

In the equation above, standardised join-count should be on the left side of the equation.

Values of standardised join-count statistics > –2.326 for BW joins were indicative of random distribution. Meanwhile, with lower values than –2.326, H_0 is rejected and in consequence, an aggregated pattern of diseased plants was determined in the field at a significance level $\alpha \leq 0.01$.

Statistical analyses. Data analyses were conducted using the SAS® System Version 8.0 for Microsoft® Windows® (SAS Institute Inc., Cary, USA).

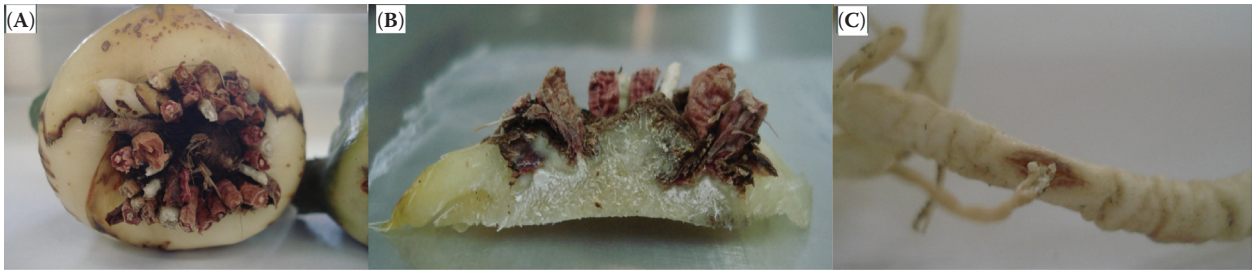

Figure 2. Rot symptoms of agave plantlets inoculated with the FsG *F. solani* strain: (**A**) rotten roots, (**B**) rotten tissue in the crown area, and (**C**) secondary rotten roots

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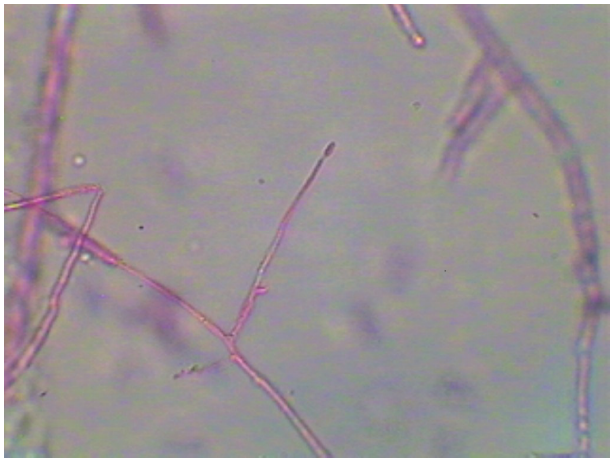

Figure 3. Long conidiophores and microconidia non-catenulate characteristic of *Fusarium solani*, observed in isolates from the reddish rot tissue of agave plants with symptoms of agave wilt

RESULTS

Pathogenicity test of F. solani strain. The agave plants inoculated with the FsG strain averaged necrosis on 56% of the length of their roots eight months post-inoculation. In addition, a reddish rot occurred on the crown (Figures 2A and 2B). The non-inoculated plants appeared to be healthy in the crown and root tissue. During the pathogenicity test, diseased plants generated new roots, but several of them were infected at sites where secondary or tertiary roots began to emerge, and these new roots often presented symptoms of root rot (Figure 2C). The aerial parts of these plants were asymptomatic. *F. solani* was reisolated from rotted tissues (Figure 3), demonstrating that FsG was pathogenic to *A. tequilana*.

Primer design for identification* of *F. solani and F. oxysporum. The more divergent region of amplified fragment with ITS1-ITS4 primers resulted in two sequences that clearly differentiated between *F. solani* and *F. oxysporum* strains (Figure 4) isolated from the tissue of agave diseased plants. Based on this zone, forward G9FsFor (5'-GGCCTGGCGTTGGGGATCG-3') and reverse G6FoRew (5'-CGCGAGTCCCAACACCAAGC-3') primers were designed to identify *F. solani* and *F. oxysporum*, respectively. Adjacent to this region for *F. oxysporum*, incompatible and compatible strains also showed variation (Figure 4), so it was useful to design FoIncRew (5'-CAATTTGGGGAACGCGAATTAA-3') and FoComRew (5'-TTTGGGGAACGCGATTTGACT-3') reverse primers to identify incompatible and compatible *F. oxysporum* strains, respectively. PCR optimization conditions for each pair of primers are shown in Table 1. The positive reaction of this procedure amplified a band of ~380 bp for any strain of *F. oxysporum* as follows: combination A (ITS1-FoComRew) identified compatible *F. oxysporum* strains; combination B (ITS1-FoIncRew) confirmed the incompatible *F. oxysporum* lab strain; meanwhile, combination C (ITS1-G6FoRew) was useful for the identification of both groups. On the other hand, *F. solani* was detected

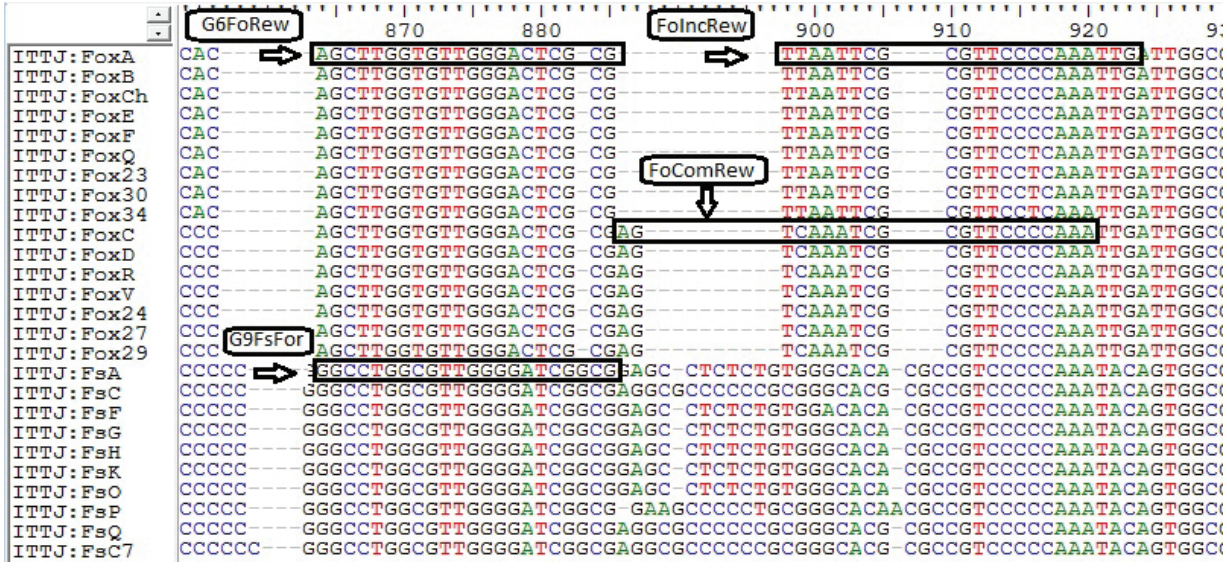

Figure 4. Diversity in sequences of *Fusarium oxysporum* and *F. solani* in the ITS2 fragment. Differences in this region were used to design primers for specific identification of isolates of *Fusarium oxysporum* general (G6FoRew), *F. oxysporum* compatible (FoComRew), *F. oxysporum* incompatible (FoIncRew), and *F. solani* general (G9FsFor)

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Table 1. Conditions of amplification of different primers to detect the presence of *Fusarium solani*, *F. oxysporum* (general), *F. oxysporum* incompatible, and *F. oxysporum* compatible strains in DNA from the stem or crown internal tissue of agave plants – Initial denaturation temperature (94°C) and time (2 min)

Primer pair	No. of cycles	Denaturation		Annealing		Extension		Final extension	
		(°C)	(min)	(°C)	(min)	(°C)	(min)	(°C)	(min)
G9FsFor-ITS4	35	94	1	58	58	72	1	72	6
ITS1-G6FoRew	35	94	1	58	58	72	1	72	6
ITS1-FoIncRew	10	94	1	66	55	72	1		
	22	94	1	64	64	72	1	72	6
ITS1-FoComRew	10	94	1	69	69	72	1		
	22	94	1	55	66	72	1	72	6

as a band of ~250 pb when combination D (G9FsFor-ITS4) was used (Figure 5). The primer combinations were validated in all *Fusarium* strains mentioned above.

Detection of frequency of F. oxysporum and F. solani in commercial fields. The analysis of internal tissues of the stem or crown from sixteen patchy groups of diseased plants indicated the presence of *F. solani* in 100% of the forty-five evaluated plants. The identity of *F. solani* was microbiologically and molecularly corroborated in the reddish rot tissue of the crown area of the plants. Additionally, in 62.5% of the sampled plants, compatible strains of *F. oxysporum* were molecularly detected; meanwhile, only in 8.3% of these plants incompatible strains *F. oxysporum* were detected.

Dispersion patterns of agave wilt. In the commercial agave fields selected to determine dispersion patterns, the incidence of diseased plants ranged from 21.75% to 82.25%. However, the distribution of the disease had a tendency to change from a random pattern in a younger plantation like that in the 2011 IV field, to an aggregated pattern in rows like in the 2011 II field, and then to a highly aggregated distribution pattern in the 2008 II field (Figures 6A–C). This gradual change of the disease distribution pattern along the cycle life of agave plants was related to a reduction of the number of contiguous diseased-healthy plants (BW) observed in the row, in comparison with those expected (E(BW)). When the incidence of agave wilt was very high, lower BW events were observed in all the fields; however, these were not statistically different from those expected, and a randomised pattern was determined by the join-count statistic (Table 2), such as is observed in the 2009-I field (Figure 6D).

DISCUSSION

Agave wilt is considered the main pathological problem in *A. tequilana* Weber var. azul and *F. oxysporum* has been reported as its principal causal agent (CRT 2010). Like other crops, *A. tequilana* is a host to pathogenic strains of *F. oxysporum* that cause a vascular wilt (Avila-Miranda *et al.* 2010); however, in this work, it has been demonstrated that *F. solani* is pathogenic to young agave plants, causing a reddish rot in roots, crown and stem tissues. This plant pathogen was detected with the use of the designed primers from the rotted tissue in roots, including plants with a range of initial symptoms, such as those with advanced foliar symptoms, and those with extreme root rot which had lost their soil anchorage

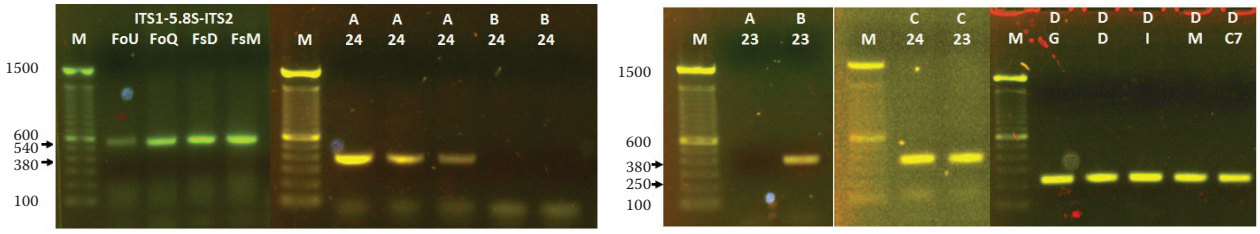

Figure 5. Validation of primer combinations to identify and discriminate between *Fusarium solani* and compatible and incompatible *F. oxysporum* strains (see the text): combination A confirmed compatible *F. oxysporum*; combination B corroborated incompatible *F. oxysporum* strains; combination C identified the strains of *F. oxysporum*, and combination D detected *F. solani* strains

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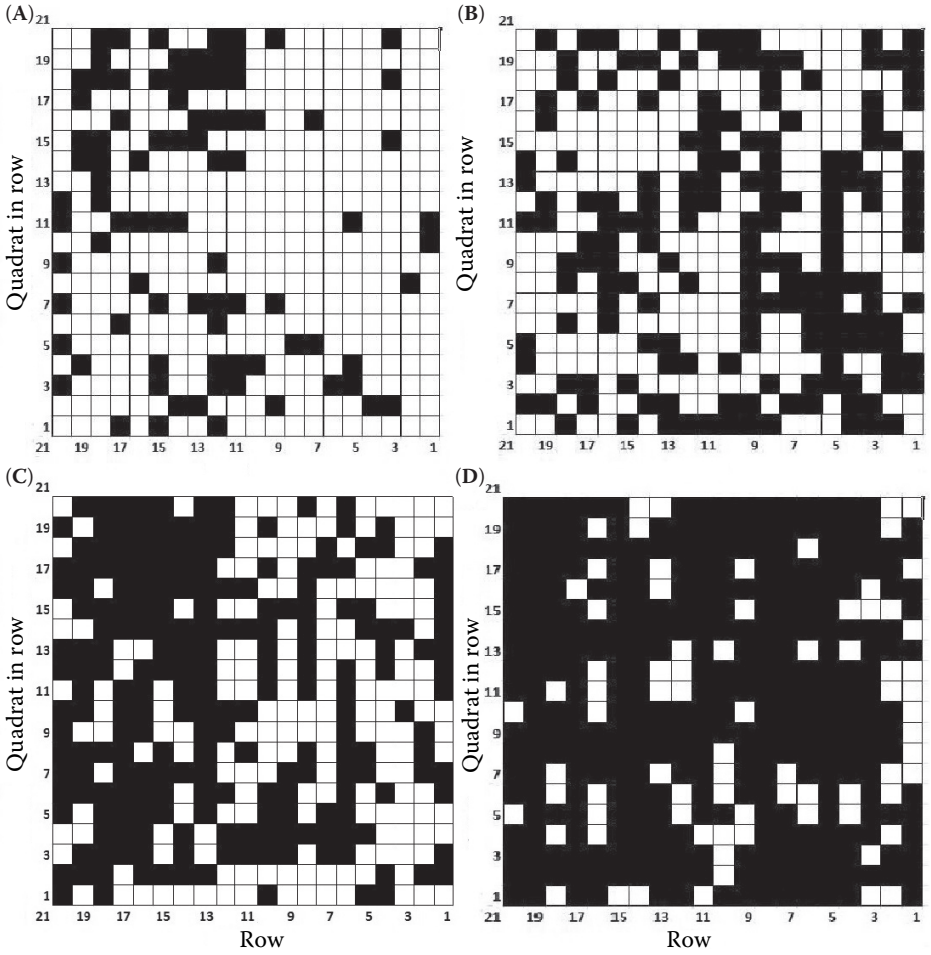

Figure 6. Examples of spatial patterns of agave wilt epidemics in areas of 20 plant rows by 20 contiguous plants: (**A**) Random pattern in the 2011 IV field, (**B**) Low contagious pattern in the sense of the row of plants in the 2011 II field, (**C**) High contagious pattern in the 2008 III field, and (**D**) A field with high incidence of agave wilt but with random pattern reported by join-count statistics in the 2009 I field

Solid squares represent diseased plants and open squares healthy plants

Table 2. Spatial association of diseased plants within a row in the field determined by the join-count statistics. High values of standardised join-count statistics (> –2.326) are indicative of positive autocorrelation ($P \leq 0.01$) and aggregated pattern of diseased plant and lower values indicate random distribution

Crop establishment year/replication	Agave wilt incidence	Observed (BW)	Expected E (BW)	Probability	Standardised join-count statistic	Distribution pattern
2008 I	59.25	127	183.50	1.23E-08	–5.576398	aggregated
2008 II	53.25	124	182.20	1.90E-11	–6.611557	aggregated
2008 III	54.50	134	188.46	1.96E-08	–5.494795	aggregated
2008 IV	37.25	133	177.65	8.6E-06	–4.297065	aggregated
2009 I	82.25	94	110.96	0.0683714	–1.488031	random
2009 II	30.50	118	161.10	0.0000382	–3.95513	aggregated
2009 III	35.00	118	172.90	9.38E-08	–5.211232	aggregated
2009 IV	40.75	155	183.49	0.0026512	–2.788062	aggregated
2010 I	62.00	153	179.06	0.0062009	–2.500501	aggregated
2010 II	67.25	154	167.39	0.1122443	–1.214679	random
2010 III	72.75	128	150.67	0.0217238	–2.019382	random
2010 IV	60.25	169	182.02	0.1077254	–1.238716	random
2011 I	45.50	143	188.46	2.34E-06	–4.578334	aggregated
2011 II	42.75	158	186.01	0.0028106	–2.7691	aggregated
2011 III	27.00	122	149.80	0.0065001	–2.483765	aggregated
2011 IV	21.75	122	130.42	0.2389373	–0.709725	random

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Figure 7. (**A**) Advanced stage of the reddish girdling necrosis symptom on the stem of agave plant associated with agave wilt disease and (**B**) plant with severe agave wilt that lost its anchorage to the soil

(Figure 7). These severe symptoms are similar to those described by Luna-Hernández (1996) for the stem rot of agave, attributed to *F. oxysporum*. However, these symptoms are different from those reported in vascular wilt diseases caused by some *F. oxysporum* f.sp. strains in different host crops, where the principal symptoms are injury to xylem vessels (Beckman 1987, 1989; Pegg 1989), or those symptoms caused by *F. oxysporum* f.spp. indicated as '*radicis-*' in their names that induce combined vascular injury and limited destruction of the parenchyma of young roots and crown tissues (Charest *et al*. 1984; Benhamou *et al.* 1989; Koyyappuratha *et al*. 2016). The severe rot symptoms in agave wilt are similar to those in the root and crown cortical tissue induced by necrotrophic soil-borne plant pathogens (MacHardy & Beckman 1981; Raaijmakers *et al*. 2009).

The changes observed in a distribution pattern of agave wilt from random to aggregated as plantations become older could be caused by both *F. oxysporum* and *F. solani* agave plant pathogens, but the ability to disperse from plant to plant of the first one is probably minor, if we consider that *F. oxysporum* induces a vascular wilt, with a specialised pathogenic process that is characterised by its penetration through the endodermis into the vascular tissue and moves up through the xylem to aboveground parts of the diseased plant, impeding the flow of water (Beckman 1987, 1989). In addition, Fusarium wilts are reported to show low transmission from plant to plant (Raaijmakers *et al.* 2009). However, this kind of pattern forming a patchy distribution of diseased plants (Figure 1) could be satisfactorily explained by the necrotrophic soil-borne plant pathogen fungi such as *F. solani* (Rupe *et al.* 2001), which cause cortical root rot, attacking preferably young roots as opposed to those lignified, and the fungus ramifies and continues to spread up the root, internally or externally, or can infect other roots in close proximity (MacHardy & Beckman 1981; Gilligan & Bailey 1997; Raaijmakers *et al*. 2009). This fact is in agreement with reports by Aceves-Rodríguez (2003), who concluded that the incidence and severity of agave wilt increased in the field when the distance between plants was decreased.

F. oxysporum strains from VCG are able to colonise the xylem vascular system and cause vascular wilt (Avila-Miranda *et al*. 2010). In this work, we present evidence that *F. solani* is very much related to agave wilt, with its independent and specific way of pathogenicity, which adds information to explain the high incidence and severity of this disease that is frequently observed in commercial agave fields. This knowledge is necessary to design better strategies of management of this disease.

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