Med. Fac. Landbouww. Univ. Gent, 59/3a, 1994

RELATIONSHIP BETWEEN THE PROTECTIVE ACTIVITIES OF SEVERAL STRAINS OF *TRICHODERMA* AGAINST DAMPING-OFF AGENTS AND THEIR ABILITY TO PRODUCE HYDROLYTIC ENZYMES ACTIVITIES IN SOIL OR IN SYNTHETIC MEDIA

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SUMMARY

Fourteen isolates of *Trichoderma* spp. were evaluated for their ability to control the damping off diseases caused by *Pythium* sp. and *Rhizoctonia solani* in bean under growth chamber conditions and for their ability to produce hydrolytic enzymes (chitinase and ß 1-3 glucanase) in culture filtrates and in soil.

Our results showed that the protective ability of the isolates of *Trichoderma* was unrelated with the *in vitro* ß 1-3 glucanase and chitinase activities released in the culture filtrates whatever the carbon source used.

On the other hand, we found a significant correlation between the ability of these isolates to control the damping-off agents and the levels of chitinase (but not ß 1-3 glucanase) activities in soil

INTRODUCTION

Biological control of soilborne plant pathogens by introduction of antagonistic microorganisms into the soil, is increasingly investigated for plant disease control.

In recent years, different strains of *Trichoderma* spp. have been tentatively used to control damping-off caused by *Rhizoctonia solani* (Kühn) and *Pythium* spp. in different crops (9, 10, 13, 15, 20, 21).

The most frequently suggested mechanisms for biocontrol by *Trichoderma* spp. include promotion of plant growth, mycoparasitism, antibiosis, and/or lysis. The involvement of cell wall degrading enzymes produced by the fungal antagonist in mycoparasitism and/or lysis has been established. Elad *et al.* (7) found that the ability of 3 isolates of *Trichoderma harzianum* to release hydrolytic enzymes (chitinase, β-1,3 glucanase and cellulase) in synthetic culture medium (containing chitin, laminarin or cellulose respectively, as sole carbon source) and in soil was correlated with their ability to control *Pythium* sp. and *Sclerotium rolfsii in vivo.*

There are only few studies about the physiology and biochemistry of the hydrolytic enzymes. All these studies were frequently carried out *in vitro*, using as carbon sources, for induction and secretion of hydrolytic enzymes, chemically well defined compounds or cell walls of fungi.

Focusing on the *in vitro* interactions between a pathogen and a biocontrol agents deliberately simplifies the experimental system. This approach does not accurately simulates the environment in which the interactions of interest normally occur, thus hampering to interfer from laboratory tests what happens under natural conditions.

Considering the potential importance of hydrolytic enzymes in screening tests for efficient antagonistic strains, our objective was to evaluate the relation between the protective activities of several strains of *Trichoderma* spp. and their ability to release chitinase and β-1,3 glucanase activities when grown in sterile loam soil or on synthetic media containing different carbon sources.

MATERIALS AND METHODS

Microbial strains

Twelve isolates of *Trichoderma* spp. (numbered 1-2-5-6-7-8-9-10-11-12-13 and 14) were supplied by CIMIC (Microbiological Research Center, Andes University of Colombia). Isolate 4 was supplied by the Bio Industry Department of the Faculty of Agricultural Sciences (Gembloux), whereas isolate 3 was originated from a commercial preparation of ORSAN Co. (France). *Pythium* sp. strain was isolated in our laboratory from sugar beet showing symptoms of damping-off .

These microbial isolates were maintained by subcultures of 7 days on Difco Malt extract agar medium (MEA) (for *Trichoderma*), and in Difco Corn meal agar (CMA) (for *Phytium*). Incubation was carried out at 25°C with a 16 h light photoperiod.

Inoculation tests

Trichoderma inocula were grown on a nutrient medium containing a mixture of wheat bran, sucrose and water (30 g/ 1.6 g/80 ml) previously autoclaved for 1 h at 120°C. Each flask was inoculated with a *Trichoderma* suspension of 10^8/ml propagules collected by scraping 7 day-old colonies on MEA with a spatula, and suspending the propagules in sterile water.

Pythium inoculum was prepared on a nutrient medium containing Vermiculite, V8 juice, and water (20 g/ 24 ml/ 80ml respectively). The medium was autoclaved for 20 min at 120°C, and was inoculated with 3 discs (5mm) of 7 day-old *Pythium* culture grown in CMA (Difco). Cultures were incubated for 7 days in a culture room as described above.

Inoculation tests were carried out in Gembloux loam soil sieved through a 4 mm-mesh screen. Sterile soil was prepared by 3 consecutive daily autoclavings for 1 h at 121°C. The soil was infested by incorporating 5% inoculum W/V *Pythium* (giving a final concentration of about 10^3 CFU/g) immediately prior to bean sowing. *Trichoderma* inoculum was incorporated both by mixing to *Pythium* infested soil (giving a final concentration of 10^6 *Trichoderma* CFU/g) and by dipping seeds for 10 min in *Trichoderma* suspension (10^6 propagules/ml). All experiments were performed in plastic pots containing 300 g of soil, each planted with 5 bean seeds (*Phaseolus vulgaris* var. Prélude) followed by incubation at 22°C±1, with a 16 h photoperiod of cool white fluorescent light.

Soil moisture holding capacity was maintained for ten days at 75 %. Stand counts started 24 h after planting and continued daily for 10 days and expressed as percentages of emergence.

Glycosidic activity of *Trichoderma* spp.

***In vitro* enzyme assays.**

For enzyme assay, *Trichoderma* spp. was grown in a synthetic media (SM) containing: $MgSO_4$. $7H_2O$ (0.2 g/l); K_2HPO_4 (0.9 g/l); KCl (0.2 g/l); NH_4NO_3 (1.0 g/l); $FeSO_4$ (2 mg/l); $MnCl_2$ (2 mg/l); $ZnCl_2$ (2 mg/l); the pH was adjusted to 6.3 before autoclaving (18); the medium was supplemented with 0.05% Tween 20 (19), and with one of the following carbon sources (each 20 mg/ml): laminarin (Sigma L-9634), colloïdal chitin (prepared from chitin Fluka N° 22720 according to Lingappa and Lockwood. (14), cellulose (AVICEL type PH10), *A. niger* cell preparation provided by Bio-Industry department, or *Pythium* mycelium prepared as follow.

Pythium was inoculated in Erlenmeyer flasks (500 ml) containing 100 ml Difco Potato dextrose broth with 4 disks of 7 day-old *Pythium* culture grown on CMA. Inoculated flasks were incubated for 96 h as described above. Mycelium was then collected through two layers of cheesecloth followed by thorough washing with sterile distilled water. Mycelium was mixed with sterile 0.05 M acetate buffer (pH 5) and homogenized with an Ultra-turrax blender for 1 min at the lowest speed. The suspension was centrifuged at 3000 r.p.m. for 10 min and the precipitate was frozen at -20° C.

Erlenmeyer flasks (250 ml) containing 50 ml liquid SM added with a carbon source were inoculated with 1 ml of propagule suspension ($2x10^7$/ml) of *Trichoderma* spp. collected from 7 day-old colonies grown in MEA at 25°C with a 16 h of photoperiod. Inoculated flasks were incubated at 25°C in a rotatory shaker at 120 r.p.m. for 5 days. The mycelium was then collected by centrifugation at 5000 r.p.m. at 4°C for 10 min and the supernatant filtered through Whatman N.1 filter paper was conserved at -20°C for enzyme assays.

Chitinase and β-1,3 glucanase activities were measured in culture filtrate according to Elad *et al.* (7).

Exo-β-1,3 glucanase was assayed by following the release of reducing sugars from laminarin according to Somogyi (25) using Nelson's reagents (17). The reaction mixture, containing 1 ml culture filtrate, and 1.6 mg laminarin dissolved in 1 ml 0.1 M citrate buffer (pH 4.7), was incubated at 37°C for 1 h. The reaction was stopped by boiling at 100°C for 10 min. Specific activity was expressed as µg glucose equivalents/µg protein.h. Protein concentration was determined according to Bradford's method (3).

Chitinase activity was assayed by measuring the amount of N-acetylglucosamine (NAGA) released from chitin according to Reissig *et al.* (23). The reaction mixture, containing 1 ml culture filtrate, 1 ml 0.1 M phosphate buffer (pH 5.1) and 1.6 mg chitin (Sigma C-3641) was incubated at 37°C for 1 h and the reaction was stopped by boiling for 10 min. Specific activity was expressed as µg of NAGA/µg protein.h.

Chitinase and glucanase activities in soil extract

Samples of 30 g containing 75% soil moisture holding capacity were placed in a flask and autoclaved at 120° C for 30 min. Soil was then inoculated to reach 10^6 propagules/g *Trichoderma* isolates, and/or 10^3 propagules/g *Pythium*. Four days later, 10 ml of 0.1 M phosphate buffer pH (5.1) was added to the soil and the mixture was shaken for 20 min (200 r.p.m.), and centrifuged at 500 r.p.m. for 30 min; the supernatant was used for enzymatic activity assay as above.

Degradation of *Pythium* mycelium

The hydrolytic activity (chitinase and glucanase) of *Trichoderma* supernatants (grown in liquid media) were tested by using *Pythium* mycelium (produced as above) as substrate. The measure of reducing sugars and NAGA were performed as above.

Data analysis

In all experiments (inoculation tests or enzymatic assays), three replicates of each treatment were used. All experiments were performed three times independently. Data were subjected to analysis of variance, (P=0.05), and means were separated with Duncan's multiple range test. Inoculation tests, were subjected to Student-Newman-Keuls test.

RESULTS

Study of the protective effect of *Trichoderma* spp. against *Pythium*.

Fourteen different isolates were tested for their ability to control damping-off, caused by *Pythium* sp. in bean, under growth chamber conditions. Treatment with *Trichoderma* spp. was achieved by both seed dressing and incorporation as wheat bran culture immediately before sowing in *Pythium* sp. infested soil.
Isolates were classified on the basis of the percentage of seed germination (Table 1). Five strains (TH-2, TH-3, TH-10, TH-11 and TH-12), reduced significantly the incidence of the disease. The most effective isolates were TH-11 and TH-3 (emergence percentage being 66.6 to 82.2 respectively), whereas four isolates (TH-1, TH-7, TH-13, and TH-14) gave no significant protection.

Enzymatic activity of *Trichoderma* spp.

Trichoderma spp. was grown in 500 ml of synthetic medium supplemented with either laminarin, colloidal chitin, cellulose or *Pythium* mycelium as sole carbon source.

After 5 days of inoculation, the β-1,3 glucanase and chitinase specific activities released in culture filtrates of *Trichoderma* spp. varied according to the carbon source and the fungal strains (Figures 1 and 2).

The highest chitinase activities were released in media containing colloidal chitin (Figure 1C) and *A. niger* cell preparation (Figure 1A) as sole carbon source. Using *A. niger* cell preparation as carbon source, the highest chitinase activities were released with isolates TH-2, TH-3 TH-7, TH-9, and TH-11 (specific activities ranging from 59 to 71 µg NAGA /µg prot. h. whereas with colloidal chitin, the significantly highest chitinase activity was released with TH-7 (93 µg NAGA/µg protein), followed by the group of isolates TH-1, TH-2, TH-3, TH-4 and TH-5. Media containing mycelium of *Pythium*, or cellulose as carbon source gave no significant chitinase activity (unpublished results) whereas a low activity was measured with laminarin (Figure 1B).

Using *A. niger* cell preparation as sole carbon source, higher β-1,3 glucanase activity was measured by the different strains of *Trichoderma* as compared with media amended with laminarin; the highest activities were measured with isolates TH-7, TH-8, TH-9, and TH-11 (Figure 2).

Table 1. Classification of isolates of *Trichoderma* according to their protective effect against *Pythium spp.*. Numbers of each column followed by the same letter are not significantly different (P= 0.05)

Trichoderma (isolate)	Mean percentage of emergence
3	76,6 a
11	68,8 a
12	61,0 b
6	56,6 b
8	54,4 b
10	52,2 b
2	51,0 b
9	44,4 bc
4	44,4 bc
14	43,2 bc
5	42,2 bc
1	37,8 c
13	33,2 c
7	30,0 c
Pythium without *Trichoderma*	26,6 c

In media containing cellulose or chitin as carbon sources, low β-1,3 glucanase activity was released by several isolates, whereas the others gave no significant activity (isolates TH-2,TH-10 and TH-14 with chitin medium and isolates TH-6 and TH-10 and TH-14 with cellulose).

The inoculation of *Pythium* into the soil did not modify the specific chitinase and glucanase activities found in the soil extracts after the incorporation of *Trichoderma*. The highest chitinase activities were found with TH-3, TH-8 and TH-11 whereas TH-1, TH-3, TH-4, TH-11 gave the highest levels of glucanase activity (Figure 3).

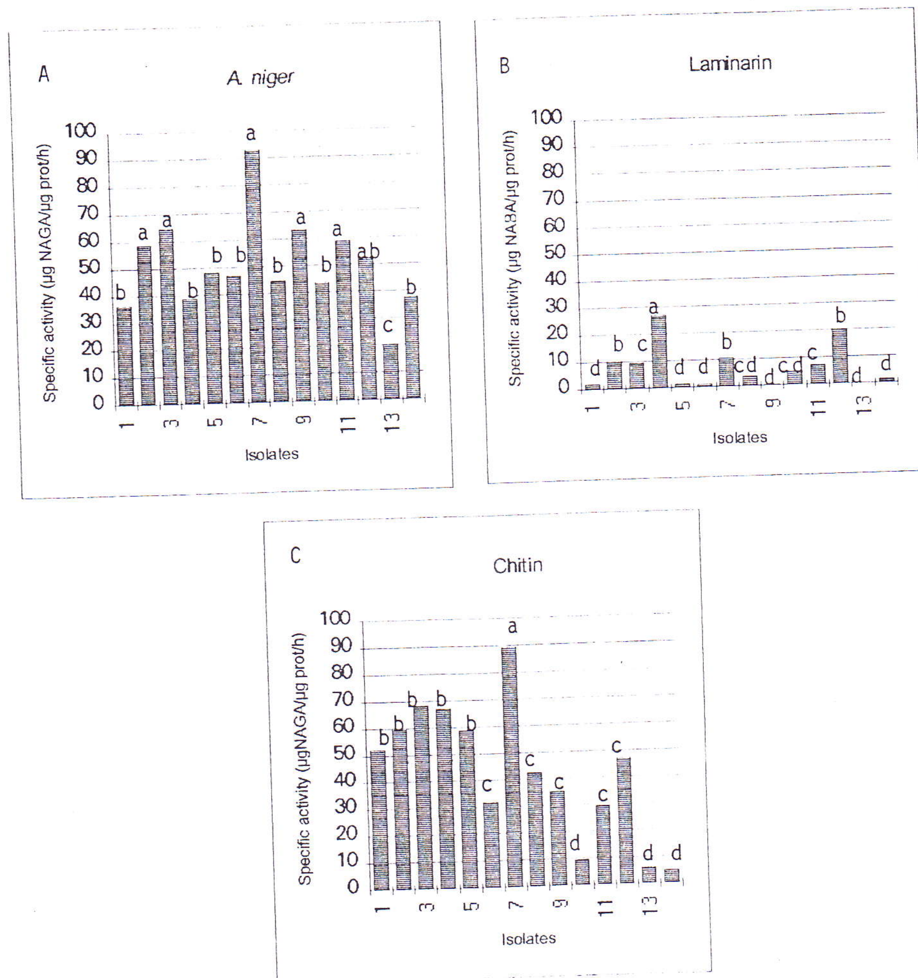

Figure 1. Chitinase activities released by *Trichoderma* spp. in culture media containing *A.niger* cell residues (A), laminarin (B) or chitin © as sole carbon source. Columns followed by a common letter are not significantly different (P = 0.05).

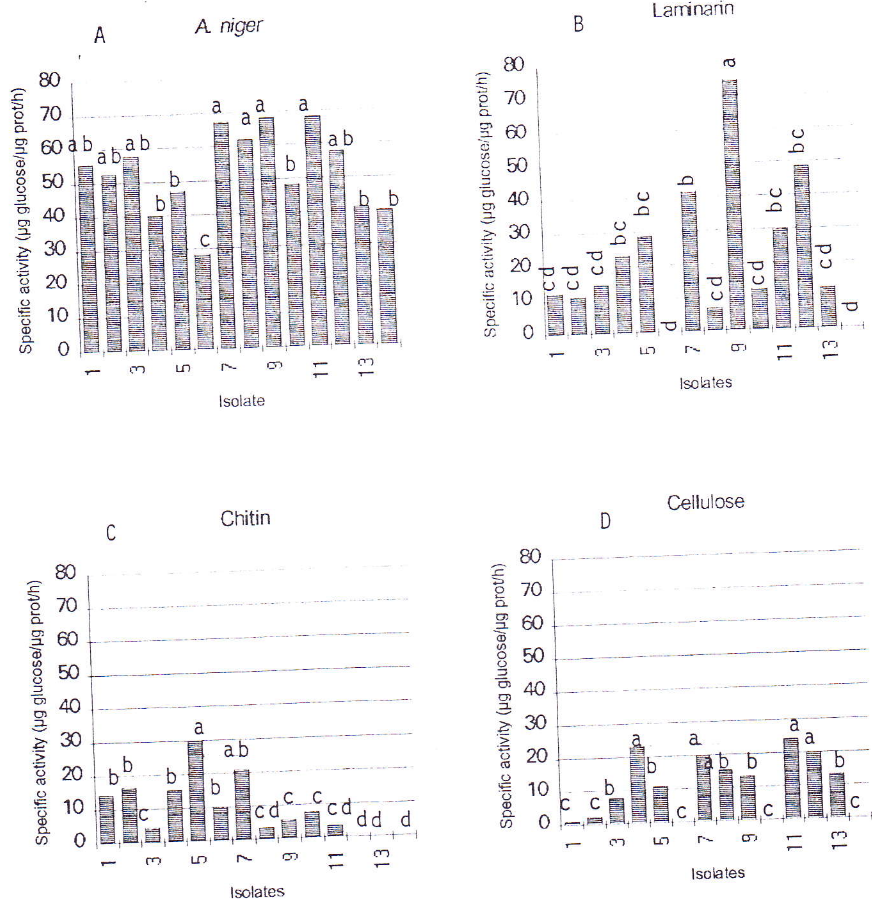

Figure 2. β-1,3 glucanase activities released by *Trichoderma* spp. in culture media containing *A. niger* cell residues (A), cellulose (B), laminarin (C) or chitin (D) as sole carbon source. Columns followed by a common letter are not significantly different (P= 0.05).

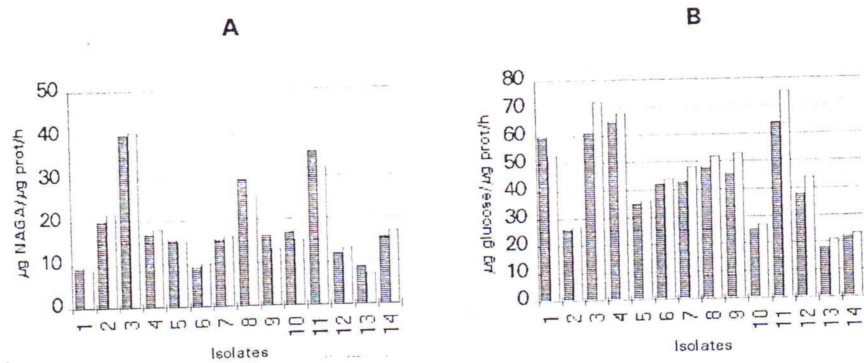

Figure 3. Chitinase (A) and β-1,3 glucanase (B) activities in soil extract inoculated with *Trichoderma* spp. alone (black columns) or with *Trichoderma* spp. plus *Pythium* (white columns). Columns followed by a common letter are not significantly different (P = 0.05).

Correlation between protective effect of isolates of *Trichoderma* and their *in vitro* glycosidic activity.

No correlation was observed between protective ability of different isolates of *Trichoderma* spp. against *Pythium* and the chitinase and β-1,3 glucanase activities released in the synthetic culture media whereas a significant correlation (r= 0.725, P=0.05) was found between their protective ability and chitinase activity in soil extract but not glucanase (Table 2).

TABLE 2: Correlation between enzymatic activity of *Trichoderma* spp. and their protective ability against *Pythium*. (P = 0.05).

Media	β-1,3 glucanase	Chitinase
SM + laminarin	0,08	0,00
SM + chitin	0,00	0,39
SM + *Pythium*	0,22	0,00
SM + *A. niger*	0,15	0,39
Soil + *Trichoderma*	0,33	0,72
Soil + *Trichoderma* + *Pythium*	0,48	0,72

DISCUSSION

The cell walls of most fungi are composed mainly of β-1,3 glucan and chitin (2) whereas the members of the Oomycetes (e.g. *Pythium* spp.) contain mainly cellulose, β-1,3, β-1,6 linked glucan (2) and little chitin (4).

Chet and Baker (6) showed that *Trichoderma* spp. produces cellulases, β-1,3 glucanases and chitinases that degrade *in vitro* glucan in the cell walls of *Pythium* spp. and chitin and β-1,3 glucan in the cell walls of *R. solani*.

The capacity to produce cell wall-degrading enzymes is however a common feature of many saprophytic fungi (5) thus bluring the interest of using the capability of producing hydrolytic enzymes as screening tests of biocontrol agents.

Mycoparasitism is considered as a mechanism involved in biological control activity of microbial strains and related to their ability to produce hydrolytic enzymes.

Strains of *Trichoderma* spp. exhibited a high variability in the enzymatic activities released in their culture filtrate. Although β-1,3 glucanase was produced in the presence of chemically pure carbon sources such as laminarin, complex substrates as *A. niger* cell residues provided the higher activities (Figure 1). Chitinase was released when chitin or *A. niger* cell residues were used as carbon sources, but no activity was found in the presence of *Pythium* mycelium as carbon source.

Our overall results showed lack of correlation between *in vitro* β-1,3 glucanase and chitinase activities of 14 isolates of *Trichoderma* spp. and their protective ability against *Pythium*. We observed strains (e.g TH- 3 and TH-11) showing high protective capability against the pathogen and presenting high hydrolytic activities in culture filtrates. Isolate TH-13 provided neither protection nor hydrolytic activity in synthetic culture media.

Other situation was observed with some strains such as isolate TH-7 which gave rise to culture filtrate with high hydrolytic activity, but presented no protective ability, or isolate TH-6 that presented low hydrolytic activity but high protective ability. This suggests that the *in vitro* level of production of cell wall-degrading enzymes is unrelated to biocontrol capability hampering any inference from laboratory tests to predict the efficiency of microbial strains under natural conditions.

On the other hand, we found a significant correlation between the ability of isolates of *Trichoderma* spp. to control damping-off caused by *Pythium*, and the levels of chitinase (not β-1,3 glucanase) that they produce in soil.

Our studies however, suggest that chitinase is not involved in the degradation of *Pythium* walls, based in the fact that there was no release of N-acetyl glucosamine from mycelium of *Pythium* by using crude extracts containing chitinase (unpublished results). This activity could be however used as a markers for screening of efficient antagonistic *Trichoderma* isolates.

The absence of correlation between the protective effect of this collection of *Trichoderma* spp. isolates against *Pythium* and their hydrolytic activities in cultures filtrates, suggests that the biocontrol of *Trichoderma* spp. reflect a more complex interaction between the pathogen-plant and antagonist. It appears that the mechanisms for biocontrol by *Trichoderma* spp. is strain dependent. Different mechanisms including mycoparasitism (7, 8, 12), but also competition for nutrients in seed exudates (9, 24), occupation of the host infection court (6) and/or antibiosis (13, 22) could be implicated in the protective ability.

Among the potential biological control agents tested in this study, we selected isolate 3 (*T. viride*) and isolate 11 (*T. koningii*) that controlled effectively damping-off in bean caused by *Pythium*.

To improve the reliability of biocontrol, several kind of amendments to biological seed treatments could be added to enhance efficacy (16), as well as seed coat precolonisation with *Trichoderma* (5), incorporation of food bases into soil (9), prior colonization (1), integrated control with toxicants (21), and combination with solid matrix priming (11). In this respect, *in vitro* studies of hydrolytic activities suggest mycelial residues of *A. niger* arisen from the industrial production of citric acid could constitute an inexpensive source of chitin and β-1,3 glucan enhancing the synthesis of hydrolytic enzymes by the biocontrol agent.

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