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The relationship between cutinases and the pathogenicity/virulence of *Fusarium solani* in potato tubers

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Summary. Cutinase activity was spectrophotometrically determined in the culture supernatants of 40 isolates of *Fusarium solani,* obtained in Iran from various biological origins and grown in a minimal medium with 0.4% cutin as the only carbon source. Enzymatic activities, which ranged from 0 to 488 nmol min^{-1} mL^{-1}, were related to the pathogenicity or virulence of the fungal isolates, determined on potato tubers using a 0–5 disease severity scale. Cutinase activity was either not detected at all or was very low in the non-pathogenic isolates, whereas it was directly correlated with the virulence of the pathogenic isolates (R_{adj}^2 = 0.97), with an increase in cutinase activity of about 100 units corresponding to a one-point increase in the disease severity scale. SDS-PAGE analysis revealed that non-pathogenic *F. solani* isolates did not produce a cutinase band, while pathogenic isolates, with various degrees of virulence, produced single or double peptide bands with molecular weights of 20–23 kDa. We conclude that enzyme activity can be used as a predictive marker of the pathogenicity and virulence of *F. solani* isolates obtained from various hosts.

Key words: *Solanum tuberosum*, phytopathogenic fungi, cutinase activity, extracellular enzymes, SDS-PAGE.

Introduction

Phytopathogenic fungi gain access to healthy plants by different means. Some pathogens, such as many rust fungi, invade their hosts via the stomata, while others penetrate the intact plant surfaces with no regard to natural openings (Koller and Parker, 1989). Cutin is a major component of the cuticle, and is a preformed structural defense that covers and protects the aerial plant surfaces against physical, chemical and biological factors in the environment, including pathogens. The cuticle is the first barrier that many pathogens must breach (Kolattukudy, 2001) and it comprises a hydrophobic cutin network of interesterified hydroxyl- and epoxy-fatty acid derivatives, overlaid and partially intermingled with wax, representing a formidable barrier (Lequeu *et al*., 2003; Nawrath, 2006). The way in which pathogens penetrate this barrier has been debated for almost a century. Penetration was long thought to be strictly mechanical, but the involvement of cutinolytic enzymes secreted by the invading pathogens has often been postulated (Koller and Parker, 1989). Phytopathogenic fungi can produce the extracellular degradative enzymes, or cutinases, that play an important role in pathogenesis, hydrolysing cutin and facilitating penetration through the cuticle (Ettinger *et al*., 1987). That fungal cutinases have a critical role in the penetration of unwounded host tissues has been demonstrated in studies using antibodies, inhibitors and cutinase-deficient fungal mutants (Shaykh *et al*., 1977; Maiti and Kolattukudy, 1979; Dickman *et al*., 1983; Dickman and Patil, 1986). Inhibition of these enzymes can therefore effectively prevent fungal infection through intact cuticles. Cutin monomers, released from the

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cuticle by small amounts of cutinase on the fungal spore surfaces, greatly increase the amount of cutinase secreted internally by the spore, with a mechanism that is still unknown (Ettinger *et al*., 1987; Sweigard *et al*., 1992). Experimentation over the last two decades suggests that cutinase has a crucial role in determining the pathogenicity and tissue specificity of a number of phytopathogenic fungi. For example, in pea, cutinase is found at the site of *Fusarium solani* f. sp. *pisi* penetration (Shaykh *et al*., 1977). To date, most work on cutinase has been performed in studies examining a fungal pathogen of pea, *F. solani* f. sp. *pisi* (Purdy and Kolattukudy, 1975; Lin and Kolattukudy, 1980; Soliday and Kolattukudy, 1983; Dantzig *et al*., 1986; Murphy *et al*., 1996). Cutinase was first purified by Purdy and Kolattukudy (1975). The virulence of *F. solani* f. sp. *pisi* isolates on pea was positively correlated with the expression level of cutinase, and the disruption of the cutinase encoding gene in *F. solani* f. sp. *pisi* lowered the virulence of the fungus on pea (Rogers *et al*., 1994). The fungus produced extracellular cutinase only when cutin, or a cutin hydrolysate, was added to the conidial suspension. Fungal activity was first detected 30–45 min after the addition of the inducers and increased for up to 6 h. After the conidia sensed that they were in contact with the plant surface, low levels of cutinase activity carried by the conidia released small amounts of cutin monomers. These monomers synthesized the great amount of cutinases needed for fungal penetration into the plant tissues (Charles *et al*., 1986). Cutinases thus have a role in surface signaling that is crucial for the differentiation of the essential infection structure and the expression of pathogenicity factors (Belbahri *et al*., 2008). These enzymes are also postulated to play a role in carbon acquisition during the subcuticular growth of *Venturia inaequalis* (Koller *et al*., 1991). In addition, cutin monomers are known to promote germ-tube and appressorium differentiation on chemically inert surfaces in both *Blumeria graminis* f. sp. *hordei* (Francis *et al*., 1996; Gilbert *et al*., 1996; Hegde and Kolattukudy, 1997; Zhang *et al*., 2005) and *Magnaporthe grisea* (Gilbert *et al*., 1996; DeZwaan *et al*., 1999; see also Skamnioti and Gurr, 2007). In the present study, the pathogenicity or virulence of 40 *Fusarium solani* isolates, obtained from various plant hosts and from the insect *Euzophera bigella* in different Iranian localities, was assessed in relation to cutinase activity.

Materials and methods

Fungal isolates and identification

The *Fusarium solani* isolates used in the study are listed in Table 1. Single-conidium cultures of all isolates were morphologically characterized both on potato-dextrose agar (PDA, Merck, Darmstadt, Germany) and on synthetic nutrient-poor agar (SNA, Nirenberg, 1976). Isolates were identified according to Gerlach and Nirenberg (1982). Sequences of the ITS region (plus 5.8S gene) and the partial translation elongation factor 1-α were generated for all isolates and confirmed that they belonged to the *F. solani* species complex (data not shown).

Pathogenicity test

Two-month-old, washed and dried potato (*Solanum tuberosum* L.) tubers were used to estimate the pathogenicity and virulence of *F. solani* isolates, because with this plant the method is relatively accurate and convenient. Using a steel rod (2 mm diameter), two holes, 5 mm deep, were formed in each of the potato tubers, one at the apical and one at the stem end. Each isolate was inoculated at both holes on three tubers (Theron and Holz, 1989). To prepare the inoculum, fungal isolates were grown for two weeks on carnation-leaf agar (CLA) at 25ºC. Conidia were washed with distilled water, counted using a haemocytometer, the suspensions were diluted to 1×10^4 conidia mL^{-1} and 200 µL of suspension at each inoculation point. After inoculation the tubers were placed in paper bags and incubated in a germinator at 10ºC and 90% relative humidity for two weeks. Tubers were then cut through the inoculation points, and the degree of rot was estimated by LEICA QWin 550 CW software (Leica Cambridge Ltd, Cambridge, England) on a 0–5 scale, basically according to Theron and Holz (1989): 1, 50–100 mm^2 of rotted area; 2, 101–150; 3, 151–200; 4, 201–250; and 5>250.

Cutin isolation

Cutin was isolated from apple as described by Walton and Kolattukudy (1972), with some modifications. Briefly, peels from red apples were boiled in oxalate buffer (4 g of oxalic acid and 16 g of ammonium oxalate per liter) for 4 h. The cuticle was collected by filtration through cheesecloth, washed several times with distilled water, dried and ground in an IKA M20 mill (IKA, Staufen, Germany). Powdered cuticle was extracted with chloroform-methanol (2:1, v:v) overnight under mild stirring. A second extraction was carried out with chloroform for 24 h. The cutin material was dried at 80ºC and then washed several times with distilled water. Pectin and cellulose were removed by treating them with pectinase (1 g L^{-1}; Sigma) and cellulase (5 g L^{-1}; Sigma) in acetate buffer (50 mM, pH = 4), at 22ºC for 48 h. Cutin was recovered by filtration, washed with distilled water and dried at 105ºC.

Table 1. Isolates of *Fusarium solani* used in the study.

Isolate	Biological origin	Location
F11	*Cucumis melo* (root)	Varamin-Ivanekey
F14	*C. melo* (root)	Varamin-Ivanekey
F17	*C. melo* (root)	Varamin-Ivanekey
F23	*C. melo* (crown)	Varamin-Ivanekey
F26	*Beta vulgaris*	Khorasan
F29	*B. vulgaris*	Shahrekord
F30	*B. vulgaris*	Karaj
F32	*B. vulgaris*	Kerman
F39	*B. vulgaris*	Khuzestan-Dezful
F41	*Pyrus malus* (root)	Isfahan-Semirom
F43	*Solanum lycopersicum*	Bojnurd
F44	*S. lycopersicum*	Bojnurd
F45	*S. lycopersicum*	Bojnurd
F46	*Pyrus malus* (root)	Isfahan-Samirom
F48	*P. malus* (root)	Isfahan-Samirom
F53	*Triticum aestivum* (root)	Lorestan-Kuhdasht
F57	*Oryza sativa* (crown)	Gilan
F59	*Cynodon dactylon* (crown)	Gilan-Kolale
F62	*Avena sativa* (root)	East Azarbaijan-Marand
F63	*A. sativa* (root)	East Azarbaijan-Marand
F64	*Glycine max* (root)	Lorestan-Chaghalvandi
F70	*G. max* (root)	Lorestan-Aleshtar
F73	*G. max* (root)	Lorestan-Aleshtar
F74	*G. max* (root)	Lorestan-Kuhdasht
F76	*Hordeum vulgare*	Khorasan
F78	*H. vulgare*	Yazd-Bafgh
F82	*H. vulgare*	Lorestan-Kuhdasht
F83	*H. vulgare*	Lorestan-Kuhdasht
F86	*Cicer arietinum* (crown)	Kermanshah
F89	*Solanum tuberosum*	Hamedan-Kabudarahang
F93	*S. tuberosum*	Ardebil-Ardebil
F94	*S. tuberosum*	Ardebil-Ardebil
F95	*S. tuberosum*	Tehran-Damavand
F97	*S. tuberosum*	Tehran-Firuzkuh
F100	*S. tuberosum*	Ardebil
F101	*S. tuberosum*	Hamedan-Kabudarahang
F106	*Helianthus* sp.	Mashhad
F107	*S. melongena*	Golestan
F111	*Euzophera bigella*	Mazandaran
F113	*S. tuberosum*	Khorasan-Shahrud

Growth medium for cutinase production

The forty strains selected as above were inoculated into 10 mL of a culture medium composed of: 0.06% $NaNO_3$, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.02% KCl, 0.01% $FeSO_4 \cdot 7H_2O$ (pH 7.2), and 0.4% cutin (w:v) as a carbon source. The cultures were incubated in a shaker-incubator at 30ºC and shaken at 100 rpm for 96 h. After development, the cultures were centrifuged at 11,000 *g* at 10ºC for 30 min. Cutinase activity was measured in the supernatant (Trail and Koller, 1993).

Cutinase assay

Cutinase activity was determined spectrophotometrically following hydrolysis of *p*-nitrophenylbutyrate (PNPB) at 405 nm. A 100 µL aliquot of the culture supernatant was added to 900 µL of the reaction mixture with the following composition: 100 µL PNPB 50 mM dissolved in 800 µL 50 mM phosphate buffer pH 7.0 and also containing 0.2% (N/P) Triton X-100 and 0.43 M tetrahydrofuran. The reaction was monitored for 1 min against a blank solution. One unit of cutinolytic activity was defined as the amount of cutinase required to release 1 µmol of *p*-nitrophenol in one minute under the specified conditions (Calado *et al*., 2002).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Culture supernatants of all 40 isolates were analysed by SDS-PAGE essentially as described by Laemmli (1970). Samples were maintained at 37°C (partially denaturing conditions) for 5 min in 1 M Tris-HCl pH 6.8 containing 10% (v:v) glycerol, 5% (v:v) β-mercaptoethanol, 2.3% (w:v) sodium dodecylsulfate and 0.2% (w:v) bromophenol blue. Electrophoresis was performed at 150 V on stacking and running gels containing 5 and 12% acrylamide, respectively. The gels were stained using Coomassie brilliant blue (G250, Merck). To determine the molecular weight of the enzyme bands, a molecular weight protein marker (SMO431, Fermentas, CinnaGen Inc., Tehran, Iran) was included.

Results and discussion

The role of cutinases in the penetration process of phytopathogenic fungi is one of the most controversial in plant pathology. Many phytopathogenic fungi (Purdy and Kolattukudy, 1973; Baker and Bateman, 1978; Koller and Parker, 1989) and bacteria (Fett *et al*., 1992) secrete

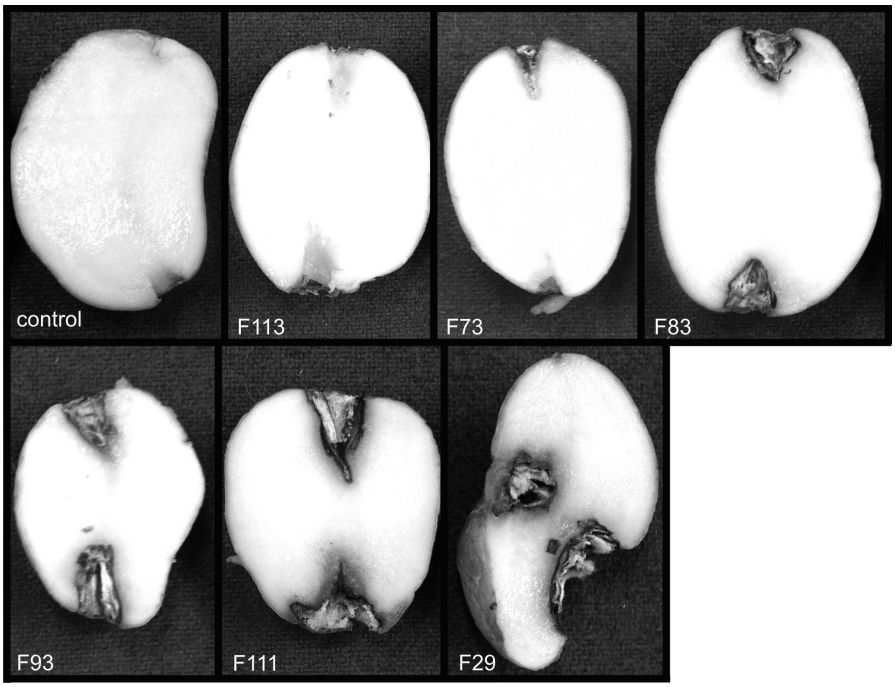

Figure 1. Pathogenicity/virulence of some representative *Fusarium solani* isolates in potato tubers. Isolates F113 caused the lowest (0) and isolate F29 the highest (5) amount of rotted area. There was no significant difference between F113 (non-pathogenic) and the control.

Table 2. Cutinase activity of *Fusarium solani* isolates grown in liquid cutin medium and their pathogenicity/virulence on potato tubers.

Isolate	Pathogenicity/ virulence[a]	Cutinase activity (nmol min^{-1} ml^{-1})
F11	3	253
F14	0	0
F17	0	37
F23	0	0
F26	0	11
F29	5	453
F30	3	255
F32	2	157
F39	3	271
F41	2	164
F43	4	353
F44	4	356
F45	1	98
F46	4	357
F48	2	171
F53	0	0
F57	3	299
F59	2	174
F62	4	375
F63	2	198
F64	2	204
F70	3	307
F73	1	114
F74	0	0
F76	2	142
F78	0	0
F82	2	208
F83	2	218
F86	2	221
F89	2	237
F93	3	311
F94	5	488
F95	4	385
F97	2	156
F100	0	0
F101	0	19
F106	4	385
F107	2	239
F111	4	389
F113	0	7

[a] 0, no rot; 1, 50–100 mm^2 of rotted area; 2, 101–150; 3, 151–200; 4, 201–250 and 5>250.

cutinases in order to break the ester bond of the cutin polymer. Cutinase activity is induced in the supernatant of *F. solani* and other plant pathogens grown in media containing cutin as a carbon source (Baker and Bateman, 1978; Lin and Kolattukudy, 1978; Koller and Parker, 1989). Cutinase production is particularly important only in fungi that penetrate their host directly. *Fusarium solani* is one of these pathogens. Disruption of the cutinase gene (*cut* A) in *Fusarium solani* f. sp. *pisi* has been reported to have a strong effect on the virulence of the pathogen towards its specific host, pea (Bakkern and Gold, 2004).

In this work, we compared *in vitro* the cutinase activity of pathogenic and non-pathogenic isolates of *F. solani*. Our previous findings (unpublished data) indicated that *F. solani* isolates were non host-specific on *Hordeum vulgare*, *Solanum tuberosum* and *Cicer arietinum*, and therefore we used potato as a general host in the pathogenicity test.

Fusarium solani isolates F14, F23, F53, F74, F78, F100 and F113, obtained from the roots and crowns of *Cucumis melo*, *Triticum aestivum*, *Glycine max*, *Hordeum vulgare* and *Solanum tuberosum* did not produce any cutinase activity (Table 2) nor did they cause any symptoms when inoculated in potato tubers (Figure 1). These isolates were therefore considered non-pathogenic. Some of them also produced putative lipase bands (between 30–40 kDa) in addition to the putative cutinase bands (data not shown).

Isolates with cutinase activities below 50 units did not cause any disease symptoms but those with high enzyme activities were pathogenic (Figure 2). A linear regression equation ($Y = -0.0303 + 0.01067x_1$) correlating pathogenicity with cutinase activity indicated that cutinase activity was directly correlated with the virulence of *F. solani* isolates ($R_{adj}^2 = 0.97$); an increase of about 100 units in cutinase activity corresponded to a one-point increase in the disease severity scale.

Isolate F111 from the insect *Euzophera bigella* not only secreted cutinase into the medium but also produced disease symptoms in the pathogenicity test on potato tubers. With this isolate both cutinase activity and disease severity were high (Table 2; Figures 1, 2). Of the four isolates obtained from *C. melo* (F11, F14, F17, F23), three were non-pathogenic, with low or no activity. All three isolates from *S. lycopersicum* (F43, F44, F45), the three from *P. malus* (F41, F46, F48), seven out of nine from gramineous host (F53, F57, F59, F62, F63, F76, F78, F82, F83), four out of five from *B. vulgaris* (F29, F30, F32, F39) and five out of eight isolates from *S. tuberosum* (F89, F93, F94, F95, F97, F100, F107, F113) were pathogenic. Isolates F29 and F94, from *B. vulgaris* and *S. tuberosum* respectively, had the highest virulence and these also had the highest cutinase activity.

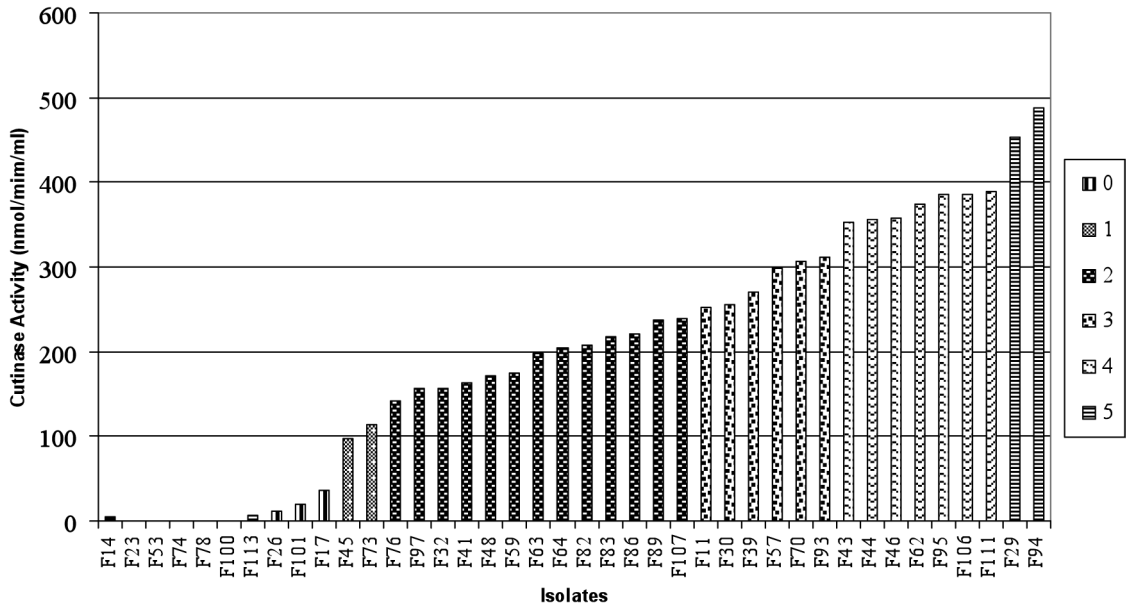

Figure 2. Cutinase activity of *Fusarium solani* isolates in relation to their pathogenicity/virulence. 0, no rot; 1, 50–100 mm^2 of rotted area; 2, 101–150; 3, 151–200; 4, 201–250 and 5>250.

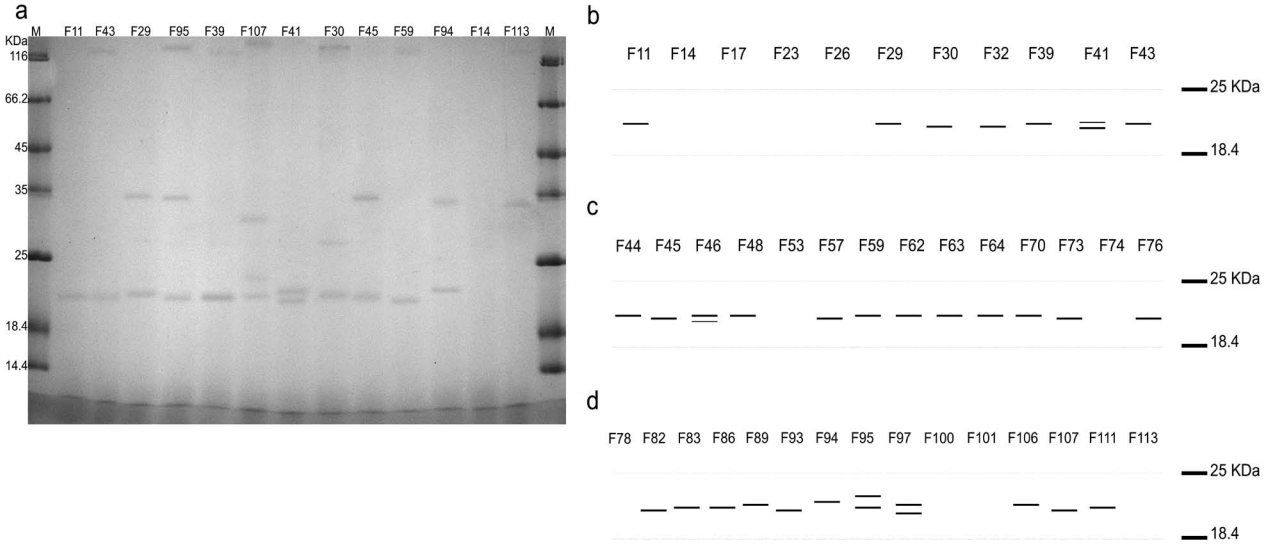

Figure 3. a, Band patterns of cutinase enzyme on SDS-PAGE. b–d, Schematic representation of SDS-PAGE band patterns of cutinase enzyme. Only cutinase bands are shown. Double peptide bands were seen in F26, F41, F46 and F97. Non-pathogenic isolates F14, F23, F53, F74, F78, F100 and F113 did not produce any cutinase bands.

These isolates were collected from Shahrekord and Ardebil respectively, which are the coldest Iranian regions, characterized by subzero temperatures and snow cover in winter.

SDS-PAGE revealed that most fungal cutinases consisted of a single peptide with a molecular weight in the range of 22–26 kDa (Kolattukudy *et al*., 1981). In *F. solani* f. sp. *pisi*, the molecular weights of the extracellular isoforms of cutinases I and II were reported to be 21.4 kDa as measured by SDS-PAGE (Purdy and Kolattukudy, 1975). In our experiment the non-pathogenic isolates F14, F17, F23, F26, F53, F74, F78, F100, F101 and F113 did not produce any cutinase band, while the other isolates, which had various degrees of virulence, produced single or double peptide bands with molecular weights in the range of 20–23 kDa. Double peptide bands were seen in isolates F41, F46, F95 and F97, where the molecular weight of the upper bands were 21.6, 21.6, 23 and 21.5 kDa, and that of the lower bands was 21.4, 21.4, 21.5, and 20 kDa respectively (Figure 3a-d). It is concluded that cutinase activity can be used as a marker to predict the pathogenicity or virulence of *F. solani* isolates on potato tubers.

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