

PECTIC ENZYME PATTERNS OF *FUSARIUM OXYSPORUM* VIRULENT ISOLATES FROM CHICKPEA IN IRAN

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Abstract

The pectic enzymes produced *in vitro* by 8 isolates (5 Highly virulent and 3 Weakly virulent) of *Fusarium oxysporum*, were detected by spectrophotometry, and characterized by polyacrylamide gel electrophoresis with substrate-containing gels (zymogram). Analysis of the polygalacturonase (PG) isozyme banding patterns (zymogram) identified two distinct phenotypes among the isolates from chickpea (*Cicer arietinum*), *F. oxysporum* zymogram group one (FOZG-1) and FOZG-2. Of the 8 isolates tested, 5 isolates had the FOZG-1 phenotype, and 3 isolates had the FOZG-2 phenotype. There were two virulent groups among these isolates: highly virulent (HV) and weakly virulent (WV). The HV isolates were all FOZG-1 and WV isolates were FOZG-2. Analysis of the pectic enzyme activity of different isolates on PZ medium demonstrated that PG and pectin lyase (PNL) were the predominant enzyme activities in FOZG1 (HV) compared with FOZG2 (WV) isolates, while the pectate lyase (PL) showed enzyme activity in both FOZG1 and FOZG2 groups. Therefore, from these results it can be hypothesized that PG and PNL enzymes may be important in pathogenicity of *F. oxysporum* isolates in Iran.

Introduction

Fusarium oxysporum is a common inhabitant of soil and has a worldwide distribution. All strains of *F. oxysporum* are successful as saprophyte and are able to grow and survive for long periods on organic materials in soil. However some strains are responsible for vascular wilt diseases on many plants of economical

importance. These pathogenic strains show a high level of host specificity and are classified on this basis into more than 120 formae speciales and races [10]. The pathogenic strains are not identifiable using morphological characters. Molecular tools have been used to characterize the diversity among pathogenic strains of *F. oxysporum* [7].

A number of systems based on variation of extractable proteins have been developed [16-17]. The most notable one is the pectic zymogram grouping system based on extracellular pectic enzyme patterns on

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polyacrylamide gel electrophoresis [11]. Cruickshank and Wade [8] initiated this technique for detection of the isozymes of pectolytic enzymes.

This work deals with the production of polygalacturonase (PG), pectin lyase (PNL), pectate lyase (PL), and examines the pectic zymograms of isolates of *F. oxysporum* sampled from infected chickpea (*Cicer arietinum*) in Iran.

Materials and Methods

Fungal Isolates and Growth Conditions

Five highly virulent (HV) and 3 weakly virulent (WV) isolates of *F. oxysporum* from aerial sections of chickpea from different geographical locations in Iran were collected [25]. Isolates were maintained in wheat seed medium (50 gr autoclaved soaked wheat seed in 500 ml flask) at 4°C. Isolates were grown in shake culture in PZ medium containing 2.64 g (NH₄)₂ SO₄, 0.34 g KH₂PO₄, 0.14 g MgSO₄·7H₂O, 10 g Citrus pectin, Sigma, 1 lit. dH₂O. pH adjusted to 4.5 [23]. After 6 days growth at 26°C, a liquid culture filtrate was obtained (as crude enzyme) by Whatman filter paper No.1 and stored at -20°C until using for enzyme assay or protein measurement.

Pectic Zymogram (Pectic Enzyme Production and Electrophoresis)

The zymogram identifications were made by electrophoresis, using culture fluids run on pectin-acrylamide gels as described by Sweetingham *et al* [23]. 10µl of culture filtrates was added to the pectin acrylamide gel. Electrophoresis was carried out at 4°C with current constant at 16 mA. After electrophoresis, the gels were incubated on 0.1 M malic acid at 25°C for 90 min. Staining of remaining pectin was performed in 0.05% ruthenium red at 4°C overnight, and then oxidized with 0.1% ammonium persulphate for 20 min.

Enzyme Assay

All isolates were grown on 10 ml of PZ medium in 25 ml Erlenmeyer flasks for 6 days at 26°C. Mycelium was removed by vacuum filtration and the filtrate was clarified by centrifugation at 14000 g for 5 min at 4°C. The supernatant was collected and placed into another Eppendorf tube for enzyme assay. Assays were repeated three times. All controls were performed using heat-denatured enzyme, at 96°C for 10 min.

Polygalacturonase (PG) activity was assayed by measuring the release of the reducing groups using the Somogi assay with Nelson's arsenomolibdate reagent [6]. The reaction mixture, containing 0.9 ml of 25% polygalacturonic acid in 25 mM citrate-phosphate buffer pH 4.5 and 0.1 ml of enzyme solution, was incubated at 40°C for 20 min. One unit of PG activity was defined as

the amount of enzyme that releases 1 µmol of galacturonic acid per minute, under the same condition.

Pectate lyase (PL) activity was assayed measuring the increased absorption of the enzymatic products at 235 nm. [6]. The reaction mixture, containing 2.5 ml of 0.6 mM CaCl₂, 60 mM Tris-HCl, 0.24% (W/V) polygalacturonic acid and 0.5 ml of enzyme solution, was incubated at 30°C for 60 min. The reaction was stopped by adding 3.5 ml of 0.5M HCl. One unit of PL was defined as the amount of enzyme that releases 1 µmol of 4,5-unsaturated Di-galacturonic acid per minute. A molar extinction coefficient of 5550M⁻¹cm⁻¹ was used for this calculation.

Pectin lyase (PNL) activity was assayed spectrophotometrically as described by Parini *et al* [19]. 100 µl of culture filtrates was mixed with 200 µl of 0.5% (w/v) pectin in 10 mM citrate phosphate buffer, pH 7.0 in a eppendorf tube. Incubation of the reaction mixture was carried out at 40°C for 30 min. The reaction was stopped by adding 700 µl of 0.5N HCl. The absorbance was measured at 235 nm. One unit of enzyme activity was defined as the amount of enzyme that increases the OD₂₃₅ by 0.045.

Protein Production

Concentration of proteins was measured by the Bradford method [4]. Bovine serum albumin was used as standard.

Results

Zymogram Grouping

The 8 isolates (5 HV and 3 WV) of *F. oxysporum* were cultured in PZ medium. The growth filtrates (crude enzymes) were used for zymogram electrophoretical identification on pectin-acrylamide gel. These isolates could be placed into two different zymogram groups, which were designated as FOZG-1 and FOZG-2 (Fig. 1). Isolates F15, F18, F23, F47, and F59 (FOZG-1) showed to have four bands, whilst FOZG-2 isolates (F2, F21, and F58) were characterized by a single band pattern. The size of this single band is similar to that of the smallest FOZG-1 band.

Enzyme Activity

The production of pectic enzymes by FOZG-1 and FOZG-2 isolates was monitored by assaying culture filtrates for PG, PNL, and PL activity.

The results showed that among FOZG-1 isolates there is a range of PG specific activity from 1.74 units in isolate F59 to 7.25 units in isolates F23, whilst in FOZG-2 isolates showed no detectable PG activity (Table 1).

Also pectin lyase (PNL) and pectate lyase (PL) activity of FOZG-1 and FOZG-2 isolates demonstrated

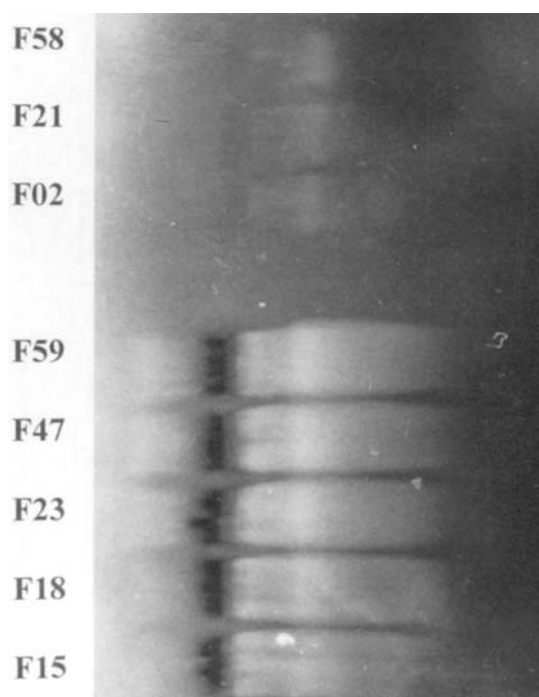


Figure 1. Zymogram patterns of different isolates of *Fusarium oxysporum*: The gel was photographed with a camera (positive contrast), the white bands show the PG activity.

Isolates F15, F18, F23, F47, and F59 are FOZG-1; and Isolates F02, F21, and F 58 are FOZG-2.

Table 1. Production of PG by *F. oxysporum* ZG-1 and *F. oxysporum* ZG-2 isolates, along with their origin

Isolate	ZG	Origin	Virulence	Protein ($\mu\text{g/ml}$)	PG activity (u/ml/min)	PG specific activity (u/ml/min)
F15	1	Orumieh	H	4.3 \pm 0.11	15.8 \pm 0.50	3.67
F18	1	Orumieh	H	4.0 \pm 0.09	13.7 \pm 0.35	3.42
F23	1	unknown	H	2.0 \pm 0.02	14.5 \pm 0.32	7.25
F47	1	Lorestan	H	7.0 \pm 0.08	24.4 \pm 0.61	3.49
F59	1	Tabriz	H	5.0 \pm 0.06	8.70 \pm 0.33	1.74
F58	2	Tabriz	W	3.0 \pm 0.02	0.00 \pm 0.00	0.00
F21	2	Orumieh	W	5.0 \pm 0.04	0.00 \pm 0.00	0.00
F02	2	Tabriz	W	3.7 \pm 0.03	0.00 \pm 0.00	0.00

that while FOZG-2 isolates have no detectable PNL activity, FOZG-1 isolates show specific enzyme activity, ranging from 0.028 (F18) to 0.750 units (F23) (Table 2). Analysis of PL specific activity among FOZG-1 and FOZG-2 isolates indicated that there is no significant difference between PL specific activity of isolates of this two groups (ranging from 0.04 to 1.30 units) (Table 3).

Protein Assay

Comparison of proteins produced by FOZG-1 and FOZG-2 isolates showed that there is no significant difference in protein production among isolates of these two zymogram groups (Table 1). The amount of proteins was used for calculation of enzymes specific activity.

Table2. Production of PNL by *F. oxysporum* ZG-1 and *F. oxysporum* ZG-2 isolates

Isolate	Protein (µg/ml)	PNL activity (u/ml/min)	PNL specific activity (u/ml/min)
F15	4.3±0.11	0.19±0.02	0.044
F18	4.0±0.09	1.13±0.04	0.028
F23	2.0±0.02	1.50±0.01	0.750
F47	7.0±0.08	0.60±0.01	0.085
F59	5.0±0.06	1.70±0.01	0.340
F58	3.0±0.02	0.00±0.00	0.00
F21	5.0±0.04	0.00±0.00	0.00
F02	3.7±0.03	0.00±0.00	0.00

Table3. Production of PL by *F. oxysporum* ZG-1 and *F. oxysporum* ZG-2 isolates

Isolate	Protein (µg/ml)	PL activity (u/ml/min)	PL specific activity (u/ml/min)
F15	4.3±0.11	2.6±0.12	0.60
F18	4.0±0.09	2.6±0.10	0.65
F23	2.0±0.02	2.6±0.09	1.30
F47	7.0±0.08	0.40±0.0	0.05
F59	5.0±0.06	0.20±0.0	0.04
F58	3.0±0.02	0.30±0.0	0.10
F21	5.0±0.04	0.40±0.0	0.08
F02	3.7±0.03	0.80±0.0	0.22

Discussion

Fusarium causes cortical rots, head blights, leaf spots, root rots, fruit rots, cankers, diebacks, and vascular wilt diseases on plants [14]. *Fusarium* blight is well known disease of chickpea in Iran and is of increasing importance in chickpea farming in west of Iran [2]. The pathogenic mechanisms of *F. oxysporum* are complex and varied and may be due to the action of different pectolytic enzymes. This fungus produces copious quantities of pectic enzymes during growth on pectin substances.

In order to identify the role of the pectolytic enzymes in pathogenesis of *F. oxysporum*, a series of experiments including zymogram grouping, assay of PG, PNL, and PL activity and protein production were performed. As reported in the literature, the pectic zymogram patterns in pectin acrylamide gel are utilized to classify the isolates of fungi [18]. This technique has been utilized for several other purposes too, such as: study of the expression of exogenous DNA in soybean [9], comparison of isoenzyme forms of different varieties of different plants [22-23], and study of

relationships among species [3]. There are some reports about usage of zymogram grouping technique to study activity change in some enzymes during senescence of wheat leaves [13-21], and also the effects of radiation on some enzyme activity in plants [15].

In this study it was demonstrated that two phenotypes or electromorphs were observed in pectic zymograms designated as FOZG-1 and FOZG-2. The zymogram pattern of FOZG-1 isolates is considerably different from that of FOZG-2 isolates which is characterized by a single band pattern. Our previous studies distinguished two virulent groups among these isolates: highly virulent (HV) (F15, F18, F23, F47, F59) and weakly virulent (WV) (F02, F21, F58) [25]. The comparison of these HV and WV isolates with zymogram grouping demonstrated that highly virulent isolates were all FOZG-1 and weakly virulent isolates were FOZG-2 (Table 1).

The predominant enzyme activity during growth of FOZG-1 isolates on PZ medium was PG and PNL, compared with FOZG-2 which have no detectable PG and PNL activity. Comparison of the level of PL activity in FOZG-1 (HV) and FOZG-2 (WV) isolates showed that this enzyme is produced by both FOZG-1 and FOZG-2 isolates. Many plant pathogenic fungi have been known to produce pectic enzymes associated with degradation of host tissues [1]. Polygalacturonase and pectin lyase are inducible in large number of fungi [12]. Pectic enzymes produced by *Fusarium oxysporum* are important for infection of hosts and disease development [24]. In this study the association of *in vitro* PG and PNL activity speculates that they may be involved in pathogenicity of *F. oxysporum* on chickpea.

The use of pectic zymogram patterns to characterize *F. oxysporum* isolates is a relatively quick, simple and reliable method which gives reproducible results and provides a photographic record. The results show that pectic zymograms are not only useful tools for classification, but also can be utilized to predict the pathogenicity of *F. oxysporum* isolates.

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