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Identification of *Alternaria* spp. associated with tomato early blight in Iran and investigating some of their virulence factors

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Abstract

Tomato is one of the major crops grown in Iran, which is attacked by a large number of pathogens. Early blight is among the most important and harmful diseases of this plant caused by *Alternaria* spp. This study was conducted to identify *Alternaria* species causing early blight disease and to evaluate different virulence factors of this fungal pathogen. The samples were collected in Khorassan-Razavi province in Iran from tomato plants showing characteristic symptoms of the disease. Morphological identification of the isolates was done on PCA medium and under controlled conditions. Six *Alternaria* species, including *A. alternata*, *A. tenuissima, A. arborescens, A. mimicula, A. interrupta* and *A. infectoria* were identified. The ITS1 and ITS4 primers were used for molecular identification of the isolates via sequence analysis. The highest frequency was observed for *A. alternata* and the least frequent was *A. infectoria.* All isolates obtained in this study were pathogenic on tomato cultivar Peto Early Ch. The results showed significant differences in pathogenicity of the isolates on tomato plants. *A. alternata* and *A. tenuissima* had the highest and *A. mimicula* and *A. infectoria* had the lowest level of pathogenicity. In the qualitative analysis of cell wall degrading enzymes (CWDEs), all tested isolates were able to produce pectinase, cellulase, amylase, protease and lipase, but production of pectinase and cellulase had direct correlation with pathogenicity of the isolates.

Keywords *Alternaria* spp. · Cell wall degrading enzymes · Pathogenicity · *Solanum lycopersicum*

Introduction

Tomato (*Solanum lycopersicum* L.)*,* is one of the most popular vegetables worldwide and the second most valuable product after potatoes, either fresh or after processing. Various species of *Alternaria* are causal agents of early blight, as a destructive disease of tomato (Kumar et al. 2008). The early blight disease is capable of causing damage at all growth stages and aerial parts of tomato, such as leaf, stem and fruit (Blancard 2012). Creation of necrotic spots in concentric rings, with a yellow chlorotic halo are common symptoms of *Alternaria* diseases (Kokaeva et al. 2015; Chaerani et al. 2007). The *Alternaria* genus was first introduced by Nees (1816), it belongs to the kingdom Mycota, phylum Ascomycota, subphylum Pezizomycotina, class Dothideomycetes, order Pleosporales,

family Pleosporaceae and is a ubiquitous fungal genus that includes saprobic, endophytic and pathogenic species (Saharan et al. 2016). This genus can grow on several substrates including seeds, leaf and fruit of plants, agricultural crops, soil, and air (Kokaeva et al. 2015). Many species of *Alternaria* are reported as the causal agent of early blight. Esfandiari (1948) has reported *Alternaria solani* for the first time in Iran. Several *Alternaria* species including *A. tomatophila*, *A. arborescens*, *A. alternata* and *A. tenuissima* are known to be the causal agents of early blight disease in Iran (Hajianfar and Zarbakhsh 2006). *A. tomatophila* is reported as an inoculation source from black currant to tomatoes and potatoes in Korea (Hong et al. 2011). *A. alternata* f. sp. *lycopersici* was recognized by Grogan et al. (1975) as a destructive species in California that causes stem canker and contaminates leaves and fresh tomatoes. Two species of *A. grandis* and *A. tomatophila* are recognized as the causal agents of early blight on tomato and potato in Brazil (Rodrigues et al. 2010). Studies from Germany revealed that *A. solani* and *A. alternata* are involved in the early blight disease (Stammler et al. 2014). *A. metachromatica* is reported

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as causal agent of early blight in Pakistan by Bashir et al. (2014).

The plant cell wall is composed of pectin, cellulose, hemicellulose, lignin and glycoproteins and acts as a defense barrier to the entrance of external factors into the cell. Plant pathogenic fungi produce several extracellular hydrolytic enzymes that enable them to infect the host tissue. These enzymes are generally known as cell wall degrading enzymes (CWDEs), which contribute to the pathogenesis of fungi by destroying wax, cuticle, cell wall and facilitate penetration and invasion of fungal pathogens into the host tissues (Yang et al. 2005; Kikot et al. 2009). Usually, destruction of plant cell wall by fungal enzymes is associated with intercellular and intracellular growth, formation of feeding organs, direct communication of fungi with food and reduction of plant defense power that causes cell death (Ten Have et al. 2002). Sensitivity of cultivars, environmental factors, toxin production potential and cell wall degreading enzymes determine the degree of disease progress (Hubballi et al. 2011). Phytopathogenic *Alternaria* spp. isolates cause plant disease by producing toxins, CWDEs and melanin (Iftikhar et al. 2015; Saharan et al. 2016). In normal conditions, degradation of cellulose is slow. This is because of crystalline nature of native cellulose. Cooperatively, contribution of cellulase enzymes, including endo−/3−1,4-glucanase, P-glucosidase, and cellobiohydrolase, causes complete hydrolysis of cellulose (Showalter 1993). Pathogenicity of a phytopathogen and degree of plant cell wall lysis depends on the amount of CWDEs produced and their activity levels specially pectinase and cellulase enzymes (Anand et al. 2008). Lipase is frequently produced by *A. brassicicola* and is one of its virulence factors (Berto et al. 1997). Also subtilisin- and tripsin-like serine proteases play an important role in virulence of *A. alternata* on tomato plants (Kokaeva et al. 2015). Cutinase (Yao and Koller 1995) and lipase (Berto et al. 1997) were detected in saprophytic and pathogenic stages of *A. brassicicola*, which are involved in penetration of the pathogen into the cabbage leaf. Pathogenicity of *A. citri* was found to be dependent on endopolygalacturonidase (Isshiki et al. 2001) and endoglucanase (Eshel et al. 2002) activity.

The objectives of this study were: (i) identification of *Alternaria* spp. associated with tomato early blight, (ii) determining pathogenicity levels of *Alternaria* spp. isolates on tomato plants, and (iii) quantitative and qualitative analysis of cell wall degrading enzymes produced by different isolates. Finally, the association between pathogenicity of the isolates and their capability in producing CWDEs was investigated.

Materials and methods

Sampling and fungal isolation Field sampling was done randomly from different parts of the farm and at the end of the growing season. From each field surveyed, five infected leaves with early blight symptoms were randomly sampled for fungi isolation. The leaves with the disease symptoms were separated from the plant and were individually placed in paper bags, transferred to the laboratory and kept in the refrigerator at 4 °C until use. The leaves were washed under a gentle stream of water and sterilized with 1% sodium hypochlorite for 3 min, then washed three times with sterilized distilled water, dried and placed onto Petri dishes containing potato dextrose agar (PDA) and incubated in dark conditions at 28 °C for seven days. Purification of the fungal isolates was performed by transferring single spores onto PDA media. Sixty-five isolates of *Alternaria* spp. were obtained from typical early blight lesions on tomato plants in the fields of Khorassan-Razavi province in Iran.

Maintenance of pure isolates Spore suspensions from pure cultures were transferred to silica gel (Perkins 1962) for long-term storage. The *Alternaria* spp. isolates could be regrown after more than one year in storage by sprinkling a few silica gel particles on V8 juice agar or other suitable media (Perkins 1962; Morris et al. 2000).

Morphological identification of *Alternaria* spp. isolates To characterize *Alternaria* spp. isolates based on the structure of conidial apparatus, all isolates were incubated at 25 °C for seven days on potato carrot agar (PCA) under an alternate light/dark cycle (8 h/16 h) (Simmons 2007). Identification was performed based on the *Alternaria* identification key descriptions, including colony properties such as growth diameter, presence or absence of aerial hyphae, sporulation patterns such as spores formation, number of spores in chain and the pattern of chain splitting, which were examined by a stereo microscope (Olympos Dp12, BX41TF, Japan). In addition, the color and size of spores, presence or absence of longitudinal and transverse septa, presence or absence of beack and surface decorations were microspically examined.

Molecular identification of the fungal isolates For molecular identification of the isolates, fungal DNA was extracted using the isolates cultured in potato dextrose broth (PDB) medium prepared by dislodging 1 ml of spore suspension (10^4 conidia/ml), obtained from seven-day-old PDA plates. Flasks containing PDB medium were agitated on a rotary shaker, at 120 rpm for 14 days at 25 °C, and mycelia were harvested and liophylized. DNA extraction was performed on freeze-dried mycelium using cetyl trimethylammonium bromide (CTAB) extraction protocol as described by Li et al. (1994). DNA concentration was investigated by 1% agarose gel electrophoresis. Observation of strong bands indicated the quantity and quality of the desired DNA extracted.

Then, polymerase chain reaction (PCR) was used to amplify DNA in 25 μl reactions using ITS1 (5'TCCGTAGGTGAACC

TGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') primers (White et al. 1990). The PCR reaction mixtures consisted of 12.5 μl PCR master mix (Ampliqon, Denmark), 3 μl DNA, 1 μl of each primer (forward and revers) and 7.5 μl sterile water to make the volume up to 25 μl. An initial denaturation at 94 °C for 1 min, followed by 35 amplification cycles consisting of 94 °C for 30 s for DNA denaturation, 55 °C for 30 s for primers annealing, and 72 °C for 45 s for DNA extension. Also, a final extension step at 72 °C for 5 min was included (Pavon et al. 2012). The PCR products were sequenced by Macrogen Co. (Seoul, South Korea). The nucleotide sequences were registered in the GenBank. Sequence similarity searches were performed using National Center for Biotechnology Information (NCBI) databases with the Basic Local Alignment Search Tool (BLAST) program. The obtained nucleotide sequences were compared with the sequences of *Alternaria* species via BLAST searches from the NCBI GenBank. The most similar sequences were downloaded and DNA sequences were manually edited using Bioedit v7.1.3 and aligned using ClustalW software (Gräfenhan et al. 2013). Phylogenetic analysis was conducted using MEGA 7.0 software (Tamura et al. 2013) by neighbour-joining analysis methods and the degree of confidence in phylogenetic branching was assessed using 1000 bootstrap resampling.

Plant growth conditions The seeds of cv. 'Peto Early Ch', which is susceptible to early blight disease (Aminian et al. 2004), were obtained from Agricultural Research Center of Khorassan-Razavi province in Iran and used in the pathogenicity testes. Surface sterilization of the seeds was performed using 1% sodium hypochlorite for 1 min. Then, the seeds were washed three times with sterile distilled water. After complete drying on sterile filter paper, the seeds were grown in a tray and transferred to the pot after germination and reaching the two-leaf growth stage. In this experiment, 45-day-old tomato plants grown in half-kg plastic pots containing 1: 1: 1 (*v/v/v*) ratio of soil, sand and leaf mold were used. The plants kept under fluorescent light (12-h photoperiod).

Pathogenicity tests The pathogenicity tests were carried out under greenhouse conditions. Single spore isolates of *Alternaria* spp. were grown on PCA for seven days at 25 ± 1 °C (Shahbazi et al. 2010) under an alternate light/dark cycle (8 h/16 h) (Simmons 2007). Conidial suspensions were obtained by flooding dishes with 10 ml of sterile distilled water, scarped and filtered through two layers of sterile cheesecloth. The concentration of conidial suspension was determined using a hemocytometer and adjusted to 1×10^6 conidia/ml. For better placement of spores on plant tissues, Tween 20 (0.05%) was added to the spore suspension. Spraying the conidial suspension was carried out in the greenhouse in cool weather on tomato cv. 'Peto Early Ch' 45-day-old plants. The plants were covered with black plastics for 72 h with relative humidity of 90% at 27 ± 2 °C. On

the control plants, sterile distilled water containing 0.05% Tween 20 was sprayed. Fifteen days after inoculation, the disease symptoms were graded using a scoring system described by Hubballi et al. (2011), including 0: No infection, 1: 0 to 10.00, 3: 10.1 to 15.00, 5: 15.1 to 25.00, 7: 25.1 to 50.00 and 9: more than 50.00% of leaf area infected. Disease index (DI) for each treatment was calculated according to the formula presented by Shafique et al. (2013):

$$DI = \frac{\text{Sum of all numerical grades} \times 100}{\text{Total number of leaves counted} \times \text{maximum category value}}$$

Qualitative measurement of CWDEs produced by *Alternaria* spp. For qualitative evaluation of CWDEs, a 6 mm diameter mycelial plug of each isolate from the margin of seven-day-old fungal colony was transferred to a culture medium. For each enzyme, an uninoculated plate served as a control. Twelve isolates of *Alternaria* were selected to be used in this assay, which showed the highest and lowest levels of pathogenicity among the isolates of each species on tomato plants, including AS40 and CH9 isolates belonging *A. alternata*, NIR12 and NEY8 isolates belonging to *A. tenuissima*, MT40 and CH1 isolates of *A. arborescens*, CH34 and MD16 isolates of *A. infectoria*, SAB21 and GHA15 of *A. mimicula* and MO23 and TB8 of *A. interrupta*. A solid medium can be used to determine the presence or absence of an enzyme and its production by fungi. Activity of cellulase, pectinase, amylase and protease was measured in five-day-old cultures (Kaur and Aggarwal 2017). Lipase activity was investigated after seven days of incubation (Ortega et al. 2013). The details of qualitative analyses of each enzyme are mentioned below.

Amylolytic activity Amylase activity was measured by growing the fungi on glucose yeast extract peptone agar (GYP) medium as described by Sunitha et al. (2013), containing 1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 16 g agar, 1 L distilled water with 0.2% soluble starch (pH = 6.0). After five days, the GYP medium was flooded with 1% iodine solution and 2% potassium iodide. Creation of a transparent halo around the colony indicated amylase activity.

Lipolytic activity Peptone-agar medium with 1% Tween 20 was used for measurement of lipase activity, as described by Sunitha et al. (2013). The medium consisted of 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$, and 16 g agar per liter of water with pH = 6. Tween 20 was sterilized separately and added to the medium after cooling (50–45 °C). After seven days, a visible precipitate around the colony due to the formation of calcium salts of lauric acid liberated by the enzyme indicated lipase activity.

Cellulolytic assay Cellulase activity was investigated using the method of Sunitha et al. (2013). The GYP medium with 0.5% carboxymethylcellulose (CMC) was used for this purpose. After 5 days incubation period at 27 ± 2 °C, each petri dish was flooded with 0.2% aqueous Congo red solution and destained with 1 M NaCl solution after 15 min. Creation of yellow color in red background of the culture medium indicated the presence and activity of cellulase.

Proteolytic activity The GYP medium, containing 0.4% gelatin with pH = 4 was used for investigating protease activity. The 8% gelatin solution was prepared using distilled water and separately was sterilized. Then, after reaching the medium to the temperature of 45–50 °C, 5 ml of sterilized gelatin solution was added per 100 ml of the medium. After five days, the media were flooded with ammonium sulfate saturated solution. The presence of a transparent halo around the colony indicated the activity and presence of protease enzyme (Sunitha et al. 2013).

Pectinolytic activity For pectinase activity, the fungi were grown on Czapek-Dox agar medium containing 0.50 g KCI, 3.0 g $NaNO_3$, 0.50 g $MgSO_4$, 0.01 g $FeSO_4$, 1.0 g K_2HPO_4, 30 g sucrose, 15.0 g agar with 2% pectin (pH = 5.6). After 3 to 5 days of fungal colony growth, the plates were flooded with freshly prepared iodine-potassium iodide solution (Hankin et al. 1971).

Quantitative assays of CWDEs produced by _Alternaria_ spp. According to previous studies on the activity of CWDEs, most enzymatic activities were carried out within 10 days after inoculation and incubation (Ortega et al. 2013; Kikot et al. 2009). Therefore, activity of pectinase and cellulase were investigated during 10 days after incubation in the medium. To determine the activity of pectinase and cellulase secreted by _Alternaria_ spp., the culture media described by MacMillan and Voughin (1964) and Abdel-Razik (1970) were used, respectively.

Activity of pectinase was measured using a medium containing 4.6 g pectin, 5 g yeast extract, 5 g peptone and 5 g KH_2PO_4 in 1 L of distilled water with pH = 7. The same medium containing 4 g of carboxymethyl cellulase was used to produce cellulose instead of citrus pectin. For quantitative analysis of pectinase and cellulase enzymes, the isolates AS40, NIR12, MT40, MO23, SAB21 and CH34 were used.

Pectinase assay For evaluating pectinase activity, the method described by Colowich (1995) was used. Pectinase activity was measured on the basis of reduction of D-galactronic acid and determination of D-galactoric content using di-nitro-salicylic acid colorimetric assay. Absorbance of the samples was measured at 540 nm. The unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of galacturonic acid per minute according to the standard curve. The standard curve was drawn based on the absorbance of different concentrations (μg/ml) of D-galacturonic acid.

Cellulase assay For investigating cellulase activity, the metod described by Wood and Bhat (1988) was used. Activity of this enzyme was measured at 550 nm. The amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity was defined as the amount of enzyme that catalyzed 1 μmol of glucose per minute during the hydrolysis reaction.

Statistical data analysis Statistical analysis of the data obtained from different tests was performed using SPSS software (version 24), and the meanings were compared using Duncan's multiple range test at 5% level.

Results

Morphological characterization of the fungal isolates In total, 75 samplese were checked out from 15 fields in 11 regions in Khorassan-Razavi province of Iran. Among them, 65 isolates of _Alternaria_ spp. were identified based on the morphological characteristics of this fungal genus described by Simmons (2007), including 22 isolates of _A. alternata_ (Fig. 1a-c), 19 isolates of _A. tenuissima_ (Fig. 1d-f), 7 isolates of _A. arborescens_ (Fig. 1g-i), 6 isolates of _A. mimicula_ (Fig. 1j-l), 4 isolates of _A. interrupta_ (Fig. 1m-o) and 7 isolates of _A. infectoria_ (Fig. 1p-r).

Molecular identification Molecular identification of the fungal isolates was performed via amplification and sequencing of the ITS region of the obtained fungal isolates. All six species produced a PCR product of about 550 bp (Fig. 2a). Among six species of _Alternaria_ identified by the morphological method, _A. interrupta_ was only identified by morphological characteristics due to the lack of any sequence for this species in NCBI. The ITS sequences of the other five species were submitted to GenBank. The accession numbers of their ITS sequences were MG 786770.1 for _A. alternata_ (AS40 isolate), MG 786766.1 for _A. tenuissima_ (NIR12 isolate), MG786771.1 for _A. arborescens_ (MT40 isolate), MG 786772.1 for _A. mimicula_ (SAB21 isolate) and MG 786617.1 for _A. infectoria_ (CH34 isolate). The sequence obtained for each fungal species had 99% similarity to the corresponding species of _Alternaria_ in the GenBank. This level of similarity is sufficient to confirm morphological identification of the isolates obtained in this study. In the phylogenetic

Fig. 1 Morphological characteristics of *Alternaria* spp. isolates obtained from tomato plants in Iran, **a-c**: *A. alternata*, colony on potato carrot agar (PCA) after 6 days (**a**), sporulation pattern with long chains (**b**), conidia in a chain with longitudinal and transverse septa (**c**); **d-f**: *A. tenuissima*, colony on PCA after 6 days (**d**), sporulation pattern (**e**), conidial chains and conidia (**f**); **g-i**: *A. arborescens*, colony on PCA after 6 days (**g**), sporulation pattern with long primary conidiophore (**h**), conidia with surface decorations (**i**); **j-l**: *A. mimicula*, colony on PCA after 6 days (**j**), sporulation pattern and the biggest conidia in beginning of chain (**k**), conidia without transverse septa (**l**); **m-o**: *A. interrupta*, colony on PCA after 6 days (**m**), sporulation pattern and sudden change in size of conidia (**n**), conidia with two different sizes (**o**); **p-r**: *A. infectoria*, colony on PCA after 6 days (**p**), sporulation pattern and shrub growth with secondary conidiophore (**q**), conidia with secondary conidiophore (**r**). Scale bar = 50 μm
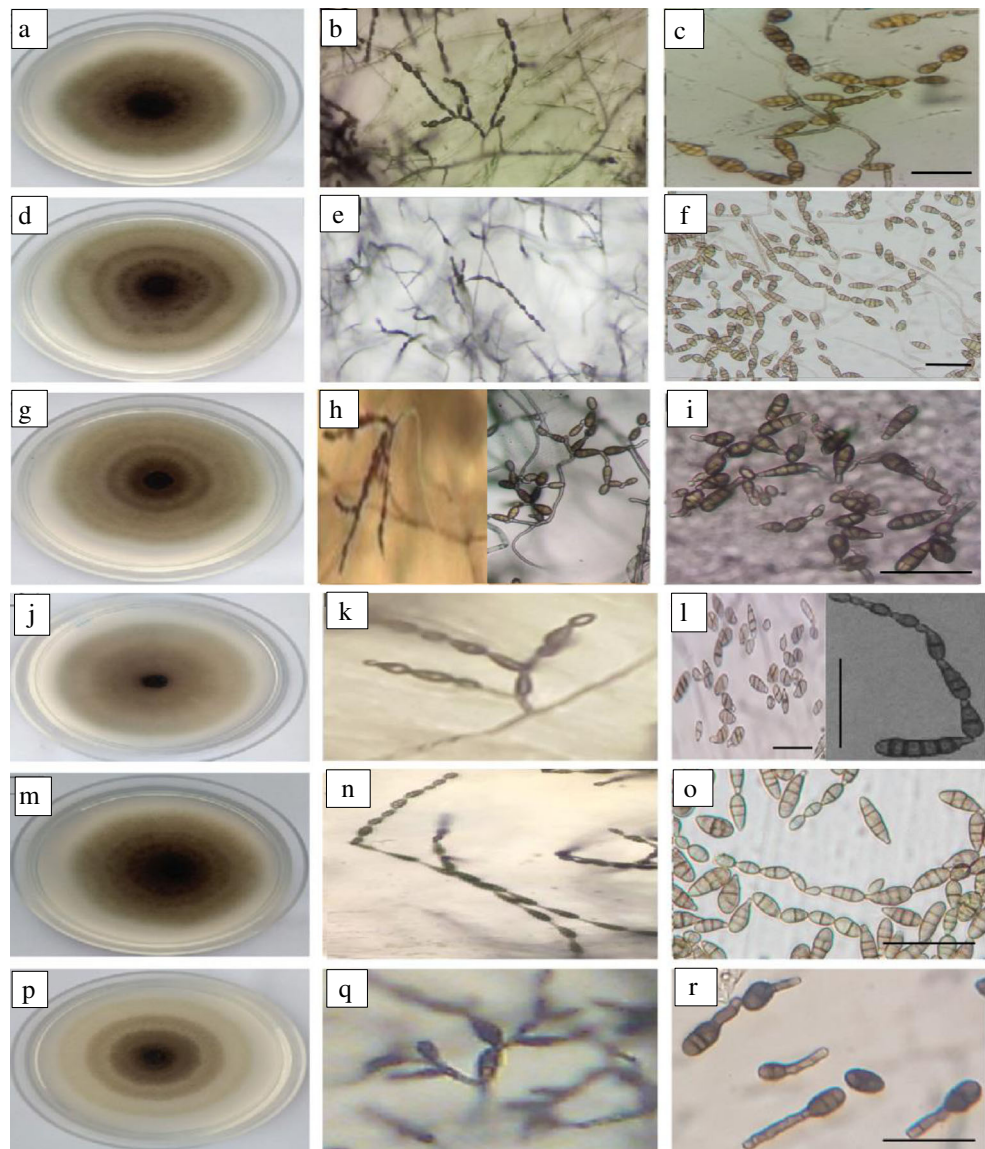
tree of the *ITS* region, six isolates of *Alternaria* spp. obtained in this study were compared with seven isolates of NCBI and each species of this pathogen showed high genetic similarity to the isolates of the same species in the GenBank (Fig. 2b).

Pathogenicity tests Pathogenicity tests using *Alternaria* spp. isolates were carried out in greenhouse conditions on 45-day-old tomato plants belonging to cv. 'Peto Early Ch'. After 5 to 7 days from the time of inoculation, the symptoms were observed in the form of browning of the tissue and then necrosis. The control plants did not show disease symptoms. In this test, the highest level of pathogenicity was related to two species, including *A. alternata* and *A. tenuissima*. The lowest pathogenicity was observed for *A. mimicula* and *A. infectoria* isolates. Two species of *A. arborescence* and *A. interrupta* showed intermediate disease progress compared to the other species obtained in this study (Fig. 3, Table 1).

Production of CWDEs

Qualitative assay Twelve isolates of *Alternaria* spp. tested were able to produce all extracellular enzymes investigated and were capable of degrading the substrates.

In the amylase activity assay, a clear zone around the colony indicated that amylase was produced and degraded the starch. Isolates AS40 and MD16 had the highest and lowest reactions, respectively. Amylase production was not significantly different among other isolates (Figs. 4a and 5a).

Fig. 2 PCR amplification products (**a**) and phylogenetic tree (**b**) of the ITS region of rDNA for the isolates of *Alternaria* spp. obtained from tomato in Iran. The phylogenetic tree is constructed via MEGA 7.0 software, using Neighbour-Joining method with 1000 bootstrap replicates. Bootstrap replication frequencies above 50% are indicated. In panel A: Lane C: negative control; Lane M, molecular size marker; Lane 1: AS40 isolate of *A. alternata*; Lane 2: NIR12 isolate of *A. tenuissima*; Lane 3: MT40 isolate of *A. arborescens*; Lane 4: SAB21 isolate of *A. mimicula*; Lane 5: CH34 isolate of *A. infectoria* and Lane 6: MO23 isolate of *A. interrupta*
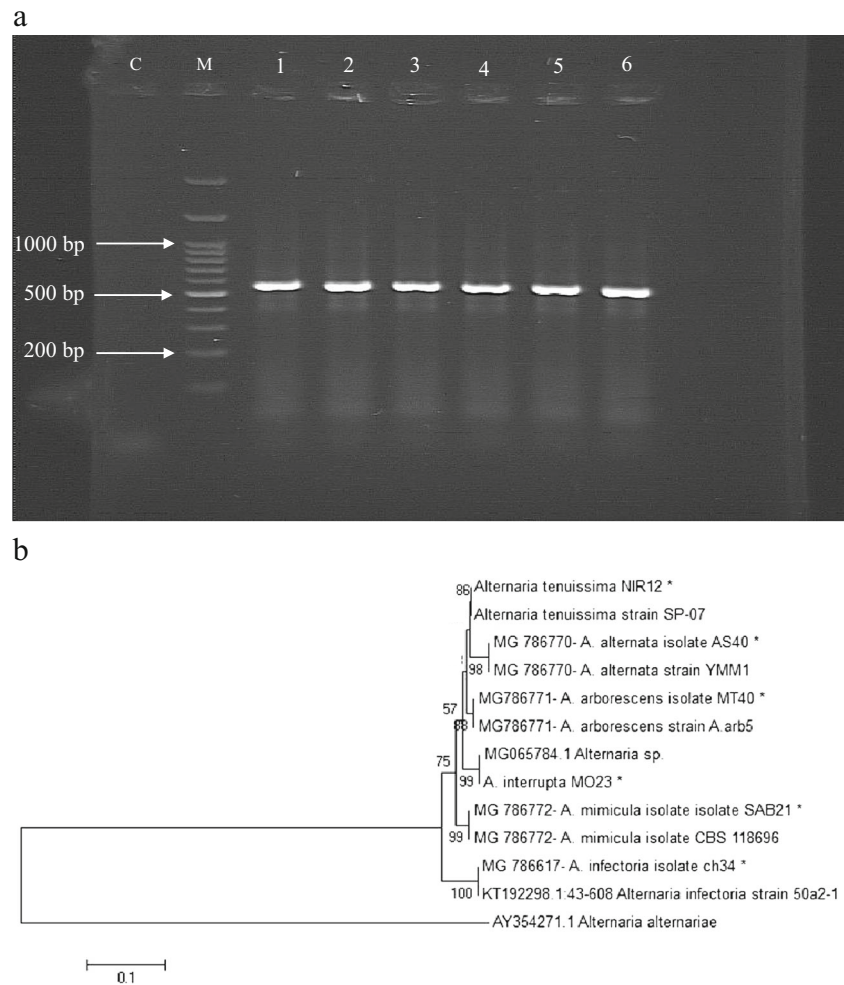
In the lipase activity experiment, the NIR12 isolate of *A. tenuissima* and the AS40 isolate belonging to *A. alternata* had the highest and the MD16 isolate of *A.infectoria* showed the lowest levels of lipase production (Figs. 4c and 5b).

Fig. 3 Disease symptoms caused by different species of *Alternaria* on tomato cv. 'Peto Early Ch'. **a**: Healthy control; **b**: symptoms caused by *A. alternata* (AS40); **c**: *A. tenuissima* (NIR12); **d**: *A. arborescens* (MT40); **e**: *A. interrupta* (MO23); **f**: *A. mimicula* (SAB21) and **g**: *A. infectoria* (CH34)
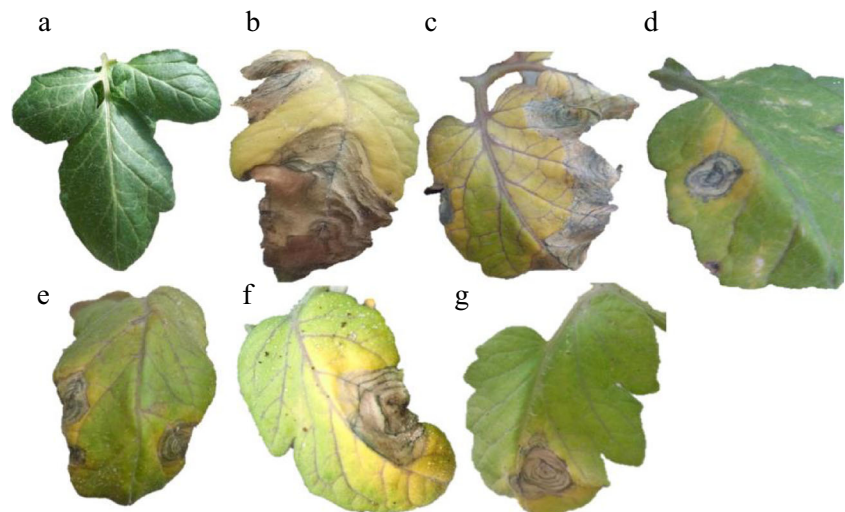
Table 1 Designation of *Alternaria* spp. isolates used in this study and their disease index (DI) on tomato cv. 'Peto Early Ch'

Number	Isolate code	Species	Geographic orign	Leaf necrosis	DI
1	AS40	*A. alternata*	Mashhad	40 a	77.77
2	AS10	*A. alternata*	Mashhad	28.33 defg	70.37
3	MD4	*A. alternata*	Mashhad	33.33 bcd	77.77
4	MT30	*A. alternata*	Mashhad	23.33 ghij	55.55
5	MT50	*A. alternata*	Mashhad	23.33 ghij	55.55
6	FF11	*A. alternata*	Fariman	28.33 defg	70.37
7	NEY15	*A. alternata*	Neyshaboor	26.66 defg	62.96
8	KA33	*A. alternata*	Kashmar	25 fghi	55.55
9	KA22	*A. alternata*	Kashmar	35 be	77.77
10	DAR5	*A. alternata*	Daregaz	23.33 ghij	55.55
11	DAR9	*A. alternata*	Daregaz	23.33 ghij	55.55
12	NIR13	*A. alternata*	Mashhad	26.66 defg	62.96
13	NIR15	*A. alternata*	Mashhad	28.33 defg	70.37
14	GHAT 1	*A. alternata*	Sabzevar	26.66 defg	62.96
15	KOH3	*A. alternata*	Sabzevar	25 fghi	55.55
16	SAR3	*A. alternata*	Sarakhs	30 def	48.14
17	TB5	*A. alternata*	Torbate Jam	26.66 defg	62.96
18	QH055	*A. alternata*	Ghochan	36.66 ab	77.77
19	QHO12	*A. alternata*	Ghochan	26.66 defg	62.96
20	QHO15	*A. alternata*	Ghochan	25 fghi	55.55
21	CH8	*A. alternata*	Chenaran	26.66 defg	62.96
22	CH9	*A. alternata*	Chenaran	23.33 ghij	55.55
23	AS20	*A. tenuissima*	Mashhad	23.33 ghij	55.55
24	AS30	*A. tenuissima*	Mashhad	26.66 defg	62.96
25	AS50	*A. tenuissima*	Mashhad	26.66 defg	62.96
26	MD6	*A. tenuissima*	Mashhad	23.33 ghij	55.55
27	MD1	*A. tenuissima*	Mashhad	25 fghi	55.55
28	MD11	*A. tenuissima*	Mashhad	28.33 defg	70.37
29	MD20	*A. tenuissima*	Mashhad	31.66 cde	77.77
30	HEY1	*A. tenuissima*	Torbate Heydarieh	30 def	77.77
31	NIR12	*A. tenuissima*	Mashhad	31.66 cde	77.77
32	NEY2	*A. tenuissima*	Neyshaboor	26.66 efgh	62.96
33	NEY3	*A. tenuissima*	Neyshaboor	23.33 ghij	55.55
34	NEY14	*A. tenuissima*	Neyshaboor	25 fghi	55.55
35	NEY16	*A. tenuissima*	Neyshaboor	31.66 cde	77.77
36	NEY8	*A. tenuissima*	Neyshaboor	21.66 hijk	55.55
37	NEY10	*A. tenuissima*	Neyshaboor	23.33 ghij	55.55
38	MT70	*A. tenuissima*	Mashhad	28.33 defg	70.37
39	MT80	*A. tenuissima*	Mashhad	30 def	77.77
40	MT100	*A. tenuissima*	Mashhad	21.66 hijk	55.55
41	DAR8	*A. tenuissima*	Daregaz	25 fghi	55.55
42	MT10	*A. arborescens*	Mashhad	26.66 efgh	62.96
43	MT40	*A. arborescens*	Mashhad	30 def	77.77
44	MT60	*A. arborescens*	Mashhad	18.33 jkl	48.14
45	MT90	*A. arborescens*	Mashhad	23.33 ghij	55.55

Production of cellulase, was significantly different among all isolates except the TB8 and CH1 isolates. Maximum cellulase activity was observed for AS40 isolate followed by NIR12 and MT40 isolates. Also, minimum activity of this enzyme was observed for MD16 and GHA15 isolates followed by TB8 and CH1 isolates (Figs. 4e and 5c).
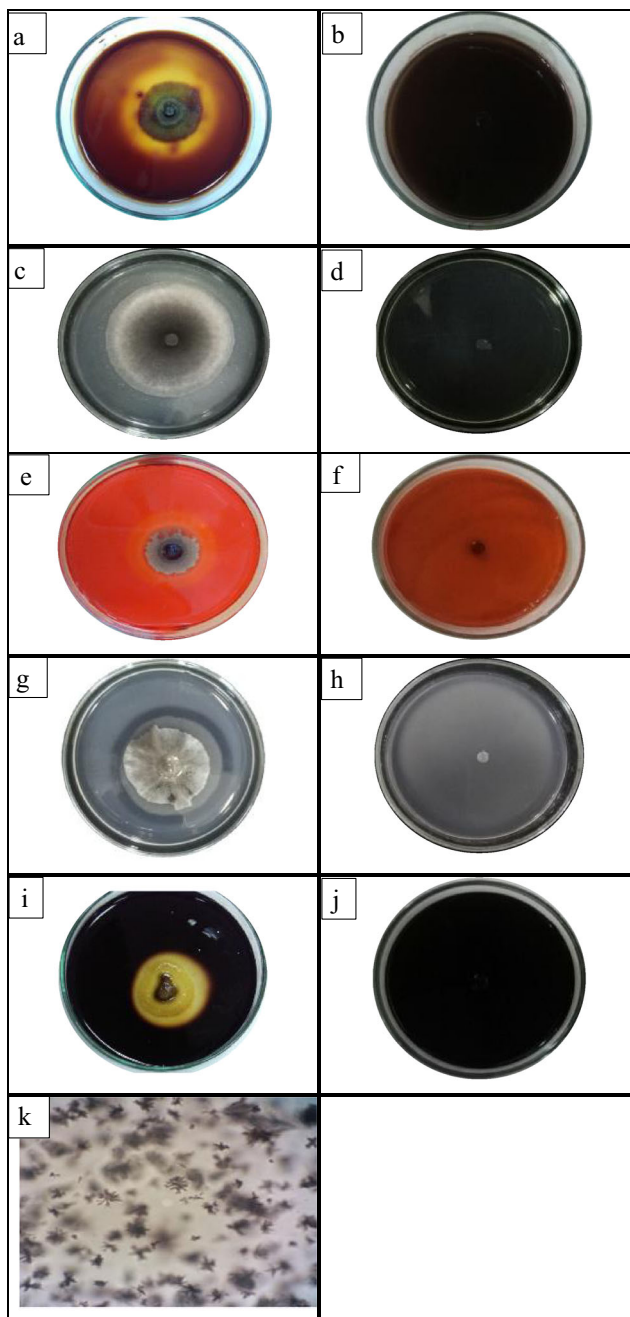
Fig. 4 Qualitative investigation of cell wall degrading enzymes production by *Alternaria* spp. isolates. Amylolitic activity on GYP medium with starch substrate (**a**); lipolytic activity on pepton agar medium with 1% tween 20 (**c**); cellolulytic activity on GYEA with 0.5% CMC (**e**); proteolitic activity on GYEA medium with 0.4% gelatin (**g**); pectinolytic activity on Czapek-Dox agar medium with 2% pectin as substrate (**i**); negative control of amylolytic, cellolulytic, lipolytic, proteolytic and pectinolytic activities, respectively (**b, d, f, h** and **j**); deposition of lauric acid salt crystals (K)

The isolates As40 and CH9, both belonging to *A. alternata*, followed by the isolate SAB21 of *A. mimicula* were maximum producers and the isolates GHA15, MD16 and NEY8 were minimum producers of protease. The isolates NIR12, MT40,

CH1, MO23 and TB8 produced similar amounts of protease and had medium reaction (Figs. 4g and 5d).

Production of pectinase was significantly different among the tested isolates. Maximum production of pectinase was observed for the isolate AS40, followed by NIR12 and minimum production of this enzyme belonged to the isolates MD16, GHA15 and CH34, respectively (Figs. 4i and 5e).

Quantitative assay The results showed that all tested species were able to produce CWDEs. Maximum production of pectinase and cellulase for all tested isolates were observed at 168 and 72 h after incubation, and then the amount of each enzyme decreased (Fig. 6a and b). The peak point for pectinase activity among different isolates varied between 6266 to 3645 µg/ml. Production of pectinase in AS40, NIR12 and MT40 isolates was the highest and the lowest pectinase activity was observed for the MO23, SAB21 and CH34 isolates, respectively (Fig. 6a). The amount of cellulase activity among isolates was between 1326.15 to 587.82 µg/ml. The highest activity of cellulase was observed for the isolate AS40 of *A. alternata*, NIR12 of *A. tenuissima* and MT40 of *A. arborescens*, respectively and the lowest cellulose activity belonged to the isolate SAB21 of *A. mimicula*, MO23 of *A. interrupta* and CH34 of *A. infectoria*, respectively (Fig. 6b).

Discussion

In this study, *Alternaria* spp. isolates associated with early blight on tomato plants in Iran were investigated. A total of 65 isolates from tomato plants with symptoms of early blight disease were collected. Morphological identification of the isolates was performed based on the morphological characteristics described by Simmons (2007). In order to confirm morphological identification, amplification and sequencing of the ITS region were performed. Also, the activity of CWDEs, involved in fungal pathogenesis, was investigated. According to previous studies, *A. solani* was the main causal agent of early blight disease in family Solanaceae (Dang et al. 2015). Nevertheless, based on the results of this study, among 65 *Alternaria* isolates identified as the causal agents of tomato early blight in Khorassan-Razavi province of Iran, 33.84% belonged to *A. alternata*, 29.23% to *A. tenuissima*, 10.76% to *A. arborescens*, 9.23% to *A. mimicula*, 6.15% to *A. interrupta* and 10.76 to % *A. infectoria*. Morphological features such as clump and shrub like growth for *A. alternata*, simple chain production with minimum branching for *A. tenuissima*, having a long initial conidiophore for *A. arborescens*, sudden change in size of spores of a simple chain for *A. interrupta*, shrub growth pattern with short chains and having the largest spore at the beginning of the chain for *A. mimicula* and having certain secondary
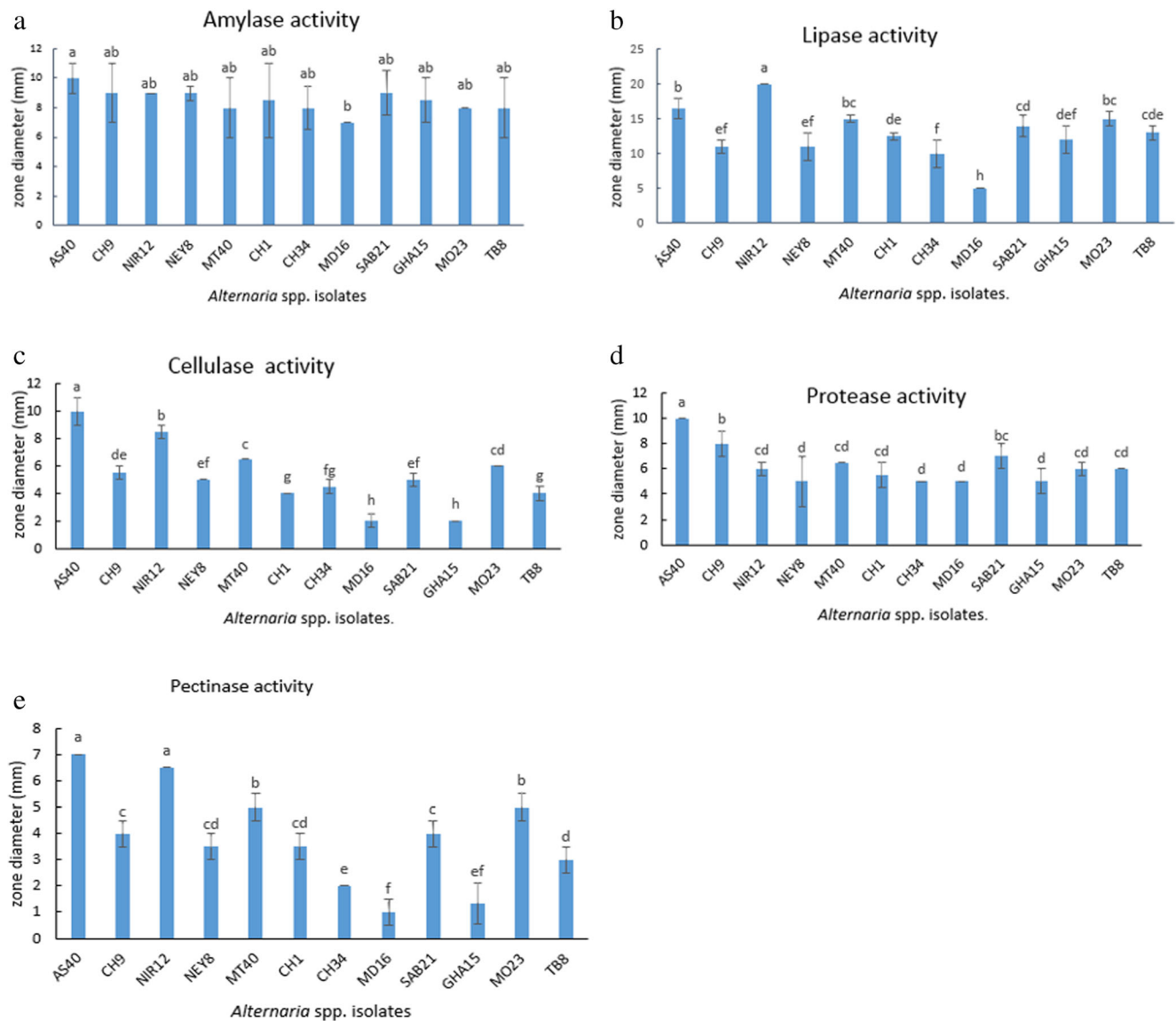
Fig. 5 Qualitative analysis of cell wall degrading enzymes produced by *Alternaria* spp., including *A. alternata* (AS40 and CH9 isolates), *A. tenuissima* (NIR12 and NEY8 isolates), *A. arborescens* (MT40 and CH1 isolates), *A. infectoria* (CH34 and MD16 isolates), *A. mimicula* (SAB21 and GHA15 isolates) and *A. interrupta* (MO23 and TB8 isolates). For each treatment three replicates were used. **a**, **b**: the means with the same letter do not have significant differences according to Duncan's multiple range test at 5% level

conidiophore for *A. infectoria* were observed, in accordance with identification key described by Simmons (2007).

One way to quickly identify fungal species is using molecular methods (Kokaeva et al. 2015). Molecular techniques are powerful tools, especially for people who are not familiar with morphological characterization of fungi (Pryor and Michailides 2002). One of these techniques is sequencing the ITS region of the ribosomal DNA (Kusaba and Tsuge 1994, 1995), that identifies *Alternaria* spp. such as *A. alternata*, *A. infectoria*, *A. tenuissima* and *A. arborescens* from each other very well (Roberts et al. 2000). The results of molecular identification confirmed morphological characterization of the fungal isolates obtained in this research and were suitable to distinguish *A. alternata*, *A. tenuissima*,

A. arborescens, *A. mimicula* and *A. infectoria* from each other. Therefore, molecular identification of these species can be used to confirm the data obtained by morphological characterization.

Four species of *Alternaria*, including *A. alternata*, *A. tenuissima*, *A. arborescens* and *A. infectoria* were previously reported on tomato plants in Iran and other countries (Hajianfar and Zarbakhsh 2006; Grogan et al. 1975). In this study, two species, including *A. mimicula* and *A. interrupta* are reported for the first time on tomato worldwide. Different *Alternaria* species can be found in different habitats and geographical regions. Therefore, they may have various metabolite profiles which are related to virulence capability of these phytopathogenic fungi. In this study, *A. alternata* isolates
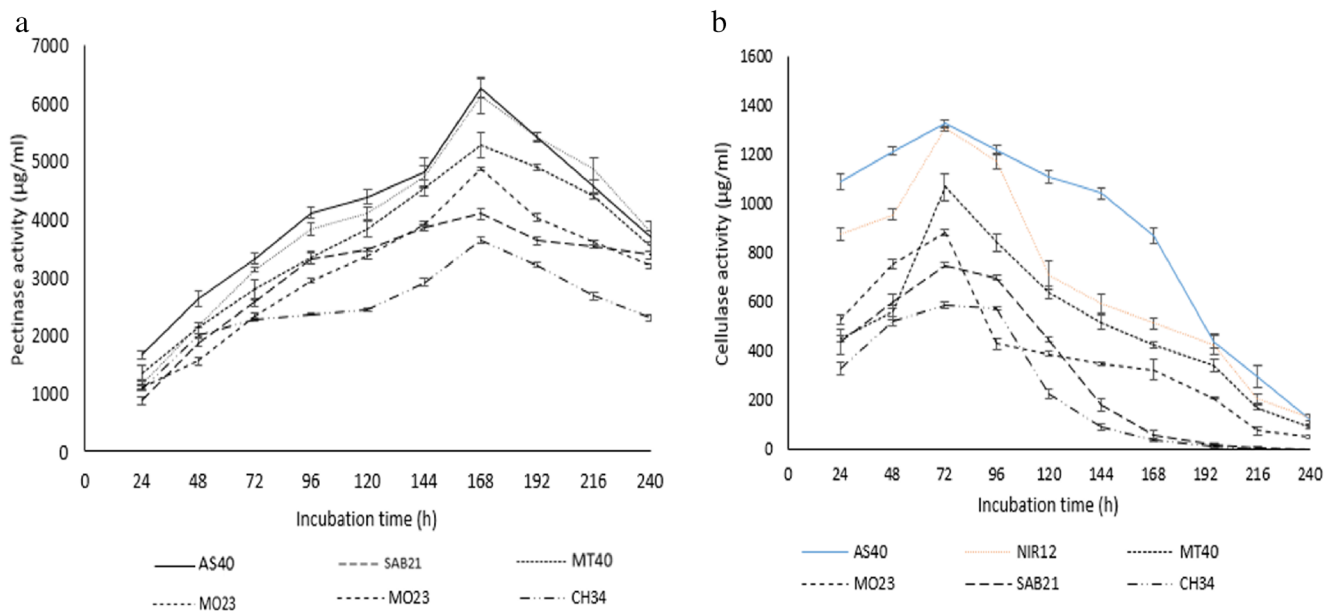
Fig. 6 Quantitative investigation of pectinase (**a**) and cellulase (**b**) activity produced by *Alternaria* spp. isolates over an incubation period of 240 h, including *A. alternata* (AS40), *A. tenuissima* (NIR12), *A. arborescens* (MT40), *A. interrupta* (MO23), *A. mimicula* (SAB21) and *A. infectoria* (CH34), obtained from tomato. For each treatment three replicates were used. **a, b**: the means with the same letter do not have significant differences according to Duncan's multiple range test at 5% level

were obtained in most of the sampled regions and showed different levels of pathogenicity on the host plant. Isolates of *A. tenuissima* and *A. infectoria* were mostly collected in the areas with cooler temperatures like Mashhad and Neyshaboor. Similarly, the isolates of *A. arborescens* were obtained from the geographic regions with cooler temperatures (Mashhad and Chenaran). On the other hand, the isolates of *A. mimicula* were mostly obtained in the areas with warmer temperatures like Sabzevar. The highest percentage of infected samples were observed in Mashhad, Chenaran and Neyshaboor, respectively. The lowest percentage of infected samples was observed in Fariman, Sarakhs, Torbate Jam, and Torbate Heydarieh (Table 1). Therefore, it can be concluded that Alternaria disease on tomato mostly occurs in the regions with cooler temperature, compared to the warmer regions which have lower level of disease occurrences. On the other hand the closer to the north of Iran, the weather becomes colder and the amount of moisture increases. Lower temperatures for *A. solani* and higher ones for *A. alternata* can increase the disease development (Stammler et al. 2014). Therefore, it can be concluded that due to the warm and dry weather of Khorassan-Razavi province and its high temperature, most of the obtained isolates were *A. alternata*, and there was no *A. solani* among the isolates obtained in this work.

The results of pathogenicity test showed that all isolates collected from infected tomato plants, were pathogenic on tomato cv. 'Peto Early Ch'. The results of statistical analysis using Duncan's multiple range test ($P < 0.05$) showed the presence of significant differences among the isolates in the levels of pathogenicity. In overall, the isolates of *A. mimicola* showed the lowest level of pathogenicity compared to other species of *Alternaria* tested. Among other species of this pathogen, we could not find any species with more pathogenicity and aggressiveness than others and it is all rather mixed.

One of the most important features of plant cell is its rigid cell wall, since this specialized structure strengthens and protects plants against various pathogens. The plant cell wall consists of three layers of the primary wall, a secondary wall and an intermediate blade. In addition to cellulose, hemicellulose and pectin also play critical roles in the construction of these walls. Plant pathogens produce a wide range of enzymes and extracellular proteins that can degrade cell wall components (Riou et al. 1991; Gibson et al. 2011). Secretion of CWDEs by fungi plays an important function in their pathogenicity or degradation ability (Archer and Wood 1995). Isolates of *Alternaria* spp. have the capability of producing wide range of toxins and enzymes as their virulence factors (Thomma 2003). In this study, production of CWDEs by *Alternaria* spp. isolates was investigated via quantitative and qualitative assays.

Jain and Dhawan (2008) demonstrated the important role of cellulase and pectinase in fungal pathogenesis. Pectic enzymes are among the most important enzymes produced by phytopathogenic fungi and bacteria, which are critical factors

in pathogenesis (Bezerra et al. 2012). Pectinase is very important for penetration of various fungi into the host tissue (Ten Have et al. 2002; Panda et al. 2004), that causes damage in the cell wall structure, increasing the access of other enzymes to cell wall, lysis and destruction of plant tissue. Pectic enzymes are the first polysaccharides produced by pathogens during infection process and host contamination (Martinez et al. 1991; Niture et al. 2006). In qualitative assay, the isolates AS40, NIR12, MT40, MO23, SAB21 and CH34 showed the highest amount of pectinase production, respectively (Figs. 4i and 5e). Similar results were observed in quantitative assay, in which the AS40 isolate with 6266 μg/ml and the isolate CH34 with 3645 μg/ml had maximum and minimum production of pectinase in liquid media. Other isolates such as NIR12, MT40, MO23 and SAB21 had 6136, 5287, 4997 and 4100 μg/ml of pectinase activity, respectively (Fig. 6a). The obtained data revealed that the isolates NIR12 and AS40 had the highest and two isolates of MD16 and GHA15 had the lowest level of pathogenicity. Therefore, it can be concluded that disease development is directly related to the rate of production and secretion of pectinase by the pathogen. This finding is in accordance with the results of Anand et al. (2008), who reported production of higher levels of pectinase in highly pathogenic isolates of *Colletotrichum capsici* and *Alternaria alternata* compared to the isolates with lower levels of pathogenicity (Kikot et al. 2009).

Cellulose is an unbranched glucose polymer that consists of β (1, 4) glucan units. It is one of the most abundant polysaccharides in plants that cause the plant to stand firmly and right (Hong et al. 2001). Many plant pathogenic fungi are able to produce cellulase for hydrolysing cellulose and its derivatives (Hubballi et al. 2011). In the qualitative analysis of cellulase activity, the dye diffusion method was performed using 12 isolates of *Alternaria* spp. and congo red was used as a dye to distinguish between the decomposed and unpaved substrate (CMC). Destruction of CMC around the fungal colony creates a clear area in the red background (Kaur and Aggarwal 2017), which indicates cellulase activity in this assay. All tested isolates were able to produce this clear area, which indicates their ability to produce cellulase in vitro. Also, similar results were obtained by Rathod and Chavan (2010), who studied production of CWDEs by *A. alternata*, *A. citri*, *A. tenuissima*, *A. crassa*, *A. dianthicola* and *A. macrospora* isolated from oilseeds. In the qualitative cellulase assay, the isolate AS40 of *A. alternata* had the maximum production of clear area. The results were similar in quantitative measurements. The isolate had maximum cellulase activity and NIR12, MT40, MO23, SAB21 and CH34 showed the lowest amount of cellulase activity, respectively, which reached to the maximum level after 72 h of incubation and then decreased (Fig. 6b). Whereas, Deep et al. (2014) reported maximum production

of cellulase from the A. *brassicicola* infecting crucifers, at 5th and 6th days of incubation.

Extracellular and intracellular lipases are produced by animals, plants and microorganisms (Murphy 2001; Griebeler et al. 2011; Andualema and Gessesse 2012). *Alternaria* is a cosmopolitan fungus associated with many types of organic materials in wet places (Iftikhar et al. 2015). Function of lipase in pathogenicity of *Alternaria* sp. has been proven (Cho et al. 2006; Berto et al. 1999). Production of lipase in different fungi depends on the composition of the medium, culture conditions, pH, temperature, type of carbon source and nitrogen (Cihangir and Sarikaya 2004). Lipase production by *Alternaria* spp. has been reported by Iftikhar et al. (2015) in vitro. In the present study, lipase activity of several isolates was investigated and reached to its maximum level later than other enzymes tested. Similar results were obtained by Khaledi et al. (2017) for *Fusarium* spp. isolates. The results of this experiment revealed that all isolates were able to produce halo due to precipitation of lauric acid salts as indicative of lipase production is accordance with the results of Rathod and Chavan (2010). The isolates of A. *tenuissima* and A. *alternata* were maximum producers of lipase, in accordance with high level of their pathogenicity on tomato plants.

Amylase assays showed that all tested isolates were able to produce this enzyme. High levels of amylase production were observed for all tested isolates. High level of amylase activity was previously reported by Shafique et al. (2010) for the isolates of A. *alternata* and A. *tenuissima,* which is in agreement with the results obtained in this research. However, to our knowledge, this is the first report on high levels of amylase production by other species of this fungal genus.

A class of enzymes that are naturally present in all living organisms and play an important role in physiological processes are proteases, which can cleave ligands or receptors at the cell surface (Dale and Vergnolle 2008). Protease is an important virulence factor in several fungal phytopathogens, including *Alternaria* spp. (Chandrasekaran and Sathiyabama 2014). Very scarce information is available on production of protease by *Alternaria* spp. isolates. Fungi possibly produce endoproteases in addition to a range of exopeptidases. García-Calvo et al. (2018) identified three proteins produced by A. *alternata,* which were involved in proteolytic processes. Our protease assay revealed that the isolates with higher level of protease activity had higher pathogenicity on the host plant, and vice versa. This result was similar to the findings of Kokaeva et al. (2015), who demonstrated the correlation between capability of *Alternaria* spp. isolates obtained from potato and tomato in producing protease and their pathogenicity levels.

In overall, this study revealed that *Alternaria* spp. isolates were pathogenic on tomato plants and had differences in their pathogenecity levels, which was correlated with their capability

in producing CWDEs. This finding is in agreement with the results of Hubballi et al. (2011), who reported that difference in the level of enzyme secretion by *A. alternata* isolates had a direct correlation with progress of the disease caused by these destructive phytopathogens. Therefore, one of the reasons for the wide host range and high pathogenicity of *Alternaria* species is their ability to produce various types of CWDEs. Finding novel methods for inhibiting the activity or production of these enzymes, as the main fungal virulence factors, could be effective in management of various diseases caused by this necrotrophic fungus.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval This research did not involve human participants and/or animals.

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