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Correlation between hydrolytic enzyme activities measured in bean seedlings after *Trichoderma koningii* treatment combined with pregermination and the protective effect against *Pythium splendens*

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Abstract

An experimental protocol consisting in the colonisation of pregerminated bean seeds dressed with *Trichoderma* sp. was used in order to study the mechanisms correlated with the protective effect against *Pythium splendens*. Seed dressed with TH-11 (*T. koningii*) for 24 h presented a higher protective effect and a higher level of seed colonisation as compared to those dressed with TH-13 (*T. longibranchiatum*). The levels of seed coat colonisation by TH-11 and TH-13 was shown to be correlated with the carboxymethylcellulase activity, as measured in the seed coats retreived from germinating dressed bean seeds. The seed coat colonisation was also associated with an increased activities of endo-1,3-β-glucanase and endochitinase measured in seed extracts, and an inhibitory effect of seed extracts on *Pythium* sporangia germination. Pretreatment of TH-13-dressed seeds with a commercial cellulase improved all parameters mentioned above, thus suggesting a role of cellulase activity in the colonisation process and the linked protective effect. The possible role of hydrolytic enzymes in the protective effects is discussed.

Introduction

Many potential biocontrol agents have already been identified, either as individual microbial strains or as complex mixtures of microorganisms among which *Trichoderma* spp. is recognized as promising to control damping-off caused by *Pythium* spp. in different crops (Adams, 1990; Chet, 1987). Most of these potential biocontrol agents appeared effective under controlled conditions but further improvement of their efficacy is often required to provide economically acceptable and reliable control under field conditions.

The complexity of the interactions between soil microorganisms (antagonists, pathogens, bulk microflora and microfauna), plants and environment, hampered the identification of the causes of the inconsistency in protection activity. The physical, chemical and biotic variables within natural environments not only affect individually each component of the biocontrol system, but also influence their interactions with each other (Hardar et al., 1984; Lewis and Papavizas, 1987).

Seed pregermination combined with *Trichoderma* spp. treatment is an attractive method for introducing the biocontrol agent onto the infection court of the host (Harman et al., 1989; Mezui M'ella et al., 1994) but also provides a convenient experimental system due to the absence of soil microflora during the occupation phase of the infection court. By combining seed pregermination and *Trichoderma* spp. treatment, Cotes et al. (1994a) reported that a successful seed coat-coloniser *Trichoderma koningii* isolate (TH-11) protected bean seeds more efficiently against *Pythium splendens* and produced more cellulase in a synthetic medium containing seed coats as sole source of carbon than a poor coloniser isolate of *Trichoderma longibranchiatum* (TH-13). Moreover, once the seed coat colonisation step was carried out, protection could be maintained even after killing *Trichoderma* sp. with a selective fungicide.

Different mechanisms, like competition for nutrients in seed exudates, occupation of host infection courts and/or mycoparasitism could be implicated in the protective effect observed. The importance of 1,3-β-glucanase and chitinase as key enzymes responsible for fungal cell and sclerotial wall lysis and degradation has been also reported (Cook and Baker, 1983). In this respect, Elad et al. (1982) found that the ability of 3 isolates of *T. harzianum* to produce hydrolytic enzymes (chitinase, 1,3-β-glucanase and cellulase) in synthetic liquid medium and in soil, was correlated with their ability to control *Pythium* sp. and *S. rolfsii in vivo*.

Based on these observations, the purpose of this study was to analyse the activities of hydrolytic enzymes (cellulase, glucanase and chitinase) in relation with the protection of bean seeds against *Pythium* in this model system of host-pathogen-protectant.

Materials and methods

Microorganisms

T. koningii (strain TH-11) and *T. longibranchiatum* strain (TH-13) were provided by the Microbiological Research Center, Andes University of Colombia. The isolate of *P. splendens* was isolated in our laboratory from a damped-off bean seedling. The methods for the growth and maintenance of these fungal strains have been previously described (Cotes et al., 1992).

Bean seeds pregermination combined with Trichoderma *sp. treatment*

Trichoderma strains were grown on malt extract agar (Difco). Conidia were harvested from 7 day-old colonies by scraping the culture surface with a spatula, and by suspending the propagules in sterile water; the suspension was then filtered through two layers of cheesescloth, and the final spore concentration was adjusted at 10^7conidia.ml^{-1}.

Bean seeds (*Phaseolus vulgaris* L. var. Prélude) were surface disinfected for 3 min with successively 3% sodium hypochlorite and ethanol and washed 3 times for 5 min with sterile distilled water. Seeds were pregerminated for 24 h on 0.7% water agar in Petri dishes kept at 25 °C with 16 h light photoperiod. Seeds were then dipped for 30 min in the spore suspension of TH-11 or TH-13 or in water (control) and pregermination was pursued for 24 h on 0.7% water agar in the same conditions of incubation. In order to kill *Trichoderma*, seeds were then dipped for 30 min in 4 ppm (a.i.) of the fungicide Sumico (25% diethofencarbe and 25% carbendazime), or in water (control).

Colonization of bean seed coats by Trichoderma *sp.*

Control or *Trichoderma*-dressed seeds were retrieved from the water agar every 6 h for 2 days, and were washed for 3 min with 0.05% Tween 20 in sterile distilled water. Seed coats were then collected, ground in a mortar and homogenized with an Ultra-Turrax blender for 1 min in 10 ml of sterile distilled water. Serial dilutions were prepared immediately and 0.1 ml aliquots were plated on 0.1% sodium desoxycholate PDA for assay of colony forming units (CFU). The number of *Trichoderma* colonies were evaluated after 5 days incubation at 25 °C under 16 h fluorescent light photoperiod. *Trichoderma* colonization was expressed as CFU. g^{-1} of seed coat.

Inoculation of P. splendens

Inoculation was then carried out by dipping the seeds for 30 min in water (control) or in suspension of *P. splendens* (10^3 propagules/ml) scraped from 7 day-old cultures grown on corn meal agar (CMA) and suspended in sterile water. Four seeds were placed on 0.7% water agar in Petri dishes and were incubated for 48 h at 25 °C in the growth chamber with 16 h light photoperiod. Symptom index was then scored according to a visual intensity scale based on the number of necrotic spots in each cotyledon [1–5 spots = 20, 6–10 spots = 40, 11–15 spots = 60, 16–20 = 80 and >20 spots (coalescent lesions) = 100]. Protection index was calculated by (SC-ST)/SC × 100 where SC = symptom index in *Trichoderma*-untreated control, ST = symptom index after treatment with *Trichoderma*.

Treatment of bean seeds with cellulase

Bean seeds pregerminated for 24 h were pretreated for 3 h by immersion in a solution 0.5% w/v of cellulase (Onozuka R-10) in 50 mM potassium acetate buffer (pH 5), or in this buffer (control). Seeds were then washed 6 times for 10 min each with sterile distilled water, and dipped for 30 min in a spore suspension of TH-11 or TH-13. Seeds were then placed for 24 h in a water agar (0.7%) to pursue the pregermination, treated with Sumico, and then inoculated with *Pythium* as indicated above.

Enzyme extraction

A weighed portion of seed coats retrieved from *Trichoderma sp.* -dressed seeds, were harvested and frozen

at −20 °C after Sumico treatment. Homogenates were prepared by grinding in a mortar using dry ice, and diluted in 50 mM potassium acetate buffer (pH 5) at a ratio of 1:2 (w/v). The extracts were then homogenized with an Ultra-Turrax blender for 2 min. After magnetic stirring (1 min) and filtration through 2 layers of muslin, the filtrates were centrifuged at 10,000 rpm for 30 min at 10 °C. The supernatants were dialysed twice for 12 h against 5 l distilled water, and then against 0.01M potassium acetate (pH 5) overnight. The dialysates were frozen at −20 °C and used as seed coat crude extract for enzyme assays.

Enzyme assays

To determine the 1,3-β-glucanase (or glucan 1,3-β-D-glucosidase) (EC 3.2.1.-) activity, the reaction mixture contained 1 ml seed coat crude extract and 20 mg laminarin (Sigma L 9634) dissolved in 1 ml 50 mM potassium acetate buffer (pH 5). Reaction mixture with heat-treated seed coat crude extract was included as control. The reaction was incubated at 37 °C for 1h and stopped by boiling for 10 min. A unit of enzymatic activity is the amount of enzyme releasing 1 μg glucose equivalents. g^{-1} tissue. h^{-1}.

The release of total reducing sugars was measured according to Somogyi (1952) using Nelson's reagents (Nelson, 1944), while free glucose was determined by using glucose oxidase reagent (Sigma) according to manufacturer's instructions. With an exo-1,3-β-glucanase (EC 3.2.1.58) activity, glucose was the only reaction product, and one unit of activity measured using the Nelson-Somogyi assay equals one unit measured by the glucose oxydase assay. The difference between the results of the two methods represents the endo-1,3-β-glucanase activity (EC 3.2.1.39).

To determine the chitinase (EC 3.2.1.14) activities, the reaction mixture contained 500 μl crude enzyme extract, 10 mg chitin (Sigma 3641) in 500 μl of 50 mM potassium acetate buffer (pH 5). In the case of endochitinase activity, 500 μl of chitobiase (EC 3.2.1.29) 10% v/v (obtained from Canadian lobster lymphatic liquid) was added to the reaction mixture to hydrolyse chitin oligomers, while addition of chitobiase was omitted for determination of exochitinase activity, and substituted by 500 μl of potassium acetate buffer. Reaction mixture with heat-treated crude enzyme extract was included as control.

The reaction was incubated at 37 °C for 10 min and stopped by boiling for 10 min. Enzymatic activity was assayed by measuring the amount of N-acetylglucosamine (NAGA) according to Reissig et al. (1955). A unit of enzymatic activity is the amount of enzyme releasing 1 μg NAGA equivalents. g^{-1} tissue. h^{-1}.

Two components of the cellulase (EC 3.2.1.4) complex were assayed. Total cellulase complex activity was measured as filter paper activity (Fpase) while carboxymethylcellulase (CMCase) was analysed using carboxymethylcellulose (Hoechst) as substrate. The reaction mixture containing 1 ml crude enzyme, 40 mg Whatman chromatography paper (No. 3001–653) (for Fpase) or 40 mg carboxymethylcellulose (for CMCase), and 1 ml 50mM citrate buffer (pH 4. 8), was incubated at 48 °C for 1 h. Reaction mixture with heat-treated crude enzyme extract was included as control. Reactions were stopped by boiling for 10 min. Fpase and CMCase activities were evaluated by measuring the release of reducing sugars from the substrates. A unit of enzymatic activity is the amount of enzyme releasing 1 μg glucose equivalents. g^{-1} tissue. h^{-1}

Degradation of mycelium of Pythium *by extracts obtained from seed coats*

The hydrolytic activity of seed coat crude extracts from *Trichoderma*-dressed seeds (TH-11 or TH-13), was tested by using *Pythium* mycelium as substrate.

Pythium mycelium was obtained by inoculating Erlenmeyer flasks (500 ml) containing 100 ml Difco Potato broth with 4 disks of 7 day-old *Pythium* culture grown on CMA. Inoculated flasks were incubated for 96 h at 25 °C with a 16 h light photoperiod. Mycelium was then collected by filtering through two layers of cheesecloth, followed by thorough washing with sterile distilled water. Mycelium was mixed with sterile 50 mM potassium acetate buffer (pH 5) and was homogenized with a blender for 1 min at the lowest speed. The suspension was centrifuged at 3000 rpm. for 10 min and the precipitate frozen at −20 °C was used as substrate in enzyme assays to measure the release of reducing sugars as indicated above.

Sporangia germination assays

Sporangia of *Pythium* were prepared by growing the pathogen on CMA at 25 °C for 4 days in Petri dishes. These plates were then flooded with sterile distilled water for 10 min (three changes of water), and placed at 10 °C for 5 days. This procedure resulted in a rapid and synchronous formation of sporangia. Two agar disks (5mm) containing sporangia were then placed in one sterile depression well slide (12 × 65 mm) and

mixed with 200 μl of either one of the following preparations: seed coats crude extracts, 0.5% w/w cellulase (Onozuka R-10) in 50 mM potassium acetate buffer (pH 5), or 50 mM potassium acetate buffer. These preparations were previously filter-sterilized through a 0.2 μm filter (Acrodisk N° 4192). Heat-inactivated (80 °C for 20 min) or autoclaved (120 ° for 20 min) seed coat extracts or cellulase preparations were used as controls.

Slides were incubated at 25 °C into Petri dishes containing a humidified filter paper in order to reach 100% RH.

Sporangia germination was determined at 6 h intervals during 24 h, by staining with lactophenol blue. Disks were examined microscopically (400×) and the percentage of germinated sporangia recorded. Sporangia were considered germinated if the lenght of the developing tube was twice that of the sporangium. Ten random microscope fields containing 15–30 sporangia per field were counted in each *Pythium* disk.

Data analysis

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

Results

Comparative study of the protection of bean seed against Pythium *with the* Trichoderma *reference strains (TH11 or TH13)*

When bean seed coats were dressed with conidia of TH-11, *Trichoderma* population increased from an initial level of 8×10^3 CFU. g^{-1} to a level of 4×10^5 CFU. g^{-1} within 48 h of incubation, whereas with TH-13, population increased from an initial level of 2.5×10^3 CFU. g^{-1} to 4×10^4 CFU. g^{-1} (Table 1).

The ability of TH-11 and TH-13 to protect bean seeds against *Pythium* sp. was related to the level of seed coat colonisation. After 24 h incubation of dressed seeds, TH-11 gave a higher protection level (100%) than TH-13 (7%), whereas the protection index provided by TH-13 rose to 76% if the incubation period was extended up to 48 h.

Effect of seed pregermination, combined with Trichoderma *dressing, on hydrolytic enzyme activity in bean tissue*

Preliminary results showed that 1,3-β-glucanase activity appeared to be exclusively an endo-glucanase activity because the release of free glucose from laminarin by extracts from seedcoats previously dressed or not with *Trichoderma* (TH-11 or TH-13) was null [unpublished data]. Chitinase activity appeared to be also an endochitinase, as no measurable NAGA was detected in the colorimetric exochitinase assay, while NAGA was released if the chitobiase is added to the reaction mixture (endochitinase assay) (but not with heat-treated crude enzyme extract in the control) [unpublished results].

Figure 1 shows that endo-1,3-β-glucanase, endochitinase and Fpase measured in seed coat extracts obtained from *Trichoderma* sp.-colonized seeds slowly increased for the whole incubation period and were higher in seed dressed by TH-11 than by TH-13.

On the other hand, the CMCase activities in TH-11 or TH-13 seed coats rapidly increased during the first steps of colonization (12 h incubation) and remained constant after 24 h incubation, the overall time course of changes being different with the two strains of *Trichoderma*. Coat extracts from seeds dressed with TH-11 and incubated for 24 h had CMCase activity around 19-fold higher than undressed control, and 2-fold higher than coat extracts prepared from TH-13-dressed seeds.

Effect of seed treatment with cellulase Onozuka on the protection effect and on seed coat colonisation by Trichoderma *sp.*

The effect of cellulase pretreatment on the protective effect of *Trichoderma* spp. was examined by dipping 24 h-pregerminated seeds for 3 h in a solution of commercial Onozuka cellulase (0.5% w/v) (containing both Fpase and CMCase activities), or in acetate buffer (control) before treating them with TH-11 and TH-13. After 24 h incubation, seeds were then treated with Sumico, inoculated with *Pythium* and incubated for 48 h before scoring the symptoms (Table 2).

In *Trichoderma* undressed-control seeds, cellulase pretreatment significantly (P = 0.05) decreased disease severity, compared to control (the protection index reached 3% in *Trichoderma*- and cellulase-untreated seeds whereas this index was near 43% in cellulase-treated seeds).

Table 1. Effect of seed dressing with *Trichoderma* spp. (TH11 or TH13) on seed coat colonisation by *Trichoderma* and seed protection against *Pythium*

Trichoderma treatment	Protection index			Seed coat colonisation (log CFU.g^{-1})	
	TH-11	TH-13	Water	TH-11	TH-13
Incubation time (h)					
0	0	0	0	3.9	3.4
12	0	0	0	4.4	3.6
24	100	7	0	4.8	3.9
48	100	76	3.2	5.6	4.6

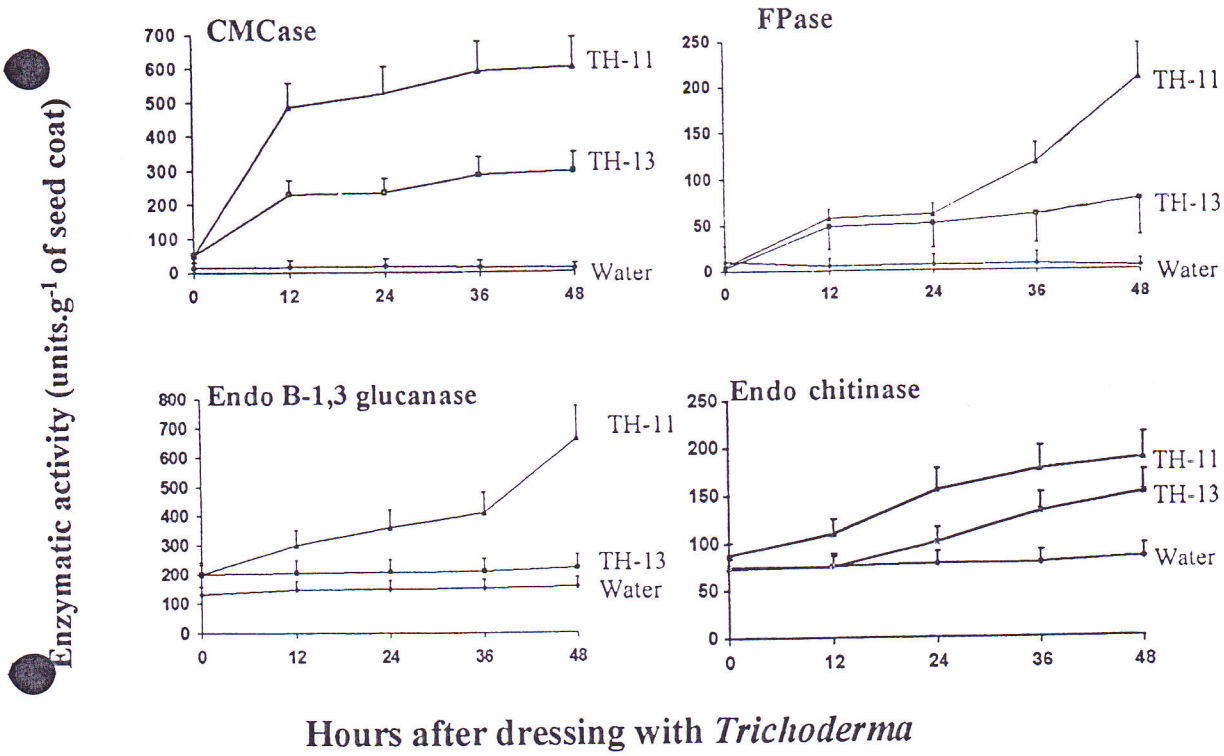

Figure 1. Time course of enzymatic activities (CMCase, Fpase, endo-1,3-β glucanase and endo-chitinase) in crude extracts of coats retrieved from seeds dressed or not with *Trichoderma* (TH-11 or TH-13). Enzymatic activities were expressed as units. g^{-1} of seed coats. Vertical bars indicate standard error of the mean of nine data.

Table 2. Effect of cellulase pretreatment on seed coat colonization by *Trichoderma* sp. and on their protective activities against *Pythium*

Seed pretreatment	Coat colonisation (CFU.g^{-1})		Protection index (%)		
	Dressing		Dressing		
	TH-11	TH-13	TH-11	TH-13	Control (water)
Water	8.10^4	8.10^3	100	6.8	3.2
Cellulase	1.10^5	7.10^4	100	83.2	43.2

TH-11 dressing without cellulase pretreatment significantly (P = 0.05) protected bean seeds against *Pythium* (protection index was 100%), while TH-13 did not protect them significantly. Seed pretreatment with cellulase, did not modify significantly seed coat colonization, and protection obtained with TH-11 dressing, while seed pretreatment with cellulase, followed by TH-13 dressing, increased seed coat colonization from a level of 8×10^3 CFU. g^{-1} to 7×10^4 CFU. g^{-1}, and protection increased from 6.8% to 83.2% in cellulase and TH-13-treated seeds (Table 2).

Effect of cellulase pretreatment on seed coat enzyme activities after Trichoderma *sp. treatment*

Very low CMCase and FPase activities (less than 30 units) were found in seed coat extracts obtained from *Trichoderma*-undressed seeds non pretreated with the cellulase Onuzuka. CMCase and FPase were about 17 and 23 times higher, respectively, in seed coat extracts obtained from *Trichoderma*-undressed and cellulase pretreated seeds, than in controls (buffer treated seed), whereas no significant differences of endo-1,3-β-glucanase and endo-chitinase activity were observed in these extracts (Table 3).

Seed coat extracts from cellulase-pretreated seeds, colonized for 24 h with TH-11 or TH-13, contained higher enzymatic activity than extracts obtained from cellulase-untretreated seeds dressed with TH-11 or TH-13 (Table 3). CMCse, Fpase and endo-1,3-β-glucanase activities were about 4, 3 or 2-fold higher, respectively, in seed coat extracts obtained from cellulase pretreated seeds dressed with TH-13, than in control dressed with TH-13 without cellulase pretreatment whereas endochitinase activities remained unchanged.

Digestion of live Pythium *mycelium*

Coat extracts prepared from seeds colonized by these *Trichoderma* strains were tested for their hydrolytic activity, using mycelium of *Pythium* as substrate.

Control reaction mixtures containing *Pythium* mycelium without crude enzymes preparation, or crude enzymes without *Pythium*, did not release measurable glucose or NAGA during the incubation.

The release of reducing sugars (expressed as glucose equivalents) from *Pythium* mycelium after treatment with coat extracts of seeds dressed with TH-11, was more than two fold that of TH-13 (Figure 2) but no NAGA release from live mycelium of *Pythium* was measured when incubated for 24 h, 48 h or 72 h with coat extracts of seeds dressed with either one of the two *Trichoderma* strains (with or without addition of chitobiase) (unpublished results).

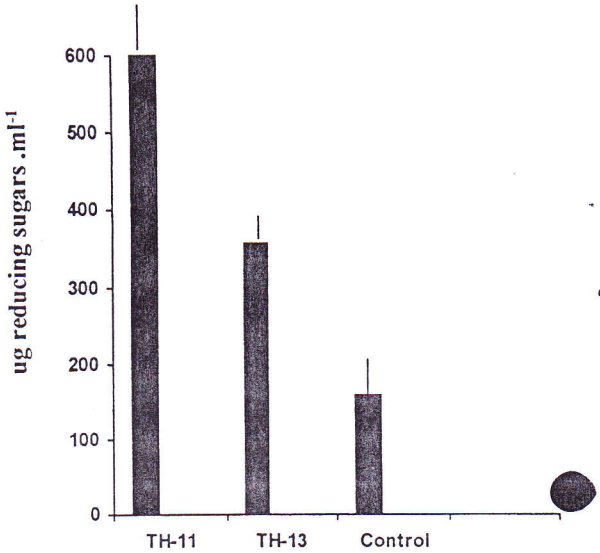

Figure 2. Release of reducing sugars from live mycelium of *Pythium* by seed coat extracts from undressed seed (control) or from seeds dressed with TH-11 or with TH-13. Vertical bars indicate standard error of the mean for nine data.

Sporangial inhibition bioassay

The inhibitory effect of coat extracts on *Pythium* sporangial germination was examined (Table 4). Application to sporangia of seed coat extracts obtained from TH-11-dressed seeds, reduced germination by about 90% as compared to sporangia treated with autoclaved extract of undressed seeds.

Seed coat extracts prepared from cellulase-pretreated seeds colonized with TH-13, or from undressed control seeds pretreated with cellulase, also significantly (90% or 80%, respectively) inhibited germination of sporangia. Inhibition was reduced by diluting the seed coat extracts and abolished after autoclaving seed coat extracts for 30 min.

Discussion

Our aim has been to contribute to the understanding of a model system of host-pathogen-protectant that might be generalized to other hosts, pathogens or situations. As model, we chose the infection of germinating bean seeds by *Pythium* sp., which leads to seed rot or damping-off when seeds are planted in a *Pythium*-infested substrate under suitable ecological conditions

Table 3. Enzymatic activities of seed coat extracts obtained from seeds pretreated with cellulase before their dressing and colonisation by *Trichoderma* sp.

Seed pretreatment	Seed dressing	Protein ($\mu g.g^{-1}$)	Hydrolytic activity (units.g tissue^{-1})			
			CMCse	FPase	Endo-1,3-β-glucanase	Endo chitinase
Buffer	TH-11	42.5	546.0	739.8	377.6	154.6
	TH-13	27.8	240.0	578.0	233.1	101.8
	Water	11.2	28.8	25.0	166.8	84.0
Cellulase	TH-11	96.3	1,021.0	2,210.0	500.0	172.0
	TH-13	60.0	954.0	1,970.0	521.0	152.8
	Water	48.0	482.0	568.3	166.0	74.3

Seed coat extracts were prepared from seeds pretreated for 3 h with a solution of Onozuka cellulase or acetate buffer (control) and then dressed with TH-11, TH-13 or sterile water, followed by incubation for 24 h. The levels of standard error of enzyme activities varies from 15 to 20% between experiments.

Table 4. Sporangia germination of *Pythium* in the presence of crude seed extracts from seeds pregerminated and dressed with *Trichoderma* (TH-11 or TH-13)

Seed coat extracts from	Sporangia germination (%)		
	Seed coat extract dilution		
	10^{-1}	10^{-2}	10^{-3}
Seed dressed with:			
TH-11	4.5	20.0	62.0
TH-11 (autoclaved)	47.4	60.0	61.5
TH-13	51.0	54.0	62.2
TH-13 (autoclaved)	61.6	62.2	65.3
Water	56.0	58.0	60.0
Water (autoclaved)	70.9	67.2	69.5
Seeds pretraeted with cellulase followed by dressing with:			
TH-13	7.3	28.5	51.0
TH-13 (autoclaved)	68.7	72.5	71.5
Water	14.6	40.7	63.4
Water (autoclaved)	73.2	69.5	62.3
50 mM acetate buffer	61.0	60.0	62.1
Cellulase	5.0	25.6	48.5
Cellulase (autoclaved)	80.0	81.5	86.7

Seed coat extracts were prepared from seeds colonised for 24 h with TH-11 or TH-13. Extracts were autoclaved for 30 min. data are representative of three independant experiments. The variation coefficient for all treatments were $<12\%$.

(Mezui M'ella, 1994) or to necrosis of cotyledons in the Petri dish assay used in this study (Cotes et al., 1992).

Possible role of carboxymethycellulase in the protection against Pythium

The level of seed coat colonization by TH-11 or TH-13 after seed dressing was shown to be correlated with the CMCase activity, as measured either in culture filtrates when these strains were grown in synthetic medium containing autoclaved seed coats as sole carbon source (Cotes et al., 1994a,b), or in the seed coats retreived from germinating dressed bean seeds.

Presumably, most of CMCase and FPase activities measured in seed coat extracts from *Trichoderma* spp.-dressed seeds, were of fungal origin, since only negligible activity was observed in seed coats extracts obtained from water controls (Figure 1). The higher CMCase activity in seed coats, after seeds were dressed with TH-11 (as compared to seeds dressed with TH-13), suggests that the ability of TH-11 or TH-13 to hydrolyse the cellulose present in seed coats could be correlated with their colonization capacity. This observation agrees with others (Ahmad and Baker, 1987), where the competitive saprophytic ability of *Trichoderma* sp., expressed as capacity to colonize the rhizosphere, was directly correlated with the amount of cellulase produced by these strains.

When pregerminated seeds were treated with Onozuka cellulase (obtained from *Trichoderma viride* cultures and containing both Fpase and CMCase activities), and were then colonized for 24 h with TH-13 (the inferior colonizer strain), seed coat colonization increased 10-fold in terms of CFU and protection after Sumico treatment increased from 7% to 83%, as compared to the cellulase-untreated control. We presume that the increased protection index by TH-13 following cellulase pretreatment rests partly on an

increased seed coat colonization. However cellulase pretreatment of control seeds without *Trichoderma* dressing also increased protection from 3% to 43%, thus suggesting that cellulase activity might have a direct inhibition effect toward *P. splendens*. It could also be possible that *Trichoderma* cellulase acts on the seed tissue by releasing toxic substances that could inhibit *Pythium* germination or by releasing plant glucans that could act as endogenous elicitors, thus inducing plant responses leading to an increased resistance towards *Pythium*. The possibility of the presence of other fungitoxic compounds in the commercial preparation of cellulase cannot be ruled out.

Possible role of 1,3-β-glucanase and chitinase found in bean tissue after dressing with Trichoderma

Combination of the pretreatment with cellulase and TH-13 dressing also increased the endoglucanase and chitinase activities whereas those remained unchanged in *Trichoderma* undressed control.

A role of such 1,3-β-glucanase and chitinase activities as defence mechanism was suggested in tomato, where *Cladosporium fulvum* induced earlier and larger increase of these enzymes in an incompatible host-parasite interaction than in compatible one (Joosten and de Wit, 1988). It has also been shown that both plant 1,3-β-glucanase and chitinase can release phytoalexin elicitors from fungal cell walls (Cervone et al., 1989; Keen et al., 1983; Kombrink and Hahlbrock, 1986).

It has been shown that plant chitinase and 1,3-β-glucanase act synergistically in the partial degradation of isolated fungal cell walls (Arlorio et al., 1992; Jones et al., 1974; Young and Pegg, 1982). In this respect, we demonstrated the ability of seed coat glycosidases (cellulase and/or endo-1,3-β-glucanase) to degrade *Pythium* cell walls and to liberate reducing sugars. Seed coat extracts from protected bean seeds (dressed with TH-11) induced a larger release of reducing sugars from *Pythium* cell walls than those from unprotected bean seeds (dressed with TH-13) (Figure 2). This is in accordance with the levels of glycosidase activity in seed coat extracts as measured using laminarin or carboxymethylcellulose as substrates.

Pythium spp. have long been classified among the few fungi whose walls are composed of cellulose and 1,3-β-glucans (Bartnicki-Garcia, 1968). Chérif et al. (1993) demonstrated the presence of chitin in cell walls of *P. ultimum*, but our study suggests that chitinase (together endo- and exo-chitinases) would not be involved in the degradation of cell walls of our *Pythium* strain, based on the fact that no N-acetyl glucosamine was released from live mycelium of *Pythium* submitted to crude coat extracts of *Trichoderma*-dressed seeds containing chitinase activity.

The interpretation of these results must remain very careful because of the lack of detailed knowledge about the immediate environment of *Pythium* in a *Trichoderma*-dressed seed to relate *in vitro* digestion studies to *in situ* events. Nevertheless, our demonstration that enzymes contained in seed coat extract are active *in vitro* towards *Pythium* mycelium, supports the idea that such enzymes might also degrade the *Pythium* cell wall during *in situ* infection.

Our model system of host-pathogen protectant do not provide any experimental evidence about the orig of the chitinase activity extracted from *Trichoderma*-dressed seeds. Basically, enzyme extraction combined all possible sources of enzymatic activities: enzymes induced in the plant, and enzymes excreted by *Trichoderma*. Cotes et al. (1994b) reported that only exo-chitinase activities were released by TH-11 and TH-13 strains of *Trichoderma*, in culture medium containing autoclaved seed coats as sole carbon source, whereas activities in bean seed coats (both in undressed control seeds or in *Trichoderma*-dressed seeds), showed only endochitinase activities, suggesting a plant origin in accordance with the characterization of such endoactivity of chitinase (Broglie et al., 1986) in bean leaves. The possibility, however, that *Trichoderma* sp. releases endochitinase under specific conditions (Lorito et al., 1993), cannot be entirely ruled out. The origin of chitinase activity should be therefore clarified by futher characterization of the enzyme(s) extracted from *Trichoderma*-dressed seeds.

Antifungal properties of seeds colonized by Trichoderma *sp.*

Our results showed that seeds colonized by *Trichoderma* spp. release a heat sensitive factor(s) in coats, which inhibits sporangia germination of *Pythium in vitro*. We have shown that enzymes found in seed coat extracts obtained from *Trichoderma*-dressed seeds could digest mycelium of *Pythium* and release reducing sugars. Seed coat extracts from bean seeds colonized with TH-11, contained more hydrolytic enzyme activities than those of TH-13 (the less effective biocontrol agent). Upon heat inactivation, such enzyme activities disappeared, together with the inhibition of *Pythium* sporangia germination. A solution of commercial

cellulase, exhibiting heat sensitive Fpase and CMCase activities, also inhibited *Pythium* sporangia germination, strengthening the hypothesis of a contribution of these enzymes in the protective effect observed.

These overall results suggest that the CMCase component of the cellulase complex is involved in the colonisation process and the linked protective effects measured by symptom index or fungitoxicity of seed extracts.

On a practical point of view, the application of antagonists by seed dressing is an attractive method for introducing a biocontrol agent onto the infection court of the host. There have been numerous reports of such treatments with *Trichoderma* for controlling soilborne plant pathogenic fungi (Harman et al., 1980; [illegible]ad et al., 1982; Lifshitz et al., 1986). The effects were highly reproducible in terms of emergence percentage, improved vigour of the plantlets and reduced post emergence symptoms, with several crop-pathogen combinations. The results of this study pave the way to a screening protocol for polyvalent microbial strains based on their ability to produce cellulase and to device a schedule of application of biological control agents that would be operational within a wide range of situations and environments.

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