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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.

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 (Bloemberg 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) (Dandur- and 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 °C ± 2 °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 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 μm 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,

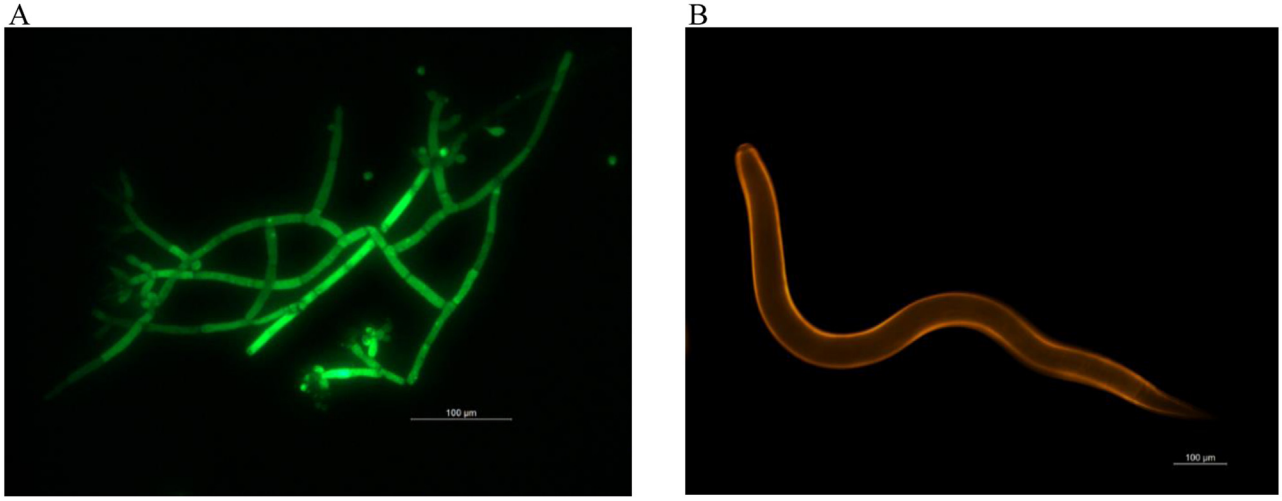

Fig. 1. (A). *Trichoderma harzianum* ThzID1-M3 hyphae expressing green fluorescent protein (GFP) and (B). *Globodera pallida* second-stage juvenile (J2) stained with PKH26 Red Fluorescent Cell Linker Kits (Sigma-Aldrich®, St-Louis, Mo) observed under inverted fluorescence microscopy (Leica DMI3000 B,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