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EFFECT OF PRECOLONIZATION OF BEAN SEEDS WITH *TRICHODERMA*, ON SYMPTOMS INDUCED BY *PYTHIUM*

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ABSTRACT

Pregerminated bean seeds were precolonized for 24-hr with 15 isolates of *Trichoderma* spp. Seed samples were then treated with the fungicide Sumico (diethofencarbe and carbendazime), in order to kill *Trichoderma* spp and were then inoculated with a suspension of *Pythium* sp. Seeds were placed thereafteron water agar and were incubated at 25°C for 48 hours. Control seeds without *Trichoderma*, treated or not with Sumico, showed 90% or 82% infection respectively, with severe symptoms. *Trichoderma* precolonization for 24 h either with or without Sumico gave 100% protection against *Pythium* with four isolates, whereas fungicide treatment nullified the biological control with three isolates, and diminished the protection with the others. However, after 48 h precolonization all *Trichoderma* isolates gave full protection, either with or without fungicide treatment.

The efficacy of the biological control of *Pythium* was linked with the rate of seed coat precolonization by *Trichoderma*. *Pythium* growth *in vitro* was stimulated by exposure to leachates of control seeds, but not of *Trichoderma*-precolonized seeds. Competition between *Trichoderma* and *Pythium* for site occupation, consumption of nutrients, consumption of attractive substances, together with possible release of toxic substances by *Trichoderma*, provide a likely explanations for the observed protection.

Pythium species are important soil pathogens involved in seed rots and preemergence damping-off of a wide variety of plant species (12,14,16,25,26,27). There have been many reports of successful use of *Trichoderma* spp to control soilborne pathogenic fungi under greenhouse conditions (1,2,3,4,6,8,17,18,20,22) but *Trichoderma* has not been used extensively so far in agricultural practice, partly because of the large amounts of inoculum required to apply the antagonist to soils, and also because of the variability in performance observed between repetitions, locations and seasons.

The application of antagonists by seed treatment is therefore an attractive method for the introduction and establishment of a biocontrol agent in the infection courts of the host (6,9,10,15,19,28,).

The objective of our study was to test the factors involved in the efficacy of potentially antagonistic isolates of *Trichoderma* spp against *Pythium* when applied as a seed precolonization, we studied:(i)the biological control potential of various isolates of *Trichoderma* against *Pythium* damping-off in soil, (ii) the efficacy of seed precolonization with *Trichoderma* isolates on seed rot induced *in vitro* by *Pythium,* (iii) the effect of seed precolonization with *Trichoderma* isolates on *in vitro* growth of *Pythium*, and on seed coat colonization.

MATERIAL AND METHODS

Fungal isolates. Various isolates of *Trichoderma* spp from different origin have been used in our study; 12 isolates numbered (1-2-5-6-7-8-9-10-11-12-13-14) were supplied by CIMIC (Microbiological Research Center, Andes University of Colombia). The Bioindustries Laboratory of the Faculty of agricultural sciences of Gembloux (Belgium) supplied isolate 4, while isolate 3 originated from a commercial preparation of ORSAN Co France. The *Pythium* strain used was isolated in our laboratory from a bean seedling showing symptoms of damping-off.

Growth chamber experiments.*Trichoderma* isolates were grown on a nutrient medium containing a mixture of wheat bran, sucrose,and water (30 g/1.6 g/80 ml) autoclaved for 1 hour at 121°C. Flasks containing this medium were inoculated with 10^8 propagules of *Trichoderma* grown on malt extract agar and were incubated in a culture room for 7 days at 25°C with 16 hours light photoperiod. *Pythium* was grown on a nutrient medium containing Vermiculite, V8 juice as additive, and water (20g/24ml/80 ml). This medium was autoclaved for 20 min at 121°C, and was then inoculated with 3 discs (5mm) of a 7 day-old *Pythium* culture grown in Corn meal agar(CM, Difco). Cultures were incubated in a culture room as described above.

Pythium inoculation tests, and biological control studies by *Trichoderma* were carried out in Gembloux loam soil sieved through a 4-mm-mesh screen. Sterile soil was prepared by autoclaving for 1 hour at 121°C on 3 consecutive days. Soil moisture holding capacity was adjusted at 75%. All experiments were performed in plastic pots containing 300 g of soil planted with 5 bean seeds followed by incubation for 7 days at 121°C, with a 16-hr photoperiod provided by cool white fluorescent lights.

The soil was inoculated with *Pythium* culture (5%V/V, giving a final concentration of about 10^3 CFU/g), inmediately prior to bean sowing. *Trichoderma* cultures (giving a final concentration of 10^6 propagules/g), were added to *Pythium* inoculated soil, immediatly prior to bean planting. Seeds were uniformly coated with a suspension of *Trichoderma* containing 10^6 propagules/ml, and were sown in the *Trichoderma* treated and *Pythium* infested soil.

After 7 days of incubation, disease incidence was evaluated on basis of the number of plants that emerged, or on basis of the number of damped-off seedlings.

Data analysis. In all experiments, treatment were replicated 6 fold; all tests were conducted in 3 independent experiments. Analysis of variance was performed, and differences were established at the significance level of P= 0.05.

In vitro biological control tests. *Trichoderma* spp were grown on malt extract agar at 25°C for 7 days, with 16 h light photoperiod. Conidia were harvested by scraping the culture surface with a spatula and suspending the spores in sterile water. Spore suspension were then filtered through two layers of chesecloth, and the final concentration was adjusted at $2x10^7$ conidia/ml.

Bean seeds were surface-sterilized for 3 min whith 3% sodium hypochlorite and ethanol, and were washed 3 times for 5 min with distilled water. Seeds were pregerminated in 0.7% water agar in Petri dishes for 24 h under growth chamber conditions. After dipping for 30 min in a spore suspension of either one of 14 different isolates of *Trichoderma* spp, they were placed for 24 h on 0.7% water in agar Petri dishes. In order to kill *Trichoderma*, seeds were then dipped for 30 min in 4 ppm of the fungicide Sumico (25% diethofencarbe and 25% carbendazime), or in water (control). Inoculation was carried out by dipping the seeds for 30 min in a suspension of *Pythium* sp (10^3 propagules/ml) scraped from 7 day-old cultures grown on Corn meal agar suspended in sterile water. Controls were dipped in sterile water. Four seeds were placed on 0.7% water agar in Petri dishes wich were incubated for 48 h at 25°C. Symptom intensity was scored according to a visual scale based on the number of necrotic spots wich had developed on each seed (1-5 spots= 20, 6-10 spots= 40, 11-15spots= 60, 16-20= 80 and >20 spots= 100). The protection index was expressed as (DC-DT)/DCx100, (DC= disease index produced by *Pythium* in control, DT= disease index produced by *Pythium* after treatment with *Trichoderma*). All treatments were carried out in 4 replicates experiments repeated 3 times; standard deviations were calculated and data are presented as mean values.

Colonization of bean seed coats by *Trichoderma* spp. The ability of isolates 8 and 11 of *Trichoderma* to colonize bean seed coats was investigated. *Trichoderma*-treated seeds were recovered from water agar substrate every 6 h during 2 days. They were washed for 3 min with 0.05% Tween 20 in sterile distilled water. Seed coats were detached and blended in a Potter for 2 min, in 10 ml of sterile distilled water. Serial dilutions were prepared, and 0.1 ml aliquots were plated onto 0.1% sodium desoxycholate PDA for assay of colony forming units (CFU). The number of *Trichoderma* colonies was evaluated after 5 days of incubation at 25°C under 16 h photoperiod fluorescent light. *Trichoderma* colonization was expressed as CFU/g of seed coats.

Effect of bean seed precolonization with *Trichoderma* on *in vitro* growth of *Pythium*. An agar disc 5 mm in diameter from a colony of *Pythium* grown for 7 days at 25°C in 0.7% water agar, was inoculated in the center of a water agar Petri dish. Two bean seeds precolonized with *Trichoderma* for 24 h, and treated or not with Sumico , were placed at opposite sides 2 cm apart the *Pythium*

inoculum. After 24 h of incubation at 25°C under 16 h light photoperiod, *Pythium* growth was measured as colony diameter in each plate. Each experiment was performed 5 times, with three replicates.

RESULTS

Effect of differents isolates of *Trichoderma* on damping-off disease of bean. The isolates differed in their ability to control *Pythium*. Comparative tests with all 14 isolates, on the basis of the percentage been of seed germination, permitted to classify isolates according to their protective effect.

Isolates 2,3,11 and 12 of *Trichoderma* applied both by soil incorporation and seed coating, reduced significantly *Pythium* induced symptoms. Isolate 3 was the most efficient biocontrol agent with 66% protection, and isolate 13 gave the weakest effect, with 3% protection only (fig. 1)

Effect of seed precolonization with *Trichoderma* on symptoms induced *in vitro* by *Pythium*. All *Trichoderma* isolates applied as seed precolonization for 24 h, reduced the incidence of *Pythium* seed rot *in vitro* on water agar. Sumico (or water)-treated controls inoculated with *Pythium* alone showed severe symptoms. Seeds precolonized with isolates 3,7,11,15 of *Trichoderma* followed or not by Sumico treatment gave 100% protection against *Pythium*. Fungicide treatment nullified the biological control of 3 other isolates (number 2,8,13), or decreased the level of protection with 9 others.

When Sumico treatment was applied after 48 h of precolonization (instead of 24 h), all 15 *Trichoderma* isolates acted as full protectants (fig. 2).

Seed coat colonization by two selected isolates of *Trichoderma* spp. The ability of *Trichoderma* strains to effectively protect bean seeds against *Pythium* sp, was linked to their capacity to colonize seed coats during the precolonization period. After 24 h, isolate 11 was a better colonizing agent of seed coat than isolate 8, and gave a higher protection level. After 48 h of precolonization, however, the level of colonization and the protection effect were similar with both *Trichoderma* isolates. (fig. 3).

Effect of bean seed precolonization with *Trichoderma* on *in vitro* growth of *Pythium*. *Pythium* growth *in vitro* was enhanced by incubation in the presence of healthy untreated seeds, compared to control dishes inoculated without seeds. In the presence of seeds precolonized with isolates 8 and 11 of *Trichoderma*, colony growth of *Pythium* was reduced.

Sumico-treated seeds, precolonization with isolate 11 of *Trichoderma* induced a larger reduction of *Pythium* growth than precolonization with isolate 8. Differences between the two isolates gradually decreased with increasing precolonization time (fig. 4).

Microscope observations of seed coats 24 h after *Trichoderma* precolonization seeds showed hyphae on the inner as well as the outer side of seed coat.

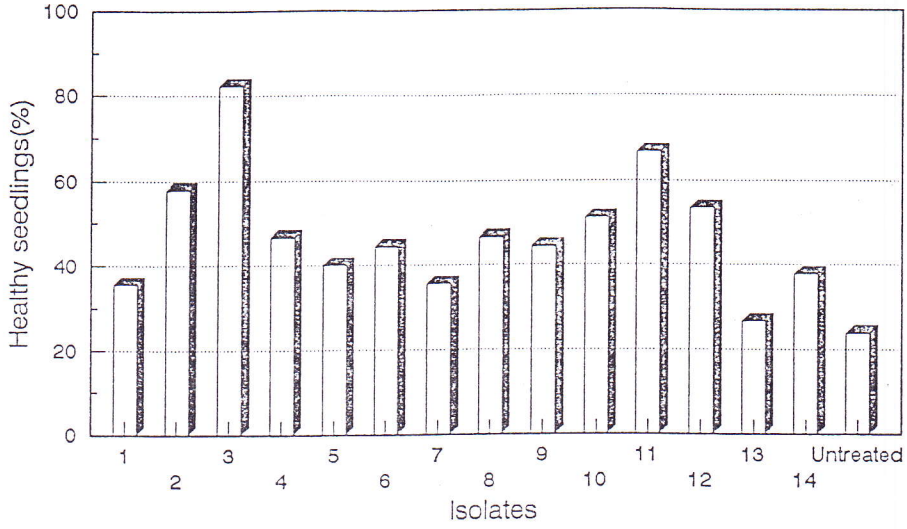

Fig 1. Effect of 14 *Trichoderma* spp isolates on emergence percentage of bean seedlings planted in soil artificially infested with *Pythium* sp. Isolates of *Trichoderma* were applied either as wheat bran preparation mixed with the soil, or as seed coating. Infested soil without *Trichoderma* was used as untreated control.

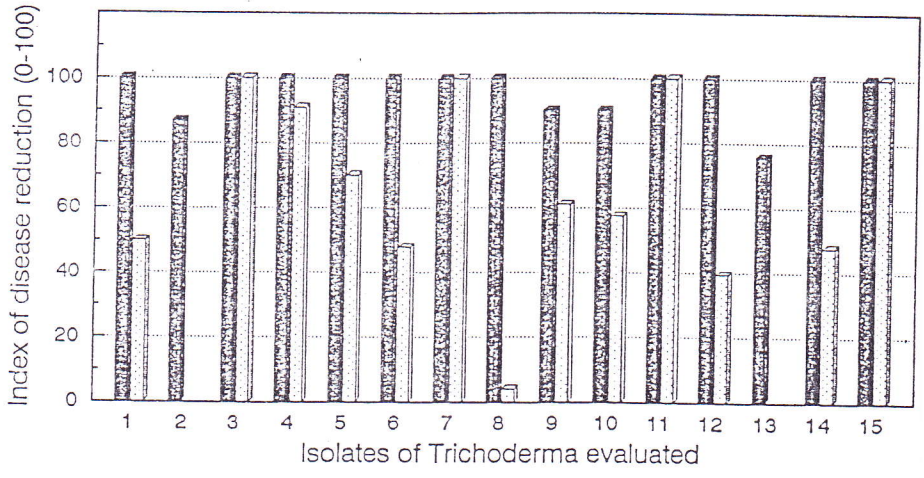

Fig 2. Effect of precolonization of bean seeds with isolates of *Trichoderma* spp, on seed rot induced *in vitro* by *Pythium* sp.
Seeds precolonized for 24 h with *Trichoderma* Seeds precolonized for 24 h with *Trichoderma* and then treated with the fungicide Sumico.

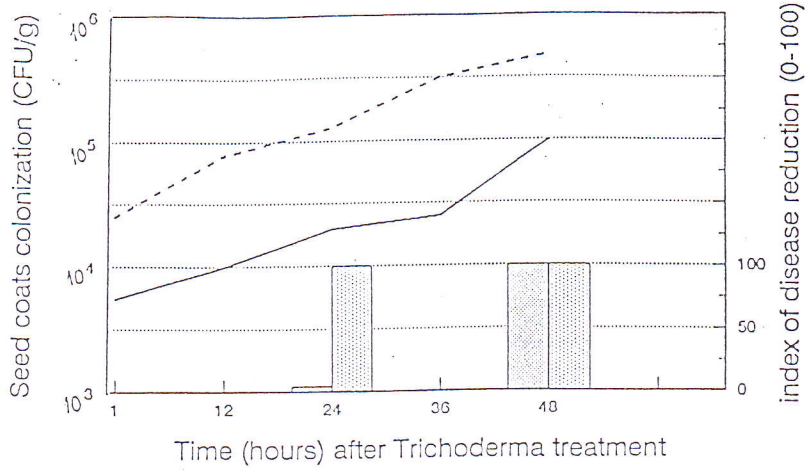

Fig 3. Relationship between seed coat colonization by *Trichoderma* spp and *Pythium* disease incidence, when seeds were treated with Sumico fungicide 24 h after *Trichoderma* spp inoculation.
Seed coat colonization by: ------ isolate 11 of *Trichoderma* sp
——— isolate 8 of *Trichoderma* sp.
Index of disease reduction ▭ isolate 11, ▭ isolate 8

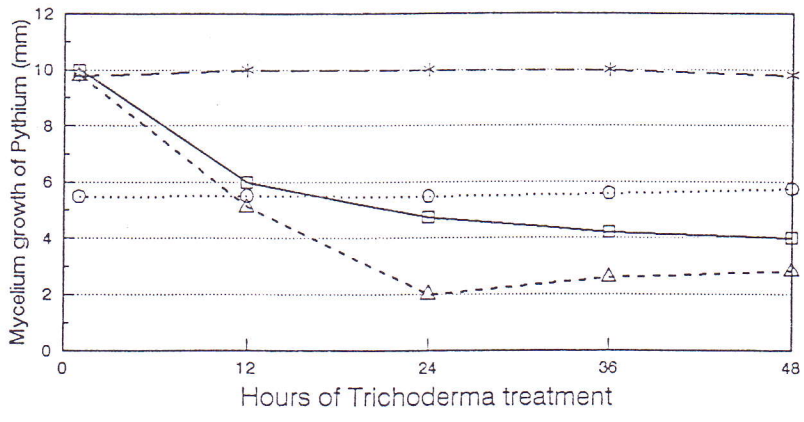

Fig 4. *In vitro* effect of seed precolonization with *Trichoderma* spp, followed by treatment with the fungicide Sumico, on the growth of *Pythium* sp.
—□— Seeds precolonized with isolate 8 of *Trichoderma* sp. Seeds precolonized with isolate 11 of *Trichoderma* . ····○···· *Pythium* growth without seeds. — ✳ — *Pythium* growth with pregerminated seeds treated with Sumico.

Discussion

Various isolates of *Trichoderma* spp incorporated into soil, or used as seed coating, reduced damping-off incidence caused by *Pythium* sp. *Trichoderma* isolates were classified according to their protection capacity against *Pythium* sp. Because of the complexity of the soil system, and of the variability in its composition and microflora, we standardized an *in vitro* method, using two *Trichoderma* selected isolates as model, in order to study the mechanisms involved in the protection obtained against *Pythium*.

With isolate 11 of *Trichoderma*, after 24 h of seed precolonization, protection obtained remained effective after killing *Trichoderma* by Sumico treatment. With isolate 8, however, the protection induced by 24 h of precolonization was nullified by fungicide application (fig. 2). Isolate 11 had a higher level of seed coat colonization than isolate 8. However, full protection was obtained after 48 h of seed precolonization with isolates 8 and 11 of *Trichoderma*, seeds being treated with fungicide or not; seed coat colonization by isolate 8 corresponded to the same level obtained after 24 h of precolonization with isolate 11.

The control of bean seed rot when *Trichoderma* was killed after 24 h precolonization was linked to seed coat colonization: Seed is the primary site of initial colonization by *Pythium* (16,19,21,25).
Seed coat colonization by *Trichoderma* could interfere with the interaction between *Pythium* and the bean host.

Infection by *Pythium* are known to be stimulated by carbohydrates exudated during seed germination (13,15,16,23), as evidenced by the fact that removal of exudates by seeds presoaking reduced disease incidence (8,23,24), whereas, addition of carbohydrates or dead seeds to the soil increased disease incidence (8). *Pythium* growth *in vitro* was stimulated by untreated germinating control seeds, but not by *Trichoderma* precolonized seeds.

We assume that partial removal of seed exudates by *Trichoderma* during seed precolonization, could be part of the protection mechanism observed in our experimental system.

Although the mechanisms of biological control of *Pythium* seed rot by *Trichoderma* are not fully known, Harman et al (1980), suggested that mycoparasitism was the main process in the reduction of *Pythium* damping-off when pea seeds were coated with *Trichoderma* (10). Our results showing the protection was maintained after killing *Trichoderma* spp after 24-48h precolonization, suggest that mycoparasitism is not a likely mechanism in the control of *Pythium* seed rot in our case.

Biological control is a complex process that could involve different mechanism. *Trichoderma* activity during precolonization, could result not only in site occupation and removal of exudates, but also in the production of toxic metabolites (17) or hydrolytic enzymes (5,7).

Our data suggest that seed precolonization with *Trichoderma* is an attractive candidate to control *Pythium* diseases in bean.

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