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Trichoderma harzianum elicits defence response genes in roots of potato plantlets challenged by *Rhizoctonia solani*

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Abstract Biological control of *Rhizoctonia solani* with *Trichoderma harzianum* has been demonstrated in several studies. However, none have reported the dynamics of expression of defence response genes. Here we investigated the expression of these genes in potato roots challenged by *R. solani* in the presence/absence of *T. harzianum* Rifai MUCL 29707. Analysis of gene expression revealed an induction of *PR1* at 168 h post-inoculation (hpi) and *PAL* at 96 hpi in the plants inoculated with *T. harzianum* Rifai MUCL 29707, an induction of *PR1*, *PR2* and *PAL* at 48 hpi in the plants inoculated with *R. solani* and an induction of *Lox* at 24 hpi and *PR1*, *PR2*, *PAL* and *GST1* at 72 hpi in the plants inoculated with both organisms. These results suggest that in the presence of *T. harzianum* Rifai MUCL 29707, the expression of *Lox* and *GST1* genes are primed in potato plantlets infected with *R. solani* at an early stage of infection.

Keywords Biological control agent · Plant defence genes · Quantitative RT-PCR · Reference genes · *Rhizoctonia* · *Trichoderma*

Abbreviations

BCA	biological control agent
β-tub	beta-tubulin
EF1-α	elongation factor 1-alpha
GAPDH	glyceraldehyde phosphate dehydrogenase
GST1	Gluthatione-S-transferase 1
Lox	Lipoxygenase
PAL	Phenylalanine ammonia lyase
PR1	Pathogenesis Related 1
PR2	Pathogenesis Related 2
R-treatment	*Rhizoctonia solani* treatment
T-treatment	*Trichoderma harzianum* treatment
T+R-treatment	*Trichoderma harzianum* and *Rhizoctonia solani* treatment
Ubc	ubiquitin conjugating enzyme-like

Introduction

Rhizoctonia solani is an important soil-borne plant pathogen affecting potato (*Solanum tuberosum*) (Banville et al. 1996). *Rhizoctonia solani*-infected

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plants may develop crown rot, root rot, or stem canker, which often leads to wilting and to plant death in severe cases. The *Rhizoctonia* disease of potato is a problem occurring throughout the world. Control of this disease has commonly relied on cultural practices and on the use of chemicals. However, cultural practices only are not always efficient and, at the present time, no effective fungicides are available, although some chemicals are recommended. In recent years, efforts have concentrated on the biological control of this pathogen using bacteria and fungal antagonists (Howell 2003; Grosch et al. 2006). These biological control agents (BCAs) represent promising alternatives to minimise the impact of chemicals on the environment by replacing these chemicals or reducing their rate of application to control plant pathogens (Chet and Inbar 1994; Harman and Bjorkman 1998) such as *Rhizoctonia solani*.

Trichoderma spp. are amongst the most studied fungal BCAs, isolated from soil and present in the rhizosphere. These fungi are opportunistic, avirulent plant symbionts, and function as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from diseases (Harman et al. 2004; Vinale et al. 2008). Depending upon the strain, the use of *Trichoderma* in agriculture can provide numerous advantages: (i) control of pathogens and of competitive/deleterious microflora; (ii) improvement of plant health and (iii) stimulation of root growth (Harman et al. 2004; Vinale et al. 2008). In addition, plant-cell wall-degrading enzymes produced by *Trichoderma*, such as xylanases and cellulases, are able to directly induce ethylene biosynthesis in plants, a well-known response to the presence of pathogens (Harman and Bjorkman 1998).

The beneficial effects of *Trichoderma harzianum* to control *R. solani* have been reported in different studies (Brewer and Larkin 2005; Vinale et al. 2006). Recently, Wilson et al. (2008) investigated the dynamics of *R. solani* on potato plants in the presence of *T. harzianum*. These authors noted a decrease in the severity of symptoms caused by *R. solani* during the first 7 days post-inoculation and attributed this observation to a transient antagonistic effect of *T. harzianum* at the early stage of the interaction. However, the molecular basis behind this protective effect remains to be elucidated. Indeed, there is extensive evidence that *Trichoderma* spp. are able to elicit the defence systems of a number of plants (Harman and Bjorkman 1998; Hanson and Howell 2004; Vinale et al. 2008). This evidence was consolidated by studies on the effect of *Trichoderma* spp. inoculations in plant proteome/gene expression by 2d electrophoresis as well as high-density oligo microarrays (Marra et al. 2006; Alfano et al. 2007; Segarra et al. 2007). Therefore, determining how *T. harzianum* affects the expression of defence response genes in potato plantlets challenged by *R. solani* is crucial to understand the mechanism of biological protection against this major pathogen.

In the recent decade, the development of the real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) technique has allowed the accurate expression profiling of RNA transcripts, and has become the most useful method for characterising gene expression in human research (Bustin 2002). This technique was used in plant-microbe interactions by McMaugh and Lyon (2003) to analyse the expression of class II chitinase in Bermudagrass following infection with the root pathogen *Ophiosphaerella narmari*. In our study, we used the QRT-PCR technique with different SA and JA-dependent genes, among the most frequently studied in the interaction between *T. harzianum* and *R. solani* on potato (Yedidia et al. 2003; Shoresh et al. 2005; Gao et al. 2006; Vinale et al. 2008).

In the present study, the time-course expression of defence response genes of potato plantlets challenged by the pathogen *R. solani* in the presence/absence of the BCA *T. harzianum* Rifai MUCL 29707 was followed during the early stage of the plant-microbe (s) interaction. The real-time QRT-PCR technology was used to assess the expression of defence response genes dependent on salicylic acid (SA): Gluthatione-S-transferase 1 (*GST1*), Pathogenesis Related 1 (*PR1*) and Pathogenesis Related 2 (*PR2*); and dependent on jasmonic acid (JA): Lipoxygenase (*Lox*) and Phenylalanine ammonia lyase (*PAL*). The study was conducted under *in vitro* conditions using a micropropagated potato (*S. tuberosum*) plantlet inoculated with *R. solani* and/or *T. harzianum*.

Materials and methods

Biological material

Propagation and maintenance of potato plantlets stock In vitro propagated potato plantlets (*S. tuberosum*,

var. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micro-propagated every 5 weeks as described in Voets et al. (2005). Plantlets were kept in boxes (90 mm×60 mm× 50 mm) on the Murashige-Skoog (MS) medium, supplemented with 20 $g\ l^{-1}$ sucrose, 3 $g\ l^{-1}$ Gel groTM (ICN, Biomedicals, Inc., Irvine, CA, USA) and adjusted to pH 5.9. Boxes were incubated in a growth chamber set at 20/15°C (day/night) with a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 15 μmol m^{-2} s^{-1}.

Culture, propagation and maintenance of T. harzianum and R. solani A culture of *T. harzianum* Rifai MUCL 29707 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL) (http://bccm.belspo.be/about/mucl.php). A plug of gel containing several conidia and mycelium was placed into a sterile 1.5 ml glass tube filled with 0.4 ml of 1% Sterile Distilled Water-Peptone (SDWP) (Duchefa, The Netherlands). The plug was swirled in the SDWP with a vortex mixer (8,000 rpm) for 15 s and the suspension was serially diluted to 10^{-2}. From the 10^{-2} dilution, 50 μl was spread in Petri plates (90 mm diam.) on 50 ml Potato Dextrose Agar (PDA) (Scharlau Chemie S.A, Barcelona, Spain). The Petri plates were incubated at 25°C in the dark for 7 days. A culture of *R. solani* Kuhn MUCL 49235 was supplied by MUCL. A plug of gel containing several bulbils and mycelium was placed on 50 ml PDA in Petri plates. The Petri plates were incubated at 25°C in the dark for 7 days.

Experimental set-up

Three treatments were considered: potato plantlets inoculated with *T. harzianum* (T-treatment) or with *R. solani* (R-treatment) alone and plants inoculated with *R. solani* and *T. harzianum* at the same time (T+R-treatment). In addition, a control treatment (i.e. potato plantlets grown in the absence of both fungi) was included. For each treatment, 10 day-old potato plantlets were inserted in Petri plates containing 50 ml of the Modified Strullu Romand (MSR) medium (Declerck et al. 1998) without sucrose and vitamins, with the roots placed on the surface of the medium and the shoot extending outside through a hole made at the base and the lid of the Petri plate (for details, see Voets et al. 2005).

The inoculation with *T. harzianum* strain was performed using a 3 mm diam plug from a 7 day-old culture of *T. harzianum*. The plug containing abundant mycelium and conidia was placed in the close vicinity of the potato roots. The Petri plates were then sealed with parafilmTM (Pechiney, Plastic Packaging, Chicago, USA, IL 60631) and the hole filled with sterilised (121°C for 15 min) silicon grease (VWR International, Belgium) to avoid contaminations (Voets et al. 2005). The inoculation with the *R. solani* strain followed the same procedure as before. A 3 mm diam plug from a 7 day-old culture of *R. solani* containing abundant mycelium and sclerotia was placed in the close vicinity of potato roots and the Petri plates were sealed as before. The inoculation with *T. harzianum* and *R. solani* strains followed the same procedure as above. A 3 mm diam plug from a 7 day-old culture of *T. harzianum* containing abundant mycelium and conidia was placed in the close vicinity of the potato roots. In parallel, a 3 mm diam plug from a 7 day-old culture of *R. solani* containing abundant mycelium and sclerotia was placed in the close vicinity of the *T. harzianum* plug and potato roots. The Petri plates were sealed as before. The control treatment consisted of potato plantlets grown in the absence of both fungi.

In all treatments, the Petri plates were covered with an opaque plastic bag and incubated horizontally in a growth chamber set at 22/18°C (day/night) with 70% relative humidity, a 16 h day^{-1} photoperiod and a PPF of 300 μmol m^{-2} s^{-1}. All the procedures described above were conducted in sterile conditions under a horizontal laminar hood. Each Petri plate was considered as an experimental unit in two experiments performed in parallel. One experiment was conducted for the analysis of real-time QRT-PCR (stability of reference genes and expression of defence response genes in potato roots challenged by *R. solani* in the presence/absence of *T. harzianum*). The other experiment was conducted by acridine orange observations to detect the presence of DNA and RNA in fresh samples of potato roots challenged by *R. solani* in the presence/absence of *T. harzianum*. Three biological replicates were used for each experiment.

RNA extraction

Three treatments (R-Treatment, T-treatment, T+R-treatment) plus a non-inoculated control were set-up as described above. Roots were harvested at 2 h, 4 h,

8 h, 24 h, 48 h, 96 h and 168 h post-inoculation (hpi) for the R-treatment and T-treatment, and 24 hpi, 48 hpi, 72 hpi, 96 hpi and 168 hpi for the R+T-treatment. Three samples per treatment were collected at each time. One control plantlet was also harvested at each time, i.e. 2 hpi, 4 hpi, 8 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi, 168 hpi. Total RNA was extracted from 50–100 mg of frozen material with Trizol® reagent Invitrogen, Carlsbad, CA, USA) with an extra chloroform purification step, and then purified using the Purelink™ Micro-to-midi total RNA purification system (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA was subsequently treated with the TURBO DNA-*free*™ kit (Ambion; Austin, USA) according to the manufacturer's instructions. Concentration and purity of total RNA were determined in a NanoDrop®-ND 1,000 UV-Vis Spectrophotometer (NanoDrop Technologies, New Zealand), using a 2 µl aliquot of the total RNA solutions. RNA purity was estimated from the A260/A280 absorbance ratio, which is an estimation of contamination mainly by proteins and phenol.

Reverse transcription

Following total RNA extraction, reverse transcription (RT) was carried out in a final volume of 20 µl, and was started by mixing 1 µg of total RNA and 0.5 µg of oligo $(dT)_{12–18}$ (Invitrogen; Carlsbad, CA, USA) at 65°C for 15 min. After 5 min incubation at 4°C, 4 µl of 5X RT buffer (250 mM TRIS-HCL, pH 8.3, 250 mM KCl, 2.5 mM spermidine, 50 mM $MgCl_2$, 50 mM DTT), 200 mM of dNTPs, 25 units of RNase inhibitor (Invitrogen; Carlsbad, CA, USA), and 200 units of M-MLV reverse transcriptase (Promega; Madison, WI, USA) were added. RT was incubated at 37°C for 1 h and inactivated at 75°C for 15 min. For each RNA sample, a reaction without RT was performed as a control for contamination by genomic DNA.

Primer design

Four reference genes: glyceraldehyde phosphate dehydrogenase (*GAPDH*), ubiquitin conjugating enzyme-like (*Ubc*), elongation factor 1-alpha (*EF1-α*) and beta-tubulin (*β-tub*) (Nicot et al. 2005), and five defence response genes: Gluthatione-S-transferase 1 (*GST1*), Lipoxygenase (*Lox*), Phenylalanine ammonia lyase (*PAL*), Pathogenesis Related 1 (*PR1*) and Pathogenesis Related 2 (*PR2*) were selected. Potato nucleotidic sequences were obtained from GenBank database (Table 1). Nine primer pairs were designed from these sequences (90–110 bp sequence length, optimal Tm at 60°C, GC% between 40% and 60%) with the Light Cycler Probe Design Software 2.0 (Roche, Montreal, QC, Canada) (Table 1). In the calibration step, an experimental control without cDNA was performed to test for primer-dimer formation, primers forming dimers being excluded.

Real-time quantitative PCR

Real-Time PCR analysis was performed using the LightCycler 2.0 (Roche, Montreal, QC, Canada). A set of standard solutions prepared from RT products was included in each run. Reactions were prepared in capillaries using the following concentrations: 7 µl of PCR water, 4 µl of 5× LightCycler® FastStart DNA

Table 1 Primer sequences of four reference genes and five defence response genes, the amplification length and the melting temperature of the amplified product

Name	Accession number	Primer sequence 5′-3′ (Forward)	Primer sequence 5′-3′ (Reverse)	Length (bp)	Tm (°C)
β-tub	Z33382	ATGTTCAGGCGCAAGGCTT	TCTGCAACCGGGTCATTCAT	101	84
EF1-α	AB061263	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA	101	84
GAPDH	AF527779	GGACATTGTCTCCAACGC	ATGAGACCCTCCACAATGC	93	84
Ubc	DQ241834	TGATGGTTACCCATTTGAGCC	ACTGGTCCTTCAGGATGTC	110	82
GST1	J03679	TTCTAGCCACCAGATTTGACC	ACATATTCCCTATATTTTTGGAGTGAGTA	97	81
Lox	Y18548	GAGTTCTCCTCATGGTGTTCGTTTA	AGTAGTCTGACACCCAACTT	101	81
PAL	X63104	GGATATGCCATCGAACTTTGAGA	ACAAATAATGGCATGGATGAGG	110	81
PR1	AJ250136	GGTGCAGGAGAGAACCTT	GGTACCATAGTTGTAGTTTGGCT	99	85
PR2	AJ009932	TATCATCAGCAGGGTTGCAAA	TCGCGAAAAATGCTATTTCTAGG	100	81

Master^{PLUS} SYBR Green I Mix, 2 µl of each forward and reverse primer (0.5 µM) and 5 µl of 1:10 diluted cDNA or standard solution as template. Capillaries were closed, centrifuged and placed into the LightCycler rotor. The following LightCycler experimental run protocol was used: denaturation programme (95°C for 10 min), amplification and quantification programme repeated 40 times (95°C for 10 s, 60°C for 20 s and 72°C for 8 s with a single fluorescence measurement), melting curve program (60–95°C with a heating rate of 0.1°C sec^{-1} and a continuous fluorescence measurement) and finally a cooling step to 40°C. In order to check PCR efficiency, standard curves (log of cDNA dilution versus C_p) using serial 10-fold dilution of cDNA were created for each pair of selected primers. A 100% PCR efficiency corresponds to a slope of −3.3. To allow a good comparison and normalisation, PCR efficiency should be between 80% and 115%. In this study, all the PCRs displayed efficiencies between 86% and 114%. For the mathematical model it was necessary to determine the crossing point (Cp) for each transcript, defined as the point at which the fluorescence rises appreciably above the background fluorescence. The Fit point method must be performed in the LightCycler software 4.1 at which Cp will be measured at a constant fluorescence level. The data were analysed by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) (for details see technical note Roche: No. LC 16/2002).

Stability of reference genes

To evaluate the effect of T-treatment, R-treatment and T+R-treatment on gene expression in roots of potato plantlets, all samples were normalised to allow comparison by the same reference genes. Normalisation was achieved using four reference genes, i.e. *GAPDH*, *β-tub*, *EF1-α* and *Ubc*. The combination of several references genes smoothes normalisation error due to the small variation in expression of a single reference gene (Vandesompele et al. 2002). The gene expression stability (*M*) was calculated using the geNorm programme (http://medgen.ugent.be/~jvdesomp/geNorm): genes with the lowest M-value are the most stably expressed (Vandesompele et al. 2002). Furthermore, to estimate the optimal number of internal control genes required for reliable normalisation, normalisation factors (NF*n*) were calculated by stepwise inclusion of the most stably expressed reference genes. Subsequently, pairwise variations (V*n*/*n*+1) were calculated for every series of NF*n* and NF*n*+1 to determine the effect of adding a $(n+1)^{th}$ gene. A great variation indicated that the newly added gene had a significant effect on normalisation and thus should preferably be included for the calculation of a reliable normalisation factor (Vandesompele et al. 2002). We therefore used the relative expression values for each cDNA sample as input for the geNorm algorithm, from which an M-value was calculated for each reference gene. This M-value reflected the expression stability of the gene compared to the other reference genes; a lower M-value means more stable gene expression and is the basis for the ranking of the genes in order of their expression stability.

Acridine orange observations

Three treatments (R-Treatment, T-treatment, T+R-treatment) plus a control were set-up as described above. Presence of DNA and RNA in fresh samples of potato roots was confirmed using acridine orange (Merck, Whitehouse Station, NJ, USA) staining. Roots were harvested 48 hpi, 96 hpi and 168 hpi for the R-treatment, T-treatment, T+R-treatment and control. Three samples per treatment and control were considered at each time. Root systems were harvested and washed in 1% acetic acid (*v*/*v*) for 30 s, rinsed with H_2O and incubated successively for 2 min in 0.1 M phosphate buffer (pH 6) and 3 min in 0.01% acridine orange in phosphate buffer (*v*/*v*). Samples were then rinsed once in 11% $CaCl_2$ (*w*/*v*) and twice in 0.1 M phosphate buffer. Roots were mounted in phosphate buffer and observed using a digital camera (model Leica DFC320; Leica Microsystems Ltd) coupled to an epifluorescence microscope (Nikon type 114) equipped with a mercury lamp (HB-10101 AF) and displayed on a 15-inch hp pavilion ze4500 screen using the image manager software: Leica IM50, version 4.0 Leica Microsystems Imaging solutions Ltd, Cambridge, UK.

Results

Stability of reference genes in the potato plants inoculated with *R. solani* and/or *T. harzianum*

GeNorm analysis In order to determine the best combination of reference genes for normalisation of

gene expression, geNorm analysis was used. The geNorm algorithm also determines the M-value and the pairwise variation (Vn/n + 1), which measures the effect of the addition of reference genes on the normalisation factor (equal to the geometric mean of the expression values of the selected reference genes). It is advisable to add additional reference genes to the normalisation factor until the added gene has no significant effect (low V-value). We used the cut-off value 0.15 proposed by Vandensompele et al. (2002) below which the addition of reference genes is not required. The analyses of M-value and pairwise variation with the geNorm software for the reference genes were performed for the three treatments (R-Treatment, T-treatment, T+R treatment) at 48 hpi, 96 hpi and 168 hpi (Fig. 1). The most stable genes (genes with the lowest M-value are the most stably expressed, Fig. 1) were *EF1-α* and *GAPDH* (M= 0.521) and *EF1-α* and *Ubc* (M=0.422) for the T-treatment and R-treatment, respectively. For the T +R-treatment the two most stable genes were *EF1-α* and *GAPDH* (M=0.164). When considering the three treatments, *EF1-α* and *GAPDH* were the most stable reference genes, followed by *Ubc*. The V graph (Fig. 1), for the T-treatment, demonstrated that three reference genes were sufficient for normalisation (as $V_{(3/4)} < 0.15$). Similar to the T-treatment, the T+R-treatment had a pairwise variation below the cut-off, so that only three genes were necessary as the internal control. For the R-treatment, the pairwise variation $V_{(3/4)}$ was > 0.15 (0.175). But we observed for this treatment that the Ct values obtained for all the four reference genes decreased at 168 hpi.

Acridine orange observations The acridine orange staining allowed the visual detection of DNA and RNA in fresh samples of potato roots. The complete root systems were analysed at 48 hpi, 96 hpi and 168 hpi for the three treatments and the control (Fig. 2). At 48 hpi, no visual difference was detected in the level of DNA and RNA staining between the three treatments and the control treatment. No differences were again observed at 96 hpi in the level of DNA and RNA staining, between the T-treatment, the T+R-treatment and the control treatment. However at this sampling time, the potato roots in the R-treatment had

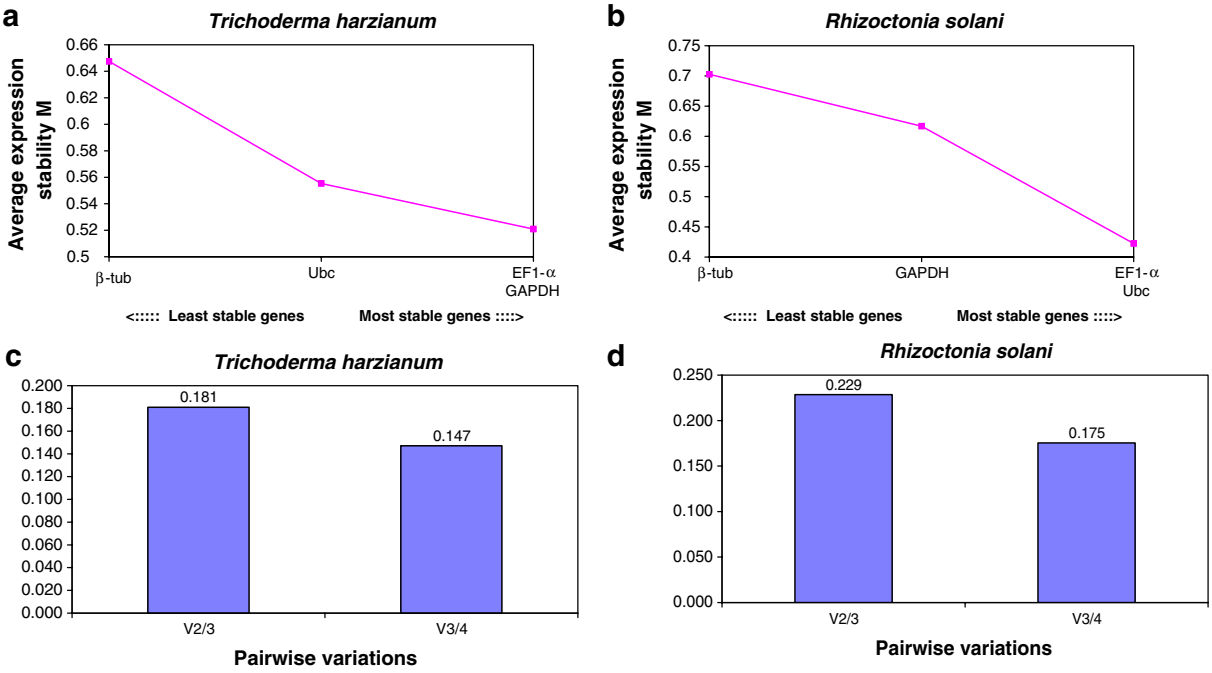

Fig. 1 Average expression stability values of reference genes and the optimal number of reference genes for normalisation by geNorm analysis: **a** and **c** Potato roots inoculated with *T. harzianum*, **b** and **d** Potato roots inoculated with *R. solani*. **a**, **b**, **c** and **d**: relative non-unit data. $V_{(n/n+1)} = n$ reference genes were sufficient for normalisation ($V_{(n/n+1)} < 0.15$) or addition of one reference gene was necessary ($V_{(n/n+1)} > 0.15$)

a visually similar level of DNA staining, while for RNA it appeared lower. At 168 hpi, only a few nuclei (DNA) were detected, while no RNA was visualised in the R-treatment. To the contrary, no difference was observed in the level of DNA and RNA staining, between the T-treatment and the control treatment at this sampling time, while for the T+R treatment, the presence of DNA and RNA was visually greater than in the R-treatment, but lower than in the control treatment. Following the geNorm analysis and acridine orange observations, we considered that the V-value (0.175) for the R-treatment was adequate for the normalisation with three reference genes (*EF1-α*, *GAPDH* and *Ubc*) for the analysis of defence response genes in potato roots. However, for the R-treatment and the time-course 168 hpi, we decided to discard the relative expression values obtained, because the Ct values obtained for the four reference genes decreased.

Expression of defence response genes in potato plantlets inoculated with *R. solani* or *T. harzianum*

To assess the expression of defence response genes in roots inoculated with *R. solani* or *T. harzianum*, 10 day-old potato plantlets were inoculated with both organisms separately. Roots were harvested at 2 hpi, 4 hpi, 8 hpi, 24 hpi, 48 hpi, 96 hpi and 168 hpi and the relative expression of *GST1, Lox, PR1, PR2* and *PAL* genes was measured (Table 2). For the T-treatment, no variation in relative expression was observed for the *GST1*, *Lox* and *PR2* genes whatever

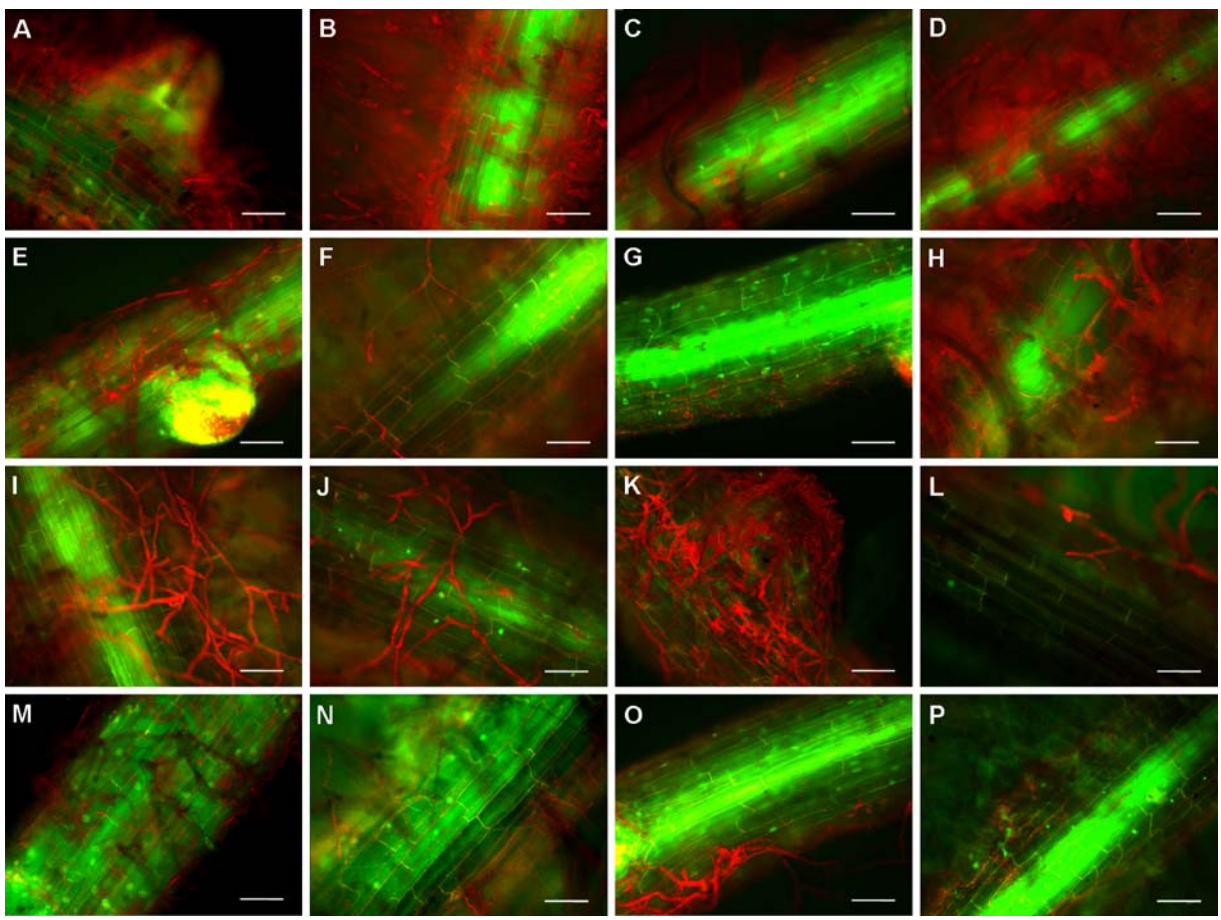

Fig. 2 Observation with the vital dye acridine orange of the potato roots in different treatments. **a**, **b**, **c** and **d**: control. **e**, **f**, **g** and **h**: Potato roots inoculated with *T. harzianum*. **i**, **j**, **k** and **l**: Potato roots inoculated with *R. solani*. **m**, **n**, **o** and **p**: Potato roots inoculated with *T. harzianum* and *R. solani*. **a**, **b**, **e**, **f**, **i**, **j**, **m** and **n**: Time-course 96 hpi. **c**, **d**, **g**, **h**, **k**, **l**, **o** and **p**: Time-course 168 hpi. Scale bar is 50 μm. Micrographs were taken at ×160 magnification

Table 2 Expression level of the five defence response genes in potato roots during the inoculation with *T. harzianum* (Th) or *R. solani* (Rs), normalised to the three reference genes in the control roots (three biological replicates)

Gene name	Treatment	Relative expression during the time-course[a]						
		2	4	8	24	48	96	168
GST1	Th	0.81 (0.22)	0.80 (0.19)	0.80 (0.21)	0.83 (0.22)	0.93 (0.27)	1.25 (0.11)	1.55 (0.23)
	Rs	0.57 (0.22)	0.76 (0.21)	0.79 (0.18)	0.74 (0.16)	1.66 (0.93)	1.26 (0.16)	7.5 (4.67)[b]
Lox	Th	0.56 (0.14)	0.74 (0.21)	0.44 (0.08)	0.65 (0.19)	0.6 (0.26)	0.76 (0.14)	0.72 (0.12)
	Rs	0.55 (0.23)	0.59 (0.18)	0.86 (0.21)	0.69 (0.17)	1.09 (0.37)	1.11 (0.11)	1.21 (0.38)[b]
PAL	Th	1.36 (0.5)	1.65 (0.38)	0.89 (0.26)	0.86 (0.17)	0.37 (0.11)	2.42 (0.18)	0.99 (0.39)
	Rs	1.49 (0.63)	1.31 (0.52)	1.21 (0.39)	3.66 (1.38)	6.63 (3.87)	2.72 (1.02)	16.09 (11.24)[b]
PR1	Th	1.15 (0.57)	0.76 (0.30)	0.26 (0.03)	1.62 (0.93)	0.83 (0.29)	2.16 (0.33)	22.25 (13.45)
	Rs	0.41 (0.45)	0.48 (0.18)	0.42 (0.33)	0.25 (0.11)	37.65 (3.60)	0.19 (0.17)	5.16 (7.18)[b]
PR2	Th	1.22 (0.37)	1.63 (0.61)	0.79 (0.21)	1.63 (0.74)	2.07 (0.62)	1.98 (0.45)	1.74 (0.04)
	Rs	1.49 (0.62)	1.85 (0.55)	1.01 (0.22)	1.07 (0.24)	13.26 (12.3)	3.19 (0.66)	24.85 (17.91)[b]

The standard error is shown in brackets. Statistical significance: relative expression ≥2

[a] Time after inoculation (h); [b] Relative expression values obtained with Ct values of reference genes not considered because they were too variable for good normalisation

the time of observation. For the relative expression of *PAL* gene, we noticed a slight up-regulation at 96 hpi with a relative value of 2.42±0.18. The relative expression of *PR1* gene increased in roots of potato plantlets at 96 hpi, with the maximum expression observed at 168 hpi (22.25±13.45). For the R-treatment, no variation in relative expression was observed for the *GST1* and *Lox* genes whatever the time of observation. For the relative expression of *PR1* and *PR2* genes, we noticed an up-regulation at 48 hpi with a relative value of 37.65±3.60 and 13.26± 12.3, respectively. The relative expression of *PAL* gene increased in roots of potato plantlets at 24 hpi, with a maximum at 48 hpi (6.63±3.87), and decreased at 96 hpi. We observed for the time-course 168 hpi, an increase of *GST1*, *PAL*, *PR1* and *PR2* gene expression. The relative expression of these genes was probably induced by the decrease of reference gene expression.

Expression of defence response genes in potato plantlets inoculated concomitantly with *R. solani* and T. *harzianum*

To assess the expression of defence response genes in potato roots challenged by *R. solani* in the presence of *T. harzianum*, 10 day-old potato plantlets were inoculated with both organisms concomitantly. Roots were harvested at 24 hpi, 48 hpi, 72 hpi, 96 hpi and 168 hpi and the relative expression of *GST1, Lox, PR1, PR2* and *PAL* genes was measured (Fig. 3). The results differed between the genes. The expression of *PAL*, *GST1*, *PR1* and *PR2* genes increased after 48 hpi, with a maximum at 72 hpi, and subsequently (i.e. starting from 96 hpi) decreased. The maximum values obtained were 15.03±5.73, 27.29±15.78, 62.83±36.35 and 79.14±39.75 for *PAL*, *GST1*, *PR1* and *PR2* genes, respectively. The expression of *Lox* gene was characterised by an induction at 24 hpi with a value of 6.03±1.19.

Discussion

Biological control (e.g. with *T. harzianum*) of the soil-borne fungal plant pathogen *R. solani* on potato has been reported in many studies over the last few years (Brewer and Larkin 2005; Vinale et al. 2006). Decrease in the severity of symptoms was observed in the early stages of infection (i.e. during the first 7 days post-inoculation) and attributed to a transient antagonistic effect of *T. harzianum* (Wilson et al. 2008). However, no studies have investigated the early (i.e. within 1 week) temporal dynamics of expression of defence gene responses in potato roots challenged by *R. solani* in the presence/absence of *T. harzianum*. This is crucial to understand the mechanism of biological protection conferred by *T. harzianum* and to develop innovative strategies to control *R. solani*. In the present study, we used the QRT-PCR to analyse and

compare the expression of defence response genes in roots of *in vitro*-produced potato plants inoculated with *R. solani* and/or the BCA, *T. harzianum*. The effect conferred by *T. harzianum* versus *R. solani* was demonstrated by the induction of the *Lox* gene expression at 24 hpi and *GST1* gene expression at 72 hpi in the roots of potato plantlets inoculated with both organisms at the same time. These results were confirmed by the acridine orange observation. Potato plantlets challenged by *R. solani* had visually greater root DNA and RNA contents in the presence of *T. harzianum* than the plantlets inoculated with *R. solani* alone.

Reliable quantification of gene expression relies on trustworthy normalisation of QRT-PCR data. The best normalisation is obtained using internal reference genes because it takes into consideration variation introduced by RNA sample quality, RNA input quantity and enzymatic efficiency in reverse transcription (Vandesompele et al. 2002). The ideal reference genes have stable expression levels that do not differ in the organs or tissues studied, and that are not influenced by the treatments. Since variation always exists for any reference gene, normalisation of gene expression based on a single reference gene can bias the results. Normalisation with multiple reference genes has become the standard, but reports that identify such genes in plant research are limited, even though algorithms are available to test the expression stability of reference gene candidates (Vandesompele et al. 2002; Pfaffl et al. 2004). For *S. tuberosum,* Nicot et al. (2005) tested seven candidate genes. The *β-tub* and *EF1-α* appeared the best reference genes for normalisation under biotic and abiotic stress conditions. For *Solanum lycopersicum*, Yan and Liou (2006) tested 18 candidate genes. The *GAPDH* and *Ubc* appeared the most adequate for normalisation with a plant pathogen. Therefore, these four reference genes (i.e. *β-tub*, *EF1-α*, *GAPDH* and *Ubc*) were selected for geNorm software analysis and the expression stability was evaluated in potato roots inoculated with *R. solani* and/or *T. harzianum*. Whatever the treatment, *EF1-α*, *GAPDH*, and *Ubc* were identified as the best candidates for normalisation with multiple reference genes.

The expression stability of the four reference genes was lower in the R-treatment than in the T-treatment and T+R-treatment. In the presence of *T. harzianum* alone, the expression stability of reference genes in potato roots was not affected, while in the presence of *R. solani* alone, this stability was markedly affected more so than in the presence of both organisms at the same time. Indeed, the Ct values obtained for all the four reference genes decreased at 168 hpi in the R-treatment, while it remained stable for the T+R-treatment. These observations suggested that *T.*

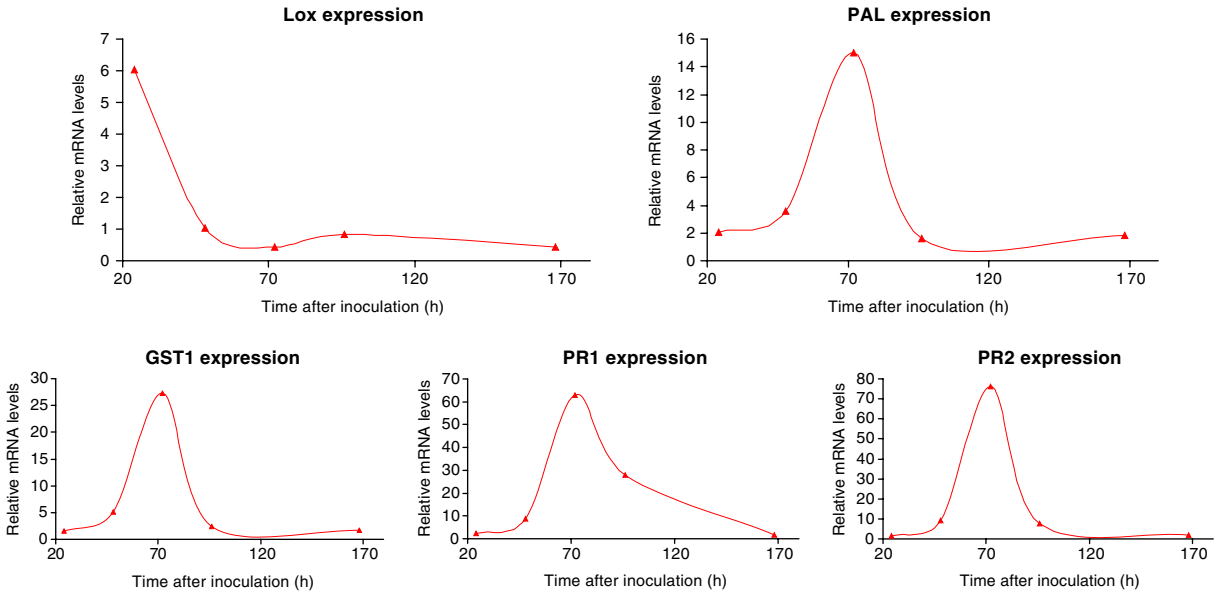

Fig. 3 Time-course of the induction of *Lox*, *PAL*, *GST1*, *PR1* and *PR2* gene expression. Expression was measured in potato roots inoculated with *T. harzianum* and *R. solani*

harzianum could confer a protective effect on potato plantlets infected by *R. solani*, at least at the early stage of infection (i.e. within 168 h), confirming the results previously obtained by Wilson et al. (2008) within a similar experimental duration. These results were supported with the vital dye acridine orange. Both single-stranded nucleic acids and double-stranded nucleic acids were detected in root tissues staining orange and green, respectively (Darzynkiewicz 1990). The amount of DNA and RNA was visibly decreased in the root tissues challenged by *R. solani* alone, while only slightly decreased when both organisms where present, and remained unchanged in the presence of *T. harzianum* alone. The reference gene EF1-α also supported the increased protection conferred by *T. harzianum* in roots challenged by *R. solani*. The EF1-α gene used as a positive control of the vitality (Van Aarle et al. 2007), had decreased expression in the R-treatment at 168 hpi, while it was not affected in the T+R-treatment. The greater impact on gene expression in the roots of plants inoculated with *R. solani* could be attributed to damage caused to the root tissues by *R. solani*, verified at 6 days post-inoculation under the scanning electron microscope using the same autotrophic culture system (de la Providencia et al. unpublished).

The induction of plant defence responses mediated by rhizosphere-colonising *Trichoderma* has been well documented (Hanson and Howell 2004; Harman et al. 2004; Shoresh et al. 2005; Vinale et al. 2008). However, in our experiment, we observed only the induction of *PR1* at 168 hpi and a slight induction of *PAL* at 96 hpi, in the plants inoculated by *T. harzianum* alone. This was in apparent contradiction with earlier studies in which a marked induction of *PAL* and hydroxyperoxide lyase (Yedidia et al. 2003), and *Lox*, *PAL*, ethylene receptor 1, ethylene-inducible CTR1-like protein kinase (Shoresh et al. 2005) were observed after a short time (24 hpi or 48 hpi). We hypothesise such differences to be attributed to the absence of root cell penetration and colonisation by the *T. harzianum* strain used in our experiment, verified at 6 days following contact under a scanning electron microscope, (de la Providencia et al. unpublished). Such invasion was observed in the studies of Yedidia et al. (2003) and Shoresh et al. (2005). However, in both these studies, the fungus was the strain *T. asperellum* T203 (a well-known inducer of plant defence responses), considered as *T. harzianum* previously. Different studies have shown that the addition of *Trichoderma* spp. metabolites may act as elicitors of plant resistance, or that their expression in transgenic plants may act also as elicitors and induce the synthesis of phytoalexins, pathogenesis-related (PR) proteins and other compounds (Elad et al. 2000; Dana et al. 2001). This suggested that the induction of *PR1* at 168 hpi in the potato roots inoculated with *T. harzianum* alone, could be due to the production of metabolites by *T. harzianum*.

Plants require a broad range of defence mechanisms to effectively fight invasion by microbial pathogens or attack by herbivorous insects. The signal molecules salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are involved in many plant-pathogen and plant-insect interactions (De Vos et al. 2005; Van Loon 2007). In our study, *R. solani* infection caused an increase in the expression of *PAL* gene in the roots of potato plantlets. The *PAL* gene is activated by the JA/ET signalling pathways in the context of induced plant defences and was shown to be a key regulatory enzyme in the synthesis of SA (Sticher et al. 1997; Kato et al. 2000). In addition, we demonstrated that *PR1* and *PR2* genes were induced at 48 hpi. PR1 is a member of the class III chitinase, and PR2 is a β-1,3-glucanase. Chitinase activity is induced in plants by various stimuli, including ET and SA (Narusaka et al. 1999; Salzer et al. 2000). However, β-1,3-glucanase is induced mainly by systemic acquired resistance (SAR) and SAR inducers, such as SA (Van Wees et al. 1999; Newman et al. 2002). To the contrary, we did not observe the induction of *Lox* gene (i.e. the first enzyme in the biosynthesis pathway of JA is lipoxygenase) in the same roots challenged by *R. solani*. These results were confirmed by Gao et al. (2006) for the induction of *PR1*, one glucanase and different chitinases genes, in tomato roots inoculated by *R. solani*. But the authors did not observe the induction of *PAL* as in our study. This suggested that potato defence response to *R. solani* attack was dependent on the signal molecules SA and ET but not JA.

In the presence of *T. harzianum* and *R. solani*, we observed an induction of the *Lox* gene expression at 24 hpi and *GST1*, *PAL*, *PR1*, *PR2* gene expression at 72 hpi in the roots of potato plantlets. This suggested that *T. harzianum* in the presence of *R. solani* induced the *Lox* and *GST1* genes in roots of potato plantlets. A similar observation was made by Yedidia et al. (2003)

with the hydroperoxyde lyase gene in cucumber with co-inoculation of *T. asperellum* T-203 and *Pseudomonas syringae* pv. *lachrymans*. A similar response was described with the *Atvsp* gene in rhizobacteria-induced systemic resistance (RISR) in *Arabidopsis* plants and termed potentiation (Van Wees et al. 1999). Taken together, our results confirm that the induction of plant resistance during the *Trichoderma* plant-pathogen interaction is similar to that elicited by rhizobacteria, which enhances the defence system but does not involve the production of PR-proteins. During this mechanism, the defensive capacity of the plant is enhanced through microbial stimulation or similar stresses and the defence responses primed by the BCA in the presence of the pathogen (Harman et al. 2004; Van Loon 2007; Vinale et al. 2008). Moreover, these observations suggest that, in the presence of *T. harzianum*, potato defence response to *R. solani* attack is dependent on the signal molecules JA/ET and SA.

In conclusion, we demonstrated that *T. harzianum* Rifai MUCL 29707 in the presence of *R. solani* induced defence response genes in potato plants grown in an autotrophic *in vitro* culture system. We demonstrated that the *Lox* gene, encoding the first enzyme in the biosynthesis pathway of JA, and the *GST1* gene encoding an auxin-responsive glutathione-S-transferase 1 (Hahn and Strittmatter 1994), were primed by *T. harzianum* Rifai MUCL 29707 in the presence of *R. solani*. We demonstrated that *T. harzianum* Rifai MUCL 29707 challenged *R. solani* in potato roots by decreasing the damage caused to DNA and RNA in infected roots. Both observations suggest that the signal molecules dependent on JA were activated in potato plantlets infected with *R. solani* in the presence of *T. harzianum* Rifai MUCL 29707, at the early stage of infection. Further studies designed to demonstrate whether the activation of these genes is required to protect the potato plantlets at the early stage of the interaction need to be carried out.

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