# Vascular wilt caused by *Fusarium oxysporum* in agave (*Agave tequilana* Weber var. azul)

Martín E. Ávila–Miranda[1], José G. López–Zazueta[1], Carlos Arias–Castro[1], Martha A. Rodríguez–Mendiola[1], Doralinda A. Guzmán–de Peña[2], José A. Vera–Núñez[2], and Juan J. Peña–Cabriales[2]*

[1]Instituto Tecnológico de Tlajomulco. km 10 Carretera Santa Cruz de la Flores–San Miguel Cuyutlán. CP 45640. Tlajomulco de Zuñiga, Jalisco, México
[2]IPN, Cinvestav–Unidad Irapuato, Departamento de Biotecnología y Bioquímica. CP 36821. Irapuato, Guanajuato, México

*Corresponding author, e–mail: jpena@ira.cinvestav.mx

*Received 15th January 2010; Accepted 18th November, 2010*

## Abstract

*Agave tequilana* Weber var. azul is a crop used to produce 'Tequila' in Mexico. The agave's wilting (marchitez in spanish) is an economically important disease associated to *Fusarium oxysporum*. The symptoms reported in the diseased plants include extensive stem and root rot that are uncharacteristic of vascular wilt associated to *F. oxysproum* in other crops. Ten agave commercial fields presenting high agave's wilting incidence in the Mexicos's Jalisco and Nayarit states were evaluated. A severity scale was used in the field to evaluate agave's wilting and, in general, a positive correlation was observed between the degree of xylem vascular damage or root rot with the wilt severity level in the field plants. Twenty–one isolates of *F. oxysporum* obtained from stem tissue of agave plants were analyzed to determine their genetic diversity using Box–PCR DNA markers. A group of ten strains with identical fingerprints was identified including isolates from distant fields. Five strains from three genetic groups were used to test their pathogenicity in agave plants propagated *in vitro* under greenhouse conditions. Wilt symptoms were observed in agave plants 200 days after inoculation of the *F. oxysporum* strains. The incidence of blocked or partially degraded xylem vessels was higher in plants inoculated with any of the *F. oxysporum* strains as compared to the control plants. The root rots symptom was not present in inoculated plants. Interestingly, several *Fusarium solani* isolates were obtained from rooting roots or stem of field diseased plants, indicating that at least two *Fusarium* species are responsible for agave's wilting disease.

**Key words:** Box–PCR, Field, *Fusaria*, Tequila, Wilting.

## Introduction

Agave (*Agave tequilana* Weber var. azul) is a crop used to produce the Mexican alcoholic beverage known as Tequila. Agave plants need at least six years to complete their life cycle. However, some important diseases reduce the number of plants that reach maturity. The agave's wilt (marchitez in spanish) is a disease mainly associated to *Fusarium oxysporum*, but until now, positive pathogenicity has not been reported in the fields or *in vitro* on agave plants. In the official Tequila's geographical indication Region, 37% of agave plants were affected by 'marchitez' during 2004

(CRT–Comité Técnico Agronómico, 2005; Eguiarte and González, 2007), and a census of agave plants, in January of 2008, identified over 40 millions diseased plants (CRT, 2008).

Symptoms reported in agave's 'marchitez' are: wilting, chlorosis (light yellow leaves), folding up of the leaf edges, drying of the older leaves from the apex to the base, extensive maroon colored rot in the crown, and finally, if the plant is pushed laterally, it easily drops because root rot (Aceves, 1999; CRT–Comité Técnico Agronómico, 2005). Thirteen years ago, *F. oxysporum* was reported as the etiologic agent of agave's stem rot (Luna, 1996). Some of the symptoms described on that report are uncharacteristic of pathogenic strains of *F. oxysporum*, which induce vascular wilt diseases in other plants, which are characterized mainly by a pathogenic process limited to the xylem tissue, but not associated to root rot (Beckman, 1987; Rodríguez–Gálvez and Mendgen, 1995; Inoue *et al*., 2002; Leslie and Summerell, 2006). Agave is clonally propagated by transplanting small plants, called 'hijuelos' that are rhizome generated, and grow up around mother plants. It is important to define if *F. oxysporum* causes vascular wilt in agave plants affected by 'marchitez' in order to generate specific disease management strategies to reduce the risk of introduction of infected vegetative material to new crop areas. In this work, xylem injury and root rot were associated to wilt symptoms of field agave plants. In addition, twenty one *F. oxysporum* isolates obtained from stems of field agave plants with 'marchitez' symptoms were analyzed using Box–PCR DNA marker to determine their genetic variability. Five *Fusarium* strains, representing three different genetic groups, were inoculated in agave plants to confirm their pathogenicity *in vitro* under greenhouse conditions.

## Material and methods

**Epidemiology, experimental site and severity scale of wilt symptoms**

Ten commercial agave fields, property of Tequila Herradura S.A. de C.V. (Table 1), with 1– to 5–years old plants were chosen as sampling sites due to the high incidence of 'marchitez' observed in their plants. Eight fields were localized in the most important production areas denominated 'Los Altos' and 'Los Valles' in the state of Jalisco and two fields from the state of Nayarit. One hundred plants per field were analyzed, *in situ*, to determine the severity of 'marchitez'. Fifteen plants, three of each category, were dug–up and analyzed for injured xylem, root rot severity and fungal isolation. The experiments were performed, under sterile conditions, in the Plant Health Laboratory at the Instituto Tecnológico de Tlajomulco, Jalisco, Mexico.

Table 1. Commercial fields selected by their high 'marchitez' incidence in three agave (*A. tequilana* Weber var. azul) production areas in Jalisco and Nayarit states, México.

| Field/Key | | County | Region/State |
|---|---|---|---|
| Tata Dios | (TAD) | Amatitán | Los Valles, Jalisco |
| Bajío Sur | (BAS) | El Arenal | Los Valles, Jalisco |
| Ojo Zarco | (OJO) | Magdalena | Los Valles, Jalisco |
| Las Lajitas | (TEU) | Teuchitlán | Los Valles, Jalisco |
| Santa Rosa | (SRO) | Tepic | Tepic, Nayarit |
| Barrosas | (BAR) | Sta María del Oro | Tepic, Nayarit |
| La Loma | (LOM) | Tepatitlán | Los Altos, Jalisco |
| El Zapote | (ZAP) | Acatic | Los Altos, Jalisco |
| La Cañada | (LAC) | Tala | Los Valles, Jalisco |
| Novillero | (NOV) | Magdalena | Los Valles, Jalisco |

The severity scale established was as follows: 0= asymptomatic plants, with extended blue–greenish leaves; 1= small greenish plants with lower leaves curled upwards in the margins; 2= yellowish plants with the most of the leaves curled upwards in the margins, and dry areas in lower leaves; 3= stunted plants, dry areas in the tip of leaves at different height level; and 4= plants with very few green areas and close to death (Figure 1).

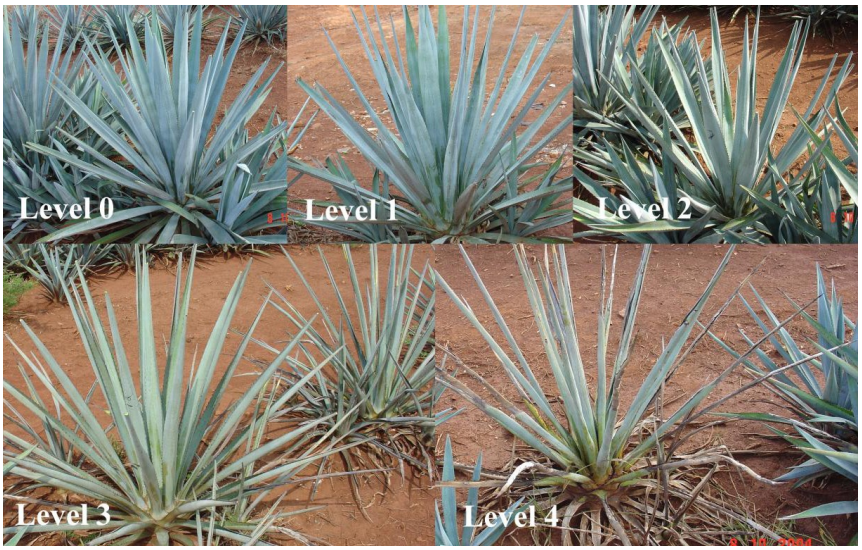


Figure 1. Severity scale of agave's 'marchitez' used for field evaluation: *level 0*, asymptomatic plants with turgent and extended blue–greenish leaves with similar foliar volume than plants in the plantation; *level 1*, small greenish plants with lower leaves curled upwards in the margins; *level 2*, yellowish plants with the most of the leaves curled upwards in the margins, and dry areas in lower leaves; *level 3*, stunted plants with dry areas in the tip of leaves at different height level; *level 4*, plants with very few green areas and close to death.

**Injured xylem vessels incidence**

Stem pieces (50µm thickness) were obtained by cross sectioning, transversal to the xylem vessels length, at four different zones of the cubic stem block of each plant. The pieces were immersed in clear lactophenol and were observed with 20X objective in a Carl Zeizz® AxioLab microscope in order to asses the number of xylem vessels in each of three ocular fields by stem piece. The percent of yellowish, blocked or degraded xylem vessels was recorded to determine the incidence of injured xylem.

**Root rot severity**

To evaluate the level of root rot, twenty pieces of primary or secondary roots per plant, of 25 cm length, were cross–sectioned in 1cm intervals with surgical blades, transversal to its length. In each transversal root section observed under a stereo microscope, necrotic root rot tissue was identified by the appearance of internal dark colored regions contrasted with the white color of the healthy root tissue. The length of the rotted root tissue was contrasted with the complete root length and expressed as percent of root rot. An average of twenty pieces was recorded to determine the root rot severity in each plant.

Data of agave's 'marchitez' field severity, expressed as percentage, from four severity levels were correlated with the incidence of injured xylem, and the severity of root rot in the same plants. The

Pearson correlation analyses was applied to explain if xylem injury or root rot variables were related to the increase of wilt severity level observed in the field.

**Fungal isolation from stem and roots**

Five small internal pieces of stem tissue from each agave plant were disinfected with 6 % sodium hypochlorite, absolute ethylic alcohol and sterile distilled water (1:1:8 v/v) for two minutes and rinsed twice in sterile distilled water. The stem pieces were plated onto potato dextrose agar media (PDA) and incubated at 28 °C to isolate the causal agent. Five days later, the fungal colonies obtained were transfered to PDA slants. Later on, mono–conidial isolation of cultures was obtained from each fungal isolate. All isolates, by triplicate, were grown on PDA at 24 °C for 10 days, and colonial color, diameter, macro–conidia, micro–conidia and chlamydospores were recorded.

Fungal isolates from rotted primary or secondary roots were isolated from 1cm segments, without epidermis, at the transition site between healthy and rotted tissue. Root segments were surface disinfected in chloride–alcohol–water (1:1:8 v/v) for two minutes then were plated on PDA+streptomycin. The fungal isolates were classified using the keys for *Fusarium* species identification by Booth (1977). Monoconidial suspensions of isolates were stored at –70°C in 14% sterile glycerol.

**DNA extraction from mycelia**

In order to obtain total DNA, aliquots of monoconidial suspensions of 25 isolates of *F. oxysporum* were grown on sterile cellophane disks on PDA media for 10 days at 27°C. Later, the mycelia were scraped off from the cellophane surface using a flame–sterilized dissection needle and transfered to 1.9mL tubes, and immediately stored at –70°C. To obtain DNA, mycelia were frozen with liquid–N and pulverized with mortar and pistil, and a modified double extraction phenol–chloroform process was performed (Lee and Taylor, 1990). To eliminate RNA, 10 Units of RNA–ase were used (Ribonuclease T2 Invitrogen®) for 30 minutes. The DNA was re–suspended in 50 µL miliQ water and its concentration was determined by spectrophotometer. The quality of the extracted DNA was determing running an electrophoresis test with 2 µL of total DNA in a 0.8% agarose gel (Turner *et al*., 1998).

**Use of Box–PCR DNA markers to generate genetic diversity groups**

In order to reduce the number of representative isolates to be used in the pathogenicity tests under greenhouse conditions, DNA fingerprints from 21 isolates were obtained with the primer Box A1R–5'CTACGGCAAGGCGACGCTGACG–3' which is one of the Rep–PCR DNA markers (Rademaker and Brujin, 1998). In this way, only one representative isolate that conforms to genetically different groups was used in the pathogenicity test. The PCR program used consisted of one cycle of seven minutes at 94°C; 36 cycles of one minute at 94°C, one minute at 53°C, five minutes at 65°C, and finally one cycle of 18 minutes at 65°C. All amplifications were carried out in a Palm–Thermocycler (Corbett Research). The electrophoresis separation was done in 19 cm gels containing 1.8% agarose (LE, Analytical Grade, Promega Corporation Wisconsin, USA) in 1X TAE. A sample of 2 µL of loading buffer (30% v/v glycerol, 0.25 w/v xilane cyanol) were added to 6.0 µL amplified DNA into a single lane. The electrophoresis conditions were 40 V, 150 mA for 21 h at 4°C using 0.5X TAEas a running buffer. The DNA fingerprints were analyzed separately to calculate the phenogram and parsimony tree by a binomial system based in the presence or absence of a given fragment. A similarity matrix, from each isolate, was computed and used as input data in the NTSYSpc, version 2.02j program (NTSYSpc, Applied Biostatistical Inc.) to obtain a dendrogram using distance index.

**Greenhouse pathogenicity test**

Strains from three genetically diverse groups were analyzed for their pathogenic capabilities. The strains from each group were selected considering the high degree of aerial wilt severity observed in the plant from which it was obtained and the high incidence of xylem discoloration. To obtain the inoculum of *F. oxysporum* isolates, these were plated in PDA Petri dishes of nine cm diameter, and were allowed to grow for two weeks. Spore suspensions with a concentration of $2x10^5$ conidia $mL^{-1}$ were obtained. Three months old agave plants, propagated *in vitro*, were used as control plants. Before inoculation, agave plants were acclimated for two weeks in the greenhouse. Later, they were transplanted to plastic pots with a mix of peat moss–perlite and sand soil (8:2 v/v), previously sterilized for one h at a pressure of 21 lb $in^{-2}$. Fifteen days later, plants were inoculated with 5 mL of $2x10^4$ conidia $mL^{-1}$ added to each pot of three equidistant points, and more sterile substrate mixture was applied in the three holes, previously made with plastic tubes at time of transplant.

Six treatments were evaluated in the experiment: in five of them, plants were inoculated with each of the five selected *F. oxysporum* strains: 1) NOV–9–A (U), 2) LAC–7–A (27), 3) BAS–1–A (Q), 4) SRO–8–B (O), 5) ZAP–6–B (24), and the sixth was the control (uninoculated plants). Plants of all treatments were maintained during 200 days under greenhouse conditions, with a temperature of 28±2 °C, manual irrigation (100 mL filtered water) three times per week, and nutrient solution (N, 250 ppm; P, 90 ppm; K, 200 ppm; Ca, 120 ppm; Mg, 50 ppm) once a week. Plants grew under natural photoperiod from June to March.

To evaluate treatments, five random selected plants were chosen at four evaluation dates (30, 60, 90 and 200 days after inoculation). The colonization of agave's vascular system by *F. oxysporum* in the root, stem and leaf tissues were determined by plating internal tissue pieces on PDA, after surface disinfection as described previously. PDA plates were incubated for five days and *F. oxysporum* isolates were identified from each replicate.

Internal tissue from plant stem was cut in thin layers, symptoms were observed microscopically, and incidence of blocked, yellowish or degraded xylem vessels, were recorded. Progressive digital pictures evaluated wilt Symptoms at 30, 60, 90 and 200 days, recording the changes in the plant appearance throughout the experiment. At 200 days, 1cm root fragments were cut from the crown of the plant, and external and internal tissue appearance were evaluated. Incidence of necrotic root induced by the *F. oxysporum* isolates on the experimental plants was also recorded.

**Statistic methods**

Statistical analysis was done using Proc Corr, Proc ANOVA and Duncan's means test using the SAS statistical program ver. 6.12 (SAS Institute Inc., 2000).

# Results

**Epidemiology**

The incidence of agave 'marchitez' in fields of the Jalisco and Nayarit states, Mexico, was from 36% to 97%. In fact, the studied fields were chosen because the plants showed 'marchitez' symptoms. Values of field severity are show in Table 2, 'Ojo zarco' site had the highest value in 4–years old plants and the values recorded in the 'El Novillero' and 'Las Lajitas' fields corresponded to young plants (one– and two–years old respectively) with 74% incidence of 'marchitez' in both fields. The high incidence in young plants is uncommon in agave fields, where the normal incubation period of 'marchitez' is of three or more years. The highest incidence average was 97% in 'Santa Rosa' (Table 2). In contrast, the lowest field severity was observed in 'El Zapote' (0.60%).

It was observed that the highest incidence value of blocked or degraded xylem vessels in field plants was recorded in 'La Loma' field with of 81% of xylem vessel damage. Values of incidence of xylem injury observed in 'El Novillero' (45%) or 'Las Lajitas' (61 %) could be considered low in comparison with the values observed in 'La Loma' or 'Santa Rosa' fields (55%). The lowest values of xylem injury associated to young plants (1 or 2 years) could be important for early diagnosis of the disease in agave cultivation, because symptoms commonly are found in older plants (Aceves, 2003). Root rot severity in the agave plants ranged from 15.9 to 64.7%. Plants from 'El Zapote', 'La Cañada' and 'El Novillero' fields showed the highest root rot severity (Table 2).

Table 2. Evaluation of severity level, wilt incidence, xylem vessel injury and root rot severity in agave (*Agave tequilana* Weber var. azul) plants from commercial production fields.

| Field key | Plant age (years) | Field severity[a] | Field incidence of wilted plants[a] | Incidence of injured xylem vessels[b] (%) | Severity of root rot[c] |
|---|---|---|---|---|---|
| TAD | 4 | 1.14 | 76 | 61 | 45.8 |
| BAS | 2 | 0.78 | 56 | 44 | 24.7 |
| OJO | 4 | 2.2 | 75 | 64 | 35.3 |
| TEU | 2 | 1.1 | 74 | 61 | 15.9 |
| SRO | 4 | 1.75 | 97 | 55 | 31.2 |
| BAR | 5 | 1.78 | 96 | 45 | 38.2 |
| LOM | 3 | 0.81 | 52 | 81 | 49.2 |
| ZAP | 4 | 0.6 | 36 | 70 | 64.7 |
| CAÑ | 4 | 1.36 | 78 | 60 | 59.3 |
| NOV | 1 | 0.93 | 74 | 45 | 51.3 |

[a]Average from 100 plants per field; [b]Average from twelve microscope 200X fields per plant; [c]Average from twenty root per plant.

The mean percentage of incidence of blocked or degraded xylem vessels observed in root plants had a straight correlation with the field severity of 'marchitez' recorded (Table 3). Interestingly, this relationship had an $r^2$ value of 0.97 for the one–year old field plants from 'El Novillero'. Similarly, the coefficient of determination was high (0.82) for 'La Cañada' field. However, it was low (–0.18) for the 'Ojo Zarco' field, which had the highest field severity level (2.2) accompanied by a severe aerial disease called 'anillo rojo'.

Table 3. Pearson correlation between field severity level in a 0–4 scale in agave (*Agave tequilana* Weber var. azul) plants and the incidence of xylem blocked veseels in stem and root rot severity.

| Symptom | Field key | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TAD | BAS | OJO | TEU | SRO | BAR | LOM | ZAP | CAÑ | NOV |
| Xilem vessel injury (%)[a] | 0.17 | 0.78 | –0.18 | 0.58 | 0.75 | 0.46 | 0.45 | 0.78 | 0.82 | 0.97 |
| Root rot[b] | 0.04 | 0.35 | 0.30 | 0.53 | 0.57 | 0.20 | 0.61 | 0.78 | 0.63 | 0.09 |

[a]Average from twelve microscope 200X fields per plant; [b]Average from twenty root per plant.

The severity level of wilt, that correlated directly with the xylem vessel injury in 'El Novillero' field and with root rot in 'El Zapote' field with an *r* of 0.78, suggest that the wilt symptom in agave plants can be an expression of injury in their xylem system or could be induced by root rot severity or both.

**Fungal isolates and DNA fingerprints**

Twenty–seven out of twenty–nine *Fusarium* isolates from the stem tissue of field plants were identified as *F. oxysporum*, according to macroscopic characteristics on PDA. This 27 isolates were part of the collection of potentially pathogenic *F. oxysporum* isolates from agave plants used in this work (Table 4).

Eleven of the isolates were obtained from symptomless plants. Box–PCR fingerprints from fifteen agave *F. oxysporum* isolates using the primer BoxA1R are shown in electrophoresis gel stained with *Ethidium Bromide* (Figure 2). Diversity was observed in the resulting fingerprints, specially in 28 or 30 isolates, but isolates F and 23 or, 22, O and N, had similar Box–PCR fingerprints, although all of them were isolated from plants of different fields.

Table 4. Wilt severity level of agave (*Agave tequilana* Weber var. azul) plants where *F. oxysporum* isolates were obtained from vascular stem tissue.

| No. | Field key | Isolate key | Plant severity level |
|---|---|---|---|
| 1 | BAS–0 | O | 0 |
| 2 | BAS–0 | M | 0 |
| 3 | BAS–0 | 34 | 0 |
| 4 | BAS–0 | F | 0 |
| 5 | ZAP–0 | 31 | 0 |
| 6 | ZAP–0 | 23 | 0 |
| 7 | ZAP–1 | 24 | 1 |
| 8 | ZAP–4 | 26 | 4 |
| 9 | TEU–0 | 22 | 0 |
| 10 | TEU–0 | 25 | 0 |
| 11 | TEU–1 | S | 1 |
| 12 | TEU–1 | J | 1 |
| 13 | TEU–2 | 30 | 2 |
| 14 | BAR–3 | N | 3 |
| 15 | SRO–2 | O | 2 |
| 16 | OJO–4 | P | 4 |
| 17 | OJO–4 | 29 | 4 |
| 18 | LAC–2 | 27 | 2 |
| 19 | LAC–2 | R | 2 |
| 20 | LAC–3 | T | 3 |
| 21 | LAC–3 | 33 | 3 |
| 22 | LAC–4 | 28 | 4 |
| 23 | NOV–00 | Ñ | 2 |
| 24 | NOV–00 | 21 | 2 |
| 25 | NOV–00 | K | 3 |
| 26 | NOV–01 | L | 3 |
| 27 | NOV–02 | U | 4 |

Dendrogram (Figure 3) obtained from fingerprints of twenty one isolates using the NTSYSpc program, version 2.02j and the correlation similarity matrix shows that genetic diversity of the *F. oxysporum* isolates is separated in three genetic groups at a coefficient value of 0.17. One group is associated to only isolate 28. The other two groups consisted of thirteen and eight isolates. The isolates of one group contained isolates obtained from fields in different agave production regions. For example, the F and J isolates were obtained from 'Los Valles' region and isolate 23 was from 'Los Altos' region. In the other group, isolates K and R were obtained from different fields of 'Los Valles' region and N and O isolates were obtained from different fields in the Nayarit region; in the third group, the isolates were obtained from 'Los Altos' region. The average distances between 'Los Altos' and 'Los Valles' regions and between the 'Los Valles' and the Nayarit regions are 200 and 180 km, respectively.

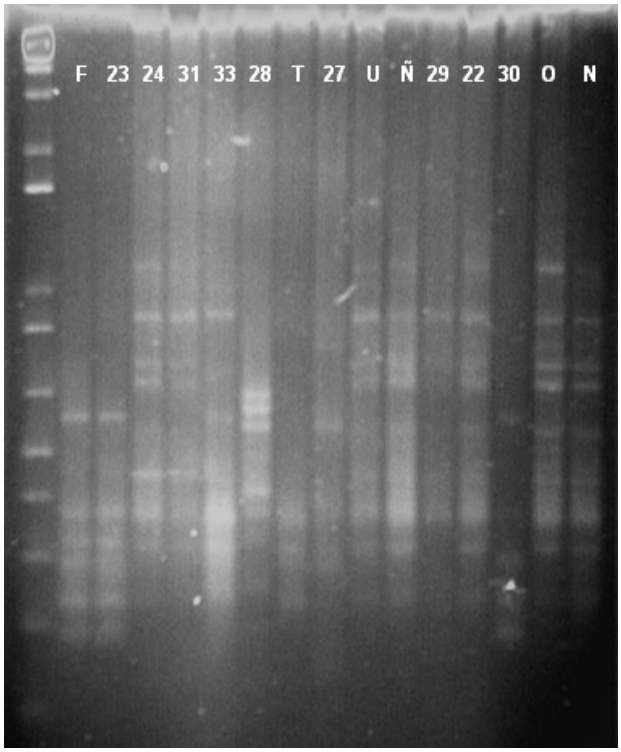


Figure 2. Finger prints from fifteen *F. oxysporum* isolates obtained from wilted agave (*A. tequilana* Weber var. azul) plants using Box–PCR DNA markers.

**Pathogenicity test**

Five isolates representing three different genetic groups of *Fusarium* were used to perform a greenhouse experiment to asses their pathogenic capacity against agave plants. *Fusarium oxysporum* strains Q, 27, O, U and 24 in *A. tequilana* plants gave interesting information, because *F. oxysporum* isolates were recovered from all plants in replicates of all treatments at 16 days after inoculation. Similarly, *F. oxysporum* isolates were recovered from stem tissue of agave plants at 90, 120 and 200 days after inoculation. Initially, the isolates were obtained from internal stem of plants; later, isolates were observed in tissue of the agave basal leaves indicating the phase of vascular colonization according to Beckman (1987). No fungal isolates were obtained from control plants on PDA+streptomycin media.

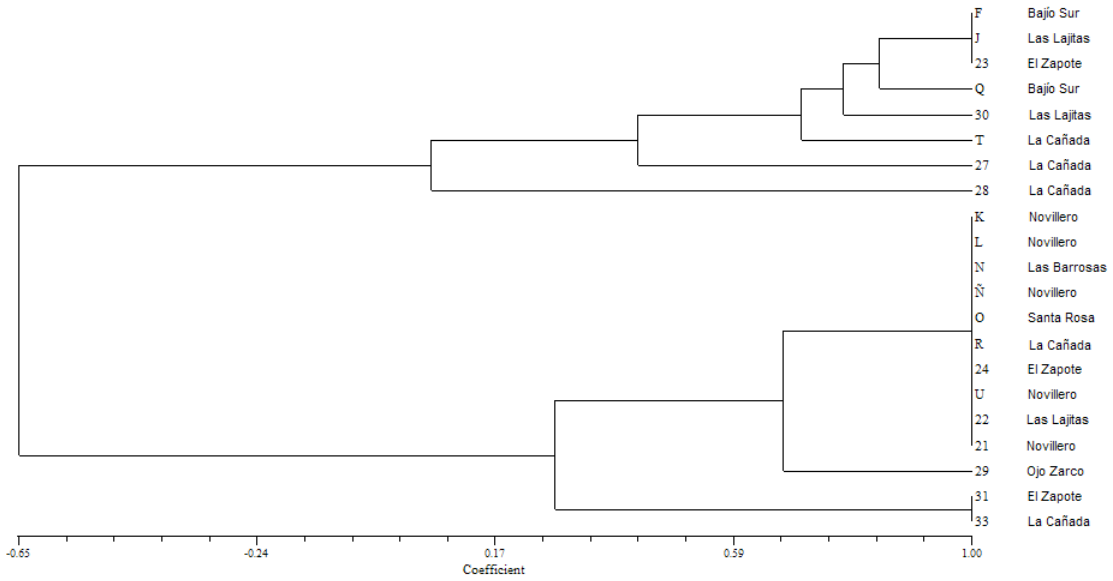


Figure 3. Dendrogram for twenty–one *Fusarium oxysporum* isolates obtained from vascular stem tissue of agave (*Agave tequilana* Weber var. azul) plants and its field of origin.

The first aerial symptoms in agave plants consisted of yellowish leaves and it was observed at 60 days after inoculation. At 90 days after inoculation, plants inoculated with strain 27 showed leaves curled upwards at the margins, which appeared earlier than the rest of the strains. Later on, at 150 days, strain dependent symptoms were revealed in the agave plants (Figure 4).

Figure 4. Yellowish leaves of agave (*Agave tequilana* Weber var. azul) plants observed at 150 days after inoculation with *Fusarium oxysporum* strains.

At the end of the experiment, 200 days after inoculation, the treatments inoculated with strains O, 24, 27 and Q had significantly more yellowish leaves than the control plants, but those inoculated with strains O and 24 showed significantly more chlorotic leaves per plant (Figure 5).

At 200 days, plants showed and increase of upwardly curled leaves than control plants, but those plants with strain–O were statically better with an incidence of 3 leaves per plant (Figure 6).

Both wilt symptoms, yellowish and upwardly curled leaves were observed in inoculated plants but were not observed in uninoculated agave plants. Control plants showed blue–greenish color leaves, normal for *A. tequilana* **(**Figure 7).

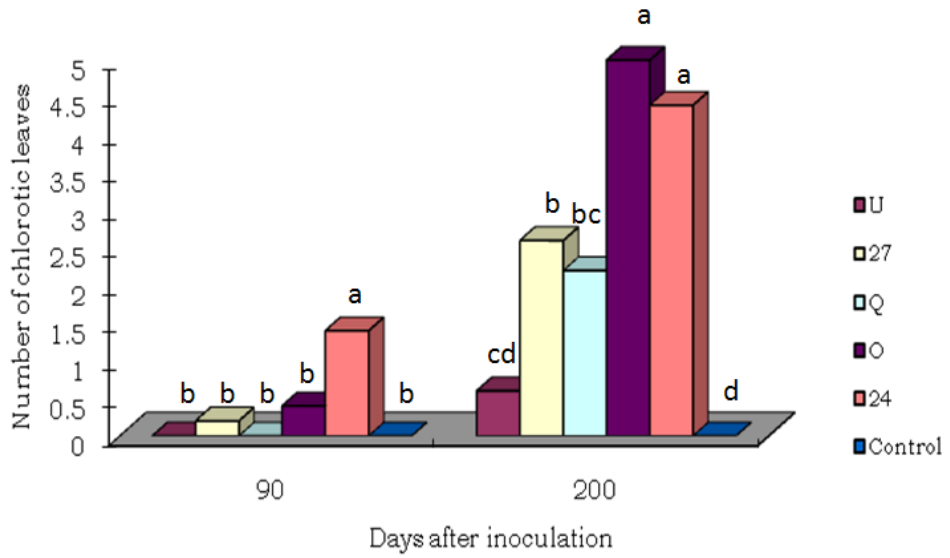


Figure 5. Chlorosis in agave (*Agave tequilana* Weber var. azul) plants observed at 90 and 200 days after inoculation with *Fusarium oxysporum* strains.

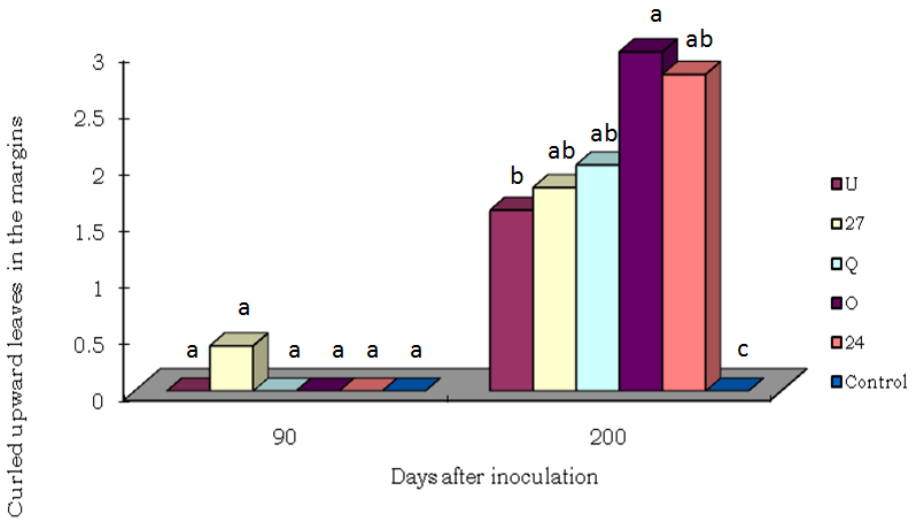


Figure 6. Number of agave (*Agave tequilana* Weber var. azul) leaves showing upward curling in their margins plants as observed at 90 and 200 days after inoculation with *Fusarium oxysporum* strains.

The incidence of blocked or partially degraded xylem from plants of the greenhouse experiment was recorded and plants inoculated with *F. oxysporum* showed significantly more injured xylem vessels than control plants. The average in this variable increased more than 60 % at 200 days in inoculated plants. Control plants presented vessels with the same yellowish appearance, but the percent of incidence was less than 20 % (from 30 to 200 days after inoculation) and it was statistically lower than the xylem injury in inoculated plants (Figure 8).

The root fragments obtained from the crown area of agave plants at 200 days after inoculation had white turgent roots, with a fibrous brown or reddish appearance. No root rot symptoms were induced by *F. oxysporum* strains in this greenhouse study. The plants showed the similar appearance of root fragments of 'O' and '24' strains treated and control plants, respectively (Figure 7).

Figure 7. Wilt symptoms of agave (*Agave tequilana* Weber var. azul) plants observed at 200 days after inoculation with *Fusarium oxysporum* strains. **A**) control plants; **B**) 'O' strain and **C**) '24' strain.

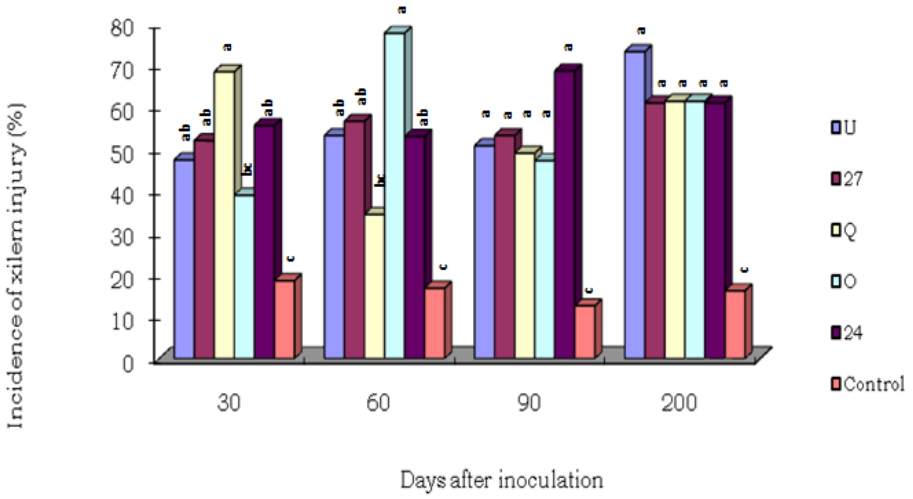


Figure 8. Incidence of xylem vessel injury in agave (*Agave tequilana* Weber var. azul) propagated *in vitro* at 30, 60, 90 and 200 days after inoculation with *Fusarium oxysporum* strains.

## Discussion

It has been reported that 12 to 38% of agave plants suffer from the 'marchitez' syndrome at varying degrees of severity (Aceves, 2003; Anónimo, 2005) in the defined official Tequila's geographical indication Region which is really a protected designation of origin in México. This condition is very important if we consider that the census of agave plants in 2007 quantified more than 500 million plants (CRT, 2008). In this work, we show that isolates of *F. oxysporum* act as a vascular wilt plant

pathogen in agave plants mainly affecting its xylem vascular system. Generally, a specific *F. oxysporum* plant pathogenic isolate has a narrow number of host plant species, and this restricted range ability is the product of a co–evolution process between plant and pathogen (Armstrong and Armstrong, 1981; Beckman, 1987; Leslie and Summerell, 2006). In the agave crop, Luna (1996) reported that *F. oxysporum* was an important agave pathogen causing stem rot. Contrary to his results, we isolated *F. solani* on of the maroon stem or root rot lesions observed in the field. Oviedo *et al*. (2004) have reported that *F. oxysporum* could establish a symbiotic relation with *Agave victoria–reginae* and Segura (1997) detected *F. oxysporum* in tissue of *Agave sisalana*. But noting is known about a *formae specialis* (f. sp.) for *A. tequilana* Weber var. azul or if any pathogenic *F. oxysporum formae specialis* can infect agave plants.

In this study, we showed that 47% of twenty one isolates of *F. oxysporum* obtained from vascular tissues of *A. tequilana* Weber var. azul had similar Box–PCR fingerprints, and others had wider diversity. Genetic similarity is common in plant pathogenic isolates of *F. oxysporum* form a specific *formae specialis* (Klister *et al*., 1991; Namiki *et al.,* 1994; Plyler *et al.,* 2000; Klister, 2001), but some plant pathogenic isolates or non pathogenic native populations have the ability to live like endophytes in non specific hosts (Summerell and Leslie, 2004; Maciá–Vicente *et al*., 2008). We consider that a specific and homologous *F. oxysporum* population pathogenic to agave crop is represented in the group of isolates obtained in this study. Lori *et al.* (2004) showed, in *F. oxysporum* f. sp. *dianthi* isolated from carnation, that genetic homology in intergenic spacer (IGS) type and vegetative compatibility groups (VCG) allow the differentiation of pathogenic isolates from native non pathogenic *F. oxysporum* isolates. Vegetative compatibility groups and ITS sequence diversity studies, with all the agave isolates, are in progress.

We consider that vascular wilt herewith described is one of the causes of agave's 'marchitez' that affects *A. tequilana* Weber var. azul. However, root or stem rots are also important in some fields with similar wilt symptoms. Thus further studies will be necessary to determinate the specific importance of both diseases.

General strategies to control monocyclic soil–borne plant diseases are based in reducing the amount of inoculum or their efficacy at the beginning of the crop in order to get efficient disease management (Madden *et al*., 2007). However, the use of pathogen infected vegetative material in clonally propagated crops like banana or garlic has been an important dispersion path for vascular wilt pathogen such as *F. oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *cepae* to new crop areas (Havey, 1995; Moor *et al*., 2001). Interestingly, propagation of agave plants is done by vegetative material ('hijuelos'). Therefore, the dispersion of his vascular pathogen is similar. Until now, the CRT recommends that agave selection of 'hijuelos' should be done by visual inspection and the elimination of those 'hijuelos' showing evident maroon rot areas. Thus, only healthy 'hijuelos' showing no apparent symptoms should be transplanted after contact fungicide treatment (CRT–Comité Técnico Agronómico, 2005). This recommendation is not enough to control stem rot because it fails to detect and eliminate asymptomatic plants with *F. oxysporum* in xylem and consequently new crop areas could be infested in few months. It is possible that asymptomatic 'hijuelos' have been planted in 'Las Lajitas' and 'El Novillero' fields, which latter developed root rots in young plants. Better strategies are needed to assure the transplant of healthy vegetative propagation material to reduce the incidence of agave's 'marchitez'.

In this study, we used a severity scale to evaluate agave 'marchitez' in the field, which permits an easy and fast method to evaluate xylem injury and root rot severity. This severity scale could be a good tool for epidemiological studies. Both parameters correlate positively with field diseases incidence and the severity of symptoms.

## Acknowledgements

We thank the Consejo del Sistema Nacional de Educación Tecnológica of México for the support of this work with the grant 704.04–P, and a fellowship for J.G. López Z. to obtain his M.Sc. degree.

## References

Aceves, R.J. de J. 1999. Diagnóstico del sistema de producción de agave con énfasis en problemas fitosanitarios. Informe de investigación. Campo Experimental Altos de Jalisco. CIRPAC–INIFAP. Jalisco, México. pp. 4.

Aceves, R.J. de J. 2003. Prevención y manejo integral de la marchitez del *Agave tequilana* Weber var. azul en Jalisco. Folleto Técnico No. 1. Campo Experimental Altos de Jalisco. CIRPAC. INIFAP. Jalisco, México. pp. 62.

Anónimo. 2005. Establecimiento de un sistema de monitoreo de enfermedades por muestreo en *Agave tequilana* Weber var. azul dentro de la denominación de origen del Tequila. Departamento de Desarrollo Rural Sustentable. CUCBA. Universidad de Guadalajara y Consejo Regulador del Tequila, A.C.

Armstrong, G.M., and Armstrong, J.K. 1981. Formae specials and races in *Fusarium oxysporum* causing wilt diseases. pp. 391–399. *In*: Nelson, P., Tousson, T.A., and Cook, R.J. (Eds.). *Fusarium*: Diseases, Biology, and Taxonomy. Pennsylvania State University. Park, USA.

Beckman, C.H. 1987. The Nature of Wilt Diseases of Plants. APS Press, USA.

Booth, C. 1977. *Fusarium*. Laboratory Guide to the Identification of the Major Species. CMI (Ed.). Kew, Ingland. 58 pp.

Consejo Regulador del Tequila (CRT)–Comité Técnico Agronómico. 2005. Plagas y Enfermedades del *Agave tequilana* Weber var. azul. Ed. Pandora. México.

Consejo Regulador del Tequila (CTR). 2008. Información estadística Enero–Julio 1995–2008. http://crtnew.crt.org.mx/images/Documentos/Inventario%20InventarioEnero2007.pdf.

Eguiarte, F.L.E., and González, G.A. 2007. De genes y magueyes estudio y conservación de los recursos genéticos del tequila y el mezcal. *Ciencias* 87: 28–35.

Havey, M.J. 1995. *Fusarium* Basal Plate Rot. pp. 10–11. *In*: Schwartz, H.F. and Mohan, S.K. (Eds.). Compendium of onion and garlic diseases. APS Press, USA.

Inoue, I., Namiki, F., and Tsuge, T. 2002. Plant colonization by the vascular wilt fungus *Fusarium oxysporum* requires FOW1, a gene encoding a mitochondrial protein. *Plant Cell* 14: 1869–1883.

Klister, H.C., 2001. Evolution of Host Specificity in *Fusarium oxysporum*. pp. 70–82. *In*: Summerell B.A., Leslie, J.F., Backhouse, D., Bryden, W.L., and Burgess, L.W. (Eds.). *Fusarium* Paul E. Nelson Memorial Symposium. APS Press, USA.

Klister, H.C., Mamol, E.A., and Benny, U. 1991. Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. *Phytopathology* 81: 331–336.

Lee S.B., and Taylor J.W. 1990. Isolation of DNA from Fungal Mycelia and Single Spores. pp. 282–287. *In*: Innis M.A., Gelfand D.H., Sninsky J.J., and White T.J. (Eds.). PCR Protocols. A Guide to Methods and Applications. Academic Press, USA.

Leslie, J.F., and Summerell, B.A. 2006. Fusarium Laboratory Manual. Blackwell Publishing, USA.

Lori, G., Edel–Hermann, V., Gautheron, N., and Alabouvette, C. 2004. Genetic diversity of pathogenic and nonpathogenic populations of *Fusarium oxysporum* isolated from carnation fields in Argentina. *Phytopathology* 94: 661–668.

Luna H.G. 1996. Pudrición del tallo de *Agave tequilana* Weber en el estado de Jalisco. México. Tesis Profesional. Universidad Autónoma Chapingo. Chapingo, México.

Maciá–Vicente J.G., Jansson H.B., Abdullah S.K., Descals E., Salinas J., and Lopez–Llorca L.V. 2008. Fungal root endophytes from natural vegetation in mediterranean environments with special reference to *Fusarium* spp. *FEMS Microbiol Ecol* 64: 90–105.

Madden, L.V., Hughes G., and Van den Bosch F. 2007. The Study of Plant Disease Epidemics. APS Press. USA.

Moor N.Y., Peeg K.G., Buddenhagen I.W., and Bentley S. 2001. Fusarium wilt of banana: A diverse clonal pathogen of a domesticated clonal host. pp. 212–224. *In*: Summerell B.A., Leslie J.F., Backhouse D., Bryden W.L., and Burgess L.W. (Eds.). *Fusarium Paul E. Nelson Memorial Symposium*. APS Press, USA.

Namiki F., Shiomi T., Kayamura T., and Tsuge T. 1994. Characterization of the formae specialis of *Fusarium oxysporum* causing wilts of cucurbits by DNA fingerprinting with nuclear repetitive DNA sequences. *App Environ Microbiol* 60: 2684–2691.

NTSYSpc, Numerical Taxonomy System, Version 2.2 for Windows, Applied Biostatistical Inc.

Oviedo, E.N., Barragan–Barragan, L.B., Gutierrez–González, P., Ramírez–Hernández, B.C., Ramírez, J.J., and Rodríguez–Garay, B. 2004. Increased photosyntethic efficiency generated by fungal symbiosis in *Agave victoria–reginae*. *Plant Cell Tissue and Organ Culture* 74: 237–241.

Plyler, T.R., Simone, G.W., Fernandez, D., and Kistler, H.C. 2000. Genetic diversity among isolates of *Fusarium oxysporum* f. sp. *canariensis. Plant Pathology* 49:155–164.

Rademaker J.L.W., and de Brujin F.J. 1998. Characterization and classification of microbes by rep–PCR genomic fingerprints and computer assisted pattern analysis. pp. 151–171. *In*: Caetano–Anollés, G. and Gresshoff, P.M. (Eds.). DNA markers protocols, applications, and overviews. Wiley–VCH, USA.

Rodríguez–Gálvez E., and Mendgen K. 1995. The infection process of *Fusarium oxysporum* in cotton root tips. *Protoplasma* 189: 61–72.

SAS Institute Inc. 2000. Statistical Analysis System, Version 6.12. Cary, NC, USA.

Segura G.W. 1977. Presencia de *Fusarium oxysporum* (Scletch) en *Agave sisalana*. pp. 11. Memorias del Seminario Nacional de Fitopatología. Araure, Venezuela.

Summerell B.A., Leslie J.F. 2004. Genetic Diversity and Population Structure of Plant Pathogenic Species in the Genus *Fusarium*. pp. 207–223. *In*: Gillings M., and Holmes A. (Eds.). Plant Microbiology. Bios, Oxford, UK.

Turner P.C., Mc Lennan A.G., Bates A.D., and White M.R.H. 1998. Instant Notes in Molecular Biology. Bios Scientific Publishers. Springer–Verlag, New York.