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The use of a GUS transformant of *Trichoderma harzianum*, strain T3a, to study metabolic activity in the spermosphere and rhizosphere related to biocontrol of *Pythium* damping-off and root rot

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Abstract

The activity of *Trichoderma harzianum* in the spermosphere and rhizosphere of different plant species was studied by use of a β-glucuronidase (GUS) transformant (strain T3a). Hereby, direct observation of micro-habitats supporting metabolic activity of *T. harzianum* is reported. Germination of conidia and mycelial growth were not supported by exudates from healthy roots of various ages. Instead, growth and activity of *T. harzianum* depended on access to dead organic substrates such as seed coats, decaying roots, and wounds, including those caused by infecting pathogens. A correlation between the GUS activity of *T. harzianum* and the biomass of *Pythium ultimum* in infected roots was established. On the basis of our observations, we suggest that the biocontrol ability of *T. harzianum* involves competition with the pathogen for substrates including the seed coat, and wounded or infected root tissue.

Introduction

The fungal antagonist *Trichoderma harzianum* has proved to be an effective biocontrol agent against a range of soilborne plant pathogens both when applied as seed treatment or when mixed into soil and soilless potting mixes (Chet, 1987; Jensen and Wolffhechel, 1995; Papavizas, 1985).

Understanding the ecology of the antagonist is critical for improvement of biocontrol and its practical implementation. However, the ecology of *T. harzianum* is poorly known, and despite extensive research, the mechanisms by which disease control is achieved are not clearly understood. Possible mechanisms are competition, antibiosis, mycoparasitism, induced resistance, and growth promotion. These mechanisms can act separately, but the emerging belief is that biocontrol results from a concurrent (Fravel and Keinath, 1991) or synergistic (Di Pietro et al., 1993; Schirmböck et al., 1994) action of several mechanisms, depending on the circumstances and the pathogen in question.

To control root diseases, an antagonist should ideally possess the ability to colonise infection sites along the rhizoplane despite competition from other micro-organisms. However, apart from one isolate (Sivan and Chet, 1989), wild-type *T. harzianum* has been reported not to be rhizosphere competent (Ahmad and Baker, 1987a; Chao et al., 1986; Green and Jensen, 1995; Papavizas, 1982). By mutation, Ahmad and Baker (1987a) introduced resistance to benomyl in strains of *T. harzianum*. Unexpectedly, these mutants also gained the ability to colonise the rhizosphere of several crops when applied to seeds in the absence of benomyl. The fact that these mutants expressed enhanced cellulolytic activity, which correlated with their rhizosphere competence, indicated that the mutants competed more efficiently for the mucigel at the rhizoplane (Ahmad and Baker, 1987b). In addition, these rhizosphere competent mutants were

found to exhibit improved biocontrol ability when tested against *Pythium ultimum* on cucumber seedlings (Ahmad and Baker, 1988). Later, Sivan and Harman (1991) improved the rhizosphere competence in a protoplast fusion progeny using the same benomyl resistant strain as one of the parents.

Until recently, studies of root colonisation by *Trichoderma* spp. have been based on indirect techniques such as determination of the number of colony forming units (CFU) per centimetre of root. However, this technique does not reflect the actual activity of the fungus (Green and Jensen, 1995; Lumsden et al., 1990). Lately, the integration of marker genes into fungal genomes has allowed fungal activity to be monitored more accurately. Eparvier and Alabouvette (1994) used β-glucuronidase (GUS) transgenic strains to examine competition between pathogenic and non-pathogenic *Fusarium oxysporum* on roots. They clearly showed that the activity of both the pathogenic and the non-pathogenic strain was reduced in the presence of the other, and that competition for actual infection sites was taking place. By use of a GUS transformant of *T. harzianum*, strain T3a, results were obtained which suggest that competition for nutrients and space in small wounds on the root surface may be important for the antagonistic effect of the organism, because it may block possible sites for pathogens to infect the plants (Green and Jensen, 1995). Lo et al. (1998) used a GUS transformed strain of the rhizosphere competent *T. harzianum*, strain T22 to study colonisation of rhizosphere and phylloplane of creeping bentgrass and interactions with *Rhizoctonia solani*.

In order to further understand the ecology of *T. harzianum*, the GUS transformed strain, T3a, was used to study metabolic activity in the spermosphere and the rhizosphere of healthy, wounded, and diseased plants. Direct observations of micro-habitats supporting metabolic activity of *T. harzianum* are reported. Additional observations support the idea that direct interactions between *T. harzianum* and pathogenic *Pythium* spp. take place in infected root tissue.

Materials and methods

Fungi and plants

T. harzianum Rifai isolate T3 was originally isolated from a *Pythium*-suppressive peat (Wolffhechel, 1989). The transformant, T3a, was derived from T3 by transformation with the *Escherichia coli* GUS gene and the hygromycin B resistance gene (Thrane et al., 1995). Peat-bran inocula of T3 and T3a were prepared according to Sivan et al. (1984).

P. ultimum Trow var. *ultimum* isolate HB2 was originally isolated from a sandy loam soil from Højbakkegaard, Tåstrup, Denmark (Wolffhechel, 1989). The fungus was stored on autoclaved barley leaves kept in sterile tap water at 4 °C. At the time of use, a barley leaf was placed on corn meal agar, CMA (Difco Laboratories, Detroit, MI, USA) to enable the fungus to grow.

The following plants were used for the experiments: Cucumber, *Cucumis sativus* L. cv. 'Langelands Kæmpe – Gigant', (Dæhnfeldt, Odense, Denmark), pea, *Pisum sativum*, L. cv. 'Ping Pong', (Dæhnfeldt), white clover, *Trifolium repens*, L., red fescue, *Festuca rubra*, L., and perennial ryegrass, *Lolium perenne*, L.

Unless otherwise stated, seeds were surface-disinfested in 2.5% NaOCl, and pre-germinated on half-strength potato dextrose agar, PDA (18.75 g PDA [Difco], 12.5 g Bacto [Difco] agar, 1 l H_2O), at 20 or 25 °C for 3 or 4 days, depending on plant species. Contaminated seedlings were discarded.

Growth conditions

Plants were grown in fine, 0–20 mm, light coloured sphagnum peat, Færdigblanding 1, (Pinstrup, Mosebrug A/S, Ryomgaard, Denmark) mixed with vermiculite, grade 2 (Skamol, Nykøbing Mors, Denmark), at 5 : 2 (wt/wt). The final pH (H_2O) was 5.5. After planting, the pots were watered with a sterile balanced nutrient solution containing (mM): $Ca(NO_3)_2$ (2.2); NH_4NO_3 (1.3); KNO_3 (1.1); $Mg(NO_3)_2$ (1.0); KH_2PO_4 (0.7); $MgSO_4$ (0.38); Fe^{3+}–EDTA (0.0016); $MnSO_4$ (0.0023); H_3BO_3 (0.0065); $ZnSO_4$ (0.0024); $NaMoO_4$ (0.0003); NaCl (0.01); $CuSO_4$ (0.0007) to a water content of 0.98 kPa (63.6% volume) and a conductivity of 3.5 mΩ^{-1}. The pots were placed in a growth chamber at 18 °C with a 16 h photoperiod and watered to the initial weight on alternate days.

Rhizosphere effect on the proliferation of T. harzianum

Both raw and steamed potting mix was used for the experiments. Steaming was carried out at 100 °C in autoclave bags (Scienceware, Pequannock, NJ, USA) for 1 h on each of three successive days. Both steamed

and raw potting mix was thoroughly mixed with inoculum of T3 to a concentration varying between 10^6 and 10^8 CFU per gram dry matter. Non-infested potting mixes were used for controls. Plants were grown in rhizoboxes (Green and Jensen, 2000) made of a 1 cm wide plastic frame, which was inserted into a fine mesh (38 μm) nylon bag. The resulting open-topped chamber measured 1 × 7 × 7 cm. The fine mesh confined plant roots, while fungal mycelia could grow through the mesh and the nutrient solution could move freely between the rhizobox and the surrounding potting mix. At the end of the experiments a firm mat of roots with adhering potting mix was present inside the rhizobox. Sixteen seedlings of white clover, red fescue, or perennial ryegrass, or eight seedlings of cucumber were planted in each rhizobox, containing 3.75 g dry matter of the potting mix. The rhizoboxes were placed in 330-ml sterile pots, that were filled with an additional 23.75 g dry matter of the potting mix.

Population densities of T3 were assessed twice within 4 to 15 days of incubation. The potting mix was sampled by removing and separating the rhizobox. Bulk potting mix was collected outside the box, and root zone potting mix was rubbed off the root mat. Samples were then suspended in 100 ml sterile water and homogenised in an Ultra-turrax T 25 (IKA-Labortechnic, Staufen, Germany) for 2 min at 13,500 rpm. Serial dilutions were made and aliquots plated on *Trichoderma*-selective medium, mTSM (Green and Jensen, 1995). The population was expressed as CFU per gram of dry matter of potting mix. The dry weight of the potting mix was determined from a 10 g sample. Five experiments were carried out using cucumber, while a sixth experiment included cucumber, white clover, red fescue, and perennial ryegrass. Each experiment differed with respect to inoculum concentration and harvest time. Each treatment in the experiments had two replicate pots.

Localisation of GUS active hyphae on the rhizoplane of healthy roots

Polypropylene centrifuge tubes (27 × 102 mm), were cut longitudinally into two halves (Ahmad and Baker, 1987a). Each half was filled with moistened potting mix, wrapped in fine mesh (38 μm) nylon, and the two half tubes were placed together and secured with rubber bands. Seeds of cucumber and pea (both non-disinfested) were soaked in water for two hours, rolled in peat-bran inoculum of the transformant T3a, and one seed was placed between the two adjoining nylon meshes at the top of each tube (Green and Jensen, 1995). Seeds without T3a were used as controls. The tubes were kept standing in a 1 l container with their bases submerged in 2 cm water and placed in the growth chamber.

Seeds or seedlings were watered from above to disperse conidia downwards. Half of the tubes were watered immediately after sowing while the other half were watered after 6 days. Two, three and nine days after watering, the tubes were taken apart, and three seedlings per treatment were placed on mTSM and incubated at room temperature for 3 days. Fungal growth from the roots was recorded. To distinguish T3a from naturally occurring strains of *Trichoderma*, the plates were incubated at 37 °C for 8 h with 0.75 ml sodium phosphate buffer, (50 mM, pH 7.0) containing 1 mM EDTA (Sigma Chemical Company, St. Louis), 0.05% (vol/vol) Triton X-100 (Bie & Berntsen, Rødovre, Denmark), and 80 μg per ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, X-gluc, (Calbiochem-Novabiochem Corporation, San Diego, CA, USA). Another three replicate seedlings were carefully placed in Petri-dishes containing 10 ml of the X-gluc solution, and incubated for 8 h at 37 °C. These seedlings were examined under a SZH stereomicroscope (Olympus, Tokyo, Japan) for the presence of blue-stained hyphae. The experiment was repeated three times and each time, a total of 36 seedlings of both cucumber and pea were examined.

Localisation of GUS active hyphae on wounded roots

Three 4-day-old cucumber seedlings were planted in 330-ml pots containing 27.5 g dry matter of potting mix, mixed with peat-bran inoculum of the transformant T3a to a concentration of 8.7×10^6 CFU per gram dry matter. Plants grown in the absence of T3a were used as controls. Five pots with T3a and 5 control pots were prepared.

After 10 days incubation, the seedlings were harvested and there roots carefully rinsed in water to remove particles of the potting mix. Localisation of hyphae on the rhizoplane and histological examination were carried out as described previously (Green and Jensen, 1995). The root systems of 30 seedlings were examined, i.e. 15 grown in the presence of T3a and 15 controls.

Localisation of GUS active hyphae on diseased roots

Fine mesh nylon (38 μm) bags were placed in 330-ml pots filled with 27.5 g dry matter of the potting mix either infested (8.7×10^6 CFU per gram dry matter) or non-infested with the transformant, T3a. The nylon bags were placed so that no potting mix was present at the inside. Six 3-day-old cucumber seedlings were positioned in each nylon bag and the potting mix gently pressed towards the sides of the bags. A surface-disinfested and pealed 1 cm thick slice of cucumber pre-colonised by *P. ultimum* isolate HB2 was placed at the side of each pot to serve as an inoculum source from which actively growing mycelium could spread to the plant roots (Green and Jensen, 2000). Plants grown in the absence of the transformant were used as controls.

After 5 days of incubation, each seedling was carefully removed from the nylon bag and the severity of disease on roots and collar was rated according to the following scale modified from Wolffhechel (1989):

0 = healthy looking, heavily branched root system;
1 = root system less branched than healthy roots (rating 0), light brown necrosis in distinct spots, often associated with the tip of side roots;
2 = only few side roots, and dark brown necrosis in distinct spots;
3 = few and small side roots, and dark brown necrosis of the whole root system, or all around the stem;
4 = plant dead.

Root systems from 24 seedlings (18 diseased and 6 controls) were stained for the presence of GUS active hyphae. A SZH stereomicroscope (Olympus) was used to locate necrosis and blue-stained hyphae of the transformant. Detailed examinations of selected samples were carried out under a Nikon Optiphot light microscope. One preliminary and one complete experiment were carried out.

Quantification of the GUS activity and the P. ultimum *biomass*

The purpose of this experiment was to quantify the qualitative observations from the previous experiment. Thus, the experimental set-up was identical to the one described above. The experiment had a complete factorial design with 4 main treatments (±T3a × ±HB2). The treatment containing both T3a and HB2 had 10 replicate pots, while the other treatments had 2 replicate pots. The experiment was carried out once.

Forty eight seedlings were harvested at day 4 and 6, respectively, and used to determine the effect of the disease severity on the GUS activity and a possible correlation with the biomass of *P. ultimum*. For the GUS assay, each individual root system was submerged into 2 ml sodium phosphate buffer (50 mM, pH 7.0) containing 0.05% (vol/vol) Triton X-100, 1 mM N-lauroyl-sarcosine (Sigma), 1 mm EDTA, and 0.02% (wt/vol) NaN_3. The assay was started by addition of 2 ml, 2 mM 4-methylumbelliferyl–β-D-glucuronide, MUG (Sigma) in phosphate buffer. After incubation for 60 min at 37 °C, the assays were quenched by transferring 50 and 500 μl of the assay solutions to 2.95 and 2.50 ml Tris stop buffer (2.5 M, pH 10), respectively. The fluorescence emitted by the enzymatic released 4-methylumbelliferone (MU) moiety was monitored on a Luminescence Spectrometer LS50B (Perkin Elmer Ltd., Buckinghamshire, England) by excitation at 365 nm and reading at 448 nm. Readings were corrected for non-enzymatic hydrolysis of MUG and converted to μmol MU per minute per gram of dry root. After determination of the GUS activity the roots were dried at 37 °C and stored at 4 °C until they were used for the ELISA.

Of the dried roots, 5 from each of the control treatments and 23 from the treatment containing both T3a and HB2 were selected randomly between root samples from day 4 and the content of HB2 quantified by the competitive indirect ELISA procedure described below. For extraction of antigens, each dried root system was placed in 0.5 ml phosphate buffered saline, PBS (NaCl, 2.92% (wt/vol); KCl, 0.02% (wt/vol); Na_2HPO_4, 0.29% (wt/vol); KH_2PO_4, 0.02% (wt/vol); pH 7.4) with 2% polyvinylpyrrolidone, PVP (Sigma) in plastic tubes containing FastDNA® Lysing Matrix (BIO 101, Inc. Vista, CA, USA) combined with both a $\frac{1}{4}''$ Ceramic Sphere (BIO 101) and a $\frac{1}{4}''$ Ceramic Cylinder (BIO 101). The root tissue was then homogenized in a FastPrep® Instrument (FP120; BIO 101) at speed setting 5 for 20 s. Then the Lysing Matrix, PVP, and root tissue were pelleted by centrifugation at 7000 rpm for 10 min.

The solid phase antigen for use in the ELISA procedure was prepared by growing *P. ultimum* isolate HB2 on 40 ml potato carrot broth, PCB (Rosendahl and Olson, 1992) in the dark at 20 °C for 4 days. At harvest, the mycelium was washed thoroughly with sterile deionized water, ground in liquid nitrogen, and

lyophilized on a Hetosicc lyophilizer (Heto, Birkerød, Denmark). Upon use, 10 mg of the lyophilized mycelium was ground in a mortar with 10 ml PBS containing 2% PVP. The PVP was pelleted by centrifugation at 7000 rpm for 10 min and the supernatant further diluted 1 : 50 in carbonate coating buffer (50 mM, pH 9.6) before being coated onto the immunoplates (see below).

Competitive indirect ELISA was carried out in Maxisorb F96 Immunoplates (Nunc, Roskilde, Denmark). The working volume was 100 µl per well, and incubation was carried out on a shaker for 1 h at approximately 22 °C. Between each step the plates were washed four times with PBS containing 0.1% (vol/vol) Tween-20 (Sigma). The supernatant containing sample antigens was diluted 1 : 5, 1 : 10, and 1 : 20 in blocking buffer (PBS containing 0.1% (vol/vol) Tween-20 and 0.5% (wt/vol) Casein [Sigma]) and incubated with an equal volume of sheep anti *Pythium*-antibody (Adgen Diagnostic Systems, Auchincruive, Scotland, UK) diluted 1 : 5000 in blocking buffer. The suspension of the lyophilized mycelium was diluted 1 : 1000 in blocking buffer and a two fold dilution series was used as the standard. After 1 h, the solutions were transferred to plates coated with the solid phase antigen. After incubation and washing peroxidase conjugated donkey anti-sheep IgG (Sigma) diluted 1 : 1000 in blocking buffer was added. After incubation the washing procedure was terminated by rinsing the wells in deionized water. Finally, a substrate solution (0.2 M potassium citrate; 3 mM H_2O_2; 0.6 mM 3,3′,5,5′-tetrametylbenzidin (Merck, Darmstadt, Germany)) was added, incubated for 15 min, and the colour reaction stopped by addition of 100 µl of 2 M H_3PO_4. The absorbance was read at 450 nm on a SpectraMax® 190 (Molecular Devices Corporation, Sunnyvale, California, USA). A blank was obtained by leaving out the primary antibody. Two replicates were made per sample. The average reading for each sample was converted to mg *P. ultimum* biomass per gram dry root.

Statistical analysis

All calculations were carried out using Statistic Analysis System, version 6.12 (SAS Institute, Cary, NC). To test for a possible rhizosphere effect on the proliferation of T3, population densities in the root zone and the bulk potting mix were compared for each experiment using the General Linear Models Procedure PROC GLM. Also, a combined four-factorial analysis of all six experiments was carried out using PROC GLM to test for possible effects of experiments, plant species, harvest time, rhizosphere effect, and their interactions.

To test the influence of the disease severity on the level of GUS activity and *P. ultimum* biomass in diseased root tissue all data were transformed logarithmically to obtain variance homogeneity. Levels of significance were calculated by PROC GLM. Correlation between data was determined by regression analysis (PROC REG).

Results

Rhizosphere effect on the proliferation of T. harzianum

For each of the six experiments and three of the plant species there were no significant differences between the population development of *T. harzianum* T3 (CFU per gram dry matter of potting mix) in root zone and bulk potting mix when a peat-bran preparation was added prior to planting (Table 1). In the case of red fescue, the population density in bulk potting mix was significantly ($P = 0.03$) higher that in the root zone. A combined analysis showed no significant differences between experiments ($P = 0.11$), and plant species ($P = 0.84$). Also, the population densities in the rhizosphere did not differ from those in the bulk potting mix ($P = 0.81$). There was a significant effect of the harvest times ($P = 0.02$) which also interacted ($P = 0.02$) with steaming of the potting mix, indicating that the population densities increased with time, but only when the potting mix had been steamed.

Localisation of GUS active hyphae on the rhizoplane of healthy roots

Roots developed between the two adjoining mesh in centrifuge tubes could be extracted without any damage and without any adhering particles of potting mix. When placed on mTSM, very few colonies appeared from roots of control plants and none of these colonies became blue when exposed to the GUS substrate (X-gluc). From seedlings treated with *T. harzianum* T3a fungal growth was seen from all parts of seeds and roots. The GUS staining on the plates revealed that this growth originated from the transformant. After the root systems of other (non-plated) *T. harzianum*-treated

Table 1. Population densities of *Trichoderma harzianum*, T3, in the root zone of different plant species compared to the bulk potting mix

Exp. no[a]	Plant	Potting mix	Root zone (CFU g^{-1} dry potting mix)[b]	Bulk potting mix (CFU g^{-1} dry potting mix)[b]	*P* values[c]
1	Cucumber	Steamed	3.611 ± 0.132	3.356 ± 0.287	0.547
2	Cucumber	Steamed	2.480 ± 0.255	3.442 ± 1.227	0.218
3	Cucumber	Steamed	3.520 ± 1.360	3.611 ± 0.545	0.906
4	Cucumber	Steamed	1.491 ± 0.989	1.046 ± 0.644	0.064
5	Cucumber	Raw	0.031 ± 0.002	0.032 ± 0.004	0.736
6	Cucumber	Raw	1.133 ± 0.107	1.073 ± 0.414	0.828
6	Ryegrass	Raw	1.272 ± 0.607	1.667 ± 0.066	0.490
6	Red fescue	Raw	1.199 ± 0.220	1.546 ± 0.243	0.030
6	White Clover	Raw	1.250 ± 0.105	1.360 ± 0.104	0.594

[a]Five different experiments were carried out using cucumber, while a sixth experiment included cucumber, perennial ryegrass, red fescue, and white clover. Each experiment differed with respect to inoculum concentration and harvest time.
[b]The present data is the average from two different harvest times.
[c]The population densities in the root zone and the bulk potting mix were compared for each experiment using the General Linear Models Procedure (PROC GLM, SAS Institute).

replicate seedlings had been subjected to GUS staining, blue stained hyphae of the transformant could only be detected in certain limited areas of varying size on the attached seed coat and on roots in its immediate vicinity (Figure 1A,B). At the cotyledon and on other parts of the rhizoplane, no staining occurred. This was the case for both cucumber and pea seedlings, whether they were harvested 2, 3, or 9 days after sowing. No staining was seen on control seedlings.

Localisation of GUS active hyphae on wounded roots

No staining was seen on roots of cucumber seedlings grown in the absence of the GUS transformed *T. harzianum*. Small colonies of blue stained hyphae occurred on different regions of the roots of cucumber seedlings grown directly in potting mix infested with the transformant. Histological examination revealed that these hyphae, as described previously (Green and Jensen, 1995), were restricted to one or a few neighbouring cells, mainly associated with small epidermal wounds such as damaged root hairs and other epidermal cells (Figure 1D).

Localisation of GUS active hyphae on diseased roots

No staining was seen on root systems of diseased plants grown in absence of the transformant. Various degrees of staining could be detected on and in the roots of *Pythium*-diseased seedlings grown in potting mix infested with the transformed *T. harzianum*. In general, the more diseased the roots were, the greater the GUS activity. However, indications were obtained in a preliminary experiment (results not shown) that a high bacterial population in the rhizophere markedly reduced the degree of staining. Microscopic examination revealed that the blue stained hyphae in most cases were associated with necrotic root tissues (Figure 1C,E). In some cases, oospores of *P. ultimum* produced in the necrotic tissue took up the dye thereby appearing blue-coloured (Figure 1E).

Quantification of the GUS activity, and the P. ultimum *biomass*

Harvest time (i.e. day 4 and 6) had no effect on the GUS activity or on *P. ultimum* biomass on roots of the cucumber seedlings. Therefore the data presented are the average from both harvest times. The background GUS activity on the rhizoplane of healthy seedlings was 0.03 μmol MU min^{-1} per gram of dry root (Table 2). On severely diseased roots (disease index 4) of seedlings grown in the absence of *T. harzianum* the background GUS activity was 0.17 μmol MU min^{-1} per gram of dry root. When the transformed *T. harzianum* was present on the rhizoplane of non-inoculated seedlings the GUS activity was 0.37 μmol MU min^{-1} per gram of dry root. The GUS activity increased significantly ($P < 0.001$)

with increasing disease index of seedlings inoculated with *P. ultimum* and grown in potting mix infested with the transformed *T. harzianum* (Table 2). Seedlings with a disease index of 1 supported a GUS activity of 1.55 increasing to 25.76 μmol MU min^{-1} per gram of dry root at disease index 4.

Reaction between the *Pythium* antibodies and components of the root tissue of cucumber seedlings caused a background of 0.01 mg per gram of dry root (Table 2). The biomass of *P. ultimum* in roots of inoculated seedlings increased significantly ($P < 0.001$) as symptoms (i.e. the disease index) on the roots became more apparent. There was a significant correlation ($r = 0.67$, $P < 0.001$) between the level of GUS activity and the *P. ultimum* biomass in the roots (Figure 2).

Discussion

In soils, nutrient availability is a limiting factor for microbial growth and activity. Consequently, most micro-organisms exist in a state of exogenous dormancy or fungistasis (Lockwood, 1977). However, this can be overcome once propagules are supplied with appropriate organic and inorganic stimuli. Stimulants most commonly introduced into the soil environment that alleviate fungistasis are components of seed and root exudates released during seed germination and root development (Nelson, 1991).

Results from this study indicate that conidia of *T. harzianum* do not germinate in response to exudates from healthy cucumber and pea roots. The GUS transformed *T. harzianum* strain T3a, grew out from all parts of healthy cucumber and pea roots when these were plated on mTSM. The absence of blue-stained GUS-active hyphae on roots of other non-plated replicate seedlings indicates that watering from above had spread conidia to the entire root system, but that germination and mycelial growth did not take place. In fact, none of the root systems in any of the experiments hosted blue-stained hyphae on undamaged and healthy areas. The most probable explanation for this phenomenon is that normal root exudation is not sufficient to support any activity of the antagonist. The low GUS activity determined on healthy roots in the quantitative assay (Table 2), is probably caused by the low metabolic activity found in resting conidia (Green and Jensen, 1995). Thrane et al., (1997) have shown that *T. harzianum* strain T3a, was able to grow superficially on healthy areas of cucumber roots. However, these results were obtained in autoclaved potting mix using agar plugs for inoculation of the antagonist. Thus, these experimental conditions were supportive for growth of *T. harzianum* due to enhanced nutrient availability and elimination of competition from naturally occurring micro-organisms, and the results are therefore not contradictory to the ones presented here. Also, Lo et al., (1998) have shown that another GUS transformed strain of *T. harzianum* (T22) can grow and proliferate on roots of creeping bentgrass. However, this strain is a specially developed rhizosphere competent mutant, which could account for its different behaviour compared to other strains of *T. harzianum*. So far, with the exception of one isolate (Sivan and Chet, 1989), wild-type strains of *T. harzianum* have not been shown to be rhizosphere competent in the sense that they do not colonise the rhizosphere of developing roots when applied to seeds (Ahmad and Baker, 1987a). Even in sterile soil, *T. harzianum* is more likely to colonise bulk soil than the root surface, when added as a seed treatment (Chao et al., 1986). In addition, Papavizas (1982) showed that *T. harzianum* did not proliferate in the rhizosphere of pea seedlings when conidia were applied directly to soil before planting. Similar results were obtained in the present study using four different plant species with quantitative and qualitative differences in root exudation (Table 1). It is generally accepted that seed and root exudates have a stimulating effect on germination of *Pythium* spores and subsequent establishment of damping-off and root rot (Nelson, 1991). Therefore, it is likely that partial removal of exudates by a biocontrol agent present in the spermosphere or rhizosphere could reduce the inoculum potential (sensu Baker, 1978) of these plant pathogens. In fact, germination of both *P. ultimum* sporangia (Chen et al., 1988; Paulitz, 1991) and *P. aphanidermatum* oospores (Elad and Chet, 1987; Tedla and Stanghellini, 1992) was reduced in the presence of pseudomonads or other soil bacteria. In contrast, our results indicate that *T. harzianum* does not respond to a normal level of root exudation. Therefore, disease control by *T. harzianum* is unlikely to be achieved by suppression of spore germination of the pathogen through competition for exudates. Similarly, Lifshitz et al. (1986) found that competition between *Trichoderma* spp. and sporangia of *Pythium* for seed exudates is not an important antagonistic mechanism. In general, fungal species like *Trichoderma* are, compared to bacteria, poor competitors for root exudates. Therefore, in raw soil or potting mix, and in the absence of a suitable food base, conidia of *Trichoderma* will most

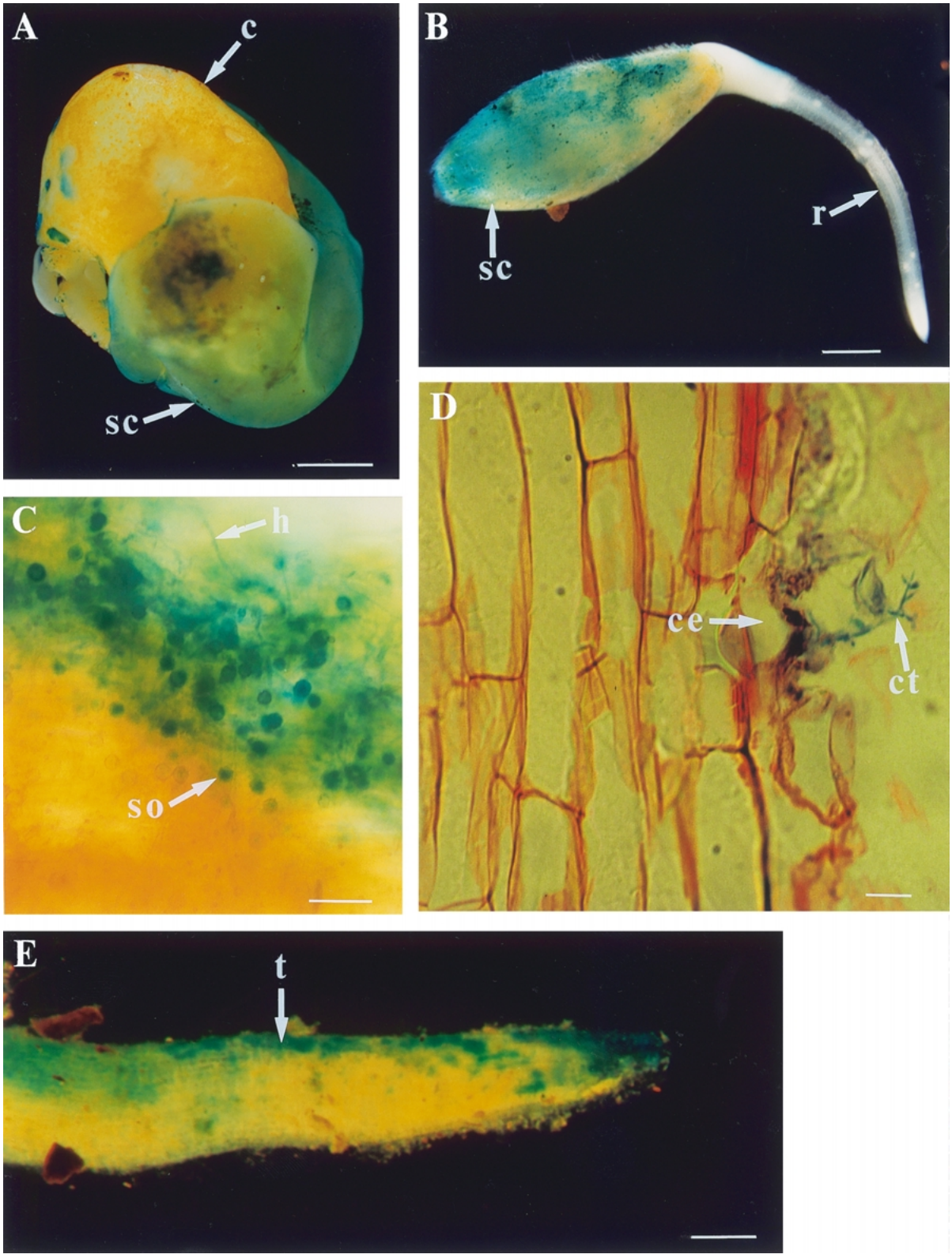
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Table 2. Relationship between disease index, GUS activity, and *Pythium ultimum* (HB2) biomass on roots of cucumber seedlings inoculated with *P. ultimum* and grown in potting mix infested with the transformant *Trichoderma harzianum* (T3a)

Treatment	Disease index[a]	GUS activity[b] (μmol MU min^{-1} g^{-1} dry root)	*Pythium ultimum* biomass[b] (mg g^{-1} dry root)
—, —	0	0.03 ± 0.00 a	0.01 ± 0.01 a
—, HB2	4	0.17 ± 0.10 b	2.06 ± 0.97 b
T3a, —	0	0.37 ± 0.22 c	0.01 ± 0.02 a
T3a, HB2	1	1.55 ± 0.97 d	0.34 ± 0.23 c
T3a, HB2	2	3.06 ± 2.84 de	0.41 ± 0.20 c
T3a, HB2	3	3.65 ± 1.90 e	1.46 ± 0.59 b
T3a, HB2	4	25.76 ± 22.27 f	2.48 ± 1.48 b

[a]The disease index relates to symptoms on roots and collar. 0 = healthy looking, heavily branched root system; 1 = root system less branched than 0, light brown necrosis in distinct spots, often associated with the tip of side roots; 2 = only few side roots, and dark brown necrosis in distinct spots; 3 = few and small side roots, and dark brown necrosis of the entire root system, or all around the stem; 4 = plant dead.

[b]The present data is the average from two different harvest times (i.e. day 4 and 6). Values in each column followed by different letters are significantly different at $P = 0.05$ according to a t-test (PROC GLM, SAS Institute).

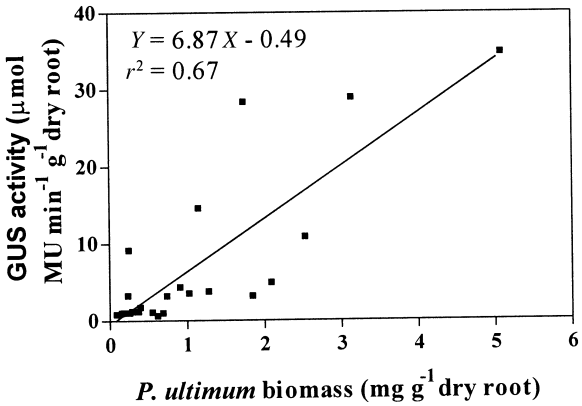

Figure 2. Correlation between the GUS activity and the *Pythium ultimum* (HB2) biomass on roots of cucumber seedlings inoculated with *P. ultimum* and grown in potting mix infested with the transformed *Trichoderma harzianum* (T3a).

likely be subjected to fungistasis when present on the rhizoplane.

Based on the GUS staining technique, this study shows that *T. harzianum*, strain T3a, when applied to seeds, actively colonised the seed coat, although the degree of colonisation was variable (Figure 1A,B). Previous results indicate that the constitutively expressed GUS gene reflects the general metabolic activity of the transformed fungi (Eparvier and Alabouvette, 1994; Green and Jensen, 1995). The activity of *T. harzianum* located on the seed coat is undoubtedly important for the ability of the antagonist to protect the germinating seed and the emerging radicle. In accordance with this, Lifshitz et al. (1986) observed that conidia of *T. harzianum* were able to germinate on pea seed coats. Since *Pythium* spp. were found to colonise the seed coat much earlier than they colonised the embryo, they suggested that the seed coat is a likely habitat for pathogen-antagonist interaction.

The present work demonstrates that *T. harzianum* colonises small epidermal wounds and root tissue infected by *P. ultimum*. When healthy cucumber seedlings were extracted from potting mix infested with the GUS transformed organism and the root systems subsequently stained for GUS activity and examined histologically, the metabolically active hyphae which were found were restricted to one or a few neighbouring cells, mainly associated with small epidermal wounds (Figure 1D). Wounding of the root epidermis probably occurred during handling of the seedlings, but minor damage to plant roots can be expected to occur under natural growth conditions. As the root grows through soil, the cuticle becomes ruptured by mechanical abrasion and by the action of soil microorganisms and animals (Cook and Baker, 1983). Also,

Figure 1. Micro-habitats in spermosphere and rhizosphere supporting metabolic activity of the antagonistic fungus *Trichoderma harzianum* visualised by use of a β-glucuronidase (GUS) transformant (T3a). Metabolically active hyphae (blue coloured) of the fungus were detected by staining for GUS activity. (A) Pea seed coated with a wheat-bran preparation of T3a and left in potting mix for 2 days. sc = seed coat colonised by T3a, c = non-colonised cotyledon. Bar = 2 mm. (B) Three day old cucumber seedling. T3a was applied as a seed treatment prior to sowing. Watering from above dispersed the conidia to the potting mix into which the radicle extended. sc = seed coat colonised by T3a, r = non-colonised radicle. Bar = 2 mm. (C) Necrotic root segment from a *Pythium*-diseased cucumber plant grown in potting mix infested with T3a. h = hyphae of T3a colonising the necrotic tissue, so = stained oogonia or oospore of *P. ultimum*. Bar = 100 μm. (D) Longitudinal section of a wounded area on a root from a cucumber plant grown in potting mix infested with T3a. ct = conidiophore of T3a with ampulliformed phialides, ce = collapsed epidermal cells. Bar = 20 μm. (E) Necrotic root tip from a *Pythium*-diseased cucumber plant grown in potting mix infested with T3a. t = patch colonised by T3a. Bar = 500 μm.

senescent and decaying rootlets will naturally occur on otherwise healthy plants (Wilhelm and Nelson, 1970). Further, when pathogens establish themselves in root tissue, parts of the root system are transformed into masses of plant debris, which in turn will be available as a source of nutrients to saprophytes. Pathogenesis is a progressive process, and our results indicate that if conidia of *T. harzianum* are present at the right place at the right time, close to the infection court on the rhizoplane, leakage of nutrients can induce germination and growth of the biocontrol agent with subsequent establishment in the infected tissue (Figure 1C,E). *Trichoderma* spp. are often found to colonise freshly fallen plant litter, which is already colonised by a range of primary saprophytes (Widden and Scattolin, 1988). *Trichoderma* spp. are also known to produce a wide range of hydrolytic enzymes and antibiotics. As such, there is no doubt that *Trichoderma* spp. are good competitors with the ability to perform secondary resource capture. It is therefore tempting to hypothesise that the biocontrol efficacy of *T. harzianum* among various mechanisms also depends on the ability to compete with the pathogen for the infected root tissue. This hypothesis is supported by the findings that GUS activity of the transformed *T. harzianum* on and in roots of plants infected by *P. ultimum* was directly proportional to the disease severity (Table 2 and Figure 2). The background GUS activity on diseased control roots was found to be significantly higher than the background activity on non-infested control roots (Table 2). This could be caused by indigenous bacteria with intrinsic GUS activity responding to the diseased roots in the same way as the GUS transformant. Similar results were obtained by Weller (1983), who found that antagonistic *Pseudomonas fluorescens* predominates on regions of wheat roots that bear take-all lesions and that consequently had a high rate of nutrient release from damaged tissues. In the case of *Trichoderma* spp., the evidence described above explains the necessity of maintaining a high and evenly distributed population of the inoculant biocontrol agent along the root at potential infection sites, to achieve a satisfactory disease control.

Trichoderma spp. are generally considered as saprophytes rather than primary colonisers of the surface of young plant roots. This implies that the antagonist is well adapted to, and can colonise, dead organic material, like the seed coat and wounds, including those caused by pathogen infection, and dying rootlets. In these special micro-habitats, *Trichoderma* spp. will be active, utilising their antagonistic mechanisms to facilitate competition for nutrients and sites. *Pythium* spp., on the other hand, are weak competitors, and their saprophytic activities are greatly restricted in the presence of other organisms (Barton, 1961; Chen et al., 1988). Competition for these micro-habitats is, in our opinion an important mechanism of action of *T. harzianum*. However, in the case of *Pythium* damping-off and root rot, successful infection does not depend on the presence of wounds on the root surface (Chérif et al., 1991), although enhanced leakage of nutrients from wounds may cause an increased stimulation of spore germination and attraction of zoospores. Even though *T. harzianum* conidia present on the rhizoplane can be activated by leakage of nutrients from the infection court, the question is, does the antagonist have the ability to stop actual disease progression within the plant, once it has been successfully infected by *Pythium*? In any case, competition by the antagonist for the infected root tissue probably reduces the build up and spread of secondary inoculum of the pathogen as indicated elsewhere (Green and Jensen, 2000).

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