JOURNAL OF HORTICULTURE AND POSTHARVEST RESEARCH
2021, VOL. 4(2), 163-176

Journal of Horticulture and Postharvest Research

Journal homepage: www.jhpr.birjand.ac.ir

University of Birjand

Chitinase production by *Fusarium* species in Iran

Maryam Afsarzadeh Laien[1] and Abbas Mohammadi[1*]

1, *Department of Plant Pathology, College of Agriculture, University of Birjand, Birjand, Iran*

ARTICLE INFO

Original Article

Article history:
Received 7 June 2020
Revised 8 July 2020
Accepted 19 July 2020
Available online 6 November 2020

Keywords:
Biodegradation
Chitin
Chitinolytic
Degenerate primer
Endochitinase

DOI: 10.22077/jhpr.2020.3444.1149
P-ISSN: 2588-4883
E-ISSN: 2588-6169

***Corresponding author:**
Department of Plant Pathology, College of Agriculture, University of Birjand, Birjand, Iran.
Email: amohammadi@birjand.ac.ir

ABSTRACT

Purpose: Chitin is a structural, long_chain polysaccharide of N-acetylglucosamine subunits with beta 1,4 glycosylated bonds and the second post-cellulose polysaccharide in nature. Chitinases enzymes are capable of degrading chitin, plays essential roles in the decomposition of chitinous wastes, can be used as a biological fungicide against phytopathogenic fungi. **Research method:** *Fusarium* isolates were recovered from soil, the spore of phytopathogenic fungi, and cereal tissues in different areas of Birjand plain during 2013-2017. A chitinase enzyme activity assay was performed using the Lugol's solution staining method and genomic replication by DECH degenerate primers. **Findings:** Eighty-three isolates of *Fusarium* presenting the chitinolytic activity were recovered from soil, cereal plant tissues from different areas of the Birjand plain in Iran. Some isolates were also recovered from barley loose smut and barberry rust spores. Chitinase enzyme activity was detected in *Fusarium solani*, *F. proliferatum*, *F. avenaceum*, *F. fujikuroi*, *F. acuminatum*, *F. semitectum*, *F. culmorum*, *F. equiseti*, *F. nygamai*, *F. diversisporum*, *F. oxysporum* and *F. longipes* by chitinase bioassay method and/or DECH primers for chitinase. **Limitations:** There were no limitations to report. **Originality/Value:** Based on the molecular data, the secreted chitinases belonged to chitinse1 or endochitinase-42 kDa, members of GH family 18. This is the first report of chitinases from *Fusarium* species in Iran.

INTRODUCTION

Chitin, a linear polymer of N-acetyl glucosamine, is synthesized by fungi, crustaceans, molluscs, insects, algae and yeasts (De Marco et al., 2000; Patel & Goyal, 2017; Synowiecki & Al-Khateeb, 2003). Chitinolytic enzymes, especially chitinases, biodegrade chitin and break down its glycosidic bonds (Fan et al., 2007). Chitinases play a significant role in the development and branching of the fungal hyphae, in spore production and germination, in cell division and degradation of the fungal cell wall, morphogenetics, and maintenance of the cell wall structure (Adams, 2004; Karlsson & Stenlid, 2008; Khoushab & Yamabhai, 2010; Patel & Goyal, 2017). In nature, the secretion of chitinases by bio-control agents in soil inhibits fungal chlamydospore germination, hyphal growth and the germination of sclerotia (Mohapatra & Parhi, 2017). Introducing of fungal chitinase gene to plants increases their resistance to phytopathogenic chitinous fungi (Mythili et al., 2018).

Chitinase production has been reported from various fungal species, including *Trichoderma harzianum* (Harman et al., 1993), *Aspergillus nidulans* (Szilagyi et al., 2018), *A. niger* (van Munster et al., 2012), *A. terreus*, *A. fumigatus* (Xia et al., 2001), *Penicillium janthinellum* (Di Giambattista et al., 2001), *Paecilomyces lilacinus* (Dong et al., 2007), *Trichothecium roseum* (Xian et al., 2012), *Metarhizium anisopliae* (Kang et al., 1999), *Verticillium chlamydosporium* and *V. suchlasporium* (Tikhonov et al., 2002).

Chitinase genes have also been reported from saprophytic or phytopathogenic *Fusarium* species, including *Fusarium acuminatum*, *F. culmorum*, *F. equiseti*, *F. oxysporum* and *F. semitectum* (Nuero, 1995). Govinda Rajulu et al. (2010) showed the producing of chitinase enzymes in some *Fusarium* species collected from India. Mathivanan et al. (1998) have described the presence of chitinase enzyme in *F. chlamydosporum* species.

In Iran, chitinolytic enzymes have been purified and identified in *T. harzianum* (Seyed Asli et al., 2004), *T. atroviride* (Harighi et al., 2006), *T. virens* and *T. longibrachiatum* (Seyed Asli et al., 2004) species. As far as it is known, it has not yet been reported the presence of chitinase genes in *Fusarium* species in Iran. The purpose of this study was to investigate the presence of chitinolytic enzymes, and the genes associated with chitinolytic enzymes in *Fusarium* isolates from Iran.

MATERIALS AND METHODS

Several *Fusarium* isolates were obtained from the Faculty of Agriculture's fungal culture collection of the Birjand University. Most of these isolates of *Fusarium* were previously isolated from the cereal's tissues in Birjand, east of Iran, during 2012-2014 (Besharati Fard et al., 2017).

During 2015-2017, additional *Fusarium* species were isolated from soil, and plant tissues in the Birjand plain. The *Fusarium* species from soil were isolated by spreading a soil suspension on minimal microbial culture medium containing 1% colloidal chitin and 200 ppm Rose Bengal (Küçük & Kivanç, 2003). The minimal medium was similar to that of Snyder and Nash (1968) medium and contained K_2HPO_4 (1.0 $g.l^{-1}$), NaCl (0.5 $g.l^{-1}$), $MgSO_4.7H_2O$ (0.5 $g.l^{-1}$), $FeSO_4.7H_2O$ (0.01 $g.l^{-1}$), Agar (15 $g.l^{-1}$) (Merck Co., Germany), 1% colloidal chitin (extracted from a crabs' body by NanoYakhteh Chemistry Co., Tehran, Iran) and 200 ppm Rose Bengal (Waksman & Fred, 1922). By removing sucrose and sodium nitrate ($NaNO_3$) from Nash & Snyder medium (Snyder & Nash, 1968), no carbon-nitrogen-containing compounds were added to the medium until chitin was used as a carbon and nitrogen source.

Spores of barley loose smut (*Ustilago nuda*), barberry rust (*Puccinia* sp.) and *Sonchus* powdery mildew (*Erysiphe* sp.) were collected from smutted grain heads and pustules of infected plants and cultured on a minimal medium containing 1% colloidal chitin without disinfecting or after surface disinfecting with 5% sodium hypochlorite for 1 minute. Many other fungal spores such as *Alternaria*, *Ustilago* from *Cynodon* and some other powdery mildew from several diseased plants were cultured, but since there is not any *Fusarium* isolation, their data was not mentioned in this paper.

Two_millilitre Eppendorf tubes containing chitin powder were autoclaved at 121°C for 15 minutes (Poshina et al., 2018). One drop of spore suspension from each isolate in sterile water was transferred to the tubes and stored up to 2 months at 27 °C for enhancement of chitin biodegradation ability.

Colloidal chitin was prepared using Hsu and Lockwood (1975) method by adding 150 ml of hydrochloric acid (HCl) to 20 g of chitin powder extracted from a crabs body for several hours, continuous washing with cold water, centrifuging and removing excess water (Hsu & Lockwood, 1975). The *Fusarium* isolates were cultured on a minimal medium containing 1% colloidal chitin and incubated at 27°C for seven days. 7-day old colonies of the *Fusarium* isolates were flooded with 1.5% iodine Lugol's solution. The presence of a bright halo around the colonies and its diameter was considered as a criterion for the ability of the fungus to biodegrade chitin (Loc et al., 2011). For all isolates, the tests were replicated three times, and the diameter of the surrounding halo was measured.

Genomic DNA was extracted by the Cetyltrimethylammonium bromide method from 7-day-old *Fusarium* colonies on potato dextrose agar (PDA) medium (Brandfass & Karlovsky, 2008; Griffiths et al., 2006). The presence of a chitinase gene was studied by replication of extracted DNA from fungal isolates using degenerate DECH (forward: 5´–TCCCARAYHCCRTTCTCCCA–3´, and reverse: 5´– AAYYTBATGGCYTAYGACT–3) and Chit-2 (forward: 5′-TCCATYGGNGGNTGGACNTG-3′ and reverse: 5′-GCRSWNGCYTCCCARAACAT-3′) primers (Afsarzadeh Laien & Mohammadi, 2018; Meng et al., 2015). The PCR reaction solution contained 10X PCR buffer (2.5 µl), $MgCl_2$ (1.2 µl of 50 mM solution), dNTPs (0.5 µl of 10 mM solution), primers (2 µl of 10 µM solution), Taq DNA polymerase enzyme (0.15 µl), and 1 µl of genomic DNA (Meng et al., 2015). All reagents were obtained from CinnaGen Co., Tehran, Iran. The final volume of each reaction reached 25 µl by de-ionized distilled water. PCR reactions were performed with Biometra thermal cycler (Analytik Jena, Germany) for chitinase amplification with 35 cycles based on the Meng et al. (2015) method. The PCR products were electrophoresed in a 1% agarose gel with 80 mA voltage.

The South Korean Macrogen company sequenced the PCR products with DECH primers. The sequences of some regions replicated by the above primers were aligned with the NCBI GenBank database using Geneious R11.1.4 (Biometrics Ltd.) software (Naim et al., 2018). The similarity of replicated regions amino acid sequences with NCBI GenBank databases were determined using Geneious R11.1.4 (Biometrics Ltd.) software (Naim et al., 2018).

The fungal isolates were identified up to species level based on the macroscopic and microscopic taxonomic morphological features using mycological keys (Leslie & Summerell, 2008). Macroscopic and microscopic features were studied including growth rate and color of colonies on PDA, micro and macroconidia, hyphal swelling and chlamydospore production. The sporulation of the fungal cultures and morphological study of fungal macroconidia was performed using carnation leaf agar (CLA) culture medium, spezieller nahrstoffarmer agar (SNA) culture medium and soil-agar culture medium (Leslie & Summerell, 2008). The molecular identification of the isolates was performed using the elongation factor (EF) gene replication (Geiser et al., 2004). South Korea's Macrogen company sequenced the product of

PCR reaction with EF primers (forward: 5´-ATGGGTAAGGA(A/G)GACAAGAC-3′ and reverse: 5´-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′). For molecular identification, the sequences were aligned with the NCBI GenBank databases using the Geneious 14.1.4 software (Naim et al., 2018).

RESULTS

Eighty-three isolates of chitinolytic *Fusarium* were recovered from soil and plant tissues from different areas of Birjand plain (Table 1; Fig. 1). Both DECH primers and the Lugol's staining method showed the presence of the chitinase gene and chitin biodegradation capability of 39 isolates *Fusarium* spp. (Table 1). Chitinase gene and chitinolytic activity was detected in the remaining 44 isolates only by DECH primers (36 isolates) or Lugol's staining (8 isolates) method (Table 1).

Table 1. Chitinolytic isolates of *Fusarium* studied in this research†

Isolate	*Fusarium* species	Source	Location	Chitinase primer	Chitinase Acc. number	EF Acc. number	Logule staining halo production	and its diameter (cm)
M1	*Fusarium proliferatum*	Soil	Birjand	DECH	ns		+	1.26
M10	*F. solani*	Soil	Birjand	DECH	ns		+	6
M13	*Fusarium* sp.	Barley loose smut spore	Amirabad	nr	ns		+	
M14	*F. proliferatum*	Barley loose smut spore	Amirabad	DECH	ns		+	1.63
M15	*F. solani*	Berberis rust spore	Darmian	DECH & ChiT-2-R	ns	MG707137	+	6
M17	*F. solani*	Soil	Birjand	ChiT-2-R	ns		+	6
M19	*F. proliferatum*	Soil	Birjand	DECH	MG707099	MG707131	+	
M2 (F58)	*F. solani*	Soil	Birjand	DECH	ns	MG707123	+	.83
M20	*F. solani*	Soil	Birjand	ChiT-2-R	ns		+	.83
M21	*Fusarium* sp.	Berberis rust spore	Darmian	DECH	ns		+	
M27	*F. solani*	Barley loose smut spore	Amirabad	DECH	ns	MG707130	+	
M28	*Fusarium* sp.	Soil	Birjand	nr	ns		+	
M3	*F. proliferatum*	Barley loose smut spore	Amirabad	nr	ns		+	
M38	*F. solani*	Soil	Birjand	DECH	ns		+	.96
M4	*F. solani*	Soil	Birjand	DECH	ns		+	.36
M46	*F. solani*	Soil	Birjand	nr	ns		+	
M47	*Fusarium* sp.	Soil	Birjand	nr	ns		+	
M51	*F. proliferatum*	Soil	Birjand	ChiT-2-R	ns		+	6
M52	*F. solani*	Soil	Birjand	nr	ns		+	
M53	*F. proliferatum*	Soil	Birjand	DECH	ns		+	2.63
M54	*Fusarium* sp.	Soil	Birjand	nr	ns		+	
M55	*F. proliferatum*	Soil	Birjand	ChiT-2-R	ns	MG707128	+	
M58	*F. proliferatum*	Barley loose smut spore	Amirabad	DECH	MG707098	MG707134	+	
M59	*F. solani*	Soil	Birjand	ChiT-2-R	ns	MG707127	+	
M6	*Fusarium* sp.	Barley loose smut spore	Amirabad	nr	ns		+	
M60	*F. proliferatum*	Soil	Birjand	DECH	ns		+	
M61	*F. solani*	Soil	Birjand	ChiT-2-R	ns	MG707136	+	
M62	*F. proliferatum*	Soil	Birjand	ChiT-2-R	ns		+	
M63	*F. proliferatum*	Soil	Birjand	DECH	MG707091	MG707135	+	

M66	*F. proliferatum*	Soil	Birjand	DECH	ns	MG707132	+	
M68	*F. proliferatum*	Soil	Birjand	DECH	ns		+	1.83
M69	*F. proliferatum*	Soil	Birjand	DECH	ns		+	1.63
M71	*F. proliferatum*	Berberis rust spore	Darmian	DECH	MG707096	MG707133	+	1.96
M72	*Fusarium* sp.	Spiny sowthistle powdery mildew nonsterile spore	Amirabad	DECH	MG707100		+	1.3
M73	*F. proliferatum*	Soil	Birjand	DECH	ns		+	1.9
M9	*F. proliferatum*	Soil	Birjand	DECH	MG707095	MG707129	+	1.4
P1	*Fusarium* sp.	Cereals tissue	Birjand	CH1	ns		+	
P10	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707084			
P11 (FAC 2)	*F. acuminatum*	Wheat root	Khusf	DECH	MG707110		+	1.36
P12 (FSE 1)	*F. semitectum*	Wheat root	Khusf	DECH	MG707118		+	6
P13 (FF 2)	*F. fujikuroi*	Wheat stalk	Giuk	DECH	MG707090		+	1.5
P14	*Fusarium* sp.	Cereals tissue	Birjand	DECH	s		nd	
P15	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707121		nd	
P16 (FS 2)	*F. solani*	Wheat stalk	Giuk	DECH	MG707092		nd	
P17 (FF 3)	*F. fujikuroi*	Wheat root	Amirabad	DECH	MG707114		nd	
P18 (FS 3)	*F. solani*	Wheat stalk	Bojd	DECH	ns		nd	
P19 (FC 1)	*F. culmorum*	Wheat root	Khusf	DECH	MG707097		nd	
P2	*Fusarium* sp.	Cereals tissue	Birjand	CH1	ns		nd	
P20	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707106		nd	
P21 (FE 1)	*F. equiseti*	Wheat root	Khusf	DECH	MG707093		+	6
P22 (FS 4)	*F. solani*	Wheat root	Birjand	DECH	MG707115		+	6
P23	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707094		+	1.3
P24 (FF 4)	*F. fujikuroi*	Wheat root	Birjand	DECH	MG707103		nd	
P25 (FS 5)	*F. solani*	Wheat stalk	Giuk	DECH	ns		nd	
P26 (FNG 1)	*F. nygamai*	Wheat root	Khusf	DECH	ns		nd	
P27 (FNG 2)	*F. nygamai*	Wheat root	Bojd	DECH	ns		nd	
P28 (FS 6)	*F. solani*	Wheat root	Amirabad	DECH	MG707116		nd	
P29 (FAC 3)	*F. acuminatum*	Wheat root	Bojd	DECH	MG707107		+	1.06
P3 (FS 1)	*F. solani*	Wheat stalk	Khusf	DECH	MG707112		nd	
P30 (FNG 3)	*F. nygamai*	Wheat root	Birjand	DECH	MG707101		nd	
P31 (FD 1)	*F. diversisporum*	Wheat root	Khusf	DECH	ns		nd	
P32 (FAC 4)	*F. acuminatum*	Wheat root	Bojd	DECH	ns		nd	
P33 (FC 2)	*F. culmorum*	Wheat root	Bojd	DECH	ns		nd	
P34 (FE 2)	*F. equiseti*	Soil	Khusf	DECH	MG707111		nd	
P35 (FO 1)	*F. oxysporum*	Wheat stalk	Giuk	DECH	ns		nd	
P36 (FS 7)	*F. solani*	Wheat root	Amirabad	DECH	MG707117		+	1.23
P37	*Fusarium* sp.	Cereals tissue	Birjand	DECH	ns		nd	
P38 (FAV 2)	*F. avenacenum*	Wheat root	Amirabad	DECH	MG707105		nd	
P39 (FL 1)	*F. longipes*	Wheat root	Khusf	DECH	ns		nd	
P4 (FAV 1)	*F. avenaceum*	Wheat stalk	Bojd	DECH	MG707109		nd	

P40 (FAC 5)	*F. acuminatum*	Wheat root	Bojd	DECH	MG707087	nd	
P41 (FAC 6)	*F. acuminatum*	Wheat stalk	Giuk	DECH	ns	nd	
P42	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707108	nd	
P43 (FL 2)	*F. longipes*	Cereals tissue	Khusf	DECH	MG707085	nd	
P44 (FSE 2)	*F. semitectum*	Wheat root	Giuk	DECH	MG707102	nd	
P45 (FS 8)	*F. solani*	Wheat root	Amirabad	DECH	ns	nd	
P46 (FAC 7)	*F. acuminatum*	Wheat root	Amirabad	DECH	MG707088	nd	
P47 (FAC 8)	*F. acuminatum*	Wheat root	Birjand	DECH	ns	nd	
P5	*Fusarium* sp.	Cereals tissue	Birjand	DECH	ns	nd	
P6 (FF 1)	*F. fujikuroi*	Wheat root	Khusf	DECH	MG707104	+	1.16
P7	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707119	+	6
P8 (FAC 1)	*F. acuminatum*	Wheat stalk	Khusf	DECH	MG707086	nd	
P9	*Fusarium* sp.	Cereals tissue	Birjand	DECH	MG707113	+	1.5

† nr: not replicated, ns: not sequenced, nd: not determined.

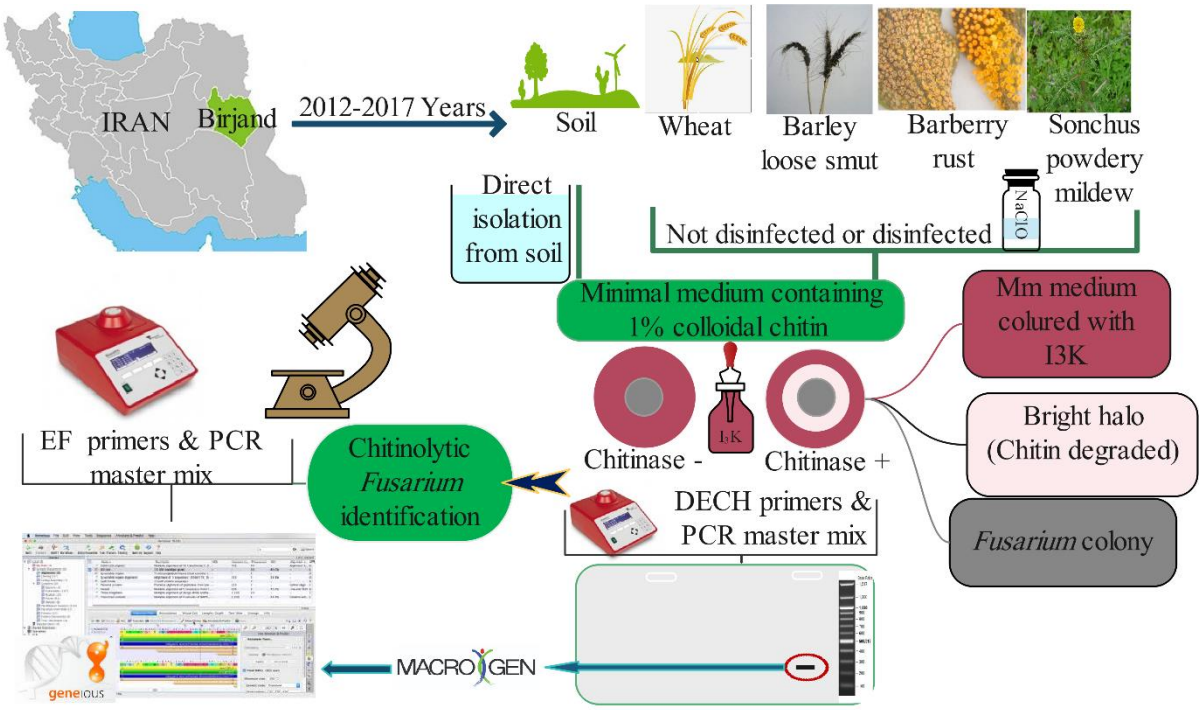

Fig. 1. Isolation and identification of chitinolytic *Fusarium* isolates from soil, plants and fungal tissues.

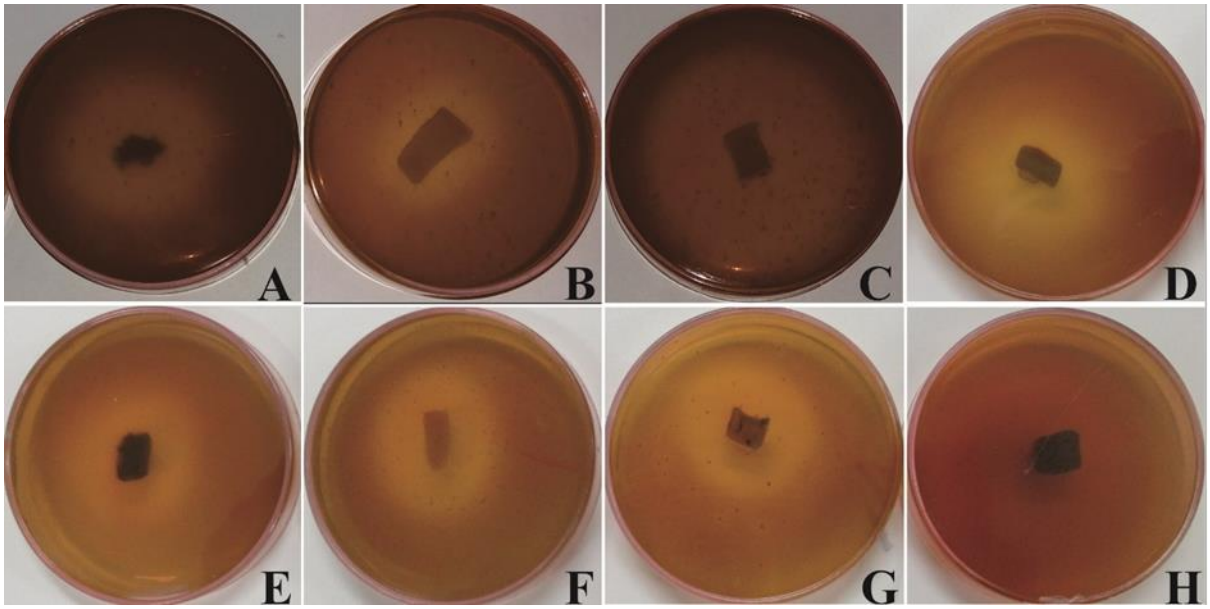

Fig. 2. Chitinase activity of (A) *Fusarium proliferatum*, (B) *F. solani*, (C) *F. equiseti*, (D) *F. acuminatum*, (E) *F. semitectum*, (F) *F. fujikuroi*, (G) *Trichoderma harzianum* and (H) control on minimal media containing 0.1% ($w.v^{-1}$) colloidal chitin and chitin powder.

Twenty-four hours after adding 1.5% Lugol's solution to the 7-day culture of isolates on a medium with at least 1% colloidal chitin, a bright halo was observed around the colony of 47 isolates. The diameter of the halo formed in the different isolate and species varied from 0.7-6 cm, a small halo around the fungal colony to the whole surface of the 6 cm Petri dishes (Fig. 2).

Two months after the preservation of *Fusarium* isolates in tubes containing chitin powder, some of them were grown on sterile chitin powder. The amount of colonization of chitin powder varied so that the growth of some isolates led to chitin powder degradation and lysing, but the control and some isolates lacking the ability to chitin degradation did not change chitin powder.

The DECH primers amplified 250 base pairs regions in chitinolytic isolates. Twenty of the chitinolytic isolates were identified to be *F. solani*. The diameter of the observed halo around fungal colonies varied from 0.7-6 cm after Lugol's staining test (Fig. 2B). DECH primers were also able to amplify the genome and confirmed the presence of chitinase gene in this species. The EF primers replicated a fragment in some of the isolates of this group whose sequences had 99.9-100 similarity with the sequence of *F. solani* species in the NCBI GenBank.

Seventeen of the chitinolytic isolates were identified as *F. proliferatum*. The diameter of the halo formed around the colonies of this species in the culture medium was 1.3-6 cm (Fig. 2A). DECH and ChiT-2-R primers also replicated a 250 and 600 bp regions in these isolates.

Eight of the chitinolytic isolates were identified as *F. acuminatum*. The diameter of the halo created after the Lugol's solution test these isolates varied between 1-1.4 cm (Fig. 2D), and DECH primers replicated the genome. EF primers also replicated a fragment in these isolates of which the sequence was similar to the sequence of *F. acuminatum* in the NCBI GenBank. Two isolates were identified as *F. equiseti* which formed a halo with a diameter of 5-6 cm in the culture in the Petri dish after Lugol's test (Fig. 2C). DECH primer was able to amplify a region of approximately 250 bp. Two isolates were found to be *F. semitectum*. The mean diameter of the halo around the colony of these two isolates was 6 cm after Lugol's test (Fig. 2E), and DECH primer could replicate 250 bp region of the fungal genome. A halo with

a diameter of 1.1-1.8 cm was observed around the colonies of 4 isolates of *F. fujikuroi* species, after staining with Lugol's solution (Fig. 2F). In this case, DECH primer replicated the genome.

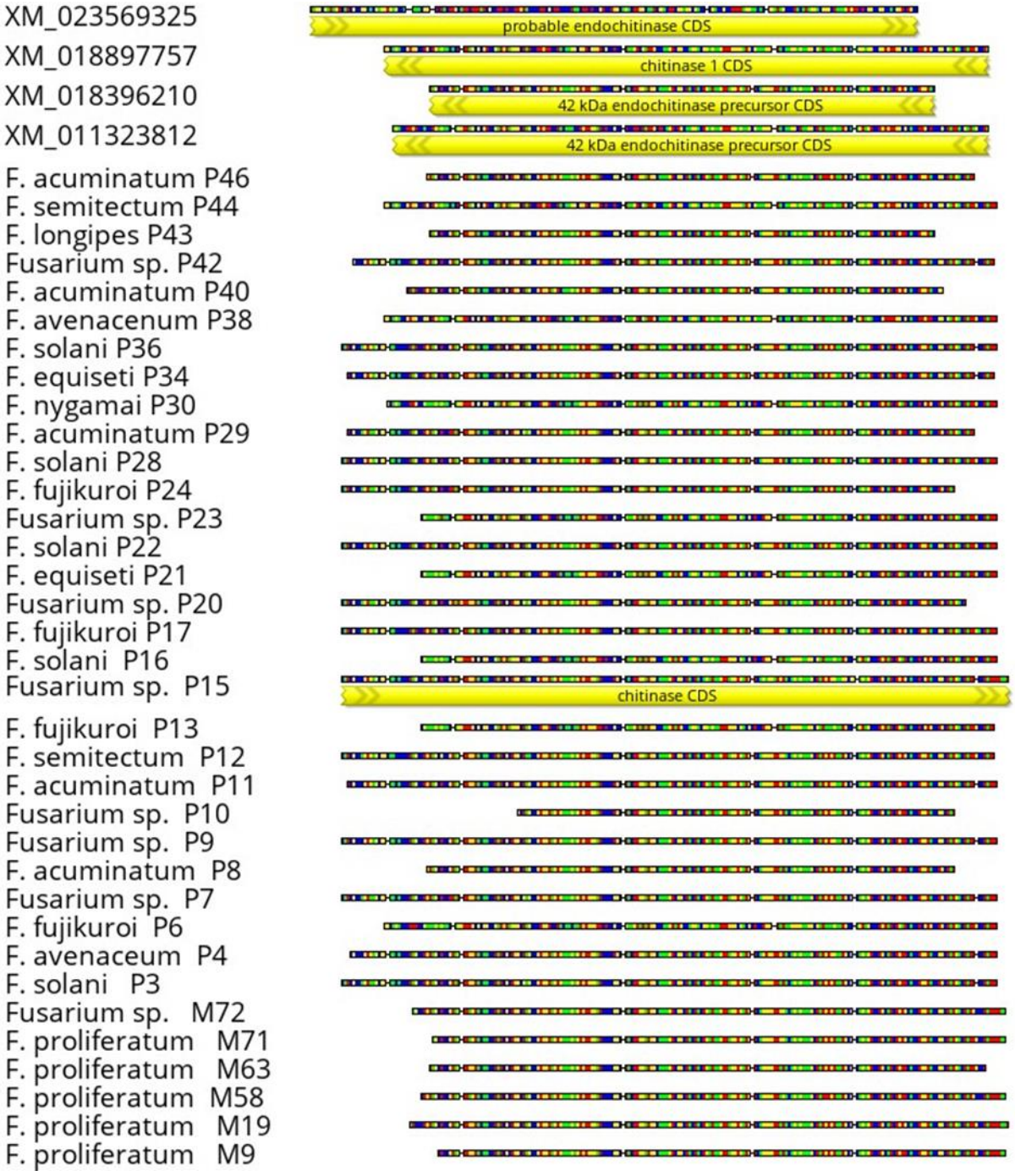

Fig. 3. Graphical display chitinase regions replicated by DECH primers similarity compared with GenBank NCBI records using Geneious software.

DECH primers were able to amplify the genome the *F. avenaceum*, *F. nygamai*, *F. diversisporum*, *F. longipes*, *F. culmorum* and *F. oxysporum* species. Chitin analysis test was not performed in the culture medium of these species, and only by the molecular method, the presence of the gene chitinase proved in these species.

DISCUSSION

The presence of chitinase genes and/or ability to produce chitinolytic enzymes have been proven in 83 isolates representing 12 species of *Fusarium*. The sequences of DECH primers replicated regions of 27 isolates had 98-99% similarity to 42 kDa endochitinase gene sequence of *F. oxysporum* f. sp. *lycopersici* (XM-018396210). This region is a part of the chitinase1 enzyme production gene. Replicated regions of some *Fusarium* isolates, had high similarity with sequences of chitinase genes in *Trichoderma* species (Fig. 3).

The sequence of the replicated region in some *F. proliferatum* isolates was consistent with 42 kDa endochitinase gene. The sequences of predicted proteins encoded by the replicated regions were 90% similar to a protected region in other *Fusarium* species (Fig. 3).

DECH primer-replicated regions of P44 (FSE2) isolate from *F. semitectum* species, was more than 99.8% similar with chitinase1 gene sequence in *F. verticillioides* isolate 7600 (XM-018897757) of the NCBI GenBank, which can confirm the presence of genes with similar structure and activity in *F. semitectum* species. The sequence of the DECH primers products in P38 isolate of *F. avenaceum* species was 90% similar with the sequence of 42-kD DNA endochitinase gene of the isolate PH-1 of *F. graminearum* species in the NCBI GenBank (XM-11323812). The sequence of the replicated regions indicates the presence of chitinase 1 or chitinase enzyme like the 42 kDa endochitinase enzyme. Further study is necessary for isolation and identification of these enzymes.

The production of this chitinase by *Fusarium* species and presence of chitinase gene in the genome of this fungus reflects the fact that some species of *Fusarium* can produce chitinase and can secrete it into the culture medium and degrade chitin in the medium. The growth of the *Fusarium* colonies in the presence of the enzyme also indicated that the enzymes did not affect *Fusarium* growth in the culture medium.

Most isolates of this study were isolated from the soil and plant tissues and a small number from vegetative or reproductive tissues of plant pathogens. The isolates from cereals are pathogenic to cereals, especially wheat (Besharati Fard et al., 2017). The results of this study showed that this isolate, which are pathogens of cereals, can produce chitinolytic enzymes in the culture medium.

The presence of chitinolytic enzymes in the pathogen isolates may be aimed to change the fungal tissue, reconstruction and destruction of the old walls and the production of new cells (Adams, 2004; Gooday et al., 1986). The presence of a bright halo around the fungal colonies after staining with Lugol's staining indicates secretion of chitinolytic enzymes into the culture medium (Fig. 4). Therefore, it may also be released inside the plant and natural media and help distribution of the fungi in the plant or soil (Homthong et al., 2016; Prasetyawan et al., 2018).

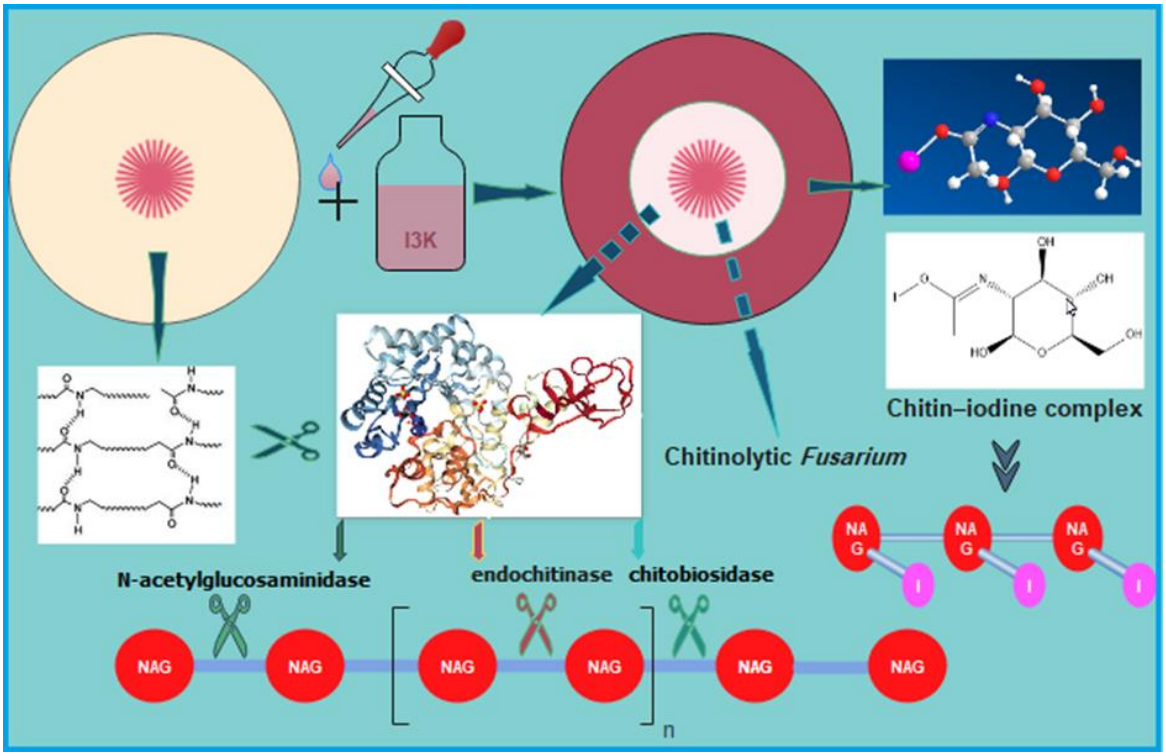

Fig. 4. Degradation of chitin that was colored by the iodine in the Lugol's solution, into simple carbohydrates that did not absorb the iodine.

Lysis of the host structure by secretion of extracellular lytic enzymes is one of the important mechanisms involved in the antagonistic activity of biocontrol agents (Mathivanan et al., 1998). The M3 and M14 isolates from *F. proliferatum* and M27 and M58 isolates of *F. solani* were recovered from *U. nuda* spores of barley. The simultaneous presence of these two fungi in infected ears could be due to concurrent infection of the ears with both fungal species. On the one hand, *F. proliferatum* and *F. solani* species can use *U. nuda* spores as a source of nutrients using chitinolytic enzymes and may be destroyed *U. nuda* spore and hyphal tissues.

We isolated *F. proliferatum* (M71), and *F. solani* (M15) isolates from spores of barberry rust, one of the most important diseases of this plant in South Khorasan. The presence of these chitinolytic species in rust pustules of barberry can indicate the infection of the barberry tissue with these species of *Fusarium* or growth of these species on barberry rust spores.

From the spores of *Sonchus* powdery mildew belonging to the genus *Erysiphe*, only one isolate (M72) of *Fusarium* was isolated, which, like the two above fungi, probably shows simultaneous infection or *Erysiphe* hyperparasitism by *Fusarium* species.

The presence of chitinase gene and the ability to produce chitinolytic enzymes have been investigated in some species of *Fusarium* (Koyama et al., 2017; Mathivanan et al., 1998). Nuero (1995) demonstrated the production of chitinase enzyme in 17 species of *Fusarium* and ability to use colloidal chitin and carboxymethyl chitin as a food source. According to the results, the activity of β-N-acetylglucosaminidase and endochitinase in *F. acuminatum* species was 127 and 22 mU/mg, 17 and 4 mU/mg in *F. culmorum*, respectively, 50 and 40 mU/mg in *F. equiseti*, respectively, 7 and 18 mU/mg in *F. oxysporum*, respectively, and 10 and 12 mU/mg in *F. semitectum*, respectively.

Govinda Rajulu et al. (2010) recovered 31 isolates of chitinolytic endophytic fungi from Indian forests, of which 12 isolates belonged to *Fusarium* species. Mathivanan et al. (1998) showed that *F. solani* species could produce chitinase enzyme in the liquid medium after six

days, which can inhibit *P. arachidis* spore germination, isolated 40 kDa chitinase from *F. chlamydosporum* species, which had a stable pH of 5 and up to 40°C. This enzyme inhibited the germination of *P. arachidis* uredospore with lysing uredospore wall and mass of the tube, reducing the development of rust pustules on groundnut leaves (Mathivanan et al., 1998).

So far, chitinases with molecular weights of 33, 42 and 44 isolated from species of *Trichoderma* genus, 27-82 kDa from *P. aculeatum* species, 46 kDa from *Ch. globosum* species and 50 kDa from *R. oryzae* species have been identified.

The sequence of *Fusarium* species in this study resembled the sequence of 42 kDa chitinase and chitinase 1 of the remaining fungal species, which are 18 chitinase genes from glycoside hydrolase family. Chitinolytic *Fusarium* isolates were evaluated for biocontrol potential of *Alternaria* and *Bipolaris* using dual culture bioassay, but no growth inhibitory and hyperparasitism was observed. The isolation and identification of chitinase enzyme from these fungi and determination of their role in the life of these *Fusarium* species should be made clear at the next steps.

CONCLUSION

Chitinase enzyme activity was detected in *F. solani*, *F. proliferatum*, *F. avenaceum*, *F. fujikuroi*, *F. acuminatum*, *F. semitectum*, *F. culmorum*, *F. equiseti*, *F. nygamai*, *F. diversisporum*, *F. oxysporum* and *F. longipes* species based on the molecular data, the secreted chitinases belonged to chitinse1 or 42 kDa, members of GH family 18. This is the first reports of chitinases from *Fusarium* species in Iran.

Acknowledgement
This article was written in partial fulfillment of a MSc thesis of the first author in plant pathology registered at the University of Birjand.

Conflict of interest
The authors declare that there is no conflict of interest.

REFERENCES

Adams, D. J. (2004). Fungal cell wall chitinases and glucanases. *Microbiology, 150*(7), 2029-2035. https://doi.org/10.1099/mic.0.26980-0

Afsarzadeh Laein, M., & Mohammadi, A. (2018). Chitinolytic fungi from the Birjand plain of Southern Khorasan Province in Eastern Iran. *Acta Mycologica, 53*(1), 1-12. https://doi.org/10.5586/am.1107

Besharati Fard, M., Mohammadi, A., & Darvishnia, M. (2017). *Fusarium* species associated with Wheat crown and root tissues in the Eastern Iran. *Archives of Phytopathology and Plant Protection, 50*(3-4), 123-133. https://doi.org/10.1080/03235408.2016.1275423

Brandfass, C., & Karlovsky, P. (2008). Upscaled CTAB-based DNA extraction and real-time PCR assays for *Fusarium culmorum* and *F. graminearum* DNA in plant material with reduced sampling error. *International Journal of Molecular Sciences, 9*(11), 2306-2321. https://doi.org/10.3390/ijms9112306

De Marco, J. L., Lima, L. H. C., de Sousa, M. V., & Felix, C. R. (2000). A *Trichoderma harzianum* chitinase destroys the cell wall of the phytopathogen *Crinipellis perniciosa*, the causal agent of witches' broom disease of cocoa. *World Journal of Microbiology and Biotechnology, 16*(4), 383-386. https://doi.org/10.1023/a:1008964324425

Di Giambattista, R., Federici, F., Petruccioli, M., & Fenice, M. (2001). The chitinolytic activity of *Penicillium janthinellum* P9: purification, partial characterization and potential applications. *Journal of Applied Microbiology*, *91*(3), 498-505. https://doi.org/10.1046/j.1365-2672.2001.01409.x

Dong, L. Q., Yang, J. K., & Zhang, K. Q. (2007). Cloning and phylogenetic analysis of the chitinase gene from the facultative pathogen *Paecilomyces lilacinus*. *Journal of Applied Microbiology*, *103*(6), 2476-2488. https://doi.org/10.1111/j.1365-2672.2007.03514.x

Fan, Y., Fang, W., Xiao, Y., Yang, X., Zhang, Y., Bidochka, M., & Pei, Y. (2007). Directed evolution for increased chitinase activity. *Applied Microbiology and Biotechnology*, *76*(1), 135-139. https://doi.org/10.1007/s00253-007-0996-7

Geiser, D. M., del Mar Jiménez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N ,.Ward, T. J., Zhang, N., Kuldau, G. A., & O´Donnell, K. (2004). Fusarium-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. *European Journal of Plant Pathology*, *110*(5-6), 473-479. https://doi.org/10.1007/978-1-4020-2285-2_2

Gooday, G. W., Humphreys, A. M., & McIntosh, W. H. (1986). Roles of chitinases in fungal growth. In M. Riccardo, J. Charles, & W. G. Graham (Eds.), *Chitin in Nature and Technology* (pp. 594), Plenum Press.

Govinda Rajulu, M. B., Thirunavukkarasu, N., Suryanarayanan, T. S., Ravishankar, J. P., El Gueddari, N. E., & Moerschbacher, B. M. (2010). Chitinolytic enzymes from endophytic fungi. *Fungal Diversity*, *47*(1), 43-53. https://doi.org/10.1007/s13225-010-0071-z

Griffiths, L. J., Anyim, M., Doffman, S. R., Wilks, M., Millar, M. R., & Agrawal, S. G. (2006). Comparison of DNA extraction methods for *Aspergillus fumigatus* using real-time PCR. *Journal of Medical Microbiology*, *55*(Pt 9), 1187-1191. https://doi.org/10.1099/jmm.0.46510-0

Harighi, M., Motalebi, M., & Zamani, M. (2006). Purification of chitinase 42 from *Trichoderma atroviride* PTCC5220. *Iranian Journal of Biology*. *19*(2), 203-214.

Harman, G., Hayes, C., Lorito, M., Broadway, R., Di Pietro, A., Peterbauer, C., & Tronsmo, A. (1993). Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Journal of Phytopathology*, *83*(3), 313-318. https://doi.org/10.1094/phyto-83-313

Homthong, M., Kubera, A., Srihuttagum, M., & Hongtrakul, V. (2016). Isolation and characterization of chitinase from soil fungi, *Paecilomyces* sp. *Agriculture and Natural Resources*, *50*(4), 232-242. https://doi.org/10.1016/j.anres.2015.09.005

Hsu, S., & Lockwood ,J. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Microbiology*, *29*(3), 422-426. https://doi.org/10.1128/aem.29.3.422-426.1975

Kang, S. C., Park, S., & Lee, D. G. (1999). Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, *73*(3), 276-281. https://doi.org/10.1006/jipa.1999.4843

Karlsson, M., & Stenlid, J. (2008). Comparative evolutionary histories of the fungal chitinase gene family reveal non-random size expansions and contractions due to adaptive natural selection. *Evolutionary Bioinformatics*, *4*, 47-60 . https://doi.org/10.4137/ebo.s604

Khoushab, F., & Yamabhai, M. (2010). Chitin research revisited. *Marine Drugs*, *8*(7), 1988-2012. https://doi.org/10.3390/md8071988

Koyama, T., Sato, F., & Ohme-Takagi, M. (2017). Roles of miR319 and TCP transcription factors in leaf development. *Plant Physiology*, *175*(2), 874-885. https://doi.org/10.1104/pp.17.00732

Küçük, Ç., & Kivanç, M. (2003). Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. *Turkish Journal of Biology*, *27*(4), 247-253.

Leslie, J. F., & Summerell, B. A. (2008). *The Fusarium laboratory manual*: John Wiley & Sons.

Loc, N. H., Quang, H. T., Hung, N. B., Huy, N. D., Phuong, T. T., & Ha, T. T. (2011). *Trichoderma asperellum* Chi42 Genes Encode Chitinase. *Mycobiology*, *39*(3), 182-186. https://doi.org/10.5941/MYCO.2011.39.3.182

Mathivanan, N., Kabilan, V., & Murugesan, K. (1998). Purification, characterization, and antifungal activity of chitinase from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*. *Canadian Journal of Microbiology*, *44*(7), 646-651. https://doi.org/10.1139/w98-043

Meng, H., Wang, Z., Meng, X., Xie, L., & Huang, B. (2015). Cloning and expression analysis of the chitinase gene Ifu-chit2 from *Isaria fumosorosea*. *Genetics and Molecular Biology*, *38*(3), 381-389. https://doi.org/10.1590/S1415-475738320150003

Mohapatra, S., & Parhi, A. (2017). Screening and characterization of rhizospheric chitinolytic bacteria for evaluation of their potential as biocontrol agent against fhytopathogenic fungi. *International Journal of Current Microbiology and Applied Sciences*, *6*(9), 3457-3463. https://doi.org/10.20546/ijcmas.2017.609.424

Mythili, J., Chethana, B., Rajeev, P., & Ganeshan, G. (2018). Chitinase gene construct from *Trichoderma harzianum* proved effective against onion purple blotch caused by *Alternaria porri*. *Indian Journal of Biotechnology*, *17*(1), 50-56.

Naim, F., Dugdale, B., Kleidon, J., Brinin, A., Shand, K., Waterhouse, P., & Dale, J. (2018). Gene editing the phytoene desaturase alleles of Cavendish banana using CRISPR/Cas9. *Transgenic Research*, *27*(5), 451-460. https://doi.org/10.1007/s11248-018-0083-0

Nuero, O. M. (1995). Production of chitinase by *Fusarium* species. *Current Microbiology*, *30*(5), 287-289. https://doi.org/10.1007/BF00295503

Patel, S., & Goyal, A. (2017). Chitin and chitinase: Role in pathogenicity, allergenicity and health. *International Journal of Biological Macromolecules*, *97*, 331-338. https://doi.org/10.1016/j.ijbiomac.2017.01.042

Poshina, D. N., Raik, S. V., Poshin, A. N., & Skorik, Y. A. (2018). Accessibility of chitin and chitosan in enzymatic hydrolysis: A review. *Polymer Degradation and Stability*, *156*, 269-278. https://doi.org/10.1016/j.polymdegradstab.2018.09.005

Prasetyawan, S., Sulistyowati, L., & Aulanni'am. (2018). Glucanase and chitinase from some isolates of endophytic fungus *Trichoderma* spp. *IOP Conference Series: Materials Science and Engineering*, *299*(1), 012026-012026. https://doi.org/10.1088/1757-899x/299/1/012026

Seyed Asli, N., Harighl, M., Zamani, M., & Motalebi, M .(2004). Study of chitinolytic enzyme production in *Trichoderma* isolates. *Iranian Journal of Biology*, *17*(3), 227-246.

Snyder, W., & Nash, S. M. (1968). Relative incidence of *Fusarium* pathogens of cereals in rotation plots at Rothamsted. *Transactions of the British Mycological Society*, *51*(3-4), 417-425. https://doi.org/10.1016/s0007-1536(68)80009-9

Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, properties, and some new applications of chitin and its derivatives. *Critical Reviews in Food Science and Nutrition*, *43*(2), 145-171. https://doi.org/10.1080/10408690390826473

Szilagyi, M., Anton, F., Pocsi, I., & Emri, T. (2018). Autolytic enzymes are responsible for increased melanization of carbon stressed *Aspergillus nidulans* cultures. *Journal of Basic Microbiology*, *58*(5), 440-447. https://doi.org/10.1002/jobm.201700545

Tikhonov, V. E., Lopez-Llorca, L. V., Salinas, J., & Jansson, H. B. (2002). Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. *Fungal Genetics and Biology*, *35*(1), 67-78. https://doi.org/10.1/006fgbi.2001.1312

van Munster, J. M., van der Kaaij, R. M., Dijkhuizen, L., & van der Maarel, M. J. (2012). Biochemical characterization of *Aspergillus niger* CfcI, a glycoside hydrolase family 18 chitinase that releases monomers during substrate hydrolysis. *Microbiology*, *158*(Pt 8), 2168-2179. https://doi.org/10.1099/mic.0.054650-0

Waksman, S., & Fred, E. (1922). A tentative outline of the plate method for determining the number of micro-organisms in the soil. *Soil Science*, *14*(1), 27-28. https://doi.org/10.1097/00010694-192207000-00004

Xia, G., Jin, C., Zhou, J ,.Yang, S., Zhang, S., & Jin, C. (2001). A novel chitinase having a unique mode of action from *Aspergillus fumigatus* YJ-407. *European Journal of Biochemistry*, *268*(14), 4079-4085. https://doi.org/10.1046/j.1432-1327.2001.02323.x

Xian, H., Li, J., Zhang, L., & Li, D. (2012). Cloning and functional analysis of a novel chitinase gene Trchi1 from *Trichothecium roseum*. *Biotechnology Letters*, *34*(10), 1921-1928. https://doi.org/10.1007/s10529-012-0989-1