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GENETIC VARIATION AMONG *FUSARIUM OXYSPORUM* F.SP. *CICERIS* ISOLATES IN PAKISTAN AS DETERMINED BY BIOLOGICAL PATHOTYPING AND VEGETATIVE COMPATIBILITY

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Abstract

Fusarium oxysporum f.sp. *ciceris* isolates 2005, 2007, 2012, 9718 and 9910 were highly virulent and produced more than 50% mortality in chickpea varieties. Of these, the isolate 9718 was found to be most aggressive. The isolates 2011, 9925, 7952, 8012, 7989 and 7802 were virulent whereas isolates 2001, 2002, 2003, 2004, 2008, 2009, 2014, 9917 and FOI were less virulent and caused less than 25% mortality.

Complementation test of various nit mutants of local isolates with each other and with international isolates revealed that the virulent isolates viz., 2005, 2007, 2011, 2012, 9718 and 9910 were compatible with the wilt causing isolates 8012 (race 5 from Spain) and 7989 (race 1A from India) and belonged to vegetative compatibility group 0280. The yellowing causing isolates 7952 and 7802 (race 0 from Spain) were not compatible with either wilt causing international isolates 8012 and 7989 or with local virulent isolates, so these belong to another VCG. The less virulent /non-pathogenic isolates 2002, 2003, 2004, 2008, 2009, 2014 and FOI were not compatible with each other and with virulent isolates.

No correlation was found between races and vegetative compatibility groups however a relationship occurred between symptoms produced by the isolates and VCG, because all the wilt causing isolates belonged to same VCG 0280. Our results suggest that two distinct VCG's are prevalent in the world and only wilt causing VCG is prevalent in Pakistan.

Introduction

Chickpea wilt caused by *Fusarium oxysporum* Schlechtend; Fr. f.sp. *ciceris* (Padwick) Matuo & K. Sato, (FOC), is the second most important disease of chickpea in Pakistan. It has reduced the share of chickpea on irrigated lands from 50% in 1950s to only 10% in 1990s (Hanif *et al,* 1999). The fungus is seed borne as well as soil borne and can survive in the soil for more than five years, moreover it has some symptom-less carriers like lentil and peas (Saxena & Singh, 1987). It is impracticable to control the disease by using fungicides and through crop rotation. Use of resistant varieties is the best way to combat the disease. But due to absence of true resistance in chickpea against wilt disease and a continuous problem of the occurrence/development of new pathogenic races (Jimenez-Diaz *et al.*, 1989), it has become very difficult to overcome the yield losses. Vegetative compatibility groups (VCG) have been used to estimate the virulence level of the isolates in *F. moniliforme* (LaMondia & Elmer, 1989). It is a very easy, economical and effective method for the characterization of the prevalent races of FOC as compared to the other modern techniques of DNA fingerprinting like RFLP, RAPD etc. The characterization of the fungus will help in geographic demarcation of FOC pathotypes in Pakistan and will facilitate the selection of appropriate host cultivars for different regions.

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Vegetative compatibility is useful to characterize diversity in fungi (Leslie, 1993). It has been used to elucidate the evolutionary relationship among races within several formae specialis of *Fusarium oxysporum* (Elias *et al.*, 1993; Marlatt *et al.*, 1996). Vegetative compatibilty has been shown to be correlated with race in a number of formae specialis, however Elias & Schneider (1991) reported that vegetative compatibility was not closely correlated with race in *F. oxysporum* f.sp. *lycopersici*. Some vegetative compatibility groups contained multiple races e.g., isolates of *F. oxysporum* f. sp. *lycopersici* of race 1,2 and 3, with a widespread geographical distribution, belonged to single VCG 0030, whereas race 1 and 2 were found in all three of the predominant VCGs. Elias *et al.*, (1993) found that isolates within a VCG were more genetically similar to one another than to isolate in different VCGs. They also concluded that different races might have evolved from within several of the VCGs found within forma specialis. Such micro evolutionary events were proposed by several models of race evolution of *F. oxysporum* (Correll, 1991; Kistler & Momol 1990). Nogales-Moncada *et al.*, (1993) have reported that the isolates of FOC from various races belong to a single VCG (0280) *F. oxysporum* f. sp. *ciceris* collected from various chickpea growing areas of the world except Pakistan.

The objectives of the present studies were to characterize *Fusarium oxysporum* f.sp. *ciceris* isolates of Pakistan and to use these informations for the diagnosis of pathotypes and vegetative compatibility groups.

Materials and Methods

Isolation, identification and maintenance of FOC: Diseased chickpea samples were collected from Thal area, Punjab, Pakistan, during a survey in March 2000. Collar regions from diseased seedlings were cut, surface sterilized with 2% sodium hypochlorite for 2 minutes, rinsed in sterilized distilled water and placed on Petri plates containing Komada's medium (KM), which is specific for *Fusarium oxysporum* (Komada, 1975). Plates were incubated at 25±2°C with photoperiod of 16 hrs light and 8 hrs of dark for 5 - 7 days. FOC colonies appeared on KM medium were sub-cultured and single spored on PDA. Identification of the isolates was confirmed on carnation leaf agar (CLA) medium (Fisher *et al.*, 1982), and later on maintained on chickpea agar meal (2% chickpea meal, 2% dextrose and 2% agar agar) and stored at -15°C for further use. Prof. R. Jimenez-Diaz (Agronomy Dept., E.T.S.I.A., Univ. Cordoba, Spain) kindly provided *Fusarium oxysporum* f. sp. *ciceris isolates* of race 0 (7952 & 7802 causing yellowing from Spain), race 1A (7989, wilt causing from India) and race 5 (8012, wilt causing from Spain).

Biological pathotyping: Pot method as described by Nene *et al.*, (1981) was used against Aug-424 (susceptible) and CM 88 (tolerant) varieties. Wilt sick soil was prepared and filled in small plastic pots. Six seeds of each variety were sown in each pot in three replications. The observations were made at five days interval after germination up to 15 days later when complete wilting was observed in virulent isolates. The experiment was repeated twice in field conditions (from 15th January to 28 February) as well as in controlled environmental conditions (Maximum temperature 30±3°C, Minimum temperature 25± 2°C, Light 4000 lux for 12 hr).

Recovery of nitrate non-utilizing mutants: Minimal agar medium (MA) was prepared as described by Hanif & Haq (1998). Chlorate containing Minimal agar medium (KMA) was used to recover nitrate non-utilizing (nit) mutants was prepared by amending MA with 1.6 g/l L-asparagine and 15 g/l $KClO_3$.

The isolate of FOC was grown on MA for 3-4 days at room temperature (25±2°C). Then 2mm discs of the fungus were picked from these plates and transferred in the center of sterilized Petri plates containing KMA and incubated at 25±2°C in dark up to 15-30 days until the appearance of fast growing chlorate resistant sectors (mutants). The sectors were picked by sterilized wire loop and transferred to MA and incubated at 25°C.

Characterization of Nit mutant: The nit mutants were characterized into three phenotypic classes on the basis of growth pattern on MA media containing different nitrogen sources (Correll, 1987).

1. Nitrate medium: MA as described above,
2. Nitrite medium: MA plus 0.5 g/L Sodium nitrite,
3. Hypoxanthine medium: MA plus 0.2 g/L hypoxanthine

To determine the physiological phenotypes, a 2-mm mycelial block of each nit mutant and the parent isolate was put on each of the three media. Plates were incubated as described above. The colony morphology was scored relative to wild type parent after 4 days (Table 1).

Table 1. Phenotypes of nit mutants of FOC by growing on different nitrogen sources.

Mutation	Mutant Designation	Growth on nitrogen sources		
		Nitrate	Nitrite	Hypoxanthine
None	Wild type	Wild Growth	Wild Growth	Wild Growth
Nitrate reductase Structural locus	Nit 1	Submerged Growth	Wild Growth	Wild Growth
Pathway specific	Nit 3	Submerged	Submerged	Wild
Regulatory locus		Growth	Growth	Growth
Molybdenum Co-factor loci	Nit M	Submerged Growth	Wild Growth	Submerged Growth

NITRATE = MM = Minimal medium with 2g/l Sodium nitrate.
NITRITE = MM, Modified with 0.5g/l Sodium nitrite.
HYPOXANTHINE = MM, Modified with 0.2g/l Hypoxanthine

Vegetative compatibility groupings: The nit mutants of local isolates were paired in all combinations with each other and with that of reference isolates in order to assign the isolates to the VCG of FOC. All pairing tests were conducted three times to confirm VCG identity.

Results and Discussion

Biological pathotyping of FOC: Based on the percentage mortality of chickpea varieties, the FOC isolates were divided into three groups i.e., highly virulent group (more than 50% mortality), virulent group (more than 25% mortality) and less virulent group (less than 25% mortality). The isolates 2005, 2007, 2012, 9718 and 9910 produced more than 50% mortality in both the varieties (Aug-424 and CM 88), so these were placed in highly virulent group (Table 2). The isolate 9718 was the most aggressive of all and showed maximum average mortality (98%) against both the varieties (Fig. 1). The

Table 2. Distribution of pathogenic isolates of *Fusarium oxysporum* f.sp. *ciceris* in the chickpea growing area of Thal (Punjab).

S. No.	Isolate	Area	Percentage mortality Aug-424	Percentage mortality CM 88	Average mortality %
1.	2001	AliKheil	2.5	0.0	1.25
2.	2002	Nurpur Thal Road	0.0	25.5	13.0
3.	2003	Hernoli	5.0	40.0	22.5
4.	2004	Chandni Chock	7.0	12.0	9.5
5.	2005	NurPurThal	95.0	51.0	73.0
6.	2007	Gohr wala (I)	94.0	74.0	84.0
7.	2008	Dule Wala	18.0	27.5	23.0
8.	2009	Shadia	9.0	6.0	7.5
9.	2011	Jandan Wala	62.0	18.0	40.0
10.	2012	Rang Pur	86.5	60.0	73.0
11.	2014	Gohr Wala (II)	5.0	12.5	9.0
12.	FOI	Faisalabad	12.0	7.0	9.5
13.	9718	Jamali	100.0	95.5	98.0
14.	9910	Jamali	93.0	55.0	74.0
15.	9917	Van Bachhran	16.0	24.0	20.0
16	9925	Unknown	59.0	22.5	41.0
17.	7952	Spain	27.0	33.0	30.0
18.	8012	Spain	40.5	8.0	26.5
19.	7989	India	21.0	59.5	40.0
20	7802	Spain	30.0	21.0	25.5

isolates 2011, 9925, 7952, 8012, 7989 and 7802 were virulent. Rest of the isolates (2001, 2002, 2003, 2004, 2008, 2009, 2014, 9917 and FOI) were less virulent as they produced less than 25% mortality. None of the isolates from Spain (both yellowing and wilt causing) did show highly virulent response against local varieties, which might be due to the difference in the genetic systems in our local desi type varieties or the isolates might be well adapted to infect the local kabuli varieties of Spain. The isolates 2002, 2003, 2008 and 7989 showed enhanced virulence against CM 88 (resistant) as compared to Aug-424 but the percentage mortality was less as compared to highly virulent isolates, might be belonging to a similar race. This indicated the prevalence of races of *F. oxysporum* f. sp. *ciceris* in Pakistan.

The isolates 2011 and 9925 showed highly virulent response against susceptible variety (Aug-424) but against CM 88 they became less virulent so their average response was virulent. Similarly the isolates 2002, 2003 and 2008 were virulent against Aug-424 but less virulent vs. CM 88. Only the isolate 7989 (race 1A from India) showed less virulent response to Aug-424 but was highly virulent to CM 88. The isolates 2001, 2004, 2009, 2014 and FOI produced less than 10% average mortality against both varieties and these might be non pathogenic isolates of FOC. The susceptible variety Aug-424 showed complete wilting after 15 days of germination and the resistant variety CM 88 wilted at 25-27 days of germination by highly virulent isolates.

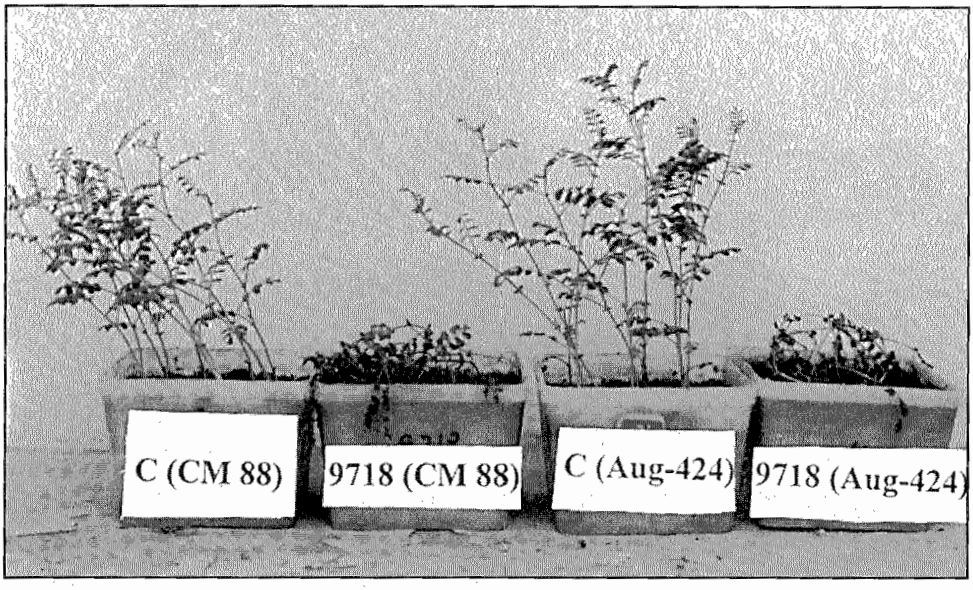

Fig. 1. Wilting of chickpea varieties Aug-424 and CM 88 by highly virulent isolate 9718.
C= Control

Vegetative compatibility groups of FOC: The isolates of FOC when grown on the minimal medium containing $KClO_3$ showed retarded growth initially for 8-10 days but after 15-20 days fast growing sectors appeared from the colonies. The retarded growth was due to the production of toxic chlorite ions, which were produced by the action of nitrate reductase on chlorate ions, which were structurally analogous to nitrate ions. These toxic conditions forced the fungus to mutate itself to inhibit the production of nitrate reductase, which formed thick sectors. Mycelia were picked from different sectors and grown on MM, where they produced thin expansive growth, which confirmed that these were not able to utilize the nitrate ions (Correll, 1987). Seventy nitrate non utilizing mutants of 20 FOC isolates were recovered and were characterized into nit 1, nit 3 and nit M based on their ability to utilize different nitrogen sources (Fig. 2). One set of each type of nit mutant was taken for further experiments and rest were discarded. No nit 3 and nit M mutants could be recovered from yellowing causing isolates 7952 /7802 and except 2002, 2003, 2004, 2005, 9910, 9925 and local isolates were nit 1 mutant. So their nit 1 mutants were only tested for complementation with nit 3 and nit M mutants of remaining isolates. No nit mutants could be recovered from isolate 2001.

Complementation test of various nit mutants of local isolates with each other and with international isolates revealed that the virulent isolates viz., 2005, 2007, 2011, 2012, 9718 and 9910 were compatible with wilt causing isolates 8012 (race 5 from Spain) and 7989 (race 1A from India), so these isolates belonged to VCG 0280 (Fig. 3). The yellowing causing isolates 7952 and 7802 (race 0 from Spain) were not compatible with either wilt causing international isolates 8012 and 7989 or with local virulent isolates, so these must be put in another VCG. Kelly *et al.*, (1994) also reported the prevalence of two main groups i.e., yellowing causing and wilt causing groups as determined by RAPD analysis.

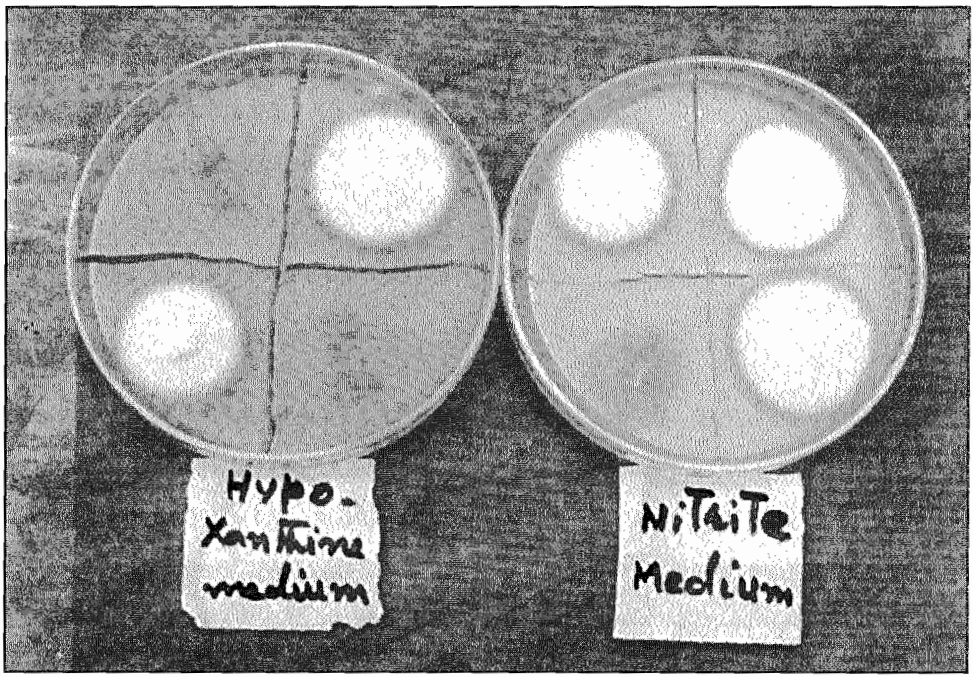

Fig. 2. Characterization of nit mutants on minimal medium containing sodium nitrite and hypoxanthine nitrogen sources (plates showing wild type and submerged growth by four nit mutants).

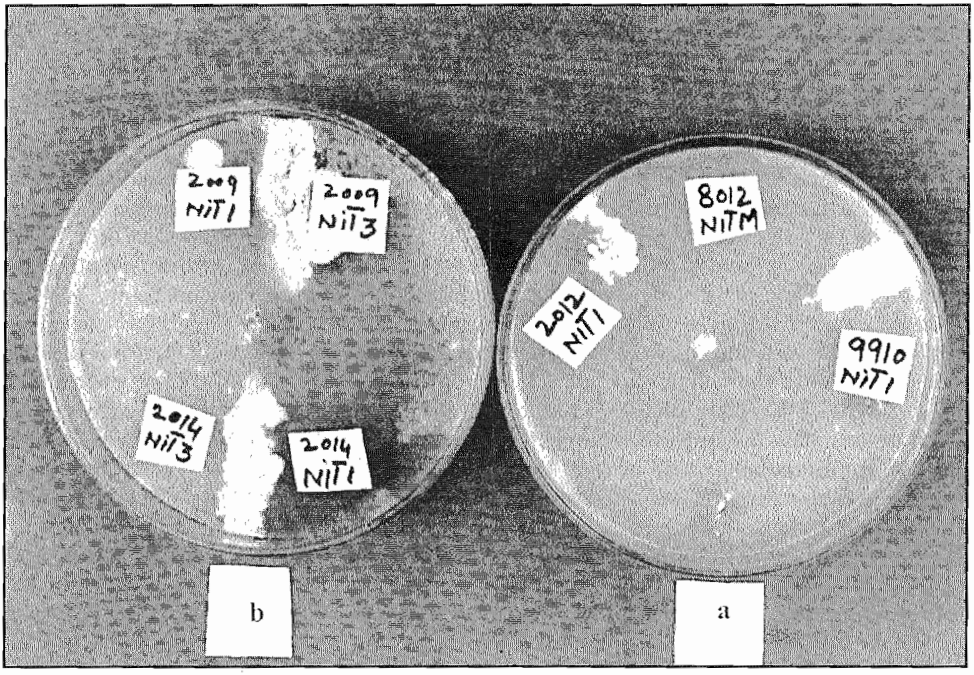

Fig. 3. a) Cross complementation of nit M mutant of 8012 isolate with nit 1 mutants of 9910 and 2012 local isolates; b) Self complementation of nit 1 mutants of 2009/2014 isolates with their nit 3 mutants.

The less virulent /non-pathogenic isolates 2002, 2003, 2004, 2008, 2009, 2014 and FOI were not compatible with each other and with virulent isolates. These isolates seemed to be genetically different to the virulent isolates. These isolates might be the non-pathogenic isolates of *F. oxysporum*.

In our results no correlation was found between races and vegetative compatibility groups because the isolates 8012 of race 5 and 7989 of race 1A belonged to single VCG. Elias & Schneider (1991) also reported that vegetative compatibility was not closely correlated with race in *F. oxysporum* f.sp. *lycopersici*. However a relationship occurred between symptoms produced by the isolates and VCG, because the wilt causing isolates belonged to same VCG (0280). Kelly *et al.*, (1994) had placed most of the yellowing causing isolates of FOC from Spain in race 'O', and all these isolates belonged to separate VCG in our experiments so a correlation occurred between race and VCG in yellowing causing isolates.

As the local virulent isolates of FOC were compatible with the wilt causing isolates of race 5 /1A and were not compatible with yellowing causing race 0 isolates, this means that races 5 and 1A might be prevailing in Pakistan and not race O, which confirmed the results of Jimenez-Diaz *et al.*, (1990) who reported that yellowing causing race 0 isolates were mainly found in Mediterranean region only.

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