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Effect of Benomyl on Chitinase and β-1,3-Glucanase Production by Free and Alginate Encapsulated *Trichoderma harzianum*

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Summary

On PDA-benomyl plates growth of *Trichoderma harzianum* was inhibited by 20 and 30 % at benomyl 1 and 2 μg/mL, respectively, and was completely inhibited at 5 μg/mL. In minimal synthetic medium (MSM) amended with different concentrations of benomyl (1.0, 3.0, 5.0, 7.0 and 10.0 μg/mL), fungal immobilisation improved chitinase and β-1,3-glucanase production at low benomyl concentrations (1, 3 and 5 μg/mL). Further increase in the production of both enzymes was obtained by immobilisation at higher benomyl concentrations (7 and 10 μg/mL). Fungal immobilisation increased bound chitinase by 15- to 30-fold at 3 and 5 μg/mL benomyl concentration, respectively. However, no effect was obtained on the bound β-1,3-glucanase. Different benomyl concentrations (0.3 to 1500 μg/mL) had no significant inhibitory effect on the activities of free or immobilised chitinase and β-1,3-glucanase. It could be suggested that either immobilised *Trichoderma* or immobilised chitinase and β-1,3-glucanase could be used in combination with benomyl to control plant pathogens.

Key words: *Trichoderma* spp., biocontrol, chitinase, glucanase, benomyl, fungicides, immobilisation, Ca-alginate

Introduction

Trichoderma is a fungus widely distributed all over the world. It is present nearly in all soils and is antagonistic against some soil-borne plant pathogens (*1–3*). Cultural practices like organic matter amendment and application of pesticides affect the survival and activity of *Trichoderma* in soil (*4,5*).

Benomyl is a potent benzimidazole fungicide [1-(butylcarbamoyl)-2-benzimidazole carbamate]. It is used for the control of many fungal plant diseases (*6*). Benomyl functions in the same way as methyl benzimidazole carbamate (MBC) fungicides by binding to fungal β-tubulin and preventing microtubular polymerisation (*7*), thus interfering with a number of cellular processes (*e.g.* mitosis, meiosis, intracellular transport of molecules or maintenance of cell shape). Moreover, carbendazim, the breakdown product of benomyl, inhibits tubulin function, which is crucial for fungal growth (*8*). The deleterious effect of benomyl on arbuscular mycorrhizae (AM) was previously reported (*9–11*). Benomyl inhibited fungal alkaline phosphatase activity of both internal and external hyphae of AM at low application level (1 μg/g soil) (*12*).

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Immobilisation of microbial cells and enzymes has become one of the most valuable tools in the field of biotechnology (*13*). Moreover, microbial entrapment gives prolonged metabolic activity when reusing the microbial cells and protects the organism from inhibitory compounds or metabolites (*14–16*).

Recently, El-Katatny *et al.* (*17*) have reported that alginate encapsulation of *Trichoderma* improved chitinase and β-1,3-glucanase production. To our knowledge no information is available on the effect of benomyl on chitinase and β-1,3-glucanase production by *Trichoderma* spp. Therefore, the objectives of this study were to measure the effect of benomyl on growth and production of chitinase and β-1,3-glucanase by *Trichoderma* and to determine if alginate encapsulation affects enzyme activity.

Materials and Methods

Trichoderma isolates

Five local isolates of *Trichoderma* spp. used in this study were isolated by Shaban (*2*) from the samples of soil collected from El-Minia Governorate, Egypt. Four isolates were identified by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany): T3 and T24 as *T. harzianum* Rifai, T18 as *T. pseudokoningii* and T21 as *T. koningii*. Isolate T23 (*Trichoderma* sp.) has not been identified at the species level. Fungal cultures were maintained on potato dextrose agar (PDA) at 4 °C.

Sensitivity of Trichoderma to benomyl on PDA plates

In vitro fungistatic activity of benomyl was studied on PDA (20 mL per 9 cm diameter Petri plates). Benomyl from Sigma was suspended in sterile distilled water and 1, 2 and 5 μg/mL were added to the medium after autoclaving, resulting in PDA-benomyl plates. Agar disks (5 mm diameter) of 6-day-old colonies of *T. harzianum* (T3) and *T. harzianum* (T24) grown on benomyl-free PDA were transferred to the center of PDA-benomyl plates. Colony radii were measured at 3 and 7 days in three replicates. Unamended PDA plates with benomyl were used as control.

Chitinase and β-1,3-glucanase production by free and immobilised Trichoderma isolates in batch culture fermentation

Production of inocula, microencapsulation and batch culture fermentation were carried out as described earlier (*16,17*). Chitin (0.5 %) was used in the cultures as sole carbon source. Both free and alginate-encapsulated cultures of *Trichoderma* isolates were screened for chitinase and β-1,3-glucanase production.

To study the effect of benomyl on enzyme production by *T. harzianum* (T3), benomyl was supplemented to the autoclaved culture medium at final concentrations of 0.0, 1.0, 3.0, 5.0, 7.0 and 10.0 μg/mL. Culture filtrates were collected after 4, 8 and 12 days of incubation. Bound chitinase and β-1,3-glucanase (intracellular enzymes) within the alginate encapsulated cells were measured after 12 days of incubation. Pellets were collected by filtration, re-suspended in 5.0 mL acetate buffer (pH=5), crushed in a mortar and then sonicated for 3 min with a cell disrupter (Cole-Parmer Ultrasonic Homogenizer-4710) operating at 50 W. The suspension was centrifuged and the bound enzyme activity was measured in the supernatant.

Effect of benomyl on enzyme activities

The effect of benomyl on the activity of free or immobilised chitinase and β-1,3-glucanase preparations was investigated. Crude enzyme was entrapped in calcium alginate using the same method described earlier. Immobilised enzyme was incubated in the presence of different concentrations of benomyl (0.3, 3, 30, 300 and 1500 μg/mL), and the retained enzyme activity was measured. Incubation was carried out for 10 min at 37 and 45 °C for chitinase and β-1,3-glucanase, respectively.

Methods used for the assay of chitinase (*18,19*) and β-1,3-glucanase (*17*) activities were described earlier.

Results

Sensitivity of Trichoderma isolates to benomyl

On PDA-benomyl plates, the growth of two *T. harzianum* (T3 and T24) isolates was inhibited by 20 and 30 % of benomyl 1 and 2 μg/mL, respectively, compared to the growth in the absence of benomyl after 7 days of incubation. However, the fungal growth was completely inhibited in the presence of benomyl 5 μg/mL.

Screening of Trichoderma isolates for enzyme production

All *Trichoderma* isolates were analysed for chitinase and β-1,3-glucanase production in minimal synthetic medium (MSM) containing chitin as a sole carbon source using free or immobilised spores (Table 1). No significant difference was observed between the different isolates, either in the free or immobilised form, for β-1,3-glucanase production. On the other hand, fungal immobilisation improved chitinase production when compared to the free cultures. T3 was the most potent isolate for chitinase production. Therefore, it was selected for further investigation.

Table 1. Screening for chitinase and β-1,3-glucanase production by free or immobilised *Trichoderma* cultures

Isolates	Chitinase activity/(pkat/mL)		β-1,3-glucanase activity/(nkat/mL)	
	Free	Immobilised	Free	Immobilised
T. harzianum (T3)	3.00	7.5	0.33	0.44
T. harzianum (T24)	1.12	4.0	0.36	0.42
T. pseudokoningii (T18)	1.10	3.9	0.38	0.39
T. koningii (T21)	1.74	3.8	0.38	0.39
Trichoderma sp. (T23)	1.70	3.9	0.35	0.40

Effect of benomyl on enzyme production by free or immobilised T. harzianum (T3)

Chitinase and β-1,3-glucanase production by free or alginate encapsulated *T. harzianum* (T3) were investigated on MSM (*17*) amended with different benomyl concentrations (0.0, 1.0, 3.0, 5.0, 7.0 and 10 μg/mL). Enzyme activities were measured in crude culture filtrates after 4, 8 and 12 days of incubation (Figs. 1 and 2). Fungal immobilisation has generally improved (up to 2-fold) enzyme production, especially for chitinase. Two patterns for the effect of benomyl on enzyme production were observed. Non-significant reduction in the activities of β-1,3-glucanase and chitinase was observed at lower benomyl concentrations (1, 3 and 5 μg/mL). On the other hand, higher benomyl concentration (7 and 10 μg/mL) significantly enhanced enzyme production. The results also indicated that the stimulatory effect was more pronounced in the presence of benomyl 7 μg/mL, especially after 12 days of incubation. Fig. 1 shows that chitinase production increases with the extention of the incubation period, except for the highest benomyl concentration (10 μg/mL). On the other hand, incubation period did not affect β-1,3-glucanase production significantly. However, in the presence of benomyl 10 μg/mL, β-1,3-glucanase production was reduced after 8 days.

The bound enzymes in free mycelium and/or in beads of *T. harzianum* (T3) were measured after 12 days of incubation. In the absence of benomyl, similar enzyme activities were recorded in both free and immobilised cultures (Table 2). Fungal immobilisation increased bound chitinase by 15- and 30-fold in the presence of benomyl 3 and 5 μg/mL, respectively. However, the immobilisation had no significant effect on the bound β-1,3-glucanase.

Table 2. Bound chitinase and β-1,3-glucanase produced by free or immobilized *T. harzianum* (T3) cultures in presence of different benomyl concentrations

γ(benomyl)/ (μg/mL)	Chitinase activity/(pkat/mL)		β-1,3-glucanase activity/(nkat/mL)	
	Free	Immo-bilised	Free	Immo-bilised
0.0	2.70	2.78	0.060	0.066
3.0	0.20	3.34	0.054	0.060
5.0	0.21	6.60	0.049	0.066

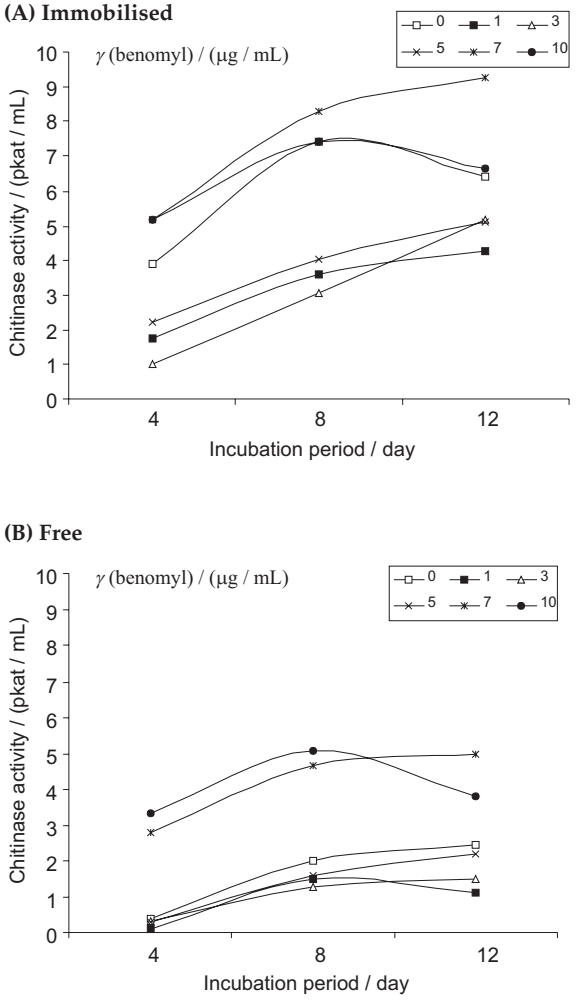

Fig. 1. Chitinase production by free or immobilised *T. harzianum* in liquid culture medium amended with different concentrations of benomyl

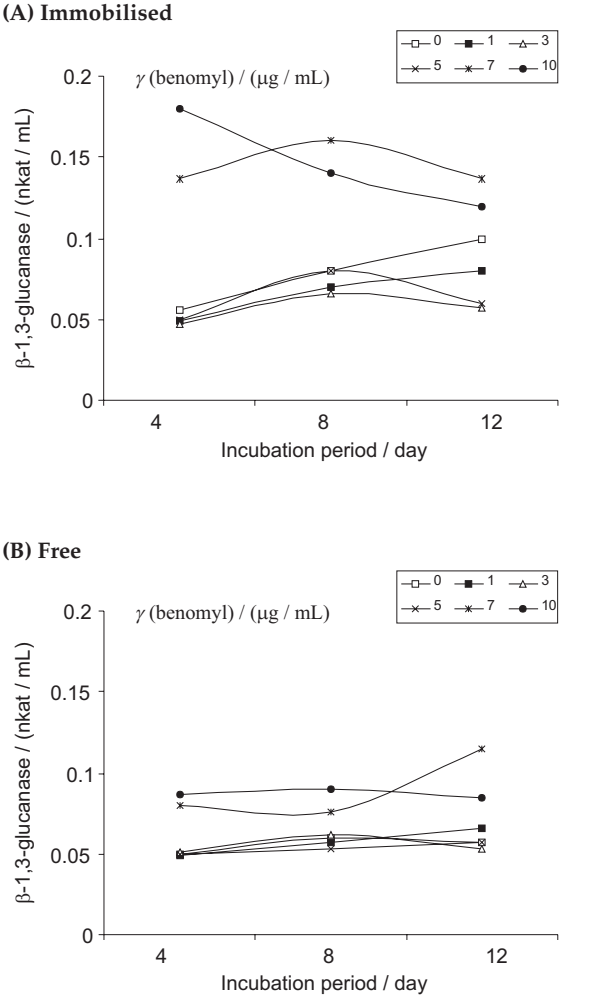

Fig. 2. β-1,3-glucanase production by free or immobilised *T. harzianum* in liquid culture medium amended with different concentrations of benomyl

Effect of benomyl on enzyme activity

The effect of different concentrations of benomyl (0.3, 3, 30, 300 and 1500 μg/mL) on the activities of free or immobilised chitinase and β-1,3-glucanase produced by *T. harzianum* (T3) was investigated (Table 3). No significant inhibitory effect on the activities of free or immobilised chitinase and β-1,3-glucanase was exhibited.

Table 3. Effect of different benomyl concentrations on the activities of free or immobilised chitinase and β-1,3-glucanase produced by *T. harzianum* (T3)

γ(benomyl)/ (μg/mL)	Relative chitinase activity/%		Relative β-1,3-glucanase activity/%	
	Free	Immobilised	Free	Immobilised
0.0	100	100	100	100
0.3	100	108	114	108
3.0	96	107	109	80
30.0	85	109	111	78
300.0	84	100	98	80
1500.0	84	100	100	78

Discussion

Chitinase and β-1,3-glucanase produced by biological control agents (BCAs) have been reported as key enzymes responsible for fungal cell and sclerotial wall lysis and degradation (*20–22*). Although there has been considerable improvement of the methods and techniques involved in the development of BCAs, the field has continuously suffered an arduous transition to wide-scale field application (*23*). One significant reason for the delay in the adoption of broad-scale biocontrol is that potential end-users doubt the efficiency of such measures as opposed to more traditional chemical control measures (*24*).

In particular, tolerance to benomyl would be useful because of the wide use of this fungicide and other fungicides in the methyl benzimidazole carbamate (MBC) group. In the application of *Trichoderma* spp. with benomyl to seedlings, it would be better to use benomyl-tolerant strains. Unexpectedly, most of the benomyl-tolerant isolates possessed improved rhizosphere competence in the absence of benomyl (*25*). However, McMahan *et al.* (*24*) reported that the application of a benomyl resistant strain of *Fusarium lateritium* alone or in combination with benomyl may provide good control of *Eutypa lata*.

We have investigated the sensitivity of *T. harzianum* (T3) to benomyl on PDA. *T. harzianum* tolerated benomyl up to 2 μg/mL, which is higher than the corresponding values of *Neurospora crassa* (*26*), *Gliocladium virens* (*27*), *T. ressei* and the wild-type *T. harzianum* (*28*). Results of PDA-benomyl test are consistent with the previously investigated wild-type strains of *Gliocladium virens* (Gl-3 and Gl-21) (*29*) and *Fusarium lateritium* (*24*), in which the concentration of benomyl 5 μg/mL completely inhibited the fungal growth on PDA. The tested isolate of *T. harzianum* (T3) in our research was moderately tolerant to benomyl and this level of tolerance to benomyl has been observed in other fungi (*30-32*). Tolerance of fungi to low levels of benomyl (0–10 μg/mL) has been previously reported and the gene conferring this tolerance has been identified in several fungal species (*26,32–34*).

Fungicide tolerance in *T. harzianum* would be useful for the integrated use of chemical and biological control agents. Induced mutant strains of the genus *Trichoderma* tolerant to MBC fungicides such as benomyl have been constructed and some of these mutants were found to produce more cellulase than the wild type strains (*35*).

Peterbauer *et al.* (*28*) investigated the effect of benomyl on the growth and the production of cellulase by *Trichoderma* spp. Although *T. reesei* produced the highest and *T. harzianum* the lowest cellulase amounts, the growth of both strains was equally inhibited by benomyl concentration of 2 μg/mL. However, sublethal doses of benomyl (0.2–0.5 μg/mL) promoted growth and stimulated cellulase production. Peterbauer *et al.* (*28*) also found that transformation of *T. reesei* with the *Neurospora crassa ben* gene coding for benomyl-tolerant β-tubulin resulted in a phenotype similar to *Trichoderma* strains exposed to sublethal doses of benomyl.

Results of this investigation showed that *T. harzianum* (T3) produced chitinase and β-1,3-glucanase at all the tested levels of benomyl with the highest enzyme production in the presence of benomyl concentration of 7 μg/mL. Production of chitinase and β-1,3-glucanase was improved significantly by alginate encapsulation. Similarly, the bound chitinase activity was significantly higher in the immobilised culture than in the free cultures. These results indicate that immobilisation did not increase only the chitinase produced externally in the culture medium but also dramatically increased the chitinase entrapped in beads. Therefore, beads act as a reservoir for enzyme and/or microbial cells, providing the surrounding environment with a sustainable supply of cells and enzymes (*36*).

The enhancement of enzyme production by immobilisation could be explained on the basis that fungal immobilisation in calcium-alginate may have gained resistance to benomyl either by detoxification or lack of uptake. Moreover, it provides physical protection, and beads may have the advantage of promoting a slow release of spores into the medium (*36–38*).

Benomyl had no effect on the activities of crude chitinase and β-1,3-glucanase. This could encourage the synergistic use of immobilised enzyme with benomyl against plant pathogens. A strong synergistic effect was observed on the inhibition of cyst germination of *Pythium* spp. by a combination of endo-β-1,3-glucanase and the fungicide Fongarid (*39*). Similarly, co-application of free biocontrol with fungicides was successfully used for the control of *Armillarea mella* and *Rhizoctonia solani* with a combination of formulations of *Trichoderma* spp. and carbon disulphide or methyl bromide, respectively (*1,3, 16,40,41*).

In conclusion, immobilised *Trichoderma* cells are suggested for use as an alternative to fungal mutants (resistant to benomyl) for combined application with benomyl against plant diseases. Further studies are needed

to clarify such interactions to improve the potential of immobilised *Trichoderma* in co-application with fungicides.

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Utjecaj benomila na proizvodnju kitinaze i β-1,3-glukanaze iz slobodnog i alginatom obavijenog mikroorganizma *Trichoderma harzianum*

Sažetak

Rast *Trichoderma harzianum* na PDA podlozi smanjen je za 20 ili 30 % uz dodatak 1 i 2 μg/mL benomila, a potpuno zaustavljen dodatkom 5 μg/mL. Fungalna imobilizacija u minimalnom sintetskom mediju (MSM) obogaćenom benomilom koncentracija 1, 3, 5, 7 i 10 μg/mL poboljšala je proizvodnju kitinaze i β-1,3-glukanaze pri malim koncentracijama benomila (1, 3 i 5 μg/mL). Daljnje povećanje proizvodnje oba enzima postignuto je imobilizacijom pri većim koncentracijama benomila (7 i 10 μg/mL). Fungalna imobilizacija po-

većala je 15–30 puta vezanu kitinazu pri koncentracijama benomila od 3 i 5 μg/mL, a nije utjecala na vezanu β-1,3-glukanazu. Različite koncentracije benomila (od 0,3 do 1500 μg/mL) nisu bitno smanjile aktivnost slobodne i imobilizirane kitinaze i β-1,3-glukanaze. Predlaže se da se za zaštitu od biljnih štetočinja primjenjuju imobilizirana *Trichoderma* ili imobilizirana kitinaza i β-1,3-glukanaza s benomilom.