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Research Article

Chitinase Activity of Biocontrol Fungi Isolated from the Golden Potato Cyst Nematode and their Antagonist Potential

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Abstract

Chitin is the most abundant polymer in the nematode eggshell. Chitinases are widely distributed in fungi and play important roles in the degradation of chitin. This study aimed to screen the chitinolytic ability of fungi isolated from *Globodera rostochiensis* and to evaluate their biocontrol potential. One hundred and fifty-four fungal isolates were compared for their ability to degrade chitin by the *N*-acetyl-glucosamine-dinitrosalicylate method. Ten isolates with high chitinase activity were selected for further characterization. Of the 10 selected isolates, three chitinase enzymes were evaluated using three substrates. Based on the results of our chitinase assay, *Beauveria bassiana*, *Lecanicillium muscarium*, *Paecilomyces* sp. and *Trichoderma atroviridae* had the highest activity. We selected these four isolates to determine the optimum pH, temperature and reaction time. Zymography was also used to demonstrate chitinase activity of the four isolates. The biocontrol potential of 10 selected isolates was assayed in water-agar *in vitro* and under greenhouse conditions. *L. muscarium* had the most potential and *Fusarium solani* was the least based on the number of parasitized juveniles and eggs in water-agar. Greenhouse trials showed *T. atroviridae* and *B. bassiana* had the highest dry root weight and tuber yield.

Keywords: *Biocontrol; Chitinase; Enzymatic Activity; Fungi; Globodera rostochiensis*

Introduction

The golden cyst nematode, *Globodera rostochiensis* [1,2] is one of the most destructive pathogens of potato in the world [3]. Biological control of plant parasitic nematodes is a compelling management strategy given the loss of nematicides, and environmental concerns regarding chemical inputs in agriculture. Fungal antagonists are considered the most applicable for biological control of nematodes [4]. Tobin *et al*. [5] reported that *Pochonia chlamydosporia* resulted in good biocontrol of the potato cyst nematode. Lopez Lima., *et al*. [6] reported that *G. rostochiensis* decreased up to 89% in the presence of *Paecilomyces* sp.

Chitinases, glucanases and proteases are considered the most important lytic mechanisms for successful biological control of nematodes [7,8] Saifullah and Khan [9] reported that *Trichoderma harzianum* enzymatically penetrated cysts and eggs of *G. rostochiensis*. Santos *et al*. [10] demonstrated that N-acetyl-yß-glucosaminidase and proteases of *P. chlamydosporia* were involved in control of the potato cyst and root-knot nematodes. Parasitism of cyst eggs by fungi leads to immobility and death of the embryos, resulting in reduction of nematode population density. The degradation of nematode eggs is caused by enzymatic action. Knowledge of various aspects of chitinolytic enzymatic systems allows the development of new generations of chitinases and the design of better strategies for biological control [11]. Fungal chitinases are necessary for hyphal growth and they are also produced by nematophagous and entomopathogenic

fungi to aid infection [12]. Because chitin is a dominant component of the eggshell in nematodes, the use of chitinase producing fungi is a promising strategy for biological control of the golden potato cyst nematode [13]. In the present study, the ability to produce chitinase by various fungi cultured from the golden cyst nematode was undertaken.

Materials and Methods

Fungal isolates: One hundred and fifty-four fungal isolates were recovered from infected eggs of *G. rostochiensis*, the golden potato cyst nematode, in Iran, in 2015. The fungi were maintained on PDA (potato dextrose agar) at 25°C. To determine the most promising isolates for biological control, the isolates were assayed for chitinase activity after 96h incubation with colloidal chitin.

***N*-acetyl-glucosamine-dinitrosalicylate assay**: Colloidal chitin was prepared following the procedure of Tikhonov., *et al* [12]. For the liquid medium enzyme assay, isolates were grown in minimal synthetic medium (MSM) ([g l^{-1}]: $(NH_4)_2SO_4$, 2.8 urea, 0.6 KH_2PO_4, 4 $CaCl_2.2H_2O$, 0.6 $MgSO_4$, 0.2 $FeSO_4.7H_2O$, 0.01 $ZnSO_4.H_2O$, 0.0028 $CoCl_2.6H_2O$, 0.0032) containing colloidal chitin (1 g l^{-1}) [14]. After 96 h growth at 25°C the culture medium was filtered through Whatman filter paper No.3 followed by filtration through 0.2-mm Millipore polydifluoropropilene membranes. The filtrate obtained was analyzed for chitinolytic activity as described below.

Chitinase activity (U mg^{-1}) was determined by measuring the release of reducing saccharides from colloidal chitin by the *N*-acetyl-glucosamine-dinitrosalicylate method described by Monreal and Reese [15]. The reaction mixture contained 200 µl 0.5% colloidal chitin in citrate phosphate buffer (0.05 M, Ph = 6.6) and 200 µl culture filtrate incubated at 37°C for 1h. Reducing sugars were determined by adding 1 ml of dinitrosalicylic acid (DNS) to the reaction mixture, heated in boiling water for 5 min, cooled to room temperature, and centrifuged at 6,000 rpm for 5 min. Absorbance was measured with a spectrophotometer (UV-770 Brite, Canada) at 540 nm. Protein concentration (mg ml^{-1}) was determined according to Bradford [16] with bovine serum albumin (Sigma-Aldrich, USA) as the standard. Chitinase specific activity was calculated from the rate of enzyme activity divided by the mass of total protein. Each reaction mixture was replicated 3 times in a completely randomized design. Chitinase activity was subjected to analysis of variance (ANOVA) by software SAS, version 9.0 (Statistical Analysis System Institute Inc., Cary, NC, USA). Of the 154 fungal isolates, 10 isolates with the highest enzymatic activity were selected for further characterization**.**

Fungal parasitism of eggs and juveniles in water-agar medium: Ten isolates with the highest chitinase activity were selected to evaluate their ability to infect eggs and juviniles in 100 mm petri dishes of water agar. For each isolate, 10 mature cysts were sterilized with 0.5% sodium hypochlorite for 3 minutes, rinsed in sterile water and placed on water-agar medium at 5 cm from a 5 mm disc of the fungus and incubated at 25ºC for two weeks in the dark. Each challenge experiment was replicated 3 times in a completely randomized design. To evaluate infection of eggs and juveniles, 10 cysts from each challenge/petri dish were suspended in 30 ml of water and crushed to release the contents. The number of parasitized eggs and juveniles per 10 cysts were calculated and subjected to analyasis of variance (SAS, version 9.0).

Evaluation of biological control under greenhouse conditions: The ability of the 10 selected isolates to biologically control of *G. rostochiensis* on potato was tested under greenhouse conditions. To produce fungal inoculum, 20g of soaked wheat seed were placed in autoclavable nylon bags with 40 ml water and autoclaved three times within 48h. Four 5 mm fungal disks from the selected isolates were used to inoculate each nylon bag. There were three replications for each isolate. The inoculated seed was incubated at 25°C in the dark. The seeds were mixed every 48h to insure thorough colonization. For the study, 20g of infected seeds and approximately 100 cysts were placed together beneath a germinated *Solanum tuberosum* 'Marphona'. Each potato was in an individual pot with 5 kg of soil. There were 3 replications for each treatment and pots were distributed in a completely randomized design. After 90 days, results were evaluated by the following parameters: root length, root dry weight, plant height, shoot dry weight, number of tubers, tuber yield, and number of cysts in 100g soil. Data was subjected to analysis of variance (SAS, version 9.0).

Fluorometric chitinase assay: The 10 selected isolates were assayed using a chitinase assay fluorimetric kit-CS1030 (Sigma-Aldrich, USA). The assay included three substrates, 4-Methylumbelliferyl N,N′-diacetyl-β-D-chitobioside, 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide and 4-Methylumbelliferyl β-D-N,N′,N′′-triacetylchitotriose for the detection of chitobiosidase, β-N-acetylglucosaminidase and endochitinase, respectively. The reaction mixture contained 90 µl substrate and 10 µl enzyme incubated for 30 min at 37°C. Fluorescence at excitation wavelength 360 nm and emission wavelength of 450 nm was measured after stopping the reaction with sodium carbonate. One unit of chitinase activity releases 1 µmole of 4-methylumbelliferone from the appropriate substrate per min at pH = 5.

The experiment was a randomized complete block design (RCB) with three replicates. Chitinase activity was subjected to analysis of variance using software SAS, version 9.0. Enzyme activity was expressed over time using the Logistic-Peak, software Slide Write, version 2.0.

Determination of the optimum conditions for chitinase production: Of the 10 fungal isolates, four were selected that had the highest chitinase activity, to determine optimum conditions for chitinase production. The optimum pH, temperature and reaction time was determined with the *N*-acetyl-glucosamine-dinitrosalicylate method. Preliminary results showed that enzyme activity was highest after 96h. Optimum conditions were determined with pHs 3, 4, 5, 6, 7, 8 and 9, temperatures 22°C, 25°C, 28°C, 31°C; for 1h, 6 h, and 24h. The reaction mixture contained 200 µl substrate (0.5% colloidal chitin in citrate phosphate buffer, 0.05 M, pH = 6.6) and 200 µl culture filtrate incubated at 37°C. The experiment was a completely randomized design with three replicates. Analysis of variance of data of the chitinase activity using of software SAS, version 9.0 was done.

Protein assay: The isolates were grown in minimal synthetic medium at 25°C in the dark for 5 days, filtered through Whatman Filter Paper No. 3 followed by filtration through a 0.2-µm Millipore polydifluoropropilene membrane. Filtered culture supernatant was lyophilized with a freeze dryer (FD-7506/5006-URT, Iran) and kept at -20°C until further use. The obtained powder was mixed with 3 ml phosphate buffered saline (PBS) ([g l^{-1}]: NaCl: 8, KCl: 0.2, Na_2HPo_4: 1.42 and KH_2Po_4: 0.24) pH = 7.4 and used as the enzyme source. Dialysis, with a molecular weight cut-off 10 was used to remove excess low molecular weight solutes. The solution was placed in dialysis tubing coated with an inert, high molecular weight hydroscopic substance that removes water from the tubing. The dialysis tubing was placed in a tall, 1000 ml graduated cylinder filled with distilled water and dialyzed overnight at 4°C with four changes of distilled water. The dialysate was collected by freeze-drying again and used for protein measurement and enzymatic assay on electrophoresis gels.

Zymogram: Chitinolytic activity based on zymography was determined using a modified method of Davis [17]: 2 ml of 30% acrylamide/methylene bisacrylamide (30:0.8, v/v) solution was mixed with 2 ml of carboxymethyl-chitin-remazol brilliant violet 5R (CM-Chitin-RBV) (Sigma-Aldrich, USA), 1 ml of 1.5 M Tris-HCl buffer (pH = 8.4) and 0.3 - 1.2g (1 - 2 M) of electrophoretically pure urea (Bio-Rad). After degassing for 1 minute, 5 ml of tetramethylethylenediamine (TEMED) (Sigma-Aldrich, USA) and 100 ml of 10% aqueous ammonium persulfate (APS) was added. The stacking gel included 0.65 ml of 30% acrylamide/methylene bisacrylamide solution, 1.25 ml of 1.5 M Tris-HCl buffer (pH = 6.8), 3.05 ml of distilled water, and 0.3 - 1.2g of urea. After degassing for 1 minute, 10 ml of TEMED and 100 ml of APS were added. The running buffer consisted of 0.5M urea in 0.3 M glycine- 0.036M Tris buffer (pH = 8.4). The gels were run at 200V for 45 min at 6°C. After electrophoresis, the gels were incubated in 0.1 M SA-buffer, pH = 4.7, for 3h at room temperature with gentle shaking (70 - 80 rpm). Chitinolytic activity was visualized as clear band zones on a blue background of nondigested CM-Chitin-RBV. Contrast between cleared zones and background was enhanced by immersing the gel in 0.1% solution of basic fuchsin in 20% aqueous ethanol for 1h at room temperature followed by washing with distilled water. Images of electrophoresis gels were recorded with GelDoc (Bio-Rad).

Results

Fungal isolates and *N*-acetyl-glucosamine-dinitrosalicylate assay: One hundred and fifty-four fungal isolates were recovered from infected eggs of the golden potato cyst nematode, *G. rostochiensis* (Table 1).

Isolate No.	Genus	Isolate No.	Genus	Isolate no.	Genus	Isolate No.	Genus
1	*Fusarium*	40	*Plectosphaerella*	79	*Fusarium*	118	*Fusarium*
2	*Chaetomium*	41	*Chaetomium*	80	*Fusarium*	119	*Alternaria*
3	*Fusarium*	42	*Alternaria*	81	*Fusarium*	120	*Fusarium*
4	*Fusarium*	43	*Fusarium*	82	*Alternaria*	121	*Fusarium*
5	*Chaetomium*	44	*Fusarium*	83	*Fusarium*	122	*Fusarium*
6	*Candida*	45	*Fusarium*	84	*Fusarium*	123	*Fusarium*
7	*Fusarium*	46	*Alternaria*	85	*Fusarium*	124	*Fusarium*
8	*Fusarium*	47	*Fusarium*	86	*Fusarium*	125	*Fusarium*
9	*Alternaria*	48	*Fusarium*	87	*Fusarium*	126	*Fusarium*
10	*Fusarium*	49	*Cylindrocarpon*	88	*Alternaria*	127	*Fusarium*
11	*Plectosphaerella*	50	*Fusarium*	89	*Alternaria*	128	*Fusarium*
12	*Fusarium*	51	*Alternaria*	90	*Fusarium*	129	*Fusarium*
13	*Fusarium*	52	*Ulocladium*	91	*Alternaria*	130	*Fusarium*
14	*Fusarium*	53	*Fusarium*	92	*Alternaria*	131	*Fusarium*
15	*Chaetomium*	54	*Fusarium*	93	*Candida*	132	*Fusarium*
16	*Chaetomium*	55	*Alternaria*	94	*Alternaria*	133	*Fusarium*
17	*Fusarium*	56	*Fusarium*	95	*Fusarium*	134	*Fusarium*
18	*Fusarium*	57	*Fusarium*	96	*Fusarium*	135	*Fusarium*
19	*Candida*	58	*Fusarium*	97	*Fusarium*	136	*Fusarium*
20	*Fusarium*	59	*Fusarium*	98	*Fusarium*	137	*Fusarium*
21	*Fusarium*	60	*Fusarium*	99	*Fusarium*	138	*Fusarium*
22	*Alternaria*	61	*Fusarium*	100	*Alternaria*	139	*Alternaria*
23	*Fusarium*	62	*Fusarium*	101	*Alternaria*	140	*Candida*
24	*Fusarium*	63	*Fusarium*	102	*Alternaria*	141	*Trichocladium*
25	*Fusarium*	64	*Fusarium*	103	*Alternaria*	142	*Fusarium*
26	*Fusarium*	65	*Fusarium*	104	*Alternaria*	143	*Fusarium*
27	*Alternaria*	66	*Fusarium*	105	*Alternaria*	144	*Candida*
28	*Alternaria*	67	*Fusarium*	106	*Fusarium*	145	*Fusarium*
29	*Fusarium*	68	*Fusarium*	107	*Fusarium*	146	*Fusarium*
30	*Alternaria*	69	*Fusarium*	108	*Fusarium*	147	*Fusarium*
31	*Fusarium*	70	*Fusarium*	109	*Fusarium*	148	*Aspergillus*
32	*Fusarium*	71	*Fusarium*	110	*Fusarium*	149	*Chaetomium*
33	*Alternaria*	72	*Fusarium*	111	*Candida*	150	*Fusarium*
34	*Fusarium*	73	*Fusarium*	112	*Fusarium*	151	*Beauveria*
35	*Fusarium*	74	*Alternaria*	113	*Fusarium*	152	*Lecanicillium*
36	*Fusarium*	75	*Fusarium*	114	*Chaetomium*	153	*Paecilomyces*
37	*Fusarium*	76	*Candida*	115	*Fusarium*	154	*Trichoderma*
38	*Alternaria*	77	*Fusarium*	116	*Chaetomium*		
39	*Fusarium*	78	*Fusarium*	117	*Chaetomium*		

Table 1: *Fungi isolated from nematode eggs and assayed by the N-acetyl-glucosamine-dinitrosalicylate method.*

***N*-acetyl-glucosamine-dinitrosalicylate assay**: To determine the most promising isolates for biological control of *G. rostochiensis*, 154 isolates were assayed for chitinase activity 96h after growth in MSM. Results of the chitinase activity showed significant differences among the 154 isolates at the p ≥ 0.01 level (Table 2). *T. atroviridae* showed the highest specific activity at 0.56 U mg^{-1}; *Candida parapsilosis* had the lowest at 0.15 U mg^{-1} (Figure 1). Ten isolates, *Alternaria alternata*, *Cylindrocarpon olidum*, *Fusarium oxysporum* (a), *C. parapsilosis* (a), *C. parapsilosis* (b), *F. oxysporum* (b), *B. bassiana*, *L. muscarium*, *Paecilomyces* sp. and *T. atroviridae* had the highest activity and were selected for further study.

Sources of variance	Degree of freedom	Specific activity
Isolate	153	0.1741**
Error	308	0.0020
Coefficient of variations (percent)		20.39

Table 2: *Analysis of variance (mean square) of chitinase activity in culture filtrate from 96h cultures of 154 isolates.*

***: Significant at 0.01 probability level.*

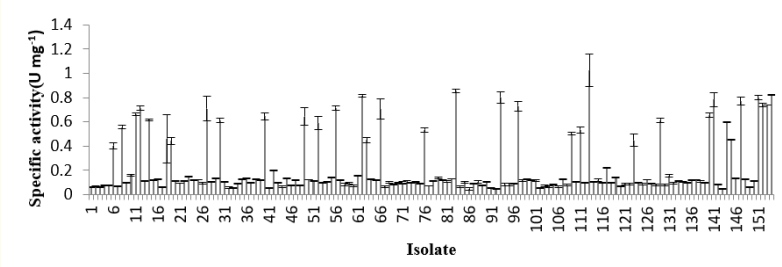

Figure 1: *Chitinase activity of culture filtrates of 154 fungal isolates by the N-acetyl-glucosamine-dinitrosalicylate assay after 96h.*

Fungal infection of juveniles and greenhouse trial: Significant differences (p ≥ 0.01) in fungal infection of eggs and juveniles were observed in water agar. *L. muscarium* had the highest level and *F. oxysporum* the least (Table 3).

Isolate	Mean no. of parasitized eggs and juveniles	Isolate	Mean no. of parasitized eggs and juveniles
L. muscarium	1110^{a}	*C. olidum.*	915^{cde}
B. bassiana	1065^{ab}	*C. parapsilosis* (a)	885^{de}
T. atroviridae	990^{ab}	*C. parapsilosis* (b)	780^{de}
F. oxysporum (a)	975^{abc}	*F. oxysporum* (b)	765^{e}
Paecilomyces sp.	975^{abc}	Control	45^{f}
A. alternata	945^{bcd}		

Table 3: *Parasitism of G. rostochiensis eggs and juveniles by selected fungi in water-agar medium.*

There were significant differences in root length, root dry weight, plant height, shoot dry weight, number of tubers, tuber yield and number of cysts in 100g soil. Isolates *T. atroviridae* and *B. bassiana* had the highest root dry weight and tuber yield under greenhouse conditions (Table 4).

Isolate	Root length (cm)	Dry weight root (g plant^{-1})	Plant height cm)	Dry weight shoot (g plant^{-1})	Number of tubers (tuber in plant^{-1})	Tuber yield (g plant^{-1})	Number of cysts in 100g soil
Nematode only	7.10^{d}	1.05^{h}	4.80^{d}	50.76^{e}	1.67^{d}	171.04^{c}	5.76^{a}
T. atrovirida	15.44^{c}	4.16^{b}	22.67^{bc}	48.47^{a}	7.67^{b}	271.60^{a}	0.48^{cd}
Paecilomyces sp.	15.12^{c}	2.99^{e}	22.33^{bc}	40.89^{b}	7b^{c}	261.43^{b}	0.47^{cd}
L. muscarium	15.09^{c}	2.62f^{g}	22.33^{bc}	30.05^{cd}	6.67^{bc}	261.77^{b}	1.42^{b}
B. bassiana	17.45^{b}	3.76^{c}	25.33^{ab}	46.74^{a}	7.33^{bc}	271.10^{ab}	0.91^{bc}
F. oxysporum (a)	15.30^{c}	2.82^{ef}	20.33^{bc}	28.56^{cd}	6.33^{bc}	256.80^{b}	1.47^{b}
C. parapsilosis (a)	15.44^{c}	2.36^{g}	20^{c}	30.48^{cd}	6.67^{bc}	262.30^{b}	1.17^{b}
C. parapsilosis (b)	16.01^{bc}	3.41^{d}	23.33^{bc}	31.76^{c}	7.33^{bc}	262.73^{b}	1.16^{b}
F. oxysporum (b)	14.39^{c}	2.47^{g}	21.67^{bc}	25.56^{d}	6.67^{bc}	254.93^{b}	0.36^{d}
C. olidum.	14.40^{c}	2.54f^{g}	20.33^{bc}	31.23^{c}	6^{c}	255.10^{b}	0.81^{bc}
A. alternata	14.49^{c}	2.31^{g}	23.33^{bc}	28.40^{cd}	6.33^{bc}	257.03$_{b}$	0.77^{bc}
Potato only	19.30^{a}	5^{a}	29.33^{a}	50.24^{a}	9.33^{a}	292.10^{a}	0^{e}

Table 4: *Effect of selected fungi on potato and number of cysts in a greenhouse trial. Nematode only is a control with no biocontrol agent and potato only has no cyst nematodes or biocontrol agent. Data represent means of three treatments.*

Means followed by the same letters in each column are not significantly different according to Duncan,s multiple range test (p≤0.05).

Enzyme assay by fluorimetric analysis: Chitinase activity was highest after 96h. The results showed the effects of different periods of incubation (1d, 2d, 3d, 4d and 5 day) on activity of Chitobiosidase, β-N-acetylglucosaminidase and endochitinase (Table 5). *B. bassiana* had the greatest amount of chitinase activity with all three enzymes (Table 5). The production of chitobiosidase and β-N-acetylglucosaminidase, for all 10 isolates began after three days of incubation, was maximum on the 4th day and decreased by day 5 (Figure 2 and 3). Activity of endochitinase began 1 day after incubation and reached its maximum on the 4th day and was lower on day 5 (Figure 4). Of the 10 isolates, the four with the highest chitinase activity were selected to determine optimum conditions for chitinase activity: *B. bassiana*, *L. muscarium*, *Paecilomyces* sp. and *T. atroviridae*.

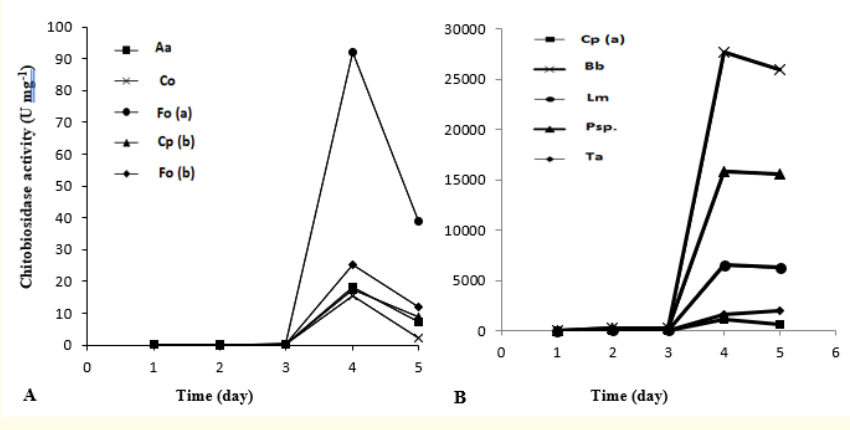

Figure 2A: *Isolates with less than 100 U mg-l chitobiosidase activity. Aa = Alternaria alternata, Co = Cylindrocarpon olidium, Fo(a) = Fusarium oxysporum, Cp(b) = Candida parapsilosis and Fo(b) = Fusarium oxysporum.*

Figure 2B: *Isolates with more than 100 U mg-l chitobiosidase activity. Cp(a) = Candida parapsilosis, Bb = Beauveria bassiana, Lm = Lecanicillium muscarium, Psp. = Paecilomyces sp. and Ta = Trichoderma atroviridae.*

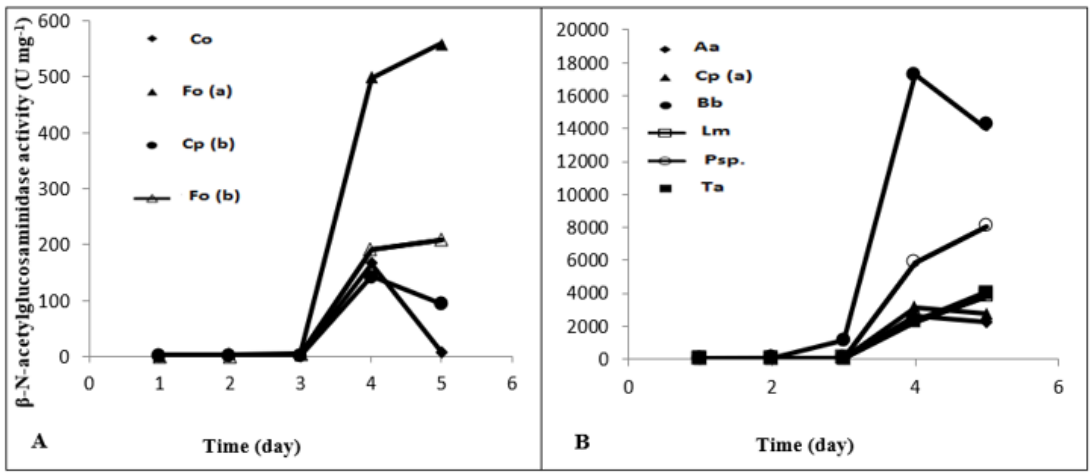

Figure 3A: *Isolates with less than 600 U mg-l β-N-acetylglucosaminidase activity. Co = Cylindrocarpon olidium, Fo(a) = Fusarium oxysporum, Cp(b) = Candida parapsilosis and Fo(b) = Fusarium oxysporum.*

Figure 3B: *isolates with more than 600 U mg-l β-N-acetylglucosaminidase activity. Aa = Alternaria alternata, Cp(a) = Candida parapsilosis, Bb = Beauveria bassiana, Lm = Lecanicillium muscarium, Psp. = Paecilomyces sp. and Ta = Trichoderma atroviridae.*

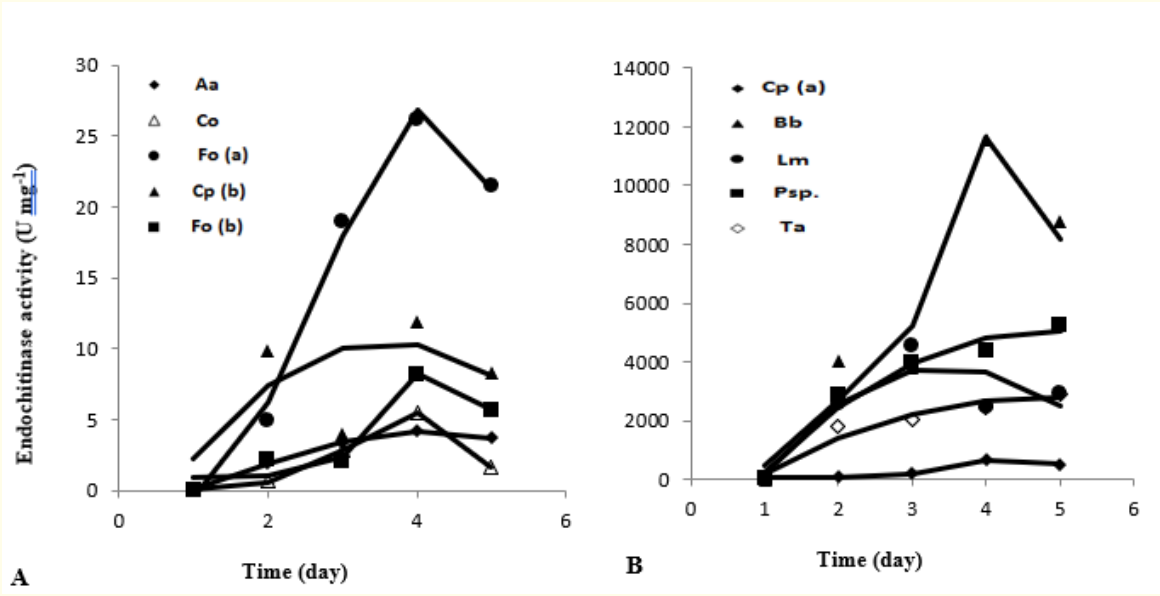

Figure 4A: *Isolates with less than 30 U mg-l endochitinase activity. Aa = Alternaria alternata, Co = Cylindrocarpon olidium, Fo(a) = Fusarium oxysporum, Cp(b) = Candida parapsilosis and Fo(b) = Fusarium oxysporum.*

Figure 4B: *Isolates with more than 30 U mg-l endochitinase activity. Cp(a) = Candida parapsilosis, Bb = Beauveria bassiana, Lm = Lecanicillium muscarium, Psp. = Paecilomyces sp. and Ta = Trichoderma atroviridae.*

Isolate	Chitobiosidase	β-N-acetylglucosaminidase	Endochitinase
A. alternata	5^{e}	988^{cd}	2^{c}
C. olidum.	4^{e}	35^{f}	2^{c}
F. oxysporum (a)	26^{e}	213^{de}	14^{c}
C. parapsilosis (a)	373^{d}	1188^{bc}	2987^{b}
C. parapsilosis (b)	5^{e}	48^{f}	7^{c}
F. oxysporum (b)	8^{e}	80^{ef}	3^{c}
B. bassiana	10841^{a}	6359^{a}	5638^{a}
L. muscarium	2604^{b}	1222^{bc}	2516^{a}
Paecilomyces sp.	6393^{ab}	2801^{b}	3300^{a}
T. atroviridae	720^{c}	1286^{b}	1835^{a}

Table 5: *Activity of three chitinase enzymes from selected isolates. Data represent the means of three isolates. Means followed by the same letters in each column are not significantly different according to Duncan's multiple range test ($p \leq 0.01$).*

Determination of the optimum conditions for chitinase production: The effects of isolate, temperature and the interaction between fungal isolate and temperature on activity of chitinase was significant at the 1% level. The optimum temperature for enzyme production for isolates *B. bassiana, Paecilomyces* sp. and *T. atroviridae* was 25°C. The optimum temperature for *L. muscarium* was 31°C (Figure 5). The effects of isolate, pH and the interaction between isolate and pH on the specific activity of chitinase was significant at the 0.01 level. The highest specific activity for the four isolates *L. muscarium, B. bassiana, Paecilomyces* sp. and *T. atroviridae* was at pH 6, 5, 5, 5 respectively. The lowest specific activity for *L. muscarium* and *Paecilomyces* sp. was pH = 3; and for *L. muscarium*, pH = 4 (Figure 6). For all four isolates, the maximum enzyme activity occurred after 1 h and the minimum activity was 24h (Figure 7).

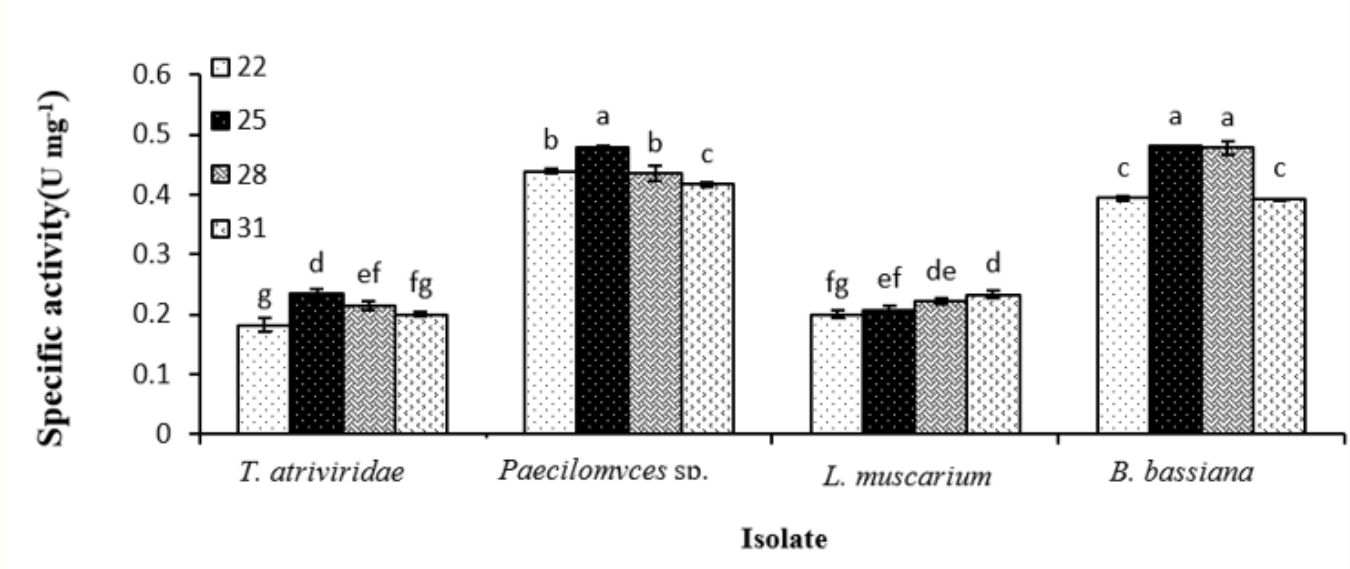

Figure 5: *Effect of temperature on chitinase activity in four selected fungal isolates.*

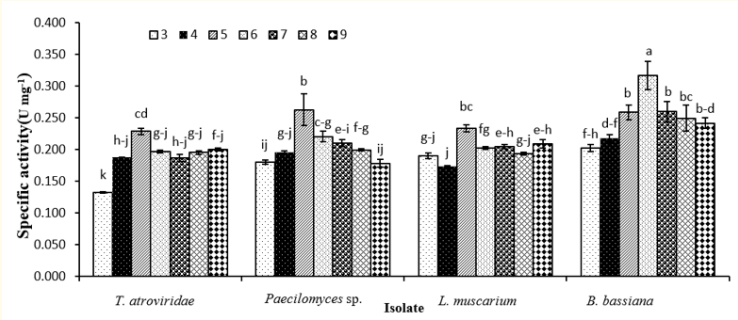

Figure 6: *Effect of pH on chitinase activity in four selected fungal isolates.*

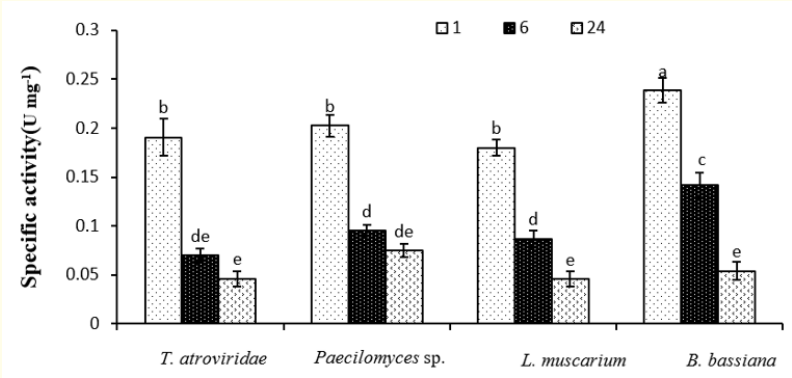

Figure 7: *Effect of reaction time on chitinase activity in four selected fungal isolates.*

Protein assay and zymograms: Zymograms of chitinase activity of isolates *B. bassiana, L. muscarium, Paecilomyces* sp. and *T. atroviridae* showed three zones of digested CM-chitin-RBV with Rfs of 0.28, 0.39, 0.48 for isolate *B. bassiana*, three zones with Rfs of 0.28, 0.39, 0.48 for isolate *L. muscarium*, two zones with Rfs of 0.39, 0.48 for isolate *Paecilomyces* sp. and four zones with Rfs of 0.16, 0.28, 0.39 and 0.48 for isolate *T. atroviridae* (Figure 8).

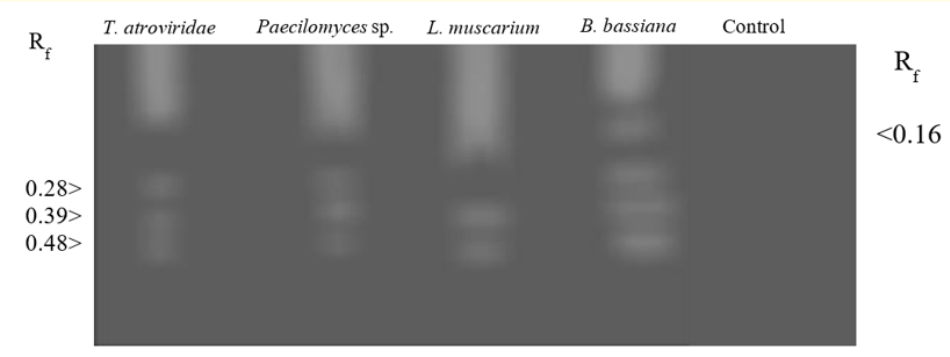

Figure 8: *Zymograph of chitinolytic activity of four slected fungal isolates.*

Discussion and Conclusion

Globodera rostochiensis is one of the most destructive pathogens of potato in the world. Since chitin is the dominant component of the nematode eggshell, and chitinases are known as degrading enzymes of chitin in a wide range of fungi, this could be an important mechanism of biological control. Chitinase may be enhanced by a combination with other bioactive peptides and lytic enzymes such as glucanase and proteases as reported in natural systems [12,18]. The aim of this study was to assay chitinase activity from selected fungi obtained from the golden potato cyst nematode to determine if chitinase, or the fungi that produced chitinase, could potentially be employed as biological control agents.

All 154 isolates had chitinase activity based on the spectrophotometric method at 540 nm. The positive biological control shown in our greenhouse experiment could be attributed to chitinase activity on the eggs as well as direct infection of the eggs and juveniles.

The highest specific enzyme activity of the four selected isolates *B. bassiana*, *L. muscarium*, *Paecilomyces* sp. and *T. atroviridae* occurred at pHs 6, 5, 5, 5. Three isolates had the highest chitinase activity at pH= 5; apparently, chitinase hydrolysis is optimum in an acidic environment. Adjusting soil pH to 5 may enhance biological control activity.

Of the four selectedisolates, the maximum enzyme activity occurred in one hour, after which chitinase activity declined. This may not be important in the soil environment as it is presumed that the fungus will produce chitinases continually.

Bands of chitinolysis in zymograms were like results by Tikhonov., *et al*. [12] that were enhanced with ionic absorption of positively charged molecules and fuchsin onto negatively charged polyanionic molecules of undigested CM-Chitin-RBV. Zymography can be a simple and helpful method in studies of complex chitinolytic systems.

In this study among the 154 isolates, four well-known biocontrol genera *Trichoderma*, *Lecanicillium*, *Paecilomyces* and *Beauveria* had the highest enzymatic activity. These four genera were previously reported by Sankaranarayanan., *et al*. [19], Safari-Motlagh and Samimi [8] and Saifullah and Khan [9] as important biocontrol agents of *G. rostochiensis*.

In this study, the biological control of *G. rostochiensis* byseveral fungal isolates showed there is a good correlation between chitinase activityand biocontrol potential.

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Bibliography

1. Wollenweber HW. "Krankheiten und Beschädigungen der Kartoffel". Arbeiten des Forschungsdienst für Kartoffelbau 7 (1923): 1-56.
2. Behrens E. "Globodera Skarbilovich, 1959, eine selbständige Gattung in der Unterfamilie Heteroderinae Skarbilovich, 1947 (Nematoda: Heteroderidae)". *Vortragstagung zu Aktuellen Problemen der Phytonematologie* (1975): 12-26.
3. Gitty M., *et al*. "Occurrence of potato golden cyst nematode in Iran and its distribution in Hamedan province". *Agricultural Biotechnology* 10 (2011): 53-61.
4. Sharon E., *et al*. "Improved attachment and parasitism of Trichoderma on *Meloidogyne javanica in vitro*". *European Journal of Plant Pathology* 123 (2009): 291-299.
5. Tobin JD., *et al*. "Effect of the fungus Pochonia chlamydosporia and fosthiazate on the multiplication rate of potato cyst nematodes (*Globodera pallid* and *G. rostochiensis*) in potato crops grown under UK field conditions". *Biological Control* 46 (2008): 194-201.

6. López Lima D., *et al*. "89% reduction of a potato cyst nematode population using biological control and rotation". *Agronomy Sustainable Development* 33 (2013): 425-431.

7. Sharon, E., *et al*. "Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*". *Phytopathology* 91 (2001): 687-693.

8. Safari Motlagh MR and Samimi Z. "Evaluation of *Trichoderma* spp. as biological agents in some of plant pathogens". *Annals of Bioloical Research* 4 (2013): 173-179.

9. Saifullah A and Khan NU. "Low temperature scanning electron microscopic studies on the interaction of *Globodera rostochiensis* woll. and *Trichoderma harzianum* rifai". *Pakistan Jouranl of Botany* 46 (2014): 357-361.

10. Santos MC., *et al*. "Biology, growth parameters and enzymatic activity of *Pochonia chlamydosporia* isolates from potato cyst and root-knot nematodes". *Nematology* 15 (2013): 493-504.

11. Gortari MC and Hours RA. "Fungal chitinases and their biological role in the antagonism onto nematode eggs. a review". *Mycological Progress* 7 (2008): 221-238.

12. Tikhonov VE., *et al*. "Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*". *Fungal Genetics and Biology* 35 (2002): 67-78.

13. Morton CO., *et al*. "Infection of plant parasitic nematodes by nematoghagous fungi - a review of the application of molecular biology to understand infection processes and to improve biological control". *Nematology* 6 (2004): 161-170.

14. Zeilinger S., *et al*. "Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host". *Fungal Genetics and Biology* 26 (1999): 131-140.

15. Monreal J and Reese ET. "The chitinase of *Sarratia marcescens*". *Canadian Journal of Microbiology* 15 (1969): 689-696.

16. Bradford MM. "A rapid and sensitive method for quantification of microgram". *Annals of Biochemistry* 72 (1976): 248-254.

17. Davis BJ. "Disc electrophoresis II. Method and application to human serum protein". *Annals of the New York Academy of Science* 121 (1964): 404-427.

18. Herrera-Estrella A and Chet I. "Chitinases in biological control". *Experientia Supplementum-Basel* 87 (1999): 171-184.

19. Sankaranarayanan, C., *et al*. "Parasitism of *Meloidogyne incognita* eggs by *Fusarium oxysporum* and other fungi". *Indian Journal of Nematology* 32 (2002): 1.

Volume 6 Issue 4 April 2020