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ADEQUACY OF A PROTOCOL FOR AMPLIFICATION OF EF-1α GENE OF *Fusarium oxysporum* f. sp. *vanillae*

Jacel Adame-García[1, 2], Felipe Roberto Flores-de la Rosa[2], Jorge Ricaño-Rodríguez [3] and Mauricio Luna-Rodríguez[4]

[1]Instituto Tecnológico de Úrsulo Galván, Tecnológico Nacional de México, Carretera Cardel-Chachalacas, C.P. 91667, Úrsulo Galván, Veracruz, México

[2]Instituto de Biotecnología y Ecología Aplicada, Universidad Veracruzana, Campus para la Cultura, las Artes y el Deporte, Av. de las Culturas Veracruzanas, Col. Emiliano Zapata, C.P. 91090, Xalapa, Veracruz, México

[3]Laboratorio de Alta Tecnología de Xalapa, Universidad Veracruzana, Médicos, Col. Unidad del Bosque, C.P. 91010, Xalapa, Veracruz, México

[4]Centro de Eco-Alfabetización y Diálogo de Saberes, Universidad Veracruzana, Campus para la Cultura, las Artes y el Deporte, Av. de las Culturas Veracruzanas, Col. Emiliano Zapata, C.P. 91090, Xalapa, Veracruz, México

E-Mail: mluna@uv.mx

ABSTRACT

Fusarium oxysporum f. sp. *vanillae* is the causal agent of stem rot in vanilla worldwide. In Mexico, this plant pathogen has been identified by their morphological characteristics and ITS sequence of rRNA, however, due to the complexity of the taxonomy of the genus, it is considered necessary to confirm this identity with the sequence of the EF-1α gene. The methodologies reported for the amplification of this gene are variable and do not show optimal results. In this study it was proposed to adapt a protocol for genomic DNA extraction and amplification of EF-1α gene for *F. oxysporum* f. sp. *vanillae*. It was observed that the modification of protocol developed for bacteria was more efficient that one based on CTAB. A decrease in the concentrations of dNTP's and primers also an increase in annealing temperature regarding a protocol reported in the literature allowed to obtain amplification products unique and good quality. Once that was confirmed that strains belong to *F. oxysporum*, sequences were deposited in GenBank.

Keywords: *Fusarium,* translation elongation factor, molecular identification, *Vanilla planifolia*, stem rot.

INTRODUCTION

Fusarium oxysporum is a ubiquitous inhabitant of soil with relevant ecological roles that place it as an endophyte (Aimé *et al*., 2013), saprophyte (Fracchia *et al.,* 2000) and pathogen (O'Donnell *et al*., 2010). In his role as phytopathogen, has been identified over 100 special forms affecting a wide variety of agricultural crops (Lievens *et al*., 2008).

Stem rot in *Vanilla planifolia* Jacks ex Andrew is the main disease in the crop producing areas and is caused by *Fusarium oxysporum* f. sp. *vanillae* (Alconero, 1968). Progress has been made in the diagnosis of this pathogen in the main producing countries worldwide as Indonesia (Pinaria *et al*., 2011) and Madagascar (Koyyappurath *et al*., 2015). In Mexico, center of origin, domestication and distribution of vanilla, Adame-García *et al*. (2015) identified highly pathogenic strains from the Totonacapan region. ITS rRNA region, which is the most widely used marker for discriminating level fungal species was used in this study (O'Donnell *et al*., 1998a; O'Donnell, 2000). However, many *Fusarium* species inside the *Gibberella* clade, have non-orthologous copies of ITS2, generating incorrect phylogenetic inferences (O'Donnell and Cigelnik, 1997; O'Donnell *et al*., 1998a) and therefore is not reliable in describing phylogenetic species.

The genus *Fusarium* has had a confused and unstable taxonomic history. A large number of factors, including the lack of clear morphological characters to separate species, variation and mutation in the culture medium and too broad species concept have led to the creation of taxonomic systems that poorly reflect the diversity of *Fusarium* species (Summerell *et al*., 2003; Geiser *et al*., 2004).

Based on the above, it has highlighted the importance of the use of translation elongation factor 1α gen (EF-1α) (Geiser *et al*., 2004). This gene encodes an essential part of the translation machinery proteins and has a large phylogenetic utility because it is (i) highly informative level *Fusarium* species; (ii) they have not been detected non-orthologous copies of this gene within the genus; and (iii) universal oligonucleotides are designed to act through the wide phylogeny of the genus. The gene was first used by Cho *et al*. (1995) and Mitchell *et al*. (1997) as to infer phylogenetic relationships among species and genus level in Lepidoptera marker.

The primers used for amplification of EF-1α gene in *Fusarium* were developed by O'Donnell *et al*. (1998b) to investigate lineages within the complex *F. oxysporum* causes Panama disease in bananas. These primers amplify a region of ~ 700 bp of the gene, flanked by three introns. This gene is consistently present in all species of *Fusarium* in single copy and displays a high level of sequence polymorphism between closely related species, even if compared with genes encoding proteins such as calmodulin, beta-tubulin and histone H3 (Geiser *et al*., 2004). Therefore, analysis of the sequence of this gene confirms the results presented so far for *F. oxysporum* f. sp. *vanillae* in Mexico.

Although the use of this gene is broad, both genomic DNA extraction and gene amplification protocols are highly variable in literature (O'Donnell *et al*., 1998; Geiser *et al*., 2004; Pinaria *et al*., 2010; Wang *et al*., 2011)

therefore, the aim of this study was to adapt a protocol for DNA extraction and amplification of EF-1α gene from *F. oxysporum* f. sp. *vanillae*.

MATERIALS AND METHODS

Obtaining *F. oxysporum* f. sp. *vanillae*

Strains previously identified as *F. oxysporum* f. sp. *vanillae* were used from the collection strains of Laboratorio de Alta Tecnología de Xalapa, under the stewardship of Dr. Mauricio Luna-Rodríguez. The strains are from Veracruz, isolated and identified by Adame-García *et al*. (2015).

DNA extraction

Two methods of DNA extraction are compared. The first consisted of a protocol used in Gram positive, Gram negative bacteria and yeast reported by Cheng and Jiang (2006) with some custom modifications. To this, there was collected approximately 500 mg mycelium of each of the isolates of *F. oxysporum* f. sp. *vanillae* of 10 to 14 days of growth on PDA, which were placed in sterile mortar to maceration and 400 µl of TE buffer (Tris / HCl 10 mM, EDTA 1 mM, pH 8.0) and 200 µl of Tris-saturated phenol 2 M pH 8.0 were added. After maceration, the solution was transferred to 1.5 ml sterilized tube and centrifuged at 8000 g for 5 min. 160 µl of the supernatant was taken and transferred to a clean 1.5 ml tube. Next were added 40 ml of TE buffer and 100 ml of chloroform, and centrifuged for 5 min at 13000 g. The lysate was purified with chloroform twice until it wasn't presented a white interphase. Subsequently 160 µl of the supernatant were taken, transferred to a clean 1.5 ml tube and 40 µl of TE and 5 µl of RNase (10 mg/ml) were added. The solution was incubated at 37 °C for 10 min and finally added 100 ml of chloroform, mixed by inversion and centrifuged for 5 min at 13000 g. 150 µl of the supernatant was transferred to a clean 1.5 ml tube. The aqueous phase contained the purified DNA.

The second protocol used involved the use of cetyltrimethylammonium bromide (CTAB) as an agent for cell lysis, a modification to CTAB method of Doyle and Doyle proposed by Stewart and Via (1993) was used, and application was made as indicated by Luna-Rodríguez *et al*. (2005) and Iglesias-Andreu *et al*. (2010) in success full tests in plants.

To assess the integrity degree of the DNA was carried out an agarose gel electrophoresis 0.8% (0.5 X TBE) in a horizontal chamber (CONSORT) and 100 V were applied consistently. Gels were stained in 100 ml of TBE 1X solution added with 2 ml of ethidium bromide (10 $mg.ml^{-1}$) for 20 min and visualized with MicroBis photo documentation system.

PCR amplification

Translation elongation factor 1α gene (EF-1α) was amplified using the EF-1 (5'-ATGGGTAAGARGACAAGAC-3') and EF-2 (5'-GGARGTACCAGTSATCATGTT-3') oligonucleotides proposed by O'Donnell *et al*. (1998a). The reaction was performed with specifications reported by Pinaria *et al*. (2010), consisting of using a reaction volume of 25 µl composed of PCR buffer 1X, $MgCl_2$ 2.5 mM, Taq DNA pol 1 U (Promega), dNTPs 1 mM, 0.25 mM of each primer (EF-1 and EF-2) and ~ 50-100 ng of genomic DNA. Amplifications were performed in a thermocycler (BioRad) under the following thermal cycling program: an initial denaturation for 1 min at 97°C; 35 cycles of denaturation at 96 °C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min. Modifications to the protocol described above to obtain a single amplification product of the expected size were performed.

Sequencing and bioinformatic analysis

The amplification products were purified with the kit Wizard ® SV Gel and PCR Clean-Up System (Promega) and sent to the Institute of Biotechnology of the UNAM for sequencing. The sequences were edited on the Bioedit 7.2.5 (Hall, 1999) software and compared by BLAST in the GenBank (NCBI) and FUSARIUM-ID (Fusarium Research Center, Geiser *et al*., 2004).

RESULTS AND DISCUSSIONS

DNA extraction using the CTAB method (Stewart and Via, 1993) was not successful, because in the final phase to precipitate the DNA with isopropanol a pink pigmentation in the precipitate was presented; showing the presence of impurities related *Fusarium* characteristic pigments, corroborated by the results of electrophoresis in 0.8% agarose gel (Figure-1). Also, in some samples DNA wasn´t obtained.

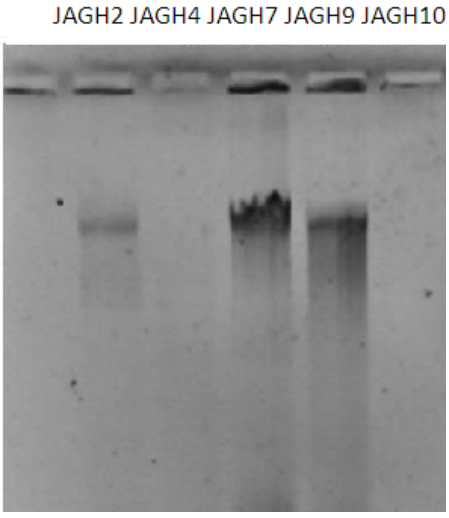

Figure-1. Agarose gel electrophoresis (0.8%) where the poor quality of the bands of genomic DNA extracted with CTAB from *F. oxysporum* f. sp. *vanillae* was observed. JAGH: code strain 2, 4, 7, 9 and 10.

In contrast, the method modified from that proposed by Cheng and Jiang (2006), proved efficient for extracting DNA of *F. oxysporum* f. sp. *vanillae*, achieving

to obtain degraded material and best quality in all the samples (Figure-2). This might be attributable to that among the modifications made to the original extraction method is the use of mortar to grind the mycelial tissue. In the original protocol cell lysis is performed with phenol alone because it is an oxidizing reagent, this directly breaks the cell and nuclear envelope of the genomic DNA. Schoffelmeer *et al*. (1999, 2001) and Fukamizo *et al.* (1996) showed that the cell wall of *F. oxysporum* has a complex structure of two layers based on different carbohydrateslike chitin, maltose, glucans and glycoproteins; giving it high mechanical resistance to the fungal wall therefore, maceration was a perfect complement to perform cell lysis with phenol. In other protocols, the grinding was realized with liquid nitrogen; however, the results were poor (González-Mendoza *et al.,* 2010). Moreover, in this protocol the DNA is purified in its final stage with chloroform to remove the whitish interface, which ensures better removal of impurities of proteins. In contrast to the method of CTAB in the modified method of Cheng and Jiang (2006), the supernatant can be used directly in molecular biology protocols, omitting the step of precipitation with ethanol or isopropanol, which reduces the risk of material loss during manual decantation.

Also, the protocol for DNA extraction here presented have another advantage, the centrifugation don´t need a specific temperature. In other protocols, cold conditions of centrifugation are essential (González-Mendoza, *et al.,* 2010; Zhang *et al.,* 2012).

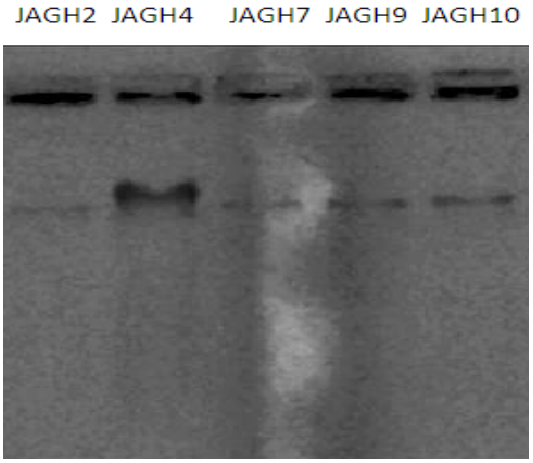

Figure-2. Agarose gel electrophoresis (0.8%) where was observed a band of genomic DNA from the five isolates of *F. oxysporum* f. sp. *vanillae*. JAGH: code strain 2, 4, 7, 9 and 10.

EF1α gene amplification was performed under thermal reaction conditions and as indicated by the proposed procedure Pinaria *et al*. (2010), without satisfactory results. By separating the amplification products on agarose gel (1.8%) it was observed over a fragment of different size than expected (~700 bp) (Figure-3).

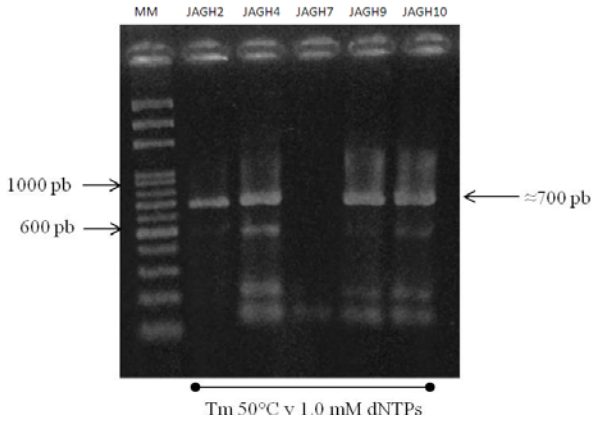

Figure-3. Agarose gel electrophoresis (1.8%) where distinct bands are observed at ~ 700 bp resulting from amplification of EF 1α gene of five *F. oxysporum* f. sp. *vanillae* strains. MM = molecular weight marker. JAGH: code strain 2, 4, 7, 9 and 10.

Therefore, it was necessary to adapt the amplification protocol. To do this, the composition of the reaction mixture was modified, decreasing the amount of dNTPs (1.0 to 0.2 mM) and 20 pM of primers per reaction. With these amplification modifications results were improved, showing less swept and better defined ~700 bp bands (Figure-4).

This showed that were still amplified distinct bands at ~700 bp, so further tests based on the gradual increase in the annealing temperature (Tm) of 50 °C to 55 °C, which was achieved were made that the number of unwanted bands began to decrease (Figure-5). The above results led to the decision to increase the Tm again to 58 °C, allowing amplify only products of ~700 bp well-defined and reproducible (Figure-6).

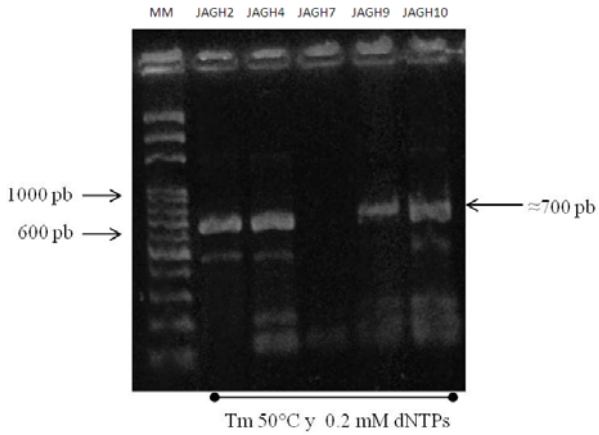

Figure-4. Agarose gel electrophoresis (1.8%) showing bands of ~700 bp resulting from amplification of EF 1α gene of five *F. oxysporum* f. sp. *vanillae* strains when were used 0.2 mM dNTPs and 20 pM of each primer. MM = molecular weight marker. JAGH: code strain 2, 4, 7, 9 and 10.

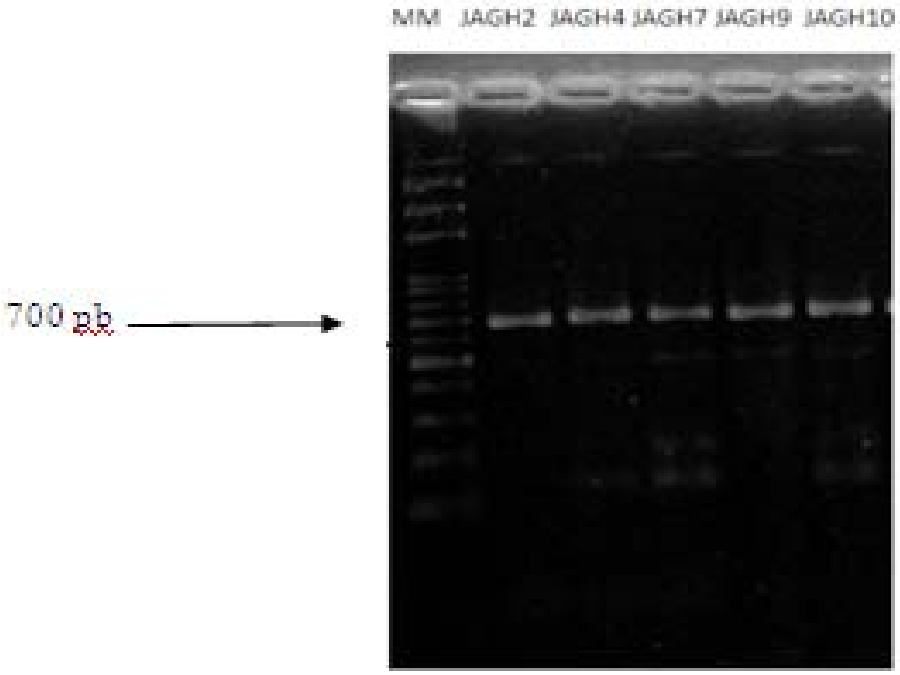

Figure-5. Agarose gel (1.8%), where minor bands at 700 pb were observed from the amplification of EF-1α gene of *F. oxysporum* f. sp. *vanillae* with Tm increasing at 55 C. 55 °C. MM = Molecular weight marker. JAGH: code strain 2, 4, 7, 9 and 10.

Once obtaining a unique amplification product, the sequence was obtained and edited in order to eliminate the regions affected by the sequencing process. The sequences were compared with the available databases and the results are shown in Table-1. The sequences were submitted to Genbank database with accession numbers KU378600 - KU378604.

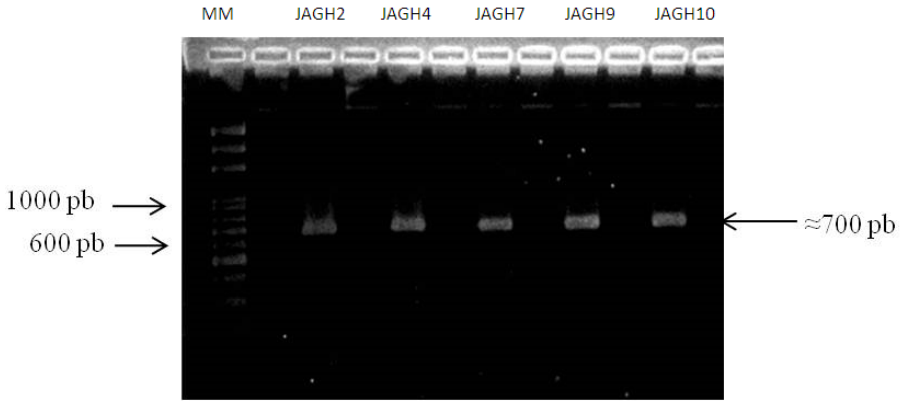

Figure-6. Agarose gel (1.8%) where well defined bands of ~700 bp were observed, produced from the amplification of EF-1α gene of *F. oxysporum* f. sp. *vanillae* strains. Tm at 58 °C. MM = Molecular weight marker. JAGH: code strain 2, 4, 7, 9 and 10.

Table-1. Identity of sequences from strains identified as *F. oxysporum* f. sp. *vanillae* compared two international databases.

Strain	Genbank			FUSARIUM ID		
	Identity	Species	Accession	Identity	Species	NRRRL
JAGH2	99%	*F. oxysporum*	EU246571	99.68%	*F. oxysporum*	38514
JAGH10	94%	*F. oxysporum*	KP964859	93.71%	*F. oxysporum*	40180
JAGH4	90%	*F. oxysporum*	LN828029	91.72%	*F. oxysporum*	26379
JAGH7	99%	*F. oxysporum*	EU091045	99.05%	*F. oxysporum*	32885
JAGH9	98%	*F. oxysporum*	KP964859	98.42%	*F. oxysporum*	40180

CONCLUSIONS

The technique proposed by Cheng and Jiang (2006) is suitable for extracting DNA from *F. oxysporum* f. sp. *vanillae*. Technical changes (decrease in the concentration of dNTPs and primers, and increased Tm) applied to the proposed Pinaria *et al*. (2010) for the amplification of EF-1α gene showed better results than the original protocol. The adequacy of this protocol will allow studies involving the sequence of the gene encoding elongation factor such as population genetics or reconstructing phylogenies.

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REFERENCES

Adame-García J, Rodríguez-Guerra R, Iglesias-Andreu LG, Ramos-Prado JM, Luna-Rodríguez M. 2015. Molecular identification and pathogenic variation of *Fusarium* species isolated from *Vanilla planifolia* in Papantla Mexico. Botanical Sciences. 93(3): 669-678. DOI: 10.17129/botsci.142.

Adame-García J., Trigos-Landa A. R., Iglesias-Andreu L. G., Flores-Estevez N., Luna-Rodríguez M. 2011. Variaciones isoenzimática y patogénica de *Fusarium* spp. asociadas con la pudrición de tallo y raíz de vainilla. Tropical and Subtropical Agroecosystems. 13: 299-306.

Aimé S., Alabouvette C., Steinberg C., Olivain C. 2013. The endophytic strain *Fusarium oxysporum* Fo47: A good candidate for priming the defense responses in tomato roots. Molecular Plant-Microbe Interactions. 26(8): 918-926.

Alconero R. 1968. Infection and development of *Fusarium oxysporum* f. sp. *vanillae* in vanilla roots. Phytopathology. 58: 1281-1283.

Cheng H-R., Jiang, N. 2006. Extremely rapid extraction of DNA from bacteria and yeasts. Biotechnology Letters. 28: 55-59.

Cho S. W., Mitchell A., Regier J.C., Mitter C., Poole R. W., Friedlander T. P., Zhao S. W. 1995. A highly conserved nuclear gene for low-level phylogenetics - Elongation Factor- 1-Alpha recovers morphology-based tree for Heliothine moths. Molecular Biology and Evolution. 12: 650-656.

Damirón-Velázquez R. 2004. La vainilla y su cultivo. Dirección General de Agricultura y Fitosanitaria. Gobierno del Estado, Veracruz, Mex.

Doyle J.J., Doyle J. L. 1990. Isolation of plant DNA from fresh tissue. Focus. 12: 13-15.

Fracchia S., Garcia-Romera I., Godeas A., Ocampo JA. 2000. Effect of the saprophytic fungus *Fusarium oxysporum* on arbuscular mycorrhizal colonization and growth of plants in greenhouse and field trials. Plant and Soil. 223: 175-184.

Fukamizo T., Honda Y., Toyoda H., Ouchi S., Goto S. 1996. Chitinous component of the cell wall of *Fusarium oxysporum*, its structure deduced from chitosanase digestion. Biosciences, Biotechnology and Biochemestry. 60(10): 1705-1708.

Geiser D. M, Jiménez-Gasco M. M., Kang S., Makalowska I., Veeraraghavan N., Ward T. J., Zhang N., Kuldau1 G. A., O'Donnell K. 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology. 110: 473-479.

González-Mendoza D., Argumedo-Delira R., Morales-Trejo A., Pulido-Herrera A., Cervantes-Díaz L., Grimaldo-Juárez O., Alarcón, A. 2010. A rapid method for isolation of total DNA from pathogenic filamentous plant fungi. Genetics and Molecular Research. 9(1): 162-166.

Iglesias-Andreu L., Luna-Rodríguez M., Durán-Vázquez M., Rivera-Fernández A., Sánchez-Coello N. 2010. Marcadores RAPDs asociados a la expresión del sexo en

Ceratozamia mexicana Brongniart (Zamiaceae). Revista Chapingo Serie Ciencias Forestales y del Ambiente. 16(2): 139-145.

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

Luna-Rodríguez M., López-Upton J., Iglesias-Andreu L.G.2005. Variabilidad morfométrica y molecular (RAPD) en una plantación de *Pinus patula* en Veracruz, México. Agrociencia. 39: 231-235.

Mitchell A., Cho S., Regier J. C., Mitter C., Poole R.W., Matthews M. 1997. Phylogenetic utility of elongation factor-1 alpha in Noctuoidea (Insecta: Lepidoptera): The limits of synonymous substitution. Molecular Biology and Evolution. 14: 381-390.

O'Donnell K. O. 2000. Molecular phylogeny of the *Nectria haematococca - Fusarium solani* species complex. Mycologia. 92: 919-938.

O'Donnell K. O., Cigelnik E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular and Phylogenetic Evolution. 7: 103- 116.

O'Donnell K. O., Cigelnik E., Nirenberg H. I. 1998a. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia. 90: 465-493.

O'Donnell K. O., Kistler H. C., Cigelnik E., Ploetz R. C. 1998b. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences of the United States of America. 95: 2044-2049.

O'Donnell O. K., Nirenberg H. I., Aoki T., Cigelnik E. 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. Mycoscience. 41: 61-78.

Pinaria A. G., Liew E.C. Y., Burgess L. W. 2010. *Fusarium* species associated with vanilla stem rot in Indonesia. Australasian Plant Pathology. 39: 176-183.

Schoffelmeer EAM, Klis FM, Sietsma JH, Cornelissen BJC. 1999. The cell wall of *Fusarium oxysporum*. Fungal Genetics and Biology. 27: 275-282.

Schoffelmeer EAM, Vossen JH, van Doorn AA, Cornelissen B. J, Haring MA. 2001. FEM1, a *Fusarium oxysporum* glycoprotein that is covalently linked to the cell wall matrix and is conserved in filamentous fungi. Molecular Genetics and Genomics. 265(1): 143-152.

Stewart C. C., Via L. E. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques. 14: 748-749.

Summerell B. A., Salleh B., Leslie J. F. 2003. A utilitarian approach to *Fusarium* identification. Plant Disease. 87: 117-128.

Taylor J. W., Jacobson D. J., Kroken S., Kasuga T., Geiser D. M., Hibbett D. S., Fisher M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology. 31: 21-32.

Tombe M., Komoto Y., Tezuka N. 1993. Identification and cultural types of *Fusarium* isolates from *Vanilla* in Indonesia. Industrial Crop Research Journal. 6: 1-5.

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-98.

Zhang S., Zhao X., Wang Y., Li J., Chen Z., Wang A., Li J. 2012. Molecular detection of *Fusarium oxysporum* in the infected cucumber plants and soil. Pakistan Journal of Botany. 44(4): 1445-1451.