
Application of pectic zymogram in the identification and genetic variation of *Fusarium* species

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The genus *Fusarium* is a species rich genus. Different media are required to study the morphological characters and classify different species, and this is a time consuming technique. Molecular and biochemical techniques have been used for identifying fungi over the last two decades, including pectic zymograms which have been used to characterize different fungi. 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 were 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.

Key words: electrophoresis, pectic enzymes, taxonomy, zymograms

Introduction

The genus *Fusarium* has a worldwide distribution and its different species are considered to be some of the most important plant disease pathogens (Nelson *et al.*, 1983). Some species produce mycotoxins on plants which contaminate seeds (Shephard *et al.*, 1995) and some such as *F. solani*, cause diseases in humans and animals (Phelps *et al.*, 1990; Eley, 1996) and are thus, hazardous to agricultural products, wildlife, livestock and humans. For this

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reason identification of the different species of *Fusarium*, including saprobic, pathogenic and toxin producing species, is of vital importance (Nelson *et al.*, 1983).

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 storage 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 (Booth, 1975).

The large variation in some of the characteristics of *Fusarium* isolates, such as pathogenicity, colony morphology of the colony, e.g. form and colour, mode of development of the chlamydospores, existence or lack of sporodochia and even the type of microconidia, have resulted in different emphasis on characters and different classification of species into intra-specific groups. The use of sub-species in the classification of *Fusarium* makes it possible to distinguish between the populations with major ecological and physiological differences, but with no major morphological differences (Sangalang *et al.*, 1995). Most of the intra specific classifications are based on pathogenic behaviour and vegetative compatibility groups (VCG). Some researchers have placed some isolates of VCG into the same race using the pectic zymogram technique (Elias and Schneider, 1991). Some pathologists have classified different races according to special geographic regions (Elias and Schneider, 1991) and others have used different molecular markers for distinction among species (Pegg *et al.*, 1995).

Many fungi, including *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 (Szeczi, 1990a,b). The aims of some research was to study the relationship between pectic enzymes and pathogenicity (Fernandez *et al.*, 1993). Other researchers have used the same method to improve the taxonomy of *Fusarium*. Szeczi (1990a) 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 movement, distribution and the activity 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.

Materials and Methods

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. *Fusarium* disease 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 hypochlorate 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 and placed on acidified PDA and incubated at 25°C 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 (Hansen and Smith, 1932). For long-term preservation, *Fusarium* isolates were grown in Bijoux bottles containing sterile dry sand and wheat bran as recommended in Butler (1980).

Macroscopic and microscopic characteristics of the pure cultures were studied and the species were identified using illustrated keys (Booth, 1977; Nelson *et al.*, 1983). The macroscopic characteristics were studied on PDA culture, the microscopic characteristics, however, were studied on CLA (Carnation Leaf Agar).

Pectic zymogram analysis

Zymogram is an electrophoretic method for measuring protheolithic activity (Leber and Balkwill, 1997) in which the enzymes are separated from one another in a polyacryl-amid gel (Kleiner and Stetler-Stevenson, 1994). A 5 mm diameter plug from each colony margin was transferred to Bijoux bottles containing 2 ml sterile liquid medium as recommended in Cruickshank and Wade (1980). The medium contained 1% citrus pectin as the sole carbon source (Sweetingham *et al.*, 1986). Cultures were grown at 25°C 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 a slurry which was kept at room temperature for 30 minutes before electrophoresis. The electrophoresis was performed using the method of Cruickshank and Wade (1980). Horizontal pectin-acrylamide gels were prepared as recommended by Sweetingham *et al.* (1986). 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 a 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 tank buffer and cold water (4-5°C) was circulated underneath the gel supporter plate during electrophoresis.

Results

Many isolates were collected from different fields in regions around Isfahan. 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 condition. *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 colonies on PDA with aerial mycelium. However, the lower surfaces of the colonies were 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 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 sporodochia, with a cream or sometimes blue colour were observed. Macroconidia were produced on CLA. They were almost similar to the macroconidia of the *F. oxysporum*, however they were wider and conspicuous wall. Their apical cell was round, the basal cell was round or foot-shaped and they usually had three and occasionally four septa. The microconidia 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 lacking, however 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 and chlamydo spores in PDA and CLA were produced in chains or columns.

The mycelia of *F. subglutinans* isolates on PDA was white and the sporulating colony was white and 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 and their basal cells were foot-shaped. In this species both polyphialides and monophialides developed, and orange colour sporodochia were observed, however, no chlamydo spores 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 chlamydo spores developed and orange sporodochia were also observed.

Zymogram patterns

To obtain the most suitable zymogram pattern, 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 thinness 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, the thinness of 50% was used. For the 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 pectic zymogram technique. The polygalacturonase (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 22 bands or isozyme loci named, a-v, belonging to polygalacturonase and

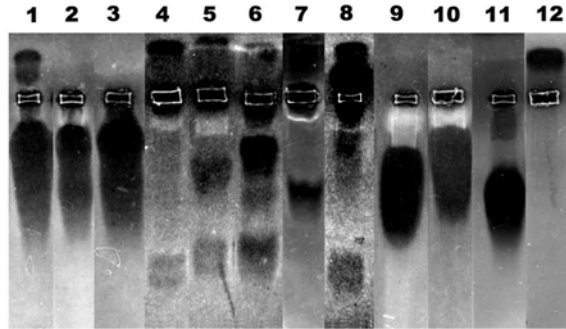


Fig. 1. Different zymogram patterns of isolates of *Fusarium* species. Lane 1, ZP1 (*F. oxysporum*); lane 2, ZP2 (*F. oxysporum*); lane 3, ZP3 (*F. oxysporum*); lane 4, ZP4 (*F. solani*); lane 5, ZP5 (*F. solani*); lane 6 (*F. solani*); lane 7, ZP7 (*F. solani*); lane 8, ZP8 (*F. solani*); lane 9, ZP9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

pectin esterase. Three bands, d, f and l, were assigned as pectin esterase and the rest of the bands corresponded to the polygalacturonase loci (Fig. 2).

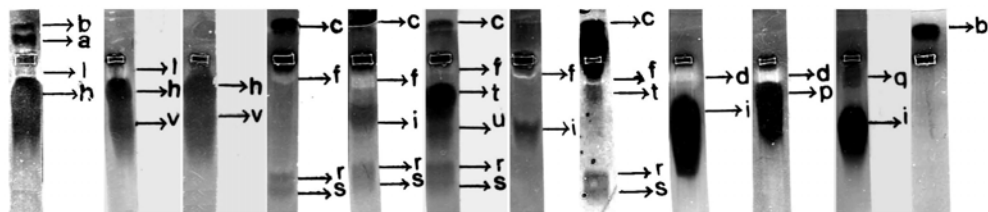


Fig. 2: Frequency of the polygalacturonase and pectin esterase isozymes observed in 12 zymogram patterns of *Fusarium* species. Three bands (d, f and l) belonged to pectin esterase and 19 bands to polygalacturonase loci.

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. subglutinans* and *F. equiseti* respectively (Table 1 and Fig. 3).

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 in patterns, however, the lack of a polygalacturonase cathodic band and the presence of strong pectin esterase in all the isolates of *F. culmorum* distinguished it from *F. oxysporum* (Fig. 3). In

Table 1. Nucleation activity for analysed bacterial strains grown at different temperatures.

Bacterial* group	INA	Bacterial** strain	Growth temperature (°C)						Optimal temperature
			2	4	10	15	20	25	
2	+	S12	-	-14	-14	-	-3	-11	20
2	+	F265	-	-	-	-	-3	-12	20
2	+	A34	-	-	-	-4	-4	-10	15-20
4	+	S294	ng	ng	ng	-5	-10	-10	15
4	+	A21	-	-	-	-4	-	-11	15
7	+	F50	-9	-12	-12	-3	-	-11	15
7	+	A56	-	-	-	-4	-4	-8	15-20
7	+	S320	-14	-	-14	-	-12	-5	25
8	+	E200	-10	-10	-10	-6	-4.5	-6	20
8	+	F69	-11.5	-12	-	-10	-4	-11	20
8	-	A96	-	-	-	-	-	-11	-
8	-	A31	-10	-10	-10	-10	-10	-9	-
10	+	F67	-14	-14	-	-4	-	-12	15
10	(+)	A12	-	-	-	-	-14	-7	25
10 ^T	-	3624	-10	-10	-10	-10	-9	-10	-
10	+	S117	-4	-3	-4.5	-4	-4	-3	2-25
11	+	S229	-2	-2	-4	-3	-2	-4	2-25
11	+	S217	-6	-5	-4.5	-5	-5	-7	4-20
11	+	F202	-	-13	-	-9	-4	-15	20
11	+	VID1	-4	-4	-4	-3	-3	-2.5	2-25
12	+	F221	-5	-5	-5	-6	-7	-7.5	2-10
12	+	S113	-4.5	-4.5	-4.5	-5	-5	-3	2-25
12	(+)	S262	-10	-10	-10	-6	-10	-8	15

+ = positive - = not frozen, ng = not grown, ^T = type strain

* Grouping of bacterial genera based on biochemical and physiological characters c.f. NEJAD *et al.* [4, 21]

** The isolates originates from: S = stem, F= litter, A = soil, E = stem (Estonia)

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). In five of the pectic zymogram patterns in *F. solani* (ZP4, ZP5, ZP6, ZP7, ZP8), a cathodic polygalacturonase band (c) in all the five patterns, and a pectin esterase band (f) and the two anodic galacturonase bands (r and s) with stable Rf were 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.

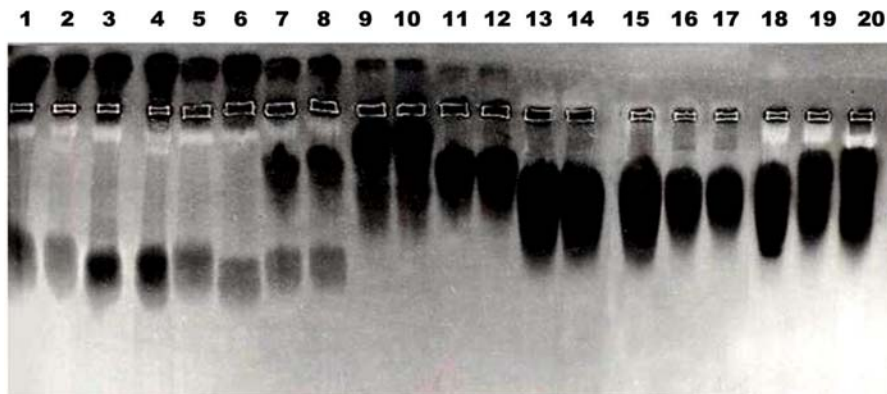


Fig. 3. Pectic zymogram patterns of isolates of *Fusarium* species. Lanes 1-8, *F. solani*; (lanes 1-6 ZP4 and lanes 7-8 ZP5), lanes 9-12, *F. oxysporum*; (lanes 9-10 ZP1 and lanes 11-12 ZP2), lanes 13-17, *F. subglutinans*; (ZP11), lanes 18-20, *F. culmorum* (lane 18, ZP9 and lanes 19-20, ZP10).

Dominant zymogram patterns (percentage 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 a 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 1).

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. 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 Boroujen area. 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, 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 to a high similarity within the species and very little similarity among the species (Table 3).

Table 2. Percentage of frozen bacterial strains (from Table 1) at different freezing temperature intervals.

Freezing temperature interval	Growth temperatures (°C)					
	2*	4*	10*	15	20	25
-2 to -5	21	25	25	48	52	22
<-5 to -7	4	0	0	13	4	12
<-7 to -16	34	37	30	17	27	61
Not frozen at -16	37	34	41	22	17	5

*Four percent of the bacterial isolates did not grow on the TSA agar plate

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).

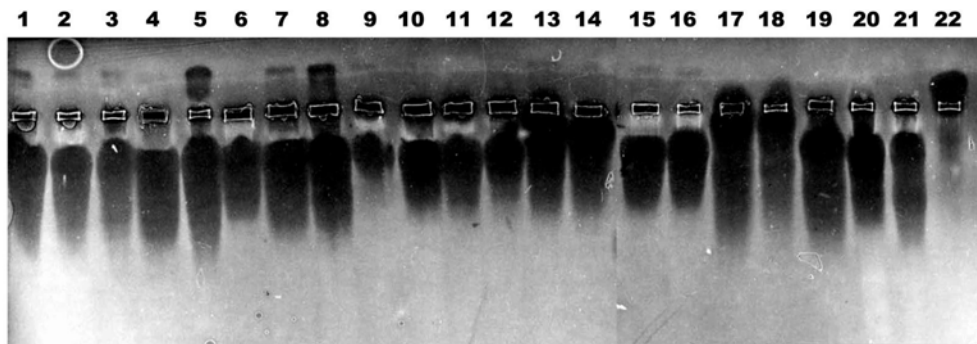


Fig. 4. Zymogram patterns of isolates of *Fusarium oxysporum*. Lanes 1-5, ZP1; lane 6, ZP2; lanes 7-8, ZP1; lanes 9-12, ZP2; lanes 13-14, ZP3; lanes 15-16, ZP2; lanes 17-19, ZP3; lanes 20-21, ZP2; lane 22, ZP3.

Table 3. Effect of growth limiting factors on growth and ice-nucleation activity of bacteria after 72 hours incubation in terms of optical density.

Bacteria*	Strain	Medium	OD	INA (°C)
<i>Bacillus</i> sp.	(S294)	TSB	1.720	-9.5
	CL	0.830	-9.5	
	NL	0.750	-12	
	PL	1.270	nf	
<i>Erwinia</i> sp.	(S113)	TSB	1.820	-7
	CL	0.412	-8.5	
	NL	0.412	-13	
	PL	1.060	-5.5	
<i>P. fluorescens</i>	(M103)	TSB	1.600	-4.5
	CL	0.820	-7	
	NL	0.660	-5	
	PL	0.790	-5	
<i>P. syringae</i>	(S229)	TSB	1.700	-2.5
	CL	0.500	-3	
	NL	0.920	-3.5	
	PL	1.255	-4	
<i>Sphingomonas yanoikuyae</i>	(E200)	TSB	1.920	-6.5
	CL	0.500	-5.5	
	NL	0.520	-13.5	
	PL	1.450	-8	
<i>Xanthomonas campestris</i>	(S117)	TSB	1.730	-5
	CL	0.480	-9	
	NL	1.070	-7	
	PL	1.250	-4	

*all strains are isolated from *Salix* stems, TSB as control froze at -16.5°C and phosphate buffer at -16°C, CL= carbon limitation, NL = nitrogen limitation, PL = phosphorus limitation, nf = not frozen.

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).

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).

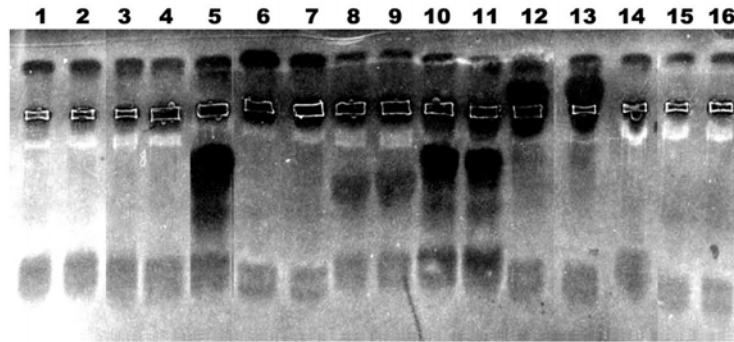


Fig. 5. Pectic zymogram patterns of isolates of *F. solani*. Lane 1-4, ZP4; lane 5, ZP6; lane 6-7, ZP4; lane 8-9, ZP5; lane 10-11, ZP6; lane 12-13, ZP8; lane 14-16, ZP4.

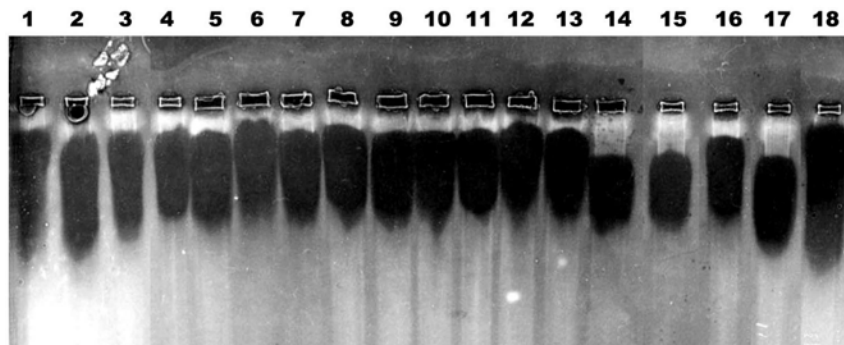


Fig. 6. Zymogram patterns of isolates of *Fusarium culmorum*. Lanes 1-13, ZP10; lanes 14-15; lane 16, ZP10; lane 17, ZP9; lane 18, ZP10.

Discussion

Classification and identification of *Fusarium* species is complicated, due to the number of species, varieties and forms in this genus (Klittich *et al.*, 1997; Palm *et al.*, 1995). there is also large morphological variation of isolates within one species (Onyike *et al.*, 1991). 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 colour, type of chlamydospore, 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 colour, the form of the colony, or the colour of the sporodochium, were variable characteristics in species. Onyike *et al.* (1991) also reported the presence of variations in isolates of species. They studied five *Fusarium* species with regard to macroscopic and microscopic characteristics and noted the variation of characteristics. In *F. equiseti*, considerable overlap in morphology of strains were 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 colour and shape are also similar in many of the species. *Fusarium oxysporum* and *F. solani* have identical colony colours 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 colour. 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.* (1989a) 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, as a good differentiating characteristic between these species and species of the section *lesiola* which are similar. Nelson *et al.* (1983) 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* as 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 colour 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 (1941).

Burgess *et al.* (1989a) 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* (1989b) 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 colour to dark violet; as a result of which the identification of the species becomes difficult.

Surve-Lyer *et al* (1995) 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 taxa 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 (Mc-Donald, 1997). 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 (1997) considered different zymogram patterns are the 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 (1993) 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_fs produced different patterns and caused variation within species. This finding is in accordance with the results obtained by Szecei (1990a) for *F. lateritium*, *F. stilboides*, *F. udum*, *F. xylarioides*, *F. moniliforme* and *F. moniliforme var. subglutinans* and Szecei (1990b) 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. Through the identification of the type of the zymogram patterns the studied species were identified. 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 (1987) 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.* (1986) 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 colour of the old colonies had changed. This conclusion has also been reached and pointed out by Cruickshank (1983). 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 behaviour. Mycologists have also studied the uniqueness of a group of isolates from specific hosts with different pathological and molecular methods. *Fusarium oxysporum* 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 (Kistler, 1997). Samak and Leong (1989) 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 (1997) 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; and 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 pathogenicity.

The pectic zymogram results from this study, which have been also 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 polyphilic 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 (Balali, 1996; Yang, 1993). 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.* (1993) 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 zymograms 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 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 (Balali *et al.*, 2000). This method was used to obtain and compare the degree of the pathogenicity of the different zymogram patterns of *R. solani* of anastomosis group 3 (AG-3) on potatoes (Balali *et al.*, 2000). Balali and Kowsari (2004) determined the degree of the pathogenicity of the different patterns resulting from *R. solani* anastomosis group 4 (AG-4) on beans. The study of the degree of the pathogenicity of the 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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