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Chitinase activity of biocontrol fungi cultured from the golden potato cyst nematode, *Globodera rostochiensis*

K. Abbasi [1], R. Wick[2†] and D. Zafari[1]

[1]*Department of Plant Protection, Bu-Ali Sina University, Hamedan, Iran*
[2]*Stockbridge School of Agriculture, University of Massachusetts, Amherst, USA*

[†]Corresponding author: rlwick@umass.edu

Abstract

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 the 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*.

G*lobodera rostochiensis*, the yellow or golden cyst nematode (Wollenweber, 1923) Behrens, 1975 is globally the most devastating and widely distributed nematode pest of potato (Gitty *et al*., 2011). The use of biological agents is a compelling management strategy given the loss of nematicides and environmental concerns regarding chemical inputs in agriculture. Among biological agents, fungal antagonists have been found with good potential to control the nematodes (Sharon *et al*., 2009). *Pochonia chlamydosporia* provides good management strategies of the potato cyst nematode (Tobin *et al*., 2008) while *Paecilomyces* sp., caused 89% reduction of *G. rostochiensis* (Lopez-Lima *et al*., 2013).

Chitinases, glucanases, and proteases are considered the most important lytic mechanisms for successful biological control of nematodes (Sharon *et al*., 2001; Safari Motlagh & Samimi, 2013). Saifullah & Khan (2014) reported that *Trichoderma harzianum* enzymatically penetrated cysts and eggs of *G. rostochiensis*. Santos *et al*., (2013) demonstrated that N-acetyl-yß-glucosaminidase and proteases of *P. chlamydosporia* were involved in the 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 a 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 (Gortari & Hours, 2008). Fungal chitinases are necessary for hyphal growth and they are also

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produced by nematophagous and entomopathogenic fungi to aid infection (Tikhonov *et al*., 2002). 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 (Morton *et al*., 2004). This study aimed to screen the chitinolytic ability of fungi isolated from *Globodera rostochiensis* and to evaluate their biocontrol potential.

Materials and Methods

Fungal isolates: One hundred and fifty-four fungal isolates were encountered from infected eggs of *G. rostochiensis* in Iran in 2015 and were maintained on potato dextrose agar (PDA) at 25°C. These isolates were assayed for chitinase activity after 96 h incubation with colloidal chitin to find out the most promising isolates for biological control.

***N*-acetyl-glucosamine-dinitrosalicylate assay:** Colloidal chitin was prepared according to the method given by Tikhonov *et al*., (2002). For the liquid medium enzyme assay, isolates were grown in minimal synthetic medium (MSM) ([g l-1]: (NH4)2SO4, 2.8; urea, 0.6; KH2PO4, 4; CaCl2.2H2O, 0.6; MgSO4, 0.2; FeSO4.7H2O, 0.01; ZnSO4.H2O, 0.0028; CoCl2.6H2O, 0.0032) containing colloidal chitin (1 g l-1) (Zeilinger *et al*., 1999). After 96 h growth at 25°C the culture medium was filtered through Whatman filter paper No.3 followed by filtration through 0.2mm 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 (Monreal & Reese, 1969). 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 37ºC for 1 h. 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 (1976) 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 juveniles in 100 mm Petri dishes of water agar. For each isolate, 10 mature cysts were sterilized with 0.5% sodium hypochlorite for 3 min, rinsed in sterile water, and placed on water-agar medium at a distance of 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 the 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 analysis 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, 20 g of soaked wheat seed were placed in autoclavable nylon bags with 40 ml

water and autoclaved three times within 48 h. 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 25ºC in the dark. The seeds were mixed every 48 h to ensure thorough colonization. For the study, 20 g 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: plant height, root length, root dry weight, shoot dry weight, number of tubers, tuber yield, and number of cysts in 100g soil. Data were subjected to analysis of variance (SAS, version 9.0).

Fluorometric chitinase assay: The 10 selected isolates were assayed via 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 an 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 96 h. Optimum conditions were determined with pHs 3, 4, 5, 6, 7, 8 and 9, temperatures 22°C, 25°C, 28°C, 31°C; for 1 h, 6 h, and 24 h. 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 37ºC. The experiment was a completely randomized design with three replicates. Analysis of variance of data of the chitinase activity using software SAS, version 9.0 was done.

Protein assay: The isolates were grown in the 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; Na2HPo4, 1.42; KH2po4, 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 (1964): 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 0.3–1.2 g (1–2 M) of electrophoretically pure urea (Bio-Rad). After degassing for 1 min, 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 0.3–1.2 g of urea. After degassing for 1 min, 10 ml of TEMED and 100 ml of APS were added. The running buffer consisted of 0.5 M urea in 0.3 M glycine - 0.036 M Tris buffer (pH=8.4). The gels were run at 200 V for 45 min at 6°C. After electrophoresis, the gels were incubated in 0.1 M SA-buffer, pH=4.7, for 3 h 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. The contrast between cleared zones and background was enhanced by immersing the gel in 0.1% solution of basic fuchsin in 20 % aqueous ethanol for 1 h 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 encountered from infected eggs of the golden potato cyst nematode (Table 1).

To determine the most promising isolates for biological control of *G. rostochiensis*, all the 154 isolates were assayed for chitinase activity 96 h after growth in MSM. Results of the chitinase activity showed significant differences among the 154 isolates at the $p \geq 0.01$ 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. Ten isolates: *Alternaria alternata*, *Cylindrocarponolidum*, *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.

Fungal infection of juveniles and greenhouse trial: Significant differences ($p \geq 0.01$) 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).

There were significant differences in plant height, root length, root dry weight, 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).

Enzyme assay by fluorimetric analysis: Chitinase activity was highest after 96 h. The results showed the effects of different periods of incubation (1 d, 2 d, 3 d, 4 d, and 5 day) on the 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. The activity of endochitinase began 1 day after incubation and reached its maximum on the 4^{th} day and was lower on day 5. 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*.

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

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

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

** Significant at 0.01 probability level

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

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)	885de
T. atroviridae	990^{ab}	*C. parapsilosis* (b)	780de
F. oxysporum (a)	975^{abc}	*F. oxysporum* (b)	765^{e}
Paecilomyces sp.	975^{abc}	Control	45^{f}
A. alternata	945^{bcd}		

Table 4. Effect of selected fungi on potato plant growth and number of cysts in a greenhouse trial.

Isolate	Root length (cm)	Dry root wt (g plant^{-1})	Plant height (cm)	Dry shoot wt (g plant^{-1})	No. of tuber (tuber in plant^{-1})	Tuber yield (g plant^{-1})	No. 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}

Means followed by the same letters in each column are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

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.

Table 5. Activity of three chitinase enzymes from selected isolates. Data represent the means of three isolates.

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}	213de	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}

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 the 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 25ºC. The optimum temperature for *L. muscarium* was 31ºC. The effects of isolate, pH and the interaction between isolate and pH on the specific activity of chitinase were 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, 5and 5, respectively. The lowest specific activity for *L. muscarium* and *Paecilomyces* sp. was pH=3; and for *L. muscarium*, pH=4. For all four isolates, the maximum enzyme activity occurred after 1 h and the minimum activity was 24 h.

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*.

Discussion

The potato cyst nematode (PCN) *Globodera rostochiensis*, the yellow species (PCN) has proved to be a major economic and phyto-quarantine pest species of potatoes worldwide; moreover, it is one of the most destructive pathogens of potato in the world. Since chitin is the dominant component of the nematode egg-shell, 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 (Herrera-Estrella& Chet, 1999; Tikhonov *et al*., 2002). 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 respectively. 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 selected isolates, 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 similar to results by Tikhonov *et al*., (2002) 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. Some of these genera were earlier reported by Sankaranarayanan *et al*., (2002), Safari-Motlagh & Samimi (2013) and Saifullah & Khan (2014) as important biocontrol agents of *G. rostochiensis*.

In this study, the biological control of *G. rostochiensis* by several fungal isolates showed that there is a good correlation between chitinase activity and biocontrol potential.

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