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Genetic diversity of *Fusarium oxysporum* f.sp. *niveum* responsible of watermelon Fusarium wilt in Tunisia and Spain

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Abstract

***Fusarium* wilt is a serious disease, caused by *Fusarium oxysporum* f. sp. *niveum* (FON) and responsible of significant yield losses in watermelon crop in Tunisia. Thirty-nine FON isolates were collected from different infected watermelon fields in Tunisia and were identified on the basis of morphological features and by using one pair of species-specific primers Fn-1/Fn-2. Genetic diversity of the twenty-six FON isolates, originated from Spain and Tunisia, was studied by applying *Inter* simple sequence *repeat* genotyping. PCR amplification appears to be efficient to identify FON isolates amplifying only a single PCR band of approximately 800 bp. The RAMS study using two, bi and four trinucleotide microsatellites primers, showed a different genetic similarity degree among FON isolates. Seventy-one bands were amplified by four ISSR primer combinations. Diversity in the banding patterns obtained by DNA fingerprinting was always >50% and allowed us to distinguish all the isolates tested, according to number and size of the fragments, which ranged from 300 to 2800 bp. The genetic similarity values are comprised between 8 and 97%. UPGMA grouped the 45 genotypes into six main clusters at a similarity index value above 0.5, showing a relative genetic homogeneity and no correlation has been found among FON isolates and their origins. The most abundant Cluster VI comprising thirty-nine FON isolates.**

Keywords: *Fusarium oxysporum* f. sp. *niveum;* genetic diversity; ISSR; molecular detection; watermelon.

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1. Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) is one of the most important vegetable crops in the world, with a yield of 109601914.00 tons in 2013 (FAOSTAT, 2016). In Tunisia, watermelon crop has, also, a high economic value with a production of about 500000.0 tons in 2013 (FAOSTAT, 2016). Watermelon Fusarium wilt (FW) is the most destructive disease to this crop and is caused by a soil-borne pathogen, *Fusarium oxysporum* f. sp. *niveum* (E.F. Sm.) Snyder & Han (Boughalleb & Mahjoub, 2006). This disease is a production-limiting disease in watermelon growing regions of the world. *F. oxysporum* f. sp. *niveum* has the ability to colonize the root cortex of watermelon plants and penetrate into the xylem resulting in an initial loss of turgor pressure, wilting causing the whole plant death (Callaghan et al., 2016). Management of FW is difficult because of the long-term survival of the pathogen in the soil and the evolution of new races (Lin et al., 2009). There are three races of *F. oxysporum* f. sp. *niveum* designated 0, 1 and 2 based on their aggressiveness or their ability to overcome specific resistance (Wehner et al., 2008). FW has also increased in watermelon production areas infected mainly by the highly virulent *F. oxysporum* f. sp. *niveum* race 2 more than *F. oxysporum* f. sp. *niveum* race 1 which was also detected (Boughalleb & Mahjoub, 2006). A major reason for this difficulty is the inability to accurately detect the presence and identity of the fungal pathogen, especially in plant tissues and soil (Zhang et al., 2005). Molecular methods have been developed to discriminate Fon from other *Fusarium oxysporum* (Lin et al., 2010) and involve a PCR (Lin et al., 2009) based on randomly amplified polymorphic DNA (RAPD) detection system. Zhang et al. (2005) developed a rapid diagnostic method using a primer set Fn1/Fn2 to differentiate Fon from *Didymella bryoniae* and a broad group of other fungi, including three other *F. oxysporum* formae speciales (Fo). This technique was rapid and reliable for their isolates. This primer set, however, was unable to differentiate Taiwanese Fon isolates from other *F. oxysporum* formae speciales. Lin et al., (2010) developed another primer set Fon1/Fon2 that was more suitable for differentiating Taiwanese *F. oxysporum* f. sp. *niveum* from *F. oxysporum* formae speciales. The set Fon1/Fon2 was also able to detect *F. oxysporum* f. sp. *niveum* in diseased watermelon tissue at early stages of wilt. In the other hand, the genetic diversity is evidently an important character of species and populations, determining their response to changes in environmental conditions, their survival and evolvement and an answer to any confusion in morphological identification (Leong et al., 2010). The Random Amplified Microsatellite (RAMS) technique has been shown to be applicable for *F. oxysporum* f. sp. *niveum* (Zhang et al., 2005). A study from India looked at SSRs that could be used in differentiation of *F. oxysporum* pathogen lineages (Mahfooz et al., 2012). Appel and Gordon (1995) demonstrated the relationship between pathogenic and non-pathogenic isolates of *F. oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Severe outbreaks of Fusarium Wilt have been observed in Tunisia, causing yield losses estimated at approximately 100% with a yield losses as high as 60% (Boughalleb & Mahjoub, 2006). A diagnostic survey was thus undertaken, and the results showed that *F. oxysporum* f. sp. *niveum* is the most species commonly isolated. Although effective, selection for traits by conventional

methods is time consuming and resource intensive. Having markers linked to traits of interest can greatly accelerate conventional breeding and allow timely release of improved cultivars. The aims of this study were to characterize *F. oxysporum* f. sp. *niveum* isolates using specific primers and their genetic diversity among *F. oxysporum* f. sp. *niveum* Tunisian population by ISSR markers.

2. Materials and methods

2.1 Fungal isolates origin

Forty-five *F. oxysporum* f. sp. *niveum* (FON) isolates originated from different regions of Tunisia and Spain were used for this study. Spanish isolates, were kindly provided by Pr. Abad-Campos P. (Universidad Politécnica de Valencia) (Table 1). The isolation, morphological identification and pathogenicity test of these isolates were done by Boughalleb and El Mahjoub (2006) who proved that these isolates are the causals agents of watermelon Fusarium wilt in Tunisia. The isolates were maintained in a collection at the laboratory of Plant Pathology, *Institut Supérieur Agronomique de chott Mariem*, Sousse, Tunisia

2.2 DNA extraction and PCR identification

Six *Fusarium* isolates (Fon 10, Fon 11, Fon 14, Fon 15, Fon 16 and Fon 17) were grown in 20 ml of potato dextrose agar (PDA) for 5 days at 28°C. Genomic DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA, USA) following manufacturer's instructions. The specific primers Fn-1 (5'-TACCACTTGTTGCCTCGGC-3') and Fn-2 (5'-TTGAGGAACGCGAATTAAC-3') sequences were amplified with PCR. Each PCR reaction mixture contained 1.25×PCR buffer, 1.25 Mm $MgCl_2$, 1 µM each dNTP, 0.5 µM of each primer, 0.1 U of DNA Taq polymerase (Dominion MBL, Córdoba, Spain), and 1 µL of template DNA. The PCR reaction mix was adjusted to a final volume of 13 µL with water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). DNA amplification was performed using PCR amplifications on a Peltier Thermal Cycler-200. The program consisted of an initial step of 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and an elongation at 72°C for 2 min. A final extension was performed at 72°C for 7 min. The volume of 5 µl of PCR products was subjected to electrophoresis in 0.7% agarose gels (agarose D-1 Low EEO; Conda). The amplification products were examined under UV light, after ethidium bromide staining, and photographed using Alpha digidoc 1000 system (Alpha Innotech Corporation, USA) gel documentation system, for scoring the bands. The 100 bp DNA ladder (Biotools, Madrid, Spain) was used as molecular size marker. Amplifications from each DNA sample were repeated at least twice. PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics). The PCR products were visualized in 1.5% agarose gels (agarose D-1 Low EEO, Conda, Madrid, Spain) and molecular weights were estimated using the GeneRuler 100 bp Plus DNA Ladder (Fermentas, Carlsbad, CA).

Table 1: Characteristics of *Fusarium oxysporum* f.sp. *niveum* isolates used for molecular identification with specific primers.

Isolates	Origin	Race
Fon1	Spain	0
Fon2	Spain	0
Fon3	Spain	2
Fon4	Spain	2
Fon5	Spain	2
Fon6	Spain	1
Fon7	Spain	1
Fon8	Spain	1
Fon9	Jebniena	-
Fon10	Testour	-
Fon11	Testour	2
Fon12	Sebbala	-
Fon13	Sebbala	-
Fon14	Oued mliz	-
Fon15	Chébika	-
Fon16	Oued Mliz	-
Fon17	Metouia	2
Fon18	Oued Mliz	1
Fon19	Oued Mliz	-
Fon20	Gafsa	-
Fon21	Skhira	2
Fon22	Skhira	0
Fon23	Jebniena	-
Fon24	Chebika	-
Fon25	Skhira	0
Fon26	Gafsa	-
Fon27	Beja	-
Fon28	Chébika	2
Fon29	Médenine	-
Fon30	Mareth	-
Fon31	Gafsa	1
Fon32	Ben Aoun	-
Fon33	Metouia	2
Fon34	Mareth	-
Fon35	Gafsa	2
Fon36	Skhira	1
Fon37	Gafsa	1
Fon38	Skhira	-
Fon39	Mareth	-
Fon40	Jebniena	-
Fon41	Gaafour	-
Fon42	Sebbala	-
Fon43	Testour	-
Fon44	Beja	-
Fon45	Gaafour	-

2.3 Genetic diversity

2.3.1 Random amplified microsatellites

Twenty-six *F. oxysporum* f. sp. *niveum* (FON) isolates has been used for RAMS test (FON1, FON 2, FON 3, FON 5, FON 6, FON 7, FON 8, FON 9, FON 12, FON 13, FON 16, FON 18, FON 20, FON 23, FON 25, FON 29, FON 31, FON 32, FON 33, FON 34, FON 35, FON 37, FON 40, FON 42, FON 44 and FON 45). Extracted DNAs were amplified using

ISSR primers. Initially, to select primers which produce polymorphic bands for the characterization of *F. oxysporum* f. sp. *niveum* isolates, a total of six ISSR primers were evaluated for their capacity to produce polymorphic, scored and reproducible DNA fingerprint patterns. The primers included were two dinucleotide, and four trinucleotide repeats with or without 5' anchors: 5'DVD $(CT)_7C$ (Mahuku et al., 2002) , 5'YHY$(GT)_7G$, 5'DHB$(CGA)_5$ (Hantula et al. 1996), 5'DDB$(CCA)_5$ (Hantula et al. 1997), 5'$(GAC)_5$, 5'$(GTG)_5$ (Pina et al., 2005) (Tib Molbiol, Berlin). Each PCR reaction contained 1 PCR buffer, 2.5 mM $MgCl_2$, 100 mM each dNTPs, 0.4 mM of each primer, 0.5 U DNA Taq polymerase (Dominion MBL, Cordoba) and 0.5–5 ng template DNA were quantified spectrophotometrically. The PCR reaction mix was adjusted to a final volume of 25 ml with water (Chromasolv Plus, Sigma-Aldrich, Steinheim). PCR amplifications were performed on a Peltier Thermal Cycler-200 (MJ Research, Waltham, Massachusetts). The program consisted of an initial step of 5 min at 95°C, followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 41°C (CT)7, 58°C (GT)7, 64°C (CCA)5, 61°C (CGA)5, 46°C (GAC)5, 56°C (GTG)5 for 1 min, and an elongation at 72°C for 2 min. A final extension was performed at 72°C for 10 min. PCR products were separated in 1.5 % agarose gels (agarose D-1 Low EEO, Conda, Madrid), stained with ethidium bromide and visualized under UV light. Gene Ruler 100 bp DNA ladder plus was used as a molecular weight marker (MBI Fermentas). All ISSR assays were repeated at least three times, and only clear and reproducible bands were considered.

2.4 Data analysis

Fragments amplified by the ISSR primers were visually scored as present (1) or absent (0); Fragments with the same size were considered equal. The genotype profiles produced by amplified RAMS markers were scored manually. The data were assembled in a unique matrix and analyzed. The similarity matrix was used to construct dendrogram. Data from the genetic distance values were used as inputs in order to generate a dendrogram using the unweighted pair-group method with arithmetic averaging (UPGMA) as implemented by MEGA software.

3. Results

3.1 PCR amplification of Fon isolates DNA

In this study, the PCR-based identification technique with the two specific primers Fn-1/Fn-2 used for some *F. oxysporum* f. sp. *niveum* isolates was able to amplify a unique DNA fragment of approximately 800 bp for all *F. oxysporum* f. sp. *niveum* isolates originated from Tunisia and from Spain as shown on Figure 1.

3.2 Genetic diversity

Six ISSR primers were tested individually using *F. oxysporum* f. sp. *niveum* isolates DNA to determine which primer exhibiting a profile of light bands in agarose gels and revealed polymorphisms level between *F. oxysporum* f. sp. *niveum* isolates. Seventy-one bands were amplified by

four ISSR primer combinations. Diversity in the banding patterns obtained by DNA fingerprinting was always >50% and allowed us to distinguish all the isolates tested, according to number and size of the fragments, which ranged from 300 to 2800 bp. The most polymorphic loci were the trinucleotide primer CGA with 22 bands differentiated with molecular sizes ranging from 400 to 2000 bp, followed by CCA which produced 20 bands between 300 and 2800 bp. However, the two other trinucleotides primers GTG and GAC generated only 15 bp each, with sizes comprised between 550 and 1500 and from 600 to 2000, respectively.

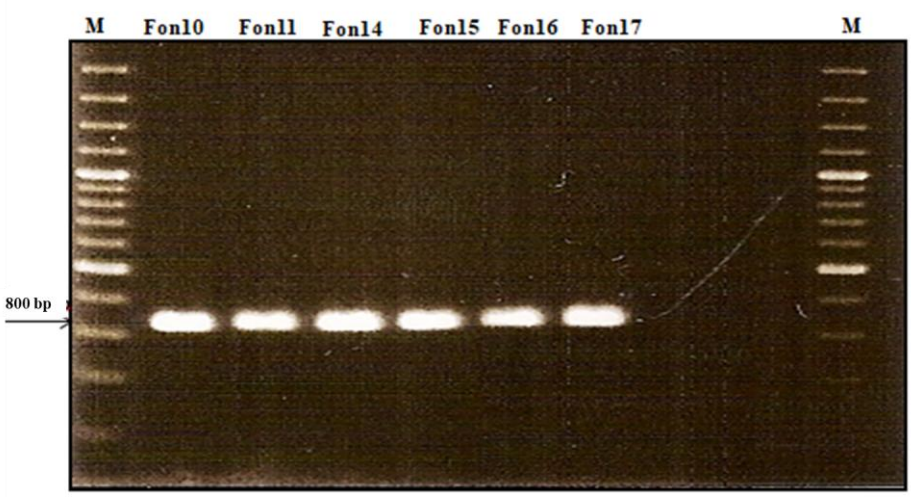

Figure 1: Agarose gel electrophoresis of PCR-amplified products using the specific primers Fn-1/Fn-2. M: 100-bp DNA ladder marker.

RAMS banding patterns generated by primers CGA (A), GTG (B), CCA (C) and GAC (D) of FON isolates. (1: Fon1; 2: Fon2; 3: Fon3; 4: Fon5; 5: Fon6; 6: Fon7; 7: Fon8; 8: Fon9; 9: Fon12; 10: Fon13; 11: Fon16; 12: Fon18; 13: Fon20; 14: Fon23; 15: Fon25; 16: Fon29 ; 17: Fon31 ; 18: Fon32 ; 19: Fon33 ; 20: Fon34 ; 21: Fon35; 22: Fon37 ; 23: Fon40; 24: Fon42; 25: Fon44 and 26: Fon 45) ; M: 100-bp DNA ladder marker. Reproducibility of amplified bands yielded by these four primers was confirmed with CGA and CCA, and the first primer was chosen to analyze the intraspecific genetic variability of *F. oxysporum* f. sp. *niveum* by Cluster analysis based on Nei's coefficient and UPGMA method. The forty-five genotypes were grouped into six main clusters at a similarity index value above 0.5 (Figure 3). Within the groups, the most abundant was Cluster VI comprising 39 isolates and the Nei's coefficient values ranged from 0.28 to 0.97, indeed, both isolates FON6 and FON7 race 1 from Spain registered the similarity coefficient of 0.97. The highest similarity value of 0.75 (97%) occurred between five FON isolates FON33 (Metouia, race 2), FON35 (Gafsa, race 2), FON37 (Gafsa, race 1), FON40 (Jbeniana) and FON42 (Sebbala)), and between the two FON isolates FON13 (Sebbala) and FON29 (Medenine). Only cluster I comprised a single genotype FON23 (Jbeniana) which could be considered as an out grouping sample. While, clusters II

consisted of two isolates FON12 (Sebbala) and FON34 (Mareth), these three isolates appeared to be distinct from all the other genotypes (Figure 2).

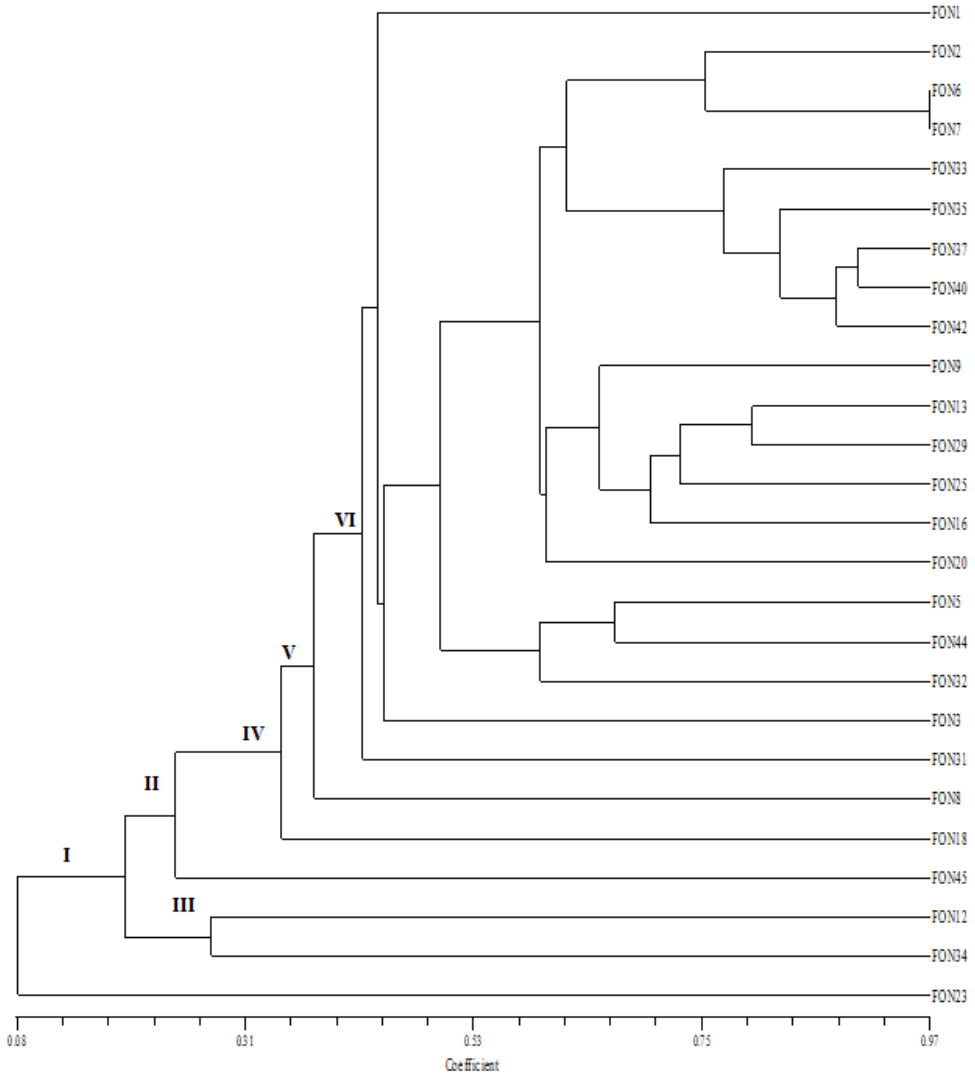

Figure 2: Dendrogram of 26 isolates of *Fusarium oxysporum* f.sp. *niveum*. Data were generated using unweighted pair group method of arithmetic means (UPGMA) using the Mega.5.1 software based on genetic distance coefficient.

Obtained results indicated that isolates FON5 and FON3 from Spain fall among Tunisian isolates, and may this indicate for a possible close relationship between these isolates and Tunisian isolates, while the rest of Spanish isolates FON1, FON2, FON4, FON6 formed a distinct cluster than Tunisian isolates suggested the existence of a highly variable genetic population in both country.

4. Discussion

The first step in Fusarium wilt management is an accurate diagnosis and detection. Control recommendations are usually made following putative diagnosis. Additional research on rapid detection techniques for *F. oxysporum* f. sp. *niveum* and economical methods for field level identification of *F. oxysporum* f. sp. *niveum* races will improve management of this reemerging disease (Everts et al., 2015). All heritable information is potentially accessible using DNA sequencing. Consequently, DNA sequence analysis is expected to provide the solution to the problem associated with the taxonomy and phylogeny of *Fusarium* species in general, and *F. oxysporum* in particular (Lin et al., 2010). In the present study, the molecular characterization by PCR using specific primers couple Fn-1/Fn-2 of Tunisian *F. oxysporum* f. sp. *niveum* isolates was successfully accomplished. And obtained result was in agreement with those reported by Zhang et al. (2005) which indicated that these primers can be beneficial for rapid detection of *F. oxysporum* f. sp. *niveum* isolates affecting watermelon, and could also be helpful in epidemiological and etiological studies to monitor the behavior of the pathogen in diseased plants. Genetic diversity within populations is an important indicator referring to a population's potential adaptation to environmental changing and to inform on the appropriate method of control (Rebib et al., 2014). Genetic control of the disease is crucial in managing these pathogens. Identification of single nucleotide polymorphism (SNP) markers linked to resistance can be a powerful tool for the introgression of valuable genes needed to develop *Fusarium*-resistant varieties. In *F. oxysporum*, DNA fingerprinting involving hybridization with SSR-containing probes has been used in the classification of some *formae specialis* (Barve et al., 2001). In this study, RAMS analysis revealed a moderate genetic diversity between *F. oxysporum* f. sp. *niveum* races. This indicated a low degree of polymorphism. Similar results were also obtained with *F. oxysporum* f. sp. *erythroxyli* (Nelson et al., 1997). The limited genetic variability observed among isolates would be expected for a pathogen that became widespread relatively quickly as a result of an increase in production of the host plant (i.e. distribution by seed) (Belabid et al., 2004). The current study suggested that some Tunisian and Spanish *F. oxysporum* f. sp. *niveum* isolates were indeed genetically similar. However, isolates from other watermelon-growing regions of the world should be tested to determine whether all the Tunisian isolates are related to other genetic groups from watermelon around the world. Since most of the markers were developed using pairs of ISSR primers, however, the use of ISSR primers in pairs, rather than individually, may be more efficient to develop this technique. By considering the cultivar and geographic origin, generally we could not find a relationship between virulence and cultivar. Hirano and Arie (2009) confirmed this result, with no stable correlation between phylogeny and pathogenic-group. The SSR primers should be particularly useful because the fungus is one of the most common *Fusarium* spp. residing in the soil

environment and that it includes pathogens, biological control agents and saprophytes. Their application should also enhance understanding relatedness of formae speciales in the *F. oxysporum* complex.

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