

ABSTRACT

Title of Thesis: ASSESSMENT OF SELECT ISOLATES OF
 TRICHODERMA VIRENS AS A POTENTIAL
 BIOCONTROL AGAINST *MELOIDOGYNE INCOGNITA*

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Three isolates of the soil-dwelling fungal organism, *Trichoderma virens* (Miller, Giddens and Foster) von Arx., were studied as potential biocontrols for the management of root-knot nematode (RKN) on bell pepper (*Capsicum annuum* L.). The study was conducted within the Moisture Replacement System (MRS), which was concurrently evaluated for its ability to serve as a culture system for *Meloidogyne incognita* (Kofoid and White) Chitwood and as an environment supportive of the growth and maintenance of *Trichoderma* isolates for biocontrol studies of this nature.

Trichoderma isolates selected for study did not suppress populations of RKN on bell pepper under the conditions of this study. A review of the literature provided a multitude of complexities potentially contributing to the final results obtained.

Investigation into these various complexities with the incorporation of the MRS will help to fully ascertain *Trichoderma*'s potential as a biocontrol and perhaps reveal viable alternatives in the management of RKN.

ASSESSMENT OF SELECT ISOLATES OF *TRICHODERMA VIRENS* AS A
POTENTIAL BIOCONTROL AGAINST *MELOIDOGYNE INCOGNITA*

By

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DEDICATION

To my husband Guy and my daughter Gabrielle

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SARE

Cosmos Club Foundation

TABLE OF CONTENTS

List of Tables.....	v
List of Figures	vi
List of Abbreviations.....	vii
I. Literature Review.....	1
II. Evaluation of the Moisture Replacement System as a method for culturing the southern root-knot nematode, <i>Meloidogyne incognita</i> ..	8
Introduction	9
Materials and Methods	10
Results.....	15
Discussion.....	17
III. Evaluation of <i>Trichoderma virens</i> survival and growth within the Moisture Replacement System.....	20
Introduction	22
Materials and Methods	25
Results.....	30
Discussion.....	32
IV. Assessment of <i>Trichoderma virens</i> for biocontrol of the root-knot nematode, <i>Meloidogyne incognita</i>	37
Introduction	38
Materials and Methods	40
Results	47
Discussion.....	52
Appendix A: Corn Meal Agar versus Potato Dextrose Agar as a medium for <i>Trichoderma</i> growth and sporulation.....	58
Appendix B: Seed Adhesive/High Humidity Experiment.....	61
Appendix C: Fresh Coat versus Dry Coat Experiment.....	69
Literature Cited.....	72

LIST OF TABLES

1. Plant growth measurements and *Meloidogyne incognita* recovered from *Capsicum annuum* L. cv. Yolo Wonder within the Moisture Replacement System. Results displayed of trials 1 and 2 represent averages of the three blocks of the completely randomized design. Results within a category with different letters were significantly different at $p=.10$ 16
2. Survival and growth data for three *Trichoderma virens* isolates evaluated as potential biocontrol organisms..... 31
3. Bell pepper plant growth responses after treatment with isolates of the biocontrol fungus *Trichoderma virens* (isolates G1-3, G1 15 and G1-21) and the root-knot nematode (RKN) *Meloidogyne incognita*. Letters are comparable within columns, but are not comparable between trials. Values represent averages of all sub samples from all blocks within a trial. Means with similar letters are not significantly different at $p=.10$ 48
4. Propagule enumeration of initial fungal slurries and CFU counts obtained via reisolation of the *Trichoderma virens* isolates (G1-3, G15 and G1-21) from the initial seed coat, the pepper seedling roots at three weeks post-planting and the pepper roots at 11 weeks post-planting.....51

LIST OF FIGURES

1.	Image of the bioassay model of the Moisture Replacement System containing 36 grow tubes.....	11
2.	Image depicting arrangement of the three blocks (bioassay models of the MRS) within the growth chamber during the second trial.....	43
3.	Number of <i>Meloidogyne incognita</i> eggs, second-stage juveniles (J2), and eggs + J2 per gram root following treatment of bell pepper with the biocontrol agent <i>Trichoderma virens</i> isolates Gl-3, Gl-15 and Gl-21, or with respective controls. Letters are comparable with each other within a category (indicated by color pattern), but are not comparable among the three categories. Bars within a category and with a common letter are not significantly different at $p=.10$	50
4.	Photo comparing differences in growth of <i>Trichoderma virens</i> isolates Gl-3, Gl-15 and Gl-21 upon Corn Meal Agar (CMA) and Potato Dextrose Agar (PDA).....	59
5.	Photo depicting layout of High Humidity treatments within the growth chamber.....	64
6.	Comparison of CFU recovery from bell pepper seeds coated with formulations of <i>Trichoderma virens</i> isolates Gl-3, Gl-15 and Gl-21. Each of the formulations tested was a combination of two factors, adhesive (gelatin or Polyox N-10) and seed coat exposure (fresh coat or post high humidity). Letters are comparable within each isolate, but not among isolates. Bars within an isolate with a different letter are significantly different at $p=.10$	66

LIST OF ABBREVIATIONS

cm	centimeters
ml	milliliters
g	gram
gm	gram
C	Celsius
hr	hour
J2	second stage root-knot nematode juveniles
CFU	colony forming units
RPM	revolutions per minute
wk	week
m ²	meters squared
L	liters
µm	micrometer

CHAPTER 1: LITERATURE REVIEW

Root-knot nematode species (*Meloidogyne* spp.) are among the most widespread agricultural pests in the world. Their extensive distribution associates root-knot disease with infection of all major crop plants as well as many other susceptible plants within agricultural and horticultural production (Sasser and Carter, 1985). Infection by root-knot nematode (RKN) occurs in the roots of plants resulting in significant reduction of yield and quality that equates to considerable financial losses for growers in agricultural and horticultural industries. In developing countries where nematode problems are prevalent, infection by nematodes greatly compounds the burgeoning problems of low agricultural productivity (Brady, 1985).

Until recently, RKN were primarily managed by a highly effective and broad-spectrum soil fumigant, methyl bromide. Methyl bromide provides a valuable management tactic against insects, nematodes, weeds and pathogens in more than 100 crops (ARS Methyl Bromide Research, 2003). In 1991, the Montreal Protocol, an international treaty on substances that deplete the ozone layer, included methyl bromide as one of the chemicals that contributes to depletion of the Earth's ozone layer. Accordingly, the production and importation of methyl bromide will be completely phased out by 2005 in developed countries and by 2015 in developing countries.

Although the number of crops benefiting from the application of methyl bromide exceeds 100, nearly 80% of preplant methyl bromide soil fumigation was applied to strawberries, tomatoes, peppers, ornamentals and nursery crops, all profitable crops in Maryland. Research by the United States Department of Agriculture (USDA) indicates that multiple alternative control measures will be required to replace the many essential

uses of methyl bromide (ARS Methyl Bromide Research, 2003). Alternatives currently under investigation include host plant resistance, biological control, alternative chemicals and innovative cultural practices, implemented either alone or in combination.

Biological control of plant-parasitic nematodes is receiving increasing attention as a viable alternative management tactic. Biological control is the total or partial destruction of pathogens by other organisms (Agrios, 1997). Under natural conditions, plant-parasitic nematodes are attacked by numerous and varied soil organisms, including fungi, bacteria, viruses, protozoans and other nematodes. This natural occurrence has led researchers to evaluate the possibilities for control by enhancing methods that exist in nature.

In recent years and under laboratory conditions, researchers at USDA have discovered that isolates of *Trichoderma virens* (Miller, Giddens and Foster) von Arx., an ubiquitous, soil-dwelling, saprophytic fungus demonstrated antagonistic activity against the nematode *Meloidogyne incognita* (Kofoed and White) Chitwood (Meyer et al., 2000). This common fungus can be found in nearly all soils and other natural habitats, especially those containing organic matter such as decaying bark (Papavizas, 1985). Typical descriptions of soil mycobiota list *Trichoderma* along with *Aspergillus*, *Penicillium* and *Fusarium* as the dominant populations (Klein and Eveleigh, 1998). This may be attributed to the diverse metabolic capability of *Trichoderma* species and their aggressively competitive nature (Gams and Bissett, 1998). Research involving *Trichoderma* species as enzymatic producers has significantly benefited biotechnology efforts (Harman and Kubicek, 1998).

Previous research on *Trichoderma* as a biological control agent has focused largely on its effectiveness in the reduction of fungal seedling diseases (Harris and Lumsden, 1997; Mao et al., 1997; Zhang et al., 1996). Commercial biological control products have been successfully developed and marketed as a result of this research (APS Biological Control Committee, 2003). The study of *Trichoderma* at USDA Agricultural Research Service (ARS) Beltsville, MD identified several isolates of *T. virens* that are particularly effective against the fungal pathogens *Pythium* and *Rhizoctonia* (R. Lumsden, personal communication). Within the past ten years research efforts involving *Trichoderma* have expanded to include evaluation of its effectiveness in reducing populations of plant-parasitic nematodes.

Some *Trichoderma* isolates have been evaluated for their antagonistic abilities against the nematode *M. incognita* (Meyer et al., 2000; Meyer et al., 2001; Zhang et al., 1996). *M. incognita* is a highly destructive plant pest in Maryland with a large impact on vegetable crops. To date, the interactions between *Meloidogyne* spp. and select *T. virens* isolates have been observed on cotton, tomato, pepper and okra, four crops that are highly susceptible to RKN infestation (Meyer et al., 2000; Meyer et al., 2001; Parveen et al., 1993; Zhang et al., 1996).

In many cases the *Trichoderma* isolate is delivered to the soil by means of a seed treatment. This is an ideal method of delivery for beneficial fungal microbes as the majority of soil borne disease occurs when a pathogen infects a seed, stem or actively growing root (Lewis, 1991). Application as a seed coat provides the biocontrol agent with the opportunity to colonize the seed surface independently and utilize the seed exudates produced during germination as a nutritive source. This initial colonization

allows the biocontrol agent to become established in the spermosphere of the germinating seed and then progress downward along the emerging radicle and into the rhizosphere. Since root-knot nematodes are root-infecting organisms, a seed coat method of application places the biocontrol agent directly in the path of the invading pathogen and increases the opportunity for antagonistic interactions.

In order to proliferate a fungal antagonist for seed application, the biology and growth requirements of the fungal organism must be carefully considered. For the rapid establishment of the fungal antagonist within the test environment, it is necessary that seed treatment formulations not only successfully apply the biocontrol agents to seeds but also provide a favorable environment for fungal proliferation within the coating formulation (Taylor et al., 1991). For these reasons, the process of delivering potential antagonists by seed has constituted a significant portion of the research directed at biocontrol agents (Harman, 1991; Lewis, 1991; Mao et al., 1997; Taylor et al., 1991; Taylor and Harman, 1990).

Of prime importance in the production of fungal seed coats is the integration of components that serve as food sources. Since *Trichoderma* is a saprophytic soil fungus, a wide range of compounds may be utilized to provide appropriate carbon and nitrogen food sources (Papavizas, 1985). Beagle Ristaino and Papavizas (1985) reported that fungal biomass, specifically chlamydospores formulated in close association with the residual nutrients of a liquid medium (molasses and brewer's yeast) were provided an adequate food source that supported growth and sporulation of the fungus in the soil. Lewis and Papavizas (1984) showed that thorough colonization of a food source (sterile moist bran) by the mycelia enabled *Trichoderma* to grow relatively unimpeded through

the soil. Harman et al., (1981) reported increased efficiency in control of damping-off when a food base compatible to the potential antagonist was added to the seed coat.

A food base can be supplied in conjunction with the seed coat by means of the liquid medium utilized as a nutrient source by the fungal isolate. As the fungal slurry is created to ultimately coat the seeds, the residual nutrients of the liquid medium are transferred with the fungal biomass that will eventually coat the seed. An inexpensive liquid medium utilized in associated research of *Trichoderma* is molasses-yeast medium (Lewis and Papavizas, 1984). This liquid media (30 g molasses and five g brewer's yeast L⁻¹ distilled water) produces a fungal biomass consisting largely of mycelial fragments and chlamydospores plus the residual nutrients of the liquid medium (Beagle-Ristaino and Papavizas, 1985). Chlamydospores are thick-walled, long-term survival propagules that may aid in the survival of the fungus under adverse conditions under which the smaller, single-walled conidia propagules may succumb (Papavizas, 1985). Earlier research on *Trichoderma* found that preparations containing chlamydospores more effectively prevented diseases than preparations containing conidia only (Lewis and Papavizas, 1984). Chlamydospore presence in biocontrol formulations is critical and their potential role in successful biological control has been further revealed through more recent research (Mao et al., 1997; Mao et al., 1998; Kerry, 2001).

Rhizosphere competence is defined as the physiological and genetic ability to proliferate along the root as it develops (Harman, 1992). The penetration of roots by infective stages of RKN takes place in the rhizosphere, a thin layer of soil no more than two mm thick surrounding the root (Stirling, 1991). Considering the habitat of RKN, a microbial pest control agent that is rhizosphere competent is of major interest in the

search for effective biocontrol. Hypothetically, a rhizosphere competent organism with antagonistic activity towards RKN may exhibit a deleterious effect upon the establishment and reproduction of the pathogen. Until the late eighties, rhizosphere competent, wild-type *Trichoderma* strains were not known to exist (Harman, 1992). At that time, only benomyl-tolerant mutants of *Trichoderma* were found to be rhizosphere competent (Ahmad and Baker, 1987). Further research into the rhizosphere competence of *Trichoderma* uncovered additional wild type strains also exhibiting the ability to colonize the roots (Sivan and Chet, 1989; Smith et al., 1990). Despite these findings, wild type *Trichoderma* isolates that are rhizosphere competent are considered rare (Harman, 1992).

Greenhouse studies have not demonstrated that *T. virens* is a consistent biocontrol agent against *M. incognita* (Meyer et al., 2000; Meyer et al., 2001; Zhang et al., 1996). Meyer et al. (2001) reported that *T. virens* suppressed nematode populations on bell pepper in greenhouse studies. However, an earlier greenhouse study, the same isolate was found to be ineffective in lowering RKN numbers on tomatoes (Meyer et al., 2000). As with all biocontrol organisms, strain selection, formulation, soil environment, target nematode, nematode population numbers, and host crop can affect results (Meyer et al., 2001). Further studies evaluating manipulation of these parameters may help to determine the potential influence of *T. virens* upon the persistence of RKN (Meyer et al., 2001).

The objectives of this thesis were planned based on the recent discovery of *Trichoderma*'s inhibitory effects against the root-knot nematode in vitro. As infestations of RKN are currently afflicting vegetable growers along the eastern shore of Maryland,

the effect of *Trichoderma* on vegetables susceptible to RKN was of particular interest. Hence, the primary objective of this study was to assess three isolates of *T. virens* (Miller, Giddens and Foster) von Arx. for their antagonistic effects upon bell pepper infected with *M. incognita* (Kofoed and White) Chitwood. The study utilized a Moisture Replacement System (MRS) bioassay unit, which had been utilized in research involving the maintenance of populations of the soybean cyst nematode (*Heterodera glycines*) (Sardanelli and Kenworthy, 1997a). Hypothesizing that this bioassay unit of the MRS would likewise be capable of supporting a population of RKN, it was integrated into this project. A second objective was to assess the long-term presence of the *Trichoderma* isolates in the rhizosphere. A third objective that was identified as the project proceeded was the need to determine the effectiveness of the seed coat application process. The fourth objective was to assess the usefulness of the MRS bioassay model as a culture system for the RKN, *M. incognita*.

ABSTRACT

CHAPTER 2: EVALUATION OF THE MOISTURE REPLACEMENT SYSTEM AS A METHOD FOR CULTURING THE SOUTHERN ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*.

An innovative bioassay system, the Moisture Replacement System (MRS), was studied as a potential means for culturing large numbers of the root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid and White) Chitwood. Each of the 36 grow tubes contained within one MRS was seeded with bell pepper (*Capsicum annuum* L. cv Yolo Wonder), a highly susceptible host to *M. incognita* nematode populations. Four weeks after seeding, each grow tube was inoculated with 2000 RKN eggs. Six weeks after inoculation each pepper plant was removed and evaluated for presence of eggs and juveniles in the soil and in the rhizosphere. Total eggs and juveniles retrieved per gram of root averaged 44,000 and 39,000 from trials one and two, respectively. Under the experimental conditions used in this study, a single MRS that contains 36 grow tubes has the capacity to produce more than 10 million RKN eggs and juveniles within the space of 0.19 m². The consistent production of high numbers of eggs and juveniles observed between trials indicates that the MRS is a reliable means of culturing RKN with minimal expenditure of time, labor, space and materials.

Introduction

Root-knot nematodes (*Meloidogyne spp.*) are serious soil pathogens of multiple crops worldwide (Taylor et al., 1982). The threat posed by these microscopic parasites has given rise to increased efforts within the agricultural research community to identify environmentally safe methods of management (Meyer et al., 2000; Meyer et al., 2001; Zhang et al., 1996).

Research involving management tactics often requires large amounts of nematode inoculum (Dukes et al., 1997). Typically, nematodes are obtained from greenhouse cultures produced in pots planted with susceptible plants. While this method is fairly uncomplicated, final populations may be inconsistent, due to the lack of uniformity that can exist from one pot to another. Problems that can affect the nematode population within the greenhouse pot environment include the potential for soil contamination and plant infestation from other organisms, and inconsistencies in the environmental parameters. Another difficulty is that cultures maintained within a greenhouse pot environment need a significant amount of space. Culture methods minimizing the need for this resource could be useful when greenhouse space is limited.

Various alternatives to typical greenhouse culturing methods have been described. For example, hydroponic culture systems involve the establishment of nematode infected tomato roots prior to placement into a funnel system (Lambert et al., 1992). This system is maintained within a culture chamber and is filled with a sterile nutrient solution that is continuously aerated. Nematode juveniles are later extracted with a seven-hour process. An alternative system, in vitro root tissue cultures, has been used for many years as a means of producing nematodes using aseptic conditions (Mitkowski and Abawi, 2002).

Nematode inoculations and culture transfers performed within a laminar flow hood result in uncontaminated sources of nematodes particularly useful in molecular and genetic studies, but are less practical when large amounts of inoculum are needed.

In 1997, a system was introduced for the culturing and bioassay of the soybean cyst nematode (SCN), *Heterodera glycines* (Sardanelli and Kenworthy, 1997a). This system, named the Moisture Replacement System (MRS), was developed to address the lack of consistent populations of SCN females that can result from the widely fluctuating soil moisture levels that may occur in standard greenhouse pot cultures. Using an enclosed bottom reservoir, the MRS provides continuous moisture to multiple grow tubes. In addition, the small size of the self-contained MRS accommodates its placement within a greenhouse, growth chamber or laboratory (Sardanelli and Kenworthy, 1997b), and direct seeding of host plants into the grow tubes eliminates any need for transplant of seedlings.

The usefulness of the MRS in the establishment and increase of SCN populations served as the foundation for evaluating the use of the MRS for culturing the RKN, *Meloidogyne incognita* (Kofoed and White) Chitwood.

Materials and Methods

A whole-plant bioassay model of the Moisture Replacement System (Figure 1) was prepared as described in Sardanelli and Kenworthy (1997a).

The host plant for this study, *Capsicum annuum* L. cv. Yolo Wonder bell pepper, was selected following preliminary trials. In those trials, two other hosts were tested, *Capsicum annuum* L. cv. Keystone Resistant Giant bell pepper and *Lycopersicon*



Figure 1. Image of the bioassay model of the Moisture Replacement System containing 36 grow tubes.

esculentum cv. Rutgers tomato. Bell pepper cv. Yolo Wonder was selected because of its longevity in those trials (data not shown). The addition of four pellets of Osmocote 15-9-12 (Scotts Company, Columbus, OH) per grow tube allowed maintenance of Yolo Wonder bell pepper for a period of 10 weeks within the MRS. In the preliminary trials, Keystone Resistant Giant bell pepper also performed well and would serve as a suitable host plant for culturing within the MRS. Rutgers tomato, although a very susceptible host, succumbed most quickly to nematode infection and was beginning to deteriorate at the end of 10 weeks. These results were similar to those found by Dukes et al. (1997).

For the study evaluating use of the MRS for RKN culture, a sandy soil medium was selected for use in the grow tubes to support pepper and *M. incognita* populations (Sasser, 1954). The soil media (pH= 4.9, 89% sand, 4% silt, 7% clay and 1.7% organic matter) was prepared by autoclaving a mixture of 90% builders sand and 10% plug and seedling mix (Scotts Redi-Earth Peat-Lite Mix, Scotts Company, Columbus, OH). Four pellets of Osmocote 15-9-12 were placed approximately five cm from the base of each 50 ml grow tube filled with the soil medium. Each grow tube was planted with one seed of bell pepper (*Capsicum annuum* L.) cv. Yolo Wonder at a depth of 1.0 cm into the soil media.

Environmental parameters within the growth chamber, but external to the MRS, were also evaluated in a preliminary setting. Growth chamber conditions were 27° C, a 12 hr photoperiod and a humidity setting of 40%. During preliminary trials it was discovered that under these conditions, the initial germination period was delayed when the bioassay MRS was placed into a growth chamber immediately after seeding. Reservoir water temperature was measured and it was discovered that the polyfoam

container used for the MRS insulated the water within the reservoir from the warming effects of the growth chamber, thereby preventing timely germination. To remedy this, immediately after seeding, the bioassay MRS was placed onto a thermostatically controlled propagation mat warmed to 26.7 °C and situated upon a utility rack with two overhead grow lights immediately after seeding (Sardanelli and Kenworthy, 1997b). The seeded MRS remained upon the rack for 10 days before transfer to a growth chamber (Convion, model BRW-36 growth chamber was used for trial one and a BRD-8 model was used for trial two). Utilization of the heating mat during this ten-day period resulted in a germination rate of nearly 100% within seven to ten days.

Soil moisture conditions for the grow tubes and the MRS units' reservoirs were monitored regularly and water was added to the reservoirs as necessary during the nine week period. Four weeks post-seeding, each pepper plant was inoculated with a 1 ml suspension containing 2,000 eggs of *M. incognita*. The suspension was pipetted into the soil medium of each grow tube to a depth of approximately two cm, approximately 1.25 cm from the base of the pepper stem (Sardanelli and Kenworthy, 1997a). The *M. incognita* population was from the Lower Eastern Shore Research and Education Center located in Salisbury, Maryland where the population was maintained within nematode infested plots. This population was verified microscopically as *M. incognita* and then increased in greenhouse stock cultures maintained at University of Maryland College Park.

At the conclusion of the ten-week period, nematode reproduction was evaluated by assessing egg populations recovered from the roots (Hussey and Barker, 1973), and by enumerating second stage juveniles (J2) present in the soil and roots (Hooper, 1986;

Hussey and Barker, 1973). Prior to nematode collection, plant height was measured from the soil surface to the uppermost portion of true stem. Following this measurement, the pepper plant, still within the grow tube, was submersed for one minute in a bucket containing approximately three L of lukewarm water. While submerged, the pepper plant was gently loosened from the confines of the grow tube, and the roots were swirled in the water to remove remaining soil. Each plant was then air-dried. The plant's shoot was excised from its roots, and root and shoot fresh weights were measured. The root was then cut into pieces two cm long and placed into a 250 ml capped flask for egg retrieval.

A modified version of the Hussey and Barker (1973) egg retrieval method was used. Approximately 100 ml of a 0.6% NaOCl dilution was added to the flask containing the root segments. The flask was placed upon a rotary shaker (200 rpm) for six minutes. The contents of the flask were then immediately sieved using a 60- μ m-pore sieve on top of a 500- μ m-pore sieve. The eggs collected on the 500- μ m-pore sieve were rinsed three times with tap water, poured into a counting dish, and diluted by addition of 100 ml of tap water. The roots collected on the 60- μ m-pore sieve also were rinsed three times with tap water and placed onto prepared funnels for extraction of J2 via a modified Baerrman funnel technique (Hussey and Barker, 1973). The soil that remained in the bucket of water following removal of the plant from its grow tube was thoroughly swirled and allowed to settle for 45 seconds before being poured through a 325- μ m-pore sieve to collect any J2 that were present in the soil. The J2 were placed onto a prepared funnel for extraction using a modified Baerrman funnel technique (Hooper et al., 1986), collected J2 from soil were diluted to 100 ml with tap water.

Mean number of eggs per root system were estimated by counting eggs from three 1 ml aliquots from the sample, calculating an average from the three aliquots and multiplying that number by 100. At the completion of 48 hours (Baerrman funnel procedure), the J2 from the roots and soil were enumerated; the number of J2 nematodes obtained from root extractions were small enough to be counted without dilution. J2 from soil were enumerated using two 1 ml aliquots withdrawn from the diluted 100 ml sample. The two soil extraction aliquots were counted and averaged to obtain the number of soil J2 per sample.

Analysis:

Two trials were performed, each consisting of three randomized blocks with four subsamples. Each trial evaluated the treatment Pepper + RKN. Data from both trials was combined for analysis, but are displayed separately in table 1. Parameters analyzed with PROC MIXED (SAS Institute) were shoot height, shoot fresh weight, root fresh weight and total eggs and J2 per gram root. Tukey's multiple mean comparison test was utilized when appropriate.

Results

Six weeks post inoculation of the grow tubes containing bell pepper, *M. incognita* populations ranged from 190,000 to nearly 300,000 nematode eggs and J2 per grow tube (Table 1). Extrapolation of those population numbers indicates that a single bioassay model that contains 36 grow tubes as a culture system has the capacity to produce more than 10 million root-knot nematode eggs and J2 within the space of 0.19 m² (Figure 1).

Table 1. Plant growth measurements and *Meloidogyne incognita* recovered from *Capsicum annuum* L. cv. Yolo Wonder within the Moisture Replacement System. Results displayed of trials 1 and 2 represent averages of the three blocks of the completely randomized design. Results within a category with different letters were significantly different at $p=0.10$.

	Shoot height (cm)	Shoot fresh weight (gm)	Root fresh weight (gm)	Eggs	J2 ¹ in the root	J2 in the soil	Total eggs and J2	Total eggs and J2 per gm root
Trial 1	7.4 a	1.5 a	7.6 a	265,958	71	26,533	292,563	43,889 a
Trial 2	6.9 a	1.9 a	5.3 b	163,583	18	29,542	193,143	39,136 a

¹J2 = second stage juvenile root-knot nematode

M. incognita populations in the two trials were compared for eggs, for soil J2, and for root J2 populations (Table 1). Eggs accounted for approximately 85 to 90 percent of the total extracted populations of eggs and J2. Root J2 accounted for less than 1 percent and soil J2 accounted for the remaining 10-15 percent.

Evaluation of nematode reproduction proceeded in three phases: enumeration of eggs, J2 in roots, and J2 in soil. For the two trials the results were fairly consistent for each of the three phases of enumeration (Table 1). In addition to total eggs and J2 per gram root, shoot height, shoot fresh weight and root fresh weight was also statistically compared between trials. The only significant difference found between the two trials was for the root fresh weight (Table 1). The higher weight in trial one may account for a tendency towards greater numbers of nematodes in the first trial.

However, one observation that demonstrates consistency of reproduction using the MRS is the average yield of total eggs and J2 per gram root (Table 1).

Discussion

Based upon the results obtained in this study, enumeration of root juveniles in future studies may be unwarranted for studies of this nature. This would eliminate one labor-intensive extraction and enumeration step.

Variability in root growth may be due to differing conditions within the two growth chambers utilized for trials one and two. Although both growth chambers were set to the same environmental parameters, the difference in chamber sizes or models (trial one utilized a larger growth chamber than used for the second trial), may have affected conditions that altered pepper growth between the two trials. Another factor possibly

affecting root weight is the age difference of the seeds utilized in the two trials. While both trials were performed with seeds from the same lot, the second trial was performed several months later.

There are several features of the MRS culture design that facilitate the establishment of a supportive environment for several generations of nematode reproduction. One of the most significant features is its capacity to maintain a constant moisture level in the soil of the grow tubes via a hydraulic gradient. Nematodes are aquatic animals requiring the presence of water for mobility and infection, and a consistently hydrated environment is essential for the promotion of survival and reproduction. As water in the soil media is either utilized by the plant or lost to evaporation, a gradient is initiated and maintained as water from a bottom reservoir is transported upwards through a nylon wick and into the soil media. By utilizing the natural cohesive forces of water a continuous state of moisture is maintained within the grow tube environment.

Other features of the MRS culture system that contribute to the significant numbers of eggs and J2 retrieved from each host plant are the ability to direct seed the host plant into each grow tube and the accessibility of healthy roots to *Meloidogyne* J2. Some culture methods rely upon transplantation of the host plant into the system near the time of inoculation, which may affect overall population numbers through root injury. Also, RKN have a preference for root cells in the region of root elongation (Dropkin, 1989). The MRS provides an aggregation of the host plant root system within the 50 ml volume of the bioassay grow tube. The profusion of fresh root tips within the exact

vicinity of the inoculation may enhance the process of infection and eventual reproduction.

The MRS is also an efficient culturing system for *M. incognita* because time, labor and resources are minimized compared to greenhouse culture. Small amounts of soil media are required to fill thirty-six 50 ml grow tubes, and approximately nine liters of water is needed to fill the reservoir. This process requires approximately one hour. After the inoculation process four weeks later, the only interventions required are daily observation of the soil for sufficient moisture and replenishment of the water reservoir when necessary, thus waste water is eliminated. The bioassay MRS may be established and maintained within a laboratory, growth chamber, or greenhouse. Even when maintained in a greenhouse setting, space is conserved. Requiring only the space necessary for a vertical utility shelf, an entire culture operation may be easily managed with resultant egg extraction numbers comparable to traditional greenhouse operations.

The MRS bioassay model is a reliable and consistent method of increasing populations of *M. incognita*. A single 50 ml grow tube has the potential to produce ca. 300,000 eggs and J2 just six weeks following a 2,000 egg inoculation. Traditional culture techniques have generally resulted in considerable variability for nematode populations due to multiple factors as mentioned in the introduction. The results obtained in this study indicate that the MRS culture system can be a highly dependable method of producing *M. incognita* inoculum.

ABSTRACT

CHAPTER 3: EVALUATION OF *TRICHODERMA VIRENS* SURVIVAL AND GROWTH WITHIN THE MOISTURE REPLACEMENT SYSTEM

The Moisture Replacement System (MRS), a bioassay system recently developed for the study of soil-dwelling nematodes, was evaluated for its ability to support the survival and increase in populations of three isolates of the fungus *Trichoderma virens* (Giddens and Foster) von Arx. on bell pepper (*Capsicum annuum* L.) in soil infested with *Meloidogyne incognita* (Kofoid and White) Chitwood. This study was performed as a preliminary step for the assessment of *T. virens* isolates as potential biocontrol organisms for the management of root-knot nematode (RKN). Adjunct studies were also performed to ascertain effective protocol for application of the isolates onto the seed and a protocol was selected for application of each isolate was applied to pepper seeds as a seed coat.

A single coated seed was planted into each grow tube of the MRS. Three weeks post-seeding, isolates were reapplied in drench form to each appropriate grow tube. One week following the drench, each grow tube was inoculated with 2,000 eggs of *M. incognita*. The survival and growth of the three fungal isolates were evaluated based on reisolation procedures by dilution plating onto a *Trichoderma* selective medium. Three reisolation procedures were performed during the study: immediately following seed coat application; three weeks post-seeding (just prior to the drench application); and eleven

weeks post-seeding. Initial reisolation procedures revealed approximately 10^5 to 10^6 colony-forming units (CFU) within each seed coat. Three weeks post-seeding, 10^5 CFU per 0.10 gm of root were reisolated. At the conclusion of the study (11 weeks post-seeding), CFU in the range of 10^5 per gram of root were reisolated. These results substantiate the use of the MRS in studies evaluating *T. virens* for the management of RKN.

Introduction

Trichoderma virens is a soil-dwelling microscopic fungus that has been studied as a potential biocontrol organism since the 1930's (Weindling, 1934). To date, *Trichoderma*'s greatest contribution as a biocontrol agent has been in the management of damping off diseases caused by the pathogens *Pythium ultimum* Trow and *Rhizoctonia solani* Kuhn (Lumsden and Locke, 1989) in bedding plants. In recent years, as *Trichoderma*'s potential for biocontrol has been promoted, research efforts involving this organism have increased. Much information is known regarding *Trichoderma*, including its ideal habitat and growth requirements (Papavizas, 1985). This information has proven useful to those studying *Trichoderma* as a management agent against *Meloidogyne* spp.

Ultimately, research involving *Trichoderma* as a potential biocontrol agent for RKN species must be field tested. However, prior to field testing a significant amount of background work must occur. Before a time and labor consuming field study can be performed, a potential biocontrol isolate must be carefully examined through controlled experiments within a laboratory or growth chamber. At first, the ability to proliferate the organism within the test environment is of particular interest.

An attractive approach to introducing a biocontrol organism is by application to a host crop's seed. The theory of this approach is that the biocontrol organism will proliferate upon the seed and transfer to the emerging root. If compatibility between the host plant and the biocontrol organism exists, the biocontrol organism ideally will colonize the entire root system, providing a protective barrier around the surface of the root (Harman, 1991).

Within a gnotobiotic test environment, seed of a host plant is coated with the biocontrol organism and the performance of the organism under the experimental conditions is observed and evaluated. This approach is further refined as the application of the seed coat and the controlled environment are modified accordingly to the organism's preferred parameters of temperature, soil media, nutrients and moisture.

Establishing the biocontrol organism within a non-native environment poses a significant challenge (Stirling, 1991). Although the many environmental factors of growth may be considered, this does not always ensure that the biocontrol organism will thrive (Meyer et al., 2000; Meyer et al., 2001). To evaluate for the survival of the biocontrol organism within the test environment, it is important to test for the presence of the organism at the time of introduction, at an appropriate interval following introduction and at the conclusion of the experiment. This periodic testing provides confirmation regarding the application technique and helps determine if the study environment is supportive. This allows for successful evaluation of the biocontrol agent's antagonistic effects (Kerry, 2001).

Presence of the biocontrol organism during and at the conclusion of the experiment also indicates the potential for rhizosphere colonizing ability. Because soil-dwelling diseases attack the root surface of the plant, a biocontrol organism capable of residing long-term on or near the root surface enhances the plants defenses against potential pathogens. Rhizosphere colonization is considered to be such a valuable characteristic of biocontrol organisms that it has developed into an important field of research (Stirling, 1991).

A previously tested bioassay system known as the Moisture Replacement System (MRS) (Sardanelli and Kenworthy, 1997a) has been evaluated and found to be an efficient and effective culturing method for the RKN, *M. incognita*. This system promotes the growth of the host plant, and the establishment and increasing density of a population of *M. incognita*. The success of the MRS is attributed to its inherent capability to maintain a constant level of soil moisture, an important criterion in the growth of many organisms. In an earlier study, this system was shown to effectively support two organisms that are interactive within a field environment, Yolo Wonder bell pepper and *M. incognita*. The objective of this study was to evaluate the MRS for its potential to establish and maintain isolates of *T. virens* in conjunction with *M. incognita* and a host plant, Yolo Wonder bell pepper. Three treatments were evaluated: 1) Pepper (P) + RKN + *T. virens* Gl-3 applied as a seed coat, 2) P + RKN + *T. virens* Gl -15 applied as a seed coat and 3) P + RKN + *T. virens* Gl 21 applied as a seed coat.

This objective was measured by assessing for the presence of *T. virens* at several intervals during the course of the experiment. As the experiment progressed the need for additional procedural information became apparent. Side experiments were designed and conducted to attain that information. They are fully described within appendices A, B, and C. The objectives for those side studies were: observation of *Trichoderma* growth upon two different substrates (Appendix A); assessment of the effect of a period of high humidity and two different seed adhesives upon the growth of *T. virens* on the pepper seed (Appendix B); and the comparison of two techniques in the application of *T. virens* upon the pepper seed (Appendix C).

While this chapter details the survival of *Trichoderma* isolates within the MRS, it is important to note that the primary objective of this overall project was to evaluate the three isolates for their biocontrol potential upon *M. incognita*. The dual nature of the overall project allowed for both components, *Trichoderma* growth and potential biocontrol effects, to be observed simultaneously. Three trials of the experiment were performed to achieve the objectives for this project. The analysis of the objective for this chapter, survival of the isolates, was determined utilizing results from the first and the second trials. The analysis of the potential biocontrol effect of *Trichoderma* isolates upon the RKN population utilized the second and third trials and will be reported and discussed in the fourth chapter.

Materials and Methods

Isolates and proliferation:

Three wild type isolates of the fungus, *T. virens* G1 3, G1 15 and G1-21 (USDA, ARS Alternate Crops and Systems Laboratory, Beltsville, MD) were used in this study. These isolates were of Maryland origin and demonstrated a high level of activity against *Pythium* and *Rhizoctonia* in previous research (R. Lumsden personal communication, 2001). Each isolate was maintained on corn meal agar (CMA, Benton-Dickson, Sparks, MD) to minimize the occurrence of potential mutations that may be observed on agars promoting faster growth, such as potato-dextrose agar (PDA, Difco, Sparks, MD). Appendix A explains how *T. virens* growth upon CMA was determined acceptable for this experiment.

Each *T. virens* isolate was cultured independently. Within a laminar flow hood an eight cm diameter portion of a week-old culture was blended within a 360 ml microblender for 30 seconds. This blended fraction was placed into a 500 ml flask containing 250 ml of a twice-autoclaved mixture of liquid molasses-yeast media (30 g molasses and five g brewer's yeast per 1 L distilled water) (Lewis and Papavizas, 1984). The culture flasks were then placed upon a rotary shaker (125 rpm) at room temperature (23 to 24° C) for five days. At the end of five days a five ml portion of the fungal culture accumulated within each flask was removed and transferred within a laminar flow hood to a newly prepared, twice-autoclaved 500 ml flask containing 250 ml of molasses-yeast media. This last step was performed to eliminate as much of the CMA as possible from the fungal culture. The flasks were placed onto the rotary shaker (125 rpm and room temperature) for an additional 15 days.

Host plant:

Capsicum annuum L. cv. Yolo Wonder bell pepper was selected as the most suitable host based on preliminary studies referred to in chapter two.

Seed coat process:

T. virens cultures were removed from the rotary shaker at the end of the 15-day period. Each was centrifuged at 13,000 rpm for ten minutes to consolidate the fungal biomass. The biomass for each isolate was collected and homogenized at 11,000 rpm for 30 seconds to produce a fungal slurry. Sterile distilled water was added to the homogenizer in small increments to achieve desired slurry consistency during homogenization. The propagules (conidia and chlamydospores) of the slurry were enumerated with a hemacytometer. Based on the calculated propagule counts, the slurry

was diluted with sterile distilled water as necessary to produce dilutions of similar concentrations across the three fungal slurries. Once the desired concentration was achieved, a mixture of eight ml of the appropriate fungal slurry was combined with two ml of gelatin (Difco, Sparks, MD) that served as the propagule adherent. Pepper seeds were coated by immersion into this mixture and then withdrawn with autoclaved forceps. They were immediately placed into their designated grow tube in the MRS at a depth of one cm and covered with the soil mixture. Appendices B and C describe how this application protocol was finalized.

MRS preparation:

The MRS bioassay model and each of the 50 ml tubes (36 total) were prepared according to procedures described by Sardanelli and Kenworthy (1997a). The reservoir of the MRS was divided into sections equal to the number of treatments using small plastic lined trays. This division of reservoir space was incorporated to eliminate any potential for cross-contamination within the reservoir water between the various treatments.

The soil mixture (89% sand, 4% silt, 7% clay, 1.7% organic matter, pH 4.9) consisted of 90% builders sand and 10% Scotts Redi-Earth Plug and Seedling Mix (Scotts Company, Columbus, OH). Soil components were first autoclaved, then combined and the resultant soil mixture moistened with tap water according to published specifications. While filling the grow tubes with the soil mixture, four pellets of Osmocote 15-9-12 (Scotts Company, Columbus, OH) were incorporated into each tube approximately five cm from the bottom.

Once planted with the coated seeds the MRS was placed onto a thermostatically controlled propagation mat preheated to 26.7 °C and situated upon a utility shelf with overhead grow lights set to a 12 hr photoperiod. The MRS remained on the propagation mat for a ten-day period allowing maximum germination of the pepper seeds to be achieved. Following the germination period, the MRS units were transferred to the growth chamber (Conviron BRW-36) with conditions set to 27° C, 40% humidity and a 12 hr photoperiod.

Drench process:

Three weeks after seeding, a slurry drench of 100,000 propagules of each fungal isolate was applied to their respective MRS grow tubes. This additional application functioned as insurance that the fungal organism was present throughout the experimental period. The drench for each isolate was prepared by the same procedure as the seed coat slurry, except it was diluted to a concentration of 100,000 fungal propagules (conidia and chlamydospore) per ml. A one ml drench of each fungal isolate was injected by pipet into the soil mixture at a depth of approximately two cm adjacent to the base of the respective seedling. The residual hole was covered with soil mix.

Nematode inoculation:

Four weeks post-seeding, grow tubes designated for inoculation with *M. incognita* received 2,000 eggs each. Inoculations were applied by pipet injection one cm into the soil mix adjacent to the base of each seedling.

Reisolation of *T. virens*:

Seed coat reisolation:

During the initial seed coat process, 15 freshly coated seeds from each fungal isolate were withdrawn from the slurry mix and reserved for reisolation studies. The 15 seeds were divided into three groups (replications) of five seeds each. Each replication was placed into an autoclaved vial and 9.9 ml of sterile distilled water was added. This dilution (1:10) was then sonicated for five minutes and vortexed for one minute. Four additional dilutions were created from each vial so that five dilutions total (1:10 to 1:100,000) were prepared for evaluation for the concentration of *T. virens*. Each vial was dilution-plated (Koch, 1994) within a laminar flow hood using *Trichoderma* medium E (TME), (Papavizas and Lumsden, 1982). Once preparation of each plate was completed, it was wrapped with parafilm and placed upon the lab bench where *Trichoderma* colony growth was observed daily for five days.

Three-week reisolation:

Three weeks after initiation of each trial, verification of *Trichoderma* presence and proliferation was conducted by reisolation procedure using two pepper seedlings from each isolate. This allowed for two repetitions of four dilutions for each fungal isolate to be produced. Each seedling used was removed from the grow tube and excess soil was removed from the seedling's root by dipping the root into tap water. The seedling's shoot was excised from the root, and the root (approximately 0.10 g) was placed into an autoclaved vial. The four dilutions (1:10 to 1:10,000) were created and standard dilution plate methods (Koch, 1994) were performed using TME as described for the seed coat reisolation.

Final reisolation:

At the conclusion of the 10-week study, two pepper plants were selected to verify *T. virens* presence for each isolate. Each plant was removed from the grow tube and excess soil was removed per the procedure used for the three-week reisolation. The shoot was excised from the root, and the root was cut into small pieces. Total root weight was measured. The root pieces were mixed and 30% of the root by weight was selected and placed into an autoclaved vial. Standard dilution plating (Koch, 1994) was performed on these selected root pieces using TME as described earlier. Two repetitions of four dilutions for each fungal isolate were produced.

Design:

The study employed a randomized complete block design. Each MRS unit (three total) served as a block (replication) within the growth chamber. Each *Trichoderma* fungal isolate was randomly assigned to a group of four grow tubes in each MRS unit. The experiment was repeated once.

Results

Reisolation of biocontrol agent:

The number of colony forming units (CFU) reisolated from the coated seeds ranged from 10^5 – 10^6 CFU per seed (Table 2). This range was considered sufficient according to results obtained by Mao et al. (1997) in studies evaluating *T. virens* G1 3 as a biocontrol agent for diseases caused by *Pythium* and *Fusarium* species. In those studies, the minimum population needed to achieve management activity similar to a pesticide was estimated to be between 10^4 and 10^6 CFU per seed.

Table 2. Survival and growth data for three *Trichoderma virens* isolates evaluated as potential biocontrol organisms.

Fungal Isolate	Fungal slurry concentration (propagules/ml)	Seed CFU reisolation ¹ (CFU/seed)	3 wk root reisolation ² (CFU/0.10 g root)	11 wk reisolation ² (CFU/g root)
<i>Trial 1</i>				
Gl-3	1.3×10^9	4.6×10^5	$1.5 \times 10^5/0.10 \text{ g}$	1.2×10^5
Gl-15	1.4×10^9	8.6×10^5	$2.5 \times 10^5/0.10 \text{ g}$	1.4×10^5
Gl-21	2.2×10^9	9.9×10^5	$7.4 \times 10^5/0.10 \text{ g}$	1.6×10^5
<i>Trial 2</i>				
Gl-3	1.5×10^9	1.1×10^6	$3.3 \times 10^5/0.10 \text{ g}$	2.7×10^5
Gl-15	1.8×10^9	7.9×10^5	$4.0 \times 10^5/0.10 \text{ g}$	1.5×10^5
Gl-21	1.5×10^9	1.1×10^6	$2.0 \times 10^5/0.10 \text{ g}$	2.0×10^5

¹ Seed CFU reisolation results for the two trials represent averages of three repetitions consisting of five dilutions each.

² Three week and eleven week reisolation results represent averages of two repetitions consisting of four dilutions each.

The CFU reisolated from the seedling roots three weeks post-seeding were similar to the amounts present during the initial seed coat reisolation (Table 2). The fact that the fungus had been maintained on an average seedling root weight of approximately 0.10 grams provided evidence that the MRS was capable of providing an environment supportive of the maintenance of the fungal organism.

At the conclusion of the study, approximately eleven weeks post-seeding, reisolation of *Trichoderma* CFU from each treatment was performed. This last reisolation effort evaluated the survival of the fungal organism over the 11-week period. Those CFU counts are reported in Table 2. For ease of comparison regarding the persistence of the three fungal isolates, final results are reported as a per gram root basis. The CFU present at the start of the study (seed coating) ranged from 460,000 and 1,100,000 propagules per seed (Table 2). Final reisolation (11-week post-seeding) evaluations revealed 120,000 to 270,000 propagules per gram root and clearly indicated a increase in the population of the organism across the three isolates. These results support the employment of the MRS in studies evaluating potential biocontrol organisms.

Discussion

There are several parameters which may have supported the successful growth and survival of the *Trichoderma* isolates within the MRS. First, the planting of freshly coated seeds directly into the pre-moistened environment of the MRS grow tubes most likely favored propagule germination. Mycelial fragments, chlamydospores and numerous conidia were observed in the seed coat slurry. As discussed in Appendix C, a freshly coated seed (versus the dried coat seed) is more likely to enhance germination of

the chlamydospores contained in the fungal slurry. The pre-moistened environment of the MRS likely promoted germination of the chlamydospores as well as the desiccant-sensitive conidia. Second, the contents of the seed coating presumably enhanced growth of the *Trichoderma* isolates. Residual nutrients of the liquid molasses yeast media and the gelatin adhesive were present in the fungal slurry used for the seed coating. Beagle-Ristaino and Papavizas (1985) reported on a study involving *Trichoderma* spp. that the residual nutrients of the liquid molasses and brewer's yeast medium, when applied in close association with the chlamydospores, provided an adequate food base to support growth and sporulation of the fungus in the soil. Results from Beagle-Ristaino and Papavizas support what was observed in this experiment. The fungal propagules most likely utilized the food source within the seed coat to augment establishment upon the seed and eventually proliferation onto the roots.

To date, documented biocontrol studies utilizing wild-type *Trichoderma* isolates have been primarily employed as a management tool for seedling diseases (Hadar et al., 1983; Lumsden and Locke, 1989; Mao et al., 1997). While numerous species of *Trichoderma* are known for their capacity to colonize the seed and upper portions of the root, it is rare to find a wild-type *Trichoderma* spp. capable of colonizing lower portions of the root. This capability is termed rhizosphere competence and is defined as the physiological and genetic ability to proliferate along the root as it develops (Harman, 1992). Rhizosphere competent organisms are those capable of colonizing the root surface or rhizosphere when applied either as a seed coat or other point source at the time of planting in the absence of bulk flow of water (Ahmad and Baker, 1987). The utilization of the MRS used in this experiment has addressed the absence of bulk flow of

water. The MRS accomplishes this by drawing water upward from a bottom reservoir and into the base of the soil mix via a nylon wick. As water either evaporates from the soil surface or is utilized by the plant, more water is pulled up through the wick and the soil mix remains hydrated due to natural cohesive forces of water. This function of the MRS eliminates the concern that the downward flow of water caused by top watering may have caused the downward movement of a fungal isolate along the root. With the MRS, the potential for rhizosphere colonization has been separated from the problematic effects of moisture maintenance.

Previous studies quantifying the rhizosphere competence of fungal organisms have typically evaluated the presence of the organisms at regular intervals along the length of the root (Ahmad and Baker, 1987; Harman, 1992). In this current study, rhizosphere competence cannot be fully confirmed for the three *Trichoderma* isolates, since competence was measured indirectly. To evaluate for the presence of the isolates at the conclusion of this study, only 30% of randomly selected segments of the root by weight was used for dilution plating. Though the root segments were considered representative of the entire root, they provided only an approximation of the presence of the fungal organism upon the entire root. Using a representative portion of the root for approximation was appropriate to determine fungal presence but cannot be used to claim colonization along the length of the root. Instead, a degree of persistence of the isolates was assumed since their presence was detected in adequate quantities that were maintained from initial application until the conclusion of the study.

Previous studies that have evaluated *Trichoderma* strains for rhizosphere competence have typically found that the rhizosphere competent strains are present in a

‘C’ shaped curve upon the root (Harman, 1992). The greatest numbers of CFU have been found near the upper portions of the root, and near the root tips with fewer CFU detected in the middle portions of the root (Ahmad and Baker, 1987; Sivan and Chet, 1989). This pattern has been attributed to the availability of food sources for the fungus present within the nutrients of the seed coat and exudates from the germinating seed that are found in the upper portions of the root, as well as exudates produced from the root tips. Considering this growth pattern, the MRS may serve as a useful bioassay tool by maximizing this characteristic growth pattern. Because each grow tube of the bioassay model is small in size (50 ml), the root tips are never far from the upper portions of the root. This proximal aggregation of the lower and upper portions of the root possibly promotes an environment rich with exudates and amendments and thus, enhances the overall proliferation of the potential biocontrol organism. It is believed that these factors influenced the survival of the three *Trichoderma* isolates evaluated in this study.

Previous studies have also reported that a fungus (biocontrol organism) may be more abundant on roots infected with nematodes compared to those that are healthy. This has been related to the release of nutrients into the rhizosphere triggered by the presence of the nematode population (Bourne et al., 1996). In this study, the evaluation of the maintenance and growth of the three *Trichoderma* isolates was done in conjunction with an evaluation of those isolates as potential biocontrol organisms for RKN. The presence of the nematodes that were introduced to the grow tubes may have enhanced the proliferation of the isolates. Another related consideration is the fact that *Trichoderma* is a saprophytic organism. As infection by the nematode ensues upon the roots, cells are sloughed from dead or decaying root tissues. This decaying material could also serve as

a food source for the fungal organism, thereby enhancing its presence along roots infected with nematodes.

Another factor that may have enhanced the growth of the fungal organism was the host plant selected for the study, Yolo Wonder bell pepper. Plant species differ in their ability to support fungal growth (Kerry, 2001). The selection of an appropriate host plant that promotes fungal growth will obviously further the chance of detecting biocontrol capabilities.

In conclusion, the MRS is suited for evaluations of *T. virens* isolates G1-3, G1-15 and G1-21 as potential biocontrol organisms for RKN. Alterations in host plant, nematode populations and soil components within future evaluations may influence the final results and are important considerations. Investigations of this nature provide valuable information as the need to discover alternative methods of nematode management becomes increasingly urgent.

ABSTRACT

CHAPTER 4: ASSESSMENT OF *TRICHODERMA VIRENS* FOR BIOCONTROL OF THE ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

Three isolates of the soil-dwelling fungus *Trichoderma virens* (Gl-3, Gl 15 and Gl 21) (Miller, Giddens and Foster) von Arx. were assessed for their ability to decrease populations of the root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood. The study was performed under growth chamber conditions incorporating the use of the Moisture Replacement System. Each of the three isolates was applied twice, first as a seed coat and later as a seedling drench. Four weeks post-seeding, pepper seedlings were either inoculated with 2,000 *M. incognita* eggs or left uninoculated (control). All treatments were harvested ten weeks post-seeding and evaluated for select plant growth characteristics and *M. incognita* population enumeration. None of the isolates consistently affected plant growth or suppressed the total number of nematode eggs plus second-stage juveniles (J2). Presence of the isolates on the roots of the pepper plants was verified at the conclusion of the study, demonstrating a capability for persistence as well as a potential for long-term rhizosphere colonization. The results indicated that although the isolates exhibited the potential for rhizosphere colonization, they were not suppressive to *M. incognita*.

Introduction

Meloidogyne incognita, the root-knot nematode (RKN), has proven to be a highly destructive plant pest in Maryland with a large impact on vegetable crops (Sardanelli et al., 1984). Many of these vegetable crops rely heavily on the application of methyl bromide for the management of RKN infestations. With the impending loss of methyl bromide from the market, growers are faced with a lack of resources from which to choose in an attempt to manage RKN effectively. This need for additional management tactics has spurred an effort to seek out environmentally sound alternatives.

An ubiquitous, soil-dwelling fungal microbe, *Trichoderma*, has long been studied as a biocontrol agent with capabilities of managing fungal seedling diseases (Hadar et al., 1983; Lumsden and Locke, 1989; Mao et al., 1997). *Trichoderma* species possess a number of characteristics that make them highly suitable for use as biocontrol agents. *Trichoderma* is a saprophytic organism capable of utilizing a wide variety of substrates to satisfy its minimal nutritional requirements. Species of *Trichoderma* are known for their production of cell wall digesting enzymes and anti-fungal substances that promote survival and colonization by antibiosis and mycoparasitism (Nevalainen and Neethling, 1998). Under suitable conditions, *Trichoderma* species exhibit a rapid growth rate. They are prolific producers of conidia, a quality highly desired in the commercial production of biocontrol agents. They are also capable of producing chlamydospores, a specialized structure ensuring continued survival under harsh environmental conditions less conducive to survival of the more sensitive conidia (Papavizas, 1985). Commercial fungal biocontrol products have been successfully developed and marketed as a result of research involving this fungus (APS Biological Control Committee, 2003).

Over the years research efforts involving *Trichoderma* have further intensified to evaluate its effectiveness in reducing plant-parasitic nematodes. Several species of *Trichoderma* evaluated against RKN species other than *M. incognita* have been found to be effective in suppressing reproduction (Windham et al., 1989), inhibiting penetration and egg hatch (Sharon et al., 2001) and decreasing the presence of galls (Parveen et al., 1993).

Previous studies indicated that antibiotics played a major role in the antagonism of *T. virens* to some plant pathogenic fungi (Lumsden and Locke, 1989; Roberts and Lumsden, 1990). An isolate of *T. virens*, G1 21, isolated from Maryland soils and studied at USDA was evaluated as a biocontrol for damping-off diseases. This isolate was found to significantly reduce the effects of disease primarily through mechanisms of antibiosis (Lumsden and Locke, 1989). Those studies led to the successful production of a biocontrol product (Soilgard©, 1999). In recent years, culture filtrates from an isolate of *T. virens*, G1 3, demonstrated antagonistic activity against *M. incognita* under laboratory conditions (Meyer et al., 2000). On further evaluation this isolate exhibited suppression of *M. incognita* on bell pepper in greenhouse studies (Meyer et al., 2001).

The objective of this research project was to further evaluate the isolate G1-3 along with two additional isolates of *T. virens* for their effectiveness as biocontrol agents against *M. incognita* on bell pepper (*Capsicum annuum* L.). These isolates were selected based on their Maryland origin and high level of activity against *Pythium* and *Rhizoctonia* in previous research (R. Lumsden personal communication, 2001).

Materials and Methods

Isolates and proliferation:

Three wild type isolates of the fungus *T. virens*, G1 3, G1 15 and G1-21 (USDA, ARS Alternate Crops and Systems Laboratory, Beltsville, MD) were used in this study. Maintenance cultures of each isolate were initiated on corn meal agar (CMA) (Difco, Sparks, MD).

Each *T. virens* isolate was cultured independently. In a laminar flow hood an 8-cm diameter portion of a week-old culture was blended in a 360 ml microblender for 30 seconds. This blended fraction was incorporated into a 500 ml flask containing 250 ml of a twice-autoclaved mixture of liquid molasses-yeast medium (30 g molasses and five g brewer's yeast L⁻¹ distilled water) (Papavizas et al., 1984). The culture flasks were then placed upon a rotary shaker (125 rpm) at room temperature (23 to 24° C) for five days. At the end of five days a five ml portion of the fungal culture within each flask was removed and transferred a laminar flow hood to a 500 ml flask containing 250 ml of molasses-yeast media, autoclaved twice. This last step was performed to eliminate as much of the CMA as possible from the fungal culture. The flasks were again placed onto the rotary shaker (125 rpm at room temperature) for an additional 15 days to promote the production of chlamydospores.

Host plant:

As the management of RKN in vegetables has become a priority, a bell pepper was selected as the test plant for this study. *Capsicum annuum* L. cv. Yolo Wonder bell pepper was selected as the most suitable host based on preliminary studies described in chapter two.

Seed coat process:

The isolates were applied twice during the experiment to each treated plant: first in seed coatings, and again in seedling drenches. For seed coating, *T. virens* cultures were removed from the rotary shaker at the end of the 15-day period. Each culture was centrifuged at 13,000 rpm for ten minutes to consolidate the fungal biomass. The biomass was homogenized at 11,000 rpm for 30 seconds, producing a fungal slurry. The propagules (conidia and chlamydospores) of the slurry were enumerated with a hemacytometer. Based on those counts, the slurry was diluted with sterile distilled water as necessary to produce dilutions of similar propagule concentrations across the three fungal isolates. Once the desired concentration was achieved (approximately 10^9 propagules/ml), a mixture of eight ml of fungal slurry was combined with two ml of gelatin (Difco, Sparks, MD), which served as the adhesive. Pepper seeds were coated by immersion into this mixture and then withdrawn with autoclaved forceps. They were immediately placed into their designated grow tubes in the MRS at a depth of one cm and covered with soil mixture. To determine colony-forming units (CFU) per seed for each isolate tested, 15 seeds were reserved and divided into three groups (replications) of five seeds each. Each replication was placed into an autoclaved vial and 9.9 ml of sterile distilled water was added. This dilution (1:10) was then sonicated for five minutes and vortexed for one minute. Four additional dilutions were created from each vial so that five dilutions total (1:10 to 1:100,000) were prepared for evaluation for concentration of *T. virens*. Each vial was dilution-plated (Koch, 1994) onto *Trichoderma* medium E (TME) (Papavizas and Lumsden, 1982). Once preparation of each plate was completed,

it was wrapped with parafilm and placed upon the lab bench where *T. virens* colony growth was recorded for a period of five days.

MRS preparation:

The MRS bioassay and each of the 50 ml grow tubes (36 total) within were prepared according to procedures described by Sardanelli and Kenworthy (1997a). The reservoir of the MRS was divided into sections equal to the number of treatments using small plastic lined trays. This division of reservoir space was incorporated to eliminate any potential for cross-contamination within the reservoir water between the various treatments.

The soil mixture (89% sand, 4% silt, 7% clay, 1.7% organic matter, pH 4.9) consisted of 90% builders sand and 10% plug and seedling mix (Scotts Redi-Earth Plug and Seedling Mix, Scotts Company, Columbus, OH). Soil mixture components were first autoclaved, then combined and the resultant soil mixture moistened with tap water according to published specifications (Sardanelli and Kenworthy, 1997a). While filling the grow tubes with the soil mixture, four pellets of Osmocote 15-9-12 (Scotts Company, Columbus, OH) were incorporated into each tube approximately five cm from the bottom. Once seeded the MRS was placed onto a thermostatically controlled propagation mat (26.7 °C) and situated upon a utility shelf with overhead grow lights set to a 12 hr photoperiod. The MRS remained on the propagation mat for a 10-day period accommodating pepper's temperature requirement for germination. Following the germination period, the MRS units were transferred to the growth chamber (Convion, models BRW-36 (trial 1) and BRD-8 (trial 2)) with conditions set to 27° C, 40% humidity and a 12 hr photoperiod (Figure 2).



Figure 2. Image depicting arrangement of the three blocks (bioassay models of the MRS) within the growth chamber during the second trial.

Drench:

Three weeks post-seeding, a slurry drench of each fungal isolate was applied the appropriate MRS grow tubes. This additional application provided that the fungal biocontrol organism would be present throughout the experimental period. The drench for each isolate was prepared by the same procedure as the seed coat slurry, except it was diluted to a concentration of 100,000 fungal propagules (conidia and chlamydospore) ml⁻¹. A one ml drench of each fungal isolate was injected by pipet into the grow tube at a depth of approximately two cm adjacent to the base of each seedling. The hole caused by pipet insertion was covered with soil mix.

Nematode inoculation:

Four weeks post-seeding, grow tubes designated for inoculation with *M. incognita* received 2,000 eggs each. Inoculations were applied by pipet one cm into the soil mix adjacent to the base of each seedling.

Reisolation of *Trichoderma*:

Three-week reisolation:

Three weeks after initiation of each trial, verification of *T. virens* presence and proliferation was conducted by reisolation technique using two pepper seedlings from each isolate. This allowed for two repetitions of four dilutions for each fungal isolate to be produced. Each seedling was removed from the grow tube. Excess soil was removed from the root by dipping the root into tap water. The seedling's shoot was excised from the root and the root (approximately 0.10 g) was placed into an autoclaved vial. The four dilutions (1:10 to 1:10,000) were created and standard dilution plate methods (Koch, 1994) were performed using TME as described for the seed coat reisolation.

Final reisolation:

At the conclusion of the 10-week study, two pepper plants per isolate were selected to verify isolate presence. Each plant was removed from the grow tube and excess soil was removed per the procedure used for the three-week reisolation. The shoot was excised from the root and the root was cut into small pieces. Total root weight was measured. The root pieces were mixed and 30% of the root by weight was selected and placed into an autoclaved vial. Standard dilution plating (Koch, 1994) was performed on these selected root pieces using TME as described earlier. For each fungal isolate, four dilutions (1:10 to 1:10,000) were completed for each of the two root samples.

Plant vigor and nematode enumeration:

At the conclusion of the study, plant height was measured from the soil surface to the uppermost portion of the true stem. Following this measurement, each pepper plant within each grow tube, was submersed for one minute in a bucket containing approximately 3 L of lukewarm water. While submerged, the pepper plant was gently loosened from the confines of the grow tube. Once each plant was extracted, its roots were carefully swirled in the water to remove remaining soil. Each plant was then placed upon a paper towel to remove excess moisture. The plant's shoot was excised from its roots. Root and shoot fresh weight were measured. The root was then cut into pieces two cm long with clean scissors and placed into a 250 ml capped flask for egg retrieval.

An assessment of egg production on the roots (Hussey and Barker, 1973) and enumeration of second stage juveniles (J2) present in the soil (Hooper, 1986) and within the roots (Hussey and Barker, 1973) was conducted. A modified version of the Hussey and Barker (1973) procedure was used to retrieve eggs from the roots. Approximately

100 ml of a 0.6% NaOCl dilution was added to the 250 ml flask containing the root segments. The flask was placed upon a rotary shaker (200 rpm) for six minutes, and the contents of the flask were then immediately sieved using a 60- μ m-pore sieve on top of a 500- μ m-pore sieve. The eggs collected on the 500- μ m-pore sieve were rinsed three times with tap water and then poured into a counting dish. The roots collected on the 60- μ m-pore sieve also were rinsed three times with tap water and then placed onto prepared funnels for extraction of J2 via a modified Baerrman funnel technique (Hooper, 1986). The soil that remained in the bucket of water following removal of the plant and roots from its grow tube was thoroughly swirled and allowed to settle for 45 seconds before being poured through a 325- μ m-pore sieve to collect any J2 that were present in the soil. The material collected on this sieve was also placed onto a prepared funnel for J2 extraction using the same modified Baerrman funnel technique.

Eggs from each root were enumerated under the stereoscope using three 1 ml aliquots from the retrieved sample that had been diluted by addition of 100 ml tap water. The three aliquot counts were averaged. At the completion of 48 hours (Baerrman funnel procedure), the J2 extracted from the roots and soil were enumerated under the stereoscope. The number of J2 obtained from root extractions were small enough to be counted without dilution. Soil extractions of J2 were enumerated using two 1 ml aliquots withdrawn from the sample that had been diluted to 100 ml with tap water. The two soil extraction aliquots averaged to obtain the final number of soil J2 per sample.

Results of the egg retrieval (modified Hussey and Barker) from the roots and the J2 retrieval from the roots and soil were combined for a 'Total egg + J2 per gram of root' result which was utilized in the analysis. Effectiveness of each *T. virens* isolate was

determined according to 'Total eggs + J2' when compared with controls. Recovery results of the juveniles from the roots and soil were combined for a 'J2 per gram of root' analysis. Eggs recovered from the roots were categorized as 'Eggs per gram of root' and analyzed.

Design and analysis:

The experiment was designed as a randomized complete block arrangement of treatments with three blocks (MRS units). Each treatment consisted of four sub samples (grow tube + pepper plant). The seven treatments evaluated were: 1) pepper alone; 2) pepper + RKN; 3) pepper + adhesive; 4) pepper + adhesive + RKN; 5) pepper + adhesive + RKN + Gl-3; 6) pepper + adhesive + RKN + Gl-15; and 7) pepper + adhesive + RKN + Gl-21. Seven dependent variables were measured: 1) shoot height; 2) shoot fresh weight; 3) shoot dry weight; 4) root fresh weight; 5) J2 per gram of root; 6) eggs per gram of root; and 7) total (eggs + J2) per gram of root. Due to interactions encountered between the data of the two trials, the data for each trial was analyzed separately using PROC MIXED (SAS Institute) followed by application of Tukey's multiple mean comparison test when appropriate.

Results

Plant growth:

Overall, no differences for shoot growth were observed among the treatments in either trial (Table 3). Only root fresh weight (trial 1- $F = 2.36$, $P = 0.0971$, trial 2- $F = 2.57$, $P = 0.0777$) varied among treatments (Table 3). In trial one, pepper alone had the highest root fresh weight and was significantly greater than the lowest root weight from seed treated with isolate Gl-21 + RKN. In trial two, the treatment containing *T. virens*

Table 3. Bell pepper plant growth responses after treatment with isolates of the biocontrol fungus *Trichoderma virens* (isolates Gl-3, Gl-15 and Gl-21) and the root-knot nematode (RKN) *Meloidogyne incognita*. Letters are comparable within columns, but are not comparable between trials. Values represent averages of all sub samples from all blocks within a trial. Means with similar letters are not significantly different at $p=0.10$.

Treatment	Shoot height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)
Trial 1				
Pepper (P) alone	8.0 a	1.6 a	0.3 a	8.5 a
P + RKN	7.4 a	1.5 a	0.2 a	7.6 ab
P + Adhesive ¹	7.7 a	1.6 a	0.3 a	7.3 ab
P + Adhesive + RKN	7.4 a	1.5 a	0.3 a	7.1 ab
P + Adhesive + RKN + Gl-3	7.2 a	1.6 a	0.3 a	5.6 ab
P + Adhesive + RKN + Gl-15	7.5 a	1.4 a	0.2 a	6.6 ab
P + Adhesive + RKN + Gl-21	6.9 a	1.6 a	0.3 a	5.0 b
Trial 2				
Pepper (P) alone	7.4 a	1.9 a	0.4 a	5.3 ab
P + RKN	6.9 a	1.9 a	0.4 a	5.3 ab
P + Adhesive	6.8 a	1.6 a	0.3 a	4.0 b
P + Adhesive + RKN	6.6 a	1.6 a	0.3 a	4.3 ab
P + Adhesive + RKN + Gl-3	7.0 a	1.4 a	0.3 a	5.0 ab
P + Adhesive + RKN + Gl-15	7.2 a	1.6 a	0.4 a	6.0 a
P + Adhesive + RKN + Gl-21	7.0 a	1.5 a	0.4 a	4.2 ab

¹Adhesive refers to the adhesive agent, gelatin (Difco, Sparks, MD).

G1 15 + RKN had the highest fresh root weight, which was significantly different from the control of pepper + adhesive, which had the lowest fresh root weight. Root fresh weights of the control treatments with and without nematodes were not significantly different within each trial. Application of the isolate G1-21 + RKN resulted in some of the lowest root fresh weights in both trials.

Nematode populations:

The application of *T. virens* isolates G1 3, G1 15 and G1-21 had no suppressive effect upon nematode populations (Figure 3). Compared to the two control treatments without *T. virens*, the J2 per gram root, eggs per gram root, and total eggs + J2 per gram root tended to be higher with all *T. virens* treatments. In trial one, treatments containing *T. virens* G1 21 resulted in significantly higher nematode numbers than any treatment (total eggs + J2 per g root, $F = 8.28$, $P = 0.0061$). In trial two, the treatment containing *T. virens* G1-15 had significantly more J2 per gram root and eggs + J2 per gram root than the control of pepper + RKN alone (total eggs + J2 per g root, $F = 2.94$, $P = 0.0909$). Most treatments resulted in similar total egg + J2 recovery in the two trials, the exception was the G1-21 treatment, which resulted in nearly a three-fold recovery difference in eggs + J2 per gram root between the two trials (Figure 3).

Reisolation of biocontrol organisms:

T. virens isolates were successfully reisolated from pepper seed coats, pepper seedling roots three weeks post-planting, and pepper roots at the conclusion of the study (Table 4).

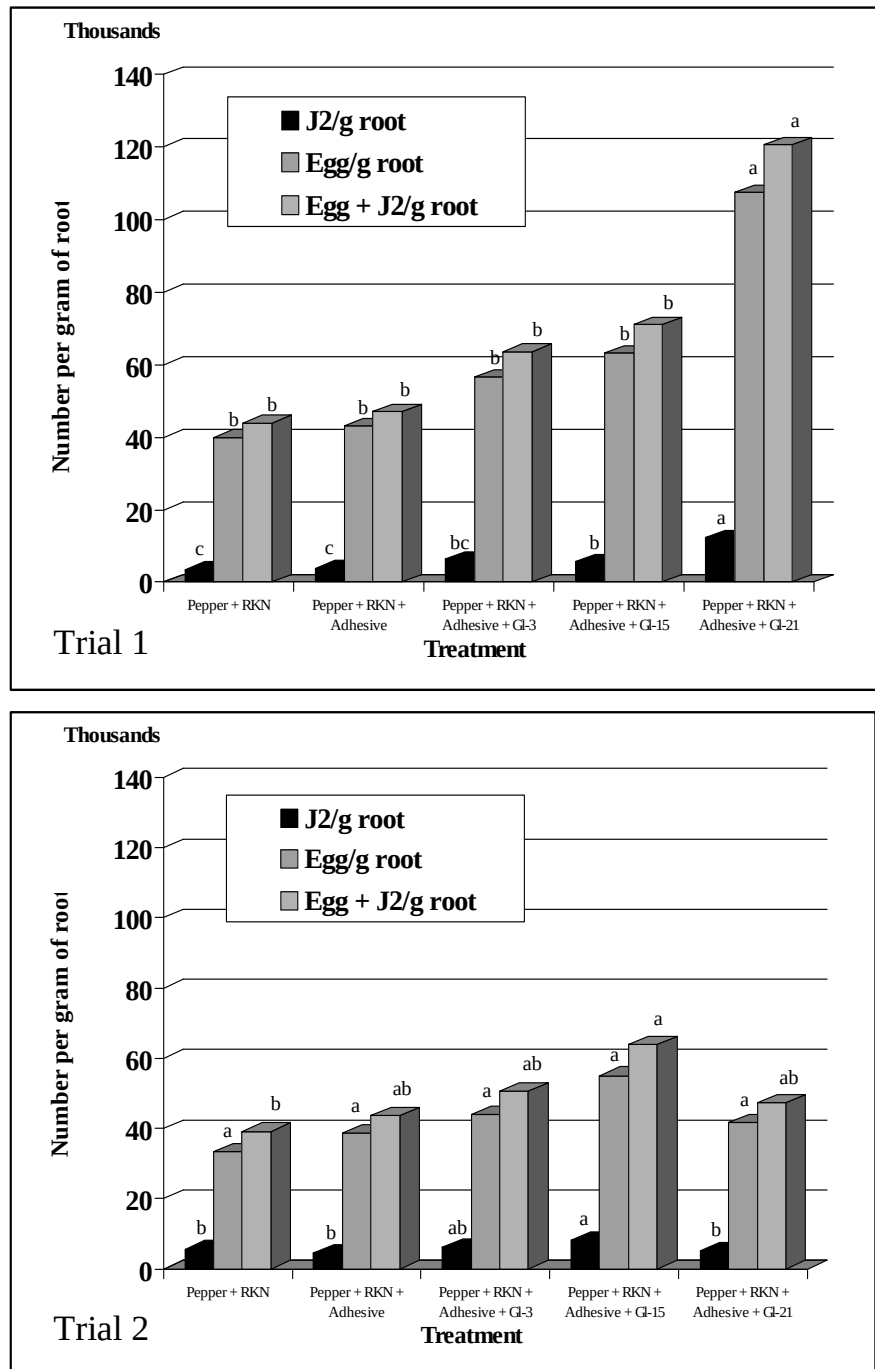


Figure 3. Number of *Meloidogyne incognita* eggs, second stage juveniles (J2), and eggs + J2 per gram root following treatment of bell pepper with the biocontrol agent *Trichoderma virens* isolates G1-3, G1-15 and G1-21, or with respective controls. Letters are comparable with each other within a category (indicated by color pattern), but are not comparable among the three categories. Bars within a category and with a common letter are not significantly different at $p=.10$.

Table 4. Propagule enumeration of initial fungal slurries and CFU counts obtained via reisolation of the *Trichoderma virens* isolates (G1-3, G1 15 and G1-21) from the initial seed coat, the pepper seedling roots at three weeks post-planting and the pepper roots at 11 weeks post-planting.

Isolate	Fungal Slurry Concentration (propagule/ml)	Seed CFU reisolation (CFU/seed)	3 week reisolation (CFU/0.10g root)	11 week reisolation (CFU/g root)
<i>Trial 1</i>				
G13	1.5×10^9	1.1×10^6	3.3×10^5	2.7×10^5
G1 15	1.8×10^9	7.9×10^5	4.0×10^5	1.5×10^5
G1 21	1.5×10^9	1.1×10^6	2.0×10^5	2.0×10^5
<i>Trial 2</i>				
G13	1.6×10^9	1.2×10^6	9.9×10^4	6.6×10^4
G1 15	1.7×10^9	9.9×10^5	3.0×10^5	6.8×10^4
G1 21	1.8×10^9	4.0×10^5	4.0×10^5	1.6×10^5

Discussion

Formulations of *T. virens* isolates G1 3, G1 15 and G1-21, applied initially as a seed coat and three weeks later as a drench, did not suppress nematode reproduction on Yolo Wonder bell pepper under the conditions of this study. These results differ from those recorded after application of *T. virens* G1 3 to greenhouse grown bell pepper cv. California Wonder for the management of *M. incognita* (Meyer et al., 2001). In that study, *T. virens* G1 3 was effective in decreasing numbers of eggs per gram of root by approximately 60% as compared to untreated controls.

It is not clear why the three isolates of *T. virens* did not suppress nematode numbers and in fact significantly increased the nematode population (G1-21, trial one) in one instance. However, a number of possible explanations exist.

Since the antagonistic effects observed with some *Trichoderma* spp. are considered related to antibiosis, environmental factors influencing antibiotic production may play a large role in the eventual effectiveness, or lack thereof, of a potential biocontrol agent. Of all environmental factors potentially influencing the production of antibiotics by *Trichoderma* spp., probably the most important is the substrate on which the fungus is grown (Howell, 1998). Studies have shown that the substrate used for culturing *Trichoderma* fungi can affect the production of inhibitory compounds (Meyer et al., 2000; Roberts and Lumsden, 1990). While corn meal agar was utilized as the substrate media in this study, future studies could evaluate culturing *Trichoderma* on additional substrates prior to determining their biocontrol effectiveness.

The soil environment has also been found to significantly affect the overall performance of *Trichoderma*. In a study where *Trichoderma* isolates were recovered

from a sandy loam soil, 10^8 conidia per gram of soil were required for suppressive effects to be observed, while only 10^4 to 10^5 conidia were required for the same effect for isolates recovered from a clay loam soil (Hadar et al, 1983). These results indicated that suppressive activity depends on factors other than the presence of large numbers of propagules (Hadar et al., 1983). Nelson et al. (1983) observed that planting media containing peat or fresh hardwood bark seldom had a suppressive effect on nematodes, even though large numbers of effective isolates were added. The soil mix used in the current study consisted of 90% builders sand and 10% of a plug and seedling mix that contained a component of peat. The components of the soil media may have interfered with the antagonistic activity of the isolates tested, resulting in the lack of suppressive effect that was observed. Further studies evaluating various soil types need to be done to fully assess *Trichoderma*'s biocontrol potential.

The efficiency of a biocontrol agent is known to be influenced by the susceptibility of the host plant. Susceptibility influences the number of nematodes that invade the roots, the number that become females and the size of the egg masses produced (Kerry, 2001). The bell pepper selected for this study was highly susceptible to infestation by nematodes as the final egg and J2 numbers demonstrated. Bourne et al. (1996) theorized that reductions in populations of RKN are likely to be greatest on plants that were poor hosts for the nematode. The high susceptibility of bell pepper to RKN provides another possible contribution to the non-suppressive effects observed in this study. Other studies that evaluated *Trichoderma* species as a biocontrol agent for nematodes utilized highly susceptible test plants (cotton and tomato) with similar final results (Meyer et al., 2000; Zhang et al., 1996).

The selection of a plant species for test purposes must also consider its suitability not only as a host for the nematode, but also for the biocontrol agent. Plant species are known to differ in their ability to support fungal growth (Bourne et al., 1996; Kerry, 2001). Different plant species secrete a variety of exudates into the rhizosphere, and those exudates function together with the rhizosphere as the substrate for the biocontrol agent. Plant species with the appropriate combination of exudates can profoundly affect a biocontrol agent's capability of proliferating within and protecting the rhizosphere. All *T. vires* isolates, G1 3, G1 15 and G1-21, were reisolated from three-week old seedlings and from 11-week old plant roots in substantial amounts, considering the initial seed coat recoveries (Table 4). These results indicate that Yolo Wonder bell pepper provided an environment that supported the growth of the biocontrol agent in the rhizosphere. Although the potential for long-term root colonization was demonstrated through the successful reisolation of the isolates at the conclusion of the study, the isolates were not suppressive to the nematode populations. According to Lumsden and Locke (1989) the ability to control disease is more likely related to production of specific metabolites or other factors than to the ability to produce fungal reproductive propagules. This is offered as yet another possible explanation why no suppressive effect of nematodes was observed in this study.

The presence of a fungal biocontrol agent may also be more abundant on roots infected with nematodes compared to those that are healthy (Kerry, 2001). This stimulation of fungal growth may result either from direct colonization of the egg mass or from the release of nutrients into the rhizosphere (Bourne et al., 1996). Root exudates may be altered by nematode infection and can lead to changes in pH of the rhizosphere

and to a selective increase of competitors or antagonists (Mai and Abawi, 1987). It is possible that the persistence of the *Trichoderma* isolates may have been partially related to the presence of the heavy nematode populations on the pepper roots. Reisolation studies in this experiment were performed primarily for verification of isolate presence throughout the duration of the study. In future studies a comparison of *Trichoderma* via reisolation from plants with and without nematode populations is needed to substantiate this concept.

Variability in root fresh weights among the treatments within each trial were not consistent between the two trials (Table 3). Variability in root growth may be due to differing conditions within the two growth chambers utilized for trials one and two. Another factor possibly affecting root weight is the age difference of the seeds utilized in the two trials. While both trials utilized seeds from the same lot, the second trial was performed several months later. It is theorized that the small size of the pepper seeds may not be able to protect against the deteriorating effects of time which might ultimately be observed in the form of less vigorous growth (Vavrina, 2003) as was observed in the root growth of this study.

The application of *T. virens* G1 21 caused some of the lowest fresh root weights, introducing the issue of phytotoxic effects caused by the biocontrol agent. It has been well documented that the close association of microorganisms with the roots of plants can directly influence the growth and development of the plant (Bailey and Lumsden, 1998). *Trichoderma* is known to produce a number of compounds generating varying levels of known antifungal and herbicidal metabolites. These characteristics have contributed to its selection as a biocontrol agent. The focus of the majority of *Trichoderma* research has

been largely directed at biocontrol effects. Only recently have the potential detrimental effects of *Trichoderma* been more closely examined (Bailey and Lumsden, 1998).

Phytotoxic effects of *Trichoderma* are dependant upon the complex combination of environmental conditions, the plant species under study (Wright, 1951) and the isolate and the quantity used (Howell et al, 1993). However, a review of the literature failed to reveal any documentation about the potential for phytotoxicity for the isolates of *T. virens* when applied in quantities utilized in this study.

T. virens isolate G1-21 is known to produce both gliotoxin and viridin secondary metabolites, which provide antifungal and herbicidal activities. In a study performed by Wright (1951), gliotoxin and viridin inhibited the germination and root growth of mustard seed whereas they were not inhibitory to root growth of red clover or wheat seedlings. In the current study, fresh *T. virens* G1-21 was applied directly to the seed. As the freshly coated seed was planted immediately into the moist conditions of the grow tube, the secondary metabolites of G1-21 may have been present upon the seed surface and may potentially have induced a toxic effect upon the pepper seed resulting in decreased root growth. Despite these results, both Lumsden et al. (1992) and Wright (1951) report that the phytotoxic effects of gliotoxin and viridin are limited in comparison with their antifungal properties. Further study would be required to confirm consistency of decreased root growth of pepper plants when pepper seeds are coated with *T. virens* isolate G1-21.

Inconsistencies in biocontrol studies between trials are not uncommon and are frequently cited as a limiting factor in the expansion of the field of biocontrol (Butt et al. 2001). An example of this type of inconsistency was demonstrated within this current

study by the isolate G1-21 (Figure 2). Although identical techniques and conditions were applied, population numbers retrieved from pepper plants receiving this treatment varied widely from one trial to the next. The potential causes of variabilities of this nature are difficult to identify. This is largely due to inadequate understanding of the ecology of the fungi. Such information is pertinent not only to better understand the ecological limits of the biocontrol organisms, but also to further develop application and management practices to provide the environment that is necessary for the organisms to persist (Cook, 1993) and succeed as a biocontrol agent.

Multiple factors such as substrate, liquid media, host susceptibility, root exudates and soil mix may have affected the final results of this study. This assessment is based upon what previous studies suggest and would provide a basis for future studies to evaluate. The many complexities of a biocontrol agent's interactions with its environment present ongoing challenges to any researcher attempting to manage research of this nature. The results of this particular study indicate that isolates G1-3, G1-15 and G1-21 of *T. virens* do not exhibit antagonistic activity against *M. incognita* on bell pepper under the various conditions used in this growth chamber experiment. This does not preclude the evaluation of additional environmental variables to fully assess the potential of *T. virens* for biocontrol.

APPENDIX A: CORN MEAL AGAR VERSUS POTATO DEXTROSE AGAR AS A MEDIUM FOR *TRICHODERMA* GROWTH AND SPORULATION

Maintenance cultures of each *Trichoderma* isolate were initiated on corn meal agar (CMA) (Benton-Dickson, Sparks, MD) per a recommendation by S.L.F. Meyer (personal communication, 2002) to minimize the occurrence of potential mutations that may be observed on agars promoting faster growth such as potato-dextrose agar (PDA) (Difco, Sparks, MD). One week after initiation, these maintenance cultures were used to initiate liquid cultures within a molasses-yeast media to promote production of viable propagules for the seed coat. Personal observations in previous studies had shown that *T. virens* isolates grown upon PDA produced a large number of propagules within a week. There was concern that CMA, a medium that produces a much smaller number of propagules within a week, would produce insufficient numbers of propagules to initiate and promote a liquid culture of the fungal isolate. To alleviate this concern, an evaluation of the ability of the isolates grown on both CMA and PDA to support initiation and proliferation of the fungal isolate within liquid culture medium was performed. Maintenance cultures of *T. virens* were grown on both agar types for one week. Liquid cultures were then separately initiated from both maintenance cultures. Observation of maintenance culture growth and hemacytometer counts of representative one ml samples from both subsequent liquid media cultures (20 days after initiation) were used to assess the two agar types. CMA was determined to be a suitable alternative to PDA. Even though side-by-side observation of the colony growth upon the two different agar types revealed apparent differences in the quantity of growth over a week (Figure 4), the eventual propagule count per ml of liquid medium was similar between the two

Figure 4. Photo comparing differences in growth of *Trichoderma virens* isolates GI-3, GI-15 and GI-21 upon Corn Meal Agar (CMA) and Potato Dextrose Agar (PDA).



agars tested (data not shown). Not only did CMA provide adequate inoculum for liquid medium culture, it also potentially minimized the occurrence of mutation and alteration of the performance of the fungal isolates.

APPENDIX B: SEED ADHESIVE/HIGH HUMIDITY EXPERIMENT

Introduction

To determine the optimal seed coat process to be utilized in this study, several adjunct studies were performed. Prompting these studies were difficulties encountered with the reisolation of *Trichoderma virens* colony forming units (CFU) from coated pepper seeds. Specifically, poor or no reisolation of *T. virens* CFU from the coated seeds was occurring. Despite confirmation that correct procedures were utilized for the seed coat and reisolation processes, a second trial attempt performed as poorly as the first. Further consultation and literature research revealed two possible theories for the poor reisolation.

One theory considered that the adhesive utilized in the fungal slurry may have been inadequate to transfer and adhere the fungal propagules to the surface of the seed coat. Gelatin had been employed as the adhesive based on previous research (S. Meyer, 2002). In an earlier seed coat study performed by Taylor et al. (1991), *T. harzianum* was assessed as a potential biocontrol agent of seedling diseases when applied as a seed coating. In that study, Polyox N-10 (Dow Chemical, Midland, MI) was one of two adhesives evaluated for adherence qualities. Based on the results from that study, Polyox N-10 was selected for use in this adjunct study. It was compared to gelatin for adhesive performance.

The second theory for the poor reisolation considered that low CFU counts resulted from an environment that was not supportive of fungal proliferation upon the seed. Taylor et al. (1991) described the incorporation of a four-day period of seed exposure to high humidity that was theorized to have enhanced the colonization of the

seed surface by the biocontrol agent. This four-day period exposed the freshly coated seeds to high humidity levels between 80% and 100% just prior to planting. This technique improved the final population of plants susceptible to the targeted seedling disease; an outcome that appeared to improve the biocontrol organism's ability to colonize and proliferate. The objective of this study was to assess the effectiveness of a period of high humidity in conjunction with a comparison of adhesives upon the final CFU reisolated from the initial seed coats.

Materials and Methods

Cultures of *T. virens* isolates Gl-3, Gl 15 and Gl-21 were initiated as described in chapter three. Seed coat slurries were created from the isolate cultures as described in the 'Seed Coat Process' section of chapter three up until the addition of the adhesive. In this adjunct study, the two adhesives Polyox N-10 (Dow Chemical, Midland, MI) and gelatin (Difco, Sparks, MD) were evaluated. At the point where the adhesive was added, each isolate's fungal slurry received two ml of either Polyox N-10 or gelatin. For each fungal slurry/adhesive treatment, thirty seeds of bell pepper (*Capsicum annuum* L. cv. Yolo Wonder) were immersed and coated as described in the 'Seed Coat Process' section of chapter three. One half of the coated seeds of each fungal slurry/adhesive treatment were withdrawn and immediately placed into autoclaved vials for serial dilution plating (Koch, 1994) on *Trichoderma* medium E (TME) (Papavizas et al., 1982) to determine CFU for freshly coated seed. The remaining one-half of the coated seeds from each adhesive treatment were withdrawn and immediately placed onto a sterilized plastic grid overlaying a tray of water and subjected to four days of high humidity. Each fungal

isolate grouping of treated seeds was covered with a plastic dome and placed into the growth chamber for a four-day period of exposure to high humidity (Figure 5). Growth chamber (Conviroon BRD 8) settings were 27°C, 73-75% humidity and a 12-hour photoperiod. At the conclusion of the four-day period, the seeds were removed and the fifteen seeds per treatment were divided into three groups of five and placed into autoclaved vials for serial dilution plating (Koch, 1994) with TME to determine CFU per seed. Fifteen seeds coated with each adhesive but not inoculated with the fungus were processed as controls.

CFU counts were enumerated for the following seed coat treatments:

- 1) Pre high humidity/fresh seed coat + gelatin + *T. virens* G13
- 2) Pre high humidity/fresh seed coat + gelatin + *T. virens* G1 15
- 3) Pre high humidity/fresh seed coat + gelatin + *T. virens* G1 21
- 4) Pre high humidity/fresh seed coat + Polyox N-10 + *T. virens* G13
- 5) Pre high humidity/fresh seed coat + Polyox N-10 + *T. virens* G1 15
- 6) Pre high humidity/fresh seed coat + Polyox N-10 + *T. virens* G1 21
- 7) Post four day period of high humidity + gelatin + *T. virens* G13
- 8) Post four day period of high humidity + gelatin + *T. virens* G1 15
- 9) Post four day period of high humidity + gelatin + *T. virens* G1 21
- 10) Post four day period of high humidity + Polyox N-10 + *T. virens* G13
- 11) Post four day period of high humidity + Polyox N-10 + *T. virens* G1 15
- 12) Post four day period of high humidity + Polyox N-10 + *T. virens* G1 21

Figure 5. Photo depicting layout of High Humidity treatments within the growth chamber.



The experiment was conducted twice. Data from each trial was analyzed separately using PROC MIXED analysis (SAS Institute) due to interactions between the treatments and the two trials identified with a combined analysis of variance. Tukey's multiple mean comparison test was utilized when appropriate.

Results and Discussion

In all cases, freshly coated seeds containing the adhesive gelatin had higher CFU than seeds coated with either gelatin or Polyox N-10 and exposed to high humidity, with one exception, in trial one, G1-15 freshly coated seeds containing gelatin did not differ from seeds exposed to high humidity containing gelatin (Figure 6). Freshly coated seeds with the adhesive Polyox N-10 had mixed responses (Figure 6). In trial one, G1-3 and G1-15 freshly coated seeds with Polyox N-10 performed the same as seeds exposed to high humidity. In trial two, G1-3 and G1-21 freshly coated seeds with Polyox N-10 performed the same as the seeds exposed to high humidity. Based upon these results and the consideration that *Trichoderma* spp. grow optimally at lower pH levels (4-5), gelatin, with a pH 5.0, was selected as the adhesive in this experiment versus Polyox N-10, with a pH 7.0.

Results of the high humidity study revealed that under the conditions of this experiment a four-day period of exposure to high humidity was detrimental to the reisolation of *Trichoderma* (Figure 6). These results contradict those documented in a similar study by Taylor et al. (1991) where exposure to high humidity appeared to improve conditions for colonization of the seed by the biocontrol agent. The most probable factor affecting these results were the humidity conditions attained for this

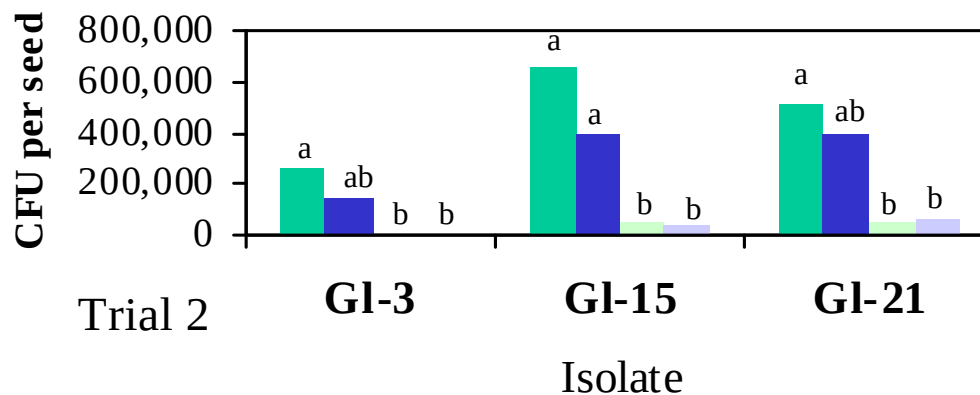
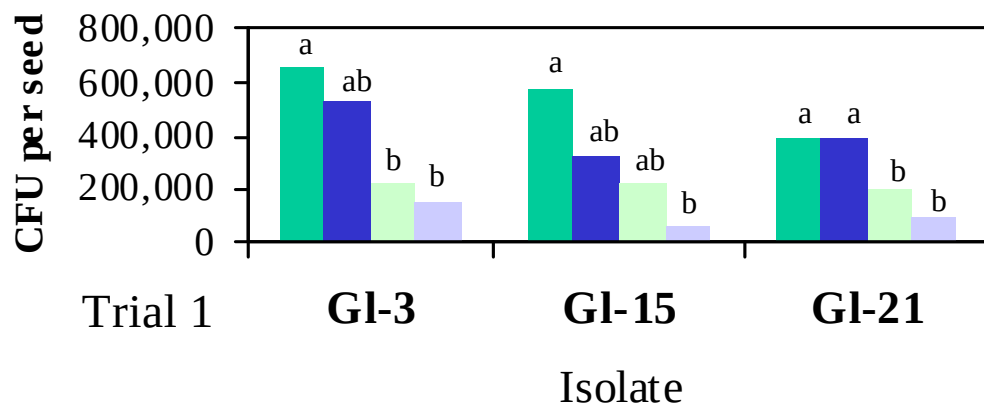


Figure 6. Comparison of CFU recovery from bell pepper seeds coated with formulations of *T. virens* isolates GI-3, GI-15 and GI-21. Each of the formulations tested was a combination of two factors, adhesive (gelatin or Polyox N-10) and seed coat exposure (fresh coat or post high humidity). Letters are comparable within each isolate, but not among isolates. Bars within an isolate with a different letter are significantly different at $p=.10$.

study. The growth chambers utilized could reach a maximum humidity level of approximately 75% whereas the conditions in Taylor's study referred to humidity levels between 80 and 100%. Therefore, it is theorized that 75% humidity was not sufficient to enhance colonization of the biocontrol agent upon the seed. This conclusion is supported by Sanogo et al. (2002), who reported on the effects of temperature and relative humidity on the germination and production of *T. stromaticum* conidia. Sanogo et al. found that at a relative humidity of 75% there was no evidence of germination or conidia production at any temperature.

The data from the high humidity study revealed a trend that the freshly coated seeds (those not exposed to the ultimately drying effects of the high humidity treatment, but instead immediately coated and dilution plated) were consistently producing higher CFU counts than seeds exposed to the four days of high humidity (Figure 6). With this trend in mind, initial procedural methods were re-evaluated. Standard protocol utilized up to the point of this adjunct study specified that all treated/coated seeds should be allowed to dry for one hour prior to placement into either the designated grow tube within the MRS or into the vial to be utilized for reisolation studies. Considering the results from this current study, the one-hour drying period specified in standard protocol may adversely affect the viability of the propagules upon the seed surface.

A study by Papavizas (1985) found that during in vitro studies approximately 75% of fresh *Trichoderma* chlamydospores germinated well on nutrient agar, whereas only 13 to 31% of chlamydospores from dried preparations germinated. Papavizas' study supports the theory that a drying period may be detrimental to eventual germination of propagules.

The conclusions of this study are as follows. For the first objective of this study, evaluating the incorporation of a period of high humidity upon reisolation of CFU from the seed, it was determined that the high humidity conditions achieved were insufficient for enhancement of colonization upon the seed. For the second objective, evaluating the adherent qualities of two adhesives, gelatin was selected as the adhesive of choice based upon higher overall CFU reisolation, and a pH more favorable for the growth of *Trichoderma*. Based on the results of this study, it was theorized that a drying period of any length of time immediately following the coating of the seed would be detrimental to the viability and final recovery of the biocontrol agent from the seed coat. The evaluation of this theory is described in Appendix C.

APPENDIX C: FRESH COAT VERSUS DRY COAT EXPERIMENT

Introduction

The study described within Appendix B demonstrated that the humidity level achieved within the growth chambers was most likely not sufficient to support the increased fungal germination rate desired. It also demonstrated that the selection of gelatin as the adhesive would be satisfactory to adhere the propagules to the seed surface. Yet, the issue of how to adjust the seed coat protocol to improve CFU reisolation of the *Trichoderma* isolates from the seed still existed. It was theorized that the one-hour drying period often incorporated into the fungal seed coat process, in combination with the overnight refrigeration of coated seeds prior to dilution plating, may be detrimental to the germination and subsequent reisolation of propagules. The objective of this adjunct study was to evaluate the CFU recovered from freshly coated seeds as compared to CFU recovered from coated seeds exposed to a one-hour drying period.

Materials and Methods

A culture of the isolate G1-15 was initiated as described in chapter three. A seed coat slurry was created from the isolate culture in the same manner as described in chapter three. Thirty seeds of bell pepper (*Capsicum annuum* L. cv. Yolo Wonder) were immersed into the fungal slurry. Fifteen seeds were withdrawn with autoclaved forceps and placed into autoclaved vials for immediate serial dilution plating (Koch, 1994) on *Trichoderma* medium E (TME) (Papavizas and Lumsden, 1982) to determine CFU per seed. Three repetitions of five seeds each were evaluated for the fresh coat treatment. The remaining 15 seeds were withdrawn with autoclaved forceps and placed onto wax

paper to dry for one hr before placement into autoclaved vials for serial dilution plating (Koch, 1994) with TME to determine CFU per seed. Three repetitions of five seeds each were evaluated for the dry coat treatment. Two treatments were designed and evaluated to test the proposed theory:

- 1) Freshly coated pepper seeds + gelatin + *T. virens* Gl-15
- 2) Dry coat pepper seeds + gelatin + *T. virens* Gl-15

The experiment was repeated. Data from both trials was combined and analyzed using PROC MIXED procedure (SAS Institute). Tukey's multiple mean comparison test was utilized when appropriate.

Results and Discussion

Final CFU counts for freshly coated seeds were significantly higher than the counts for coated seeds allowed to dry for one hour prior to reisolation procedures. Average CFU of the fresh coat treatment was 5.9×10^5 , significantly different ($p=.10$) from the average CFU retrieved from the dry coat treatment, 2.2×10^5 . These results indicate that the drying period negatively affected the viability of the fungal propagules on the seed coat. Thus, inclusion of a drying period immediately following the seed coat application of the fungal organism would inhibit germination of the fungal propagules within the grow tube and lessen the chance of detecting an antagonistic effect. Additionally, the drying period that may occur during refrigeration of seed prior to conducting reisolation procedures may also inhibit germination of the propagules upon the nutrient media used for seed coat reisolation studies. This would result in an inaccurate estimation of fungal propagules per seed.

The results observed during this study were incorporated as part of the new protocol of using only freshly coated seeds without any drying exposure. This protocol was utilized in the assessment of the biocontrol capabilities and persistence of *T. virens* within the MRS.

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