Use of GFP-tagged *Trichoderma harzianum* as a tool to study the biological control of the potato cyst nematode *Globodera pallida*

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ABSTRACT

The fungus *Trichoderma harzianum* strain ThzID1-M3 isolated in the Palouse region of the state of Idaho and transformed to express green fluorescent protein (GFP) was used as a biomarker to study the biological control processes of the potato cyst nematode *Globodera pallida*. Experiments were conducted to evaluate the effect of *T. harzianum* ThzID1-M3 on *G. pallida* infection and reproduction at 45 and 75 days after inoculation respectively; and to observe, through microscopy rhizosphere chamber, the colonization of *G. pallida* second-stage juveniles and potato rhizoplane by *T. harzianum* ThzID1-M3 10 days after inoculation. Significant reduction of *G. pallida* infection in potato roots was observed when soil was amended with *T. harzianum* ThzID1-M3. *Globodera pallida* cysts recovered from soil and *G. pallida* reproduction rate were significantly reduced by 49% and 60% respectively, when soil was amended with *T. harzianum* ThzID1-M3 compared to the non-amended soil. Microscopic observations showed that *T. harzianum* ThzID1-M3 colonized *G. pallida* second-stage juveniles and cysts, and proliferated in the rhizoplane and rhizosphere of potato. To our knowledge, this is the first report on the study of *Trichoderma* spp. marked with GFP against a potato cyst nematode. The use of GFP-tagged *T. harzianum* has the potential to monitor the biological control processes of *G. pallida* and the tools advanced in this study should facilitate the design of novel strategies to control this economically important nematode pest of potato.

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1. Introduction

The potato cyst nematode *Globodera pallida* Behrens (1975) and Stone (1972) is a globally regulated pest of potato (*Solanum tuberosum*). *Globodera pallida* is a quarantine pest in the state of Idaho, where it was first found in the United States in 2006 (Hafez et al., 2007). At high levels of infestation in a field, *G. pallida* can reduce tuber yields up to 80% (Talavera et al., 1998; Vasyutin and Yakovleva, 1998). *Globodera pallida* is characterized by the development of a globose cyst, which is the dead female body containing several hundred nematode eggs (Brodie et al., 1993). Cysts can survive in soil for many years (Perry, 1999) and the pest is usually spread over long distances by contaminated soils, tubers or by contaminated farm machinery (Evans and Stone, 1977).

Trichoderma harzianum, a soilborne filamentous fungus, has been extensively evaluated as a biocontrol agent and found effective against many soilborne plant pathogens (Bae and

Knudsen, 2000, 2006; Dandurand et al., 2000; Dandurand and Knudsen, 1993; Hadar et al., 1979; Harman et al., 2004; Knudsen et al., 1991; Knudsen and Bin, 1990; Sharon et al., 2001; Sivan et al., 1984; Weindling, 1932). Mechanisms of biocontrol used by *T. harzianum* against soilborne pathogens consist in: (1) mycoparasitism (Barak et al., 1985; Chet et al., 1981; Papavizas, 1985); (2) antibiosis and production of hydrolytic enzymes (Howell, 1998; Lorito et al., 1996); (3) competition (Papavizas, 1985); (4) root colonization (Altomare et al., 1999; Harman et al., 2004); or (5) plant growth promotion and induced resistance in host (Altomare et al., 1999; Howell et al., 2000; Kleifeld and Chet, 1992; Windham et al., 1986; Yedidia et al., 1999, 2000, 2003).

Trichoderma harzianum strain ThzID1 was obtained from Palouse silt loam soil on the University of Idaho Plant Science Farm in Moscow, Idaho (Knudsen and Bin, 1990) and has been reported to colonize sclerotia of *Sclerotinia sclerotiorum* (Knudsen et al., 1991; Bae and Knudsen, 2006; Kim and Knudsen, 2009). *Globodera pallida* reproduction rate was significantly reduced by *T. harzianum* ThzID1 during two consecutive greenhouse experiments (Dandurand and Knudsen, 2016). Bae and Knudsen (2000) transformed *T. harzianum* ThzID1 with three exogenous genes

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encoding green fluorescent protein (GFP), enabling rapid observation of its hyphae, mycelia and conidia. Both wild-type (*T. harzianum* ThzID1) and GFP-transformed (*T. harzianum* ThzID1-M3) strains colonized sclerotia of *Sclerotinia sclerotiorum* at levels above those of indigenous *Trichoderma* spp. in untreated controls, and there were no significant differences in colonization levels between wild-type and co-transformant strains; however, the presence of the GFP and GUS marker genes permitted differentiation of introduced *Trichoderma* from indigenous strains (Bae and Knudsen, 2000).

GFP is a powerful tool for monitoring bacterial and fungal activities *in situ* and does not require any substrate or additional cofactors to fluoresce (Bloembergen et al., 1997; Lorang et al., 2001; Lu et al., 2004). Orr and Knudsen (2004) demonstrated that the use of GFP-transformant of *T. harzianum*, along with epifluorescence microscopy, is a useful tool to distinguish active hyphal biomass, the form of the fungus that is functional for biological control, from inactive propagules such as conidia or chlamydospores that are enumerated by plate counts.

We hypothesized that the use of *T. harzianum* ThzID1-M3 would allow direct observation of the biocontrol processes of *G. pallida*. *Trichoderma harzianum* ThzID1-M3, as a biomarker, should reveal the mechanisms employed to reduce *G. pallida* infection and reproduction in potato. Biocontrol of *G. pallida* has been previously described using a wild-type strain (*T. harzianum* ThzID1) (Dandurand and Knudsen, 2016). This study was focused on elucidating the processes involved in the biocontrol of *G. pallida* using *T. harzianum* ThzID1-M3.

2. Materials and methods

2.1. Propagation of *G. pallida* and *T. harzianum* ThzID1-M3

Globodera pallida, obtained from an infested potato field in Shelley ID, was propagated on the susceptible potato cultivar 'Desiree' under greenhouse conditions of $18^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and 16:8-h light: dark period (Dandurand and Knudsen, 2016; Kooliyottil et al., 2016). After 16 weeks of growth, cysts were recovered from soil using the Fenwick method of flotation (Fenwick, 1940) and stored at 4°C prior experimental use. The identity of *G. pallida* was confirmed by morphological and molecular methods (Skantar et al., 2007). Cysts, with 53% hatching ability, 70% viability and $304.2 \text{ eggs cyst}^{-1}$, were surface-sterilized in a solution of 0.5% NaOCl for 5 min and rinsed thoroughly with sterile distilled water.

Ten cysts were put inside a sterile nylon mesh bags (McMaster Carr, Elmhurst, IL) with a $250 \mu\text{m}$ of mesh opening. The nylon mesh was sealed along the edges with a hand sealer (Sealer 8" F-200, Sealer sales Inc., Northridge, CA), and were placed in sterilized distilled water for hydration for 3 days before amending to soil.

Trichoderma harzianum ThzID1-M3, isolated from Palouse silt loam soil on the University of Idaho Plant Science Farm in Moscow, ID (Knudsen and Bin, 1990) and transformed to express green fluorescent protein (GFP) (Bae and Knudsen, 2000), was maintained on potato dextrose agar (PDA) for 1 week at 22°C . The identity of *T. harzianum* ThzID1-M3 was confirmed by morphological and molecular methods (Bae and Knudsen, 2000; Barnett and Hunter, 1998; Kim and Knudsen, 2008), and was observed under fluorescence microscopy (Leica DMI3000 B, Leica Microsystems, Wetzlar, Germany) for GFP activities (Fig. 1A). Oat kernels were used as growth substrate for *T. harzianum* ThzID1-M3. Two-hundred grams of oat kernels placed in a 1000-ml Erlenmeyer flask were mixed with 200 ml of distilled water and autoclaved twice at 121°C . After 24 h, the mixture was inoculated with five *T. harzianum* ThzID1-M3 fungal plugs (7-mm diameter) from PDA plate and was incubated at 22°C for 20 days prior to experimental use.

2.2. Plant material culture and growth

Potato tubers (*S. tuberosum*) cv. 'Russet Burbank', classified as certified disease free (from the Nuclear Potato Seed Program, University of Idaho), were used for experiments conducted in clay pots. Tubers were sterilized for 5 min in 0.5% NaOCl, rinsed thoroughly in sterile distilled water, dried, and left for 1 week under dark conditions to break dormancy prior to planting. For experiments conducted in microscopy rhizosphere chambers (Micro-Rocs), potato plants (*S. tuberosum*) cv. 'Russet Burbank' were grown from tissue culture plantlets in standard media (Murashige and Skoog, 1962) for 1 week prior to transplanting.

2.3. Effect of *T. harzianum* ThzID1-M3 on *G. pallida* infection and reproduction in potato

Experiments were conducted using Prosser fine sandy loam soil, which was air-dried and sieved through a 5-mm mesh. A 2:1 sand: soil mixture (56% sand, 35% silt, 8% clay, pH 7.0) was autoclaved twice for 90 min at 121°C prior to experimental use. A 15-cm diameter size Terra Cotta clay pot (The Home Depot, Atlanta,

Fig. 1. (A). *Trichoderma harzianum* ThzID1-M3 hyphae expressing green fluorescent protein (GFP) and (B). *Globodera pallida* second-stage juvenile (J2) stained with PKH26 Red Fluorescent Cell Linker Kits (Sigma-Aldrich®, St-Louis, Mo) observed under inverted fluorescence microscopy (Leica DMI3000 B, Leica Microsystem, Wetzlar, Germany).

GA) was used for experiments, each clay pot contained 1.5 kg soil mix. Two experiments at 45 and 75 days were conducted to evaluate the effect of *T. harzianum* ThzID1-M3 on *G. pallida* infection and reproduction in potato roots. Proliferation of *T. harzianum* ThzID1-M3 in cysts, roots and soil, was also evaluated. There were two treatments for each time period: (1) the non-amended control consisted of *G. pallida* cysts applied to non-amended soil; and (2) *G. pallida* cysts applied to soil amended with *T. harzianum* ThzID1-M3. Treatments included five replicates in a completely randomized design. Five grams of oat kernels colonized by *T. harzianum* ThzID1-M3 fungal inoculum (10^6 conidia g^{-1} oat kernel) was mixed into 1.5 kg of soil and distributed to a clay pot. A cyst bag was placed at 5-cm below the soil line and covered with 2-cm soil before placing one potato tuber and covering with soil. *Globodera pallida* was applied at an initial rate (Pi) of 2.03 egg g^{-1} soil. Pots were maintained at $18^\circ C \pm 2^\circ C$, 60% relative humidity, and 16:8-h light: dark period under greenhouse conditions. Pots were watered twice daily in the amount of 100 ml of water, and fertilized three times a week using Jack's classic garden fertilizer 20-20-20 (JR Peters Inc., Allentown, PA) applied at a rate of 0.5 g liter $^{-1}$ water.

2.4. Microscopic observation of *T. harzianum* ThzID1-M3 biocontrol activity

One-week-old tissue culture potato plantlets were transplanted into Micro-Rocs chambers (Advanced Science Tools, LLC, Pullman, WA), and were inserted between the cover glass and the filter paper, the adjacent slot was filled with soil consisted in 85% peat and organic materials mixture (Sunshine Mix, SunGro®, Vancouver, Canada). Potato plantlets were left to grow for 2 weeks under greenhouse conditions at $18^\circ C \pm 2^\circ C$, 16:8 light: dark conditions, watered once daily with 25 ml of water and fertilized three times a week at a rate of 0.5 g l $^{-1}$ of water using Jack's classic garden fertilizer 20-20-20 (JR Peters Inc., Allentown, PA). To obtain second stage hatched juveniles, 20 cysts were placed inside a hatching chamber, made of 30- μm nylon mesh, attached to a 2-cm diameter plastic pipe and placed inside a corning centrifuge tube (Sigma-Aldrich®, St-Louis, Mo). Two milliliters of fresh sterilized water, 1 ml of potato root diffusate (PRD), 1.5 mg ml $^{-1}$ of gentamicin and 0.05 mg ml $^{-1}$ of nystatin were added into the corning centrifuge tube, followed by incubation at $22^\circ C$ for 2 weeks. Hatched juveniles (J2s) were cleaned of eggs shells and other debris by using sucrose gradient centrifugation (30–60%) (Kooliyottill et al., 2016). Hatched J2s were collected and incubated overnight in a solution containing 100 μg ml $^{-1}$ of ampicillin and streptomycin (w/v) on a shaker at room temperature. Hatched J2s were further treated with benzethonium chloride (0.125 g ml $^{-1}$) (Sigma-Aldrich®, St-Louis, Mo) by continuous shaking at room temperature for 20 min on a rocker (Standard Analog Rocker, VWR, Radnor, PA); washed 8 times by centrifugation (4000 rpm for 10 min) and re-suspended in sterile distilled water (modified from Upadhyay et al., 2013). Collected J2s were stained using PKH26 Red Fluorescent Cell Linker Kits, 4×10^{-6} M (Sigma-Aldrich®, St-Louis, Mo) according to manufacturer's protocol, and incubated for 10 min at room temperature in dark with intermediate shaking (3 times) for 15 s and washed 5 times in sterile distilled water. The stained J2s (Fig. 1B) were collected by centrifugation at 4000 rpm for 10 min and transferred to 0.01% sterile agarose (modified from Sobczak et al., 2005), and kept at $-4^\circ C$ until use. Potato plantlets were inoculated with 1-week old *T. harzianum* ThzID1-M3 on PDA discs according to the treatment. Three 7-mm diameter PDA discs were crushed and mixed with sterile distilled water and pipetted over potato root surfaces through the Micro-Rocs cover glass at a rate 2 ml containing 3.2×10^5 conidia ml $^{-1}$. After 2 weeks, potato plantlets were inoculated with 100 μl of agarose solution

containing 2 J2s μl^{-1} (Kooliyottill et al., 2016). There were two treatments, as follows: (1) *G. pallida* J2s only applied to non-amended soil; and (2) *G. pallida* J2s applied to soil amended with the biocontrol *T. harzianum* ThzID1-M3. Treatments were arranged in a completely randomized design, replicated five times and the experiment was repeated. Inoculated plantlets were maintained under greenhouse conditions at $18^\circ C \pm 2^\circ C$, 16:8 light: dark conditions for 10 days, and were placed under inverted fluorescence microscopy (Leica DMI3000 B, Leica Microsystems, Wetzlar, Germany) at day 5 and 7 to observe the interactions of *T. harzianum* ThzID1-M3 with: (1) the potato rhizoplane; (2) *G. pallida* J2s; and (3) roots infected by *G. pallida* J2s.

2.5. Nematode infection assessment

Acid fuchsin (Sigma-Aldrich®, St-Louis, Mo), an acidic dye, was used to stain *G. pallida* juveniles in potato roots in order to determine the level of root infections (Byrd et al., 1983) using a dissecting microscope (Leica M80, Leica Microsystems, Wetzlar, Germany). After counting, roots were dried and weighed in a scale (Thermo Fisher Scientific, Waltham, MA) and juvenile nematode density g $^{-1}$ root was determined.

2.6. Nematode reproduction assessment

Cysts were extracted from soil using the Fenwick flotation method (Fenwick, 1940). Extracted cysts were crushed in 100 μl of sterile distilled water and eggs were counted under a dissecting microscope (Leica M80, Leica Microsystems, Wetzlar, Germany). Nematode reproductive factor (Rf) was calculated based on the ratio of the final egg population density (Pf) over the initial egg population density (Pi) per gram of soil.

2.7. *Trichoderma harzianum* ThzID1-M3 proliferation assessment

To assess the presence of *T. harzianum* ThzID1-M3 in cyst, potato rhizoplane and rhizosphere, molecular characterization of *T. harzianum* ThzID1-M3 was performed and consisted in DNA extractions followed by PCR analysis, culture media plating technique, and fluorescent microscopy observations of fungal colonization in *G. pallida* second-stage juveniles, cysts and potato rhizoplane.

DNA materials were extracted from root segments and cyst samples by using the DNeasy plant DNA extraction kit (Qiagen Inc., Venlo, Netherlands) according to manufacturer's protocol. DNA was extracted from soil samples using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA) according to manufacturer's protocol. DNA materials extracted from root, cyst, and soil samples was processed using EasyStart PCR Mix-in-a-Tube (Thermo Fisher Scientific Inc., Waltham, MA) according to manufacturer protocol. DNA samples were amplified through a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). Primers for *Trichoderma* spp. were designed from GenBank (National Center for Biotechnology Information [NCBI], Bethesda, MD) based on the conserved regions of the consensus sequence (Kim and Knudsen, 2008). Forward and Reverse primers were obtained from Sigma-Aldrich® (Sigma-Aldrich®, St-Louis, Mo). Forward primer sequence (TGP4-F) was as follows: 5'-CTCCCAACCCAATGTGA AC-3'; Reverse primer sequence (TGP4-R) was as follows: 5'-GCGAGTGTGCAAAAT ACTG-3'; and the amplicon size based on the consensus sequence was 466 bp.

To further assess the presence of *T. harzianum* ThzID1-M3, roots were sterilized in 0.5% NaOCl, washed thoroughly in sterile distilled water and cut into 1-cm segment. Five 1-cm root segments were plated in *Trichoderma* selective media (TSM) (Elad et al., 1981), replicated four times, and were incubated for 1 week

at 22 °C. *Globodera pallida* cysts, from recovered cyst bags, were sterilized in 0.5% NaOCl and washed thoroughly in sterile distilled water. Ten cysts were placed in a 1.5 ml microtube and were crushed in 500 µl sterile distilled water. Serial dilution method was used to quantify the presence of *T. harzianum* ThzID1-M3 at 1:10³, 1:10⁴, 1:10⁵ and 1:10⁶-fold dilution. Diluted solutions were spread in TSM and were incubated for 1 week at 22 °C. For rhizosphere assay, ten grams of soil samples were mixed in 90 ml of sterile distilled water to yield a 10-fold dilution. One ml of the 10:1 dilution was added to 9 ml of sterile distilled water to yield 1:10³ to 1:10⁶-fold dilution, were spread in TSM and incubated for 1 week at 22 °C. Besides GFP activities, the production of conidia from effused conidiophores, or from conidiophores aggregated into fascicles or pustules was used as a morphological characteristic of *Trichoderma* spp. for identification in TSM-grown culture (Barnett and Hunter, 1998).

To visualize *T. harzianum* ThzID1-M3 proliferation in *G. pallida* second-stage juveniles, cysts and potato rhizoplane, an inverted fluorescence microscope (Leica DMI3000 B, Leica Microsystems, Wetzlar, Germany), equipped with a Leica DFC340 FX digital fluorescence camera, a Leica AF6000E fluorescence software, a rhodamine and a GFP filters, was used to observe *T. harzianum* ThzID1-M3 hyphae and mycelia.

2.8. Statistical analysis

All data analyses were performed using SAS package 9.4 (SAS Institute Inc., Cary, N.C.). Analysis of variance using generalized linear mixed effects procedure (Proc Glimmix) was performed to analyze root weight, *G. pallida* infection and reproduction in potato roots with significant differences occurring at level of $P \leq 0.05$. Means were separated by the Tukey-Kramer adjustment.

3. Results

3.1. Nematode infection

At 45 days post inoculation (DPI), average *G. pallida* infection in non-amended soil was 2.49 fourth-stage juveniles (J4) g⁻¹ root, while in amended soil with *T. harzianum* ThzID1-M3 the infection level was 0.40 J4 g⁻¹ root, resulting in significant reduction in infection rate by 84% ($P \leq 0.05$) (Table 1). *Globodera pallida* juveniles (second [J2], and third [J3] stage) and adult females were observed in the non-amended soil treatment, however they were not detected in soil amended with *T. harzianum* ThzID1-M3 (Table 1). Despite this decrease, no significant difference was observed in fresh potato root weight ($P \geq 0.05$) (Table 1). In the infection assay conducted in Micro-Rocs chambers, no interaction between treatment and experiment effects were detected, therefore experimental data from both experiments were combined. Average *G. pallida* J2s in non-amended control was 751.30 J2s g⁻¹ root, while in amended treatment with *T. harzianum* ThzID1-M3 the average of *G. pallida* J2s was 244.60 J2s g⁻¹ root, resulting in significant reduction in infection rate by 67% ($P \leq 0.05$) (Table 2). Average potato dry root weight in non-amended soil was

Table 2

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* second-stage juveniles (J2) root infection and root weight using Micro-Rocs chambers.

Treatment	Root weight ^a (g)	<i>G. pallida</i> J2 g ⁻¹ root
Non-amended control	0.13	751.30
<i>T. harzianum</i> ThzID1-M3	0.21	244.60

Means of root weight and *G. pallida* J2 g⁻¹ root are significant ($P \leq 0.05$) according to Tukey-Kramer adjustment test of the Glimmix procedure.

^a Dry root weight.

0.13 g, while in amended soil with *T. harzianum* ThzID1-M3 dry root weight was 0.21 g, resulting in significant increase in potato dry root weight by 62% ($P \leq 0.05$) (Table 2).

3.2. Nematode reproduction

At 75 days post inoculation (DPI), average final *G. pallida* egg density (Pf) was 6.40 eggs g⁻¹ soil and reproduction rate (Pf/Pi) was 3.15 in non-amended soil. In soil amended with *T. harzianum* ThzID1-M3 Pf was 2.56 eggs g⁻¹ soil and Pf/Pi was 1.26, resulting in significant reduction in nematode reproduction by 60% ($P \leq 0.05$) (Table 3). Average number *G. pallida* cysts in non-amended soil was 55 cysts root⁻¹ ball, while in amended soil with *T. harzianum* ThzID1-M3 the average was 28 cysts root⁻¹ ball, resulting in significant reduction of *G. pallida* cysts by 49% ($P \leq 0.05$) (Table 3). No significant difference was observed in fresh potato root weight ($P \geq 0.05$) (Table 3) whether or not soil was amended with *T. harzianum* ThzID1-M3.

3.3. *Trichoderma harzianum* ThzID1-M3 proliferation

Electrophoresis analysis of PCR products revealed the presence of *T. harzianum* ThzID1-M3, visible by the occurrence of white band in agarose gel, in: (1) *G. pallida* cyst, where non-amended treatment was compared to amendment with *T. harzianum* ThzID1-M3 (Fig. 2A); (2) potato rhizoplane, where non-amended treatment was compared to amendment with *T. harzianum* ThzID1-M3 (Fig. 2B); and (3) potato rhizosphere, where amended treatment with *T. harzianum* ThzID1-M3 is shown (Fig. 2C). Through plate assay, *G. pallida* cysts were found to be heavily colonized by *T. harzianum* ThzID1-M3 at an average of 1.41×10^5 conidia forming unit per cyst. Fluorescent microscopic observations of *G. pallida* cyst surfaces revealed heavy presence of *T. harzianum* ThzID1-M3 hyphae and mycelia, indicating signs of fungal parasitism (Fig. 3A, B). However, despite the heavy colonization of cysts, no colonized eggs were observed in crushed cysts. Examinations of plate assay revealed that *T. harzianum* ThzID1-M3 colonized 10% of the potato rhizoplane. Fluorescent microscopic observations using Micro-Rocs chambers revealed that *T. harzianum* ThzID1-M3 was able to extensively colonize the potato rhizoplane and establish hyphal growth inside the cortex of the root system (Fig. 3C). *Trichoderma harzianum* ThzID1-M3 was observed to colonize and kill *G. pallida* juveniles (J2) prior to root infection (Fig. 3D). *Globodera pallida* juveniles (J2) were observed inside infected potato root in non-amended treatment (Fig. 3E) but

Table 1

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* juveniles root infection and root weight at 45 days.

Treatment	Root weight ^a (g)	<i>G. pallida</i> J2 g ⁻¹ root	<i>G. pallida</i> J3 g ⁻¹ root	<i>G. pallida</i> J4 g ⁻¹ root	<i>G. pallida</i> adult female g ⁻¹ root
Non-amended control	57.80	0.02	0.20	2.49	0.05
<i>T. harzianum</i> ThzID1-M3	52.54	0	0	0.40	0

Means of root weight are not significant ($P \geq 0.05$), however means of *G. pallida* J4 g⁻¹ root are significant ($P \leq 0.05$) according to Tukey-Kramer adjustment test of the Glimmix procedure.

^a Fresh root weight.

Table 3

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* cyst density, final egg population density (Pf), reproduction factor (Rf) as a ratio of Pf over initial egg population density (Pi) (Pi = 2.03), and root weight at 75 days.

Treatment	Root weight ^a (g)	Cyst root ⁻¹ ball	Cyst g ⁻¹ soil	Pf g ⁻¹ soil	Rf
Non-amended control	59	55	0.04	6.40	3.15
<i>T. harzianum</i> ThzID1-M3	63	28	0.02	2.56	1.26

Means of root weight are not significant ($P \geq 0.05$), however means of cyst root⁻¹ ball, cyst g⁻¹ soil, Pf g⁻¹ soil and Rf are significant ($P \leq 0.05$) according to Tukey-Kramer adjustment test of the Glimmix procedure.

^a Fresh root weight.

not in roots colonized with *T. harzianum* ThzID1-M3. Through plate assay, potato rhizosphere was found to be heavily colonized by *T. harzianum* ThzID1-M3 at a rate of 10^5 CFU g⁻¹ soil.

4. Discussion

4.1. Effect of *T. harzianum* ThzID1-M3 on *G. pallida* infection and reproduction

Trichoderma harzianum ThzID1-M3 significantly reduced *G. pallida* infection and reproduction in potato roots. Similarly, amendment of soil with *T. harzianum* ThzID1 (non-transformed strain) reduced *G. pallida* reproduction rate by 43% and 48% during two consecutive potato crop cycles in greenhouse experiments (Dandurand and Knudsen, 2016). However, mechanisms behind such reduction in *G. pallida* reproduction rate were not fully understood. The use of *T. harzianum* ThzID1-M3 as a biomarker tool allows us to understand the interaction between the fungus and its target in order to define possible mechanisms behind the biocontrol of *G. pallida* in potato. Enabled with a constitutive GFP tag, which does not alter the biocontrol efficacy of the fungus, we have been able to track the physical presence of *T. harzianum* mycelia in *G. pallida* cysts, second-stage juveniles (J2), and in the rhizoplane and rhizosphere of potato. *Trichoderma harzianum* ThzID1-M3 was able to colonize both *G. pallida* cysts and second-stage juveniles (J2), however no egg colonization was observed. Colonization process was characterized by hyphal growth of *T. harzianum* toward *G. pallida* second-stage juveniles (J2), followed by surface contact and penetration of *T. harzianum* hyphae inside the nematode body resulting in larval death. Similarly, *T. harzianum* colonization process has been previously described in *Globodera rostochiensis* (Saifullah and Thomas, 1996; Trifonova, 2010), in

Meloidogyne javanica (Sahebani and Hadavi, 2008; Saifullah and Khan 2014; Sharon et al., 2001) and in *M. incognita* (Goswami et al., 2008). Other fungal species showed similar colonization process when applied against other plant-parasitic nematodes (Kiewnick and Sikora, 2006; Tobin et al., 2008; Zhang et al., 2014), and by expressing GFP in the fungus *Clonostachys rosea*, Zhang et al. (2008) showed the antagonist process of the saprophytic nematode *Panagrellus redivivus* started from the adherence of conidia to nematode cuticle for germination, followed by the penetration of germ tubes into the nematode body and subsequent degradation of the nematodes.

Although eggs were not observed to be colonized by *T. harzianum* ThzID1-M3, colonization of cysts could allow for close proximity of the biocontrol agent to the hatched juveniles, and lead to colonization of the infectious stage of the nematode prior to penetration into roots, as observed in the Micro-Rocs experiment. Potential mechanisms used by *Trichoderma* spp. to attack its prey that could be at play in the Micro-Rocs experiment include: (1) a positive chemotrophic growth towards its host; (2) direct contact with the prey; (3) formation of an appressorium-like penetration structures; and (4) production of tissue degrading hydrolytic enzymes that allows hyphal penetration and subsequent death of the prey, and its consumption by *Trichoderma* (Herrera-Estrella and Chet, 2004).

4.2. Effect of *T. harzianum* ThzID1-M3 in the potato rhizoplane and rhizosphere

Trichoderma harzianum ThzID1-M3 was able to colonize potato rhizoplane and rhizosphere. Under fluorescence microscopy, *T. harzianum* ThzID1-M3 was observed colonizing potato root surface and penetrating the root cortex. Rhizoplane colonization by *T.*

Fig. 2. Conventional PCR results of the *Trichoderma* genus-specific primer set (TGP4) on genomic DNA samples. Lane 1, low mass marker (Invitrogen); lane 2, positive control of *T. harzianum* ThzID1-M3; from lane 3 to 8 the presence of *T. harzianum* ThzID1-M3 represented by white band in amended treatment. (A) Cyst PCR result; (B) Root PCR result; and (C) Soil PCR result.

Fig. 3. (A) and (B). *Globodera pallida* cysts colonized by *Trichoderma harzianum* ThzID1-M3 observed under inverted fluorescence microscopy using Micro-Rocs chambers; (C). Section of a potato root colonized by *T. harzianum* ThzID1-M3 observed under inverted fluorescence microscopy using Micro-Rocs chambers, hyphal growth was detected inside and outside root cortex; (D). *Trichoderma harzianum* ThzID1-M3 hyphal attacking *G. pallida* second stage juvenile (J2) prior root infection; and (E). *Globodera pallida* second-stage juveniles (J2) inside potato root observed under inverted fluorescence microscopy using Micro-Rocs chambers.

harzianum ThzID1-M3 contributes to the protection of the infection court and also favors *T. harzianum* ThzID1-M3 growth and proliferation in the rhizosphere, and the ability to colonize plant roots has often been stressed as an important requirement for *Trichoderma* spp. to act as biocontrol agents (Harman et al., 2004; Sivan and Chet, 1989). Previous studies described similar rhizoplane and rhizosphere colonization by *T. harzianum* (Ahmad and Baker, 1987; Chao et al., 1986; Orr and Knudsen, 2004). Lu et al. (2004) showed the GFP-tagged *Trichoderma* was able to colonize the outer layer of the roots of cucumber seedlings, as well as inoculated soil. Harman (2006) showed that *Trichoderma* spp. can colonize the root epidermis and outer cortical layers and increased plant growth and nutrient uptake, as we observed in colonization assays using Micro-Rocs chambers. Other studies suggest potential induction of plant systemic resistance triggered by the presence of *Trichoderma* spp. in plant roots leading to a broad spectrum protection against plant pathogens (Yedidia et al., 1999, 2000, 2003).

5. Conclusions

Trichoderma harzianum ThzID1-M3 was found to be useful to monitor the biocontrol interactions between *T. harzianum* and *G. pallida*. These are the first findings on the biocontrol process of *T. harzianum* marked with GFP on *G. pallida*. *Trichoderma harzianum* was able to reduce *G. pallida* infection and reproduction in potato roots through hyphal colonization of cysts and juveniles, although no colonization of eggs was observed. *Trichoderma harzianum* was also able to establish hyphal colonization in the potato rhizoplane

and rhizosphere, possibly providing long term protection of the infection court. The tools advanced in this study should help develop an effective method to investigate the ecological properties of *T. harzianum* against plant pathogenic microorganisms.

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