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MAL and AM conceived the study and processed all legal documents for collection; MAL conducted field sampling and isolation of strains; MAL and AM conducted microscopy and taxonomic analyses, chitin biodegradation tests, DNA extraction, PCR amplification and sequencing; AM analyzed and interpreted data; MAL and AM drafted the manuscript

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ORIGINAL RESEARCH PAPER

Chitinolytic fungi from the Birjand plain of Southern Khorasan Province in Eastern Iran

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Abstract

Fungal chitinases play important roles in the decomposition of wastes, mycoparasitism, and biocontrol of nematodes and plant pathogens through chitin biodegradation. This study was conducted during 2013–2017 to investigate the presence of chitinase genes in *Trichoderma* and *Clonostachys* species from the Birjand plain, and to evaluate their ability to degrade chitin. Fungal spores and soil suspensions were cultured on minimal medium containing 1% colloidal chitin from crab bodies to isolate chitinolytic fungi. Chitinolytic ability of the isolates was evaluated on this medium by staining with 1% Lugol's iodine solution and screening for the production of a bright halo around the colonies. Fifty-two isolates capable of degrading chitin were recovered. DNA extracted from the isolates was amplified using Chit2 or DECH degenerative primers that are related to the chitinase gene, and their sequences were aligned using the NCBI GenBank database. The Chit2 and DECH primers amplified 600-bp and 250-bp fragments, respectively, and according to sequence alignment, the isolates had sequences similar to that of the *chi18* chitinase genes. Morphological and molecular characterization allowed identifying the isolates as belonging to the species *Trichoderma harzianum* ($n = 41$), *T. longibrachiatum* ($n = 1$), *T. virens* ($n = 3$), *T. brevicompactum* ($n = 1$), *Clonostachys rosea* ($n = 5$), and *C. rogersoniana* ($n = 1$), some of which may potentially be used as biocontrol agents of pathogenic nematodes and fungi. This is the first report of isolation of fungi capable of chitin biodegradation from the South Khorasan Province in Eastern Iran.

Keywords

biodegradation; chitin; chitinase; *Clonostachys*; *Trichoderma*

Introduction

Chitin is a structural, long-chain polysaccharide of *N*-acetylglucosamine subunits with beta 1,4 glycosylated bonds and the second postcellulose polysaccharide in nature [1]. This compound is found in the exoskeleton of arthropods, flatworms, protozoans, and crustaceans [2]. The fungal cell wall also contains chitin and chitosan [3]. Chitin forms about 20–58% of the total weight of shell waste, which is responsible for generating over 100 billion tons of chitin wastes annually [4].

Chitinolytic enzymes are capable of decomposing the insoluble chitin polymer [5]. Since chitin is resistant to degradation because of its crystalline structure, its decomposition usually requires the activity of more than one type of enzyme. Chitinases include exochitinases, endochitinases, *N*-acetylhexosaminidase, and transglucosylase enzymes. The products of chitinase activity can control microbes and tumors, wound healing, sewage treatment, and drug delivery [6–8].

Chitinases are found in higher plants, some invertebrates, arthropods, and nematodes [9,10]. Bacteria and fungi also widely synthesize chitinases. Genomic studies

have shown that fungi have about 25 different chitinases [5]. Fungi associated with plants, such as phytopathogenic and mycorrhizal fungi, are also capable of producing chitinases [11,12]. Chitosanases are also isolated from bacteria and fungi [13,14].

Although the purpose of the existence of such a wide variety of chitinases in fungi is not fully understood yet, they are known to provide various functions including controlling hyphal development, spore production and germination, cell division, and parasitism [15,16]. Other functions of chitinases in nature are associated with morphogenesis, protection, preserving the structure of the fungal cell wall, nutrition, and pathogenicity [17]. Fungal chitinases also play an important role in the decomposition of chitin in the carbon and nitrogen cycles [18]. Fungi that produce chitinolytic enzymes have been exploited for biocontrol of pathogenic plant fungi and nematodes [19, 20]. The synthesis of chitinase has been studied in *Talaromyces emersonii*, *Neurospora crassa*, *Beauveria bassiana*, *Choanephora cucurbitarum*, and *Phascolomyces articulosus* [21–23].

Chitinase-producing fungi are studied much less than are chitinolytic bacteria. Currently the only commercial application of fungal chitinases is an enzyme isolated from the *Trichoderma harzianum*. *Penicillium janthinellum* has been used in vitro to produce chitinase [24]. Liu et al. [25] isolated the 46-kDa endochitinase gene from a biocontrol species, *Chaetomium globosum*. Expression of this gene is influenced by the cell wall of pathogenic fungi of plants, and seems to be involved in biocontrol activity of this fungus. *Metarhizium anisopliae* produces at least six different chitinases. *Aspergillus fumigatus* also has multichitinolytic enzymes of which the functions of five different chitinases have been identified [26]. A 42-kDa chitinase enzyme has been purified from *Piromyces communis* by Sakurada [27]. Pinto et al. [28] purified a chitinase with a molecular weight of 30 kDa from *M. anisopliae*. A chitinase with a 43-kDa molecular weight has also been isolated from *T. harzianum* [29]. *Isaria japonica* species produce two chitinases, P-1 and P-2, with molecular weights of 43.273 kDa and 31.134 kDa, respectively [30]. Souza et al. [31] purified a 43-kDa endochitinase from *Colletotrichum gloeosporioides*. Two 67-kDa isozymes of the *N*-acetyl-β-D-glucosaminidase group have been isolated from *Fusarium oxysporum* [32].

To date, no study has been conducted to investigate the presence of chitinase genes in the fungal species occurring in Southern Khorasan Province and Birjand plain, a desert region in Eastern Iran. The current study was conducted to identify the presence of chitinase genes in the fungal isolates from Southern Khorasan Province.

Material and methods

Fungal isolates were collected from soil and infected plant tissues from the Birjand plain (South Khorasan Province, Iran) during 2013–2017, and investigated for chitinase production. Chitin degrading fungi were identified using the methods described by Agrawal and Kotasthane [33], which includes spreading spore suspensions on minimal medium (K_2HPO_4, 1.0 g/L; NaCl, 0.5 g/L; MgSO4·7H_2O, 0.5 g/L; $FeSo_4$·7H_2O, 0.01 g/L; agar, 15 g/L; water, 1 L), containing 1% colloidal chitin from crab bodies (Nano Yakhteh Co., Tehran, Iran) as a source of carbon and nitrogen, and 0.5% Rose Bengal as a colony growth inhibitor. The protocol of Murthy and Bleakley [34] was used for the preparation of colloidal chitin.

Isolates were cultured on the minimal medium and then transferred to the minimal medium containing 1% colloidal chitin. To increase and stabilize the ability of the isolates to decompose chitin, tubes containing 0.1 g of pure chitin powder were prepared and sterilized in an autoclave at 121°C for 15 minutes. A spore suspension from each isolate was prepared in sterile water, one drop of each suspension was individually transferred to the tubes containing the pure chitin powder, and incubated for 1–2 months at 27°C.

Analysis of biodegradation ability

Chitin biodegradation by the fungal isolates was carried out according to the methods previously described by Loc et al. [35] using a minimal medium containing 1% colloidal

chitin with Lugol's iodine staining solution. The isolates were cultured for 5–7 days at 27°C. Hydrolysis of colloidal chitin was detected by staining using 1.5% Lugol's solution. The appearance of a bright halo around the colonies was indicative of the ability of the isolates to biodegrade chitin.

Hyperparasitic activity

Hyperparasitism of the fungal isolates was evaluated by coculturing of the *Trichoderma* and *Clonostachys* isolates with two strains of *Bipolaris* spp. and a strain of *Alternaria alternata* on potato dextrose agar (PDA) medium. Interaction of the fungal hypha was evaluated by light microscopy.

Molecular studies

The presence of chitinase enzyme genes was investigated by amplification of extracted DNA from fungal isolates by polymerase chain reaction (PCR) using the Chit2 degenerative primer set (forward primer: 5′-TCCATYGGNGGNTGGACNTG-3′ and reverse primer: 5′-GCRSWNGCYTCCCARAACAT-3′) and DECH degenerative primer set (forward primer: 5′-TCCCARAYHCCRTTCTCCCA-3′, and reverse primer: 5′-AAYYTBATGGCYTAYGACT-3′) [36]. DNA was extracted from the fungal isolates using 600–800 µL of cetyltrimethyl ammonium bromide (CTAB) 2× extraction buffer and 1 µL of proteinase K (Cinnagen Co., Iran) added to the fungal cells and incubated 65–70°C for 60 min. An equal volume of chloroform was added to each tube and centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was transferred to the new Eppendorf tubes, 600 µL of cold ethanol was added and centrifuged for 10 min at 13,000 rpm. After removing the supernatant, the pellets were air-dried and then dissolved in distilled water [37]. The Chit2 and DECH degenerate primers amplified the target areas of the extracted DNAs and the PCR products were sequenced by Macrogen Co. (South Korea). The sequences of the amplicons were aligned using the National Center for Biotechnology Information (NCBI) GeneBank database, and a phylogenetic tree was generated using Geneious R11 software (Biomatters Co., New Zealand).

Results

In this study, the presence of chitinase genes and the ability to express chitinolytic enzymes by the *Trichoderma* and *Clonostachys* isolates collected from the Birjand region of Eastern Iran were investigated using molecular screening for the chitinase genetic markers and in vitro analysis of enzymatic activity on solid culture medium containing chitin. The culture medium used in this study contained 1% colloidal chitin as a source of carbon and nitrogen. The components of the minimal media used in this study was similar to the Czapek-Dox agar culture media [38], but colloidal chitin was used to replace sucrose as the carbon source. The $NaNO_3$ in the Czapek-Dox media was also been replaced with NaCl in the current study, so that chitin was used also the source of nitrogen.

Several fungal colonies grew on the medium after culturing suspensions prepared from the collected soil samples. Colonies with aerial and well-developed mycelia were transferred to 2-mL Eppendorf tubes containing pure sterilized chitin, to evaluate their ability to use chitin as a sole nutrient source and to grow well on wet chitin powder. In some cases this led to changes in the color of the chitin or to degradation of the chitin powder (Fig. 1A). After 1–2 months of maintaining the cultures in pure chitin, a bright halo was detected around the colonies of some isolates that had been transferred to minimal medium containing chitin and stained with Lugol's iodine solution. The development of halos varied among the isolates and species (Fig. 1B,C). The isolates that produced a bright halo biodegraded chitin, while isolates with no clear halo around their colonies did not produce chitinase enzymes (Fig. 1D). The size of the halo was relative to the enzymatic activity with the larger halos being produced by the fungal

isolates with greater enzymatic activity (Fig. 1B). The halos that were formed around the colonies were caused by the degradation of complex carbohydrate chitin that was colored by the iodine in the Lugol's solution, into simple carbohydrates that did not absorb the iodine [39]. The DECH and Chit2 primers amplified 250-bp and 600-bp fragments of the target DNA, respectively, from the *Trichoderma* and *Clonostachys* isolates (Fig. 1H).

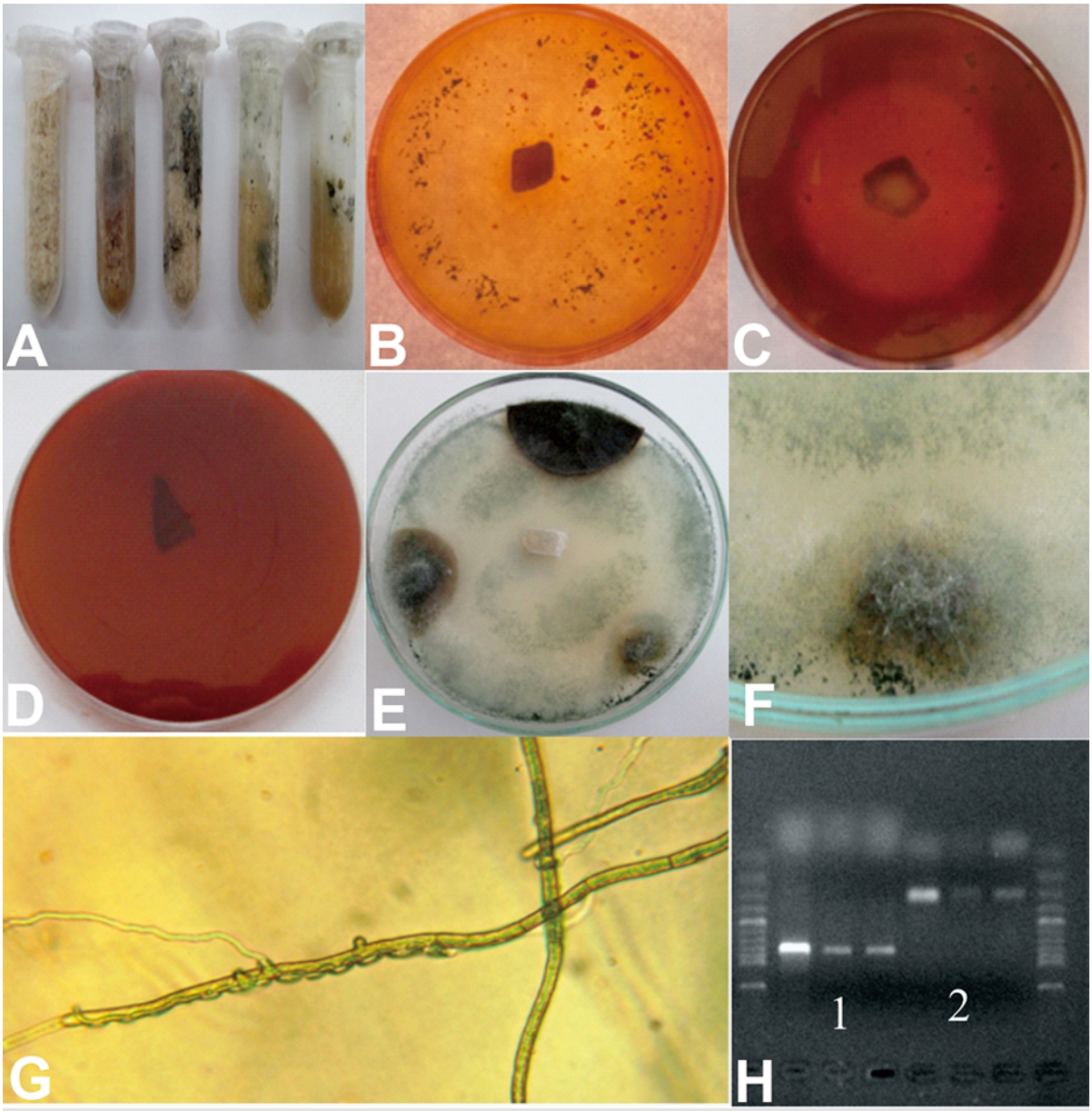

Fig. 1 Determination of chitinase activity of *Trichoderma* (**A**) and *Clonostachys* (**B,C**) isolates on minimal media containing 0.1% (w/v) colloidal chitin and chitin powder. Control (**D**). Hyperparasitism of *Trichoderma* isolates on *Alternaria alternata* hypha (**E–G**). Agarose gel electrophoresis of PCR products (**H**) of Chit2 (1) and DECH (2) primers.

Discussion

The development of a halo around the colonies of the *Trichoderma* and *Clonostachys* isolates was indicative of the ability to degrade chitin. Based on the staining using Lugol's solution, 46 and six isolates of *Trichoderma* and *Clonostachys*, respectively, were able to degrade chitin (Tab. 1). Since the *Trichoderma* fungi exhibited rapid growth and fully occupied the culture media, the entire surface of the culture media developed a light color (Fig. 1B). The above-mentioned *Trichoderma* species were able to decompose much of the chitin in the culture medium, but for the remaining *Clonostachys* isolates,

Tab. 1 Chitinolytic fungi isolated during this study.

Number	Isolate	Organism	Isolation source	Location	GeneBank accession number
1	M11	*Trichoderma harzianum*	Soil (*Agaricus bisporus* baiting method)	Birjand	
2	M12	*T. harzianum*	Soil	Birjand	
3	M16	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	
4	M23	*T. harzianum*	Barley loose smut (not disinfected)	Faculty of Agriculture farms, Amirabad)	
5	M24	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	
6	M25	*T. harzianum*	Fruiting bodies of the nonidentified fungus	Birjand	
7	M26	*T. harzianum*	Fruiting bodies of the nonidentified fungus	Birjand	
8	F46	*T. harzianum*	*Tilletia tritici* spores (disinfected)	Faculty of Agriculture farms, Amirabad)	MG601052
9	M37	*T. harzianum*	Soil	Birjand	
10	M39	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	
11	M40	*T. harzianum*	Soil	Birjand	
12	F49	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	MG601053
13	M49	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	
14	M5	*T. harzianum*	*Puccinia graminis* (*Berberis vulgaris* Rust) spore; not disinfected	Darmian	
15	F51	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	MG601054
16	M64	*T. harzianum*	Barley loose smut (not disinfected)	Faculty of Agriculture farms, Amirabad)	
17	M67	*T. harzianum*	Soil (*A. bisporus* baiting method)	Birjand	
18	P56	*Trichoderma virens*	Soil (*A. bisporus* baiting method)	Birjand	MG601050
19	R1	*T. harzianum*	Soil	Birjand	
20	R10	*T. harzianum*	Almond	Shukatabad orchard	MG601048
21	R11	*T. harzianum*	Basil	Chahkand farm	
22	R12	*T. harzianum*	Soil	Birjand	
23	R13	*T. harzianum*	Almond	Shukatabad orchard	
24	R14	*T. harzianum*	Pistachio	Sarayan	MG601047
25	R15	*T. brevicompactum*	Wheat	Gayok farm	MG601046
26	R16	*Clonostachys rogersoniana*	Almond	Mohammad Shahr	
27	R17	*C. rosea*	Apple	Mud orchard	MG601045

Tab. 1 Continued

Number	Isolate	Organism	Isolation source	Location	GeneBank accession number
28	R18	*T. harzianum*	Inside the water jumble (soil)	Mohammad Shahr	
29	R19	*T. harzianum*	Basil	Chahkand farm	MG601044
30	R2	*T. harzianum*	Soil	Birjand	
31	R20	*T. harzianum*	Basil	Chahkand farm	
32	R21	*T. harzianum*	Common fig	Aliabad orchard	
33	R22	*C. rosea*	Walnut	Chahkand	MG601043
34	R23	*C. rosea*	Berberis	Mud orchard	
35	R24	*C. rosea*	Jujube	Bojd orchard	MG601042
36	R25	*T. harzianum*	Sesame	Amirabad	
37	R26	*T. harzianum*	Soil	Birjand	MG601041
38	R27	*T. harzianum*	Radish	Sarbishe	
39	R28	*T. longibrachiatum*	Sugar beet	Qaen	MG601040
40	R29	*T. harzianum*	Pear	Chahkand orchard	
41	R3	*T. harzianum*	Common fig	Aliabad orchard	
42	R30	*T. harzianum*	Barley	Bojd farm	
43	R31	*T. harzianum*	Okra	Shukatabad farm	
44	R32	*T. harzianum*	Pine	Amirabad	
45	R33	*T. virens*	Pear	Chahkand orchard	
46	R4	*T. harzianum*	Soil	Birjand	
47	R5	*T. virens*	Pomegranate	Shukatabad – orchard	MG601049
48	R6	*T. harzianum*	Soil	Birjand	
59	R7	*T. harzianum*	Tomato	Birjand	
50	R8	*T. harzianum*	Inside the water jumble (soil)	Khusf	
51	R9	*T. harzianum*	Grape	Mohammad Shahr	

the area around the colony was indistinguishable from the other parts of the media. For the *Clonostachys* isolates, the size of the halos were smaller compared with those of the *Trichoderma* isolates (Fig. 1B). The degradation of colloidal chitin obtained from crab body in the culture medium demonstrated that these fungi were able to use chitin as a source of carbon and nitrogen. However, as the results also indicated, the chitinolytic ability of the fungal species differed. The greatest chitin degradation capability was observed in the *T. harzianum* isolates (Fig. 1B and Fig. 2).

Based on sequence analysis of the amplified regions of the *T. harzianum* isolates R10 (MG601048), R14 (MG601047), R19 (MG601044), R26 (MG601041), F46 (MG601052), F49 (MG601053), and F51 (MG601054), *T. brevicompactum* isolate R15 (MG601046), and *T. longibrachiatum* isolate R28 (MG601040), portions of the endochitinase gene *Chi18* were replicated by the DECH and Chit2 primers. In *T. virens* isolates P56 (MG601050) and R5 (MG601049), a portion of the *ech1* gene of class V chitinases was amplified (Tab. 1). The sequence of the amplified regions of this gene had similarity to the sequence of the *T. harzianum* gene *chi18-5* deposited in the NCBI GenBank (Fig. 3). The 42-kDa protein encoded by this gene is secreted extracellularly and is an orthologue of *ech42* [40]. The *ech42* gene-locus is

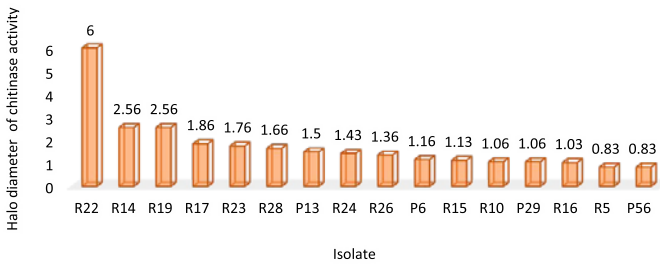

Fig. 2 Diameters of the bright halos around the colonies of the fungal isolates on minimal medium containing 1% colloidal chitin after staining with Lugol's solution.

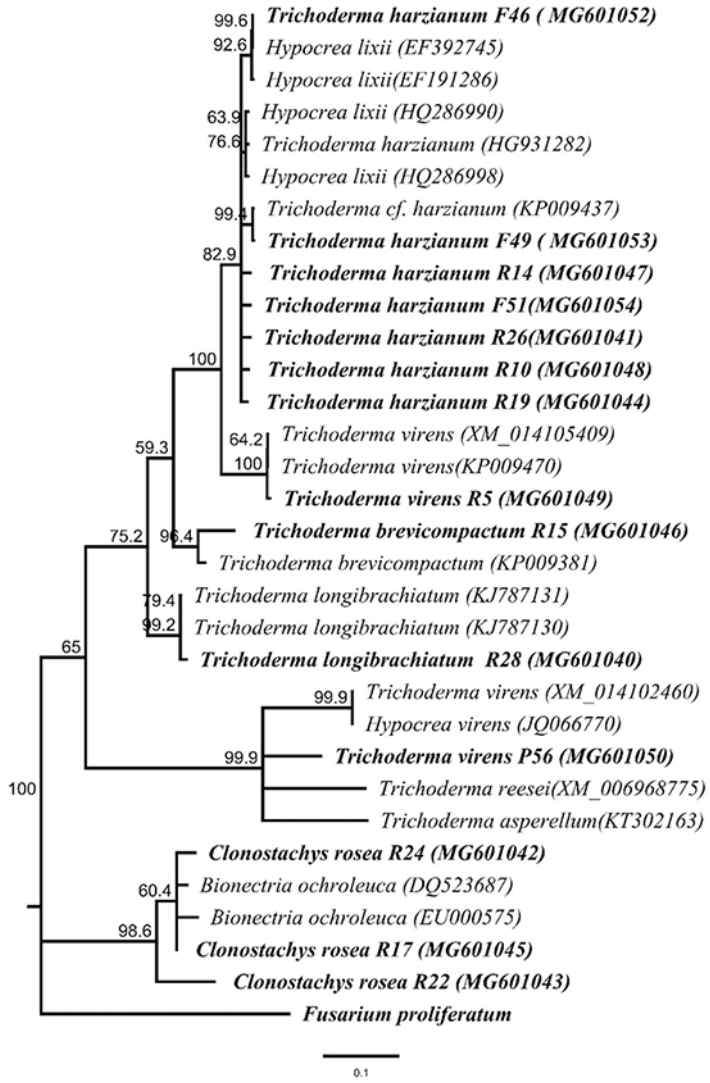

Fig. 3 Neighbor-joining phylogenetic tree based on chitinase gene sequences. The relationships between *Trichoderma* and *Clonostachys* strains are shown. Bootstrap percentages (based on 1,000 replications) higher than 50% are shown the at branch points.

conserved among ascomycetes, and is used as a marker for *Trichoderma* phylogeny [41]. The activity of this gene is increased in the presence of chitin and appears to be one of the genes important in mycoparasitic activity. In addition to being one of the most well-protected *Trichoderma* genes, *ech42* is also one of the most important genes involved in the interaction of *Trichoderma* and fungal pathogens [40]. The chitinase gene *ech42* is expressed at a high rate when *Trichoderma* is grown in medium containing chitin, and has a lethal effect on the cell wall of many other fungi, especially *Botrytis cinerea* [42]. During the interaction of mycorrhizal fungi and plant pathogens, *ech42* is expressed at a very high level. Genes *chit33*, *ech42*, *chit42*, and *nag1*, which play a role in the early stages of mycoparasitic interactions, have all been identified in *Trichoderma* [43]. *Trichoderma chitt33* encodes a 33-kDa protein and its sequence has high homology with fungal and herbal chitinases. Studies have shown that the activity of this single-copy gene is more closely associated with the saprophytic and mycoparasitic characteristics of *Trichoderma* than to the morphological characteristics [43]. Expression of *chitt33* is reduced in the presence of glucose. Studies by Kovacs et al. [44] showed that *T. longibrachiatum* has the highest production levels of extracellular chitinases under conditions of fermentation. Seven unique chitinases have been isolated from *T. harzianum*, which include two *N*-acetylglucosaminidase (73 and 102 kDa), four endochitinases (31, 33, 42, and 53 kDa) and a 40-kDa ketobiosidase [12]. The species *T. reesei* produces 18 different chitinases with a molecular weights ranging from 40 to 180 kDa [45].

In the *C. rosea* isolates R22 (MG601043), R24 (MG601042), R17 (MG601045), and R23, and an *C. rogersoniana* isolate R16, *crchi1* and *ech42* were PCR amplified using the primers. The sequences of these amplified regions were similar to the sequence of EU000575 of *crchi1*, and the sequence of DQ523687 of *ech42* present in GenBank was the same (Fig. 3). The *crchi1* gene contains three introns, is 1,746 bp in size, encodes a 44-kDa protein, and is from a family of 18 chitinases found in the mycoparasitic species *C. rosea* [46]. The expression of this gene is inhibited in the presence of glucose, but various sources of chitin, such as the cell wall of *Rhizoctonia solani*, exacerbate its expression.

In the phylogenetic tree based on the sequence of amplified regions using DECH and Chit2 primer set, the isolates of this study were grouped into different clades, and the sequences of replicated regions could be used to separate these species from each other (Fig. 3). The DECH primer set amplified the chitinase genes in species of *Trichoderma*, and *Clonostachys*. The amplified regions belonged to different regions of the chitinase gene, depending on the particular fungal species. Sequence comparison of these areas indicated variation in the chitinase genes of different species. This is the first report of the presence of chitinase gene in these fungal species in Birjand in the eastern area of Iran.

Isolate M5 was recovered from the nondisinfected pustule of *Puccinia graminis*, rust pathogen of seedless barberry (*Berberis vulgaris* L. var. *asperma*) in the Darmian

region. This isolate was a hyperparasite of *Bipolaris* and *Alternaria* (Fig. 1E–G), but its effects on *P. graminis* was not evaluated. It may be an epiphytic fungus of seedless barberry, or a hyperparasite and biocontrol agent of *P. graminis*.

Isolate M36 from loose smut of wheat fungal spores (*Ustilago tritici*), and isolate M64 from the fungal spores of smut of barley (*U. hordei*) were isolated by spreading *Ustilago* spores onto minimal medium agar containing chitin (Tab. 1). M36 was isolated from disinfected spores may be a hyperparasite of *U. tritici*.

Several isolates were recovered from soil using the *Agaricus bisporus* baiting method (Tab. 1). Because these isolates caused infection in *Agaricus*, they were shown to have apparent hyperparasitic activity on *A. bisporus*.

Several studies have been conducted on the enzymes of *Trichoderma* species from Iran. Seyed Asli et al. [47] investigated the production of chitinase enzyme in *Trichoderma* species from different regions of Iran and reported the presence of three endochitinase genes in the *T. harzianum*. Shahbazi et al. [48] enhanced the activity of the chitinase gene of *T. harzianum* using gamma-inducible inductive mutations in native isolates of *Trichoderma* fungi. Mostafanezhad et al. [49] studied the biocontrol of the disease, and the induction of defensive compounds in *Meloidogyne javanica*-infected tomato plants using several *Trichoderma* isolates, and concluded that three isolates of 125, 126, and Bi10 from *T. harzianum* showed the highest activity of the chitinase enzyme.

Baharvand et al. [50] studied the production of chitinolytic enzymes from *T. viride* mutant-isolates and showed that the highest levels of extracellular protein was detected in *T. viride* isolates M7 and M8, and that the highest activity of the chitinase enzyme was found in *T. viride* mutant-isolates M19, M1, M5, M4, and M6. The results indicate that β-1,4-*N*-acetylglucosaminidase and acetylglucosamine enzymes are present in different isolates. The highest enzyme activity was observed in *T. viride* mutant-isolate M19, which has the highest levels of endochitinase enzymes (31 and 42 kDa), and enzyme β-(1,4)-*N*-acetylglucosaminidase (73 kDa). The induction of gamma-ray mutations in *Trichoderma* fungi by these researchers led to improve production of chitinase enzymes for the biological control of plant diseases.

Yazdanpanah-Samani et al. [51], by examining the heterologous expression of the 36-kDa chitinase enzyme from *T. atroviride*, showed that the best expression conditions at the time of induction are an optical density (OD_{600}) 0.3, and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) with an incubation time of 6 h. Kavari et al. [52] investigated the expression of the chitinase enzyme by several isolates of *Trichoderma* fungi and its effect on the biological control of a root nematode of tomatoes, *M. javanica*, and isolates T.BI, T6, T65, which demonstrated enzymatic activities of 19.2, 18.3, and 17 units per mL, respectively, and were reported to be the most active isolates. Ataei et al. [53] evaluated the activity of chitinase 42 against *Candida albicans* and showed that the enzyme had significant inhibitory activity against this fungus compared with that against a control group.

Although in many species of chitinase producing fungi, especially in the fungal pathogens of insect, the role of chitinase in fungal infiltration in the host body is not well understood, but in *Trichoderma* species, these enzymes play a role in the penetration into the host. Also, in *C. rosea*, the role of these enzymes has been shown to aid in entering the host body. Clearly, the reactions and levels of resistance of various fungal species to these enzymes differ depending upon the particular species [54,55]. Xian et al. [56] isolated *Trchi1* chitinase gene from *T. roseum*, and transferred the gene into a tobacco plant, which increased the plant's resistance to *A. alternata* and *Colletotrichum nicotiana*. Zhang et al. [57] isolated *tachi* from *T. asperellum* and transferred the gene into soybean plants resulting in increased resistance of the plants to *Sclerotinia sclerotiorum*. *Tachi* encoded a 44-kDa chitinase from family 18 of glycoside hydrolase.

The *Trichoderma* and *Clonostachys* species isolated in this study contained chitinase genes, produced chitinolytic enzymes, were hyperparasitic to *A. alternata* and *Bipolaris* mycelia, and may potentially be used as a biocontrol agent against pathogenic plant fungi that have chitinous hyphae, or be used as industrial chitinase producing isolates.

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