BIOLOGICAL CONTROL OF *FUSARIUM SOLANI, RHIZOCTONIA SOLANI* AND THE PALE CYST NEMATODE *GLOBODERA PALLIDA* WITH *TRICHODERMA HARZIANUM* THZID1

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

The fungi *Trichoderma* spp. have been extensively studied and received considerable attention as biological control agents against many plant pathogenic fungi and nematodes. The goal of our study was to evaluate the ability of *Trichoderma harzianum* to reduce diseases caused by *Fusarium solani*, *Rhizoctonia solani*, and the pale cyst nematode *Globodera pallida* in potato. In addition, proliferation of *T. harzianum* mycelia through soil media and potato root system have been determined. The introduced fungus *T. harzianum* ThzID1 significantly reduced disease incidence in potato roots caused by *R. solani*, however disease reduction caused by *F. solani* was not significant. The added ThzID1-M3 significantly reduced *G. pallida* cysts formation in soil, and also reduced *G. pallida* juvenile nematodes in potato roots. *Trichoderma harzianum* ThzID1-M3 mycelia significantly proliferated in soil media, over *G. pallida* cyst surface, and potato roots. In conclusion, *T. harzianum* ThzID1-M3 showed promise as a potential biocontrol agent.

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CHAPTER 1

LITERATURE REVIEW

Rationale for the use of biological control agents in agriculture

Plant diseases as a threat to food security

About 795 million people are undernourished worldwide, down 167 million over the last decade and 216 million less than in 1990-1992 (FAO, 2015). Although, socioeconomic and political factors are often pointed to be the main causes of food insecurity, plant diseases aggravate and amplify the food crisis problem, and when left unresolved, can provoke major famine for a country and disrupt global food trade. Worldwide losses caused by plant diseases were estimated at US$ 300 billion from 1988 to 1990 (Fletcher et al., 2006). It is conservatively estimated that diseases, insects and weeds together annually interfere with the production of, or destroy between 31 and 42 % of all crops produced worldwide (Agrios, 2005). Of the average 36.5 % of total losses, 14.1% are caused by diseases, 10.2% by insects, and 12.2% by weeds (Agrios, 2005). According to Sasser and Freckman (1987), crop losses from nematodes range between 8 to 20% on major crops around the world and can cause considerable crop damage with annual losses estimated at US$ 87 billion worldwide. The total global potential loss due to pests varied from about 50% in wheat, 80% in cotton production, 26-29% for soybean, 31% for maize, 37% for rice and 40% for potatoes (Oerke, 2006). Overall, weeds produced the highest potential loss (34%), with animal pests and pathogens being less important (losses of 18 and 16%) (Oerke, 2006). Pests reduce crop productivity in various ways; according to Boote et al. (1983) pests can be classified by their impacts into the categories stand reducers (damping-off pathogens), photosynthetic rate reducers (fungi, bacteria, viruses), leaf senescence accelerators (pathogens), light stealers (weeds, some pathogens), assimilate sappers (nematodes, pathogens, sucking arthropods) and tissue consumers (chewing animals, necrotrophic pathogens). About 65% of U.S. crop losses are due to nonindigenous pathogens, amounting to an estimated cost of US$ 137 billion annually (Pimentel et al., 2000).

Biological control as an ecological approach for sustainable agricultural production

Agriculture has the capability to feed 8-10 billion people while substantially decreasing the proportion of the population who go hungry (Waggoner, 1995; Kates, 1996; Sen,

1981; Plucknett, 1993), but there is little consensus on how this can be achieved by sustainable means. As agricultural production intensifies, environmental concerns over the use of pesticides in general are increasing which have led to reassessment of the chemicals currently on the market, often resulting in their withdrawal (Dingham, 1993; Thomas, 1996).

Agricultural intensification has severe impacts on farmland biodiversity (Geiger et al., 2010; Benton et al., 2003). Delivery of ecosystem services are constrained by the declines in biodiversity (Hooper et al., 2005). The services considered most at risk from agricultural intensification are: (1) biological pest control (Tscharntke et al., 2005), (2) crop pollination (Biesmeijer et al., 2006), and (3) soil fertility (Brussaard et al., 1997). The soil biota considered at present to be most at risk are macrofaunal shredders of organic matter, bioturbators of soil, specialized bacteria like nitrifiers and nitrogen fixers, and fungi-forming mycorrhizas (Brussaard et al., 1997). Agricultural intensification in Europe have had deleterious and measurable effects on bird populations, for example increase in cereal yield alone explained over 30% of the variation in bird population decline (Donald et al., 2001).

In plant pathology, the term ‘biological control’ or its abbreviated synonym ‘biocontrol’ refers to the use of microbial antagonists to suppress diseases caused by plant pathogens. Biocontrol agents are often antagonistic to the activities of plant pathogens, and can be described as follows: (1) direct antagonism, characterized by hyperparasitism or predation; (2) mixed-path antagonism, characterized by the production of antibiotics, lytic enzymes and secondary metabolites; (3) indirect antagonism, characterized by physical competition for resources and niche occupation, or (4) induction of host resistance. The first historical record of biological control dates back to around AD 300 when predatory ants were used to control pests in citrus orchards (van Lenteren, 2005; van Lenteren and Godfray, 2005).

Advantages of biological control, as outlined by Bale et al. (2008), in comparison with pesticides include: (1) actively searches for their prey and increased pest control over time, (2) unlikely development of resistance to a control agent, (3) biological control agent attacks only specific target species, and (4) pest control is not limited to the area within which the biocontrol agent is applied. The main limitation of biological control is that it is

slower to suppress pest populations than most pesticides as parasitized organisms may take several days to die; and also, biocontrol agents require a period of time to establish an economic level of pest suppression (Bale et al., 2008). To be successful in the long-term, biological control agents must be studied and evaluated for understanding their interaction with the pathogen or nematode target, the crop and the soil ecosystem (Deacon 1991, 1994; Kerry, 1995; Kerry and Hominick, 1999; Whipps, 1997).

Successes of biological control products are dominated by bacterial and fungal strains that are easy to grow in culture and are proven to be effective against a range of pathogens (Whipps and Davies, 2000). Many of the biological control products are not actually registered and marketed as biological control agents in order to avoid the associated costs to obtain data on toxicology, efficacy, environmental impact and fate (Whipps and Davies, 2000).

Nevertheless, success stories of biological control abound in literature and the number of biological control products in the market is considerable. The biocontrol of cotton-cushion scale *Icerya purchasi* on citrus crops in California in the 1880s by an imported ladybird (the vedalia beetle *Rodolia cardinalis*) and dipteran parasitoid (*Cryptochaetum iceryae*) is widely regarded as the first major success of biological control (DeBach, 1964). There are over 80 products near to or on the market that have biological control activity against plant pathogens (Whipps and Davies, 2000). Examples of biological control agents include *Bacillus subtilis* against *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium* spp.; *Pseudomonas cepacia* against *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*; *Trichoderma* spp. against *Pythium*, *Sclerotinia* and *Verticillium* spp. (Whipps and Davies, 2000).

Linford (1937) began research into the use of biological control agents against plant parasitic nematodes, and since then a plethora of biological control agents have been used or evaluated against plant parasitic nematodes. Some examples of biocontrol agents in the group of bacteria include: *Agrobacterium radiobacter* against *Globodera pallida* (Hackenberg et al., 1999); *Bacillus subtilis* against *Heterodera cajani* (Siddiqui and Mahmood, 1995); *Serratia marescens* against *Globodera rostochiensis* (Cronin et al., 1997b); *Pseudomonas fluorescens* against *Globodera rostochiensis* (Cronin et al., 1997a). Some examples of biological control agents in the group of fungi include: *Acremonium* sp.

against *Globodera*/*Heterodera* spp. (Crump, 1998); *Arthrobotrys* spp. against *Meloidogyne* spp. (Jaffee et al., 1998); *Cylindrocarpon destructans* against *Meloidogyne javanica* and *Globodera* sp. (Freitas et al., 1995; Crump and Flynn, 1995); *Fusarium oxysporum* against *Meloidogyne incognita* (Hallman and Sikora, 1996); *Trichoderma harzianum* against *Heterodera cajani* (Siddiqui and Mahmood, 1996); *Verticillium chlamydosporium* against *Globodera* spp. (Crump, 1998), *Heterodera schachtii* (Jalali et al., 1998), *Meloidogyne* spp. (Kerry, 1995). The importance of fungi in the decline of cereal cyst nematodes below the economic threshold has been clearly demonstrated (Kerry, 1975, 1984; Kerry et al., 1982; Kerry and Crump, 1998).

Potato production in the state of Idaho and its challenges

Idaho is the nation's largest producer, packer and processor of potatoes. Idaho has been the number one potato-producing state for the past 50 years, producing about 29% of the U.S. potato crop, 40% of U.S. processed potato products and one-third of the nation's fresh potato shipments (USDA/NASS, 2014). The final value of Idaho's 2013 potato crop was US$ 1.02 billion with an estimated production of 13.1 billion pounds and the value of production of U.S. potato was estimated to US$ 3.9 billion with an estimated production of 44.7 billion pounds of potatoes (USDA/NASS, 2014).

Tuber and root diseases are serious threats for potato production worldwide. Fusarium dry rot of seed tubers can cause crop losses up to 25%, while more than 60% of tubers can be infected in storage (Wharton et al., 2007). Extensive yield losses, from 10% to 30% on marketable size tubers as a result of Rhizoctonia disease have been reported (Carling et al., 1989; Read et al., 1989). Hodda and Cook (2009), from a recent detection of *Globodera* spp. in Australia, estimated cumulative losses to Australian agriculture over 20 years may exceed $ 370 million without action to prevent spreading and entry to new areas. Following the withdrawals of various pesticides from market, development of pesticides resistance and ecological impacts of pesticides usage in agriculture, biological control represents a key component of a system approach to integrated pest management to counteract pesticide resistance, withdrawal of chemicals and minimize the usage of pesticides.

The plant pathogenic fungus *Fusarium solani*

The genus *Fusarium*

The genus *Fusarium* was introduced by Link (1809) with the primary character being the presence of the distinctive canoe-shaped conidia. Members of this genus can cause diseases in plants, humans, and domesticated animals (Boonpasart et al., 2002; Goldschmied et al., 1993; Krcmery et al., 1997). Many *Fusarium* spp. have both an anamorph (asexual stage) and teleomorph (sexual stage). The International Code of Botanical Nomenclature (Article 59.1) indicates that the correct name in such settings is the teleomorph name, although reference to an anamorphic name alone is permissible under some conditions (Article 59.5). But sexual stages associated with *Fusarium* spp. are not commonly observed under field conditions, thus most scientists and plant pathologists refer solely to the anamorph stage.

Fusarium solani (Martius) Snyder and Hansen (1940), sexual stage *Haemanectria haematococca* (Berkeley and Broome) Samuels and Nirenberg, is regularly isolated from soils in a variety of environments, high frequency from soils in rainforest habitats (Summerell et al., 1993) and occupies a wide host range. Cultures of *Fusarium solani* in potato dextrose agar (PDA) usually are white to cream and macroconidia are relatively wide, straight to slightly curved with 3 to 7 septates having rounded ends. *Fusarium solani* occurs in the form of chlamydospores in naturally infested soil (Booth, 1971). *Fusarium solani* is often found associated with wounds or with localized infections caused by species of *Pythium*, *Phytophthora*, *Botryosphaeria*, *Macrophomina*, *Rhizoctonia* or by other species of *Fusarium*. Futhermore, *F. solani* often attacks hosts weakened by unfavorable conditions or following nematode damage or virus infections, and is also associated with the damping-off of the seedlings including tree seedlings (Booth, 1971). Some economically important plants with significant diseases caused by *F. solani* include avocado (Darvas et al., 1987), beans (Silbernagel et al., 1990), citrus (Dandurand, 1990; van Rensburg, 2001), corn (Naik et al., 1982), cowpea (Sajise, 1988), orchids (Benyon, 1996), peas (Grunwald et al., 2003; Kraft et al., 1983; Kraft et al., 1989), peppers (Fletcher, 1994) and potato (Secor and Gudmestad, 1999). *Fusarium solani* has a cyanide hydratase enzyme that may be useful for bioremediation of sites contaminated with

cyanide (Dumestre et al., 1997; Barclay et al., 1998; Barclay et al., 2002). Not all *F. solani* isolates are plant pathogens and some of them live as saprophytes in soil.

Fusarium dry rot, caused by *F. solani*, affects potatoes worldwide. This disease affects tubers in storage and planted seed tubers. Seed tubers become infected through wounds during storage or preparation for planting. *Fusarium* spp. can survive for several years in soil. Fusarium dry rot of seed tubers can reduce crop establishment by killing developing potato sprouts, and crop losses can be up to 25%, with more than 60% of tubers possibly infected in storage (Wharton et al., 2007). Symptoms associated with Fusarium dry rot are dark depressions on the surface of the tuber, large lesions of dead tissue spread in concentric rings and the presence of mycelia and conidia around necrotic tissues. Fusarium dry rot may lead to secondary infections by soft rot bacteria when the tubers are stored under conditions of high relative humidity (Wharton et al., 2007).

Fusarium spp. metabolites

Plant pathogenic *Fusarium* spp. produce a wide range of secondary metabolites during the infection stage in host plants, disrupting host defense responses and suppressing plant growth (Glenn, 2007). Plant responses to *Fusarium* metabolites or phytotoxins can disrupt physiological process such as: (1) inhibition of root or shoot growth; (2) inhibition of seed germination; (3) changes in leaf color such as chlorosis; (4) cell death; and (5) suppression or activation of defense responses (Nishiuchi, 2014). Two major *Fusarium* metabolites, fumonisins and trichothecenes, induce apoptosis-like programmed cell death and can contribute to the virulence of the pathogen on some plants (Nishiuchi, 2014).

Cultural control of Fusarium dry rot

Wharton et al. (2007) outlined some of the following procedures to prevent dry rot: (1) plant only certified seed; (2) store potato seeds at 40ºF to 42ºF and 85 to 90% relative humidity, and kept ventilated; (3) minimize potato seeds injury; (4) disinfect seed cutting and handling equipment; (5) avoid potato seed storage near potential source of inoculum.

Chemical control of Fusarium dry rot

Several chemical products are currently available and offer broad-spectrum control for Fusarium dry rot, and include: (1) Tops MZ (thiophanate-methyl as active ingredient); and (2) Maxim MZ (fluodioxinil as active ingredient) (Wharton et al., 2007).

Biocontrol of *Fusarium* spp. by *Trichoderma* spp.

Trichoderma harzianum controlled Fusarium wilt in cotton and muskmelon when applied in both naturally or artificially infested alluvial Vertisol and sandy-loam soils, respectively (Sivan and Chet, 1986). *Trichoderma harzianum*, applied as a seed coating or as wheat-bran/peat, reduced *Fusarium* spp. infection in tomato (Sivan et al., 1987). Mousseaux et al. (1998) showed that the application of *T. harzianum* reduced seedlings mortality, caused by *Fusarium oxysporum*, in Douglas-fir by 50%. *Trichoderma harzianum* has been demonstrated as an effective biocontrol agent against *F. solani* in peanut, in both naturally infested and artificially contaminated fields (Rojo et al., 2007).

The plant pathogenic fungus *Rhizoctonia solani*

Rhizoctonia solani was first described in 1815 by DeCandolle and was designated as *R. crocorum*, while *R. solani*, the most important species of *Rhizoctonia*, was described in 1858 by Julius Kühn. There are presently about 120 species, varieties or formae speciales described within *Rhizoctonia* spp. *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris*) is a soil-borne basidiomycete fungus that has a wide range host and attacks many economically important crops worldwide.

Classification and identification of *Rhizoctonia solani*

Isolates of this fungus are classified into genetically isolated anastomosis groups (AGs). Anastomosis in *R. solani* is defined as a manifestation of somatic, or vegetative, incompatibility between hyphae of different but related strains (Anderson, 1982) and consists in a collection of closely related isolates grouped together based on their capability to anastomose with one another. Anastomosis grouping may be used to investigate the prevalence of number of clones existing in a field and their distribution (Carling, 1996) and different AG groups cause different symptoms, for instance on potato. Current diagnostic features of *R. solani* are as follows: (1) hyphal pigmentation of different brownish tones; (2) branching near the distal septum of cells in young vegetative hyphae; (3) constriction of hyphae and the formation of septa near the point of origin of hyphal branches; (4) presence of dolipore septa; and (5) more than 2 nuclei in cells close to the tips of young vegetative (Parmerter and Whitner, 1970; Sneh et al., 1991).

Mode of survival and growth of *Rhizoctonia solani*

Rhizoctonia solani responds rapidly to nutrient-stress by remobilizing the mycelial resources into sclerotia. Sclerotia are formed primarily from compact masses of monilioid cells, with barrel-shaped cells averaging 20-22 µm wide and 30-35 µm long and having the same number of nuclei as undifferentiated hyphae (Tu and Kimbrough, 1975). Light influences sclerotial formation and morphology, and some isolates produce sclerotia in light but not in dark (Durbin, 1959). By placing mycelial mats on a bed of sterile glass beads leached with water, Christias and Lockwood (1973) quantified the sclerotia produced within 4 days, accounting for 39% of the original mycelial dry weight, 26% of the original carbohydrate content and 50% of the original nitrogen content. In response to nutrient-stress *R. solani* not only remobilized its mycelial nutrients into sclerotia, but also incorporated further nutrients from soil. *Rhizoctonia solani* can survive for many years in soil as sclerotia or as a saprophyte, colonizing soil organic matter. In certain cases, the teleomorph of *R. solani* (*Thanatephorus cucumeris*) can produce basidiospores that will cause disease and also serve as a source for rapid and long-distance dispersal of the fungus (Gonzalez, 2011). *Rhizoctonia solani* has a natural role as a saprotroph in soils. It can grow extensively from a food base into soil and the growth can continue even after the food base has been removed (Blair, 1943). But, the proliferation of *R. solani* was suppressed if the soil was supplemented with finely ground organic matter such as dried grass or wheat straw, which promoted the activities of other microorganisms (Deacon, 1996).

Infection process of *Rhizoctonia solani*

Unable to make vegetative spores, *R. solani* is prevalent mostly as, mycelium or sclerotium. Sclerotia are the primary survival structures and source of inoculum. Sclerotia are formed in soil or on plant residues and have been reported to survive for several years (Sherwood, 1970). Both basidiospores and sclerotia will germinate and form mycelia prior to infection. Mycelium also plays a role in dispersal and survival through rapid growth and soil colonization (Papavizas, 1970) and is relatively persistent, especially on colonized debris (Papavizas and Davey, 1962). Mycelial growth is stimulated by plant exudates (Kerr and Flentje, 1957; Flentje et al., 1963; De Silva and Wood, 1964). The growth stimulation rate correlates with higher amino acids, carbohydrates, phenols and organic

acids concentrations present in the exudates of younger plants as compared to older plants (Nour El Dein and Sharkas, 1964; Martinson, 1965; Reddy, 1980). Following mycelia stimulation by root exudates, hyphae that are flattened and firmly attached to the plant surface can be observed (Christou, 1962; Armentrout and Downer, 1987), then the attached hyphae start to follow the anticlinal walls of the contiguous epidermal cells. Three to five hours later side branches are formed at right angles, which are characteristic for infection structures of *R. solani* (Armentrout and Downer, 1987). Complex infection structures (appressoria) are firmly attached to the epidermis by the hyphal branches and their swollen tips. Later the swollen tips form infections pegs, which will penetrate the cuticle and the epidermal cell wall (Flentje, 1957; Fukutomi and Takada, 1979). The peg allows the fungus to exert maximal hydrostatic pressure on the plant surface. Once inside the root system, the hypha returns to normal diameter, establishing the primary hypha, and rapidly invading the epidermal tissue and the outer layers of the cortex, growing intracellularly as well as in the intercellular spaces depending on the strain (Christou, 1962; Ruppel, 1973; Weinhold and Motta, 1973; Fukutomi and Takada, 1979; Kemaing and Hanchey, 1980). As the infection proceeds, *R. solani* produces extracellular hydrolytic enzymes capable of degrading cell walls. Substances produced by *R. solani* includes pectinolytic and cellulolytic enzymes, such as endopectin lyase, which have been reported to be associated with tissue degradation in later stages of infection (Marcus et al., 1986). At least 10 different extracellular enzymes have been identified to be produced by *R. solani* (Lister et al., 1975; Bertagnolli et al., 1996). The infection process of *R. solani* is designated as ectotrophic behavior because the mode of entry is through mechanical penetration of intact tissue, although penetration can also be through wounds. *Rhizoctonia solani* AG 3 provokes damping-off in potato seedlings and can be manifested as pre-emergent by preventing germination or as a post-emergent, root-rot disease that kills the sprout and produces sclerotia (black scurf) on potato tubers reducing their marketability.

The *Rhizoctonia* disease complex of potatoes comprises two distinct phases: infection of growing plants (*Rhizoctonia* canker) and infestation of daughter tubers by sclerotia (black scurf). Most notably AGs 3, 4, 5 and 8, are capable of doing moderate to extensive damage to potato plants, while other AGs are capable only of inflicting minor injury or no damage at all (Carling and Leiner, 1990). Damage to the plants including light brown to

black lesions of varying size form on sprouts, stolons and roots. On tubers, the disease symptoms are characterized by a change in tubers number, size distribution, and a degradation of quality due to the presence of sclerotia on tubers surface (Black scurf) (Carling et al., 1989).

Chemical and cultural controls of *Rhizoctonia* disease

Fungicides are commonly used to control seed-borne inoculum of potatoes, these include Tops MZ (thiophanate-methyl as active ingredient), Maxim MZ (fludioxinil as active ingredient) and Moncoat MZ (flutolanil as active ingredient) (Wharton et al., 2007). Planting seed potatoes with as little as 5% of the tuber covered with sclerotia often leads to serious damage (Powelson et al., 1993), thus using scurf-free seed tubers will prevent the occurrence of *Rhizoctonia* disease. The use of crop rotations will promote soil microbial life, resulting in an increase in competition, predation and parasitism, which will in turn reduce the prevalence of *Rhizoctonia* inoculum (Van Bruggen et al., 1996). It has been reported that *R. solani* is relatively sensitive to elevated temperatures. Pullman et al. (1981) demonstrated that solarization reduced the number of propagules of *R. solani* from 4 in 100g soil to under undetectable levels.

Biocontrol of *Rhizoctonia* spp. by *Trichoderma* spp.

Hadar et al. (1979) showed that *T. harzianum* directly attacked the mycelium of *R. solani* when both fungi were grown together on a glucose plus minerals medium. Additionally, the authors detected production of the enzymes β-(1-3) glucanase and chitinase when *T. harzianum* was grown on a mineral medium containing laminarin or chitin as carbon sources. In greenhouse experiment, *T. harzianum* effectively controlled damping-off of bean, tomato and eggplant seedlings (Hadar et al., 1979). Similar biocontrol success was obtained in many studies when exposing *Trichoderma* spp. to the mycelium of *R. solani* (Elad et al., 1980; Chet and Baker, 1981; Brewer and Larkin, 2005).

The pale cyst nematode *Globodera pallida*

The pale cyst nematode or potato cyst nematode (PCN) is the name commonly given to two species of cyst nematode that attack potato, namely *Globodera pallida* (Stone, 1972; Behrens, 1975) and *Globodera rostochiensis* (Wollenweber, 1923; Behrens, 1975) that

originate from Andean regions of South America (Evans et al., 1975; Baldwin and Mundo-Ocampo, 1991). *Globodera pallida* and *G. rostochiensis* were introduced to Europe around the mid or late 19th century on potatoes imported for breeding purposes. Since then, they have spread into most of the potato growing areas of Europe, South America, and in some potato-growing areas of USA, Canada, Mexico, Pakistan, India, Morocco, Sri Lanka, the Philippines, and Japan (Jatala, 1994). The genus *Globodera* is well-described by Skarbilovich (1959) and Behrens (1975) who noted the following characteristics: female nematodes are nearly spherical when fully grown; cuticle with a lace-like pattern; vulval lips absent; vulva located in a cavity below outline of body, with vulval slit less than 15µm long, too short for passage of eggs; in mature females vulva with tubercles on dorsal and ventral wall of vulval cavity; anus terminal or sub-terminal, without fenestration; vaginal remnants, under-bridge and bullae rarely present; cysts circumfenestrate and all eggs retained in body. Cyst nematodes are considered sedentary endoparasites and their distribution determined by the roots of the host plant.

After fertilization, eggs proceed through embryogenesis and second-stage juveniles (J2) hatch. *Globodera rostochiensis* and *G. pallida* require host root exudates to stimulate hatch (Rawsthorne and Brodie, 1986), the stimulatory effect of potato root diffusates (PRD) can be detected up to 80 cm away from roots and persists in soil for a long time after plant removal (Malinowska, 1996). A five-minute exposure for 4 weeks to PRD induces 43% hatch in *G. pallida* and similar exposure for about 5 weeks induces 60-70% hatch in *G. rostochiensis* (Forrest and Perry, 1980; Perry and Clarke, 1981; Perry and Beane, 1982). *Globodera pallida* is adapted to lower temperatures than *G. rostochiensis* as hatching of *G. pallida* begins at lower temperatures (Mugniery, 1978). After hatching, the infective J2 locates host roots, penetrates, and initiates the formation of a syncytium (fusion of cells), which provides the nutrients necessary for continued development and reproduction, and once feeding begins the J2 swells and becomes sedentary (Koenning and Sipes, 1998). Further growth and development of the J2 led to the third-stage juvenile J3, where the sex of the juvenile can be determined. The cyst nematodes are sexually dimorphic species, adult males are vermiform and motile, whereas adult females are swollen and sedentary (Raski, 1950). At the fourth-stage juvenile J4, spermatogenesis takes place, and the male may produce several thousand sperms, exit the roots, and seek

females (Shepherd and Clark, 1983). The J3 females continue to swell in the roots, becoming lemon-shaped to globose, then ruptures the root epidermis with her posterior end, exposing her vulva to the rhizosphere (Raski, 1950). After insemination, females begin production of up to 600 eggs (Brodie et al., 1993), and her body dies and forms a cyst which encapsulates the eggs.

Discovery of *Globodera pallida* in the state of Idaho

The pale cyst nematode *G. pallida* is a quarantine pathogen of potato (*Solanum tuberosum* L.) in many countries. In March 2006, cyst nematodes were discovered in tare soil from a potato processing facility in eastern Idaho, United States. The nematodes were found during a routine inspection conducted jointly by Idaho State Department of Agriculture and the USDA Animal and Plant Health Inspection Service through the Cooperative Agricultural Pest Survey program. Six cysts from the new detection were received by the USDA-ARS Nematology Laboratory for species identification. Subsequent extensive sampling traced the nematode to two fields in northern Bingham County, Idaho (Hafez et al., 2007). The morphology of the cysts and second-stage juveniles and molecular analyses established the identity of the species as the pale cyst nematode *G. pallida* (Stone, 1973; Behrens, 1975). Morphological characteristics used for identification included cyst shape, characteristics of cyst terminal cone including nature of fenestration, cyst wall pattern, anal-vulva distance, number of cuticular ridges between anus and vulva (Hafez et al., 2007).

Following the initial PCN detection in 2006, Canada, Mexico and South Korea shut off importation of potatoes from Idaho, while Japan cut off importation of potatoes from the entire U.S (USDA, 2016). The Mexico and Canada export markets have both been re-opened with the exception of potatoes from PCN-regulated areas; the South Korea market was reopened in June 2010 with the exception of potatoes originating from Bingham and Bonneville Counties, Idaho; and the Japan market remains closed to Idaho potatoes but negotiations are actively underway to re-gain market access (USDA, 2016).

Control of *Globodera pallida* spread in Idaho

The discovery of *G. pallida* in Idaho constitutes a significant threat to the Idaho potato industry, Animal and Plant Health Inspection Service (APHIS) and the Idaho State Department of Agriculture (ISDA) have implemented a regulatory program designed to

prevent the pest's spread to other fields. The program outlines restrictions on the movement of plants and soil, requires sanitation procedures for equipment, and enforces limitations on planting potato (USDA, 2016). In 2016, the PCN program deregulated a 75-acre Bingham County associated field after it completed a release protocol comprised of a sequence of surveys with negative laboratory results for PCN, changing the current regulated area to 9,853 acres, of which 2,897 acres are infested fields (USDA, 2016). The PCN program also allowed the use of the chemical methyl bromide (MeBr) for soil fumigation from 2007 to 2014, because of environmental concerns, the use of MeBr has been suspended. MeBr depletes the stratospheric ozone layer, the amount produced and imported in the U.S. was reduced until it was phased out in January 1, 2005, under the Montréal Protocol on substances that deplete the ozone layer and the Clean Air Act (CAA). With chemical application becoming more tenuous, research has been focus on the development of biological control agent as a viable option to control *G. pallida*.

Biocontrol of plant parasitic nematodes.

The fungus *Paecilomyces lilacinus* strain 251 is registered for biological control of nematodes in several countries (Atkins et al., 2005). *Paecilomyces lilacinus* reduced numbers of *Meloidogyne incognita* J2 in field-grown tomato by 70% and 41% when applied at transplant and 2 weeks after transplanting, respectively (Martez et al., 1996). Kiewnick and Sikora (2006) demonstrated that a single pre-plant application of *P. lilacinus* at a concentration of 1×10^6 CFU/g soil significantly reduced *M. incognita* in tomato roots. Goswami et al. (2008) showed that *P. lilacinus* suppressed galling of tomato by *M. incognita* by 39% when applied at transplant.

The fungus *Pochonia chlamydosporia* was first reported in 1974 as a parasite of nematode eggs in the UK, subsequently becoming one of the most studied potential biological control agents of nematodes (Kerry, 1997, 2000; Kerry and Hirsch, 2011; Manzanilla-López et al., 2013). *Pochonia chlamydosporia* has been effective in the biological control of root-knot and cyst nematodes in glasshouse pots (Kerry, 1995, 2001). *Pochonia chlamydosporia* infects nematode eggs through the development of appressoria at the hyphal tip or laterally, and all eggs that are colonized by the fungus are destroyed within a few days of infection (Kerry and Hirsch, 2011). *Pochonia chlamydosporia* significantly reduced *Globodera pallida* and *G. rostochiensis* multiplication rates in potato

roots by parasitizing adult females and colonizing the potato rhizoplane (Tobin et al., 2008).

Kerry and Crump (1977) showed that the fungus *Verticillium chlamydosporium* killed 50% of *Heterodera Avenae* eggs on barley roots. Three species of fungi, *Catenaria auxiliaris*, *Nematophthora gynophila* and a Lagenidiaceous fungus have been found attacking female sugar beet cyst nematodes *Heterodera schachtii*, causing the breakdown of the nematode cuticle, preventing cyst formation and replacing the female body contents with a mass of resting spores (Kerry, 1980; Kerry and Crump, 1980).

The nematophagous fungus *Dactylella oviparasitica* has been shown to parasitize and kill sedentary juveniles of *Heterodera schachtii* and reducing nematode population by 94-97% (Olatinwo et al., 2006; Becker et al., 2013).

Trichoderma longibrachiatum was found to decrease significantly *Heterodera avenae* infection in wheat by colonizing and degrading *H. avenae* cysts through extracellular chitinase activity (Zhang et al., 2014). In field experiment, *T. harzianum* reduced galling on tomato roots by 47% compared to untreated plots (Goswami et al., 2008). Low temperature scanning electron microscopic (LTSEM) studies revealed that *T. harzianum* infected mature potato cysts nematode eggs by penetrating directly the cyst wall or via natural opening of mouth, the penetration was either chemical or mechanical (appressorium) (Saifullah and Khan, 2014).

Racke and Sikora (1992) showed that isolates *Agrobacterium radiobacter* and *Bacillus sphaericus* at densities of 9.7×10^8 and 3.16×10^9 cfu ml^{-1}, respectively, caused up to 41% reductions in root infection caused by *G. pallida* in potato. Similar results were obtained by Hackenberg et al. (1999) when using *A. radiobacter* against *G. pallida*, and a number of other biocontrol agents have shown promises for controlling *Globodera* spp. such as: *Serratia marescens* against *G. rostochiensis* (Cronin et al., 1997b); *Pseudomonas fluorescens* against *G. rostochiensis* (Cronin et al., 1997a); *Verticillium chlamydosporium* against *Globodera* spp. (Crump, 1998). It has been demonstrated that lipopolysaccharides (LPS) of *Rhizobium etli* strain G12 induce in potato roots systemic resistance (up to 37%) to infection by *G. pallida* (Reitz et al., 2000).

The biological control agent *Trichoderma harzianum*

The genus *Trichoderma*

The genus *Trichoderma*, a common soil-inhabiting fungus, is probably best known for its biological control activity against a number of soilborne phytopathogens. Nearly all temperate and tropical soils contain 10^1-10^3 culturable propagules per gram (Harman et al., 2004). *Trichoderma* is a member of the filamentous ascomycete (teleomorph *Hypocrea*, Ascomycota, Dikarya), and is also among the most commonly isolated biotrophic and saprophytic fungus, and frequently found on other fungi, on dead wood and bark, or in soil and the rhizosphere. Jaklitsch (2009, 2011) showed that generally *Trichoderma* anamorphs are considerably more common on plant material than the teleomorphs. In nature, the asexual forms of the fungi persist as clonal, often heterokaryotic, individuals from populations that probably evolve independently in the asexual stage (Harman et al., 2004). Although the full diversity of *Trichoderma* species is unknown, 83% of *Trichoderma* species occurs on wood and bark of trees and shrubs, 2% may be found on gramineous or herbaceous hosts, 2% on soil and forest debris and 13% specifically on other fungi (Atanasova et al., 2013a).

Weindling (1932) was the first to describe the mycoparasitic activity of *T. lignorum* against a number of soilborne phytopathogens. Since then, *Trichoderma* spp. have been widely and in many cases positively tested as biocontrol agent against a number of plant pathogens (Hadar et al., 1979; Sivan et al., 1984; Sivan and Chet, 1986; Harman et al., 2004; Rojo et al., 2007). Knudsen et al. (1991) showed that colonization of sclerotia of *Sclerotinia sclerotiorum* by *T. harzianum* reduced the pathogen's inoculum-producing ability.

Trichoderma spp., although effective as a biocontrol, can be influenced by other microbes and be less effective. Recent studies have shown that nearly all of 75 molecularly defined *Trichoderma* species are able to reduce development of phytopathogenic fungi in approximately 70% (Monfil and Casas-Flores, 2014). However, fungus-feeding nematode, organic compounds or other soil microorganisms could have a detrimental effect on growth of *Trichoderma* spp. in the soil. Bae and Knudsen (2001) showed that the addition of *Aphelenchoides* sp., a fungivorous nematode, in both heat-treated (80ºC) and untreated field soil, significantly reduced radial growth and recoverable

populations of *T. harzianum*. Dandurand et al. (2000) showed that the presence of glucosinolate, organic compounds produced by *Brassica napus* seed meal, inhibited colonization of sclerotia of *S. sclerotiorum* in soil by *T. harzianum*. Bae and Knudsen (2005) observed that soil microbial biomass influence the growth and proliferation of *T. harzianum*, their results conclude that higher levels of microbial soil biomass result in increased interactions between introduced *T. harzianum* and soil microorganisms, and that microbial competition in soil favors a shift from hyphal growth to sporulation in *T. harzianum*, potentially reducing its biocontrol activity.

Trichoderma metabolism

The carbon sources most frequently used by *Trichoderma* species seem to be glucose, fructose, mannose, galactose, xylose, trehalose, and cellobiose (Manczinger et al., 2002). Production of secondary metabolites are among the most favorite weapons against soilborne fungal plant pathogens. Secondary metabolites produced by *Trichoderma* species inhibit the growth of other microorganisms by releasing low-molecular-weight diffusible compounds or antibiotics, such as harzianic acid, alamethicins, peptaibols, 6-penthyl-α-pyrone, viridian, gliovirin, gliotoxin, heptelidic acid (Benítez et al., 2004; Schuhmacher et al., 2007; Zeilinger and Omann, 2007). In order to withstand toxic environments, including antibiotics produced by themselves or phytopathogens, plant antimicrobial compounds, and synthetic chemicals, *Trichoderma* species have developed transporters for removing unwanted or harmful substances. The most important transporters belong to the MFS (Major Facilitator Superfamily) and ABC groups. Both transporters are described as group of proteins involved in the transport of a wide range of compounds, including external elements and toxins (Silva et al., 2014).

Biocontrol mechanisms used by *Trichoderma* spp.

Numerous studies showed that several genes, enzymes and other effectors were found to be involved in mycoparasitic response among different species and in different stages of their predation (Seidl et al., 2009; Reithner et al., 2011; Omann et al., 2012; Catalano et al., 2011; Atanasova et al., 2013b).

A number of processes of *Trichoderma* mycoparasitism were studied and grouped into four stages: (1) waiting for a prey (ambushing); (2) recognition of the presence of a potential prey (sensing); (3) induction of the biochemical tools to besiege the prey

(hunting); and (4) actual attack and eventual killing and feeding on the prey (Druzhinina et al., 2011).

Two principal groups of fungal antagonistic relationships are classified: (1) biotrophic interactions, where the pathogenic fungus feeds from its host without provoking its death via a highly specific interaction, these mycoparasites are not successfully used as biological control agents; (2) necrotrophic interactions, where interactions are more violent against a broad range of phytopathogenic fungi, these mycoparasites, which include the genus *Trichoderma*, are effective biological agents and are widely used (Monfil and Casas-Flores, 2014).

Trichoderma **metabolites**

Trichoderma spp. might recognize the other fungi by the secretion of small peptides or other molecules that are released as a response to proteases secreted by the *Trichoderma* spp. (Druzhinina et al., 2011). Montero-Barrientos et al. (2011) demonstrated that NADPH oxidases (Nox) produce reactive oxygen species (ROS) as defense response in *Trichoderma* spp. The authors overexpressed the *nox1* gene in *T. harzianum* T34, leading to an increase in ROS production during its interaction with *Pythium ultimum*, which resulted in a more effective control of this pathogen. The authors also showed that the overexpressed *nox1* gene led to an increased production of protease, cellulase and chitinase enzymes. *Trichoderma* spp. produce a wide range of secondary metabolites (SM) which are small organic molecules that are not essential for their growth, development and reproduction. Rubio et al. (2009) showed that the disruption of *Thctf1* gene that encodes for the production of 6-pentyl-alpha-pyrone (6-PP), a well-studied SM, reduced antimicrobial capacity against *Fusarium* spp. Malmierca et al. (2012) showed that the absence of *tri4* gene which encodes for the production of trichothecenes, a mycotoxin from the group sesquiterpenoid-derived secondary metabolites, reduced the biocontrol activity of *Trichoderma* against *Botrytis cinerea* and *Rhizoctonia solani*. *Trichoderma* spp. produce siderophores to convert insoluble iron in the soil into soluble iron by chelation, and make it available for uptake. Segarra et al. (2010) showed that competition for iron has been associated with the suppression of *Fusarium* spp. by *Trichoderma*.

Rhizosphere colonization by *Trichoderma*

Rhizosphere colonization by *T. harzianum* contributes to the protection of the infection court and also favors *T. harzianum* growth and proliferation in soil, and the ability to colonize plant roots has often been stressed as an important requirement of *Trichoderma* to act as biocontrol agents (Harman et al., 2004). In sterile soil, *T. harzianum* was detected in the rhizosphere of the upper half of pea roots (Chao et al., 1986). Ahmad and Baker (1987) described the ability of *Trichoderma* strains to grow and compete with other microorganisms to colonize the root surface and compete with them for nutrients secreted by root exudates. Sivan and Chet (1989) showed that *T. harzianum* strain T-35 significantly reduced disease caused by *Fusarium oxysporum* by colonizing the rhizosphere of cotton plants. Proteomic and transcriptomic studies showed that colonization of plant roots by *Trichoderma* provoke a systemic change in the expression of plant genes regulating stress responses, isoprenoid oxylipins and ethylene biosynthesis, photosynthesis, photorespiration, and carbohydrate metabolism (Vargas et al., 2009). Druzhinina et al. (2011) reported that the rhizosphere is among the preferred ecological niches for *Trichoderma* spp. which provides opportunities for both biotrophy and saprotrophic nutrition on plant root exudates, therefore contributing to the growth and proliferation of *Trichoderma* into the soil.

Influence of *Trichoderma* on soil fertility and plant growth

Ahmad and Baker (1987, 1988) reported that *T. harzianum* promotes better seedling emergence and plant growth. Studies have shown that corn roots colonized by *T. harzianum* T22 require 40% less nitrogen fertilizer than corn roots lacking the fungus. Other studies reported that T22 can solubilize a range of plant nutrients such as rock phosphate, Fe^{3+}, Cu^{2+}, $Mn^{4+}$and Zn^{0} that may be unavailable to plants in certain soils (Altomare et al., 1999; Harman et al., 2004). Benitez et al. (2004) reported that *Trichoderma* can produce organics such as citric, gluconic or fumaric acids that lower soil pH and thereby permit the solubilization of phosphates. Bjorkman et al. (1998) suggested that *T. harzianum* T22 induced growth promotion of maize by colonizing the rhizosphere and limiting or even reversing the effects of oxidative damage to the roots, the authors also reported that the addition of *T. harzianum* T22 to hypochlorite damaged seedlings restored seedling vigor. Mastouri et al. (2010) demonstrated that T22 confers tolerance to

abiotic stresses such as heat and cold shock resulting in greater seed germination and improved seedling vigor. Proteomic and transcriptomic studies showed that colonization of plant roots by *Trichoderma* provoke a systemic change in the expression of plant genes regulating stress responses, isoprenoid, oxylipins and ethylene biosynthesis, photosynthesis and photorespiration rates, and carbohydrate metabolism (Vargas et al., 2009).

Trichoderma spp. as inductors of systematic resistance in plants

In contact with pathogenic and nonpathogenic microorganisms, plants initiate a wide range of defense mechanisms. Two main mechanisms have been identified: (1) systemic acquired resistance (SAR); and (2) induced systemic resistance (ISR). SAR is triggered by local infection, providing long-term systemic resistance with the activation of PR genes that requires the involvement of the signal molecule salicylic acid (SA) (Durrant and Dong, 2004; Vallad and Goodman, 2004). ISR is triggered by colonization of roots by some specific nonpathogenic rhizobacteria, and requires the participation of the signal molecule jasmonic acid (JA) followed by ethylene signaling pathway (Van Loon et al., 1998; Vallad and Goodman, 2004). A number of studies have found that *Trichoderma* spp. have the ability to turn on specific disease-resistance genes in plants, such as *PAL1* in cucumber roots colonized by *T. asperellum* (Yedidia et al., 2000, 2003; Harman et al., 2004; Shoresh et al., 2010). Calderón et al. (1993) demonstrated that suspension cell cultures of grapevine treated with an elicitor from *T. viride* showed a hypersensitive-like response characterized by cell plasmolysis accompanied by localized cell death due to a large increase in endogenous levels of H_2O_2. Yedidia et al. (1999), in an experiment with cucumber plants, showed evidence that *T. harzianum* was able to penetrate the root system without causing extensive damage and trigger plant host defense reactions by increasing the level of plant peroxidase and chitinase activities. Thus *Trichoderma*-inoculated plants may be sensitized to respond faster and to a greater extent to potential pathogen attacks. Viterbo et al. (2005) revealed that a mitogen-activated protein kinase (MAPK) is essential for *Trichoderma* in order to induce full systemic resistance in cucumber against the bacterial pathogen *Pseudomonas syringae.* It has also been shown that *Trichoderma* spp. induce the surrounding plant cells to deposit cell wall material and

produce phenolic compounds that limit *Trichoderma* growth inside the root, which prevents it from damaging plant cells (Shoresh et al., 2010).

Trichoderma harzianum strain ThzID1-M3

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). *Trichoderma harzianum* ThzID1 has been extensively studied and evaluated as a potential biocontrol agent against plant pathogenic fungi. Knudsen et al. (1991) showed that polyethylene glycol (PEG)-treated pellets with *T. harzianum* ThzID1 showed significantly greater proliferation of hyphae in soil than untreated pellets. *Trichoderma harzianum* ThzID1 also demonstrated the ability to colonize sclerotia of *Sclerotinia sclerotiorum* (Knudsen et al., 1991; Bae and Knudsen, 2006; Kim and Knudsen, 2009). Dandurand and Knudsen (1993) showed that the application of the granular formulation of *T. harzianum* ThzID1 to pea seeds reduced root-rot caused by *Aphanomyces euteiches* f. sp. *pisi* in growth chamber experiments and also increased plant top weights compared to non-coated seeds. Mousseaux et al. (1998) showed that *T. harzianum* ThzID1 significantly reduced proliferation of *Fusarium* spp. in Douglas-fir seedlings, while promoting seed germination and growth. *Trichoderma harzianum* ThzID1 hyphal growth has been modeled in an individual-based approach from image analysis of hyphal biomass accumulation in soil, and results showed the ability to predict fungal growth in natural habitats (Knudsen et al., 2006).

Trichoderma harzianum ThzID1 was co-transformed with genes encoding green fluorescent protein (GFP) from jellyfish *Aequorea victoria*, β-glucuronidase (GUS) and hygromycin B (*hygB*) resistance, using polyethylene glycol-mediated transformation (Bae and Knudsen, 2000). The co-transformant *T. harzianum* ThzID1-M3 morphology was similar to that of the wild type; however, mycelial growth rate on agar was reduced (Bae and Knudsen, 2000). Wild-type and transformant strains both 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). The GFP tool has been used by other researchers also to monitor activities of biocontrol agents. Spellig et al. (1996) used GFP in *Ustilago maydis*, the causative agent of corn smut

disease, to study host-pathogen interactions in vivo. Vanden Wymelenberg et al. (1997) used GFP in *Aureobasidium pullulans* for quantification on leaf surfaces, providing a powerful method for ecological studies of plant-microbe relationships in nature. Maor et al. (1998) used GFP for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. Orr and Knudsen (2004) demonstrated that the use of GFP, 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. Baehler et al. (2005) used the GFP tool to study the production of antifungal compounds in the biocontrol agent *Pseudomonas fluorescens*. Kim and Knudsen (2008, 2011) developed a specific PCR primer/probe set for *T. harzianum* ThzID1-M3, which exhibited high precision and reproducibility, and results showed that while microscopy combined with image analysis can provide useful information on the spatial and temporal dynamics of colonization, real-time PCR can provide a more precise assessment of the extent of sclerotial colonization over time and can more easily be used to sample entire sclerotia.

The biocontrol efficacy of *T. harzianum* ThzID1-M3 has been evaluated against biotic and abiotic components in soil. Knudsen and Bin (1990) determined the effects of temperature, soil moisture and wheat bran on growth of *T. harzianum* ThzID1 from alginate pellets and showed that temperature had a significant positive effect on radial growth rate but that matric potential and bran effects were not significant. Bae and Knudsen (2001) showed that *Aphelenchoides* sp, a fungivorous nematode, may be a significant biotic constraint on activity of *T. harzianum* ThzID1-M3 in the field. Furthermore, Bae and Knudsen (2005) showed that higher levels of microbial soil biomass result in increased interactions between introduced *T. harzianum* ThzID1-M3 and soil microorganisms, and further that microbial competition in soil favors a shift from hyphal growth to sporulation in *T. harzianum* ThzID1-M3, potentially reducing its biocontrol efficacy.

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CHAPTER 2

Biological control of *Fusarium solani* and *Rhizoctonia solani* with *Trichoderma harzianum* ThzID1 in potato

ABSTRACT

Trichoderma harzianum strain ThzID1 isolated in the state of Idaho was evaluated as potential biocontrol agent against *Fusarium solani* and *Rhizoctonia solani* in potato (*Solanum tuberosum* L.) seedlings, two well-known root-rot soilborne phytopathogens in potato and many other crops and trees. The objectives of this study were to assess the influence of *T. harzianum* ThzID1 on root-rot disease reduction and on growth of potato plants. Separately, *T. harzianum* ThzID1, *F. solani* and *R. solani* were maintained in potato dextrose agar (PDA) and then grown on sterile oat kernel at 25ºC for 20 days. Experiments were conducted under greenhouse conditions for 40 days at 25ºC ± 2ºC with 15:9-hr light and dark hours. Potato tubers cv 'Russet Burbank' were used as hosts for the fungi and a sterile mixture 2:1 sand and silt loam was used as soil medium for potato tuber growth. Treatments for each experiment (*T. harzianum* ThzID1 vs *F. solani*, and *T. harzianum* ThzID1 vs *R. solani*) were designed as follows: control (no fungi added), ThzID1 (only), phytopathogen (only), and ThzID1 applied together with phytopathogen. Treatments were replicated five times in a completely randomized block design and experiments were repeated. Experiments were evaluated after 40 days. Results in both experiments showed disease development in potato roots in treatments with the phytopathogen (only) ($P < 0.05$) compared to the control treatments. *Trichoderma harzianum* ThzID1 applied together with *R. solani* was able to significantly reduce disease incidence in roots ($P < 0.05$) compared with *R. solani* only. Amendment with *T. harzianum* ThzID1 only showed no significant increase on potato growth compared with the control treatment ($P > 0.05$). However, when applied with *F. solani*, *T. harzianum* ThzID1 was unable to reduce disease incidence ($P > 0.05$) compared to the *F. solani* treatment only. In this experiment *T. harzianum* ThzID1 application significantly decreased potato growth compared to the control treatment ($P < 0.05$). We demonstrated that the introduced mycoparasitic fungus *T. harzianum* ThzID1 shows potential for biocontrol of *R. solani*. However, more studies are required for elucidating the potential of *T. harzianum* ThzID1 for biocontrol of *F. solani*.

INTRODUCTION

Fusarium solani (Martius) Snyder and Hansen (1940) is a deuteromycota and well-known soilborne fungus responsible for diseases in potato (*Solanum tuberosum* L.). Commonly known as Fusarium dry rot, it affects both tubers in storage and seedlings, and it reduces crop yield by destroying potato roots and sprouts. Symptoms associated with Fusarium dry rot include dark depressions on the surface of the tuber, root-rot in seedlings, and presence of secondary pathogens (*Pectobacterium* spp., *Pythium* spp.) in rotten tissues (Wharton, 2007).

Rhizoctonia solani Kühn (1858) is a basidiomycete and common soilborne fungus. Members of this species are important plant pathogens responsible for root-rot diseases in many crop species worldwide. *Rhizoctonia* spp. are divided into a complex sub-specific groups called anastomosis groups (AG) (Parmeter et al., 1969; Adams and Butler, 1979). *Rhizoctonia solani* isolates AG 3 disease symptoms in potato include death of pre-emergent sprouts (damping-off), cankers on underground stem parts and stolons characterized by brown and black sunken lesions, diminished root systems, and production of progeny tubers with sclerotia, often referred to as the "dirt that won't wash off". Resistant varieties are currently unavailable (El Bakali and Martín, 2006; Fox, 2006; Ikeda et al., 2012).

The use of biocontrol agents has become an important alternative to agrochemicals for control of plant diseases in agriculture. *Trichoderma harzianum*, a soilborne filamentous fungus, has been widely used and studied as biological control agent against many soilborne plant pathogens (Weindling, 1932; Hadar et al., 1979; Sivan et al., 1984; Knudsen et al., 1991; Harman et al., 2004). Biocontrol mechanisms used by *T. harzianum* against soilborne pathogens include mycoparasitism, considered as a direct attack of one fungus upon another through mechanical and chemical actions, can be referred as direct antagonism (Chet et al., 1981; Barak et al., 1985; Papavizas, 1985); antibiosis, by the production of antibiotics and hydrolytic enzymes (Lorito et al., 1996; Howell, 1998); competition for space, enzyme substrate, nutrients and oxygen (Papavizas, 1985); and plant growth promotion and induced resistance (Kleifeld and Chet, 1992; Windham et al., 1986; Howell et al., 2000; Yedidia et al., 1999, 2000, 2003). *Trichoderma harzianum* has been shown to control Fusarium wilt in cotton and muskmelon (Sivan and Chet, 1986),

reduce *Fusarium* spp. infection in tomato (Sivan et al., 1987), reduce seedling mortality caused by *Fusarium oxysporum* in Douglas-fir (Mousseaux et al., 1998), control *Fusarium solani* in peanut (Rojo et al., 2007), and *T. harzianum* has shown potential for biocontrol of *Sclerotinia sclerotiorum* (Knudsen et al., 1991). Hadar et al. (1979) showed that *T. harzianum* directly attacked the mycelium of *R. solani* when both fungi were grown together in vitro. In greenhouse experiments, *T. harzianum* effectively controlled damping-off of bean, tomato and eggplant seedlings (Hadar et al., 1979). Similar biocontrol success was obtained in many other studies when exposing *Trichoderma* spp. to the mycelium of *R. solani* (Elad et al., 1980; Chet and Baker, 1981; Brewer and Larkin, 2005).

The objectives of this study were to evaluate the potential of *T. harzianum* ThzID1 as biological control agent to reduce the level of root-rot disease caused by *F. solani* and *R. solani* on potato seedlings, and also to assess the effect of *T. harzianum* ThzID1 on growth of potato plants. We hypothesized that the addition of *T. harzianum* ThzID1 with the potato seed piece would reduce the level of root infection by *F. solani* and *R. solani* and enhance potato plant growth.

MATERIALS AND METHODS

Trichoderma harzianum ThzID1, *Fusarium solani* and *Rhizoctonia solani*

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). *T. harzianum* ThzID1, *F. solani* and *R. solani* were allowed to grow for 1 week on potato dextrose agar (PDA) plates with streptomycin (25 mg/L) at 25ºC. Oat kernels were used as growth substrate for ThzID1, *F. solani* and *R. solani*. Two hundred grams of oat kernels were placed in 1000-ml Erlenmeyer flasks, followed by the addition of 200 ml of distilled water. Flasks were autoclaved twice for 1 hour at 121ºC and allowed to cool for 24 hours. Flasks were inoculated separately with five fungal plugs of 7 mm diameter from PDA plates followed by incubation at 25ºC for 20 days.

Soil preparation

The soil media consisted in a mixture of 2:1 sand and silt loam, and autoclaved twice for 1 hour at 121ºC. The soil mixture was allowed to cool down for 1 week.

Potato tubers

Potato tubers cv 'Russet Burbank', classified as certified disease free (from the Nuclear Potato Seed Program, University of Idaho), were sterilized for 1 minute in 0.4% NaOCl, rinsed thoroughly in sterile distilled water, dried, and left for 1 week at room temperature under 24 hours of darkness for stimulating sprout germination before planting.

Experimental treatments

Experiments were conducted in 10-cm diameter clay pots. There were two experiments: *T. harzianum* ThzID1 vs *F. solani* and *T. harzianum* ThzID1 vs *R. solani*. There were four treatments for each experiment: (1) untreated control, (2) ThzID1 (only), (3) phytopathogen (only), and (4) ThzID1 applied together with phytopathogen. Treatments were replicated five times in a completely randomized block design and both experiments were repeated. Five grams of oat kernel containing fungi inoculum were added to 1.5 kg of soil and mixed very well together in the clay pot before adding one potato tuber according to the treatments. *Trichoderma harzianum* ThzID1 was applied at a rate of 6.20×10^6 conidia/g of oat kernel, *F. solani* at a rate of 4.51×10^8 conidia/g of oat kernel, and *R. solani* at a rate of 5.09×10^8 hyphae/g of oat kernel. Pots were maintained at 25ºC ± 2ºC, 60% relative humidity, and 15:9-hr light: dark photoperiod in a greenhouse environment. Pots were watered twice daily in the amount of 500 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 of water.

Measurement

Experiments were evaluated 40 days after planting. For each treatment, stems were cut, measured and weighed; roots were washed in tap water, dried for 30 minutes and weighed. Biomass were calculated as the sum of root and stem weights. Root disease was estimated and the damage was categorized numerically as follows: 0 = no damage, no lesions or rot; 1 = minor damage, one to several lesions less than 5 mm long; 2 = intermediate damage lesions greater than 5 mm long, some roots girdled, and much dead tissue; 3 = major damage, lesions large, and most root tissue dead; 4 = all roots rotted and dead, or no roots present (Carling and Leiner, 1990).

Statistical analysis

This study was conducted as a completely randomized block design. All analyses were performed using SAS package 9.4 (SAS Institute Inc., Cary, N.C.). Analysis of variance (ANOVA) using proc GLM (generalized linear model) was performed for analyzing biomass and length of roots and stems, and multiple comparisons by Tukey's HSD method were done, with significant differences occurring at $P < 0.05$ level. Non-parametric analysis using proc npar1way was performed for analyzing root disease index, and multiple comparisons by proc npar1way DSCF were done, with significant differences occurring at $P < 0.05$ level.

RESULTS

Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Fusarium solani*

The first experiment resulted in failure. No disease was observed, thus biocontrol activity of *T. harzianum* ThzID1 could not be assessed (Table 2.1).

Table 2.1 Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Fusarium solani*.

Treatment*	Root length** (cm)	Root weight (g)	Stem length (cm)	Biomass (g)	Disease index***
Untreated control	13.00[a] (0.63)	45.00[a] (7.91)	68.41[a] (2.44)	249[a] (25.69)	0.40[a] (0.24)
ThzID1	14.00[a] (0.71)	45.32[a] (4.91)	63.62[a] (3.11)	254[a] (15.20)	0.40[a] (0.24)
F. solani	14.00[a] (1.64)	35.15[a] (1.33)	62.71[a] (3.72)	237[a] (6.72)	1.80[a] (0.37)
ThzID1+*F. solani*	12.60[a] (1.43)	34.80[a] (2.65)	65.19[a] (2.38)	245[a] (16.11)	1.00[a] (0.45)

*Data from the first experiment.

**Means with the same letter are not significantly different ($P > 0.05$) as determined by Tukey's studentized range (HSD). Standard error of the mean is shown in parenthesis.

***For root disease index, non-parametric procedure with pairwise two-sided multiple comparison analysis (Dwass, Steel, Critchlow-Fligner method) was used to evaluate level of significance of each treatment.

The second experiment showed significant increase in potato root length, root weight, stem length and biomass in untreated control when compared to *T. harzianum* ThzID1 applied only, *F. solani* applied only, and both fungi applied together ($P < 0.05$). *Trichoderma harzianum* ThzID1 applied only or together with *F. solani* showed no significant increase in potato root length, root weight, stem length and biomass when compared to *F. solani* applied only ($P > 0.05$). Non-parametric analysis showed

significantly less disease in untreated control when compared to *F. solani* applied only and *F. solani* applied together with *T. harzianum* ThzID1 ($P < 0.05$). *Trichoderma harzianum* ThzID1 applied only showed significantly less disease when compared to *F. solani* applied only and *F. solani* applied together with *T. harzianum* ThzID1 ($P < 0.05$). However, *T. harzianum* ThzID1 applied together with *F. solani* showed no significant reduction in disease when compared to *F. solani* applied only ($P > 0.05$) (Table 2.2).

Table 2.2 Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Fusarium solani*.

Treatment*	Root length** (cm)	Root weight (g)	Stem length (cm)	Biomass (g)	Disease index***
Untreated control	19.75[a] (0.63)	75.33[a] (3.10)	44.33[a] (2.14)	209[a] (7.57)	0.40[a] (0.40)
ThzID1	10.40[b] (0.93)	17.90[b] (4.36)	20.76[b] (2.08)	57[b] (2.09)	0.80[a] (0.37)
F. solani	8.00[b] (2.49)	10.10[b] (5.26)	16.29[b] (4.26)	50[b] (26.30)	3.20[b] (0.37)
ThzID1+*F. solani*	5.60[b] (1.43)	5.22[b] (2.85)	13.45[b] (1.27)	27[b] (10.41)	3.60[b] (0.40)

*Data from the second experiment.
**Means with the same letter are not significantly different ($P > 0.05$) as determined by Tukey's studentized range (HSD). Standard error of the mean is shown in parenthesis.
***For root disease index, non-parametric procedure with pairwise two-sided multiple comparison analysis (Dwass, Steel, Critchlow-Fligner method) was used to evaluate level of significance of each treatment.

Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Rhizoctonia solani*

The first experiment resulted in failure. No disease was observed, thus biocontrol activity of *T. harzianum* ThzID1 could not be assessed (Table 2.3).

Table 2.3 Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Rhizoctonia solani*.

Treatment*	Root length** (cm)	Root weight (g)	Stem length (cm)	Biomass (g)	Disease index***
Untreated control	13.75[a] (1.11)	51.98[a] (2.89)	63.58[a] (2.67)	223[a] (6.69)	0.80[a] (0.37)
ThzID1	16.20[a] (1.39)	41.73[b] (1.26)	65.22[a] (3.05)	218[a] (13.71)	0.20[a] (0.20)
R. solani	14.67[a] (0.33)	36.55[b] (2.04)	59.54[a] (3.90)	207[a] (4.00)	0.80[a] (0.20)
ThzID1+*R. solani*	16.60[a] (1.03)	50.65[a] (1.61)	56.08[a] (1.82)	229[a] (2.47)	0.80[a] (0.37)

*Data from the first experiment.
**Means with the same letter are not significantly different ($P > 0.05$) as determined by Tukey's studentized range (HSD). Standard error of the mean is shown in parenthesis.
***For root disease index, non-parametric procedure with pairwise two-sided multiple comparison analysis (Dwass, Steel, Critchlow-Fligner method) was used to evaluate level of significance of each treatment.

The second experiment showed no significant differences in potato root length, root weight, stem length and biomass for the four treatments evaluated ($P > 0.05$). No disease was observed in untreated control. *Trichoderma harzianum* ThzID1 applied only showed significant disease reduction when compared to *R. solani* applied only ($P < 0.05$). *Trichoderma harzianum* ThzID1 applied only showed no significant disease reduction when compared to *T. harzianum* ThzID1 applied together with *R. solani* ($P > 0.05$). *Trichoderma harzianum* ThzID1 applied together with *R. solani* showed significant disease reduction when compared to *R. solani* only ($P < 0.05$) (Table 2.4).

Table 2.4 Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Rhizoctonia solani*.

Treatment*	Root length** (cm)	Root weight (g)	Stem length (cm)	Biomass (g)	Disease index***
Untreated control	16.00[a] (1.79)	16.50[a] (1.17)	26.53[a] (1.00)	124[a] (12.08)	0[a] (0)
ThzID1	10.00[a] (0.00)	18.80[a] (4.07)	25.43[a] (0.41)	98[a] (20.40)	0.20[a] (0.20)
R. solani	12.33[a] (0.33)	17.77[a] (3.19)	27.49[a] (1.03)	101[a] (9.71)	1.40[b] (0.24)
ThzID1+*R. solani*	12.80[a] (1.36)	20.93[a] (1.56)	27.97[a] (0.26)	127[a] (3.51)	0.60[a] (0.24)

*Data from the second experiment.
**Means with the same letter are not significantly different ($P > 0.05$) as determined by Tukey's studentized range (HSD). Standard error of the mean is shown in parenthesis.
***For root disease index, non-parametric procedure with pairwise two-sided multiple comparison analysis (Dwass, Steel, Critchlow-Fligner method) was used to evaluate level of significance of each treatment.

DISCUSSION

Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Fusarium solani*

The first experiment resulted in failure, therefore no biocontrol activity of *T. harzianum* could be assessed. One explanation for this failure could be explained by possible cross-contamination of *F. solani* to untreated control and *T. harzianum* applied only.

The second experiment showed significant increase in potato root length, root weight, stem length and biomass in untreated control when compared to *T. harzianum* ThzID1 applied only, *F. solani* applied only, and both fungi applied together ($P < 0.05$). However, *T. harzianum* ThzID1 applied only or together with *F. solani* showed no significant increase in potato root length, root weight, stem length and biomass when compared to *F. solani* applied only ($P > 0.05$). Our results differed from other studies that showed *T. harzianum* as a plant growth promoter. *Trichoderma harzianum* has been reported to

promote better seedling and plant growth in bean, cucumber, maize, radish, and tomato (Ahmad and Baker, 1987, 1988; Bjorkman et al., 1998). Windham et al. (1986) reported that the addition of *Trichoderma* spp. to autoclaved soil increased radish plant growth when compared to untreated controls. Chang et al. (1986) showed that *T. harzianum* applied at a rate of 10^5 CFU/ g soil increased significantly the dry weights of bean, radish, tomato, pepper, and cucumber compared to untreated control. Dandurand and Knudsen (1993) showed that the application of the granular formulation of *T. harzianum* ThzID1 to pea seeds increased plant top weights compared to non-coated seeds. Contreras-Cornejo et al. (2009) showed that *T. virens* enhanced biomass production and promoted lateral root growth in Arabidopsis. Explanations for this inconsistency in our result when compared to other studies, could be that not enough time was allowed for *T. harzianum* ThzID1 to colonize completely the potato rhizosphere in order to observe significant increase in potato growth. Additionally, soil water content level might have delayed *T. harzianum* ThzID1 growth in potato rhizosphere. We suggest that the use of potato plantlets, grown previously in tissue culture medium, for planting in pots instead of tubers might allow rapid root colonization by *T. harzianum* ThzID1, and therefore we could observe significant increase in potato growth. We also suggest to increase the rate of application of *T. harzianum* ThzID1 up to 10^7 CFU/ g soil, increase the number of replicates up to ten, maintain adequate soil water content, and to extend evaluation of treatments up to 60 days.

The second experiment showed that potato plants treated with *F. solani* only displayed significant increase in root damage when compared to untreated control ($P < 0.05$). *Trichoderma harzianum* ThzID1 applied only showed no significant increase in root damage when compared to untreated control ($P > 0.05$). *Trichoderma harzianum* ThzID1 applied only showed significantly less root damage when compared to *F. solani* applied only ($P < 0.05$). However, *T. harzianum* ThzID1 applied together with *F. solani* showed no significant reduction in root damage when compared to *F. solani* applied only ($P > 0.05$). Our results differed from other studies that showed *T. harzianum* as a biocontrol of *Fusarium* spp. Sivan and Chet (1986) showed that *T. harzianum* controlled Fusarium wilt in cotton and muskmelon. *Trichoderma harzianum*, applied as a seed coating or as wheat-bran/peat, reduced *Fusarium* spp. infection in tomato (Sivan et al., 1987). Datnoff et al. (1995) showed that *T. harzianum* significantly decreased disease incidence caused by *F.*

oxysporum in tomato. Mousseaux et al. (1998) showed that the application of *T. harzianum* ThzID1 reduced seedlings mortality, caused by *F. oxysporum*, in Douglas-fir by 50%. *Trichoderma harzianum* has been demonstrated as an effective biocontrol agent against *F. solani* in peanut, in both naturally infested and artificially contaminated fields (Rojo et al., 2007). However, studies revealed that antagonistic potential may vary among *Trichoderma* spp. and strains (Bell et al., 1982; Schubert et al., 2008). Knudsen and Bin (1990) suggested that reduced availability of oxygen in wetter soil might adversely affect *T. harzianum* ThzID1 hyphal proliferation in the soil. Kredics et al. (2003) showed that enzymatic activities of *T. harzianum* strain T66 and T334 reacted differently as influenced by the effects of temperature, water potential, pH, presence of pesticides, metal ions and antagonistic bacteria in the soil. Rosa and Herrera (2009) demonstrated that *T. atroviride* strain CH 304.1 provided the highest biocontrol effects against *Rosellinia necatrix* than *T. harzianum* strain CH 252. Marzano et al. (2013) reported that the phytotoxin fusaric acid produced by *Fusarium oxysporum* f. sp. *lycopersici* inhibits *T. harzianum* strain ITEM 908 (Th908) growth, and its biocontrol effect against *F. oxysporum* was found to be poor.

Explanation for this inconsistency in our result when compared to other successful biocontrol studies, could be that not enough time was allowed for *T. harzianum* ThzID1 to colonize completely the potato rhizosphere in order to observe significant reduction in root damage. We suggest that the use of potato plantlets, grown previously in tissue culture medium, for planting in pots instead of tubers might allow rapid root colonization by *T. harzianum* ThzID1, and therefore we could observe significant reduction in root disease incidence. *Trichoderma harzianum* ThzID1 should be inoculated with potato plantlets one week before applying *F. solani* in the soil in order to favor rapid rhizosphere colonization by *T. harzianum* ThzID1. We also suggest to increase the rate of application of *T. harzianum* ThzID1 up to 10^7 CFU/ g soil, increase the number of replicates up to ten, maintain adequate soil water content, and to extend evaluation of treatments up to 60 days.

Influence of *Trichoderma harzianum* ThzID1 on root-rot caused by *Rhizoctonia solani*

The first experiment resulted in failure, therefore no biocontrol activity of *T. harzianum* could be assessed. One explanation for this failure could be explained by possible cross-contamination of *R. solani* to untreated control and *T. harzianum* applied only.

The second experiment showed no significant differences in potato root length, root weight, stem length and biomass when comparing all the treatments ($P > 0.05$). Therefore, the activity of *T. harzianum* ThzID1 as a plant growth promoter could not be assessed. One explanation for this inconclusive result could be related to the application rate of *T. harzianum* ThzID1 and to the timeline for the evaluation of the experiment. Therefore, we suggest that the use of potato plantlets, grown previously in tissue culture medium, for planting in pots instead of tubers might allow rapid root colonization by *T. harzianum* ThzID1, and therefore we could observe significant increase in potato growth. We also suggest to increase the application rate of *T. harzianum* ThzID1 up to 10^7 CFU/ g soil, increase the number of replicates up to ten, maintain adequate soil water content, and to extend evaluation of treatments up to 60 days.

The second experiment showed no root damage in untreated control. However, significant increase in root damage was observed in *R. solani* applied only when compared to *T. harzianum* ThzID1 applied only and *T. harzianum* ThzID1 applied together with *R. solani* ($P < 0.05$). *Trichoderma harzianum* ThzID1 applied together with *R. solani* significantly reduce root damage when compared to *R. solani* only ($P < 0.05$). Similar results were obtained by other studies that illustrated *T. harzianum* as an effective biocontrol agent against *R. solani*. Hadar et al. (1979) showed that *T. harzianum* applied in the form of wheat bran culture to *R. solani*-infested soil significantly controlled damping-off of bean, tomato, and eggplant seedlings. Elad et al. (1980) showed that wheat bran preparations of *T. harzianum* inoculum significantly decreased diseases caused by *R. solani* in beans, cotton, and tomatoes. Chet and Baker (1981) demonstrated that *T. hamatum* applied at a rate of 10^6 propagules per gram of soil, effectively controlled *R. solani* on radishes and beans. Lewis and Lumsden (2001) showed that the application of *T. hamatum* and *T. virens* reduced damping-off of eggplant, zinnia, cucumber, and cabbage caused by isolate R-23 of *R. solani*. Mechanisms used by *T. harzianum* to control *R. solani* include: (1) direct parasitism (mycoparasitism), *T. harzianum* directly attack the mycelia of *R. solani* (Hadar et al., 1979); (2) production of lytic enzymes such as chitinase and glucanase (Lorito, 1998), *Trichoderma* spp. enzymatic productions showed antifungal activity towards a broad spectrum of fungal pathogens (*Rhizoctonia*, *Fusarium*, *Alternaria*, *Ustilago*, *Venturia*) (Tronsmo, 1991; Lorito et al., 1993; Lorito et al., 1994);

and possibly (3) induced systemic resistance (ISR) in plant, De Meyer et al. (1998) showed the participation of induced plant defense by *T. harzianum* T39 effectively controlled *Botrytis cinerea* in tomato, lettuce, pepper, bean, and tobacco. We conclude that *T. harzianum* ThzID1 showed promise for biocontrol of *R. solani* in potato roots.

Considerations for the use of *Trichoderma harzianum* ThzID1 in agriculture

In order to recommend to a farmer to use *T. harzianum* ThzID1 in its field to control *F. solani* or *R. solani*, we will have to ensure that *T. harzianum* ThzID1 pass the attributes to be a successful biocontrol agent. Jatala (1986) listed some of the attributes of a successful biocontrol agent, as followed: (1) the biocontrol agent candidate must not be pathogenic to plants, humans, or other animals. *Trichoderma harzianum* ThzID1 has been evaluated for more than 20 years as a biocontrol agent and it has not been reported as plant pathogenic (Knudsen and Bin 1990; Knudsen et al., 1991; Dandurand and Knudsen, 1993; Mousseaux et al., 1998; Dandurand et al., 2000). *Trichoderma harzianum* ThzID1 has not been evaluated for pathogenicity in humans or animals. However, it has been reported that *Trichoderma* spp. have been associated with 12 cases of human infections, half of which were peritonitis (Munoz et al., 1997), caused by *T. harzianum* (Guiserix et al., 1996) and *T. koningii* (Campos-Herreros et al., 1996). But, isolates of *Trichoderma* spp. application in agriculture has not been reported to cause diseases in humans or animals (Harman et al., 2004); (2) the biocontrol agent candidate must reduce or suppress plant pathogenic fungi populations to a level below the damage threshold. *Trichoderma harzianum* ThzID1 in greenhouse experiment showed potential to reduce level of *Sclerotinia sclerotiorum* infection in pea roots (Knudsen et al., 1991) and to reduce root-rot caused by *Aphanomyces euteiches* f. sp. *pisi* in peas (Dandurand and Knudsen, 1993); (3) the biocontrol agent candidate must be capable of adapting to or tolerating varying soil-environmental and structural conditions long enough that it can locate and kill the plant pathogens or (inundative application) or of establishing itself to levels detrimental to the pathogens (augmentative application). Soil temperature affected radial growth rates of *T. harzianum* ThzID1 hyphae from alginate pellets and soil moisture between -0.03 and -0.5 Mpa did not significantly affect radial growth rates (Knudsen and Bin, 1990); (4) it should be biologically competitive with other existing or introduced parasites in the host ecosystem and also highly reproductive so that sufficient infective propagation units are

produced to maintain a high population. *Trichoderma harzianum* ThzID1 demonstrated the ability to rapidly colonize and reproduce at the expense of sclerotia of *Sclerotinia sclerotiorum* (Knudsen et al., 1991; Bae and Knudsen, 2006; Kim and Knudsen, 2009).

The potential for biological control of fungal plant pathogens using *T. harzianum* ThzID1 is considerable, but more evaluation of this biocontrol agent must be done in both greenhouse and in the field in order to make final recommendation to farmers for agricultural application. These evaluations should include experiments that would determine the optimum application rate of *T. harzianum* ThzID1 for optimal control of fungal pathogens in soil, trials that would test *T. harzianum* ThzID1 to control not only *F. solani* or *R. solani* but other fungal pathogens such as *Pythium* spp. in multiple cropping systems, and experiments to evaluate possible induced systemic resistance in plant by *T. harzianum* ThzID1.

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CHAPTER 3

Biological control of the pale cyst nematode *Globodera pallida* with *Trichoderma harzianum* ThzID1-M3 in potato

ABSTRACT

Trichoderma harzianum strain ThzID1-M3 isolated in the state of Idaho and transformed to express green fluorescent protein (GFP) was evaluated as a potential biocontrol agent against the pale cyst nematode (PCN) *Globodera pallida* in potato (*Solanum tuberosum* L.). *Globodera pallida* is a quarantine pest in the state of Idaho, globally regulated, and represents a major threat to Idaho potato industry.

The objectives of this study were to: (1) evaluate *T. harzianum* ThzID1-M3 as a biocontrol agent to reduce *G. pallida*, (a) infection and (b) reproduction in potato; (2) determine the ability of *T. harzianum* ThzID1-M3 to, (a) colonize nematode cysts, (b) proliferate in soil and (c) colonize the rhizoplane of potato. *Trichoderma harzianum* ThzID1-M3 maintained in *Trichoderma* selective media (TSM) was allowed to grow massively on sterile oat kernels at 25ºC for 20 days. *Globodera pallida* cysts were inserted inside nylon mesh bags at a rate of 0.73 eggs/g of soil and were hydrated for 3 days before application in clay pots with potato tubers. Experiments were conducted under greenhouse conditions at 18ºC ± 2ºC with 16:8 hr light: dark periods. The first experiment was conducted for 45 days to evaluate the level of *G. pallida* infection in potato roots, while the second experiment was conducted for 75 days to evaluate the level of *G. pallida* reproduction and the proliferation of *T. harzianum* ThzID1-M3 in cysts, roots and soil. Experimental treatments were as follows: potato only as the untreated control, *T. harzianum* ThzID1-M3 (only), PCN (only), and *T. harzianum* ThzID1-M3 applied together with PCN. Treatments were replicated five times in a completely randomized block design. Potato tubers cv ‘Russet Burbank’ were planted in a sterile sand and silt loam mixture (2:1).

Globodera pallida infection in potato roots were assessed using acid fuchsin staining method and *G. pallida* juveniles were counted under dissecting microscope. *Globodera pallida* reproduction in potato rhizosphere were assessed using the Fenwick method for cyst extraction from soil and *G. pallida* eggs were counted under dissecting microscope. *Trichoderma harzianum* ThzID1-M3 colonization in cyst, root and soil samples were

assessed using cultural plating in selective media and PCR analysis of extracted DNA from samples.

Results showed significant reduction in nematode infection in potato by 87% ($P < 0.05$). Significant reduction of *G. pallida* cysts in soil by 49.45% ($P < 0.05$) was observed. Significant reduction in *G. pallida* reproduction rate (Pf/Pi) by 60% was also observed ($P < 0.05$). *Trichoderma harzianum* ThzID1-M3 was able to colonize *G. pallida* cysts, the rhizoplane of potato, and to proliferate in soil. The introduced fungus *T. harzianum* ThzID1-M3 shows potential as a biocontrol agent for *G. pallida*.

INTRODUCTION

Globodera pallida Stone (1972) and Behrens (1975), a potato cyst nematode also known as the pale cyst nematode (PCN), is a root-parasitic nematode attacking potato plants (*Solanum tuberosum* L.) and other *Solanum* spp. In highly infested fields, *G. pallida* can reduce potato tuber yields up to 80% (Talavera et al., 1998; Vasyutin and Yakoleva, 1998) and provoke development of secondary diseases from damaged roots (Nicol et al., 2011). *Globodera pallida* is characterized by the development of cysts, which are the remains of the dead female body used to store nematode eggs (up to 600) after fertilization by adult males (Brodie et al., 1993). *Globodera pallida* cysts can survive in soil for many years (Perry, 1999). Spread of this pest is usually by contaminated soils, tubers, plants for transplanting or by farm machinery (Evans and Stone, 1977). Originated in the Andeans regions of South America, *G. pallida* was introduced into Europe in the 19th century on potatoes imported for breeding purposes (Evans et al., 1975; Baldwin and Mundo-Ocampo, 1991). Since then, *G. pallida* has spread into most potato growing areas of Europe, Canada, Mexico, Pakistan, India, Morocco, Sri Lanka, the Philippines, and Japan (Jatala, 1994).

In 2006, *G. pallida* was discovered in the U.S only in the state of Idaho. Subsequent sampling traced PCN to two fields in northern Bingham County, Idaho (Hafez et al., 2007). Idaho is the largest producer, packer, and processor of potatoes in the United States with a final value of $ 1.02 billion (USDA/NASS, 2014). The presence of *G. pallida* cysts in the most productive potato areas of Idaho represents a major threat to the Idaho potato industry. Hodda and Cook (2009), from a recent detection of *Globodera* spp. in Australia,

estimated cumulative losses to Australian agriculture over 20 years could exceed $ 370 million if no action to prevent spread of PCN was taken. Following *G. pallida* discovery in Idaho, Animal and Plant Health Inspection Service (APHIS) and the Idaho State Department of Agriculture (ISDA) designated the PCN as a quarantine pathogen of potato, and implemented a regulatory program designed to prevent the spread of the parasitic nematodes to other potato fields and to eradicate PCN from infested fields (USDA, 2008). The fumigant methyl bromide (MeBr) was allowed for use under Quarantine and Pre-shipment (QPS) within the comprehensive regulatory program for eradicating *G. pallida* in infected fields (USDA, 2008). However, due to environmental concerns, MeBr application was discontinued in 2015.

Trichoderma harzianum, a soilborne filamentous fungus, has been extensively used, evaluated and studied throughout the world as a biocontrol agent against many soilborne plant pathogens (Weindling, 1932; Hadar et al., 1979; Sivan et al., 1984; Knudsen et al., 1990, 1991; Dandurand and Knudsen, 1993; Bae and Knudsen, 2000, 2001, 2005; Dandurand et al., 2000; Harman et al., 2004). Mechanisms of biocontrol used by *T. harzianum* against soilborne pathogens consist in parasitism, referred as direct antagonism through mechanical and chemical actions (Chet et al., 1981; Barak et al., 1985; Papavizas, 1985); antibiosis and production of hydrolytic enzymes (Lorito et al., 1996; Howell, 1998); competition for space, nutrients and oxygen (Papavizas, 1985); root colonization by hyphal expansion (Altomare et al., 1999; Harman et al., 2004); plant growth promotion and induced resistance in host (Kleifeld and Chet, 1992; Windham et al., 1986; Howell et al., 2000; Altomare et al., 1999; Yedidia et al., 1999, 2000, 2003). Saifullah and Khan (2014) observed *T. harzianum* colonizing mature potato cyst nematodes by penetrating the cyst wall using chemical and physical (appresorium) mechanisms. *Trichoderma harzianum* showed the ability to colonize the root-knot nematode *Meloidogyne javanica* eggs, second-stage juveniles (J2), and overall demonstrated its potential to reduce root galling caused by *M. javanica* in tomatoes (Sharon et al., 2001; Sharon et al., 2007; Naserinasab et al., 2011; Lamovsek et al., 2013). Additionally, specific activities of resistance-related enzymes, peroxidase (POX), polyphenol oxidase (PAL), and phenylalanine ammonia lyase (PPO) increased significantly in *T. harzianum* inoculated tomato plants (Sahebani and Hadavi, 2008).

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). Bae and Knudsen (2000) transformed *T. harzianum* ThzID1 with three exogenous genes encoding GFP, *hyg* B resistance, and GUS. Stable transformant *T. harzianum* ThzID1-M3 was then produced. ThzID1-M3 was able to express green fluorescent protein (GFP) when placed under epifluorescence microscopy in around 550 nm wavelength excitation and processed with analytic imagery software. *Trichoderma harzianum* ThzID1-M3 has been revealed fundamental for quantifying proliferation of introduced *T. harzianum* into the soil (Orr and Knudsen, 2004). The GFP tool has been used by other researchers also to monitor activities of biocontrol agents. Maor et al. (1998) used GFP for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. Baehler et al. (2005) used the GFP tool to study the production of antifungal compounds in the biocontrol agent *Pseudomonas fluorescens*.

The objectives of this study were to: (1) evaluate *T. harzianum* ThzID1-M3 as a biocontrol agent to reduce, (a) *G. pallida* infection and (b) reproduction in potato; (2) determine the ability of *T. harzianum* ThzID1-M3 to colonize, (a) nematode cysts, (b) proliferate in soil and (c) colonize the rhizoplane of potato. We hypothesized that the addition of *T. harzianum* ThzID1-M3 with the potato seed piece reduces the level of infection and reproduction of *G. pallida*.

MATERIALS AND METHODS

Trichoderma harzianum ThzID1-M3

Trichoderma harzianum ThzID1-M3 was grown on *Trichoderma* selective medium (TSM) for 1 week at 25ºC. TSM, modified from (Elad et al., 1981), consisted of the following components (g/liter distilled water): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.15; NH_4NO3, 1.0; glucose, 3.0; chloramphenicol, 0.25; rose bengal, 0.15; streptomycin sulfate, 0.025; and agar, 20. Oat kernels were used as a growth substrate for *T. harzianum* ThzID1-M3 by placing 200 g of oat kernels in a 1000-ml Erlenmeyer flasks, adding 200 ml of distilled water, autoclaving twice for 1 hour at 121ºC, and allowed to cool down for 24 hours prior to inoculation with five fungal plugs (7mm diameter) from TSM plate. The fungus was incubated at 25ºC for 20 days prior to experimental use.

***Globodera pallida* cysts**

Cysts, with 53% hatching ability and 108.50 eggs/cyst, were surface-sterilized in a solution of 0.03% NaOCl for 5 minutes and rinsed thoroughly with sterile distilled water (Nour et al., 2003). Ten cysts were put inside a sterile nylon mesh bags (Sefar Nitex®, Buffalo, NY) with a 250 µm of mesh opening and 34% of open area screen. The nylon mesh was sealed with an impulse hand sealer (Sealer 8" F-200, Sealer sales Inc., Northridge, CA) along the edges, and were placed in sterilized distilled water for hydration for 3 days before amending to soil.

Soil mixture

Silt loam soil was obtained from Prosser, Washington, mixed in a 2:1 sand to silt loam, and autoclaved twice for 1 hour at 121ºC. The soil mixture was used 1 week after autoclaving.

Potato tubers

Potato tubers cv 'Russet Burbank', classified as certified disease free (from the Nuclear Potato Seed Program, University of Idaho), were sterilized for 1 minute in 0.4 % NaOCl, rinsed thoroughly in sterile distilled water, dried, and left for 1 week under dark conditions to break dormancy prior to planting.

Biocontrol experiments

Experiments were conducted in a 15-cm diameter size Terra Cotta clay pot (The Home Depot, Atlanta, GA). There were two experiments. The first experiment was conducted for 45 days to evaluate the level of *G. pallida* infection in potato roots. The second experiment was allowed to grow for 75 days prior to evaluation of reproduction by *G. pallida*. At this time, proliferation of *T. harzianum* ThzID1-M3 in cysts, roots and soil, was also evaluated. There were four treatments for each experiment: (1) untreated control, (2) ThzID1-M3 only, (3) PCN only, and (4) ThzID1-M3 applied together with PCN. Treatments included five replicates in a completely randomized block design. Five grams of oat kernels colonized by *T. harzianum* ThzID1-M3 fungal inoculum 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 of soil before placing one potato tuber and covering with soil. *Trichoderma harzianum* ThzID1-M3 was applied at a rate of 10^6 conidia/g of oat kernel. Pots were maintained at 18ºC ± 2ºC, 60% relative humidity, and 16:8-hours light: dark

period under greenhouse conditions. Pots were watered twice daily in the amount of 500 ml liters 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 of water. At the end of each experiment, stems were cut, measured and weighed; roots were cleaned and weighed; and root, cyst, and soil samples for each treatment were collected for further analysis.

***Globodera pallida* infection in potato roots**

Acid Fuchsin (Sigma-Aldrich®, St-Louis, Mo), an acidic dye, was used to stain *G. pallida* juveniles in potato roots to quantify and determine the infection level (modified from Byrd et al., 1983). Roots were washed, chopped into 1-2 cm segments, and placed in a 200-ml beaker with 90 ml of tap water. Twenty milliliters of chlorine bleach (8.25% NaOCl, Clorox Company, Oakland, CA) were added to give 1.5% NaOCl, and root segments were allowed to remain in this solution for 4 minutes. Root segments were washed thoroughly with running water for 45 seconds, and then immersed in tap water for 15 minutes to remove any NaOCl residual. Root segments were transferred to a beaker containing 50 ml of tap water to which 2 ml of stock stain solution (3.5 g acid fuchsin, 250 ml acetic acid, and 750 ml distilled water) were added, and boiled for 30 seconds in a microwave oven. Stain solution was allowed to cool down to room temperature, drained and root segments were rinsed in running tap water. Roots were de-stained in boiling 30 ml of glycerin (99% concentrated, Sigma-Aldrich®, St-Louis, Mo) acidified with 5 M HCl, and cooled down. Root segments were observed under a dissecting microscope (Leica M80, Leica Microsystems, Wetzlar, Germany) for *G. pallida* counting.

***Globodera pallida* reproduction in potato rhizosphere**

Extraction of cysts from soil was performed using a modified Fenwick flotation plastic cylinder (Fenwick, 1940). Soil samples were air dried for 3 weeks prior to extraction so that cysts would float during extraction. For each replicate, the air-dried soil sample was poured over a No. 20 USA Standard Testing Sieve (W.S Tyler Inc., Mentor, OH) of 0.841 mm opening, placed on top of plastic cylinder and water was poured over the soil sample. Water, organic matter and cysts flew onto a No. 60 USA Standard Testing Sieve (W.S Tyler Inc., Mentor, OH) of 0.250 mm opening, and soil remained at the bottom of the cylinder. Cysts and debris were collected and rinsed into a container, and later poured in a

petri dish and counted under a dissecting microscope (Leica M80, Leica Microsystems, Wetzlar, Germany). Cysts were detected floating or sunk in the water sample and were sorted and placed in a sterile glass petri dish.

Extracted cysts were evaluated for egg counting. Ten cysts were sterilized in 0.3% NaOCl, washed with sterile distilled water and placed in a 1.5-ml microtube. Cysts were crushed in 100 µl sterile distilled, 10 µl of the solution were pipetted in a slide and observed under a dissecting microscope (Leica M80, Leica Microsystems, Wetzlar, Germany) for egg counting. Reproduction factor or nematode multiplication rate (Pf/Pi) was calculated based on the ratio of the final egg population density (Pf) over the initial egg population density (Pi) per gram of soil.

***Trichoderma harzianum* ThzID1-M3 colonization in root, cyst and soil samples**

To assess the presence of *T. harzianum* ThzID1-M3 in potato roots, *G. pallida* cysts and soil samples, 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 potato rhizoplane and *G. pallida* cysts.

Root segments were ground in a sterilized porcelain mortar and DNA was extracted using the DNeasy plant DNA extraction kit (Qiagen Inc., Venlo, Netherlands) according to manufacturer protocol. DNA from root samples was eluted twice in 50 µl of AE buffer and stored at -20ºC before PCR amplification. Ten cysts were placed in a 1.5-ml microtube, crushed in sterile distilled water, and DNA was extracted using the DNeasy plant DNA extraction kit (Qiagen Inc., Venlo, Netherlands) according to manufacturer protocol. DNA from cyst samples was eluted twice in 100 µl of AE buffer and stored at -20ºC before PCR amplification. DNA was extracted from soil samples using PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA) according to manufacturer protocol. DNA from soil samples was eluted once in 50 µl of solution C6 and stored at -20ºC before PCR amplification.

DNA 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, 2007). Forward and Reverse primers were obtained from Sigma-Aldrich® (Sigma-Aldrich®, St-Louis, Mo). Forward primer sequence (TGP4-F) was as follows: 5'-CTCCCAAACCCAATGTGA 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. Each PCR reaction mixture contained 0.8 µl of TGP4-F, 0.8 µl of TGP4-R, 10 µl of PCR Master Mix, 7.8 µl of PCR water, and 1 µl of genomic DNA as template. The PCR cycling protocol consisted of the following: 94ºC for 60 s; 94º C for 60 s; 51ºC for 60 s; 72ºC for 60 s; and repeat for 35 cycles. PCR products were assessed for the presence of *T. harzianum* ThzID1-M3 using a Mini-Sub® Cell GT Cell electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA) in a 1.5% agarose gel (Sigma-Aldrich®, St-Louis, Mo), for 30 minutes at 75 volts.

Roots were sterilized in 1% NaOCl, washed thoroughly in sterile distilled water and cut into 1-cm segments. Five 1-cm root segments were plated in *Trichoderma* selective media (TSM) to stimulate *T. harzianum* ThzID1-M3 growth and were replicated four times. Plates were incubated at 25ºC for 1 week and *T. harzianum* ThzID1-M3 was identified through microscopy observation (Leica M80, Leica Microsystems, Wetzlar, Germany), and average percentage of root colonization was calculated for each treatment. 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 (Barnett and Hunter, 1998; Gams and Bissett, 2002).

Globodera pallida cysts, from recovered cyst bags, were sterilized in 0.3% 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 and assess the presence of *T. harzianum* ThzID1-M3 at $1{:}10^3$, $1{:}10^4$, $1{:}10^5$ and $1{:}10^6$ fold-dilution (Dandurand and Knudsen, 2002; Madigan et al., 2012). Three hundred µl from each diluted solution was plated in TSM, spread over media surface and incubated at 25ºC for 48 hours to allow fungal development. *Trichoderma harzianum* ThzID1-M3 was assessed and counted through microscopy observation (Leica M80, Leica

Microsystems, Wetzlar, Germany) using morphological characteristics of *Trichoderma* spp. for identification (Barnett and Hunter, 1998; Gams and Bissett, 2002).

Serial dilution method was used to quantify and assess the presence of *T. harzianum* ThzID1-M3 in soil samples (Dandurand and Knudsen, 2002; Madigan et al., 2012). Ten grams of soil samples were mixed in 90 ml of sterile distilled water to yield a 10-fold dilution (10:1) and was vortexed. One ml of the 10:1 dilution was added to 9 ml of sterile distilled water to yield $1:10^3$ to $1:10^6$ fold-dilution. Five hundred µl from each diluted solution was plated in TSM, spread over media surface and incubated at 25ºC for 48 hours to allow fungal development. *Trichoderma harzianum* ThzID1-M3 was assessed and counted through microscopy observation (Leica M80, Leica Microsystems, Wetzlar, Germany) using morphological characteristics of *Trichoderma* spp. for identification (Barnett and Hunter, 1998; Gams and Bissett, 2002).

Green fluorescent proteins (GFP) activities in *T. harzianum* ThzID1-M3 from the surface of root segments and individual *G. pallida* cyst were detected using a Leica DM 2500 microscope (Leica Microsystems, Wetzlar, Germany) equipped with 100 W halogen illumination, 5 fluorescence filters and a bandpass filter of 515-560 nm light excitation that was used to observe GFP of *T. harzianum* ThzID1-M3.

Statistical analysis

This study was conducted as a completely randomized block design. All analyses were performed using SAS package 9.4 (SAS Institute Inc., Cary, N.C.). Proc GLIMMIX procedure was performed to analyze root weight, biomass, *G. pallida* infection and reproduction, and colonization of soil, cysts and roots by *T. harzianum* ThzID1-M3 with significant differences occurring at level of $P < 0.05$.

RESULTS

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* infection

Significant reduction in infection by *G. pallida* J2, J3, J4 and adult females occurred in the *T. harzianum* ThzID1-M3 treatment compared to PCN treatment only ($P < 0.05$). No significant differences were observed in root weight for all treatments ($P > 0.05$), however *T. harzianum* ThzID1-M3 applied only or together with PCN showed significant decrease in biomass ($P < 0.05$) compared to control treatment (Table 3.1).

Table 3.1 Influence of *Trichoderma harzianum* ThzID1-M3 on potato growth and *Globodera pallida* root infection

Treatment*	Root weight** (g)	Biomass (g)	J2/g root	J3/g root	J4/g root	Adult female/g root
Untreated control	71.34[a] (8.5)	250[a] (25.4)	0	0	0	0
ThzID1-M3	52.96[a] (3.4)	179[b] (10.8)	0	0	0	0
PCN	57.80[a] (5.4)	224[ab] (2.9)	0.02(0.02)	0.20(0.14)	2.49[a] (1.46)	0.05(0.05)
PCN+ThzID 1-M3	52.54[a] (7.1)	185[b] (11.1)	0	0	0.40[b] (0.21)	0

*Data from the first experiment at 45 days.
** Means with the same letter are no significantly different ($P > 0.05$) as determined by Tukey-Kramer adjustment for multiple comparison. Standard error of the mean is shown in parenthesis.

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* reproduction

Globodera pallida cysts were significantly reduced in soil by 49.45% when applied with *T. harzianum* ThzID1-M3 compared to PCN treatment only ($P < 0.05$). Significant reductions were observed for nematode reproduction rate (Pf/Pi) by 60% compared to PCN treatment only ($P < 0.05$). No significant differences were observed for root weight and biomass of potato plants ($P > 0.05$) (Table 3.2).

Table 3.2 Influence of *Trichoderma harzianum* ThzID1-M3 on potato growth and *Globodera pallida* reproduction

Treatment*	Root weight** (g)	Biomass (g)	Cyst/g soil	Pi/g soil	Pf/g soil	Pf/Pi
Untreated control	61[a] (12.21)	255[a] (38.95)	0	0	0	0
ThzID1-M3	54[a] (5.51)	249[a] (12.26)	0	0	0	0
PCN	59[a] (7.39)	250[a] (19.29)	0.04[a] (0)	0.72[a] (0)	6.40[a] (1.86)	8.85[a] (2.57)
PCN+ThzID1-M3	63[a] (6.31)	265[a] (14.62)	0.02[b] (0)	0.72[a] (0)	2.56[b] (1.69)	3.54[b] (2.33)

*Data from the second experiment at 75 days.
** Means with the same letter are no significantly different ($P > 0.05$) as determined by Tukey-Kramer adjustment for multiple comparison. Standard error of the mean is shown in parenthesis.
Pi: initial nematode population density (eggs/g soil)
Pf: final nematode population density (eggs/g soil)
Pf/Pi: Nematode reproduction rate

Colonization of *Globodera pallida* cysts by *Trichoderma harzianum* ThzID1-M3

Trichoderma harzianum ThzID1-M3 was able to colonize *G. pallida* cyst surfaces. The average of ThzID1-M3 conidia forming unit per cyst was estimated at 1.41 x 10^5 CFU/cyst. Under fluorescence microscopy (Leica Microsystems, Wetzlar, Germany), *T.*

harzianum ThzID1-M3 hypha were detected growing over individual cyst surface, but no colonization of eggs was observed (Figure 3.1). PCR analysis confirmed the presence of *T. harzianum* ThzID1-M3 in cyst DNA samples, visible by the occurrence of white band in agarose gel after electrophoresis (Figure 3.2).

Colonization of potato roots by *Trichoderma harzianum* ThzID1-M3

Trichoderma harzianum ThzID1-M3 was able to colonize potato roots. Average root colonization by plate assay was 10% and no significant differences of colonization were observed between *T. harzianum* ThzID1-M3 treatment only and *T. harzianum* ThzID1-M3 applied with PCN ($P > 0.05$). Fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) revealed *T. harzianum* ThzID1-M3 hyphal growth over the surface of roots (Figure 3.3). PCR analysis confirmed the presence of *T. harzianum* ThzID1-M3 in root DNA samples, visible by the occurrence of strong white band in agarose gel after electrophoresis (Figure 3.4).

Colonization of soil media by *Trichoderma harzianum* ThzID1-M3

Trichoderma harzianum ThzID1-M3 was able to proliferate in the soil after 75 days. Average soil colonization was 10^5 CFU/g of soil for both treatments with no significant differences among treatments ($P > 0.05$). PCR analysis confirmed the presence of *T. harzianum* ThzID1-M3 in soil DNA samples, visible by the occurrence of white band in agarose gel after electrophoresis (Figure 3.5).

DISCUSSION

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* infection

The addition of *T. harzianum* ThzID1-M3 with PCN showed significant decrease of nematode populations inside potato roots ($P < 0.05$) compared to PCN treatment alone. This result suggests that *T. harzianum* ThzID1-M3 was able to disrupt the nematode life cycle by direct or indirect interactions with *G. pallida*. Other studies using different fungal biocontrol agents and plant-parasitic nematodes showed similar results on infection control. The biocontrol fungus *Paecilomyces lilacinus* reduced numbers of *Meloidogyne incognita* J2 in field-grown tomato by 70% and 41% when applied at transplant and 2 weeks after transplanting, respectively (Martez et al., 1996). Saifullah and Thomas (1996) described similar interactions between *T. harzianum* and the potato cyst nematode *G.*

rostochiensis where the fungus was able to colonize both cysts and eggs resulting in larval death, whereas in these experiments cysts but not eggs were colonized by *T. harzianum* ThzID1-M3. Kiewnick and Sikora (2006) demonstrated that a single pre-plant application of *P. lilacinus* at a concentration of 1 x 10^6 CFU/g soil significantly reduced *M. incognita* in tomato roots. Goswami et al. (2008) showed that *P. lilacinus* suppressed galling of tomato by *M. incognita* by 39% when applied at transplant. *Trichoderma longibrachiatum* was found to decrease significantly *Heterodera avenae* infection in wheat by colonizing and degrading *H. avenae* cysts through extracellular chitinase activity (Zhang et al., 2014). In field experiment, *T. harzianum* reduced galling on tomato roots by 47% compared to untreated plots (Goswami et al., 2008).

We concluded that *T. harzianum* ThzID1-M3 shows promise as a biocontrol agent to reduce *G. pallida* infection in potato roots. Future biocontrol experiment using *T. harzianum* ThzID1-M3 should be focus on field application trials in order to test the efficacy of *T. harzianum* ThzID1-M3 against *G. pallida* in a competitive soil microbial ecosystem.

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* reproduction

Our result showed significant reduction of cyst formation in soil amended with *T. harzianum* ThzID1-M3 ($P < 0.05$); cysts reduction in soil was 49.45% compared to the PCN only treatment. Significant reduction in the reproduction rate (Pf/Pi) by 60% was also observed ($P < 0.05$) by amendment with *T. harzianum* ThzID1-M3. Similarly, Tobin et al. (2008) showed that the fungus *Pochonia chlamydosporia* was able to significantly reduce nematode reproduction rate (Pf/Pi) in potato cyst nematode *Globodera* spp. in two field experiments at a rate of 48% and 51% respectively. The ability of *T. harzianum* ThzID1-M3 to significantly reduce *G. pallida* reproduction rate is an important strategy in the reduction of the level of inoculum in soil and eventually in time will contribute to the eradication of this pest in infested potato fields.

We concluded that *T. harzianum* ThzID1-M3 shows promise as a biocontrol agent to reduce *G. pallida* cysts in soil. Future biocontrol experiment should focus on field application trials in order to test the efficacy of *T. harzianum* ThzID1-M3 against *G. pallida* in a competitive soil microbial ecosystem.

Trichoderma harzianum ThzID1-M3 colonization of *Globodera pallida* cysts

Our result showed that *T. harzianum* ThzID1-M3 was able to colonize *G. pallida* cysts, although no egg colonization was observed. Similarly, Zhang et al. (2014) reported the colonization and dissolution of *Heterodera avenae* cysts by the hyphae and the metabolite of *T. longibrachiatum*, but no direct egg colonization was observed. However, Saifullah and Thomas (1996) showed that *T. harzianum* was able to colonize both cysts and eggs of *G. rostochiensis*. Even though, 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 (Sharon et al., 2001; Suárez et al., 2004; Naserinasab et al., 2011; Szabó et al., 2012; Lamovsek et al., 2013). Through direct parasitism of cyst and eggs, activities of chitinolytic enzymes of *Trichoderma* spp. are thought to be responsible for reduction in nematode infestation in plant roots (Sharon et al., 2001; Suárez et al., 2004; Szabó et al., 2012).

We concluded that *T. harzianum* ThzID1-M3 showed the ability to colonize *G. pallida* cysts. However, future biocontrol experiment should be focused on examining the ability of *T. harzianum* ThzID1-M3 to colonize *G. pallida* eggs, which is an important mechanism for disrupting the nematode life cycle.

Trichoderma harzianum ThzID1-M3 colonization of potato rhizoplane and rhizosphere

Trichoderma harzianum ThzID1-M3 was able to colonize potato rhizoplane and rhizosphere as confirmed by PCR analysis of root and soil DNA samples, and no significant differences between *T. harzianum* ThzID1-M3 applied only and *T. harzianum* ThzID1-M3 applied together with PCN were observed ($P > 0.05$). Rhizosphere colonization by *T. harzianum* contributes to the protection of the infection court and also favors *T. harzianum* growth and proliferation into the soil, and the ability to colonize plant roots has often been stressed as an important requirement of *Trichoderma* spp. to act as biocontrol agents (Harman et al., 2004).

Similar results showed that *T. harzianum* was detected in the rhizosphere of the upper half of pea roots (Chao et al., 1986). Ahmad and Baker (1987) described the ability of *Trichoderma* strains to grow and compete with other microorganisms to colonize the root

surface and compete with them for nutrients secreted by root exudates. Knudsen and Bin (1990) reported the addition of a food base had a positive effect on proliferation of *T. harzianum* ThzID1 in soil, thus enhancing its biocontrol spatial outreach into the soil. Harman (2006) showed that *Trichoderma* spp. can colonize the root epidermis and outer cortical layers and increased plant growth and nutrient uptake.

Studies have shown that *Trichoderma* spp. have the ability to turn on specific disease-resistance genes in plants, such as *PAL1* in cucumber roots colonized by *T. asperellum*, in a process known as induced systemic resistance (ISR) (Yedidia et al., 2000, 2003; Harman et al., 2004; Shoresh et al., 2010). Calderón et al. (1993) demonstrated that suspension cell cultures of grapevine treated with an elicitor from *T. viride* showed a hypersensitive-like response characterized by cell plasmolysis accompanied by localized cell death due to a large increase in endogenous levels of H_2O_2. Yedidia et al. (1999), in an experiment with cucumber plants, showed evidence that *T. harzianum* was able to penetrate the root system without causing extensive damage and trigger plant host defense reactions by increasing the level of plant peroxidase and chitinase activities. Thus *Trichoderma*-inoculated plants may be sensitized to respond faster and to a greater extent to potential pathogen attacks. Future biocontrol research need to focus on revealing the process of induced systemic resistance in plant by *T. harzianum* ThzID1-M3, elucidating the mechanisms behind ISR activation, and determining ISR potential against *G. pallida*.

We conclude that *T. harzianum* ThzID1-M3 showed potential for biocontrol of *G. pallida* in potato roots by possibly disrupting the nematode's life cycle, colonizing *G. pallida* cysts, potato roots and soil. Further research is needed to understand the interactions between *T. harzianum* ThzID1-M3 and *G. pallida* in order to implement an effective and stable biocontrol strategy against this important pest of potato.

Considerations for the use of *Trichoderma harzianum* ThzID1-M3 in agriculture

Our results demonstrated that *T. harzianum* ThzID1-M3 showed promises for biocontrol of *G. pallida*. Our experiments showed that *T. harzianum* ThzID1-M3 was able to reduce *G. pallida* infection and reproduction in potato, to reduce *G. pallida* cysts in soil, and to colonize the rhizosphere and rhizoplane of potato. However, the activity of *T. harzianum* ThzID1-M3 as a plant growth promoter could not be assessed in our experiments. One explanation for this inconclusive result could be related to the

application rate of *T. harzianum* ThzID1-M3 and to the timeline for the evaluation of the experiment. Therefore, we suggest that the use of potato plantlets, grown previously in tissue culture medium, for planting in pots instead of tubers might allow rapid root colonization by *T. harzianum* ThzID1-M3, and therefore we could observe significant increase in potato growth.

Before making final recommendations to the farmers to use *T. harzianum* ThzID1-M3 as a biocontrol agent against *G. pallida*, we should: (1) test the efficacy of *T. harzianum* ThzID1-M3 on field application trials in order to test its behavior in a competitive soil microbial ecosystem; (2) examine the ability of *T. harzianum* ThzID1-M3 to colonize *G. pallida* eggs, which is an important mechanism for disrupting the nematode life cycle; (3) determine the process of induced systemic resistance (ISR) in plant by *T. harzianum* ThzID1-M3, elucidating the mechanisms behind ISR activation, and determining ISR control potential against *G. pallida.* Only after these exhaustive field trials that we can make final recommendations to the farmers, and for now we are compiling evidence that showed *T. harzianum* ThzID1-M3 as a potential biocontrol agent.

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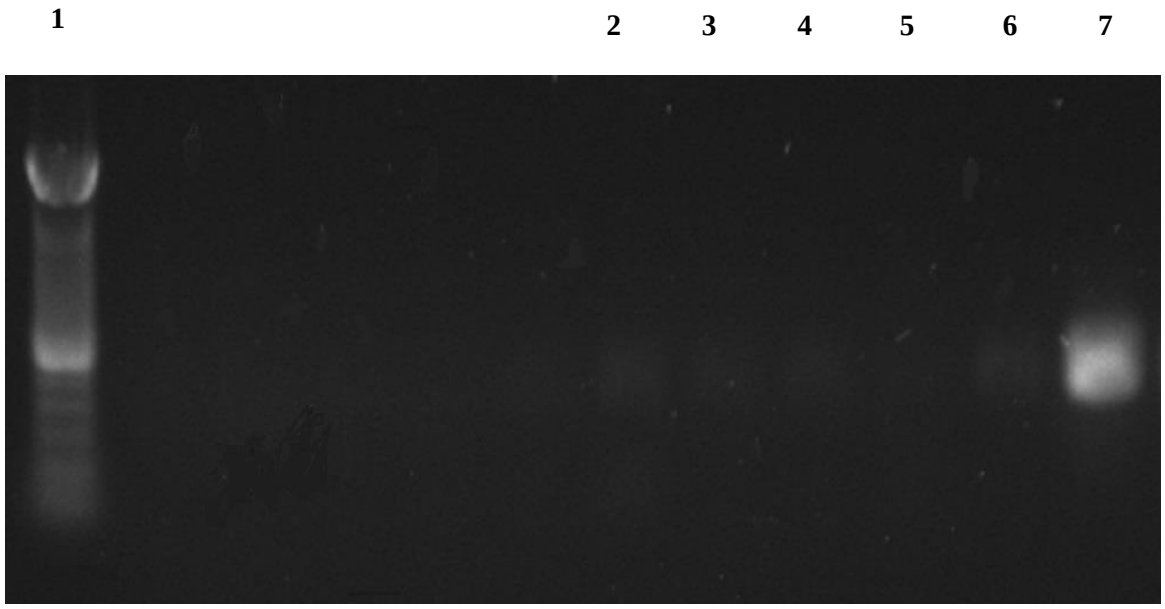

Fig. 3.1. Conventional PCR result of the *Trichoderma* genus-specific primer set (TGP4) on genomic DNA of cyst samples. Lane 1, low mass marker (Invitrogen); lane 2 to 5 represent the presence of *T. harzianum* ThzID1-M3 on DNA cyst samples; lane 7, positive control of *T. harzianum* ThzID1-M3.

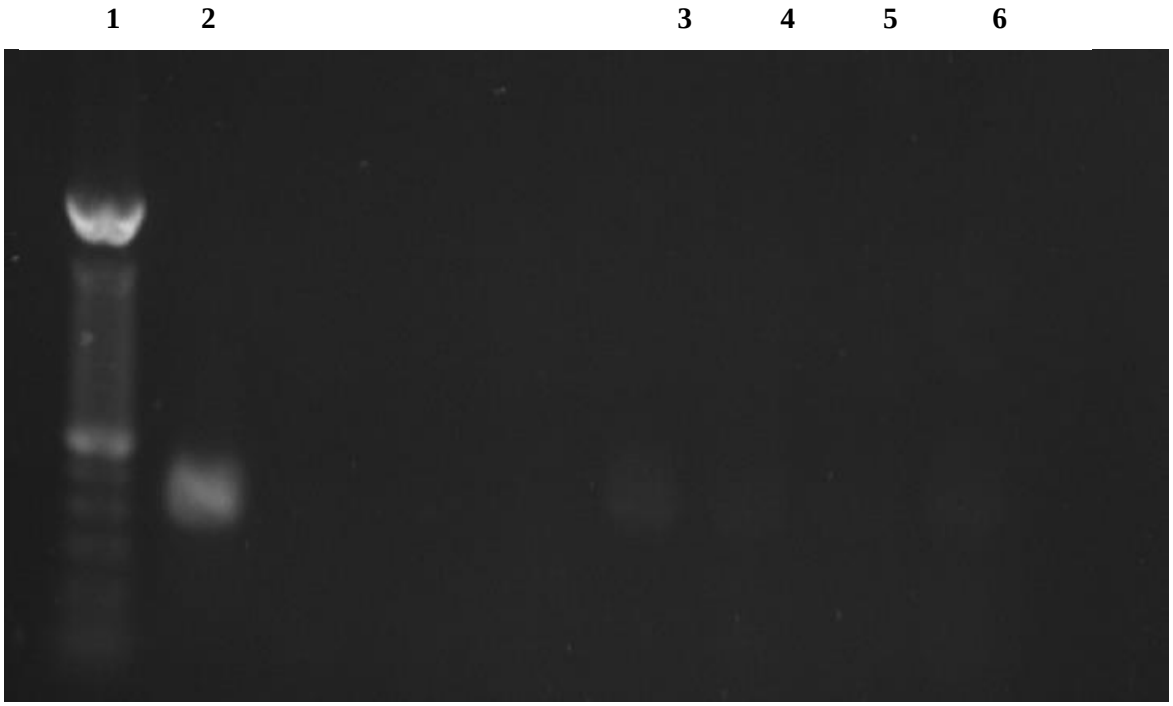

Fig. 3.2. Conventional PCR result of the *Trichoderma* genus-specific primer set (TGP4) on genomic DNA of root samples. Lane 1, low mass marker (Invitrogen); lane 2, positive control of *T. harzianum* ThzID1-M3; lane 3 to 6 represent the presence of *T. harzianum* ThzID1-M3 on DNA potato root samples.

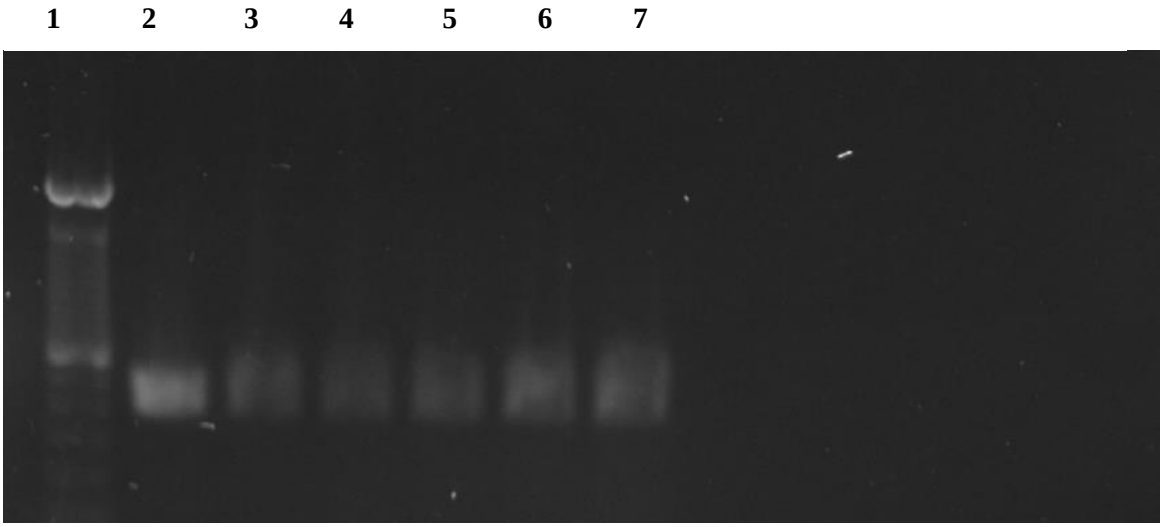

Fig. 3.3. Conventional PCR result of the *Trichoderma* genus-specific primer set (TGP4) on genomic DNA of soil samples. Lane 1, low mass marker (Invitrogen); lane 2, positive control of *T. harzianum* ThzID1-M3; lane 3 to 7 represent the presence of *T. harzianum* ThzID1-M3 on DNA soil samples.

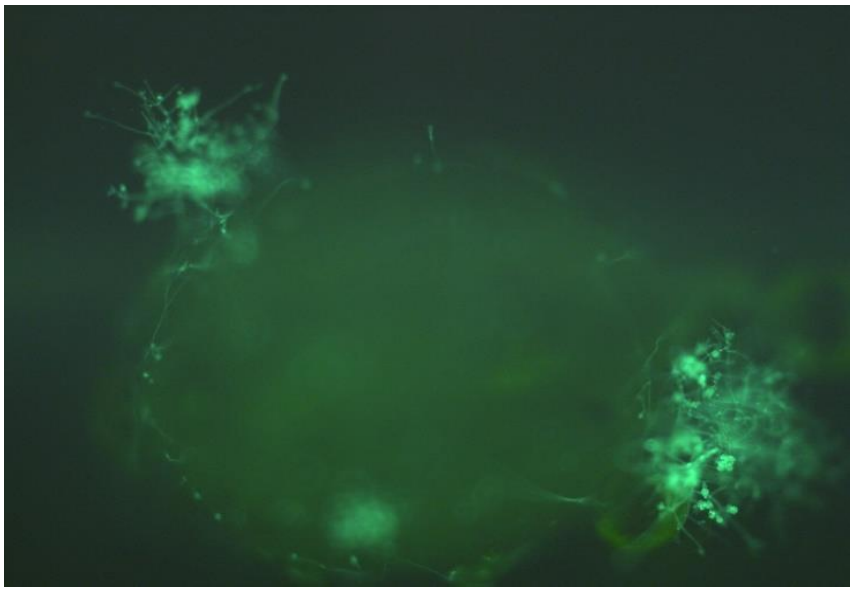

Fig. 3.4. *Globodera pallida* cyst colonized by *Trichoderma harzianum* ThzID1-M3. Observed under fluorescence microscopy (Leica DM 2500, Leica Microsystem, Wetzlar, Germany).

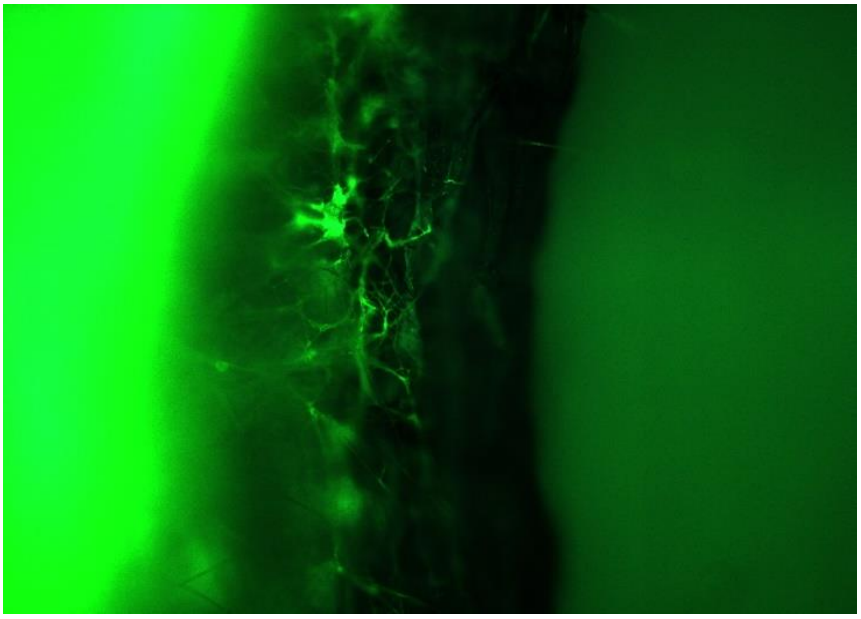

Fig. 3.5. Potato root segment colonized by *Trichoderma harzianum* ThzID1-M3. Observed under fluorescence microscopy (Leica DM 2500, Leica Microsystem, Wetzlar, Germany).

CHAPTER 4

Microscopic observations of biocontrol activity of *Globodera pallida* by *Trichoderma harzianum* ThzID1-M3 in Microscopy Rhizosphere Chambers, Micro-ROCs

ABSTRACT

Globodera pallida, the pale cyst nematode (PCN), has been classified as a quarantine pest in the state of Idaho, U.S, where it was first detected in 2006 in a potato processing facility. *Globodera pallida* can significantly reduce yield of potato in highly infested fields. The fungus *Trichoderma harzianum* shows promise for biological control of plant pathogenic nematodes. *Trichoderma harzianum* strain ThzID1-M3, engineered to express green fluorescent protein, GFP, was tested as a biocontrol agent against PCN second stage juveniles (J2s). The objectives of this study were to determine the mechanisms used by *T. harzianum* ThzID1-M3 to reduce *G. pallida* second stage juveniles (J2s) infection in potato roots and to colonize the potato rhizoplane. The experiment was conducted using plastic rhizosphere chambers designed to allow microscopic observation of roots. The dimensions of the chambers were 70 mm x 50 mm x 22 mm, and a corrosion resistant cover glass formed the observation area. Tissue culture potato ('Russet Burbank') germlings were inserted between the cover glass and the filter paper, and the adjacent slot was filled with the soil medium. There were two treatments: *G. pallida* second stage infectious juveniles (J2s) only applied to non-amended soil (control) and *G. pallida* J2s applied to soil amended with the biocontrol fungus *T. harzianum* ThzID1-M3. Each treatment was replicated five times and arranged in a completely randomized design and the experiment was repeated. *Trichoderma harzianum* ThzID1-M3 was applied as crushed PDA over the potato root surface and left to grow for 1 week before inoculating infectious second stage juvenile nematodes (J2s) over the potato root surface. Results showed that *T. harzianum* ThzID1-M3 was able to colonize the potato root cortex and *T. harzianum* ThzID1-M3 was able to colonize and kill *G. pallida* (J2s) prior to root infection which significantly reduced by 67% infection compared to the non-amended control ($P < 0.05$). We conclude that *T. harzianum* ThzID1-M3 has the potential for biological control of *G. pallida* by directly attacking second stage juveniles that are migrating to roots and reducing the number of J2s that infect the root.

INTRODUCTION

Globodera pallida known as the pale cyst nematode (PCN) is a quarantined pest in the state of Idaho, where it was first discovered in the U.S in 2006 (Hafez et al., 2007). *Globodera pallida* is a root-parasitic nematode that attacks potato (*Solanum tuberosum* L.) and other *Solanum* spp. *Globodera pallida* infestation in the field could reduce potato tubers yields up to 80% (Talavera et al., 1998; Vasyutin and Yakoleva, 1998) and also favors the development of secondary diseases that further damage potato roots and tubers (Nicol et al., 2011). One characteristic of *G. pallida* is the development of a cyst, which is the dead female body containing several hundred nematode eggs (Brodie et al., 1993). *Globodera pallida* cysts can survive into soil for many years (Perry, 1999) and the pest is usually spread by contaminated soils, tubers, plants for transplanting or by farm machinery (Evans and Stone, 1977).

Linford (1937) began research into the use of biological control agents against plant-parasitic nematodes and since then a plethora of biological control agents have been used or evaluated against plant-parasitic nematodes. Examples of fungi biocontrol agents include: *Acremonium* sp. against *Globodera*/*Heterodera* spp. (Crump, 1998); *Arthrobotrys* spp. against *Meloidogyne* spp. (Jaffee et al., 1998); *Cylindrocarpon destructans* against *Meloidogyne javanica* and *Globodera* sp. (Freitas et al., 1995; Crump and Flynn, 1995); *Fusarium oxysporum* against *Meloidogyne incognita* (Hallman and Sikora, 1996); *Trichoderma harzianum* against *Heterodera cajani* (Siddiqui and Mahmood, 1996); *Verticillium chlamydosporium* against *Globodera* spp. (Crump, 1998), *Heterodera schachtii* (Jalali et al., 1998), *Meloidogyne* spp. (Kerry, 1995). The importance of fungi in the decline of cereal cyst nematodes below the economic threshold has been clearly demonstrated (Kerry, 1975, 1984; Kerry et al., 1982; Kerry and Crump, 1998).

Trichoderma harzianum, a soilborne filamentous fungus, has been extensively evaluated throughout the world as an effective biocontrol agent against many soilborne plant pathogens (Weindling, 1932; Hadar et al., 1979; Sivan et al., 1984; Knudsen et al., 1990, 1991; Dandurand and Knudsen, 1993; Bae and Knudsen, 2000, 2001, 2005; Dandurand et al., 2000; Sharon et al., 2001; Harman et al., 2004). Mechanisms of biocontrol used by *T. harzianum* against soilborne pathogens consist in mycoparasitism, referred as direct antagonism through mechanical and chemical actions (Chet et al., 1981;

Barak et al., 1985; Papavizas, 1985); antibiosis and production of hydrolytic enzymes (Lorito et al., 1996; Howell, 1998); competition for space, nutrients and oxygen (Papavizas, 1985); root colonization by hyphal expansion (Altomare et al., 1999; Harman et al., 2004); plant growth promotion and induced resistance in host (Kleifeld and Chet, 1992; Windham et al., 1986; Altomare et al., 1999; Howell et al., 2000; 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). Bae and Knudsen (2000) transformed *T. harzianum* ThzID1 with three exogenous genes encoding GFP, *hyg* B resistance, and GUS. *Trichoderma harzianum* ThzID1-M3 was able to express green fluorescent protein (GFP) when placed under epifluorescence microscopy in around 550 nm wavelength excitation and processed with analytic imagery software (Leica Microsystem, Wetzlar, Germany). *Trichoderma harzianum* ThzID1-M3 has proved to be fundamental for quantifying and observing proliferation of introduced *T. harzianum* in soil (Orr and Knudsen, 2004). The GFP tool has also been used by other researchers to monitor activities of biocontrol agents. Maor et al. (1998) used GFP for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. Baehler et al. (2005) used a GFP-transformed *Pseudomonas fluorescens* to study its production of antifungal compounds.

The objectives of this study were to determine the mechanisms used by *T. harzianum* ThzID1-M3 to reduce *G. pallida* second stage juveniles (J2s) infection in potato roots and to colonize the potato rhizoplane. We hypothesized that the inoculation of potato plantlet roots with *T. harzianum* ThzID1-M3 reduces the level of infections by *G. pallida* second stage juveniles (J2s).

MATERIALS AND METHODS

Trichoderma harzianum ThzID1-M3

Trichoderma harzianum ThzID1-M3 was allowed to grow on potato dextrose agar (PDA) for 1 week at 25ºC. PDA contained 24 g of potato dextrose, 17 g of agar, and 25 ppm of streptomycin.

Potato root diffusate (PRD)

Potato tissue culture plantlets, cv 'Russet Burbank' classified as certified disease free (from the Nuclear Potato Seed Program, University of Idaho), transplanted to a 15-cm Terra Cotta clay pots (The Home Depot, Atlanta, GA), were maintained at 18ºC and 16:8 light: dark conditions, watered daily in the amount of 500 ml of water for 5 weeks and were used to obtain potato root diffusate (PRD). Two hundred ml of distilled water were poured through the pot and the flow through was collected in a 100 ml beaker. The PRD was filtered and sterilized using bottle top filter of 0.2 µm size and frozen at -20ºC until used.

Potato tissue culture

Nodal stem segments (1-cm long) from potato plant, 'Russet Burbank' cultivar classified as certified disease free (from the Nuclear Potato Seed Program, University of Idaho), were cut in a sterile environment and placed inside a mini-jar containing 6 to 7 ml of propagation medium. The propagation medium contained the following ingredients per liter of distilled water (modified from Chronis et al., 2014): MS salt, 4.3 g; D-gluconic acid, 5 g; sucrose, 30 g; agar, 5.5 g, pH was adjusted between 5.6 and 5.7, the solution was microwaved for 7 min to allow dissolution of the agar, autoclaved for 30 min. at 121 ºC and was allow to cool at room temperature before use. Potato tissue culture plantlets were incubated at room temperature for 1 week before use.

Egg hatch

Globodera pallida cysts were surface sterilized with 0.5 % NaOCl solution for 5 minutes, rinsed thoroughly with sterile distilled water and were hydrated for 3 days in sterile distilled water at room temperature in a shallow petri dish to allow for gas exchange. The cysts were then collected and put inside a hatching chamber, made of 30-µm nylon mesh (Sefar Nitex®, Buffalo, NY) with a 250 µm of mesh opening and 34% of open area screen, attached to a 2-cm diameter plastic pipe and placed inside a corning centrifuge tube. Two ml of fresh sterilized water, 1 ml of potato root diffusate (PRD), 1.5 mg/ml of gentamicin and 0.1 mg/ml of nystatin were added into the corning centrifuge tube, followed by incubation at 18 ºC, after 2 weeks, juveniles were collected as described below.

***Globodera pallida* second stage juvenile nematodes (J2s)**

Hatched juveniles (J2s) were cleaned of eggs shells and other debris by using sucrose gradient centrifugation (30-60%). Hatched J2s were collected and incubated overnight in a solution containing 100 µg/ml of ampicillin and streptomycin (w/v) on a shaker at room temperature. Hatched J2s were further treated with benzethonium chloride (0.125%) by continuous shaking at room temperature for 20 minutes 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 (200/100µl) were stained using PKH26 Red Fluorescent Cell Linker Kits (used for microscopy observation) (Sigma-Aldrich®, St-Louis, Mo) for general cell membrane labeling and designed to maintain cell viability, while maximizing dye solubility and staining efficiency during the labeling step. J2s were added to diluted PKH26, 4×10^{-6} M (Sigma Aldrich®, St-Louis, Mo), and incubated for 10 min at room temperature (22ºC) in dark with intermediate shaking (3 times) for 15 secs and washed 5 times in sterile distilled water. The stained J2s 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 in -4ºC until use.

Microscopy Rhizosphere Chambers (Micro-ROCs)

Microscopy Rhizosphere Chambers (Advanced Science Tools, LLC, Pullman, WA) are designed to fit standard microscopes. The dimensions of the chamber are 70 mm x 50 mm x 22 mm. The observation area consists of high quality # 1.5 corrosion resistant cover glass in 28 mm x 57 mm of dimension. Tissue culture potato plantlets 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).

Plant inoculation

Potato plantlets were inoculated with fully grown *T. harzianum* ThzID1-M3 on PDA discs according to the treatments. Three 7-mm 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. After 2 weeks, potato plantlets were inoculated with 100µl of agarose solution containing 200 J2s (Dinh et al., 2014).

Experimental treatments

The two treatments, as follows: (1) *G. pallida* second stage infectious juveniles (J2s) only applied to non-amended soil (control); 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. Potato plantlets were grown in growth chamber under a cycle of 16:8 hours of light: dark at 18 ± 2 ºC (daytime) and 14 ± 2 ºC (night time) for 5 days prior to evaluation.

Effect of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* root infection

Treatments were evaluated using an inverted fluorescence microscope (Leica DMI 3000 B, Leica Microsystems, Wetzlar, Germany) for observations of roots colonized by *T. harzianum* ThzID1-M3 and infected by *G. pallida*.

Acid Fuchsin (Sigma-Aldrich®, St-Louis, Mo), an acidic dye, was used to stain *G. pallida* juveniles (J2s) in potato roots in order to quantify and determine the level of root infections (modified from Byrd et al., 1983). Roots were washed, chopped into 1-2 cm segments, and placed in a 200-ml beaker with 90 ml of tap water. Twenty milliliters of chlorine bleach (8.25% NaOCl Clorox®, Oakland, CA) were added to give 1.5% NaOCl, and root segments were allowed to remain in this solution for 4 minutes. Root segments were washed thoroughly with running water for 45 seconds, and then immersed in tap water for 15 minutes to remove any NaOCl residual. Root segments were transferred to a beaker containing 50 ml of tap water to which 2 ml of stock stain solution (3.5 g acid fuchsin, 250 ml acetic acid, and 750 ml distilled water) were added, and boiled for 30 seconds in a microwave oven. Stain solution was allowed to cool down to room temperature, drained and root segments were rinsed in running tap water. Roots were de-stained in boiling 30 ml of glycerin (99% concentrated, Sigma-Aldrich®, St-Louis, Mo) acidified with 5 M HCl, and cooled down. Root segments were observed under a dissecting microscope (Leica M80, Leica Microsystems, Wetzlar, Germany) for *G. pallida* counting.

Statistical analysis

All analyses were performed using SAS package 9.4 (SAS Institute Inc., Cary, N.C.). Proc GLIMMIX procedure was performed to analyze *G. pallida* J2s in potato roots with significant differences occurring at level of $P < 0.05$.

RESULTS

Microscopic observations of *Trichoderma harzianum* ThzID1-M3 interactions with the potato rhizoplane and *Globodera pallida*

Observations under fluorescence microscopy (Leica M80, Leica Microsystems, Wetzlar, Germany) showed that *T. harzianum* ThzID1-M3 was able to extensively colonize the potato root surface, and *T. harzianum* ThzID1-M3 was also able to establish hyphal growth inside the cortex of the root system (Figure 4.1). Microscopic observations also showed that *T. harzianum* ThzID1-M3 colonized and killed *G. pallida* juveniles (J2s) prior to root infection (Figure 4.2), while *G. pallida* (J2s) applied to non-amended soil showed an increased root infection by *G. pallida* infectious J2s (Figure 4.3).

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* root infection

The first and second experiments were consistent and no interactions between treatments and experiments were detected, therefore both data were combined. Results showed a significant reduction (67%) of *G. pallida* juveniles (J2s) in potato roots colonized by *T. harzianum* ThzID1-M3 compared to *G. pallida* (J2s) applied to non-amended soil ($P < 0.05$). Additionally, a significant increase in root dry weight (62%) was observed in amended soil with *T. harzianum* ThzID1-M3 compared to *G. pallida* (J2s) applied to non-amended soil ($P < 0.05$) (Table 4.1).

Table 4.1 Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* infectious J2s in potato roots

Treatment	Root dry weight (g)	J2s/g root
G. pallida J2s	0.13[a] (0.03)	751.3[a] (289.9)
G. pallida J2s + ThzID1-M3	0.21[b] (0.04)	244.6[b] (149.4)

*Means with the same letter are no significantly different ($P > 0.05$) as determined by Tukey-Kramer adjustment for multiple comparison. Standard error of the mean is shown in parenthesis.

DISCUSSION

Trichoderma harzianum ThzID1-M3 interactions with the potato rhizoplane and *Globodera pallida*

The ability of *T. harzianum* ThzID1-M3 to successfully establish in the root zone is an important aspect in its biocontrol effectiveness against a broad-range of soilborne plant pathogens. Rhizosphere colonization by *T. harzianum* contributes to the protection of the infection court and also favors *T. harzianum* growth and proliferation in soil, and the ability to colonize plant roots has often been stressed as an important requirement of

Trichoderma to act as biocontrol agents (Harman et al., 2004). In sterile soil, *T. harzianum* was detected in the rhizosphere of the upper half of pea roots (Chao et al., 1986). Rhizosphere competence is the ability used *Trichoderma* strains to grow and compete with other microorganisms to colonize the root surface and compete with them for nutrients secreted by root exudates (Ahmad and Baker, 1987; Harman et al., 2004; Shoresh et al., 2010). Sivan and Chet (1989) showed that *T. harzianum* strain T-35 significantly reduced disease caused by *Fusarium oxysporum* by colonizing the rhizosphere of cotton plants. During root colonization, *Trichoderma* grows intercellularly, but the epidermis, cortex and vessels remain intact or minimally altered (Yedidia et al., 1999; Salas-Marina et al., 2011). To successfully colonize roots, *Trichoderma* spp. are to overcome and detoxify allelochemicals and phytoalexins secreted by plants (Ruocco et al., 2009). Few *Trichoderma* spp. have been isolated as true endophytes (Holmes et al., 2004; Bailey et al., 2006, 2009), and other *Trichoderma* spp. were found in association with cacao tree (*Theobroma cacao*) in South America such as *T. theobromicola* from Peru and *T. paucisporum* from Ecuador (Samuels et al., 2006).

Yedidia et al. (1999) showed that plants respond to the presence of other organisms by activating defense mechanisms. Two main mechanisms have been identified: (1) systemic acquired resistance (SAR); and (2) induced systemic resistance (ISR). SAR is triggered by local infection, providing long-term systemic resistance with the activation of PR genes that requires the involvement of the signal molecule salicylic acid (SA) (Durrant and Dong, 2004; Vallad and Goodman, 2004). ISR is triggered by colonization of roots by some specific nonpathogenic rhizobacteria, and requires the participation of the signal molecule jasmonic acid (JA) followed by ethylene signaling pathway (Van Loon et al., 1998; Vallad and Goodman, 2004; Segarra et al., 2007). A number of studies have found that *Trichoderma* spp. have the ability to turn on specific disease-resistance genes in plants, such as *PAL1* in cucumber roots colonized by *T. asperellum* (Yedidia et al., 2000, 2003; Harman et al., 2004; Shoresh et al., 2010). Calderón et al. (1993) demonstrated that suspension cell cultures of grapevine treated with an elicitor from *T. viride* showed a hypersensitive-like response characterized by cell plasmolysis accompanied by localized cell death due to a large increase in endogenous levels of H_2O_2. Yedidia et al. (1999), in an experiment with cucumber plants, showed evidence that *T. harzianum* was able to

penetrate the root system without causing extensive damage and trigger plant host defense reactions by increasing the level of plant peroxidase and chitinase activities. Thus *Trichoderma*-inoculated plants may be sensitized to respond faster and to a greater extent to potential pathogen attacks. Viterbo et al. (2005) revealed that a mitogen-activated protein kinase (MAPK) is essential for *Trichoderma* in order to induce full systemic resistance in cucumber against the bacterial pathogen *Pseudomonas syringae.* It has also been shown that *Trichoderma* spp. induce the surrounding plant cells to deposit cell wall material and produce phenolic compounds that limit *Trichoderma* growth inside the root, which prevents it from damaging plant cells (Shoresh et al., 2010). Vargas et al. (2009) showed that colonization of plant roots by *Trichoderma* provoke a systemic change in the expression of plant genes regulating stress responses, isoprenoid oxylipins and ethylene biosynthesis, photosynthesis and photorespiration rates and carbohydrate metabolism. Naserinasab et al. (2011) reported that in addition to *Trichoderma* direct antagonism, induction of defense-related enzymes involved in peroxidase pathway contributed to enhance resistance against invasion of *Meloidogyne javanica* in tomato.

The mechanism used by *Trichoderma* to attack its prey involves sequential and cooperative events, including a positive chemotrophic growth towards its host, direct contact with the prey, formation of an appresorium-like penetration structures, 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, 2003). Sahebani and Hadavi (2008) showed that different concentrations of *T. harzianum* decreased *M. javanica* infections in tomato roots by causing direct and indirect effect on nematode eggs, juveniles and inducing resistance in plant.

Influence of *Trichoderma harzianum* ThzID1-M3 on *Globodera pallida* root infection

Trichoderma harzianum ThzID1-M3 significantly reduced *G. pallida* root infection. Similarly, the biocontrol fungus *Paecilomyces lilacinus* reduced numbers of *Meloidogyne incognita* J2 in field-grown tomato by 70% and 41% when applied at transplant and 2 weeks after transplanting, respectively (Martez et al., 1996). Kiewnick and Sikora (2006) demonstrated that a single pre-plant application of *P. lilacinus* at a concentration of 1 x 10^6 CFU/g soil significantly reduced *M. incognita* in tomato roots. Goswami et al. (2008) showed that *P. lilacinus* suppressed galling of tomato by *M. incognita* by 39% when

applied at transplant. *Trichoderma longibrachiatum* was found to decrease significantly *Heterodera avenae* infection in wheat by colonizing and degrading *H. avenae* cysts through extracellular chitinase activity (Zhang et al., 2014). In field experiment, *T. harzianum* reduced galling on tomato roots by 47% compared to untreated plots (Goswami et al., 2008). Additionally, results showed a significant increase in potato dry root weight in *T. harzianum* ThzID1-M3 amended soil compared to *G. pallida* (J2s) applied to non-amended soil. Similarly, Ahmad and Baker (1987, 1988) reported that *T. harzianum* promotes better seedling emergence and plant growth. Dandurand and Knudsen (1993) showed that the application of the granular formulation of *T. harzianum* ThzID1 to pea seeds reduced root-rot caused by *Aphanomyces euteiches* f. sp. *pisi* in growth chamber experiments and also increased plant top weights compared to non-coated seeds. Mousseaux et al. (1998) showed that *T. harzianum* ThzID1 significantly reduced proliferation of *Fusarium* spp. in Douglas-fir seedlings, while promoting seed germination and growth.

We conclude that *T. harzianum* ThzID1-M3 has the potential for biological control of *G. pallida* by directly attacking second stage juveniles that are migrating to roots, consequently reducing the number of infectious J2s. Future biocontrol research need to focus on revealing the process of induced systemic resistance in plant by *T. harzianum* ThzID1-M3, elucidating the mechanisms behind ISR activation, and determining ISR potential against *G. pallida*.

Considerations for the use of *Trichoderma harzianum* ThzID1-M3 in agriculture

Before making final recommendations to the farmers to use *T. harzianum* ThzID1-M3 as a biocontrol agent against *G. pallida*, we should: (1) test the efficacy of *T. harzianum* ThzID1-M3 on field application trials in order to test its behavior in a competitive soil microbial ecosystem; (2) examine the ability of *T. harzianum* ThzID1-M3 to colonize *G. pallida* eggs, which is an important mechanism for disrupting the nematode life cycle; (3) determine the process of induced systemic resistance (ISR) in plant by *T. harzianum* ThzID1-M3, elucidating the mechanisms behind ISR activation, and determining ISR control potential against *G. pallida.* Only after these exhaustive field trials that we can make final recommendations to the farmers, and for now we are compiling evidence that showed *T. harzianum* ThzID1-M3 as a potential biocontrol agent.

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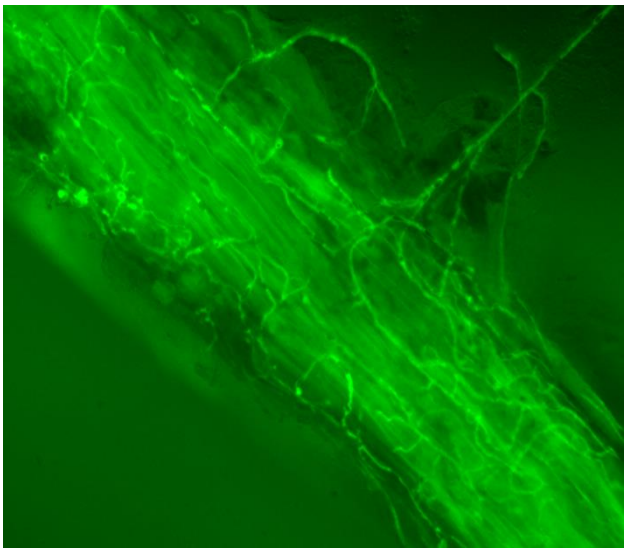
A)



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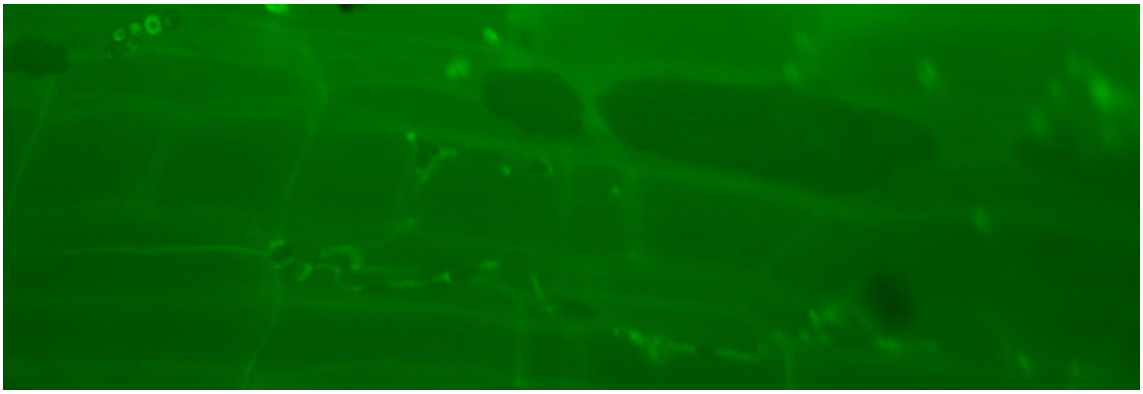


Fig. 4.1. Sections of a potato root colonized by *Trichoderma harzianum* ThzID1-M3 observed under inverted fluorescence microscopy (Leica DMI 3000 B, Leica Microsystem, Wetzlar, Germany). A. *Trichoderma harzianum* ThzID1-M3 hyphal growth over and inside the potato root cortex. B. Closer-look of *Trichoderma harzianum* ThzID1-M3 hyphal growth over the potato root surface.

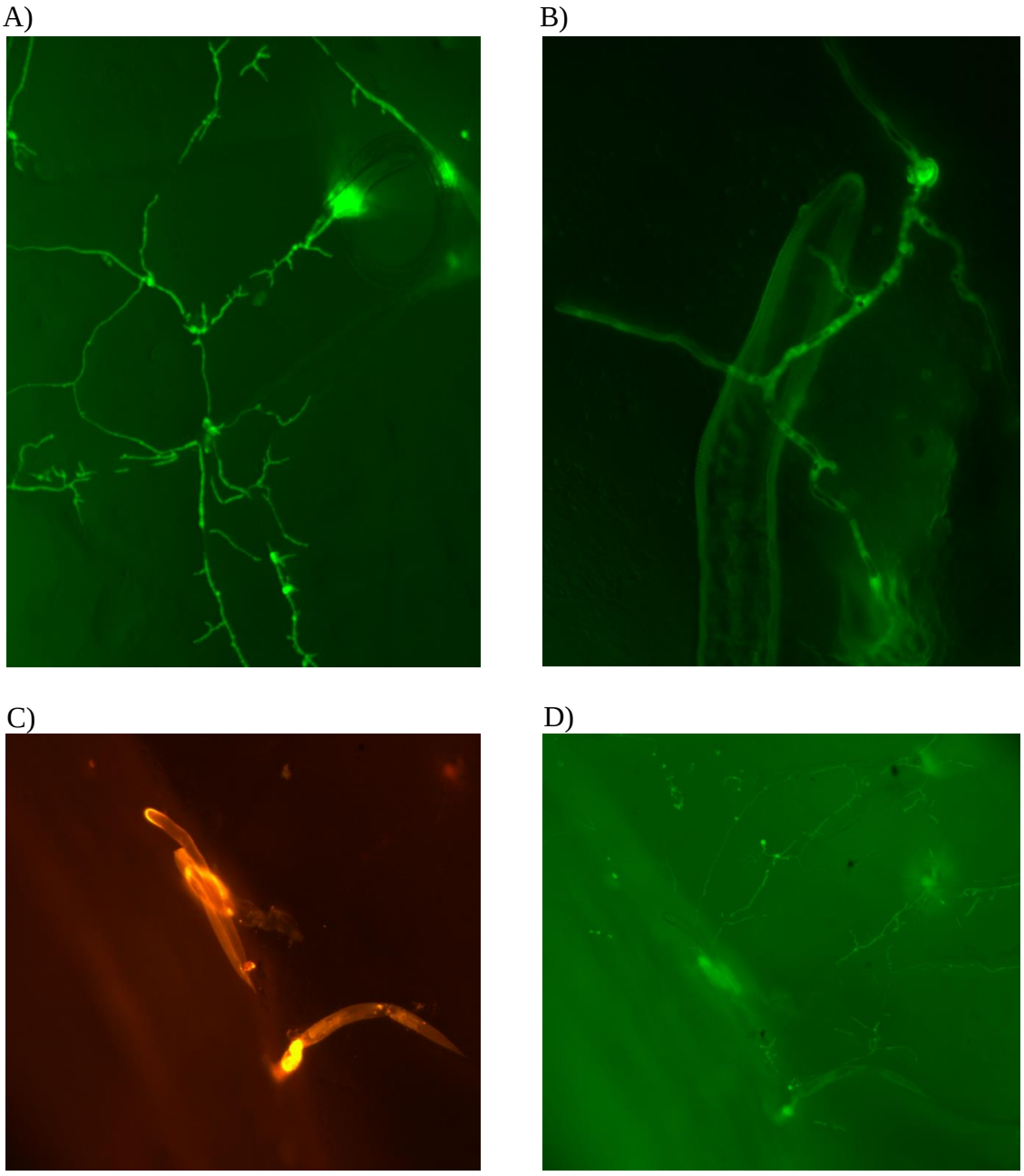
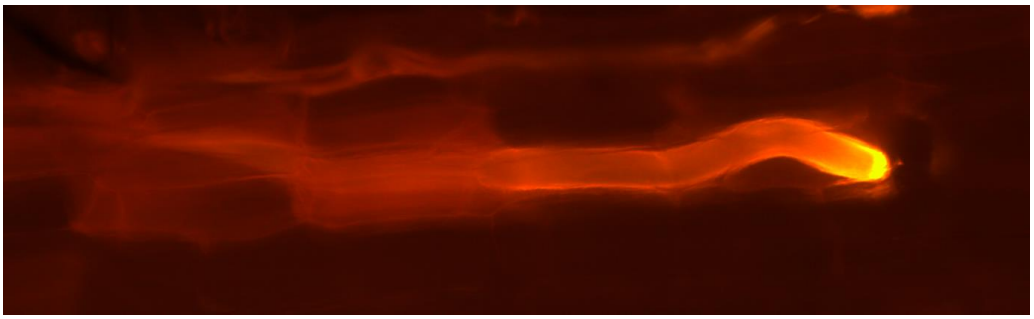


Fig. 4.2. A. *Trichoderma harzianum* ThzID1-M3 hyphal attacking a *Globodera pallida* second stage juvenile (J2). B. Closer-look of *Trichoderma harzianum* ThzID1-M3 hyphal attacking a *Globodera pallida* second stage juvenile (J2). C. Dead *Globodera pallida* second stage juveniles (J2s) over root surface, and D. the same dead *Globodera pallida* second stage juveniles (J2s) colonized by *Trichoderma harzianum* ThzID1-M3 hyphae.

A)



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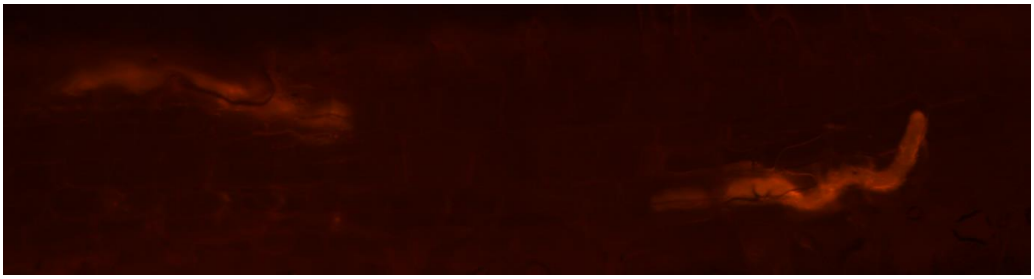

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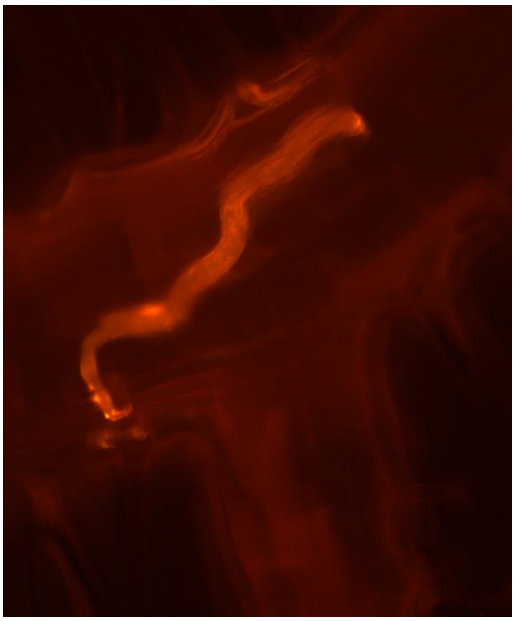

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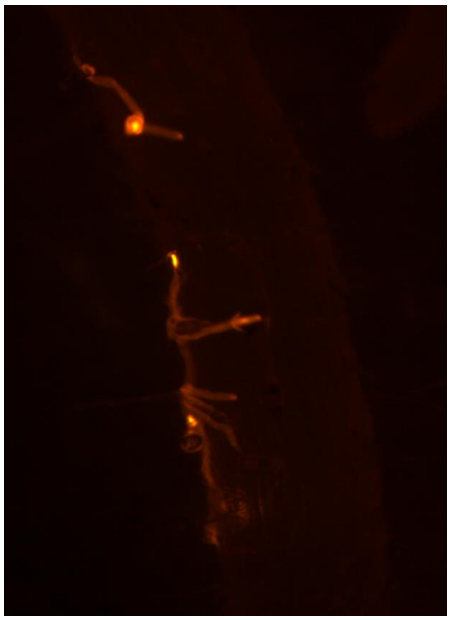

Fig. 4.3. *Globodera pallida* second stage juveniles (J2s) stained with Red Fluorescence PKH26 (Sigma-Aldrich®, St-Louis, Mo) inside potato root (A, B, C, D). Observed under inverted fluorescence microscopy (Leica DMI 3000 B, Leica Microsystem, Wetzlar, Germany).