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Identification of *Fusarium solani* F. sp. *pisi* the Cause of Root Rot in Chickpea and Assessment of its Genetic Diversity Using AFLP in Northeast Iran

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Abstract: Twenty isolates of *Fusarium solani* f. sp. *pisi* were obtained from diseased chickpea plants collected from different chickpea growing areas in northeast Iran. A pathogenicity test was performed for all isolates. Result indicated that the *Fsp.* isolates differed in their virulence on the susceptible cultivar Jam. AFLP was used to evaluate the genetic variation among 20 isolates of *Fsp*. The 4 primer combinations produced 330 scorable bands of which 110 were polymorphic (34%). The dendrogram constructed using UPGMA method, distinguished 4 main groups at 65% similarity level that multi dimensional analysis confirmed it. No clear trend was detected between clustering in the AFLP dendrogram and geographic origin. The AFLP analysis in this present study showed an apparent link with result of pathogenicity tests.

Key words: Chickpea, fusarium root rot, AFLP, pathogenicity

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most important pulse crops cultivated in many countries of Africa and Asia. In addition to its importance as food crop, it is valued for its beneficial effects in improving soil fertility and thus profitability and sustainability of production systems (Hannareddy and Dubey, 2006).

Fusarium root rot caused by *Fusarium solani* Mart. amend. Sacc. f. sp. *Pisi* Synder and Hansen *(Fsp)*, is one of the major factors limiting chickpea production worldwide (Westerlund *et al*., 1984). The disease is widespread in chickpea-growing areas of the world and is reported from different countries, causing significant economic losses (Trapero *et al*., 1985; Westerlund *et al*., 1984). The pathogen persists in the soil as chlamydospores that can survive for several seasons and is capable of colonizing crop residue and roots of most crops grown in rotation with chickpea and some weeds (Mohammadi and Banihashemi, 2006). As a result of the persistence of the pathogen in the soil, the use of resistance cultivars is one of the most practical and cost-effective strategies for managing *Fusarium* rot, but deployment of resistant varities has not been extensive because of undesirable agronomic characteristics (Jimenez *et al*., 1974). Besides, the high pathogenic variability in *Fusarium solani* (Telomorph: *Nectria haematococca*) may limit the effectiveness of resistance (Suga *et al*., 2000). Determination of both host specifity and genetic diversity in *Fsp*. population are important in breeding for resistance. Pathogen diversity can be studied using a wide array of techniques. The amplified fragment length polymorphism (AFLP) method is based on selective amplification of restriction fragments generated from total genome DNA (Vos *et al*., 1995). It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The AFLP method generates dominant markers, which are less useful for studies that require precise assignment of allelic states, such as heterozygosity analysis.

Nevertheless, because of their rapidity, replicability and high resolusion, AFLP markers have emerged as a major type of genetic markers with a broad range of application, especially in analysing genetic variation (Muller and Woffenbarger, 1999). AFLP fingerprinting has been shown to have potential for high-resolution differentiation of genetically related *Fusarium* sp. isolates from different crops (Abd-Elsalam *et al*., 2003; Abdel-Satar *et al*., 2003; Blabid *et al*., 2004). Little is known about the genetic diversity of the *Fsp* isolates in northeast Iran or about the host range of this pathogen. The objectives of the present study were to, characterize *Fsp* associated with chickpea black root rot disease in northeast Iran by morphological characterization and pathogenecity test and to, use AFLP markers with a range of primer pairs to find sufficient variation to draw

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conclusions about the genetic relationship within *Fsp* isolates and determine their correlation to pathogenicity.

MATERIALS AND METHODS

Fungal isolates and pathogenicity test: Chickpea plants with yellowing and wilting symptoms were collected from major chickpea growing area in northeast Iran during 2004-2005 growing season. Segments of diseased roots were surface-sterelized and cultured on PDA medium. were cultured Single-spore from germinated macroconidia using serial dilution method was prepared as described by Cother *et al*. (1977). The isolates were identified according to identification keys of *Fusarium* sp. (Burgess *et al*., 1994; Gerlach and Nirenberg, 1982). To determine colony morphology, growth rates and pigmentation, isolates were grown on PDA and incubated under a 12 h dark/light cycle both at 25 and 30°C. Isolates were transferred to SNA test tube slants and makarti glasses containing sterile sand for short-term and long-term storage, respectively. Inoculum was prepared as described by Armengol *et al*. (2000) and spore suspensions were adjusted to 1×10^6 microconidia mL^{-1}.

The *in vitro* pathogenicity test was carried out under greenhouse conditions (at 20±5°C with a 12/12 h light/dark photoperiod) on chickpea cultivar, Jam. Seeds were surface-sterelized using 1% sodium hypochlorite for 5 min, rinsed in distilled water and grown (10 seeds/pot) for 2 weeks in plastic pots containing 550 g of sterilized sand. The 15-days old seedling (around 8-10 cm long) were carefully uprooted and the roots washed under running tap water to remove excess sand. The roots of seedling were then dipped into 10 mL of the inoculum for 1.5 min and transfered to soil mixture consisting of field soil, sand and compost (1:1:1 by volume) that was moistened and autoclaved for 1 h for 2 consecutive days. As a control, seedlings with similarly prepared root tips were dipped in distilled water for 1.5 min. The expriments was carried out using a randomized complete block design with four replication. Host range was defined by inoculation of the isolates on eight different cultivars including (pea, bean, mung bean, lentil, soybean, melon, watermelon, tomato). The disease severity was recorded 45-50 days after inoculation using a 1-9 scales described by Bhatti and Kraft (1992). Isolate identities were confirmed through re-isolation.

DNA extraction: Pure cultures of all isolates of *Fsp* were grown on potato dextrose broth for 10 days at 25±1°C. Cultures were filtered through a double layer of sterile muslin and the mycelium was washed with distilled water. Lyophilized tissue was pulverized in liquid nitrogen. Total genomic DNA was extracted using a modified CTAB (Cetyldimethylethyl Ammonium Bromide) as described previously (Weising *et al*., 1995). Total DNA was dissolved in 50-200 of Tris- EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) depending on the size of the DNA pellet and quantified by measuring the nucleic acid/ protein absorbance ratio using a spectrophotometer (Biometra-Germany).The quality of the extracted DNA was visually checked on 1% agarose gels. Dissolved DNA was stored at -20°C until used. DNA extracts were adjusted to a final concentration of 100 ng μL^{-1} for AFLP analysis.

AFLP analysis: Genomic DNA was subjected to AFLP analysis based on the method described by Vos *et al*. (1995) with slight modification. All primers and adapters used in different steps were obtained from Sib Enzyme (Russion). AFLP templates were prepared by simultaneous digestion of approximately 500 ng of DNA with 5U each of *Eco*RI and *Mse*I. Ligation of the restriction fragments to the adaptors was performed in the same step. A 1:5 dilution of the restricted and adapter-ligated DNA was used as a template in the pre-amplification reactions. *Eco*RI and *Mse*I primers without selective nucleotides (*Eco*RI + 0 and *Mse*I + 0) were employed for pre-amplification. Electrophoresis of 5 μL aliquots of the pre-amplification products in a 1% agarose gel coincide with DNA size marker confirmed successful amplification. For the final selective amplification, the 1:20 diluted pre- amplification DNA was amplified based on Touch down PCR using four primer pairs including: *Eco*RI +AC/ *Mse*I +AG, *Eco*RI +AG/*Mse*I +AG, *Eco*RI +AG/ *Mse*I +AC, *Eco*RI +AG/ *Mse*I +CG. The amplified fragments were separated electophoretically in a denaturing 6% polyacrylamid gels. A glass plate was treated with Bind Silane in order to chemically cross-link the gel to the glass plate. Equal volumes of formamide loading dye and amplified product were loaded into each lane. Prior to loading the gel, samples were heated at 95°C for 5 min and then snap-cooled on ice to prevent DNA secondary structures fro reanneling. Gels run at constant power(500 W, at 50°C) and then stained by a modification of the silver staining method of Sanguinetti *et al*. (1994).

Data analysis: For each isolates, the DNA fingerprints were manually scored by considering 1 for the presence or 0 for the absence of specific AFLP bands. Only major, distinct and very clear bands were scored. Monomorphic bands were not scored. Cluster analysis was performed on the similarity matrix employing the Unweighted Pair Group Method Using Arithmetic Means (UPGMA) algorithm provided in the computer program NTSYSpc, version

2.02e (Ronlf, 1998). Also, multi dimensional scaling was performed using Statistica (version 5.5 A) software (Statessoft, 1995).

RESULTS

Morphological characterization: Twenty isolates of *Fsp* obtained from diseased chickpea plants collected from different agroclimatic regions in northeast Iran. All isolates were purified through single-spore method and on the basis of morphological characters were identified as *Fusarium solani* f. sp. *Pisi.* The morphological concept of *Fsp* in culture were characterized by production of slightly curved, usually 4-5 septate macroconidia with a blunt apical cell and foot-shaped apical cell from usually cream-colored but sometimes green sporodochia, abundant production of resistant chlamydospores reflecting its common soil habitat, production of 1-2 celled microconidia that varied in shap and long unbranched monophialides, predominantly cream-colored mycelia that might vary in pigmentation.

Pathogenicity test: All isolates proved pathogenic on susceptible chickpea cultivare Jam under artificial inoculation. Characteristic symptoms such as nonvascular yellowing, black collar. Yellowing of lower leaves and root rot were observed. The result of host specifity range tests conducted with 8 different cultivars showed that all the pathogenic isolates caused root rot only in chickpea and pea. Hence, the *formae special* of isolates identified as *Fusarium solani* f. s*p. pisi.* The disease development on chickpea cultivare Jam revealed variability in disease severity among isolates. the percentage of root rot with necrotic spots and yellowing or wilting chickpea seedling showed significant differences at p = 0.05. Based on this character (virulence rate) the isolates were classified in 4 groups; highly, moderately, weakly and very weakly virulent. The highly virulent isolates (H1,H3, FS2, FS7, CH, FS25, FS9, N2, FA, MP) caused 100% root rot and decreased growth or death of plants. The moderately virulent isolates (FS25, FS18, FS31) lead to 50% root rot and wilting seedlings and decrease of secondary roots. The weakly virulent isolates (FS35, FS20, FS36, FSF, FS14, FS16) caused about 20% wilting or 25% hypocotyl covered with necrotic spots. One isolate (FS28) showed the lowest level of virulence among all isolates tested that caused less 10% root rot and minor yellowing seedling.

AFLP analysis: Genetic variation was detected among 20 isolates of *Fsp* using AFLP marker. Sixteen *Eco*RI+NN/*Mse*I+NN primer combination were tested on five isolates from different regions. Finally, Four primer pairs were selected for the diversity screening including *Eco*RI +AC/ *Mse*I +AG, *Eco*RI +AG/ *Mse*I +AG, *Eco*RI +AG/ *Mse*I +AC, *Eco*RI +AG/ *Mse*I +CG based on the clarify of the bands obtained, the number of fragments amplified and the polymorphism rate observed. A total of 330 bands were amplified from four primer combination, of which 110 bands (34%) were polymorphic, with an average of 27.5 polymorphic bands per primer combinations. On the basis of this molecular marker, the estimated genetic diversity index for each primer combination was ranged from 0.29- 0.38.

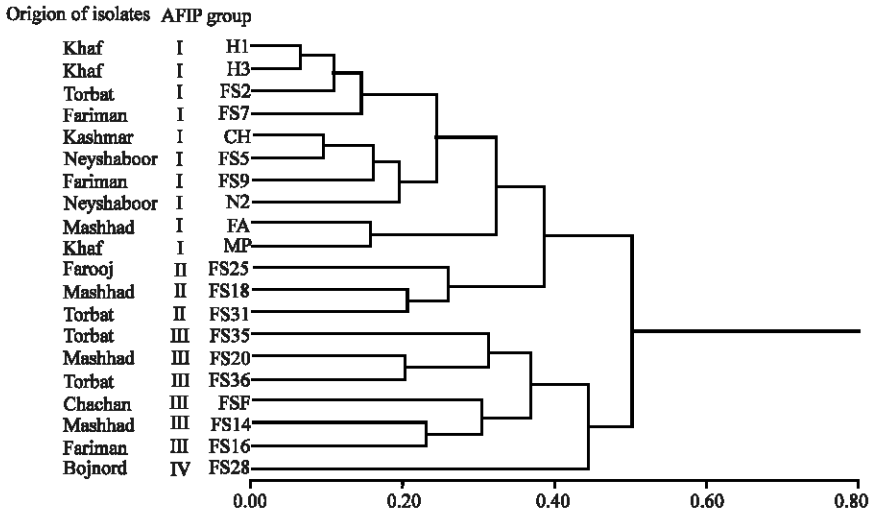

Fig. 1: Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 20 *Fusarium solani* f. s*p. pisi* isolates based on genetic distance

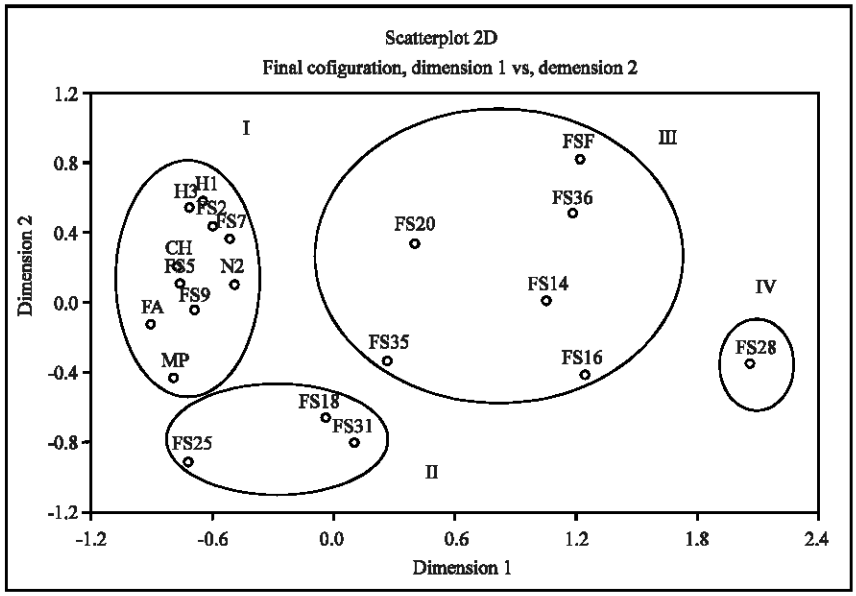

Fig. 2: Multi- dimensional display generated based on matrix of genetic distance by STATISTICA among 20 *Fusarium solani* f. sp. pisi isolates

Also, the pair-wise genetic distance of the isolates varied from 0.06-0.78. The least genetic distance belonged to isolates H1 and H3 from Khaf region while the highest distance belonged to isolates MP and FS28 from Khaf and Bojnord. DNA fingerprinting of 20 *Fsp* isolates with AFLP-primers were analysed by Jaccard similarity coefficient to generate a dendrogram.

UPGMA analysis of the AFLP banding patterns distinguished four main groups, joined by a single node at the 65% similarity level. The genetic groups were named from I to IV (Fig. 1). Moreover, multi dimensional analysis confirmed it (Fig. 2). The first group, AFLP 1, included 10 isolates (H1,H3, FS2, FS7, CH, FS25, FS9, N2, FA, MP). Isolates of this group originated from different regions. The isolates of this group were highly virulent. The second group, AFLP II, included 3 isolates (FS25, FS18, FS31) that were moderately virulent. The third group, AFLP III, included 7 isolates (FS35, FS20, FS36, FSF, FS14, FS16) that were weakly virulent. The forth group included only one isolate, i.e., Fs28, which is the lowest level of virulence.

DISCUSSION

Morphology of the *Fsp* isolated from cultured diseased chickpea agreed well with systems provided by Burgess *et al.* (1994), Gerlach and Nirenberg (1992). Besides, the variability of virulence of *Fsp* has been determined in this study by conventional pathogenecity test. The isolates studied exhibited different virulence level and distinguished 4 groups. Although, most plant pathogenic *Fusarium* species have a broad host range, many species, including *F. solani*, were thought to cause disease only on a narrow range of host plant. Hence, the idea of *formae specials* in *F. solani*, meant to indicate the ability of causing disease only on particular hosts (Suga *et al.*, 2000). In the present study, defined of host specific range showed that the pathogen caused root rot only in chickpea and pea and was identified as *Fusarium solani* f. sp. *pisi*. AFLP is a powerful tool in molecular fingerprinting and for studying relationship among fungal isolates at the population, species and superspecific level (Abd-Elsalam *et al.*, 2003). From the four primer combinations scored in this study, 330 AFLP bands were reliably detected. Of these, 110 were polymorphic (a 34% polymorphism rate). AFLP analysis can be useful in the identification of genetic diversity among *Fsp* isolates.

The AFLP analysis in the present study showed a clear link with the pathogenicity and molecular characterization of the isolates. The pathogenicity test separated *Fsp* isolates to 4 different groups based on disease severity on susceptible cultivar Jam by a 1-9 scale. Also, AFLP results revealed the four genetic groups. In addition, we showed that there was no correlation between AFLP analysis and geographic origine of the isolates. Understanding the genetic relationships of *Fusarium* sp. is very important. A plant pathologist diagnoses a *Fusarium* sp. and expects to be able to make inferences about its biology, pathogenecity, economic importance and methods of control, based on the identification. Thus, correct species identification and assessment of genetic diversity have important

implication for the control of disease worldwide. The present study generated significant information in terms of pathogenic and genetic variability of *Fsp* which could be used further for development of area specific resistant varieties of chickpea. This study also highlights the facts that both pathogenic virulence analysis and AFLP markers are useful tools for analysing the structure of the pathogen population, but furthere studies are needed to make them complementary to each other .

In summary, we identified an optimal set of AFLP primer combinations suitable for the evaluation of genetic variation among *Fsp* isolates. The largest number of polymorphic bands was produced using primer combination *Eco*RI-AC/*Ms*eI-AG and the lowest was produced with *Eco*RI-AG/*Ms*eI-CG. Our molecular data that is genetic distance in isolates elucidated initial information on the occurrence of a relatively high level of genetic diversity affecting within *Fsp* isolates associated with chickpea root rot disease. As a result AFLP marker is a versatile and vigorous technique in studies on the genetic variation in natural population of *Fusarium* species.

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