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Investigation on Genetic Diversity of *Fusarium oxysporum* Schlecht Isolated from Tuberose (*Polianthes tuberosa* L.) based on RAPD Analysis and VCG Groups

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Abstract

Fusarium rot disease of Tuberose (*Polianthes tuberosa* L.), caused by the vascular wilt pathogen *Fusarium oxysporum*, is a major production constraint in Tuberose growing regions of Iran. Samples collected from different fields in Dezful Khuzestan province, and 110 isolates of *F. oxysporum* recovered. Genetic diversity among F. oxysporum isolates determined using vegetative compatibility groups (VCGs) and Random Amplified Polymorphic DNA (RAPD) techniques. In the first experiments, 45 isolates selected randomly to VCG test. *Nit* mutants generated on MMC and Czapeck media each containing 3% $KClO_3$. The isolates assigned to into four VCG groups. These isolates also characterized by RAPD technique into six genetic groups at 61% similarity level. Cluster analysis of the RAPD data showed a close agreement with VCG grouping in some cases. In addition, Pathogenicity test revealed that all isolates were pathogenic. This research is the first report of genetic diversity of *F. oxysporum* on Tuberose in Iran.

Keywords: *Fusarium oxysporum; Polianthes tuberosa;* Vegetative compatibility groups; RAPD

Introduction

Tuberose is an important flower from the aesthetic and commercial point of view. It is famous among the commercially grown flower is due to its potential for cut flower trade, long vase life and essential oil industry [1]. Many fungal diseases have affected tuberose. Among the fungal diseases, tuber rot indicated by *Fusarium oxysporum* Schlecht is a serious disease in Tuberose [2]. Muthukumar [3] reported occurrence of tuber rot (F. oxysporum) in Tamil Nadu, India. For the first time, *F. oxysporum* has been isolated from infected Tuberoses in Iran. For this reason, new techniques need for rapid and sensitive detection of this fungus. Virulence is an exceptionally useful trait for the characterization of diversity among *F. oxysporum* isolates. Vegetative compatibility groups are another useful tool for identifying diversity among isolates [4]. All isolates within a subgroup, VCG, readily form heterokaryons with each other, whereas isolates from different groups do not. Isolates that are vegetative compatible is much more likely genetically similar than vegetative incompatible [5]. Various genetic marker systems such as RAPD can be used to determined genetic diversity among different isolates within a species. The aim of this work is assessing the genetic variation among isolates of *F. oxysporum* within each VCG and between different VCGs of pathogen and relationship between RAPD analyses and vegetative compatibility groups and assessing the agreement between all two methods in differentiating among isolates.

Material and Methods

Fungal isolates and media

A total of 110 isolates of *F. oxysporum* from different fields of Dezful, Khuzestan province was recovered from Tuberose plants showing typical root and tuber rot or Fusarium wilt symptoms during 2010-2011. Laboratory isolation was performed immediately after returning from the fields. Small pieces of tubers and root surface were sterilized by 1% NaOCl, rinsed twice in sterilized distilled water, and transferred to Nash& Snyder selective medium [6]. After incubation for about a week at 25 ± 5°C, a single spore culture was prepared from each isolate and transferred to Potato Dextrose Agar (PDA) medium. After that, small pieces of fungal colony were transferred to Carnation Leaf Agar (CLA) medium. Species identification was performed using Burgess Identification key and internet key of FusKey [7].

Generation and characterization of *nit* mutants

In order to investigation of VCG groups, 45 isolates selected randomly. Basal medium and Minimal medium used as described by Correll and Leslie [4]. Selection of nit mutant followed as Correll and Leslie [4] and Puhalla [8] methods. Inducement of nit mutant was selected using MMC medium containing different percentage of chlorate and Czapeck chlorate containing 3% $KClO_3$. Phenotype classes of *nit* mutants were determined by growing colony base on consumption rate of nitrogen on the culture medium, which contain four different nitrogen nutrient including Nitrate medium, Nitrite medium, Hypoxanthine, and Ammonium medium. The plates incubated at room temperature. Result from that three phenotypes (*nit1, nit3*, and *nitM*) were saved from each isolate [4].

VCG assignment

First, VCG assignment was based on a complementation reaction between *nitM* (or *nit3*) and *nit1* mutants on minimal medium as

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described by Jacobson and Gordon [9]. Pairs of isolates that exhibited vigorous aerial growth at the contact site of the two *nit* mutant-mycelia, indication the formation of a heterokaryon, were determined as vegetatively compatible, and assigned to the same VCG. Pairing test conducted in 5 cm Petri dishes the setup of the test was as follow: in each Petri dish, mycelia discs were placed at the corners of a 1.5×1.5 cm square. At the ends of one diagonal were the *nitM* (or *nit3*, if *nitM* was not available) mutants of the two isolates to be paired, and on the ends of the other diagonal were the *nit1* mutants of the two isolates, respectively. All possible pairs were tested for all isolates for which *nitM* or *nit3* mutants were available [4].

Pathogenicity test

Pathogenicity test was conducted for 45 isolates. Inoculum consisted of wheat seeds inoculated with fungal spores harvested from 5-day-old culture on PDA [10]. Plastic pots (1 liter capacity, 12 cm side) were filled with a mixture containing 50:50:50 (vol/vol/vol) of sand:loam:compost. Two pots used for each fungal isolate. For inoculation, the soil around the tuber was poll over carefully, and 3-5 infected wheat seeds placed beside the seedlings at the three-true-leaf stage. Controls consisted of tubers planted in autoclaved soil without fungal inoculum. Plants were grown in a greenhouse with natural daylight at 26-30°C. An initial symptom of disease was recorded three weeks after inoculation and symptoms assessed weekly.

DNA preparation and RAPD analysis

Total DNA was extracted using from lyophilized mycelium by method as described by Raeder and Broda [11]. RAPD profiles were obtained from 20 isolates of *F. oxysporum*. PCR amplification of DNA fragments was carried out using 13 arbitrary primers including OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07 OPA-08, OPA-09, OPA-10, OPA-11, OPA-12, and OPA-13 that are listed in table 1. Each amplification reaction was set at 25 µl volumes containing: 2.5 µl 10XPCR Buffer, 0.75 µl $MgCl_2$, 16 µl water, 1 µl primer, 2.5 µl dNTP, 2 µl template DNA, 0.3 µl tag Polymerase and 2.5 µl PCR Buffer. The reaction mixture was overlaid with mineral oil (10 µl) to suppression of evaporation during PCR cycling. The thermocycler (Biometra, Germany) programmed for an initial denaturation step at 94°C followed by 35 cycles. Each cycle consisted of 94°C for 1 min, a primer annealing step at 36°C for 1 min, primer extension at 72°C for 2 min and a final extension at 72°C for 5 min. The reaction products subjected to electrophoresis on 1% agarose gel containing 3 µl DNA safe green (Sinagen, Iran). In this study, DNA ladder 100 bp (Fermentas) was applied. Electrophoresis was performed for 1 h and 20 min in 90 V and then directly was visualized over a UV light sources. Gel image was acquired with a Gel Doc system (Tekno Gen, Iran) [12].

DNA analysis

To record RAPD patterns, a binary 0/1 matrix was prepared on which each intense RAPD band scored as one when present or zero when absent. Dendrogram was constructed using the software NTSISpic 2.01 version. The genetic distance data was subjected to cluster analysis by the program SHAN and clustering method of UPGMA and Jakard similarity coefficient.

Medium	MMC			Czapeck	Total
Chlorate %	1.5	3	5	3	
nit1	0	83	30	188	301
nit3	0	67	19	85	171
NitM	0	23	5	47	75
Total	0	173	54	320	547

Table 1: The numbers of nit mutants of *F. oxysporum*.

Results

In this study, 143 isolates of Fusarium were collected from infected plants in different parts of Dezful. Isolates were including 110, 27, and 6 at three species of *F. oxysporum*, *F. solani*, and *F. equiseti* respectively. Thus, *F. oxysporum* considered as predominant species of root and tuber rot. Macroscopic and Microscopic morphology of *F. oxysporum* was accordance to the description of Nelson et al. [6]. Colonies were fast growing, on PDA, 4.5 cm in 4 days, aerial mycelium white with orange sporodochia; reverse hyaline to dark blue or dark purple. Conidiophores were short, single with lateral monophialides in the aerial mycelium, later arranged in branched clusters. Macroconidia was fusiform, curved, pointed at the tip, three septate and basal cells pedicellate. Microconidia were abundant, mostly nonseptate, and ellipsoidal to cylindrical, straight, or often curved. Chlamydospores were hyaline, terminal or intercalary, rough-walled or smooth.

Assessment of genetic diversity of this fungus using vegetative compatibility groups showed any sector in MMC containing 1.5% potassium chlorate. Thus, *nit* mutants were selected by adjustment of the $KClO_3$ addition concentration as 2, 3, 4, and 5. The most mutants were obtained from MMC medium containing 3% potassium chlorate (Figure 1) and 173 mutants recovered (Table 1). However, growth of some isolates did not limit. Therefore, Czapeck chlorate with 3% $KClO_3$ was applied in order to sectors frequency. In this medium, the production of sectors was considerable. Therefore, this medium was applied for most of the isolates (Figure 2). Determination of phenotypic classes of *nit* mutants also revealed that the *nit1* phenotype recovered most frequently (54.51% of all mutants), followed by nit3 (30.96%) and *nitM* (13.57). Complementation between nit mutants, indicated by the development of the dense aerial growth, when the mycelia of the nit mutant colonies came in contact, and anastomosed to form a heterokaryon. Complementation occurred more rapidly, and the growth of the resulting heterokaryon was more robust in pairing of *nitM* with *nit3* or *nit1* than in pairing of *nitM* with *nit3* mutants.

Figure 1: Heterokaryon growth on minimal medium.

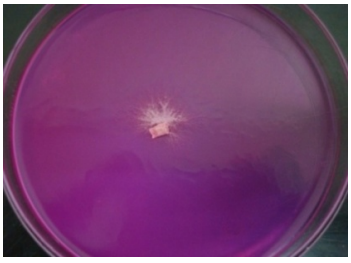

Figure 2: Sector production in Czapeck chlorate medium.

Eventually 45 selective isolates of *F. oxysporum* grouped into four VCGs. They consisted of one large VCG a (28 isolates), and three other VCG b (10 isolates), VCG c (3 isolates), and VCG d (4 isolates) (Table 2). Self-incompatibility did not observe between complementary nits recovered from single isolates of *F. oxysporum*.

The pathogenicity of isolates was examined on seedlings at the three-true-leaf stage. A total of 45 isolates from different VCG groups were virulent on Tuberose plants. Isolates caused root and tuber rot, wilt and yield decreasing. Based on the symptoms, isolates F36 and F18 were the most virulent isolates and F37 was the less. In addition, fungi that isolated from infected plants cultured on Nash& Snyder medium for proving the results. Severity of pathogenicity of isolates did not assess, and there was no data for compression results.

Of the 13 arbitrary primers tested, seven primers generated reproducible banding pattern selected for RAPD-PCR (Table 3).

Isolate	VCG groups	Pathogenicity	Isolate	VCG groups	Pathogenicity
F35	VCG a	virulent	F11	VCG a	virulent
F36	VCG a	virulent	F24	VCG a	virulent
F65	VCG a	virulent	F40	VCG a	virulent
F37	VCG a	virulent	F25	VCG a	virulent
F73	VCG a	virulent	F29	VCG a	virulent
F78	VCG a	virulent	F113	VCG b	virulent
F97	VCG a	virulent	F16	VCG b	virulent
F99	VCG a	virulent	F34	VCG b	virulent
F66	VCG a	virulent	F51	VCG b	virulent
F59	VCG a	virulent	F3	VCG b	virulent
F52	VCG a	virulent	F4	VCG b	virulent
F98	VCG a	virulent	F105	VCG b	virulent
F92	VCG a	virulent	F2	VCG b	virulent
F87	VCG a	virulent	F15	VCG b	virulent
F1	VCG a	virulent	F9	VCG b	virulent
F21	VCG a	virulent	F20	VCG c	virulent
F5	VCG a	virulent	F81	VCG c	virulent
F100	VCG a	virulent	F95	VCG c	virulent
F18	VCG a	virulent	F27	VCG d	virulent
F54	VCG a	virulent	F8	VCG d	virulent
F53	VCG a	virulent	F31	VCG d	virulent
F22	VCG a	virulent	F48	VCG d	virulent
F45	VCG a	virulent			

Table 2: Pathogenicity and VCG groups of 45 selective isolate of *F. oxysporum*.

Primer	sequence	Polymorphic bands	Total bands	Polymorphism %	The size of band (bp)
OPA-01	5´-CAGGCCCTTC-3´	8	9	88	200-1000
OPA-02	5´-TGCCGAGCTG-3´	3	5	60	100-700
OPA-03	5´-AGTCAGCCAC-3´	-	-	-	-
OPA-04	5´-AATCGGGCTG-3´	-	-	-	-
OPA-05	5´-AGGGGTCTTG-3´	-	-	-	-
OPA-06	5´-GGTCCCTGAC-3´	-	-	-	-
OPA-07	5´-GAAACGGGTG-3´	-	-	-	-
OPA-08	5´-GTGACGTAGG-3´	3	4	75	100-700
OPA-09	5´-GGGTAACGCC-3´	3	5	60	100-1500
OPA-10	5´-GTGATCGCAG-3´	4	5	80	100-1000
OPA-11	5´-CAATCGCCGT-3´	5	6	83	100-1200
OPA-12	5´-TCGGCGATAT-3´	-	-	-	-
OPA-13	5´-CAGCACCCAC-3´	4	4	100	100-700

Table 3: Primers used in this study and major random amplified polymorphic DNA fragments generated from the *F. oxysporum* isolates.

Primers of OPA -03, OPA-04, OPA-05, OPA-06, OPA-07, and OPA-12 did not produce a considerable number of amplification products for comparison. A total of 38 distinct bands, even in repeated amplifications, amplified using the DNAs from 20 *F. oxysporum* isolates as templates; 30 of these bands were polymorphic. The size of RAPD bands ranged from 100-1500 bp. The primer OPA-01 had the most reproducible bands and primer OPA-13 had the less.

These isolates were classified into six groups based on Jakard similarity coefficient using UPGMA method. These groups including group I (F48 and F4), group II (F73, F113, F11, F97, F24, F18, F100, F8, F59, F22, F36 and F25), group III (F27 and F99), group IV (F20), group V (F21) and group VI (F37 and F66) (Figure 3). Result showed that two isolate F22 and F59 with the most genetic similarity belonged to the same VCG groups.

In this research, we studied comparison between VCG and genetic groups. Results from RAPD and VCG test were almost similar together in some cases. Isolates F22 and F59, with the most similarity, belonged to group II and VCG a. In "group I," result from RAPD did not accordant to VCG test and F48 and F4 belonged to VCG d and VCG b respectively. Among group II with 12 members, accordance of results observed for 10 members that all belonged to VCG a. Nevertheless, F133 and F8 belonged to VCG b and VCG d respectively. In addition, two members placed in different VCG groups in "group III" (VCG a, and VCG d). Among group VI results were complete accordance together and both isolates belonged to VCG a, On the other hand, this marker could be shown parts of diversity. Isolate F20, belonged to VCG c and group IV and quite separated in every cut off the line among the isolates. Probably it is from the different form special. However, for proving this case, sequencing should apply. "VCG a" was the largest group among all groups and more than half the isolates belonged to this group. This group formed the immense cluster in level of 61% similarity and indicated predominance of this group (Figure 4). It was

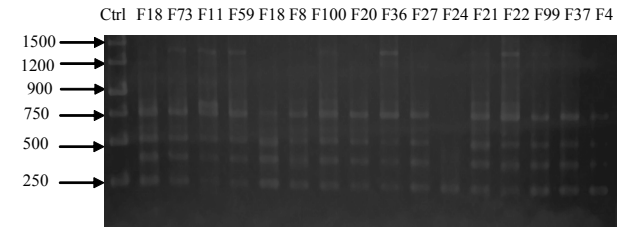

Figure 3: An example of amplification of genomic DNA using RAPD technique among isolates of *F. oxysporum* with OPA-09.

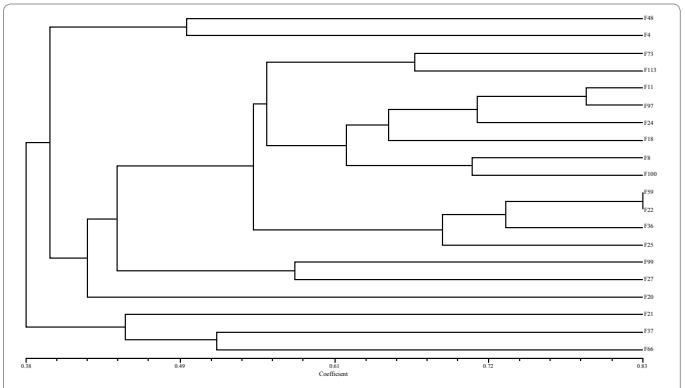

Figure 4: UPGMA clustering analysis of RAPD data of 20 isolates of *F. oxysporum* by Jakard coefficient.

also true about genetic group II that the majority of its isolates belonged to VCG a, and probably all originated from a common ancestor. In addition, isolates with the high pathogenicity belonged to the same VCG and genetic group. It was clear that there was a relation between pathogenicity and genetic groups, But regarding severity of diseases had not been measured in this research this require increasing the number of experiments, isolates and comparison between statistical data with measuring of diseases severity with results from RAPD. Comparison between results showed that RAPD and VCG could show a degree of genetic diversity. Population of fungus had low genetic diversity that one of this reason is absent of sexual reproductive that can say isolates are from the same ancestor.

Discussion

During field investigation, symptoms were similar to which were reported in the different areas of the world that including tuber rot, dwarf, wilt, and yield decline [2]. This research is the first report of *F. oxysporum* on Tuberose in Iran. Muthukumar [3] reported incidence of tuber rot in Tamil Nadu, India. Also, Roy [13] in India and Tzeng and Tung [14] in Taiwan reported occurrence leaf spot caused by *F. equiseti* on tuberose. In investigating of VCGs groups, there was not any sector on MMC with 1.5% potassium chlorate after 7-10 days. However, MMC with 3% potassium chlorate had the most sectors frequency. In addition, Czapeck chlorate with 3% chlorate caused increasing of sector production. Rahkhodayi [15] during the investigation of VCG groups of *F. oxysporum* and *F. solani* showed that growth of sectors limited in the MMC with 3% chlorate. Jelodar [16] resulted like this research with Czapeck medium. In her experiment, any sectors did not produce in MMC medium with 1.5 and 3% chlorate after 14 days. Sectors Growth in MMC with 5% chlorate limited, but most of the isolates showed wild growth type when transformed in minimal medium. Amani et al. [17] in analyzing of VCG groups of *F. oxysporum*, causal agent of banana wilt in Sistan and Blochestan province, resulted that chlorate resistance sectors produced in MMC and PDC including 3.5-4% potassium chlorate. In addition, 71.5 percentages of sectors obtained from PDC medium [17]. Harveson and Rush [18] in research about genetic diversity of *F. oxysporum* isolated from sugar beet and Zhou and Everts [19] in similar research on watermelon failed to produce sector on MMC medium with 1.5% chlorate and had to increase chlorate to 4.5% [18,19].

Investigation on genetic diversity of *F. oxysprum* using RAPD marker showed that population of diseases had low genetic diversity. In addition, results from the RAPD and VCG test were almost similar together. Results from RAPD did not similar to VCG among group I and isolates F48 and F4 belonged to VCG d, and VCG b respectively. Accordance of results was observed for 10 members that all belonged to VCG a (group II) with 12 member, but F113 and F8 belonged to VCG b, and VCG d respectively. Also among group III, two members were placed in different VCG groups. Results were complete accordance together among group VI; On the other hand, this marker can show parts of diversity. This is because of this marker cannot able to cover entire the genome. Also, some of morphological and physiological traits did not observe in RAPD technique. Probably increasing of numbers of primers and isolates may be a suitable alternative. These results were similar to research by Dastjerdi et al. [20] about genetic diversity of *F. oxysporum* on sugar beet that proved members of a VCG group placed in the same genetic group. In the present research regarding with group II, as the largest group, similar results will obtain. Baghai Yavari et al. [21] showed that results from clustering analysis, confirmed results from RAPD in investigating of Fusarium wilt of potato. However, in investigating of genetic diversity of Iranian isolates of *F. oxysporum* by Zamani et al. [5] there was no relation between results from RAPD and VCG test. Isolate F20 that belonged to VCG c and group IV separated in every cut off the line among the isolates and probably it is from the different Form Special. Nevertheless, for proving this case, sequencing should apply. Baysal et al. [22] also determined molecular traits of *F. oxysporum* f.sp. *melongenae* using RAPD and ISSR and can get high diversity between isolates. However, molecular discrimination of *F. oxysporum* complicate by the observation that different isolates classified into single forma specials may have independent evolutionary origins. Although the RAPD technique successfully uses in many studied for detection and identification of genetic diversity of *F. oxysporum* isolates, it suffers from well-known limitation of poor reproducibility and inters laboratory transferability.

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