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IDENTIFICATION AND GENETIC VARIATION OF *FUSARIUM* SPECIES IN ISFAHAN, IRAN, UNING PECTIC ZYMOGRAM TECHNIQUE*

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Abstract – The genus *Fusarium* is a species rich genus. Different media are required to study the morphological characters and classify different species, a time consuming technique. In this study pectic zymograms were used to identify species and form species of *Fusarium* isolates. Fusarium isolates (318) were obtained from different areas and hosts in Isfahan Province, Iran. Isolates were identified to species based on morphological characters. A pectic enzyme solution was prepared for each isolate using liquid media containing citrus pectin as the sole carbon source. Electrophoresis was performed using acrylamide gel containing 0.2% citrus pectin as the enzyme substrate. The gels were incubated in 0.1M malic acid before staining overnight in 0.02% ruthenium red, to visualize enzyme electrophoretic patterns. Several zymogram phenotypes were obtained for polygalacturonase and pectin esterase. In total, 12 zymogram patterns were determined for the 318 isolates tested. The results showed that there is considerable intraspecific variation in *Fusarium* species. There were 3, 5 and 2 zymogram electrophoretic patterns for *Fusarium oxysporum*, *F. solani* and *F. culmorum* respectively. However, there was only one zymogram pattern for *F. subglutinans* and also one for *F. equiseti*. Although the intraspecific variation based on pectic zymograms was not correlated to the form species of *Fusarium*, species of *Fusarium* could be distinguished using this technique as there was no common zymogram pattern among species.

Keywords – *Fusarium*, pectic enzymes, zymograms

1. INTRODUCTION

The genus *Fusarium* has a worldwide distribution. It's different species are considered to be some of the most important plant disease pathogens [1], with some species producing mycotoxins on plants which contaminate the seeds [2] and some such as *F. solani*, cause diseases in humans and animals [3, 4] and are thus hazardous to agricultural products, wildlife, livestock and humans. For this reason identification of the different species of *Fusarium*, including saprobic, pathogenic and toxin producing species, is of vital importance [1].

The genus *Fusarium* consists of populations that are quite variable. For this reason, identification of its different species requires special culture media and methods, as well as standard incubation conditions. High variability in species, especially under different environmental conditions, has caused taxonomists to consider some special criteria to be important in the classification of species. For this reason, different methods and keys have been presented for the identification of the species [5].

The large variation in some of the characteristics of *Fusarium* isolates such as pathogenicity, colony morphology of the colony, e.g. form and color, mode of development of the chlamydospores, existence or lack of sporodochia and even the type of microconidia, have resulted in different emphasis on character and different classification of species into intra-specific groups. The use of sub-species in the

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classification of *Fusarium* makes it possible to distinguish between the populations with major ecological and physiological differences, but with no major morphological differences [6]. Most of the intra specific classifications are based on pathogenic behavior and vegetative compatibility groups (VCG). Some researchers have placed some isolates of VCG into the same race using the pectic zymogram technique [7]. Others have classified different races according to special geographic regions [7] and many have used different molecular markers for distinction among species [8].

Many fungi, including the *Fusarium* species, release extra-cellular enzymes which break down the pectin of the cell walls of many plants. Pectic enzymes of pathogens cause root rotting and wilting of plants. Different patterns resulting from pectic enzymes have been studied in different *Fusarium* isolates [9, 10]. The aims of some research were to study the relationship between pectic enzymes and pathogenicity [11]. Other researchers have used the same method to improve the taxonomy of *Fusarium*. Szeczi [9] has used the pectic zymogram method to clarify the taxonomy of *F. lateritium* and its related species. Results revealed differences among *F. stilboides, F. xylarioides* and *F. lateritium* regarding R*f* value of pectin esterase (PE) and polygalacturonase (PG) bands. In this study, the isolates of *Fusarium* recovered from different areas and hosts were also identified using the pectic zymogram technique.

2. MATERIALS AND METHODS

a) Sampling, isolation and identification

Diseased potato, tomato, bean, onion, cucumber, watermelon, cantaloupes and cotton plants were collected from different areas in Isfahan (Lenjan, Feridan, Broujen, Najaf-Abad and Baraan) in the spring and summer of 1999 and 2000. Symptoms such as wilting, vascular discoloration and root rotting were considered as indices for sample collection.

The collected samples were cut into small pieces (2-3 cm) and washed in running tap water for 20 minutes, surface sterilized in 10% sodium hypochlorite for 2 minutes, rinsed in sterilised distilled water and air dried on sterile filter paper. The disinfected pieces were cut into 3-5 mm pieces, placed on acidified PDA and incubated at 25ºC in the dark. After two or three days the fungal colonies were observed under a microscope and hyphal tips of *Fusarium*-like fungi were transferred to PDA. The pure cultures of the isolates were obtained using a single-spore culture technique [12]. For long-term preservation, *Fusarium* isolates were grown in Bijoux bottles containing sterile dry sand and wheat bran as recommended in Butler [13].

Macroscopic and microscopic characteristics of the pure cultures were studied and the species were identified using illustrated keys [1, 14]. The macroscopic characteristics were studied on PDA culture, the microscopic characteristics, however, were studied on CLA (Carnation Leaf Agar).

b) Pectic zymogram analysis

Zymogram is an electrophoretic method for measuring protheolithic activity [15] in which the enzymes are separated from one another in a polyacryl-amid gel [16]. A 5 mm diameter plug from each colony margin was transferred to Bijoux bottles containing 2 ml of sterile liquid medium as recommended in Cruickshank and Wade [17]. The medium contained 1% citrus pectin as the sole carbon source [18]. Cultures were grown at 25ºC in the dark without shaking for 6-9 days. Mycelium was then removed by filtration using sterile Watman filter paper. One hundred µL of each culture filtrate was mixed with 0.01 g Sephadex G-200 to make slurry which was kept at room temperature (23-25°C) for 30 minutes before electrophoresis. The electrophoresis was performed using Cruickshank and Wade's method [17]. Horizontal pectin-acrylamide gels were prepared as recommended by Sweetingham *et al*. [18]. The volume of 10 µL of culture filtrate (Sephadex slurry) from each sample was loaded into the wells and 2-3

µL bromophenol blue were applied to each of the first and last wells as tracking dye. Cotton cloth was used as a wick at the cathodic and anodic ends of the gel. Power was supplied using a constant 12-14 mA per gel and electrophoresis was stopped when the tracking dye had migrated 5 cm toward the anodic end. An aqueous solution of boric acid (7.2 g/l) and tetraborate dehydrate (15.75 g/l) was used as a tank buffer and cold water (4-5ºC) was circulated underneath the gel supporter plate during electrophoresis.

3. RESULTS

A total of 318 isolates were characteristic of *Fusarium*. Many isolates produced canoe-shaped macroconidia from early stages of growth and the rest developed macroconidia after temperature shock or under light conditions. Based on morphological characters, *Fusarium oxysporum, F. equiseti, F. subglutinans, F. solani* and *F. culmorum* were identified from the samples, with *F. oxysporum* having the highest frequency.

Isolates of *F. oxysporum* from different hosts produced white colored colonies on PDA with aerial mycelium. However, the lower surface of the colonies was pink or light to dark violet. Canoe-shaped macroconidia with a long apical cell and a foot-shaped basal cell formed with 3 to 5 septa. Uni or bi-cellular, ovoid to ellipsoid microconidia were abundant. Microconidia formed in groups on CLA medium from short or sometimes branched monophialides. Chlamydospores were mostly single or rarely in chains on PDA or CLA in two-week old cultures. On some PDA cultures macroconidia were produced from orange sporodochia.

Fusarium solani isolates on PDA were cream or white and in rare cases the lower surface was light violet. Ring-shaped spordochia, with a cream or sometimes blue color were observed. Macroconidia were produced on CLA. They were quite similar to the macroconidia of the *F. oxysporum*, however they were wider and a conspicuous wall. Their apical cell was round, the basal cell was round or foot-shaped and they usually had three or occasionally four septa. The micrconidia were also abundant, mono or bi-cellular and oval or elliptical in shape. The conidia on CLA were formed in clusters on the elongated phialides.

Colonies of *F. culmorum* had yellow aerial mycelia with a red or brown base on PDA. Microconidia were, however, lacking short thick macroconidia with flattened apical cells, and basal cells that tapered to a point were produced. Macroconidia in CLA were mostly 5-(6-7) septate. Macroconidia were produced from branched or non-branched monophialides, while chlamydospores in PDA and CLA were produced in chains or columns.

The mycelia of *F. subglutinans* isolates on PDA was white, as well as in the sporulating colony, however in some samples, violet or pink, thus being similar to *F. oxysporum*. In this species microconidia were abundant and oval or elliptical and mostly mono-cellular. Macroconidia were very long, thin and bent. Conidia had three to five septa with foot-shaped basal cells. In this species both polyphialides and monophialides developed and orange color sporodochia were observed, however, no chlamydospores developed.

Cultures of *F. equiseti* on PDA were white, red or brown when old. In this species no microconidia was observed, however, well defined macroconidia with a very conspicuous, bent, somewhat elongated and needle like apical cell, with five to six conspicuous septa were observed. Chains of chlamydospores developed and orange sporodochia were also observed.

Zymogram patterns

To obtain reproducible and clear bands, the conditions with respect to: the duration of the growth of the fungus in the liquid culture medium, pH of the culture medium, environmental temperature, the amount of citrus pectin in the gel, the concentration of the liquid culture loaded on the gel and the mA

intensity of the electrophoresis current were determined. In *F. oxysporum, F. culmorum and F. equiseti* the high concentration of galacturonase (PG) enzyme necessitated dilution of the amount of the enzyme. In this way the amount of citrus pectin of the gel was reduced and a thinness of 25% was chosen for the samples to be loaded on the gel. The suitable volume of the liquid culture medium was determined to be 3 ml and the growth period between 6-8 days. In the case of *F. solani* isolates, due to the suitable concentration of the produced enzyme, the loaded sample was not diluted. A 2ml volume of the liquid culture and an 8-day growth period was adopted. In the case of *F. subglutinans* isolates, a thinness of 50% was used. For better separation of the bands, the suitable intensity of electrophoresis current was chosen as 12 and 14 mA respectively.

A total of 318 *Fusarium* isolates obtained from different hosts were studied using a pectic zymogram technique. The polyglacturonase (PG) isozymes were observed in all, and pectin esterase (PE) in many of the isolates. The patterns obtained from these 318 isolates were classified into 12 zymogram patterns based on the electrophoretic phenotypes. The 12 zymogram patterns identified in the isolates were designated as ZP1 to ZP12 (Fig. 1). There were 15 bands or isozyme loci, belonging to PG and PE (Table 1). Three bands, d, f and l were assigned as PE and the rest of the bands corresponded to the PG loci (Fig. 2). The faint and non reproducible bands were not considered in zymography analysis.

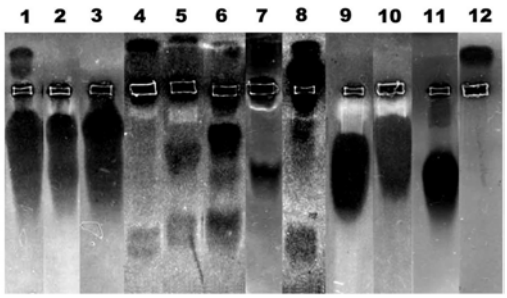

Fig. 1. Different zymogram patterns of isolates of *Fusarium* species. Lane 1, ZP1 (*F. oxysporum*); lane 2, ZP2 (*F. oxysporum*); lane 3, ZP3 (*F. oxysporum*); lane 4, ZP4 *(F. solani*); lane 5, ZP5 *(F. solani*); lane 6 (*F. solani*); lane 7, ZP7 (*F. solani*); lane 8, ZP8 (*F. solani*); lane 9, ZP9 (*F. culmorum*); lane 10, ZP10 (*F. culmorum*); lane 11, ZP11 (*F. subglutinans*); lane 12, ZP12 (*F. equiseti*)

Table 1. Zymogram patterns and frequency of different pectic isozymes polygalacturonase (PG) and pectin estrase (PE) based on R*f* value in five *Fusarium* species

R*f*	Band	Izozyme	*F.oxysporum*			*F. solani*					*F.culmorum*		*F.subglutinans*	*F.equiseti*
			ZP_1	ZP_2	ZP_3	ZP_4	ZP_5	ZP_6	ZP_7	ZP_8	ZP_9	ZP_{10}	ZP_{11}	ZP_{12}
0.12	*h*	PG	+	+	+	-	-	-	-	-	-	-	-	-
0.22	*v*	PG	-	+	+	-	-	-	-	-	-	-	-	-
0.40	*r*	PG	-	-	-	+	+	+	-	+	-	-	-	-
0.46	*s*	PG	-	-	-	+	+	+	-	+	-	-	-	-
0.20	*i*	PG	-	-	-	-	+	-	-	-	+	-	+	-
0.14	*t*	PG	-	-	-	-	-	+	+	+	-	-	-	-
0.28	*u*	PG	-	-	-	-	-	+	-	-	-	-	-	-
0.10	*p*	PG	-	-	-	-	-	-	-	-	-	+	-	-
0.08	*q*	PG	-	-	-	-	-	-	-	-	-	-	+	-
0.04	*l*	PE	+	+	-	-	-	-	-	-	-	-	-	-
0.08	*f*	PE	-	-	-	+	+	+	+	+	-	-	-	-
0.06	*d*	PE	-	-	-	-	-	-	-	-	+	+	-	-
	a	PG	+	-	-	-	-	-	-	-	-	-	-	-
	b	PG	+	-	+	-	-	-	-	-	-	-	-	+
	c	PG	-	-	-	+	+	+	+	+	-	-	-	-

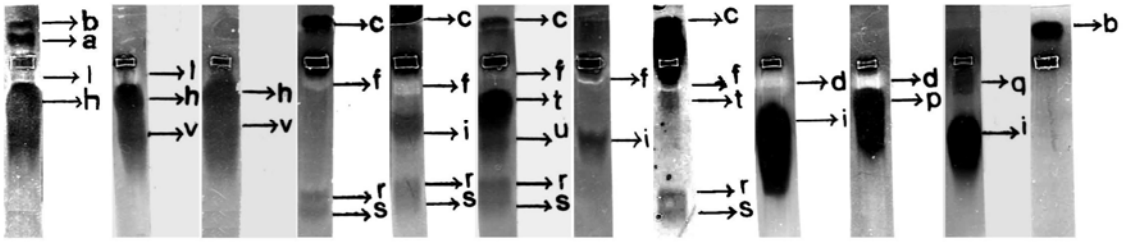

Fig. 2. Frequency of the polygalacturonase and pectin estrase isozymes observed in 12 zymogram patterns of *Fusarium* species. Three bands (d, f and l) belonged to pectin estrase and 12 bands to polygalacturonase loci. (Faint and non reproducible PG bands have not shown in data and not considered in R*f* value analysis)

Comparison among the 12 zymogram patterns revealed that ZP1, ZP2, and ZP3 belonged *to F. oxysporum*; ZP4, ZP5, ZP6, ZP7, and ZP8 to *F. solani*; ZP9 and ZP10 to *F. culmorum* and ZP11 and ZP12 to *F. subglutinas* and *F. equi*seti respectively (Table 2 and Fig. 3).

Table 2. Zymogram patterns observed for the isolates of *Fusarium* species tested

Fusarium species	No. of isolates	ZP1	ZP2	ZP3	ZP4	ZP5	ZP6	ZP7	ZP8	ZP9	ZP10	ZP11	ZP12
F.oxysporum	194	+	+	+	-	-	-	-	-	-	-	-	-
F. solani	71	-	-	-	+	+	+	+	+	-	-	-	-
F. culmorum	23	-	-	-	-	-	-	-	-	+	+	-	-
F.subglutinans	10	-	-	-	-	-	-	-	-	-	-	+	-
F. equiseti	20	-	-	-	-	-	-	-	-	-	-	-	+

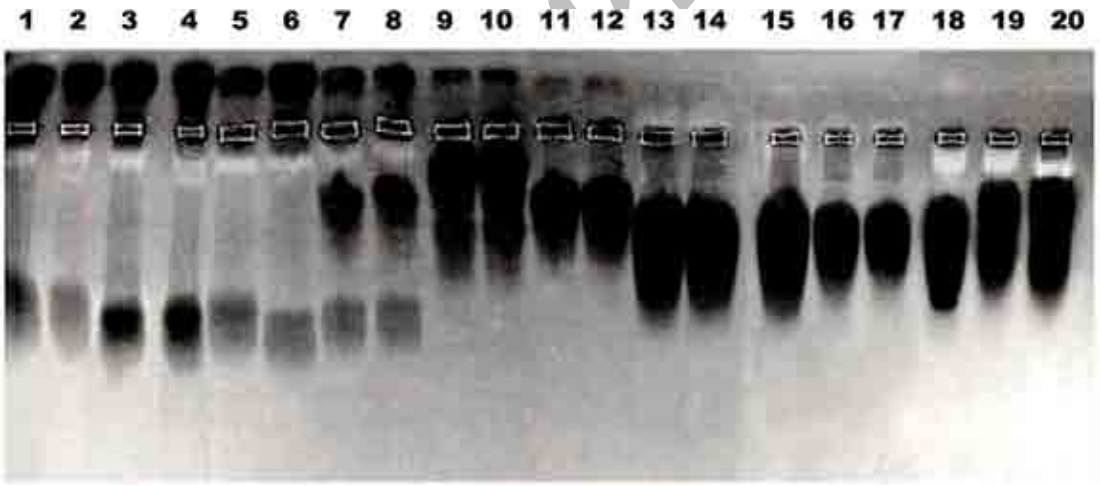

Fig. 3. Pectic zymogram patterns of isolates of *Fusarium* species. Lanes 1-8, *F. solani*; (lanes 1-6 ZP4 and lanes 7-8 ZP5), lanes 9-12, *F. oxysporum*; (lanes 9-10 ZP1 and lanes 11-12 ZP2), lanes 13-17, *F. subglutinans*; (ZP11), lanes 18-20, *F. culmorum* (lane 18, ZP9 and lanes 19-20, ZP10). Lanes 1 & 2 bean isolates; lanes 3, 4, & 5 tomato isolates; lanes 7, 8, 9 & 10 tomato isolates and lane 20 wheat isolate

All isolates within each species showed distinguishable pectic zymogram patterns, so that the difference in the magnitude of activity and movement of the PE and PG bands among different species was quite noticeable. *Fusarium oxysporum* and *F. culmorum* had similar patterns, however the lack of a PG cathodic band and the presence of strong PE in all the isolates of *F. culmorum* distinguished it from *F. oxysporum* (Fig. 3). In some of the isolates of *F. oxysporum* two distinguishable cathodic PG bands were observed (Fig. 4). Also many isolates from *F. oxysporum* produced a PE band with lower intensity than the PE band in *F. culmorum*. The zymogram patterns from *F. solani* isolates showed strong similarities (Fig. 3). All five of the pectic zymogram patterns in *F. solani* (ZP4, ZP5, ZP6, ZP7, ZP8), contained a

cathodic PG band (c), and a PE band (f) and the two anodic PG bands (r and s) with stable R*f* were also present (Fig. 5). The zymogram patterns in *F. subglutinans* and *F. equiseti* were similar in all the cases and did not indicate any differences within the species.

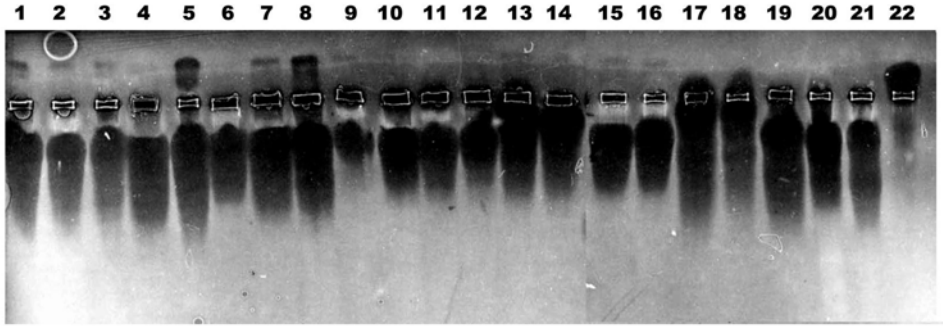

Fig. 4. Zymogram patterns of isolates of *Fusarium oxysporum*. Lanes 1-5, ZP1; lane 6, ZP2; lanes 7-8, ZP1; lanes 9-12, ZP2; lanes 13-14, ZP3; lanes 15-16, ZP2: lanes 17-19, ZP3; lanes 20-21, ZP2; lane 22, ZP3. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 21 & 22 potato isolates; lanes 10, 15, 16, & 17 tomato isolate; lanes 11, 12, 13 & 14 onion isolates, lane 18 watermelon isolate, lane 19 cantaloupe isolate and lane 20 cotton isolate

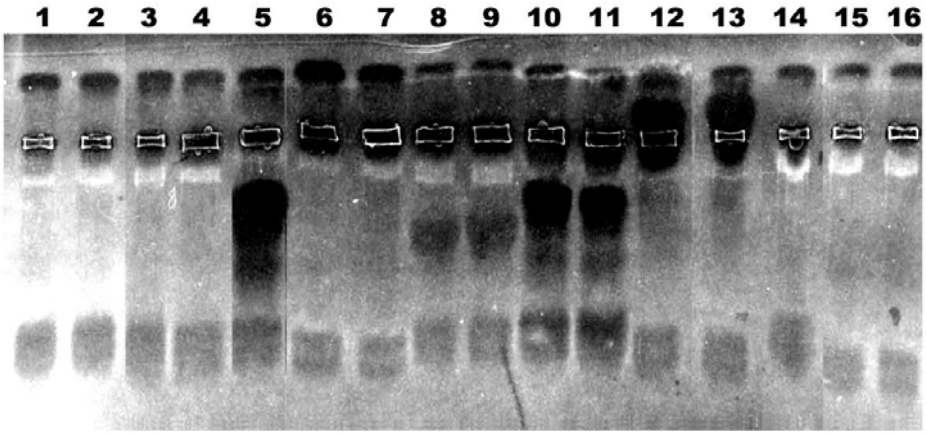

Fig. 5. Pectic zymogram patterns of isolates of *F. solani*. Lane 1-4, ZP4; lane 5, ZP6; lane 6-7, ZP4; lane 8-9, ZP5; lane 10-11, ZP6; lane 12-13, ZP8; lane 14-16, ZP4 (ZP7 is not shown in this gel)

Dominant zymogram patterns (% related isolates) were obtained for *F. oxysporum*, *F. solani and F. culmorum*. ZP1 with 44% frequency was the dominant pattern for *F. oxysporum*, but ZP2 and ZP3 each contained 32% and 24% of the isolates of *F. oxysporum* respectively. ZP4 and ZP5 contained 62% and 20% of the isolates for *F. solani* respectively. ZP6, ZP7 and ZP8 contained fewer isolates and were presented as single patterns. ZP10 was found to be the dominant pattern for *F. culmorum* and contained 64% of the isolates. However ZP9 contained 36% of the isolates (Table 2).

The different patterns, in different regions, were obtained for the three above-mentioned species and the dominant pattern for each case was determined. Lenjan, Fereydan and Bara'an regions had all the patterns of *F. oxysporum* and Falavarjan and Boroujen regions had the two patterns ZP1 and ZP2. The Lenjan region had the most diverse patterns of *F. solani* from Falavarjan and Bara'an the two patterns ZP4 and ZP5 and from Fereydan the two patterns of ZP4 and ZP8 were obtained. None of the patterns of *F. solani* were obtained from the Boroujen area. The Falavarjan region had both patterns of *F. culmorum;* Minadasht had only the ZP10 pattern and from the Lenjan, Feryedan and Boroujen regions none of the patterns of *F. culmorum* were obtained. The dominant patterns for *F. oxysporum*, *F. solani* and *F.*

culmorum for all the isolates were designated as ZP1, ZP4 and ZP10 respectively.

The presence or absence of each one of the alleles (bands) in different patterns and the type of the band (PG or PE and anodic or cathodic) were determined for each ZP, and the amount of R*f* was calculated. The results indicated the similarity of existing alleles in the isolates within species, and very little similarity among different species (Table 2). The percentage of genetic similarity of the 12 zymogram patterns (ZP1–ZP12), based on the different existing alleles and their R*f*'s were calculated. The results indicated a high similarity within the species and very little similarity among the species (Table 3).

The different *F. oxysporum* isolates from potatoes, tomatoes, watermelons and cotton, plus isolates from onions and cantaloupes were compared with respect to different patterns. No similarity was observed among the zymogram patterns obtained from different hosts and no pattern belonged to a specific host, thus, each one of the patterns can be found in different hosts and different hosts can have different types of patterns (Fig. 4). Different *F. solani* isolates were also separated based on the hosts and were compared with different patterns of this species. The patterns of this species also did not belong to any specific host and each host had the different patterns (Fig. 5).

Table 3. Genetic similarity (%) revealed for zymogram patterns (ZP) based on the total isozyme bands for *Fusarium* species

Zymogram pattern	ZP1	ZP2	ZP3	ZP4	ZP5	ZP6	ZP7	ZP8	ZP9	ZP10	ZP11	ZP12
ZP1	100											
ZP2	50	100										
ZP3	50	66	100									
ZP4	0	0	0	100								
ZP5	0	0	0	88	100							
ZP6	0	0	0	80	67	100						
ZP7	0	0	0	57	91	38	100					
ZP80	0	0	0	88	0	0	0	100				
ZP9	0	0	0	0	0	0	0	0	100			
ZP10	28	40	0	0	0	0	0	0	0	100		
ZP11	0	0	0	0	0	0	0	0	0	0	100	
ZP12	33	0	50	0	0	0	0	0	0	0	0	100

The different isolates of *F. culmorum* were also identified based on the hosts and were compared with the two patterns of this species. The results like the two previous species indicated to lack of correspondence between the patterns and hosts (Fig. 6).

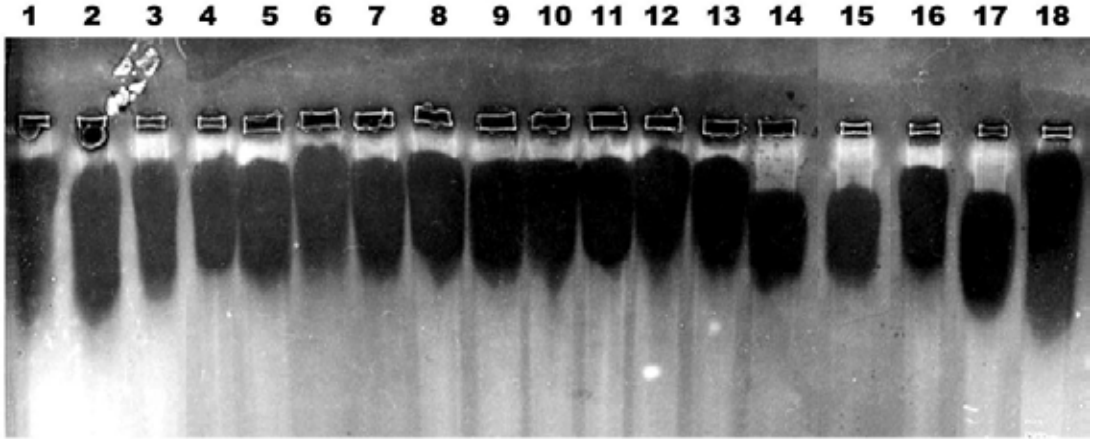

Fig. 6. Zymogram patterns of isolates of *Fusarium culmorum*. Lanes 1-13, ZP10; lanes 14-15; lane 16, ZP10; lane 17, ZP9; lane 18, ZP10. Lanes 1, 2, 5, 6, 7, 8, 10, 11, 14, 15, 16, & 17 potato isolates; lanes 3 & 4 bean isolates; lane 9 onion isolate; lanes 12 & 13 tomato isolates and lane 18 wheat isolate

4. DISCUSSION

Classification and identification of *Fusarium* species is complicated due to the number of species, varieties and forms in this genus [19, 20]. There is also a large morphological variation of isolates within one species [21]. In this study, *Fusarium oxysporum*, *F. solani*, *F. culmorum*, *F. subglutinans* and *F. equiseti* were obtained from different regions and different hosts and showed considerable morphological variation. The isolates from each species varied in color, type of chlamidospore, shape and number of septa in macroconidia and microconidia and type of phialides. This morphological variation meant that some morphological characteristics were unreliable for the identification of the species. For example, characteristics such as color, the form of the colony, or the color of the sporodochium were variable characteristics in species. Onyike *et al*. [21] also reported the presence of variations in isolates of species. They studied five *Fusarium* species with regard to macroscopic and microscopic characteristics and and noted the variation of characteristics. In *F. equiseti*, considerable overlap in the morphology of strains was observed.

Fusarium oxysporum (section elegans) showed common characteristics with *F. solani* (section martiella) and *F. subglutinans* (section Lesiola) making distinction among them difficult. *Fusarium culmorum* (section discolor) also had many similar characteristics with other species of section discolor. *Fusarium equiseti* (section gibbosum) had similar morphological characteristics with *F. semitectum* (section arthosporiella). Microconidia of taxa also varied from being circular to oval; therefore not useful for the identification of species. Colony characters such as color and shape are also similar in many of the species. *Fusarium oxysporum* and *F. solani* have identical colony colors and cannot be distinguished from each other on colony characteristics. Colonies of species of section discolor are also similar and the species cannot be distinguished from one another through color. In some cases, there is greater overlap among species. For example *F. oxysporum* has numerous morphological similarities with *F. nygamai*, which makes the distinction between these two species difficult. Burgess *et al*. [22] mentioned the morphological overlap between the two species of *F. solani* and *F. oxysporum.* The presence of long phialides in *F. solani* and short phialides in *F. oxysporum* were the best characteristics for differentiating between these two species. Also, they considered the lack of the formation of microconidia in chains a good differentiating characteristic between these species and species of the section Liseola which are similar. Nelson *et al*. [1] confirmed the morphological similarity between *F. subglutinans* and *F. oxysporum*; and considered the presence of branched phialides and the lack of chlamydospores in *F. subglutinans* a distinguishing character from *F. oxysporum*.

During the work with five species of *Fusarium* in this study, it was observed that changes in temperature, pH, the nutritional substrate and light resulted in changes in morphological characteristics such as color and colony. In some cases, changes in the shape of macroconidia, the development of microconidia and the presence or lack of chlamydospores were observed. Such variations have been considered by Snyder and Hansen [23].

Burgess *et al* [22] also observed morphological changes due to the effects of different environmental conditions and considered constant environmental conditions necessary for taxonomic studies in *Fusarium*. They believe some characters gradually disappeared with changes in the culture conditions or some character did not develop. In another study, Burgess *et al* [24] did not consider morphological measurements reliable in the classification of *Fusarium* species because different substrates and different environmental conditions cause considerable variation in them. They also consider different environmental conditions such as culture medium, light and temperature as the causes of changes in pigment production of *F. oxysporum* from no color to dark violet; as a result of which the identification of the species becomes difficult.

Surve-Lyer *et al* [25] stated that identification of fungi is generally carried out on the basis of differences in morphology. However, species and populations can be undistinguishable morphologically, but distinguishable from the genetic point of view. Biochemical and genetic methods such as the analysis of isozymes, in addition to the determination of the boundaries of taxons with similar morphology of cultures, can be used for the identification of the genetic variation within and among the species of fungi. The majority of these methods have been based on the multi allelic nature of loci. In an analysis that is based on the repetition of the alleles, in order to have a reliable estimate, it is necessary to have a large number of samples [26]. For this reason, in the present study, many samples consisting of 318 isolates have been used.

In this study *Fusarium* species were identified using a pectic zymogram technique. Deacon [27] considered different zymogram patterns are as a result of the presence of mutations in locations where the enzymes are coded on the DNA. Due to changes in the amino acids resulting from mutation, electrophoretic movements of the enzymes change and manifest the existing genetic variation in a population. Yang [28] concluded that expression of the pectic enzymes in the *R. solani* samples is governed by different loci and it seems there is a relation between these loci.

In this work the *Fusarium* isolates from different hosts grouped into 12 zymogram patterns on the basis of electrophoresis of the pectic enzymes. The isolates from an individual species produced similar pectic zymogram patterns. The bands with different R*f*s produced different patterns and caused variation within species. This finding is in accordance with the results obtained by Szeczi [9] for *F. lateritium*, *F. stliboides*, *F. udum*, *F. xylarioides*, *F. moniliforme* and *F. moniliforme var. subglutinans* and Szeczi [10] for *F. graminearum* and *F. culmorum*. The existence of specific zymogram patterns for each species can be considered as a simple and fast method for the identification of the species. In this method a large number of isolates were prepared simultaneously and their electrophoretic patterns obtained. The studied species were identified through the identification of the type of the zymogram patterns. The overlap between the species under study was low and they were easily distinguishable. Therefore, if other *Fusarium* species are studied, it should be possible to distinguish between them. Cruickshank and Pitt [29] obtained the same result for some *Penicillium* species. They consider the isolation of these species through the morphologic methods, growth characteristics or the secondary metabolites difficult. For this reason, they consider the zymograms as a valuable tool for classifying species. Sweetingham *et al*. [18] also considered this method fast and easy for the identification of the *Rhizoctonia* isolates, the results of which are repeatable by other workers.

The zymogram patterns from the five species under study did not change in the case of cultures stored for long periods, whereas their morphological characteristics did. For example, the color of the old colonies had changed. This conclusion has also been reached and pointed out by Cruickshank [30]. He has also considered the application of pectic zymogram in the case of the old cultures which have lost some of the morphological characteristics.

Because of the classification difficulties in *Fusarium*, species have often been named based on pathological behavior. Mycologists have also studied the uniqueness of a group of isolates from specific hosts with different pathological and molecular methods. *Fusarium oxysoporum* has a large variation in characters and is a destructive pathogen and has therefore received considerable interest. Seventy special forms of this species have been identified [31]. Samak and Leong [32] on the same basis have considered *F. solani* to comprise 3 Formae specialis. For this reason attention was given to the hosts from which the isolates were obtained in this study. Comparisons were also made among the morphological characteristics of the isolates from the individual hosts. The results of these comparisons indicated that there is no difference among the morphological characteristics of these isolates, and if there were any differences among these isolates, they were not related to their hosts. Kistler [31] considered the Formae specialis as

isolates which are morphologically similar and undistinguishable, but cause diseases on different plants. He also considered the classification of strains on the basis of hosts as the cause of subdivisions within species. This problem brings about the question of whether the isolates with common hosts are genetically more similar as compared to those that cause diseases on different hosts; in other words, the isolates from common hosts are monophyletic and have been derived from a specific and unique genotype. He suggested that this subject could be studied using genetic markers independent of pathogeniticy.

The pectic zymogram results from this study, which have also been obtained from different isolates and different hosts, showed that, isolates belonging to one species recovered from one host had different pectic zymogram patterns. This result is more evident in the case of *F. solani* with 5 different patterns and is in accordance with the hypothesis of the polyphyletic nature of the Formae specialis. It is important to note that in the zymogram method, the production of pectic enzymes is an inductive process; therefore there is a possibility of the effects of the conditions of the culture medium and the nutritional substrates in this process [28, 33]. In this study different concentrations of pectic enzymes and different intensities of the bands in different conditions confirmed the above conclusion.

A PL (pectin liase) band was not observed in any species studied. According to Fernandez *et al*. [11], due to the effects of the pH, PG and PL enzymes rarely appear together. The other important point to note is that the enzymes under consideration in this study show only a part of the existing genomes. Therefore, although it is possible that similarity of zymogrames is an indication of a closer relation, sometimes it is not adequate evidence for the identification of the species boundaries.

The study of the genetic variation among and within species and subspecies using a pectic zymogram marker can be helpful in identifying the relation among genotypes and pathogenic phenotypes. Also it can help in the understanding of how different species have evolved through time; and which factors were responsible for the changes. Probably the presence of plants and their genotypes are important factors in the induction of these changes [34]. This method was used to obtain and compare the degree of pathogenicity of the different zymogram patterns of *R. solani* of anastomosis group 3 (AG-3) on potatoes [34]. Balali and Kowsari [35] determined the degree of the pathogenicity of the different patterns resulting from *R. solani* anastomosic group 4 (AG-4) on beans. The study of the degree of the pathogenicity using representative isolates of different zymogram patterns resulting from this study on different hosts could probably indicate such a relation that could be the subject for future studies.

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