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Production of Chitinase and β-1,3-glucanase by *Trichoderma harzianum* for Control of the Phytopathogenic Fungus *Sclerotium rolfsii*

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Summary

Twenty four isolates of *Trichoderma* were screened for β-1,3-glucanase and chitinase activity. Out of these organisms, a strain identified as *T. harzianum* Rifai secreted highest activities. *In vitro* production of chitinases and β-1,3-glucanases by *T. harzianum*, a mycoparasite of phytopathogenic fungi, was examined under various culture conditions. Enzymes production was significantly influenced by the carbon source incorporated into the medium and was stimulated by acidic pH from 5.5 to 6.0. Glucose or GlcNAc (0.5 %) addition along with chitin for chitinase and laminarin for β-1,3-glucanase, repressed production of these enzymes, while the polysaccharides as sole carbon source enhanced production of the respective enzymes. Production of both enzymes was also enhanced by polysaccharides contained in the mycelium of *S. rolfsii. T. harzianum* culture filtrates, possessing chitinase and β-1,3-glucanase activities, were capable of hydrolysing dried or fresh mycelium of the phytopathogenic fungus *S. rolfsii*. Growth of *S. rolfsii* was significantly inhibited (up to 61.8 %) by enzyme preparations from *T. harzianum*.

Key words: Trichoderma harzianum, chitinase, β-1,3-glucanase, antagonism, *Sclerotium rolfsii*, inhibition

Introduction

Intensified use of fungicides has resulted in the accumulation of toxic compounds potentially hazardous to humans and environment *(1)* and also in the buildup of resistance of the pathogens *(2)*. In order to tackle these national and global problems, effective alternatives to chemical control are being investigated and the use of antagonistic microbes seems to be one of the promising approaches *(3)*. Antagonism may be accomplished by competition, parasitism, antibiotics, or by a combination of these modes of action *(4,5)*. Parasitism involves the production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi *(6)*. The importance of β-1,3-glucanase and chitinase as key enzymes responsible for fungal cell and sclerotial wall lysis and degradation has been reported *(1)*. These enzymes have been shown to be produced by several fungi and bacteria and may be an important factor in biological control *(7–9).*

Various modes of action have been associated with the ability of *Trichoderma* spp. to control plant pathogens *(10)* including substrate competition, the ability to colo-

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nize the ecological niche favored by the pathogen, antagonism by antibiotics *(11)* or cell-wall degrading enzymes *(12)*. Several fungicides based on *Trichoderma* spp. have been commercialized in the last few years. However, there is still considerable interest in finding more efficient mycoparisitic fungi especially within *T. harzianum* strains, which differ considerably with respect to their biocontrol effectiveness *(8)*.

The plant pathogen basidiomycete *S. rolfsii* causes stem and pod rots which are major constraints to groundnut production in many groundnut-growing regions: *e.g.* in the USA southern blight of peanuts is a problem in all peanut-producing states and has to be controlled primarily by the use of fungicides. Furthermore, the fungus causes disease in over 500 plant species. Recently, the fungus has also been found in Europe on different hosts including juglans and sunflowers *(13,14)*.

With a view to establish that the mechanism in the process of parasitism of *S. rolfsii* by a newly isolated strain of *T. harzianum* involves the release of hydrolytic enzymes by the latter, the objectives of this study were *(i)* to determine the physiological conditions which stimulate the *in vitro* production of chitinases and β-1,3-glucanases and *(ii)* to assess the role of these enzymes in parasitism and in antagonism.

Materials and Methods

Microorganisms and cultivation

Trichoderma spp. (24 isolates) and the phytopathogenic fungus (*Sclerotium rolfsii*) were obtained from the Botany Department collection, Faculty of Science, University of Minia, Egypt. Isolates T18, T21, T3 and T24 were identified as *T. pseudokoningii* Rifai, *T. koningii* Oudemans and two different strains of *T. harzianum* Rifai, respectively, by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Potato dextrose agar (PDA; Biolife) was used to maintain cultures of pathogenic and antagonistic isolates. The minimal synthetic medium (MSM) used for the cultivation of *Trichoderma* spp. contained the following components (in grams per liter): $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4 \cdot 7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source (0.5 %) and the pH was set to 6.3 (50 mM phosphate buffer).

Preparation of dried mycelium

Erlenmeyer flasks (250 mL) containing 100 mL of potato dextrose broth were incubated with 1 cm^2 discs of potato dextrose agar (PDA) of actively growing mycelium of *S. rolfsii.* The inoculated flasks were incubated at 30 °C for 7 days. The mycelium was then collected by filtration through Whatman No.1 filter paper, washed with distilled water and homogenized in distilled water using a laboratory homogenizer (VIRTIS 23, Virtis Company, Gardener N.Y. 12525, Germany). The suspension was centrifuged three times (6000 x g for 10 min) after washing with distilled water. The mycelium was stored in a lyophilized state and used as C-source.

Screening of Trichoderma isolates for enzyme production

Spore suspension inoculum of all *Trichoderma* spp. (1.0 x 10^6 spores per mL of culture medium) were used and inoculated into duplicate 100 mL flasks containing 20 mL of unbuffered mineral synthetic medium (MSM) supplemented with dried mycelium as the sole carbon source (5 g L^{-1}). The cultures were grown at 30 °C for 5 days without shaking. Culture filtrates were centrifuged at 4 °C for 10 min at 5000 x g and the clear supernatants were either immediately tested for enzyme activity or stored at –20 °C until assayed.

Conditions for enzyme production by T. harzianum Rifai (T24)

T. harzianum was grown on buffered MSM medium (phosphate buffer pH=6.3) supplemented with 0.5 % of the appropriate carbon source (chitin or laminarin; Sigma), inoculated with agar discs inoculum (3 discs, 5 mm/10 mL buffered liquid culture medium) of cultures grown on different agar-plates (PDA, Czapeks-agar, Malt extract-agar and Yeast extract-agar). Cultures were incubated at 150 rpm on a rotary shaker for 5 days at 30 °C and harvested as described above. In subsequent experiments the effect of shaking (0, 50, 100, 120 and 150 rpm), inoculum age (2–8 days) and incubation time were investigated.

Effect of cultivation conditions on enzyme production

T. harzianum was cultivated on MSM medium supplemented with 0.5 % of the substrates tested. Combination of crushed chitin + glucose (0.5 % + 0.5 %), chitin + GlcNAc (0.5 % + 0.5 %) and uncrushed chitin were tested for chitinase production, and a combination of laminarin + glucose (0.5 % + 0.5 %) for β-1,3-glucanase. Subsequently, various concentrations of chitin or laminarin (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 %) were compared and *S. rolfsii* dried (0.5 %) or fresh (5 %) mycelium were investigated as carbon sources. The effect of different nitrogen sources (organic or inorganic) on chitinase and β-1,3-glucanase production by *T. harzianum* was tested in MSM containing chitin or laminarin (0.5 %). Nitrogen sources were added to the medium at a concentration equivalent to 0.339 Ng/L, whereas, urea, peptone-casein and corn steep solid were supplied at 0.5 % concentration. To study the effect of pH the initial pH of the medium was adjusted to values from 4 to 8 using 50 mM of acetate buffer (4 to 5.5 pH) or phosphate buffer (6–8.5 pH). To study the effect of temperature, the fungus *T. harzianum* was grown on MSM pH=6.0 for chitinase and pH=5.5 for β-1,3-glucanase at 20, 25, 30, 35, 40, 45 and 50 °C.

Enzyme activity assays

Chitinase activity was assayed using the colorimetric method described by Molano *et al. (15)* with minor modifications *(16)*. The assay mixture contained 1 mL of 0.5 % pure chitin (Sigma, suspended in 50 mM acetate buffer pH=5.2) and 1 mL of enzyme solution. The reaction mixture was incubated for 7 h at 37 °C with shaking and was stopped by centrifugation (5000 g/min) for 10 min and the addition of 1 mL of dinitrosalicylate

(DNS) reagent *(17)*. β-1,3-glucanase was assayed similarly incubating 500 μL of 5.0 % (w/v) laminarin in 50 mM acetate buffer (pH=4.8) with 200 μL enzyme solution at 45 °C for 30 min and determination of the reducing sugars with DNS *(17)*. The amount of reducing sugars released was calculated from standard curves for GlcNAc and glucose, and the activities of chitinase and β-1,3-glucanase were expressed in pkat (pmol/s) and nkat (nmol/s), respectively.

Hydrolytic activity of T. harzianum culture filtrate

To test the hydrolytic activity of the culture filtrate of *T. harzianum* on the phytopathogenic fungus *S. rolfsii*, the mycoparasite was grown for 5 days on MSM containing chitin as carbon source. Culture filtrates were incubated with phytopathogenic fungus dried (1 mg/mL) or fresh (10 mg/mL) mycelium or chitin (1 mg/mL) in 100 mL flask and the release of reducing sugars (Glucose, GlcNAc) was monitored. 100 μL of toluene was added to the reaction mixture before incubation at 37 °C for 24 h. GlcNAc reducing sugars were measured in 1 mL of the mixture according to Reissig *et al. (18)* using p-dimethylamino-benzaldehyde (DMAB) reagent. Total reducing sugars were measured using DNS *(17)*. Heat-inactivated culture filtrate containing fungal mycelia were used as controls.

Antifungal activity of T. harzianum metabolites in culture filtrates

Agar plates (PDA; 10 % volume fraction) were prepared with *T. harzianum* culture filtrates (sterilized by filtration) or with water (control). *S. rolfsii* was inoculated in the center of agar plates using 5-mm mycelial discs and incubated at 30 °C for 3 days. The radial diameter of the colonies was measured at right angles every day, for six replicate plates per treatment, measuring daily growth rate, and percent of inhibition was calculated.

Results

Out of 24 fungal isolates the best enzyme producer was T24 identified as *T. harzianum* Rifai which exhibited highest activities of both chitinase (29.3 pkat/mL) and β-1,3-glucanase (5.70 nkat/mL; Table 1). This strain was thus used for all further experiments and referred to as *T. harzianum.*

Inoculation of shake flasks with agar disks gave higher enzyme activities from *T. harzianum* than spore suspensions. The best medium for pre-cultures was PD-Agar giving 1.4-3.5 times more of chitinase and 1.1–2.5 times more of β-1,3-glucanase than any other agar-plate used under the same conditions (Table 2). The most favorable inoculum age for shaken flasks was three days, older inoculum lead to decrease in the enzyme activities. Chitinase and β-1,3-glucanase production was significantly influenced by the shaking speed and best results were obtained at 150 rpm.

Production of chitinase using a medium containing chitin reached a maximum after 7 days of growth and decreased thereafter (Table 2). In contrast, synthesis of β-1,3-glucanase increased rapidly after 24 h peaking at the fourth day. Maximum chitinase activity (7 days) and β-1,3-glucanase activity (4 days) were always reached after the same period of time irrespective of the carbon source used (data not shown). The temperature optimum for the production of both enzymes was around 30 °C and practically no growth was observed above 40 °C (Table 2). Highest chitinase production on chitin was measured when the initial pH was set to 6.0 while the optimum for β-1,3-glucanase production on laminarin was at pH=5.5 (Fig. 1). Out of several carbon sources tested highest chitinase activity was found in cultures supplied with crushed chitin (59.8 pkat/mL, Fig. 2), while uncrushed chitin gave lower activities (37.4 pkat/mL).

The levels of chitinase found when *T. harzianum* was incubated with chitin were more than twofold higher than with glucose and growth on N-acetylglucosamine (GlcNAc) resulted in lower enzyme production than

Table 1. Screening of 24 isolates of *Trichoderma* spp. for chitinase and β-1,3-glucanase activities

Isolate No.	Chitinase activity	β-1,3-Glucanase activity
	pkat/mL	nkat/mL
T1	4.3 ± 0.7	0.83 ± 0.1
T2	11.9 ± 0.8	0.86 ± 0.1
T3	18.7 ± 1.1	1.25 ± 0.2
T4	6.7 ± 0.9	1.10 ± 0.1
T5	18.7 ± 1.2	1.11 ± 0.1
T6	10.4 ± 1.0	0.94 ± 0.2
T7	14.6 ± 0.9	0.83 ± 0.1
T8	9.6 ± 0.5	1.06 ± 0.1
T9	10.9 ± 0.7	1.22 ± 0.1
T10	10.4 ± 0.6	0.89 ± 0.2
T11	13.9 ± 1.1	1.08 ± 0.1
T12	10.8 ± 0.6	0.75 ± 0.1
T13	10.3 ± 0.9	0.83 ± 0.1
T14	13.7 ± 1.2	0.89 ± 0.2
T15	10.1 ± 0.7	0.89 ± 0.2
T16	14.7 ± 0.8	0.78 ± 0.1
T17	19.0 ± 1.0	0.86 ± 0.1
T18	19.5 ± 0.9	0.89 ± 0.1
T19	11.7 ± 0.8	0.61 ± 0.1
T20	8.3 ± 0.7	1.11 ± 0.2
T21	12.6 ± 0.8	1.14 ± 0.1
T22	11.6 ± 0.9	0.83 ± 0.1
T23	12.3 ± 0.8	0.83 ± 0.1
T24	29.3 ± 1.2	5.70 ± 0.2

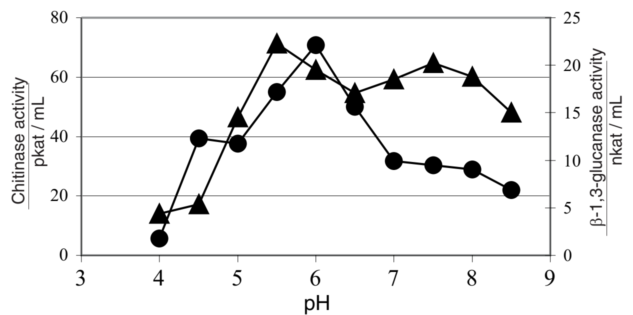

Fig. 1. Effect of initial pH on chitinase and β-1,3-glucanase production (using 0.5 % chitin or laminarin as carbon source, respectively) by *T. harzianum* (● chitinase, ▲ β-1,3-glucanase)

was obtained with other non-chitinous substrates. Chitinase production was higher in the presence of glucosamine (GlcN)-containing medium than in media containing GlcNAc. Repression was highest when glucose (5 %) or GlcNAc (5 %) was added to the chitin-containing medium at the onset of the experiment (14.8 and 13.9 pkat/mL, respectively, data not shown). Chitinase synthesis was repressed when other polymers such as cellulose and chitosan were used as a substrate. In the presence of chitin as sole carbon source, *T. harzianum* produced nearly three times more chitinase than in the presence of laminarin. For β-1,3-glucanase, laminarin (β-1,3-glucan) was the most favorable substrate. The production of β-1,3-glucanase decreased by 41.5 % when laminarin was combined with glucose (8.6 nkat/mL, data not shown). In the presence of laminarin as sole carbon source, *T. harzianum* produced nearly one and a half times higher activity of β-1,3-glucanase than in the presence of chitin. Absence of carbon source gave zero chitinase activity, whereas, some of β-1,3-glucanase was produced without carbon source (2.1 nkat/mL). Raffinose as carbon source gave the lowest activity of both chitinase or β-1,3-glucanase (Fig. 2).

Chitinase and β-1,3-glucanase production depended on substrate concentration and above 1 % (w/v) chitin there was no further increase of chitinase synthesis, whereas, β-1,3-glucanase synthesis reached a peak at 0.75 % laminarin (Fig. 3).

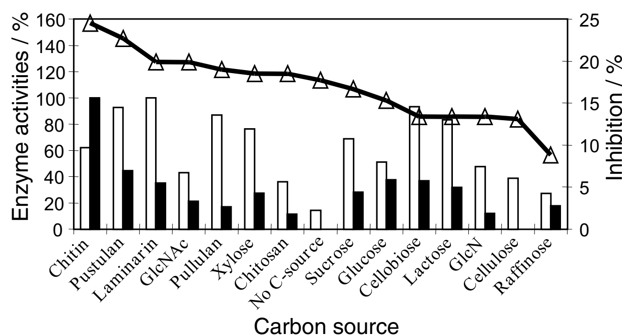

Fig. 2. Effect of different carbon sources on chitinase and β-1,3-glucanase production by *T. harzianum*, and on inhibition of *S. rolfsii* (100 % β-1,3-glucanase activity correspond to 14.7 nkat/mL and 100 % chitinase activity correspond to 59.8 pkat/mL) (❑ β-1,3-glucanase, ■ chitinase, Δ % inhibition)

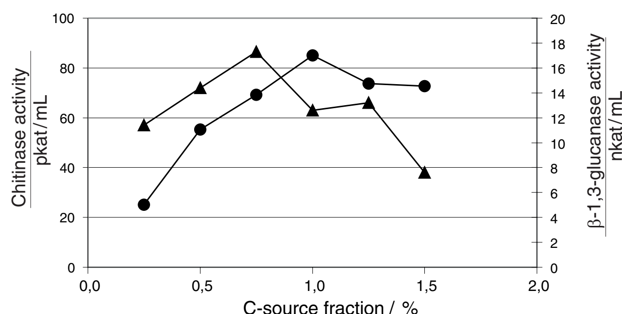

Fig. 3. Effect of chitin and laminarin concentration on chitinase and β-1,3-glucanase production by *T. harzianum*, respectively (● chitinase, ▲ β-1,3-glucanase)

Table 2. Consecutive improvement of *T. harzianum* culture conditions* for chitinase and β-1,3-glucanase production by *T. harzianum*

Parameters	Chitinase activity %	β-1,3-Glucanase activity %
Inoculum:		
Agar	100	100
Spores	87.4	98.2
Agar plate:		
PDA	100	100
Czapeks-A	51.2	94.2
Malt-A	73.0	96.3
Yeast-A	28.9	39.7
Shaking:		
No shaking	38.6	80.8
50 rpm	74.0	45.5
100 rpm	91.9	87.0
120 rpm	95.2	89.4
150 rpm	100	100
Inoculum age/day:		
2	67.1	32.0
3	100	100
4	63.6	56.1
5	64.3	45.7
6	61.5	56.3
7	57.0	58.8
Incubation/day:		
1	07.0	14.5
2	39.4	37.7
3	42.4	60.9
4	43.7	100
5	52.3	90.6
6	74.9	65.5
7	100	61.4
8	92.8	58.6
9	82.1	62.1
10	64.2	53.4
Incubation temp./°C		
20 °C	24.4	38.3
25 °C	93.8	93.7
30 °C	100	100
35 °C	31.0	10.2
40 °C	16.3	7.3
Nitrogen source:		
No N-source	57.4	7.9
$NaNO_3$	58.4	66.7
NH_4NO_3	75.1	77.1
$(NH_4)_2SO_4$	75.5	58.0
NH_4Cl	55.8	59.7
Urea	–	65.0
Peptone-casein	32.5	100
Corn steep solid	100	90
NH_4-tartrate	58.7	54.4

* For carbon sources, pH see Figs. 1–3

Corn steep solid, was the most stimulative for chitinase production, followed by $(NH_4)_2SO_4$ or NH_4NO_3 (Table 2). Peptone-casein gave the least degree of enzyme activity, whereas urea gave no enzyme activity when

used as a nitrogen source. In contrast, peptone-casein was the best nitrogen source for β-1,3-glucanase production, followed by corn steep solid and then NH_4NO_3 (Table 2). A significant activity of chitinase was produced by the fungus in culture medium amended with chitin as carbon source and absence of nitrogen source (no N-source).

Significant activities of β-1,3-glucanase and chitinase were produced by *T. harzianum* in culture media amended with dried or fresh mycelium of *S. rolfsii* (Fig. 4). Fresh mycelium of *S. rolfsii* was more preferable than dried mycelium. Levels of enzyme production by *T. harzianum* started to decrease after the fourth day of incubation when fresh mycelium of the phytopathogenic fungus was used as carbon source, whereas the levels of enzymes increased when *T. harzianum* was incubated with dried mycelium of the phytopathogenic fungus at the same period. Levels of enzyme in the presence of the mycelium of phytopathogenic fungus as carbon source reached nearly the same levels as in the presence of chitin or laminarin (chitinase or β-1,3-glucanase, respectively).

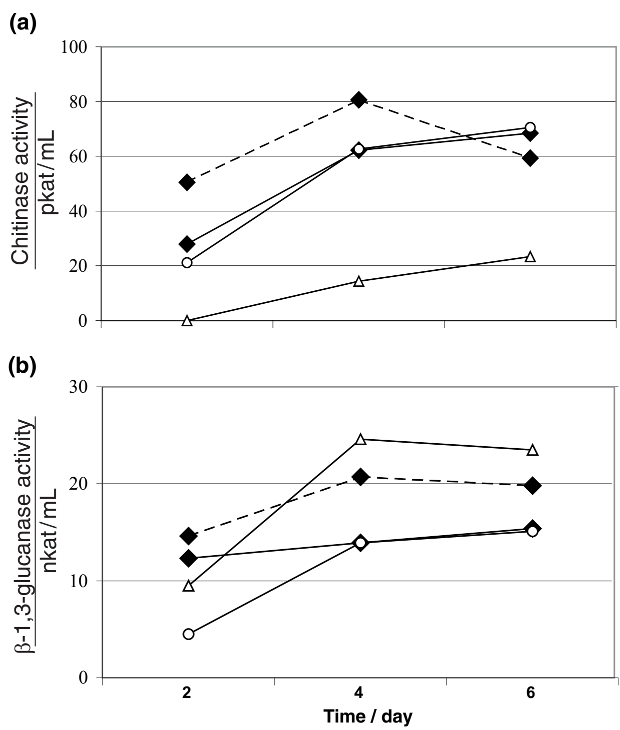

Fig. 4. Effect of incubation time and different carbon sources on chitinase (a) and β-1,3-glucanase (b) production by *T. harzianum* (- -◆- - *S. rolfsii* dried mycelium, —◆— *S. rolfsii* fresh mycelium, -○- Chitin, Δ Laminarin)

Incubation of fresh or dried mycelium of the phytopathogenic fungus *S. rolfsii* with *T. harzianum* culture filtrates resulted in a high release of reducing sugars (Fig. 5). *S. rolfsii* dried or fresh mycelium was very sensitive to hydrolysis by *T. harzianum* crude enzymes.

Different carbon sources for the cultivation of *T. harzianum* had noticeable effects on colony diameter of *S. rolfsii*, when grown on a medium incorporating *T. harzianum* culture filtrates (Fig. 2). Of the tested carbon sources (0.5 %), *S. rolfsii* growth was most restricted (24.5 %) by enzymes produced on chitin-containing medium, whereas raffinose carbon source gave the least degree of inhibition (8.8 %). 1 % chitin concentration in the cultivation medium increased the inhibition to 33.7 % and a sample with heat inactivated enzymes gave still 27.5 % inhibition (data not shown). Concentration (5x) of the culture filtrate by ammonium sulfate precipitation (75 %) increased inhibition to 61.8 and 45 % for a sample with heat inactivated enzymes (data not shown).

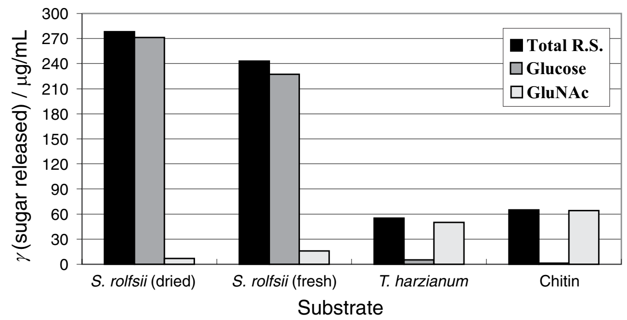

Fig. 5. Release of total reducing sugars (R.S.), glucose and N-acetyl glucosamine from *S. rolfsii* (dried and fresh mycelium), *T. harzianum* and chitin by the *T. harzianum* enzymes

Discussion

A wide range of prokaryotic and eukaryotic microorganisms have the potential to produce cell-wall-degrading enzymes when chitin or isolated fungal cell wall material are present in the growth medium *(19,20)*. The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic fungi *(21–23)*. *Trichoderma* spp. attach to the host hyphae by coiling, hooks or apressorium-like structures and penetrate the host cell walls by secreting hydrolytic enzymes such as a basic proteinase *(24)*, β-1,3-glucanase and chitinase *(25)*.

Production of extracellular β-(1,3)-glucanases, chitinases, and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls *(24,26,27)*. These observations, together with the fact that chitin, β-1,3-glucan and protein are the main structural components of most fungal cell walls *(28)*, are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens *(29)*. *Trichoderma* spp. (especially *T. harzianum* and *T. viride)* exhibit considerable variability among strains with respect to their biocontrol activity and host range *(30)*. Thus, new strains, which are more effective in biocontrol, could have considerable industrial potential. Screening twenty-four *Trichoderma* isolates we found a strain identified as *T. harzianum* Rifai to be the best producer of both chitinase and β-1,3-glucanase.

The physiological age of the mycelium used for inoculation of shake flasks seems to be an important factor for enzyme production. Chitinase and β-1,3-glucanase production by *T. harzianum* was highest by using 3-day-old mycelium as inoculum. Ulhoa and Peberdy *(31)* found in their study on chitinase production using washed mycelium of another *T. harzianum* strain that both mycelium harvested at 18 h (exponential phase) and at 24 h (early stationary phase) showed similar production of extracellular chitinase. In our results, enzyme production using agar discs was higher than using spore suspensions of *T. harzianum,* which is in agreement with the results from Seyedi-Rashti *(32)* on the xylanase production by *T. harzianum.*

Most of the chitinolytic enzyme systems reported in the literature are inducible *(31,33,34)*. Monreal and Reese *(34)* suggested that the most probable inducers of chitinase in *Serratia marcescens* are soluble oligomers derived from chitin, but not the monomer (GlcNAc). Ulhoa and Peberdy *(31)* suggested that products of chitin degradation also regulate chitinase synthesis in *T. harzianum* 39.1. In agreement with these findings we found high chitinase activity only in cultures supplied with chitin but not with other polymers such as cellulose and chitosan, which is further indicative of induction. Interestingly, removal of the acetyl group produces a non-susceptible substrate (chitosan) and substitution of the N-acetyl group with OH (= cellulose) similarly yields a non-substrate. Previously, in *T. harzianum* 39.1 neither chitobiose nor N-acetylglucosamine promoted enzyme production *(31)*.

It was found that chitinase activity increased with increasing chitin concentration up to 1 %. Ulhoa and Peberdy *(31)* suggested that chitinase production was substrate concentration dependent, above 0.5 % (w/v) chitin there was no further promotion of synthesis. Elad *et al. (8)* reported that chitinase secretion into the growth medium by *T. harzianum* was increasing up to concentrations of 1 %.

Chitinase production decreased with glucose or GlcNAc (0.5 %) addition along with 0.5 % chitin. This is consistent with the results obtained by Mahadevan and Crawford *(35)*, who found that various hexoses and pentoses repressed enzyme production by *Streptomyces lydicus* WYEC108. The same results were obtained for β-1,3-glucanase production when glucose (0.5 %) was added along with 0.5 % laminarin, which decreased the activity to nearly 50 % when compared to laminarin alone. Production of β-1,3-glucanase under otherwise inducing conditions was inhibited by the addition of glucose *(36)*.

The enzyme β-1,3-glucanase is commonly produced by fungi as a constitutive enzyme *(37)*. Results of this study indicated highest production of β-1,3-glucanase in the presence of laminarin (β-1,3-glucan), pustulan (β-1,6-glucan) and pullulan (1,6-glucan) in descending order of efficacy, suggesting that the induction patterns of the enzyme may vary in response to the glucan structure and that β-1,3-glucanase induction depends on the type of linkage *(36)*. Significant activities of enzyme were secreted in the presence of cellobiose, lactose or xylose. Enzyme production increased up to 0.75 % of laminarin concentration, but decreased at higher concentrations. β-1,3-glucanase was in contrast to chitinase, only partially repressed in the presence of sucrose or glucose, suggesting that a certain activity of this enzyme is produced constitutively. Elad *et al. (8)* reported that *T. harzianum* secreted β-1,3-glucanase when grown on laminarin or starch (as a component of wheat bran) and not on glucose, concluding that the enzyme is produced inductively.

A low level of β-1,3-glucanase and no chitinase was measured when the mycoparasite was deprived of a carbon source (no C-source) and its growth was low. Although the exact reasons for this remain to be established, it seems probable that in conditions of carbon starvation and poor growth the fungus actively secrets some levels of hydrolytic enzymes. This type of control has been demonstrated in other fungi such as *Neurospora crassa (38)* and *S. glucanicum (39)*. Alternatively, the production of β-1,3-glucanase in carbon-deprived media could be related to metabolism, such as mobilization of wall glucans *(38)* and to some morphogenetic functions and changes *(40)*. No production or low level of chitinase activity in deprived carbon source or glucose medium confirms that chitinase enzyme is produced inducibly not constitutively.

Chitinase and β-1,3-glucanase production was favored by acidic pH (pH=6.0 and 5.5, respectively). Acidic pH was also reported to be an important growth parameter in the production of chitinases and β-1,3-glucanases in mycoparasite *T. harzianum (8)* and the thermophilic *Streptomyces* spp. *(41)*, respectively. Ulhoa and Peberdy *(31)* found that the production of chitinase was markedly affected by pH, with the optimum at pH=6.0.

Levels of enzyme production by *T. harzianum* incubated with fresh or dried mycelium of *S. rolfsii* reflected the high content of chitin and glucan in the cell wall of that phytopathogenic fungus. Levels of enzymes produced by *T. harzianum* decreased within six days of incubation with fresh mycelium; however, there was a continuous increase in enzymes production with dried mycelium. This effect might be a result of some metabolites produced by the phytopathogen against the antagonist.

Crude culture filtrates of *T. harzianum* possessing β-1,3-glucanase and chitinase activities had the ability to release reducing sugars (glucose, GlcNAc) from dried or fresh mycelium of the phytopathogenic fungus *S. rolfsii*. These results are in agreement with those of Tweddell *et al. (42)*, who demonstrated that lytic enzymes of *Stachybotrys elegans* degrade *R. solani* mycelium. The culture filtrates of *T. harzianum* showed lytic activity on purified cell walls of *Saccharomyces cerevisiae* and *Botrytis cinerea (43)*. Also, "autolysis" of *T. harzianum* (fresh mycelium) might be the reason of released reducing sugars.

Culture filtrates have been used by Calistru *et al. (44)* to demonstrate the possible presence and role of fungal metabolites in the process of antagonist behavior of *Trichoderma* spp. Doi and Mori *(45)* reported antifungal potential of culture filtrates of two *Trichoderma* spp. on wood decay fungi and filtrates of *T. harzianum* were found to be suppressive of the white-rot pathogen, *S. cepivorum (46)*. In this study, culture filtrates of *T. harzi-*

anum inhibited the growth rate of *S. rolfsii*. Calistru *et al. (44)* suggested that *Trichoderma* culture filtrates had only marginally curtailed pathogen growth, in contrast with, *e.g.* metabolites of *Gliocladium virens*, which completely inhibited the growth of *S. cepivorum (47)*. Obviously, in the 1:10 dilution used, *Trichoderma* metabolites did not have any inhibitory effect. The same dilution was used in this study and inhibition with *T. harzianum* reached more than 30 %. The macroscopic and microscopic (SEM) observations, of plates with the pathogens cultured with filtrates of *Trichoderma* spp. suggested that *Trichoderma* spp. were able to induce morphological alterations in both *Aspergillus flavus* and *Fusarium moniliforme (44)*.

Inhibition of *S. rolfsii* depended on the carbon source used and correlated with the level of chitinase activity, rather than β-1,3-glucanase activity (Fig. 2). However, there was still some inhibition observed at low levels of both chitinase and β-1,3-glucanase. Previously, a purified endochitinase has shown antifungal activity; however, strong synergism was observed with chitobiosidase, β-1,3-glucanase and also with mycotoxic metabolites *(48)*. Future investigations will focus on the roles of mycotoxic substances secreted by the new *T. harzianum* isolate to study the synergisms between these compounds and enzymes in the biocontrol process.

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Trichoderma harzianum proizvođač hitinaze i β-1,3-glukanaze za suzbijanje fitopatogene gljivice *Sclerotium rolfsii*

Sažetak

U dvadesetčetiri izolata vrste *Trichoderma* ispitana je aktivnost β-1,3-glukanaze i hitinaze. Od ispitivanih izolata, soj utvrđen kao *T. harzianum* Rifai pokazuje najveću aktivnost. Proizvodnja hitinaze i β-1,3-glukanaze *in vitro* s pomoću *T. harzianum*, koji je mikoparazit fitopatogenih gljiva, ispitana je pri različitim uvjetima uzgoja. Na proizvodnju enzima znatno je utjecao izvor ugljika u podlozi, te kiseli pH u rasponu od 5,5 do 6,0. Dodatak glukoze ili GlcNAc (0,5 %), zajedno s hitinom za hitinazu i laminarinom za β-1,3-glukanazu, smanjivao je proizvodnju tih enzima, dok su polisaharidi, kao jedini izvor ugljika, omogućili njihovu veću proizvodnju. Polisaharidi koji se nalaze u miceliju *S. rolfsii* povećavaju proizvodnju obaju enzima. Filtrati kulture *T. harzianum* s hitinaznom i β-1,3-glukanaznom aktivnosti mogli su hidrolizirati i suhi i svježi micelij fitopatogene gljivice *S. rolfsii.* Enzimski pripravci iz *T. harzianum* inhibiraju rast *S. rolfsii* do 61,8 %.