Notas Científicas

Survival in soil and detection of co-transformed *Trichoderma harzianum* by nested PCR

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Abstract – The objective of this work was to evaluate the survival of two *Trichoderma harzianum* co-transformants, TE 10 and TE 41, carrying genes for green fluorescent protein (*egfp*) and for resistance to benomyl, during four weeks in a contained soil microcosm. Selective culture media were used to detect viable fungal material, whose identity was confirmed by the observation of the fluorescent phenotype by direct epifluorence microscopy. PCR using two nested primer pairs specific to the *egfp* gene was also used to detect the transformed fungi. Although it was not possible to reliably detect the *egfp* gene directly from soil extracts, an enrichment step involving selective culture of soil samples in liquid medium prior to DNA extraction enabled the consistent detection of the *T. harzianum* co-transformants by nested PCR for the duration of the incubation period.

Index terms: biological control, transformation, *egfp*, benomyl resistance.

Sobrevivência em solo e detecção de co-transformantes de *Trichoderma harzianum* por PCR "nested"

Resumo – O objetivo deste trabalho foi avaliar a sobrevivência de dois co-transformantes de *Trichoderma harzianum*, TE 10 e TE 41, expressando o gene da proteína de fluorescência verde (*egfp*) e resistência a benomil, por um período de quatro semanas em microcosmo de solo, sob condições controladas. Foi utilizado um meio seletivo para detecção de material fúngico viável, o qual foi confirmado por observação quanto ao fenótipo de fluorescência em microscópio de epifluorescência direta. O fungo transformado foi detectado por PCR "nested", utilizando-se dois pares de primers específicos para o gene *egfp*. Foram utilizados meios líquidos enriquecidos no cultivo de amostras de solo, permitindo uma detecção consistente de co-transformantes de *T. harzianum*, uma vez que não foi possível a detecção do gene *egfp* por PCR de amostras de DNA extraídas diretamente de solo.

Termos para indexação: controle biológico, transformação, *egfp*, resistência a benomil.

Trichoderma species and strains have received much attention because of their potential as biocontrol agents of many plant pathogenic fungi. Results from field trials testing the biocontrol effect of *Trichoderma harzianum* indicate that this fungus functions efficiently under different environmental conditions, protecting several crops and controlling various plant pathogens (Chet, 1987). However, many antagonistic microorganisms do not show consistent biocontrol effects when released to the environment, making the careful monitoring of these strains an essential part of their evaluation.

Phenotypic or genotypic monitoring of random environmental isolates requires large sample sizes and is potentially difficult and laborious. Introduction of heterologous markers to an antagonist allows easy differentiation from indigenous fungi in the environment (Thrane et al., 1995). Lo et al. (1998) used a co-transformed strain of *T. harzianum* expressing hygromycin resistance and β-glucuronidase (GUS) activity as tools for assessing population development, colonization, and interactions with plant pathogens. Green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, is recognised as a sensitive and non-invasive reporter and an efficient marker (Chalfie et al., 1994).

The objective of this work was to evaluate the survival of two *Trichoderma harzianum* co-transformants,

TE 10 and TE 41, carrying genes for green fluorescent protein (*egfp*) and for resistance to benomyl, during four weeks in a contained soil microcosm.

The co-transformants, TE 10 and TE 41 (Inglis et al., 1999), were grown on potato dextrose agar (Difco, Detroit, Michigan, USA) containing 1.5 μg mL^{-1} benomyl at 28ºC for about five days until conidiation. Five mililiters conidial suspension ($5.0x10^7$ conidia mL^{-1}) of TE 10 and TE 41 were mixed with 50 g non-sterile soil (sandy loam from the Brazilian cerrado) in 100 mL plastic pots with lids to preserve humidity. Controls consisted of pots containing soil treated with 5 mL sterile water. Pots were then incubated at 28ºC for four weeks. One gram of soil from each pot was sampled 24 hours after inoculation and at weekly intervals for four weeks (Lo et al., 1998). Soil samples were diluted in 10 mL 0.1% tween 80 and plated on *Aspergillus* complete medium (ACM) (Pontecorvo, 1952), supplemented with 1.5 μg mL^{-1} benomyl, 20 μg mL^{-1} tetracycline and 100 μg mL^{-1} ampicillin until colony formation at 28ºC. Three plates were prepared for each sample and colonies examined directly for EGFP fluorescence using an inverted microscope (Axiovert, Carl Zeiss, Oberkochen, Germany) with excitation filter of 450–490 nm and emission filter at 520 nm.

Soil samples (200 mg) were inoculated into 100 mL YG liquid medium (2.5% glucose; 0.5% yeast extract; 3 μg mL^{-1} benlate; 20 μg mL^{-1} tetracycline; 100 μg mL^{-1} ampicillin) and incubated at 28ºC and 150 rpm for 48 hours. Mycelia were harvested by filtration, washed with distilled water and frozen in liquid nitrogen. Total DNA was extracted using a modified miniprep method (Porteous & Armstrong, 1993). Mycelium (100 mg) was placed in 800 μL extraction buffer (25 mM EDTA; 250 mM NaCl; 0.5% SDS; 200 mM Tris-HCl pH 8.0) and subjected to three alternating cycles of one minute in liquid nitrogen and one minute at 65ºC. The nucleic acids were extracted twice with phenol/chloroform then precipitated with isopropanol and resuspended in 100 μL milliQ water. To remove any soil PCR inhibitor, 20 μL nucleic acid extract was treated with RNase A and DNA, then purified using a glass binding kit (Geneclean; Bio 101 Inc., Vista CA, USA). Attempts were also made to extract DNA directly from soil samples (200 mg) using the above protocol.

To detect the presence of the *egfp* gene by PCR, four *egfp* specific primers were used in two sets. The first primer pair consisted of EGFP1: 5' CACCGGGGTGGTGCCCATCC and EGFP2: 5' GGCGGCGGTCACGAACTCCAG. Each 25 μL PCR reaction in a reaction buffer (50 mM KCl; 0.1% tween 20; 10 mM Tris-HCl pH 9.2) which also contained 1.5 mM $MgCl_2$, 0.35 μM each primer, 30 μM dNTP's, 2U *Taq* DNA polymerase and approximately 20 ng DNA. The reaction mixture was overlaid with 30 μL mineral oil and placed in a thermocycler (Model PTC-100, MJ Research, Watertown, Massachusetts, USA) using an initial denaturing time of 3 min at 94ºC, followed by 30 cycles of denaturation for 1 min at 94ºC, annealing at 62ºC for 1 min and extension for 1 min at 72ºC. A final extension for 5 min at 72ºC was also utilized. A 5 μL product from the first reaction was used in a second reaction, identical to the first, but with the primers replaced by the nested pairs EGFP3: 5' TGAACCGCATCGAGCTGAAG and EGFP4: 5' CAGCAGGACCATGTGATCG. After the amplifications, the products were separated and visualized in 1.5% agarose gels stained with ethidium bromide (0.5 μg mL^{-1}).

The two *T. harzianum* co-transformants were both found to be recoverable from soil using simple selective culture techniques, where the possession of a heterologous gene for resistance to the fungicide benomyl by these transformants enabled direct selection. Moreover, the green fluorescent phenotype allowed in vivo identification, by epifluorescence microscopy, of the transformants on the recovery plates. Almost no contaminating fungi were recovered during the tests showing different growth characteristics to the applied *Trichoderma* transformants.

Numbers of transformant colony forming units (CFU) recovered from pots, including time 0 samples, were higher than the conidium concentration originally applied ($5x10^6$ conidia g^{-1}) (Table 1). This discrepancy is probably due to uneven mixing of conidia and soil at the time of inoculation. Despite this, numbers of recovered CFU were found to rise during the period of monitoring, most likely due to the ability of the transformants to grow

Table 1. Recovery of transformed colony forming (CFU) of *Trichoderma harzianum* strains TE 10 and TE 41 from soil *Aspergillus* complete medium supplemented with 1.5 μg mL^{-1} benomyl.

Week	CFU recovered per gram soil ($x10^6$ colonies)[(1)]	
	TE 10	TE 41
0	1.5±0.7	1.0±0.0
1	1.5±0.7	2.0±0.0
2	1.0±0.2	1.5±0.5
3	4.5±2.1	4.5±2.1
4	5.5±2.1	7.5±0.7

[(1)]Number (mean±standard deviation) of CFU recovered are expressed as the mean of three replicate plate counts, with adjustment for dilution factor used.

and sporulate in the soil medium. For both transformants TE 10 and TE 41, analysis of EGFP fluorescence allowed instant confirmation of the recovered colonies identity, in which 100% of recovered benomyl-resistant colonies were brightly fluorescent when examined microscopically.

Pots inoculated with wild-type *T. harzianum* strain 1051, a second *Trichoderma* strain, designated TVC and uninoculated pots were used to provide negative control soil samples for PCR. In these controls, no amplification products were detected with any combination of *egfp*-specific primers.

PCR detection of the *egfp* gene in DNA samples, extracted directly from inoculated soil, proved to be highly inconsistent, even from freshly inoculated samples. This difficulty is probably related to inhibition of PCR by soil components that may be difficult to remove by standard extraction protocols (Tsai & Olson, 1992; Romanowski et al., 1993; Jansson, 1995; Thrane et al., 1995). Soil DNA extracts used were usually brown colored, indicating significant levels of persistent contaminants (Porteous & Armstrong, 1993). Problems faced in direct extraction of amplifiable DNA from soil led to the adoption of a liquid culture enrichment step prior to PCR. DNA minipreps of these enriched samples were found to be consistent and effective templates for PCR detection of the *egfp* gene using both primer pairs in nested PCR (Figure 1).

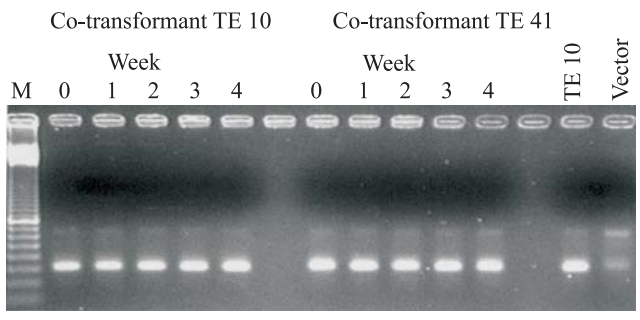

Figure 1. Nested PCR of *Trichoderma harzianum* co-transformants TE 10 and TE 41 to detect the *egfp* gene during four weeks of monitoring, where M is the 100 bp ladder marker. Positive amplification controls were DNA extracted from a pure culture of TE 10 and plasmid pEGFP/gpd/tel (Vector). Negative (No DNA and wild type *T. harzianum* 1051 genomic DNA) controls were included in all reactions sets, and yielded no PCR products. The strongly amplified band corresponds to the 307 bp product from primer set two and the weak band of higher molecular weight is residual amplification of the 650 bp product of primer set one.

Both standard culture techniques and PCR monitoring of the *egfp* gene indicated the ability of the two *T. harzianum* transformants to persist in soil for at least four weeks in a culturable state. Counts of fluorescent colonies suggest that the introduced traits are stable in the two co-transformants studied, which are therefore potential candidates for biocontrol trials against *C. perniciosa* on cocoa.

Acknowledgements

To Conselho Nacional de Desenvolvimento Científico e Tecnológico for a Master degree studentship for the first author and for a post-doctoral scholarship for the third author.

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Received on October 14, 2003 and accepted on January 26, 2004