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Investigation of *Fusarium verticillioides* on the Basis of RAPD Analysis, and Vegetative Compatibility in Iran

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Abstract

Genetic diversity among 41 isolates was determined using vegetative compatibility groups from different regions of Fars (Firozabad, Ramjerd, Drodzan, Shiraz, Pasargad, Sepidan and Mravdasht) and Khuzestan (Ezh, Ramhormoz and Ahodasht) provinces. Nitrate non-utilizing (Nit) mutants were generated on Czapeck media, containing 5% $KClO_3$. Phenotypic classes of nit mutants were determined to 17 different Vegetative Compatibility Groups (VCGs) on media containing nitrate, hypoxanthine and ammonium. The largest group contained 18 isolates and others contained two and one, respectively. 24 isolates were selected for RAPD test, to represent 17 vegetative compatibility groups. A set of seven random primers revealed a total of 36 alleles. High level of genetic diversity was observed among *F. verticillioides* isolated in the region. 29 alleles (80.55) showed high polymorphism in all the isolates. Genetic relatedness was calculated based on genetic distance matrix, and 11 groups were clustered based on UPGMA and Dice coefficient.

Keywords: *Fusarium verticillioides*; RAPD; Vegetative compatibility

Introduction

Rice (*Oryza sativa* L.) is one of the most important grains consumed by almost half of the world's population [1]. Root rot and crown rot are significant diseases of rice that yield the most losses in various rice-growing areas [2]. Bakanae disease caused by *Fusarium verticillioides* (syn. *F. moniliforme*) was first described in Japan, and now is widely distributing in Asia. In addition, the disease has been reported to be on the rice tracts of European countries and America [3,4]. The typical symptoms are the abnormal elongation of seedling and the fungus which not only causes considerable damage on many plants, but also is parasitic on plants without the production of visible symptoms [2]. The pathogen has a wide host range and is widespread throughout the world. It's also known to cause stalk rot and leaf blight [5]. Knowledge of the genetic structure of the *F. verticillioides* population might be useful, in order to establish effective strategies for controlling the disease [6]. There are a number of techniques used to observe genetic variation among fungal pathogens. One of the techniques is VCG which is based on the ability of the mycelium to anastomose to form heterokaryon, for the aim of determining genetic relatedness [7]. The VCG technique is particularly suitable for population genetic studies of F. verticillioides, since the field isolates of this fungus belong to many VCGs [8]. Several molecular techniques are available for investigating genetic variability within plant pathogenic fungi population, such as Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) [9]. Random Amplified Polymorphic DNA (RAPD) analysis has many advantages, as a means of characterizing genetic variability such as speed, low cost, and minimal requirement of DNA. RAPD markers are especially suitable for haploid plant pathogenic fungi, in which the dominance of amplified fragments does not affect genetic analysis [10]. This technique has been successfully used to assess genetic variability within many plant pathogenic fungi [11], including *Fusarium* section *Liseola* [12-14]. In this research, the characterization of *F. verticillioides* isolates (RAPD analysis and vegetative compatibility) and the relationship between pathogenicity and genetic markers, such as RAPD and vegetative compatibility group, are reported.

Materials and Methods

Sampling, isolation, and the maintenance of fungal isolates

Rice plant with brown and black symptoms on the root and crown were randomly collected during the growing seasons of 2009-2010, from different parts of rice-growing areas in Fars, a city in the southwest of Iran-namely Firozabad, Ramjerd, Drodzan, Shiraz, Pasargad, and Sepidan-and Khuzestan, located in the southeast of Iran-namely Ezeh, Ramhormoz and Ahodasht.

Diseased samples were cut into pieces of 2-5 mm, surface sterilized by dipping into domestic bleach solution (containing 1% of NaOCl) for 2-3min, washed three times by sterile distilled water, dried by sterile filter paper, and finally plated on Potato Dextrose Agar (PDA) or Nash & Snyder selective medium samples, which were incubated in an incubator at 23°C for 3 days. Isolates were seen to be single spored by harvesting conidia from a colony by a sterile needle, and through being streaked them on an agar plate containing 2% water. Conidia were incubated as above and single germinated conidia subcultured onto PDA plates and incubated with, for 2-3 weeks. Single-conidial isolates were stored on sterile filter paper at 20°C. The identification of the fungal isolates was performed based on their morphological characteristic on Carnition-Leaf Agar (CLA) and PDA media. Afterwards, isolates were identified by Berges identification key internet [15].

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Vegetative compatibility

Vegetative compatibility tests were conducted; following the protocols described by Puhalla [7] and Correll et al. [16]. Nitrate non-utilizing (nit) mutants were generated on Czapeck chlorate, containing 5% $KClO_3$ through using Potato dextrose agar+chlorate (PDC) medium containing various percents of chlorate, which were assigned to different physiological phenotypes (nit1, nitM, and nit3), on the basis of their growth on Minimal Medium (MM) amended with different nitrogen sources (nitrate medium, nitrite medium, hypoxanthine, and ammonium medium). Plates were incubated at 26°C in the dark.

Complementation test and the determination of VCGs

To identify vegetative self-incompatible isolates among all the rest, each nitM isolate was placed against nit1 or nit3 isolate and self-compatibility was observed by the dense growth (the formation of a heterokaryon), at the line of contact between the two mutants. Complementation was carried out between self-incompatible isolates, as described by Jacobson and Gordon [17]. NitM, each of the isolates was placed in the center and several blocks of nit1 and nit3 from the same isolate were placed at the distance of 2 cm from the nitM block. The growth of aerial mycelium in confrontation between two colonies was an indicator of vegetative self compatibility [16]. Isolates that formed heterokaryons were assigned to the same Vegetative Compatibility Group (VCG).

Fungal culture and DNA preparation

Liquid culture was initiated by adding 5 mm mycelial discs from the growing edge of single conidial colonies grown on PDA to 250 mm Erlenmeyer flasks, containing 50 ml of PDB (Potato Dextrose Broth) medium, and then were incubated at 28°C on an orbital shaker for two weeks. Mycelia from cultures were collected by vacuum filtration, washed by sterile distilled water, lyophilized, and finally ground in liquid nitrogen. Total genomic DNA was extracted from the powdered sample of mycelium, by using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) procedure by Von Korf et al. [18]. The DNA was resuspended in 50 µl of TE buffer and stored at -20°C.

RAPD primer and amplification condition

Seven arbitrarily chosen primers from Operon primer kit were tested for PCR amplifications of *F. verticillioides* DNA (Table 1). The preliminary amplification determined the optimal concentration of the component in the PCR reaction mixture. Amplifications were performed in a total volume of 25 µl containing 2 µl 10X PCR Buffer, 1.7 µl $MgCl_2$,15 µl water, 1 µl primer, 2 µl dNTP, 3 µl template DNA, and 0.3 µl tag polymerase. Each reaction was overlaid with one drop of mineral oil. The program included an initial denaturation at 94°C for 5 min, 45 cycles with denaturation at 94°C for 2 min, annealing 34°C for 1 min, an extension step at 72°C for 2 min, and a final extension at 72°C for 15 min. Negative controls (no template DNA) were used for each set of experiments, to test for the presence of nonspecific reaction. The PCR products were electrophoresed on 1.2% of agarose, containing 3 µl DNA, stained by ethidium bromide and visualized in a UV transilluminator.

Primer	Sequence
OPA-516	5'- AGCGCCGACG -3'
OPA-02	5´- TGCCGAGCTG-3´
OPA-515	5´-GGGGGCCTCA-3´
OPA-542	5´-CCCATGGCCC-3´
OPA-548	5´- GTACATGGGC-3´
OPA-540	5'- GCCCCTTTAC-3'
OPA-07	5´- GGTCCCTGAC-3´

Table 1: Sequence of the primers used.

Isolate	Location	VCG
FK69	Marvdasht	VCG1
FK78	Ramjerd	VCG1
FK62	Shiraz	VCG1
FK98	Droodzan	VCG1
FK19	Ramjerd	VCG1
FK17	Ramjerd	VCG1
FK43	Firozabad	VCG1
FK50	Marvdasht	VCG1
FG17	Ahodasht	VCG1
FK16	Marvdasht	VCG1
FK63	Marvdasht	VCG1
FK34	Sepidan	VCG1
FG18	Ezah	VCG1
FG3	Ezah	VCG1
FG6	Ahodasht	VCG1
FG22	Ezah	VCG1
FG23	Ezah	VCG1
FG24	Ezah	VCG1
FK84	Firozabad	VCG2
FK86	Firozabad	VCG2
FG21	Ezah	VCG3
FG19	Ezah	VCG4
FK4	Marvdasht	VCG5
FK80	Droodzan	VCG6
FK37	Pasargad	VCG7
FK114	Marvdasht	VCG8
FK89	Droodzan	VCG9
FK46	Pasargad	VCG9
FK99	Droodzan	VCG10
FK38	Droodzan	VCG11
FK13	Ramjerd	VCG11
FK42	Firozabad	VCG12
FK104	Marvdasht	VCG12
FK64	Pasargad	VCG13
FK61	Shiraz	VCG14
FK3	Firozabad	VCG15
FK91	Droodzan	VCG16
FK75	Firozabad	VCG17

Table 2: Location, Virulence and Vegetative compatibility group of isolates of *Fusarium verticillioides.*

RAPD product scoring and data analysis

Data were compiled as binary 0/1 matrix by the presence (1) or the absence (0) of a band at a particular position. Only major RAPD bands were considered in statistical analysis. Dandrograms were produced by cluster analysis using the Unweighted Pair-Grouped Method by Arithmetic average (UPGMA), using the software Ntysys 2.01 version.

Results

Identification

The identification performed is based on the morphological characteristics. Aerial mycelium was white to pale orange or violet in almost half of the entire colony. Macroconidia observed on CLA, were slightly curved and septated. Microconidia were formed in chains (3 to 35 spores) on monophialides on KCl medium. Based on these

mycological characteristics, 80 isolates were identified *as Fusarium verticillioides*.

Vegetative compatibility group

38 isolates were selected from a total of 80 of *F. verticillioides*, for the aim of VCG determination. No fast growing sector was produced, even after 14 days in Minimal medium cholorate (MMC) and PDC medium containing 1.5% and 3.5% of potassium chlorate. As a result, the concentration of $KClO_3$ was increased to 6% and finally 41 nitrate non-utilizing mutants were obtained. The growth of many isolates was not limited. So, in order to increase efficiency, Czapeck chlorate containing 5% of $KClO_3$ was used. In this medium, the production sectors were considerable. With this medium, 405 mutants were isolated in total. A total number of 446 mutants of *F. verticillioides* were obtained with 49.32% of nit1, 30.02% of nit3 and 20.4% of nitM. Complementation among nit mutants was 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 (Figure 1). All the isolates were grouped into 17 VCGs based on pairing complementary nit mutants, mainly with nit3 and NitM. The largest one contained 18, and the rest contained 1 or 2 members. These results indicated high genetic diversity caused by sexual reproduction.

RAPD analysis

Genetic variability among the 24 isolates from Khuzestan and Fars was assessed by RAPD analysis. In order to compare the overall similarity among these isolates at DNA level, 7 primers were used, which generated polymorphic bands (Figure 3). The data from polymorphic bands were analyzed by UPGMA. Among the seven primers, the most informative primer was OPA-2, which included 7 bands and it showed high polymorphism, while primer OPA-7 was low which had 3 fragments.

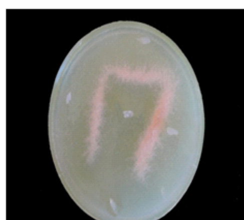

Figure 1: Heterokaryon growth on minimal medium.

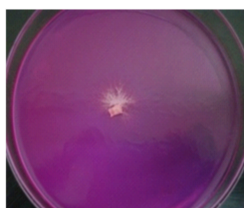

Figure 2: Sector production in Czapeck chlorate.

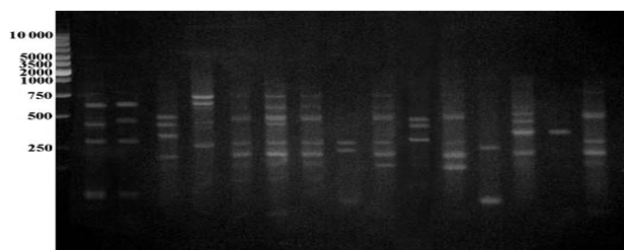

Figure 3: An example of amplification of genomic DNA using the Random amplified polymorphic DNA technique. Numbers represent different *Fusarium verticillioides* isolates. M, Molecular marker.

Discussion

The two goals of this study were to determine genetic diversity by VCG and RAPD techniques, and to assess the ability of RAPD, as compared to the VCG to genotype isolates of *Fusarium verticillioides.* VCG assignment was based on complementation reaction between NitM (or nit3) and nit1 mutants on minimal medium. In this investigation, there was no sector on MMC and PDC with 1.5 % and 3% of potassium chlorate after 7-10 days, but MMC and PDC having 6% of potassium chlorate and 41 mutants were generated. To save time, Czapeck chlorate with 5% of chlorate was used, which increased the sector production. Jelodar [19] reached results similar to those of Czapeck medium. In her experiment, no sectors were produced in MMC medium with 1.5 and 3% of chlorate, after 14 days. Sectors growth in MMC having 5% of chlorate was limited, but most of isolates showed wild growth type, when they were transformed in minimal medium. Afterwards, she used Czapeck chlorate having 5% of chlorate.

Different isolates of *F. verticillioides* were divided into 17 groups. The largest group was VCG1, including 18 members and other groups had 2 or 1. No specific pattern was observed between geographical origin and VCG. The occurrence of isolates of different VCG in the same location indicates that there is considerable genetic diversity among these isolates.

RAPD analysis, a method widely used to characterize isolates, indicated a high level of genetic variability among isolates of fungi [20]. RAPD analysis was compared with the classification of pathogenicity groups through the use of 7 primes, but the results did not show good correlation between amplification patterns and pathotype classification.

Investigation on genetic diversity of *F. oxysprum*, using RAPD marker showed that the population of diseases had high genetic diversity. Also, the results showed that RAPD fingerprinting cannot provide, in most cases, the same resolution for genotyping as VCG. In most cases, RAPD divided a VCG into different haplotypes, while in other cases; a RAPD haplotype contained more than one VCG. It is, however, likely that greater resolution could have been obtained with RAPDs, by increasing the number of bands scored. In spite of the high sensitivity it did provide, the VCG technique alone for strain or clone identification would not be recommended. While VCG assays only one marker, RAPD analyses a number of markers that can be simply increased, in order for us to meet specific needs. The increase can be done by using primers. As a result, more detailed population genetic analyses can be conducted. In addition, the RAPD technique is more time- and labour- efficient than VCG typing. Isolates from two provinces were present in the same VCG, or the same RAPDs. This might be the result of gene flow between two provinces, or from a common gene pool of *Fusarium* isolates, that become established in the two provinces. The results indicate that there is high genetic variability among isolates of *Fusarium verticillioides* in two provinces, as identified by RAPD analysis that sexual reproduction can be one of the reasons [21]. In this study, the relationship between the VCG and RAPD results were very low; so that, only two isolates with the highest genetic similarity FG22 and FG17 were in same VCG and RAPD group.

Huang et al. [21] also found high genetic diversity among isolates of this pathogen in Israel; also they could find close relationship between VCG and RAPDs. But Zamani et al. [22], in their studies on the genetic diversity of Iranian isolates of *F. oxysporum* had no relation between results from RAPD and VCG test, too.

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