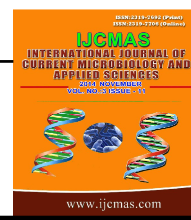
Int.J.Curr.Microbiol.App.Sci **(2014) 3(11) 157-165**



International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 11 (2014) pp. 157-165**
http://www.ijcmas.com

Original Research Article

Discrimination of different pathogenic *Fusarium oxysporum* isolates based on virulence and intergenomic-spacer sequences (IGS)

Mohamed E. SELIM[1*] and Abdel Fattah M. El Zanaty[2]

[1]Agricultural Botany Department, Faculty of Agriculture, Menoufiya University,
[2]Genetics Department, Faculty of Agriculture, Menoufiya University, Shibin El-Kom, Egypt
**Corresponding author*

A B S T R A C T

Fusarium oxysporum is one of the most dominant fungal inhabitants of the soil worldwide. Some isolates belonging to species in the genus *Fusarium* are plant pathogenic organisms as they can cause wilting, yellowing and yield losses. Conversely, many isolates belonging to the same species of *Fusarium* are non-pathogenic and in some cases even beneficial for the host plant. This variation makes the discrimination and determination of pathogenicity and virulence potential of *Fusarium* isolates important and relevant to plant health. Traditionally, bioassays conducted under greenhouse conditions were used for predicting whether isolates of *Fusarium* are pathogenic to specific host plant or not. However, such bioassays and pathogenicity tests are complex and time consuming. Molecular discrimination has become a good alternative to bioassay tests in the process of characterization of *Fusarium* isolates as well as for many other organisms. The objective of this study was to find a possible marker for potential virulence in highly pathogenic verses weak pathogenic *F. oxysporum* isolates. Using two specific primers for *F. oxysporum* species, PN22 and PNfo, the intergenic-spacer (IGS) amplicons were generated for 11 different *F. oxysporum* isolates which were isolated from 5 different governorates in Egypt during 2010 and 2011 seasons. Sequencing of IGS amplicons revealed that the phylogenetic diversity of the examined isolates. The results showed that the most virulent isolates had higher similarity to each other when compared to the less virulent isolates. Conversely, the results indicated that there is a correlation between IGs regions and the geographic distribution of the *Fusarium* isolates. The isolates which were isolated from the same governorate were more similar to each other than those isolated from other governorates. In conclusion, PCR amplification of intergenomic-spacer fragments can be used as a tool for discrimination of *F. oxysporum* isolates and for initial separation of geographic relationships between isolates.

Keywords

Fusarium oxysporum, Pathogenicity, PCR, Virulence

Introduction

Fusarium oxysporum Schlechtend.: Fr. is a fungal species complex that is frequently encountered in soils and in plant roots worldwide. It comprises of soil borne saprophytic, root endophytic and plant pathogenic isolates. Plant pathogenic isolates can cause severe damage to host plants. Based on host specificity, *Fusarium*

isolates are grouped into formae speciales (f. spp.), which are sometimes further divided into races based on cultivar specificity (Olivain and Alabouvette 1997, 1999; Olivain *et al.,* 2003; Armstrong and Armstrong, 1981; Di Pietro *et al.,* 2003; Michielse and Rep, 2009). So far, about 120 formae speciales and races have been described (Armstrong and Armstrong 1981; Gordon and Okamoto, 1992; Alabouvette *et al.,* 2001). Some of these forma specialis may cause diseases in specific plant species, while the other formae speciales belonging to same species may not be harmful and may even develop a neutral or beneficial mutualistic interrelationship with the host plant (Mandeel and Barker 1991; Hallmann and Sikora 1994a, 1994b; Sikora and Fernandez, 2005; Sikora *et al.,* 2007). Some of the non-pathogenic strains can also colonize a host plant without causing disease symptoms. These endophytic isolates may play a beneficial role, since they have been shown to increase plant resistance to fungal pathogens, plant parasitic nematodes or insects (Dababat *et al.,* 2008).

To detect the presence of virulent isolates of *Fusarium* wilt in the soil or tissue of potential hosts in a certain growing region, the characterization of the local *F. oxysporum* populations is highly important. Pathogenicity tests are often used for testing of virulence levels of *F. oxysporum* isolates on specific cultivars rather than to determine the formae specialis. Such bioassays are time consuming and can also be difficult to interpret due to variability in results obtained because of inconsistencies in biotic and abiotic test conditions. Therefore, finding new and rapid alternatives for the classification and characterization of *F. oxysporum* isolates are highly relevant.

Molecular identification techniques have been shown to be among the most promising and useful tools for *Fusarium* species and isolate identification due to accuracy as well as consistency of the results (Lievens *et al.,* 2008). The molecular characterizations of *F. oxysporum* isolates have, however, shown some drawbacks, i.e. complications which can be observed by polyphyletic analysis due to the nature of many formae speciales. Such isolates belonging to different formae speciales may be more related than isolates belonging to the same forma specialis (Kistler, 1997; Lievens *et al.,* 2008). Van der Does and Rep (2007) postulated in many cases, that the ability of a *Fusarium* isolate to infect particular plant species depends on specific genes encoding for host determining small secreted proteins that distinguish virulent from avirulent strains.

The main objectives of this study were to characterize various isolates of *Fusarium oxysporum* isolated from tomato with respect to their virulence under greenhouse conditions and to compare the phenotypic results with phylogenetic analysis based on the intergenomic-spacer region sequences (IGS).

Materials and Methods

Fungal isolates

Fungal isolates of *Fusarium oxysporum* were isolated during the 2010–2011 growing season from five different governorates in the Nile delta region of Egypt (Table1). Four governorates were located within the delta while the fifth governorate, Ismailia, was located in the east, adjacent to the delta (Figure 1). *Fusarium* isolates were obtained from the cortical tissues of naturally infected tomato roots (*Solanum lycopersicum*), which showed the typical symptoms of *Fusarium* wilt disease. Roots were cut into 1 cm sections, surface sterilized by soaking into ethanol 70% for 3 min, rinsed 3 times in

autoclaved water and briefly dried between sterile filter paper. The surface sterilized roots segments were subsequently mounted onto potato dextrose agar (PDA), amended with 150 mg^{-1} streptomycin and 150 mg^{-1} chloramphenicol to inhibit bacterial growth. Emerging mycelium from the root sections, which had the morphological characteristics of the genus *Fusarium,* were isolated and further propagated on PDA media. Each fungus was then purified using the single spore colony technique (Choi *et al.,* 1999). Purified isolates were reared on PDA plates and incubated at 27°C in the dark for 2 weeks and formed spores were extracted and used for inoculation purposes. For long-term storage, several small agar plugs (≈10 mm in diam.) were disked using a sterilized cork borer and transferred into micro tubes prior to freezing at -80°C.

Virulence test

Fungal inoculum for virulence test in the greenhouse was prepared by placing agar plugs of the *Fusarium* isolates on PDA plates and incubation them for 2 weeks. The mycelia and spores were then scraped from the surface, suspended in water and sieved through 3 layers of cheesecloth under aseptic conditions. The number of colony forming units (CFU) in the spore suspension was counted using a haemocytometer (Thoma, Germany) and the concentration was adjusted using sterilized tap water.

Seeds of tomato, super strain B cv, were sown in 96-well plastic trays filled with autoclaved beetmooth substrate. After 3 weeks growth, the seedlings were transplanted into plastic pots filled with 3 kg of a soil and sand mixture (1:2, w/w). After transplanting, a 3 ml fungal spore suspension ($1x10^9$ spores per ml) was injected into three holes of 2 cm deep made by plastic rod in the seedling rhizosphere. Control plants were treated with 3 ml sterile tap water. Each treatment was replicated four times arranged in a randomized complete design. Eight weeks after inoculation, disease severity was estimated using an index of leaf damage (ILD) by following the formula of a scale of 0 to 4 (0 = asymptomatic leaves, 1 = leaves wilted, 2 = leaves with moderate yellowing, 3 = leaves with necrosis and 4 = dead leaves) (Beye and Lafay, 1985). The ILD was calculated according to the following formula, ILD= ∑ grades/max grade.

DNA extraction

For DNA isolation from the *Fusarium oxysporum* isolates, 500 ml flasks containing 200 ml of potato dextrose broth (PDB, Defco, Germany) were inoculated with one fungal agar plug each and incubated at 27°C on a rotary shaker at 100 rpm for 2 weeks. Then, mycelia and spores were sieved through 3 layers of cheesecloth, collected and stored at -80°C. The frozen samples were then lyophylized using a Christ-LMC-1 (Beta 1-8-K) freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany). The mycelium was subsequently ground in a 15 ml plastic round bottom tube, placed in liquid nitrogen, using a pre-cooled metal spatula and vortex. DNA was extracted from 20 mg of the mycelium powder using the illustra DNA extraction kit (GE Health care, UK).

IGS- amplification and sequence analysis

The 10 isolates of *Fusarium oxysporum* were characterized by amplifying the ribosomal intergenic-spacer (IGS) region as described by Edel *et al.* (1995 and 1997) and subsequent sequencing. For amplification, the primers PNFo (5`- CCCGC CTGGCTGCGTCCGACTC-3´), and PN22 (5`- CAAGCATATGAC TACTACTGGC-3´) were used. The PCR mix contained 10

µL 5X Green Go Taq reaction buffer (Promega), 2 µl dNTPs (10 mM), 1 µl PNFo primer, 1 µl PN22 primer, 0.25 µl Tag polymerase (0.625 units) and 37.75 µl water and 2 µl fungal DNA as template.

Polymerase Chain Reaction (PCR) was performed in a Bio-RAD, C1000 thermo cycler by an initial denaturation at 95°C for 10 min, followed by 34 cycles of 95°C for 1 min, 52°C for 2 min and 72°C for 3 min, and with a final extension cycle of 72°C for 4 min. The amplified fragments of approximately 1.5 kb were isolated by agarose gel electrophoresis and purified using the GFX DNA purification kit (GE Healthcare, UK) and subsequently sequenced from both directions by GATC Biotech (Konstaz, Germany), using PNFo and PN22 as primers.

Sequences were analysed and aligned using the CLC Main Workbench software, version 6.9 (Aarhus, Denmark). As a reference, sequence fragments of the pathogenic *F. oxysporum* f.sp. lycopersici, isolate 4287 (Broad Institute, USA) were used.

Results and Discussion

Pathogenicity test

Twelve isolates, collected from the roots of diseased tomato and showing the typical morphological characteristics of *Fusarium oxysporum* wilt were selected for the assessment of the virulence level. The results showed that all the tested isolates were pathogenic toward tomato plants and caused symptoms corresponding to *Fusarium* wilt disease on tomato. Moreover, results revealed that the isolates varied regarding to their virulence under the bioassay conditions (Figure 2). The results also illustrated that the index of leaf damage (ILD) ranged from 31 to 94% with isolates 12 and 1, respectively, indicating that there was significant variability in virulence among the isolates.

Furthermore, results showed that *F. oxysporum* isolates with a different virulence were detected in all geographical locations and apparently, virulence could not be related to geographical location.

Molecular identifications

IGS-sequencing

The IGS region of the isolated fungi was amplified using PNFo and PN22 as primers, since these two primers are considered specific for *Fusarium oxysporum* (Edel *et al.,* 1995 and 1997). The successful amplification of the IGS region indicates that these isolates are all *F. oxysporum*, thus confirming successful morphological characterization.

The similarity and differences between the IGS sequences among the highly and weakly virulent of *F. oxysporum* isolates were evaluated by aligning the amplified intergenomic spacer (IGS) sequences of 10 pathogenic *Fusarium oxysporum* isolates, together with the pathogenic *F. oxysporum* f.sp. *lycopersici*, isolate 4287 (Broad Institute, USA). The results showed that with PN22 as a sequence primer, the sequence similarity between isolates 9 and 10 which had the same virulence level under greenhouse condition was 100% (Figure 3). Similar results were detected between isolates 1 and 6 as well as between isolates 7 and 12. While the similarity between isolates 2 and 3 reached up to 65%. Similar results were observed again with IGS sequences which are sequenced with the PNfo primer (Figure 4).

The twelve isolates of *Fusarium oxysporum* were isolated from infected tomato plants

grown in fields located in 5 different governorates in Egypt. All isolates showed identical morphological and microscopic characteristics corresponding to *Fusarium oxysporum* the causal agent of tomato wilt. The pathogenicity test under greenhouse conditions showed that the isolates varied in their virulence levels toward tomato as shown in Figure 2. The most virulent isolates (1 and 2) caused up to 93% leaf damage, whereas the leaf damage index only reached 30% within the least virulent isolates, 11 and 12. The classical identification carried out here together with the virulence assay confirms that all isolates could be classified as f. sp. *lycopesici*. Although belonging to the same forma specialis, the actual virulence can nevertheless vary significantly among the various isolates.

The molecular identification by amplification of the IGS regions by using specific primers confirmed that all isolates belonged to the species *F. oxysporum*. Further phylogenetic analysis of the IGS sequences suggested that there is a correlation between virulence and IGS sequences. It may be that to a certain extent, the sequence composition of the IGS is a reflection of virulence. Thus, the similarity of IGS sequences between isolates 9 and 10 which had a similar level of virulence on tomato, recorded 100% and 88% with PN22 and PNfo as sequence primer, respectively (Figures 3 and 4). Similar results were recorded again with isolates 2 and 3 which had a similar pathogenicity potential under greenhouse conditions and have up to 74% similarity with PNfo primer. On the other hand, the results demonstrated that, the similarity among the isolates belonging to the same location (same governorate) was very high while it reached to 100% between isolates 7 and 12 with both primers, PNfo and PN22. Those two isolates were originally isolated from Kafr-El-sheikh. This was theoretically expected since small changes in sequence of the IGS region may reflect the life style of the isolate (Weider *et al.,* 2005). IGS regions contain various regulatory elements that govern the transcriptional efficiencies of the rRNA-encoding genes. Differences in virulence and the accompanied plant defense responses may well require modifications in the regulation of the expression of rRNA-encoding genes in order to maintain an optimal growth rate. It was reported that the rate of transcriptional production of pre-rRNA was directly proportional to the number of enhancers located in the IGS (Cluster *et al.,* 1987; Grimaldi and Di Nocera, 1988). Genotypes composed of longer IGS LVs may benefit from higher rDNA transcriptional rates via more enhancer and promoter sites in the sub-repeat region of the IGS and thus exhibit faster development (i.e. higher growth rates). Furthermore, Allard *et al.* (1990) suggested that selection is acting directly on the sequence variability in the transcription units (i.e., sub repeats within the IGS). Zhang *et al.* (1990) determined that the high adaptability associated with a few specific alleles may result from adaptively favourable nucleotide sequences in either the transcription units or the IGS and that adaptability in barley depends more on the quality (i.e., sequence and length variation) rather than the quantity (i.e., CN) of rDNA present.

Nevertheless, molecular identification techniques for determining *Fusarium oxysporum* pathogenicity and virulence are still complicated due to the polyphyletic nature of many formae speciales, meaning that isolates belonging to different formae speciales may be more related than isolates belonging to the same formae specialis (Kistler, 1997; Lievens *et al.,* 2008). Jimenez-Gasco and Jimenez-Diaz (2003) could however demonstrate the correlation

between the molecular identification of *Fusarium oxysporum* f.sp. ciceris and its pathogenic races 0,1A,5, and 6 based on specific primers and PCR assays. They were able by using specific SCAR primers and PCR assay to identify and differentiate isolates of *F. oxysporum* and assign the pathogenic races belonging to f.sp. *ciceris*.
In this study, we present that sequencing the IGS PCR fragments of 10 different *F. oxysporum* isolates is a very useful tool in the way of identification of virulence of *Fusarium* oxysporum isolates. Further studies with virulence effector genes, secreted in xylem (SIX), and more varied virulent *Fusarium* isolate is highly candidate for confirming the results of IGS primers and PCR assays.

Table.1 List of the 12 *Fusarium oxysporum* isolates and their codes which were isolated from diseased tomato plants during the 2010–2011 growing season from the different 5 governorates

Governorate	Isolates Number	Isolates Code
Minufiya	3	1-5-6
Gharbiya	3	9-10-11
Qalubiya	2	4-8
Kafr El-Sheikh	2	7-12
Ismailiya	2	2-3

Figure.1 Location of *Fusarium* isolations from the 5 different governorates in Egypt during the 2010–2011 growing season

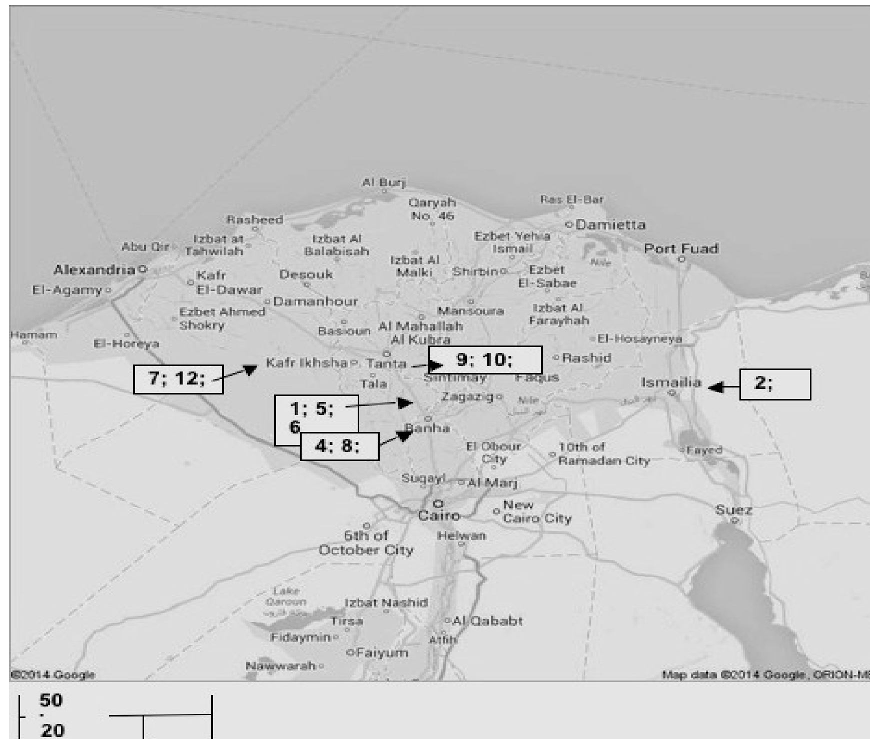


Figure.2 Virulence of the twelve collected *Fusarium oxysporum* isolates collected from 4 governorates in the Nile delta and one governorate in Ismailiya and compared to the control plants (c). Error bars presented by standard deviation indicate the virulence with significant differences among isolates of *Fusarium*

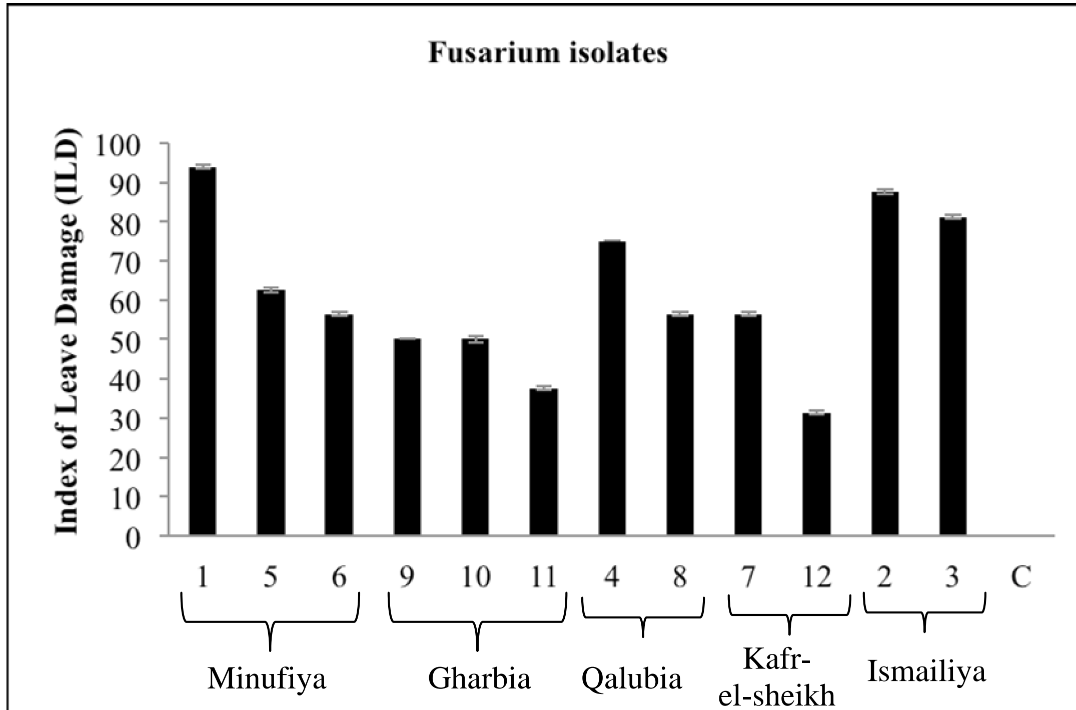


Figure.3 Dendrogram of the sequences of the amplified IGS fragments with PN22 primer obtained from 10 pathogenic *Fusarium oxysporum* isolates, 1,2,3,4,6,7,8,9,10,12 and aligned with one pathogenic *F. oxysporum* f.sp. *lycopersici*, isolate 4287. Bars indicate the genetic similarity [%] amongst taxa based on the Dice coefficient of similarity

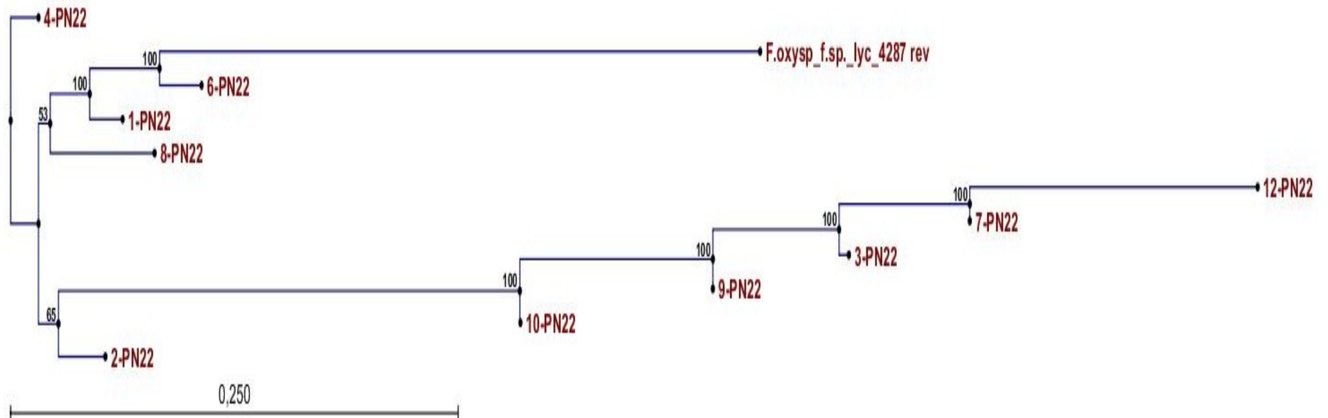
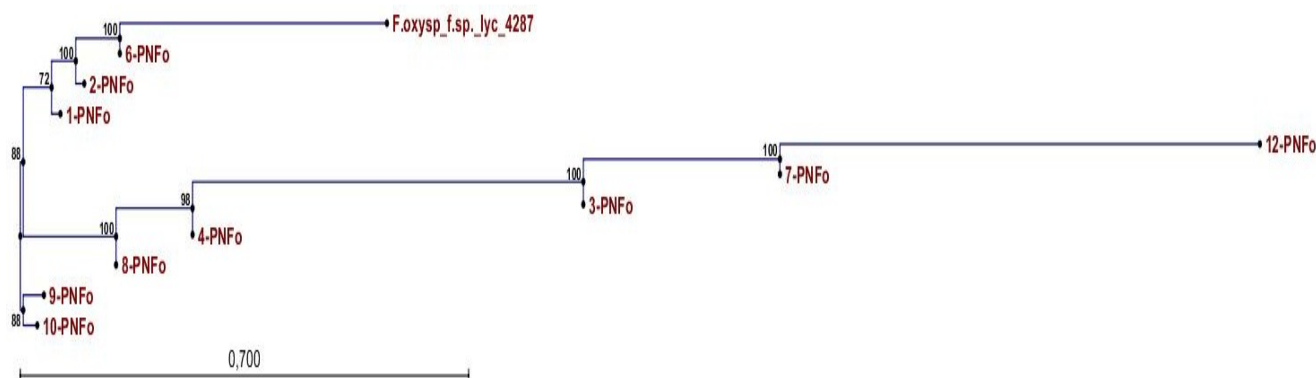


Figure.4 Dendrogram of the sequences of the amplified IGS fragments with PNfo primer obtained from 10 pathogenic *Fusarium oxysporum* isolates, 1,2,3,4,6,7,8,9,10,12 and aligned with one pathogenic *F. oxysporum* f.sp. *lycopersici*, isolate 4287. Bars indicate the genetic similarity [%] amongst taxa based on the Dice coefficient of similarity



References

Alabouvette, C., Edel, V., Lemanceau, P., Olivain, C., Recorbet, G., Steinberg, C. 2001. Diversity and interactions among strains of *Fusarium oxysporum*: Application and biological control. In: Jeger, M.J., Spence, N.J. (Eds). Biotic interactions in plant-pathogen associations. CAB International, London, England, Pp. 131–157.

Allard, R.W., Saghai-Maroof, M.A., Zhang, Q., Jorgensen, R.A. 1990. Genetic and molecular organization of ribosomal DNA (rDNA) variants in wild and cultivated barley. *Genetics,* 126: 743–51.

Armstrong, G.M., Armstrong, J.K. 1981. *Formae speciales* and races of *Fusarium oxysporum* causing wilt diseases. In: Nelson, P.E., Toussoun, T.A., Cook, R.J. (Eds). *Fusarium:* Diseases, biology, and taxonomy. Pennsylvania State University Press, University Park, London. Pp. 391–399.

Beye, I., Lafay, J.F. 1985. Study of selection criteria for the general resistance in Verticillium wilt of tomato. *Agronomie,* 5: 305–311.

Choi, Y.W., Hyde, K.D., Ho, W.H. 1999. Single spore isolation of fungi. *Fungal Diversity,* 3: 29–38.

Cluster, P.D., Marinkovic, D., Allard, R.W., Ayala, F.J. 1987. Correlations between development rates, enzyme activities, ribosomal DNA spacer-length phenotypes, and adaptation in *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA.,* 84: 610–14.

Dababat, A.A., Selim, M.E., Saleh, A.A., Sikora, R.A. 2008. Influence of *Fusarium* wilt resistant tomato cultivars on Root colonization of the mutualistic endophyte *Fusarium oxysporum* strain 162 and as biological control of root-knot nematode. *J. Plant Dis. Protect.,* 115(6): 273–278.

Di Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J., Roncero, M.I.G. 2003. *Fusarium oxysporum*: exploring the moleculr arsenal of a vascular wilt fungus. *Mol. Plant Pathol.,* 4: 315–325.

Edel, V., Steinberg, C., Avelange, I., Laguerre, G., Alabouvette, C. 1995. Comparison of 3 molecular methods for the characterization of *Fusarium oxysporum* strains. *Phytopathology,* 85: 579–585.

Edel, V., Steinberg, C., Gautheron, N., Alabouvette, C. 1997. Populations of non-pathogenic *Fusarium oxysporum* associated with roots of four plants species compared to soil borne populations. *Phytopathology,* 87: 693–697.

Gordon, T.R., Okamoto, D. 1992. Population structure and the relationship between pathogenic and nonpathogenic strains of *Fusarium oxysporum. Phytopathology,* 82: 73–77.

Grimaldi, G., Di Nocera, P.O. 1988. Multiple repeated units in *Drosophila melanogaster* ribosomal DNA spacer stimulate rRNA precursor transcription. *Proc. Natl. Acad. Sci. USA.,* 85: 5502–5506.

Hallmann, J., Sikora, R.A. 1994a. Occurrence of plant parasitic nematodes and nonpathogenic species of *Fusarium* in tomato plants in Kenya and their role as mutualistic synergists for biological control of root knot nematodes. *Int. J. Pest Manage.,* 40: 321–325.

Hallmann, J., Sikora, R.A. 1994b. Influence of *Fusarium oxysporum,* a mutualistic fungal endophyte, on *Meloidogyne incognita* of tomato*. J. Plant Dis. Protect.,* 101: 475–481.

Jimenez-Gasco, M.M., Jimenez-Diaz, R.M. 2003. Development of a specific polymerase chain reaction-based assay for the identification of *Fusarium oxysporum* f.sp. ciceris and its pathogenic races 0, 1A, 5 and 6. *Phytopathology,* 93: 200–209.

Kistler, H.C. 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. *Phytopathology,* 87: 474–479.

Lievens, B., Rep, M., Thomma, B.P.H.J. 2008. Recent developments in the molecular discrimination of formae speciales of *Fusarium oxysporum*. *Pest Manag. Sci.,* 64: 781–788.

Mandeel, Q., Baker, R. 1991. Mechanisms involved in biological control of *Fusarium* wilt of cucumber with strains of non-pathogenic *Fusarium oxysporum*. *Phytopathology,* 81: 462–469.

Michielse, C.B., Rep, M. 2009. Pathogen profile update: *Fusarium oxysporum. Mol. Plant Pathol.,* 10: 311–324.

Olivain, C., Alabouvette, C. 1997. Colonization of tomato root by a non-pathogenic strain of *Fusarium oxysporum*. *New Phytol.,* 137: 481–494.

Olivain, C., Alabouvette, C. 1999. Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. *New Phytol.*, 141: 497–510.

Olivain, C., Trouvelot, S., Binet, M., Cordier, C., Pugin, A., Alabouvette, C. 2003. Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and non-pathogenic strains of *Fusarium oxysporum*. *Appl. Environ. Microbiol.,* 69: 5453–5462.

Sikora, R.A., Fernandez, E. 2005. Nematode parasites of vegetables. In: Luc, M., Sikora, R.A., Bridge, J. (Eds). Plant parasitic nematodes in subtropical and tropical agriculture. CABI Publishing, UK. Pp. 319–392.

Sikora, R.A., Schafer, K., Dababat, A.A. 2007. Modes of action associated with microbially induce in planta suppression of plant-parasitic nematodes. *Australas Plant Path.,* 36: 124–134.

Van der Does, H.C., Rep, M. 2007. Virulence genes and the evolution of host specificity in plant pathogenic fungi. *Mol. Plant Microbe. Interact.,* 20: 1175–1182.

Weider, L.J., Elster, J.J., Crease, T.J., Mateos, M., Cotner, J.B., Markow, T.A. 2005. The functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. *Ann. Rev. Ecol. Evol. Sys.,* 2005: 219–242.

Zhang, Q., Saghai Maroof, M.A., Allard, R.W. 1990. Effects of adaptedness of variations in ribosomal DNA copy number in populations of wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Proc. Natl. Acad. Sci. USA.,* 87: 8741–8745.