Techniques

A Tool for Monitoring *Trichoderma harzianum*: II. The Use of a GUS Transformant for Ecological Studies in the Rhizosphere

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This work was supported by the Danish Agricultural and Veterinary Research Council (SJVF) grant 13-4591.
We thank J. Hokenhull, C. Thrane, and M. Lübeck for valuable comments on this manuscript, and E. de Neergaard for helping with the histological techniques. We also thank M. Krause Pedersen for technical assistance.
Accepted for publication 28 August 1995.

ABSTRACT

Green, H., and Jensen, D. F. 1995. A tool for monitoring *Trichoderma harzianum*: II. The use of a GUS transformant for ecological studies in the rhizosphere. Phytopathology 85:1436-1440.

Population development, activity, and stability of a β-glucuronidase (GUS)-transformed strain of *Trichoderma harzianum* were studied in the rhizosphere of cucumber plants grown in sphagnum peat. When compared to the wild-type isolate, there was no significant difference in terms of population development (CFU/g of sphagnum peat). In a treatment with a combination of the wild-type isolate and the transformant, the proportion of GUS-active colonies on plates was constant throughout the experiment. On that basis, we concluded that the transformant was genetically stable when grown in a natural potting mixture. GUS was extracted from infested rhizosphere peat and assayed spectrophotometrically. The activity curve was inversely related to the population curve. Quantification of activated conidia by fluorescein diacetate staining indicated that GUS activity correlated with the activity of the fungus. Hyphae of *T. harzianum* stained with 5-bromo-4-chloro-3-indoyl-β-D-glucuronide were easily detected on the roots, especially around small wounds, such as damaged epidermal cells. Colonization of wounds may be important for the antagonistic effect of the organism, because it may block a possible way for pathogens to infect root tissue. Our results demonstrate the suitability of using a GUS transformant for detecting and monitoring a specific strain of *T. harzianum* deliberately released into the environment.

Additional keywords: biocontrol, biomass, GUS transgenic, population studies.

The fungal antagonist *Trichoderma harzianum* Rifai is an effective biocontrol agent against a range of important aerial and soilborne plant pathogens (4,13,17). To obtain a reliable effect of the organism under different environmental conditions, we need to know more about the ecology of this antagonist.

In microbial ecology, numerous methods for assessing populations, activity, and biomass have been developed (20), but a recurrent problem has been the inability to detect, monitor, and recover specific microorganisms either naturally present or deliberately released into the environment. This has become even more of a concern now that regulatory agencies in many countries require that microbial biocontrol products, containing either naturally occurring or genetically modified organisms, be assessed in terms of their risk to the environment into which they are released.

Dilution plating on selective media often has been used for quantitative isolation of *Trichoderma* spp. from soil (6,7,19). However, a major drawback to this method is that the majority of colonies arise from conidia rather than hyphae (27), which makes a correlation to fungal activity difficult. Even on selective media problems with background microflora are common, and in all cases, it is difficult to distinguish between introduced and indigenous *Trichoderma* spp. These problems have been partially overcome by utilizing fungicide-resistant strains (1,2,18).

More recently, molecular approaches have enabled reporter and marker genes to be integrated into the fungal genome. Such strains can be monitored after introduction into natural environments. The GUS (β-glucuronidase) reporter gene from *Escherichia coli* seems very promising for use in ecological studies, because background activity is either absent or low in most plants and fungi investigated (11,28). Furthermore, the enzyme is fairly stable and can be assayed easily by different methods (9,11). GUS transformants of several plant pathogenic fungi have been used for detection and biomass quantification in infected plant tissue (5,14,16).

The objectives of this study were to develop methodologies that facilitate ecological studies of a biocontrol fungus in soil and soilless potting mixtures. This paper for the first time reports the use of the GUS gene as a marker for monitoring the presence, the population development, and the activity of an introduced strain of *T. harzianum* in the rhizosphere.

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MATERIALS AND METHODS

Fungi and plants. *T. harzianum* isolate T3 was originally isolated from *Pythium*-suppressive peat (29). The transformant T3a was derived from the wild-type isolate T3 and transformed with the *E. coli* GUS gene and the hygromycin B resistance gene (24). Peat-bran inocula of the fungi were prepared according to Sivan et al. (22). Cucumber plants, *Cucumis sativus* L. "Langelands Kaempe-Gigant" (Daehnfeldt, Denmark), were used for the experiment.

Potting mixture. Fine, 0 to 20 mm, light-colored sphagnum peat (Pinstrup, faerdigblanding 1, Mosebrug A/S, Denmark) was mixed with vermiculite, grade 11 (Skamol, Nykobing Mors, Denmark) at 3:2 (wt/wt). The final pH (H_2O) was 5.5.

Media. *T. harzianum* was maintained on potato-dextrose agar (PDA, Difco Laboratories, Detroit). Dilution plating was carried out on a modification of a *Trichoderma*-selective medium (mTSM) (7) in which rose bengal had been substituted with 0.5 ml of Triton X-100 per liter.

Population studies. The potting mixture was steamed for 1 h on three successive days and thoroughly mixed with the peat-bran inoculum (0.001 g/g dry matter). The following treatments were prepared: T3, T3a, mixture of T3/T3a (1:1, wt/wt), and a noninfested control. Due to contamination during production, the peat-bran inoculum of transformant T3a contained 3% of the wild-type isolate T3.

To facilitate the separation of rhizosphere peat from bulk peat, plants were grown in rhizoboxes (1 × 7 × 7 cm) consisting of a plastic frame and a fine-mesh (30 μm) nylon chamber. The fine mesh confined plant roots, whereas fungal mycelia could grow freely between the rhizobox and the surrounding peat.

Cucumber seeds were surface-sterilized in 2.5% NaOCl and pregerminated on weak PDA (18.75 g of PDA, 12.5 g of Bacto [Difco] agar, and 1 liter of H_2O). Eight 3-day-old seedlings were planted in each rhizobox, containing 3.75 g dry matter of the potting mixture. The rhizoboxes were placed in 330-ml pots that were filled with an additional 23.75 g dry matter of the potting mixture. The pots were watered with a balanced nutrient solution, including micronutrients, to a water content of –1.47 kPa (64.5% volume) and a conductivity of 3.5 mMho. 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.

The first samples were taken on the day of mixing, and on days 2, 4, 7, and 14, the rhizoboxes were pulled up, and the sphagnum peat was separated from the roots and used for fluorescein diacetate (FDA) staining of conidia and hyphae, enzyme extraction, and dilution plating on mTSM. The plates were incubated for 4 days at room temperature.

To determine the percentage of colonies originating from the transformant, a ring was cut in each colony with a cork borer (6 mm), and 7 μl of 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc, Sigma B-0522 [Sigma Chemical Company, St. Louis]) at 1 mg/ml of extraction buffer (26) was added to each ring. The plates were incubated in darkness at 37°C for 2 h, and the number of blue colonies was counted. Approximately 60 colonies were tested per treatment.

FDA staining of conidia and hyphae. Approximately 1.4 g (dry weight) of rhizosphere peat was sampled, homogenized in 100 ml of 50 mM sodium phosphate buffer, pH 7.0, and diluted 1:100, and 10 μg of FDA per ml in acetone was added (23). After 5 min, 5 ml of the suspension was filtered through a black, 8-μm polycarbonate membrane filter (Nuclepore, Costar Scientific Corporation, Badhoevedorp, the Netherlands) and placed in a drop of 2.5% (vol/wt) 1,2-diazabiclyclo-[2.2.2]-octane (Sigma D-2522) in phosphate buffer to reduce fading. The slide was viewed under a Nikon Optiphot epifluorescent microscope (Nikon, Tokyo) equipped with an excitation filter at 420 to 485 nm and a barrier filter at 520 nm. Between 100 and 200 conidia were examined, and the slide was searched for phialides and mycelia fragments.

Activity studies (GUS assay). Approximately 1.4 g (dry weight) of rhizosphere peat was frozen at –20°C, homogenized in liquid nitrogen, and suspended in 30 ml of extraction buffer (26). The suspension was mixed in an Ultra-turrax T 25 (IKA-Labortechnik, Staufen, Germany) for 5 min at 20,500 rpm on ice and filtered through a cellulose filter (1-F-1445, Bie & Berntsen, Rodovre, Denmark) followed by a filter 0.2-μm Minisart NML cellulose acetate filter (Satorius, Göttingen, Germany). A solution of *p*-nitrophenyl-β-D-glucuronide (4 mg/ml, Sigma N-1627) was added at a rate of 0.2 ml/ml of filtrate, which was incubated in the dark at 37°C for 1 h, and the reaction was stopped with 0.5 ml of 1 M Na_2CO_3 (21). Optical density was measured at 405 nm on a Hitachi 150-20 spectrophotometer connected to a Hitachi 150-20 data processor (Nissei Sangyo American, Ltd., Mountainview, CA). The total protein content was determined using the method of Bradford (3).

Localization of hyphae on the rhizoplane and histological examination. The root systems of the harvested cucumber plants were incubated at 37°C for 16 h in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.05% (vol/vol) Triton X-100, and 80 μg of X-gluc per ml (16). The roots were examined under an Olympus SZH stereomicroscope (Olympus, Tokyo) for blue-stained hyphae.

Regions with localized hyphae were cut out and imbedded in LR Gold (Bio-Rad A2034, Richmond, CA) according to Hurek and Villiger (10). Longitudinal sections (12 μm) were cut on a microtome. Sections with intensive-colored hyphae were counterstained with safranin for 15 s. In all cases, sections were mounted in DPX (Fluka 44581, Buchs, Switzerland), dried at room temperature, and viewed under a Nikon Optiphot microscope. A total of 15 roots was examined.

Rhizosphere competence assay. Polypropylene centrifuge tubes (27 × 102 mm) were cut longitudinally into two halves (2). Each half was filled with nonsterile moistened potting mixture and wrapped in fine-mesh (30 μm) nylon. The two half-tubes were put together and secured with rubber bands. Cucumber seeds were soaked in water for 2 h, rolled in peat-bran inoculum of T3a, and one seed was placed between the two adjoining nylon mesh at the top of each tube.

The tubes were placed in a 1-liter container with the base submerged in 2 cm of water to ensure upward water movement only. The container was placed in a growth chamber at 18°C with a 16-h photoperiod. After 10 days, the tubes were taken apart, and hyphae on the rhizoplane were localized as described above. The experiment was repeated twice with three replicates per treatment.

Statistical analysis. The population and activity experiments were repeated several times, with three replicates per treatment. Due to variation in the initial amount of inoculum added to each experiment, the present data were derived from one representative experiment. Statistical analysis was carried out using SAS (SAS Institute, Cary, NC). Data from the dilution plating was transformed logarithmically, and the similarity of the slopes was analyzed using PROC GLM. PROC ANOVA was used to analyze whether the percentage of colonies originating from the transformant varied on different days. PROC REG was used to test the correlation between GUS activity and the percentage of active conidia.

RESULTS

Population studies. The populations of *T. harzianum* (CFU/g dry matter of sphagnum peat) decreased slightly from day 0 to day 2 and then increased dramatically up to day 7, after which it began to level out (Fig. 1). The curves had an almost sigmoid shape. There was no significant difference ($P = 0.7573$) between

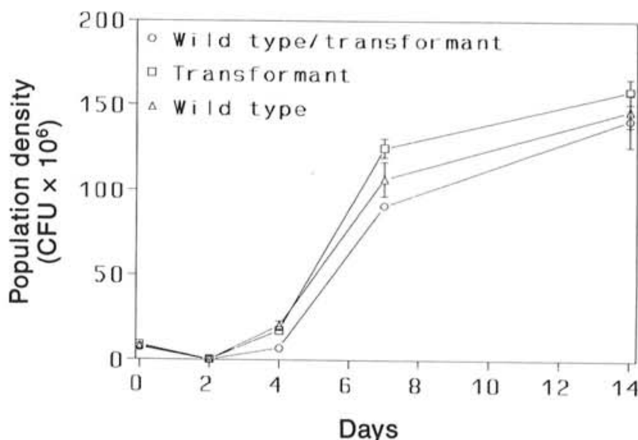

Fig. 1. Population development of *Trichoderma harzianum* wild-type isolate T3, transformant T3a, and T3/T3a mixture measured by dilution plating and expressed as CFU/g dry matter of sphagnum peat. Bars represent the standard error. There was no significant difference between the curves.

the population curves of the mixture (T3/T3a), the wild-type (T3), and the transformant (T3a).

The test for GUS reaction carried out directly on the plates after dilution plating was reliable, and because the colonies did not have to be transferred, the method was much faster than the microtiter assay described by Couteaudier et al. (5). Wounding the mycelium with a cork borer allowed the enzyme to leak out rather than having to wait for the substrate to diffuse into the mycelium.

Wild-type (T3) colonies showed no GUS activity. Ninety-seven percent of the colonies originating from the treatment containing only the transformant (T3a) were GUS positive (Fig. 2). The remaining 3%, which were wild-type contaminants of the initial inoculum, showed no GUS activity. Of the colonies originating from the treatment containing a mixture of T3 and T3a, 44% were GUS positive. There was no significant difference between the percentage of positive colonies recovered at each day throughout the experiment.

FDA staining of conidia and hyphae. The transformation of a relatively inactive (resting) conidium into a physiologically active one, prior to germination, is generally accompanied by water absorption and swelling (15). Resting conidia measured 3.0 × 2.7 μm and reached a size of 5.8 to 7.7 μm in the active state.

On day 2, 20% of the conidia were resting, 50% had become active, and 30% had germinated (Fig. 3). On day 4, when formation of conidia had begun, the percentage of active conidia was 53%, but although the germ tubes were much longer, the number of germinated conidia had decreased to 10%. After 7 days, only 16% were active, 3% had germinated, and a few clumps of mycelium were seen. Many detached phialides were observed, indicating intensive formation of conidia. On day 14, less than 1% of the conidia were active and only sparse germination had occurred. Very few clumps of mycelia and detached phialides were observed. Throughout the experiment, actual biomass estimation based on measurement of hyphal lengths was impossible due to the scattered appearance of hyphal fragments.

Activity studies (GUS assy). GUS activity (optical density per mg of protein extracted from infested sphagnum peat) increased dramatically during the first few days of the experiment. For the treatment containing only T3a, maximum activity was reached after 2 days, while for the treatment with the mixture of T3a/T3, the maximum was reached after 4 days. Subsequently, the activity curves decreased until they reached the same level at day 14 as at day 0. Apart from day 7, the extract from the T3/T3a mixture showed approximately half the GUS activity compared to the extract from the treatment with only the transformant (Fig. 4). When GUS activity for T3a was plotted against the proportion of both swollen and germinated conidia (Fig. 5), a strong correlation was seen ($r = 0.873$).

Localization of hyphae on the rhizoplane and histological examination. Blue-stained hyphae of *T. harzianum* (T3a) were easily detected on roots of plants grown in peat infested with the GUS transgenic organism. No staining was seen on root systems from

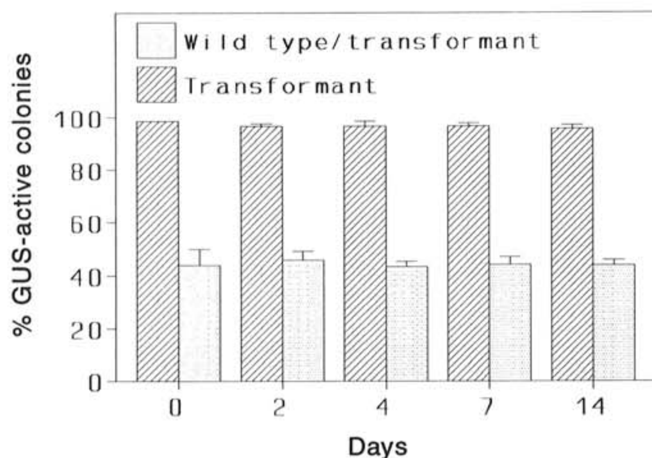

Fig. 2. The percentage of *Trichoderma harzianum* colonies from dilution plating that had a positive β-glucuronidase (GUS) reaction. Colonies originating from the wild-type isolate showed no reaction. Bars represent the standard error. Columns with the same hatching are not significantly different.

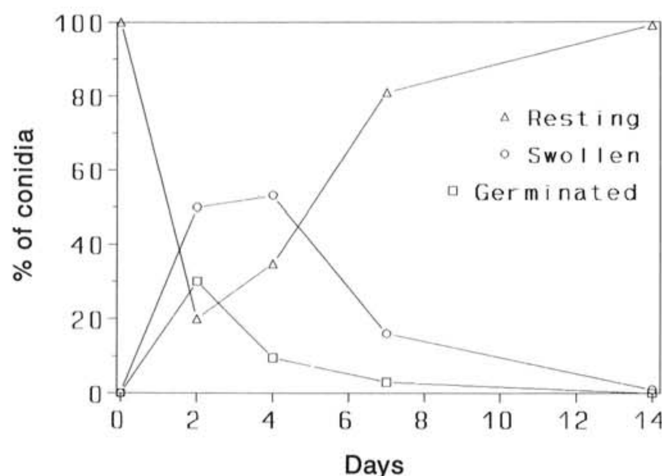

Fig. 3. Time course study of swelling and germination of *Trichoderma harzianum* conidia (percentage) in moist sphagnum peat on different days. The conidia were stained with fluorescein diacetate.

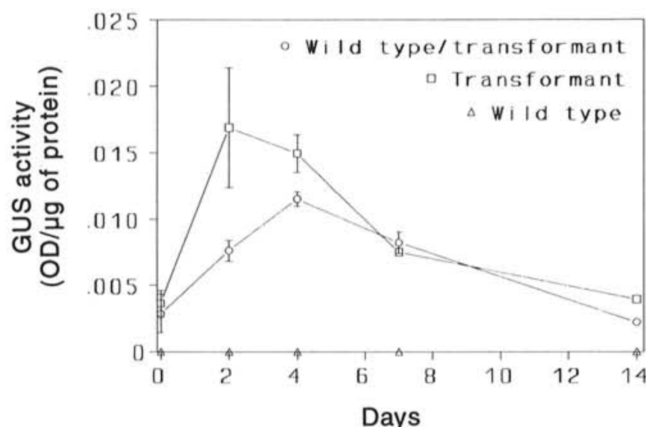

Fig. 4. Time course study of β-glucuronidase (GUS) activity of the protein extracted from *Trichoderma harzianum*-infested sphagnum peat. Bars represent the standard error.

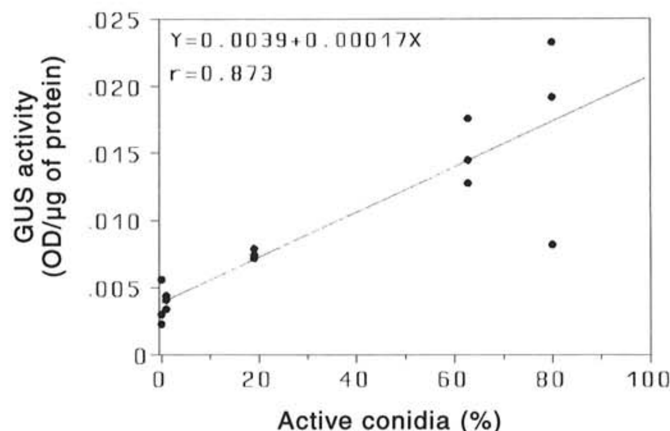

Fig. 5. β-glucuronidase (GUS) activity in extract from the treatment containing the *Trichoderma harzianum* transformant alone plotted against the sum of swollen and germinated conidia.

control plants grown in noninfested peat or peat infested with the wild-type (T3). The occurrence of stained hyphae varied from almost no hyphae on the total root systems to many small colonies of mycelia randomly spread over different regions of the roots. There did not seem to be a preference for the root tip or any other region. Histological examination revealed that the hyphae were restricted to one or a few neighboring cells, mainly associated with small wounds such as damaged root hairs and other epidermal cells. In a few cases, hyphae were found growing inside cortical cells, but with the exception of an occasional dead root, hyphae were never found in vascular tissue. Typical conidiophores with ampulliform phialides were seen occasionally on the surface of the root. The intensity of the staining varied from light, almost translucent turquoise in cortical cells to dark blue at the epidermis.

Rhizosphere competence assay. The method described is an easy way to study rhizosphere competence, because the blue-stained hyphae on roots free of soil or peat are readily visible to the naked eye or under the stereomicroscope. The occurrence of stained hyphae can clarify whether the organism has any preferred regions on the root surface. However, in this experiment, no hyphae were observed on the rhizoplane of roots emerging from seeds coated with *T. harzianum* (T3a) conidia. This confirmed earlier observations that our wild-type (T3) isolate is not rhizosphere competent (H. Wolffhechel and D. F. Jensen, *unpublished data*).

DISCUSSION

Population studies (CFU/g of sphagnum peat) of the transformant (T3a) and the wild-type (T3) isolates showed that the transformant grew and increased in the rhizosphere just like the wild-type. This further confirms previous results from Thrane et al. (24), who showed that strain T3a phenotypically resembles the wild-type (T3). In addition, the proportion of colonies originating from the transformant was constant throughout the population studies, indicating that the transformant is genetically stable when grown in a natural potting mixture.

Comparison between results of the activity and population studies show that GUS activity reached its maximum after 2 to 4 days and then dropped while the population curve went up. A possible explanation for this is that GUS production was high when the conidia were preparing for germination and during the period of mycelial growth. After a few days, the nutrients released by steaming the peat were used up by the organism (N. Heiberg and H. Green, *unpublished data*), causing a decrease in metabolic activity, including GUS production. At this point, mycelial growth stopped, accompanied by formation of conidia (i.e., increase in CFU). This hypothesis is strongly supported by the results of the FDA staining, in which the number of active and germinated conidia was highest after 2 days. At day 4, formation of conidia began, and after 14 days, mostly resting conidia were left in the sphagnum peat. Although scattered swelling, germination, and formation of conidia seemed to take place at any time thereafter, this had no detectable influence on the population density and GUS activity. Rather, it indicated a constant turnover of the total biomass. The GUS activity detected at this point (day 14) was mainly due to the presence of the enzyme in the conidia. Both Thrane et al. (24) and Liljeroth et al. (14) were able to extract and detect low amounts of GUS from conidia.

If present, X-gluc–stained hyphae of the transformed strain were clearly visible on root tissue. However, in the rhizosphere competence assay no hyphae were observed on the rhizoplane, confirming the results of Ahmad and Baker (2) that wild-type *T. harzianum* is not rhizosphere competent. The result is further supported by work done by H. Wolffhechel and D. F. Jensen (*unpublished data*), in which T3 was not found to be not rhizosphere competent. However, it was possible to locate hyphae of the transformant on root systems of plants that had been growing in peat infested with the organism. These hyphae were found especially in association with small wounds, such as damaged epidermal cells. These observations support the hypothesis that this ability is important for the antagonistic effect of *T. harzianum* as competition for the nutrients leaking from the cells, and actual colonization of the wound may block a possible way for pathogens to infect the plant. Thus, a combination of histochemical techniques and the GUS marker technique may be feasible for the development of a procedure to study niche competition by pathogen and antagonist. The resulting information would be valuable in elucidating the mechanisms of antagonism.

Many microbial ecologists have considered chemical determination an attractive possibility for biomass estimation, but most of the substances chosen as biomass indicators do not distinguish single species from the total microbial population. However, the integration of marker genes into the genome of microorganisms enables the production of compounds with the necessary degree of specificity.

There have been several reports of the application of the GUS marker technique for biomass quantification of plant pathogens in infected plant tissue. Oliver et al. (16) used a GUS-transformed strain of *Cladosporium fulvum* to distinguish between compatible and incompatible reactions in tomato plants. Liljeroth et al. (14) found a positive correlation between GUS activity and lesion size in barley roots infected with a transformed strain of *Bipolaris sorokiniana.* They also showed a positive correlation between GUS activity and the content of the fungal biomass indicator ergosterol in infected host tissue. Couteaudier et al. (5) found a reduction of GUS activity in flax roots exposed to a GUS-transformed pathogenic strain of *Fusarium oxysporum* in association with a nonpathogenic strain, which they interpreted as the ability of the nonpathogenic strain to inhibit successful colonization by the pathogenic strain.

Jenkinson and Ladd (12) argued that any substance chosen as a biomass indicator should be present in live cells in constant, known concentrations. However, quantitative measurements of fungal biomass should always be interpreted with care because the content of any biomass indicator can vary under different conditions (25).

In this, as well as in the above mentioned examples (5,14,16), production of GUS is controlled by a constitutive promoter, which makes it probable that the production of the enzyme correlates with the general physiological condition of the organism, varying with different phases of the life cycle and under the influence of different environmental conditions. Therefore, the level of enzyme production, rather than being an indication of the biomass, qualifies as an expression of the activity of the fungus. This is strongly supported by the fact that GUS activity in peat samples correlated well with the proportion of activated conidia (Fig. 5). In accordance with this, Eparvier and Alabouvette (8) considered that a constitutively expressed GUS gene in *F. oxysporum* f. sp. *lini* reflects the metabolic activity of the strain. Thus, they found that the color intensity of X-gluc–stained hyphae indicated the activity of the fungus on flax roots. Jefferson (11) and Wilson et al. (28) found that the bacteria, fungi, and plants tested had, with some exceptions, only negligible background GUS activity. This makes it possible to study fungal activity in complex systems by extraction of the enzyme, but one should be aware that in nonsterile soil, background activity may occur due to the presence of bacteria or fungi with intrinsic GUS activity.

Our results demonstrate the suitability of using a GUS-transformed strain for monitoring the presence, the population development, and the activity of a specific strain of *T. harzianum* deliberately released into the environment.

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