



Fusarium sp. ASSOCIATED WITH *Vanilla* sp. ROT IN NAYARIT, MEXICO

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ABSTRACT

Vanilla production is threatened by fungi, which cause diseases such as *Fusariumoxysporum*. This research aimed to identify *Fusarium* associated with rot of wild *Vanilla* in Nayarit, México, by morphological, molecular and pathogenic characteristics. Morphological characterization was based on colony color, development of microconidia, macroconidia and chlamydospores on specific media, and growth rate. Molecular analysis was by sequencing the ITS1-5.8S-ITS2 region of rDNA gene. Pathogenicity test was conducted in healthy leaves and stems of wild *Vanilla*. The colonies were classified on four groups by pigmentation. Strains of fast, moderate and slow growth were observed. Of the 40 isolates, morphological analysis showed that 38 corresponded to *Fusariumoxysporum* and two to *Fusariumsolani*. The sequences of the ITS1-5.8S-ITS2 region of 38 strains had a similarity of 98% to 100% with *Fusariumoxysporum*, the remaining two strains with *Fusarium* sp. Nineteen strains originated lesions on leaves from the fourth day and up to 90 days in stems. It was found that *F. oxysporum* is mostly the causal agent of rot of wild *Vanilla* in Nayarit, México.

Keywords: pathogen vanilla, stem and root necrosis, *Fusariumoxysporum* f. sp. *vanillae*.

1. INTRODUCTION

The vanilla crop belong to *Vanilla* genus of the Orchidaceae family; this family includes more than 800 genera and over 25, 000 species (Bory *et al.*, 2008). The *Vanilla* genus has more than 107 species of which only *V. planifolia*, *V. pompona* and *V. tahitiensis* are cultivated (Soto and Dressler, 2010).

In Nayarit state from Mexico, vanilla has been established in small wild populations. *Vanilla* plants from Nayarit were identified as *V. pompona* (Soto and Dressler 2010); the identification was made based on their morphological markers. Productive potential of *V. pompona* it makes of this specie an alternative crop for the state, because their fruits have industrial, alimentary, therapeutic and cosmetic importance.

The *Vanilla* species are attacked by different fungal diseases like root rot, vascular wilt, necrosis and anthracnose caused by *Fusarium oxysporum*, *Chlamidospora vanillae*, *Sclerotium* sp. y *Colletotrichumvanilla*, respectively (Pinaría *et al.*, 2010). Of these fungi, *F. oxysporum* infected a broad range of hosts (Talubnak and Soyong, 2010) and caused the most important vanilla disease. *F. oxysporum* distributed on all vanilla producer regions worldwide (Bhai and Danesh, 2008). Was been reported losses for more of 80% of *V. planifolia* (Pinaría *et al.*, 2010; Vijayan *et al.*, 2012).

Mexico is the origin and distribution center of all vanilla genotypes around the world (Bory *et al.*, 2008;

Lubinsky *et al.*, 2008), however, *Fusarium* root rot has been only reported on *V. planifolia* (Adame-García *et al.*, 2015). For development of an efficient management of this disease it's necessary that the knowledge about the biology and ecology of pathogen be extensive (Gangadhara *et al.*, 2010) and Mexico it's a better place for understand the evolution of this pathogen.

The report made by Adame-García *et al.* (2015) show that very aggressive strains are present on cultivars of *V. planifolia* in the Totonacapan region, center of domestication of vanilla, and that this pathogens can to threat the production. Therefore, if vanilla (tentatively *V. pompona*) can be a crop of economic importance for Nayarit, Mexico, it's necessary an exploration about the presence of *F. oxysporum* f. sp. *vanillae* and other *Fusarium* species associated with vanilla, that was the aim of this study.

2. MATERIAL AND METHODS

2.1 Sampling

During 2014 root samples of wild vanilla plants from seven farms in the municipalities of Xalisco and Ruiz, Nayarit State, Mexico were collected. The collected material had yellowing of the lower stem and leaves with slight necrosis and apparently healthy material (Table-1).

**Table-1.** Geographical location of collecting sites.

Municipality	Property	Geographic localization	Altitude (masl ^x)
Xalisco	Malinal A	N:21.37223 W:105.04379	639
	Malinal B	N:21.37172 W:105.04180	717
	Malinal C	N:21.36900 W:105.03957	716
	El Italiano	N:21.37707 W:104.98861	985
	Oreja de Ratón	N:21.42811 W:104.88986	1005
Ruíz	Cordón del Jilguero A	N:21.9103 W:104.95421	531
	Cordón del Jilguero B	N:21.9103 W:104.95421	531

^xmsnm= meters above sea level

2.2 Monosporic isolates

Healthy and damage roots were cleaned with sterile distilled water, and cut into pieces of 3 to 5 mm. In roots with damage, the fragments were obtained between healthy and diseased tissue (grow area). The segments were treated with a solution of sodium hypochlorite 2% for 5 min twice, washed in three steps of sterile distilled water and transferred to Petri dishes containing potato dextrose agar medium (PDA). The inoculated dishes were incubated at 25° C in an incubator Scorpion Scientific XMT6-808 for five days in the dark.

From colonies, which showed white-cream color to a dark violet, small fragments were taken and transferred to new Petri dishes with PDA. To ensure the genetic purity of the developed colonies suspensions of microconidia were made for spore cultures. Subsequently, the cultures were transferred to PDA culture media, CLA (Carnation Leaves Agar) and SNA (Agar Spezieller Nährstoffarmer).

2.3 Morphological identification

The identification of the isolates was carried out using keys Nelson *et al.* (1983) and lab manual Leslie and Summerell (2006) from colonies of each isolate grown on PDA, SNA and CLA media. Ten days colonies grown on PDA were reviewed by its color characteristics and growth rate. The microscopic features of colonies, microconidia, macroconidia and chlamydospores, were observed in 10 and 15 days grown in the SNA and CLA media, respectively.

2.4 DNA extraction and amplification of the region ITS1-5.8 S- ITS2

DNA was extracted according to the technique of hexadecyltrimethylammonium bromide (CTAB) (Stewart and Via, 1993). Each fungal strain was developed in PDA for eight days. For each culture dish, 2 ml of STE buffer (100 mM NaCl, 10 mM Tris.HCl, 1 mM EDTA, pH 8) was added to resuspend the mycelium. A portion of the

cell suspension was transferred to a sterile mortar frozen. 600 µl of extraction buffer (2% CTAB, 1.42 M NaCl, 20 mM EDTA, 1 M Tris HCl pH 8, 2% PVP-40, and ascorbic acid) were added and the mycelium was crushed with a hand of mortar. The homogenate was transferred to a microcentrifuge tube, and 800 µl of chloroform-isoamyl alcohol (24:1 v/v) solution were added. The solution was stirred until a homogeneous mixture and centrifuged at 1000 g for 5 min. Then were collected 500 to 600 µl of the supernatant into a sterile tube and 700 µl of isopropanol were added. Samples were incubated for 5 min at room temperature and then centrifuged at 14,000 g for 20 min. The supernatant was poured off and the pellet dried by inverting the tubes on paper towels. The pellet was resuspended in 100 µl of nuclease-free water.

For amplification of region ITS1-5.8S-ITS2 of rRNA gene, recommendations of Ochoa *et al.* (2007) were followed. The reaction mixture was formulated with 20 pM of each oligonucleotide ITS1 (GTA GGT GAA GCT 5'TCC GCG G-3) and ITS4 (TCC GCT TAT TGA 5'TCC GC3 TAT), 2.5 mM MgCl₂, 0.25 mM dNTPs, 1x Taq DNApol buffer and 1 U of Taq DNA pol (Promega). The final reaction volume was 25 µl. The thermal program consisted of an initial denaturation at 95 °C for 15 min and then 35 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min; and finally, an extension at 72 °C for 10 min. The fragments were analyzed by horizontal electrophoresis on agarose gel 1.6%, stained in TBE buffer solution added with 2 µl of ethidium bromide (10 mg/ml) and visualized under UV light.

2.5 Sequencing and analysis of amplified

The amplification products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega). Sequencing was performed through the service offered by the Institute of Biotechnology at the Autonomous University of Mexico (UNAM). The sequences were edited with BioEdit 7.1.3.0 (Hall, 1999)



software. Sequences data were analyzed in GenBank at the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) system.

2.6 Phylogenetic analysis

The obtained sequences were aligned with the Multiple Alignment procedure (Bootstraps = 1000; Open Gap Penalties = 15; Gap Penalties Extend = 3) implemented on BioEdit Software 7.1.3.0 (Hall, 1999). The maximum likelihood tree was sought by RxmlGui software with GTR like evolution model. A sequence of *F. verticillioides* (FvX94166) was used like outgroups. Two sequences from strains previously identified like *F. oxysporum* f. sp. *vanillae* were incorporated in the analysis.

2.7 Pathogenicity tests

Pathogenicity was evaluated in leaves and stem cuttings rooted. To ensure the health of plant material a microbiological analysis it was made. Each strain was considered as a treatment, only pathogenic and nonpathogenic strains were identified.

a) Pathogenicity test on leaves: The leaves were washed with running water, they were disinfected with sodium hypochlorite solution 2% for 2 min and two rinses with sterile distilled water were performed. In each leaf, four fragments of mycelium (~5mm diameter) grown on PDA medium for 10 days incubation at $25 \pm 1^\circ \text{C}$ were placed. Two fragments were placed in areas where incisions of 0.5 cm in length were made and two in zones without incisions. The same scheme was used in leaf where free PDA fragments of *Fusarium* were placed. Two leaflets were used for each strain. The samples were placed in humid chambers retaining 100% relative humidity at $25 \pm 1^\circ \text{C}$ for 12 days. It was monitored daily for the initial stages in which the strains were able to generate

symptoms. As a confirmatory test was re-isolated *Fusarium* sp. from those samples where the characteristic symptomatology of the disease was observed and compared with the initial strain.

b) Pathogenicity tests on rooted stems: The experiment was developed in a set of black bags (10x15 cm) with 500 g of soil inoculated with 10 ml of spore suspension (10^6 CFU ml^{-1}) of *Fusarium* sp. The initial spore suspension was prepared by adding 2 ml of Tween 20 (20%) to the Petri dish with *Fusarium* culture. Each suspension was poured into test tubes with 8 ml of sterile distilled water and mixed using a vortex mixer; then, dilutions were made to concentration of 10^6 CFU ml^{-1} determined by a Neubauer chamber. In addition a control solution without fungal inoculum containing 2 ml of Tween 20 (20%) and 8 ml of sterile distilled water was prepared.

Stem cuttings rooted ~30 cm in length with two knots were used. The cuttings were planted two days after inoculation of the fungus to the substrate. The assay was conducted with three replicates for each treatment. The presence or absence at 60 days after implantation was determined. As a confirmatory test was re-isolated *Fusarium* sp. from those samples where the characteristic symptomatology of the disease was observed and compared with the initial strain.

3. RESULTS AND DISCUSSIONS

3.1 Isolation and obtaining pure fungal cultures

In each location a different number of strains was obtained, to which were assigned a key composed of letters and numbers; the letter indicate the place from origin follow by the number of root fragment from that was isolated. In total 40 *Fusarium* sp. strains were obtained (Table-2).

Table-2. Identification keys by place, organ and root fragment from that was isolated the *Fusarium* strains.

Property	Fragments	ID key
Malinal A	10	A1, A2, A3, A4, A7, A8, A9, A10
Malinal B	10	B1, B2, B3, B3 ₂ , B7, B10
Malinal C	16	C10, C16
El Italiano	29	R1-3, R1-5, R1-7, R1-9, R1-10, R1-15, R1-20, R1-24, R1-25, R1-26, R1-28, R1-28b
Oreja de Ratón	26	R2-1, R2-7, R2-9, R2-10, R2-11, R2-12, R2-16, R2-21, R2-25, R2-26
Cordón del Jilguero A	10	BCA1
Cordón del Jilguero B	10	BCB1

3.2 Morphological identification of *Fusarium* sp.

The colonies had different chromaticism in PDA medium, so that, for identification were separated into four groups: white, white-brown, pink-with and purple color, where the last were the most abundant.

This proves that in the genus *Fusarium* an important factor is the production of pigments, since some species are sensitive which can cause varying widely in coloration (Leslie and Summerell, 2006). The isolates obtained showed a coloration in several purple hues,



which, according Britz *et al.* (2005), it is characteristic of *Fusarium* because secondary metabolites as bikaverin, reddish pigment mycelium may turn from dark violet to white because the nitrogen source contained in the medium or the acidity thereof (Wiemann *et al.*, 2009). In the case of *F. oxysporum* isolates, initially colonies with white aerial mycelium and changes over time to pale pink or violet, consistent with those reported by Diaz *et al.* (2007) presented and also described by Brown *et al.* (2012) for *F. verticillioides*.

In addition to temperature, the pH and light, *Fusarium* growth is determined by the nutritional components of the culture medium among which nitrogen and carbon (Kavanagh, 2005). On PDA, *Fusarium* sp. isolates produced abundant and dense mycelium on the surface of the medium, according Leslie and Summerell (2006), this is because this substrate is rich in carbohydrates that allow the development of these fungal structures. However, the strains showed different growth patterns to 10 days of incubation, so that were classified into three groups: 1) fast-growing strains reached 8 cm or more 2) moderate, 7 to 8 cm and 3) slow, minor strains to 7 cm. Thus, 40 strains obtained more than 50% of the colonies showed rapid growth.

On SNA medium, the 40 strains developed macroconidia with three to five septa. 38 colonies in these structures were observed slightly thin, concave, basal cell with a foot-shaped, coinciding with the descriptors for *F. oxysporum*. The macroconidia of remaining two strains were thicker and basal cell cylindrical shape, characteristics of *F. solani* (Leslie and Sumerell, 2006). Macroconidia are important structure in identifying species of the genus *Fusarium*, the morphology of this structure in some cases it is sufficient to identify to species (Agrios, 2005). However, O'Donnell *et al.* (2015) showed that the morphological type Elegans is not unique for *F. oxysporum*, because it's present in others phylogenetic species.

In all isolates of *Fusarium* sp., microconidia are seen abundantly arranged in monophialides with false heads, characteristics that match 12 species of *Fusarium* reported by Leslie and Summerell (2006), however only *F. oxysporum* and *F. solani* have been associated with vanilla, where only the first has behaved like a fungal pathogen (Pinaría *et al.*, 2010). Besides, other species of *Fusarium* with false heads in monophialides have more than one type of grouping microconidia or may present also polyphialides. To distinguish between these two species, length of phialide was used as a reference, thus short phialides 38 strains and two long phialides were found, which confirmed *F. oxysporum* and *F. solani*, respectively.

Microconidia were oval without septa. Leslie and Summerell (2006) describe microconidia of *F. oxysporum* like oval, elliptical or kidney shape, without the presence of septa, they are presented in false heads aerial mycelium short and monophialides; while for *F. solani* refer oval shape, ellipsoid, kidney apparently fusiform and usually without septa, although occasionally present one

or two, and are presented in false heads with long monophialides end.

In CLA medium at 17 days is observed abundantly chlamydospores development in most of the isolates. These structures exhibited spherical shape and interleaved with terminal location in the hyphae, simply appearing in pairs and only two isolated shown chlamydospores in short chain of three to four. It is noteworthy that the two strains that showed this characteristic matching the two strains identified as *F. solani* by the macro and microconidia features. Chlamydospores are important for the description of several species of *Fusarium*, based on their presence or absence can determine the species of this genus, but also the layout and how they are presented can be taken as a characteristic for identification (Leslie and Summerell, 2006). These structures have the ability to survive dormant in the soil for several years under unfavorable conditions even in the absence of host plants and remain dormant until they find appropriate to germinate (Freeman and Maymon, 2000; Morse *et al.*, 2000; Hennessy *et al.*, 2005).

3.3 Molecular analysis

ITS amplification generated 550 bp products, consistent with the results of Fraire-Cordero *et al.* (2010) and Ochoa *et al.* (2007) for *Fusarium* fungi. BLAST analysis of sequences of 38 isolates of strains morphologically as *F. oxysporum*, showed a similarity of 98% to 100% with sequences *F. oxysporum* f. sp. *vanillae* (KM005088.1, KM005087.1, KM005086.1, KM005084.1, KM005080.1) and *F. oxysporum* (KM979995.1, KM980005.1, KM979515.1, KP276620.1, LC026138.1, KM979515.1, KM579585.1, KM980005.1, KP276620.1) while the sequences of the isolated B7 and R2-1 identified by morphology as *F. solani*, shown similarity values with 78% (JQ316463.1) and 89% (EU091028.1) with sequences reported as *Fusarium* sp. and *F. oxysporum*, respectively. The sequences were compared with the amplified *F. oxysporum* f. sp. *vanillae* obtained by Adame-García *et al.* (2011) from samples of Veracruz, Mexico.

The fact that by morphological identification has been determined the presence of two strains of *F. solani* in roots of vanilla and this has not been fully corroborated by molecular analysis could be due to the high morphological variability that *F. oxysporum* in culture media. Therefore, based on this variation Chandra *et al.* (2011) recommend resorting to DNA tests, which have made significant progress in the identification of plant pathogenic fungi, especially *Fusarium*. In this regard, Riveros *et al.* (2001), through the analysis of ITS, they found that a strain identified as *F. solani* morphologically, genetically associated in the group of *F. oxysporum*, a finding consistent with the results obtained in this work. It is considered desirable to use the sequence of more variables genes for the elucidation of the identity of problem strains (Geiser *et al.*, 2004; Rahjoo *et al.*, 2008).



The resulting tree maximum likelihood analysis showed that the sequences obtained starting from *F. solani* strains are grouped separately in a single clade, while *F. oxysporum* sequences were located in two major clades. This suggests that these strains are actually different

species; however, both the morphological analysis and the ITS1-5.8 S-ITS2 region were not suitable for elucidation of identity (Figure-1). The distribution of *F. oxysporum* strains into clades of tree has not a geographic separation.

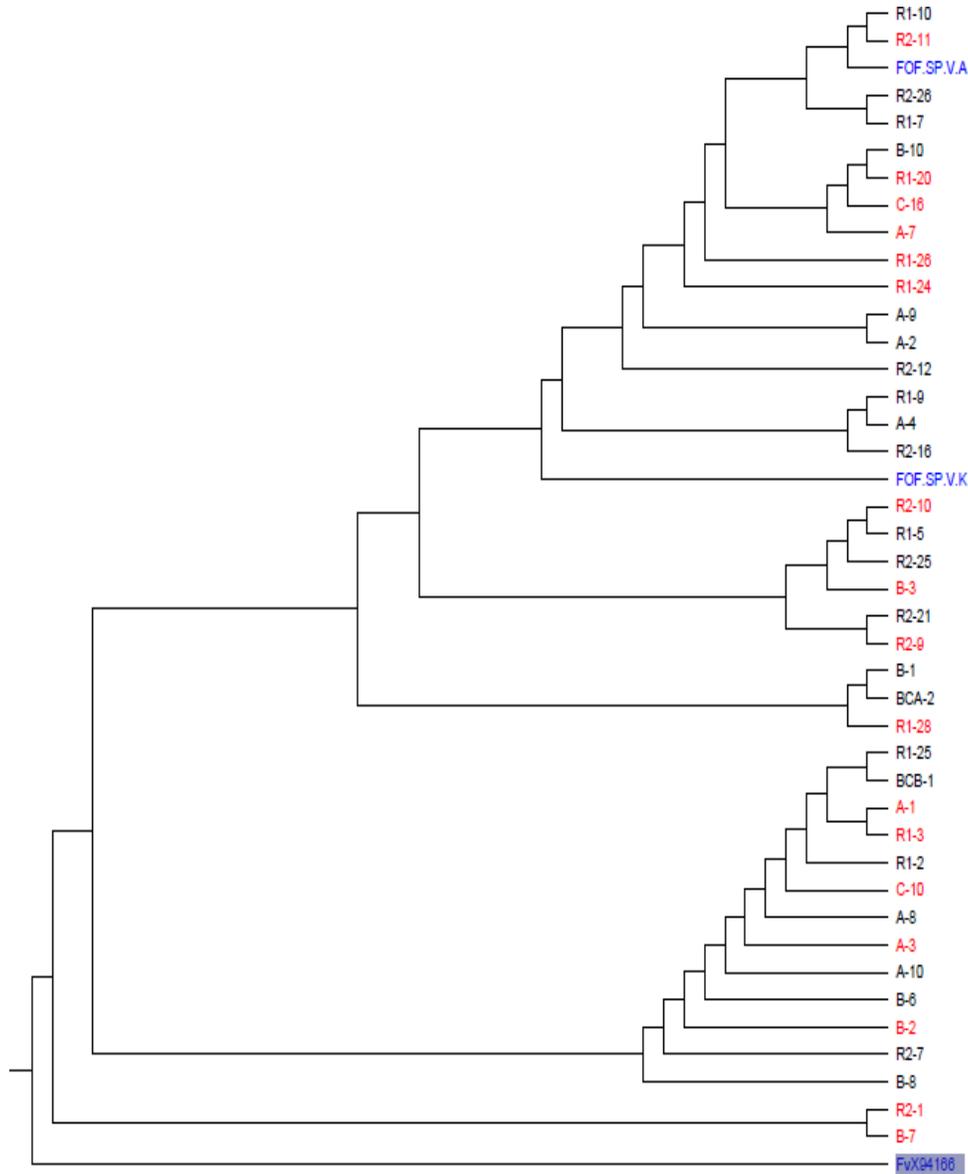


Figure-1. Maximum Likelihood tree that shows phylogenetic relationships among *Fusarium* sp. isolated from *Vanilla* sp. root. Terminals on red are pathogenic strains on leaf and stem tests. R2-1 and B-7 strains were identified as *F. solani* by morphological features. They are phylogenetically separated to *F. oxysporum* clades. FvX94166 (*F. verticillioides*) outgroup.

It has been found that phylogenetic analyzes within the complex *F. oxysporum* are not resolved according to the host they infect, strains belonging to the same special form have a polyphyletic distribution within the species, such as *F. oxysporum* f. sp. *vanillae* (Pinarria *et al.*, 2015). Inami *et al.* (2014) disclose that this may be due to horizontal gene transfer is carried out from a pathogenic

strain to a nonpathogenic, where this shares the genes that cause the disease, also known as SIX (secreted in xylem) genes and there recent evidence that the transfer of these genes gives specificity to a host strains of *F. oxysporum* (Laurence *et al.*, 2015).

3.4 Pathogenicity tests on leaves



Pathogenicity tests showed that 19 of the 40 strains of *Fusarium* generated rot. The development of infection was evident at four days after inoculation. Infections are characterized by the presence of aqueous necrotic lesions with whitish mycelium on the leaf surface. The remaining 21 strains showed no leaf damage, so not considered pathogenic fungi.

The strains showed differences in the pathogenic response in the day to the damage were observed. Thus three groups were formed: the first consists of the A7, C16, and C10 R2-1 strains that showed damage from the fourth day post inoculation (dpi), which are considered more virulent strains; the second consists of the R2-9, R2-10, R2-11, B3 and B32 strains, where damage was evident from the fifth dpi; and the third batch of the respective strains made from 6 to 11 dpi. In general it was found that 50% of the strains were able to develop *Fusarium* rot on leaf tissue of *Vanilla* sp.

Adame-García *et al.* (2011) reported that 12 days are sufficient to determine if a strain is pathogenic or not; in this study it is found that by testing pathogenicity of strains vanilla leaves can differentiate pathogenic and nonpathogenic strains. Sometimes the environment is very favorable for the fungi's growth, and the fact that the soil is its natural reservoir, its ability to survive in latency and prompt conidial dispersion are reasons that can explain their presence in leaves as a resident and not as a pathogen (Agrios, 2005). However, although *F. oxysporum* be known as causing root rot and stem vanilla, does not exclude that it can be isolated from rots leaves. Pinaria *et al.* (2010) point out that stems rot and severe infections in leaves and roots of vanilla in Indonesia it is due to *F. oxysporum*, which has led to significant losses.

It was considered that *F. oxysporum* fungus is causal agent of root rot in vanilla and *F. solani* is an endophytic hedge, thus which grow into the plant tissue without causing any symptoms (Pinaria *et al.*, 2010). The results of this study as to what obtained by Adame-García *et al.* (2015) shown that not only *F. oxysporum* is pathogenic for vanilla, but *F. solani* is also pathogenic, although it is isolated less frequently and less aggressive than *F. oxysporum*.

3.5 Pathogenicity tests on stems

Rooted *Vanilla* sp. stems inoculated with the fungus spore suspension showed mild symptoms of infection at three months after inoculation; the first sign of damage appreciated by *Fusarium* was a slight chlorosis at the base of the plant. Adame-García *et al.* (2015) found that at 30 days of inoculation *F. oxysporum* generated necrotic lesions on stems of plants of *V. planifolia*; necrotic lesions had a watery appearance with chlorotic halos that developed descending manner which caused the wilting of the plant and therefore death.

Coincidentally, the strains that caused rot leaves were the same that caused disease in stems, confirming that these strains are pathogenic to vanilla, results consistent with those obtained by Adame-García *et al.* (2011), who found differences in pathogenicity of strains, and that it was consistent both leaves and stems rooted in.

Pinaria *et al.* (2010) and Santa *et al.* (2012), indicate that, in vanilla, are several species of *Fusarium* but only *F. oxysporum* f. sp. *vanillae* is related to vanilla stem rot in Indonesia and Colombia. Studies in Asian and African countries have determined the presence of different fungi and associated oomycetes to rots, among which are: *Phytophthora meadii*, *Fusarium oxysporum*, *Calospora vanillae*, *Sclerotium* sp, *Colletotrichum gloeosporioides*, *Colletotrichum vanilla* and *Cylindrocladium quinquiseptatum* and others (Thomas and Bai, 2000; Bhai and Dhanesh, 2008).

Basal rot is not a seasonal disease and is widely distributed in areas that produce vanilla. There are reports of this infection in India (Vijayan *et al.*, 2012), China (Tombe and Liew, 2011), Indonesia (Pinaria *et al.*, 2010), among others. It has been estimated that this disease has caused losses of up to 80% on fields (Pinaria *et al.*, 2010). The attack of this fungus grows stronger when high levels of humidity present in the soil, too much shade, high plant density and lack of sanitary practices (Bhai and Dhanesh, 2008).

4. CONCLUSIONS

The morphological identification determined that of 40 isolated strains, 38 were identified as *F. oxysporum* and two as *F. solani*. Molecular analysis was inconclusive in identifying strains of *F. solani* since these showed greatest similarity to strains identified as *Fusarium* sp. and *F. oxysporum*.

Of the 40 strains, 19 had capacity to generate damage both the foliage and stem. In the leaves, the symptom of infection appeared four days after the inoculation of the fungus and in stems, at 90 days. Sheet infections are characterized by the presence of necrotic lesions watery with white mycelium on the surface, and stems began with a slight chlorosis at the base followed by necrotic lesions watery with chlorotic halos that were developed down manner causing wilt and therefore the death of the plant.

By correspondence with the host is determined that *F. oxysporum* f. sp. *vanillae* is the causal agent for the root and stem rot of wild *Vanilla* sp. on Nayarit, Mexico.

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