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Evaluation of Trichoderma atroviride as a potential biological control agent of Cryphonectria parasitica /

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EVALUATION OF *TRICHODERMA ATROVIRIDE* AS A POTENTIAL BIOLOGICAL CONTROL AGENT OF *CRYPHONECTRIA PARASITICA*

A Thesis Presented

by

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Dedicated to Merlyn, Edna, Velda, Claudette, Iris, and the memory of Eda.

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ABSTRACT

EVALUATION OF *TRICHODERMA ATROVIRIDE* AS A POTENTIAL BIOLOGICAL CONTROL AGENT OF *CRYPHONECTRIA PARASITICA*

FEBRUARY 1998

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In the search for other methods with which to control the fungus *Cryphonectria parasitica*, which causes chestnut blight on American chestnut (*Castanea dentata*) trees in North America, the antagonist *Trichoderma atroviride* was isolated. The study was to (1) evaluate the ability of the antagonist to suppress growth and pathogenicity of *C. parasitica*; (2) determine the best carrier medium which would enhance the logevity and efficacy of *T. atroviride*; (3) characterise the mode of suppresive action of *T. atroviride* on *C. parasitica*.

Trichoderma atroviride stopped the growth of *C. parasitica* in *in vitro* tests on agar media. The pathogen on dual culture plates was not reisolated when overgrown by the antagonist. Later tests on bark and wood tissue and seedlings of American chestnut, in and outside of the greenhouse, indicated that prophylactic treatments with a water supension of *T. atroviride* spores significantly reduced the development of cankers. Post treatment with the antagonist limited canker development in the bark wood test and on

potted seedlings treated outside. Severe heat in the greenhouse appeared to have affected the antagonist's ability to control the pathogen.

Enhancement of spore longevity by Wilt Pruf® and mineral oil over water suspensions was not significant. Efficacy of the treatment was not enhanced by either of the two media. No conclusions could be made on effectivity of Phyton 50® and lanolin in the enhancement of spore longevity efficacy of *T atroviride*.

Observations by light and scanning electron microscopy showed no coiling around or penetration of the hyphae of the pathogen. Tests for volatile antibiosis were negative. However, growth of the pathogen placed directly in cell free culture filtrates was significantly limited. Heat treatments of the filtrates indicated that some of the active metabolites were heat labile and others were heat stable. Well plate tests using the same filtrates failed to produce similar results, perhaps because of the lower concentration of filtrate used. Possible mode of antagonism may therefore be a combination of activity of non-volatile metabolites when in high concentrations and competion for food resources.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Chestnut Trees and the Introduction of the Blight

The American chestnut (*Castanea dentata* [Marsh.] Borkh.) played a very important role in the lives of North Americans. The chestnut was a major component of the hardwood forests of the Eastern United States (Anagnostakis, 1987). The nut was an important source of nutrition for people and domesticated animals as well as to the wildlife in the eastern forests (Kuhlman, 1978). The wood was used to build homes, barns, telephone poles, shipmasts, and railroad ties. Tannins extracted from the wood were used in the leather industry (Anagnostakis, 1987). The tree was also widely used in shade-tree plantings, and it was in this capacity that the chestnut blight disease was first observed on American chestnut trees lining an avenue at the New York Zoological Garden (Anagnostakis, 1987). In 1905, approximately 98% of the chestnut trees in the New York Zoological Garden were infected (Kuhlman, 1978). The fungus causing chestnut blight was highly virulent and resulted in symptoms such as bark cankers which girdled the trees, wilting of foliage and the formation of epicormic sprouts below the cankers (Anagnostakis, 1987).

The fungus was first identified and named *Diaporthe parasitica* by William A. Murrill (Murrill, 1906). The genus changed to *Endothia* in 1913, (Anderson and Babcock, 1913; Anderson, 1914), and more recently *Cryphonectria* (Murr) (Barr, 1979). The fungus produces yellow-orange pycnidia which push through the epidermis of the

host tissue and exude yellow-orange tendrils of conidia. Red perithecia found pushing through the epidermis of the cankers produce ascospores which are shot into the air (Griffin and Elkins, 1986; Anagnostakis, 1987). Fungal mycelium of *C. parasitica* invades healthy tissue via a wound and produces enzymes which degrade the phloem and cambium tissue (McCarroll and Thor, 1978). The fungus was apparently introduced from Asia, and this hypothesis has been supported by the fact that Chinese and Japanese chestnut are resistant to the disease (Metcalf, 1908). Chestnut blight was also found in China and Japan (Shear and Stevens 1913; Shear and Stevens, 1916).

Despite the large effort to combat the fungus, *C. parasitica* moved very rapidly out of New York State and by the mid 1940s had spread to all areas of the American chestnut range. Dissemination of conidia is facilitated by insects, birds, other mammals and splashing rain (Griffin and Elkins, 1986; Agrios, 1988). Ascospores are forcible ejected from perithecia and carried by the wind (Griffin and Elkins, 1986; Agrios, 1988). The American chestnut tree however, has managed to survive by sprouting from stumps, forming understory trees and the cycle repeated.

The European chestnut, *Castanea sativa* Mill., is very similar to the American chestnut and is very important as a crop plant. In 1938 chestnut blight was found in Italy where it occurs on European chestnut and various oak species (Gravatt, 1952). The disease also occurs in France, Switzerland, Yugoslavia (Gravatt, 1952), Spain, Turkey, Greece, Hungary and USSR (Griffin and Elkins, 1986). The chestnut blight epidemic in Europe was similar to that in the United States, though the European chestnut (*Castanea sativa*) is more resistant to the fungus (Berry, 1960).

When the disease was found in Europe it was thought that the chestnut population would be reduced as it was in North America. However, in Italy in the early 1950s some trees with cankers were observed to be healing (Biraghi, 1951). On a closer look Biraghi (1953) found that *Endothia parasitica* was restricted to the outer layer of the tree bark, apparently the result of heavy callousing of the host bark in and around cankers. However, Biraghi concluded that the decrease in blight symptoms was due to the trees accquiring resistance to the fungus (Griffin and Elkins, 1986). The fungal strains of *C. parasitica* subsequently isolated by Grente (1965) was demonstrated to have reduced virulence and were termed hypovirulent.

1.2 Hypovirulence and Fungicides

Hypovirulence in *C. parasitica* is the result of infection by a cytoplasmic, double-stranded ribonucleic acid (dsRNA) virus (Day et al., 1977). This virus is transmitted by hyphal anastomosis which allows the virus to move from the infected strains to the healthy virulent strains (Day, 1978). To effectively establish hypovirulent populations, transmission of the hypovirulent strains was accelerated in Europe by frequent human-mediated inoculations (Anagnostakis, 1987).

The similarity between the chestnut blight epidemics in Europe and North America lead scientists to believe that hypovirulence could be an effective method of control in the United States. Initial inoculations of European hypovirulent strains on American chestnut seedlings (Anagnostakis and Jaynes, 1973) were very promising so field experiments at the Connecticut Agricultural Experiment Station farm in New Haven, were begun in 1973

using hypovirulent strains from France and Italy (Anagnostakis, 1987). The hypovirulent strains were able to control natural infections where the hypovirulent strains were applied. However, cankers beginning elsewhere on the trees were not hypovirulent and were able to colonize and kill the trees (Anagnostakis, 1987).

Hypovirulent strains produce low amounts of pycnidia and conidia compared to the virulent strains (Elliston, 1978; Griffin et al., 1983), therefore the spread of hypovirulence has been slow but successful in Europe. However, hypovirulent strains found in the U. S. A. and used in experiments to control chestnut blight have been unsuccessful (Tattar et al. 1996; MacDonald, personal communication). Failure of hypovirulence to spread in the U. S. A. may be due to several factors including environmental conditions, greater susceptibility of American chestnut to the blight, and the variation in characteristics of both virulent and hypovirulent strains existing in the U. S. A. (Tattar et al., 1996).

Fungicides were found to give limited protection to American chestnut from the blight disease (Jaynes and Van Alfen, 1974; Elkins et al., 1978; Payne et al., 1983). With the systemic fungicide methyl-2-benzimidazolecarbamate (MBC), Jaynes and Van Alfen (1974) were able to provide limited protection to American chestnut from blight. However, to be effective, a large number of applications would be necessary. This would be impractical due to cost and possibly phytotoxicity to trees, and induction of fungal resistance to the fungicide could occur. Benomyl soil injections were also found to be unsuitable (Elkins et al., 1978).

1.3 Another Approach to the Biological Control of *C. parasitica*

Another approach to the treatment of chestnut blight is the use of antagonistic microorganisms. McCabe (1974) applied a compress of compost wrapped in a building-paper sleeve, to the canker. The application resulted in the control and healing of the canker 6 months after application. Weidlich (1978) showed that use of muddy soil as a compress wrapped in a polyethylene sleeve controlled the canker on American chestnut after only 3 months. In the same study, autoclaved muddy soil was not effective in controlling the progress of the cankers. A total of fifty-three organisms were isolated from the muddy soil including bacteria, actinomycetes and a number of fungi, one of which was found to be an antagonist and identified as a *Trichoderma* sp. Magnini (1981) and Turchetti and Gemignani (1981) also reported that the use of sphagnum peat and soil produced results similar to those of Weidlich (1978) and McCabe (1974) when used on graft unions of European chestnut. The control attained by Turchetti and Gemignani (1981) on European chestnut was attributed to thermostable antifungal metabolites and antagonistic microorganisms such as *T. viride*. They also showed that *C. parasitica* was parasitised by *T. viride*. In Austria, where the chestnut blight was first reported by Donaubauer (1964), hypovirulent forms of *C. parasitica* have not been found and a search for other possible antagonistic organisms was conducted (Wilhelm et al., 1992). *Bacillus subtilis* sp. was extracted from xylem fluid, and shown to be capable of suppressing chestnut blight when applied as a prophylactic treatment to the bark. In Europe there is a commercially available tree wax containing *B. subtilis* which is used to protect chestnut grafts (Heiniger and Rigling, 1994; Turchetti, 1987). *Bacillus subtilis* has also been used

as an antagonist against the fungus *Ceratocystis ulmi* (*Ophiostoma ulmi*), the causal agent of Dutch elm disease (Turchetti, 1987).

Trichoderma spp. are regarded as non-pathogenic saprophytic fungi commonly associated with fallen branches, decaying tree stumps and soil (Rifai, 1969). Many species of *Trichoderma* are mycoparasites (Barnett and Binder, 1973) and are known to be effective antagonists of *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kühn, (Elad, et al. 1983; Elad, et al. 1984; Benhamou and Chet, 1993; Benhamou and Chet, 1996), *Pythium ultimum*, (Besnard and Davet, 1993), *Armillaria gallica* (Dumas and Boyonoski, 1992), *Polymyxa betae* Keskin, (D'Ambra, and Mutto, 1986), *Botrytis cinerea* (Elad and Kirshner, 1992), *Chondrostereum ulmi*, and *Ceratocystis ulmi* (Ricard, 1983). The antagonistic ability of *Trichoderma* spp. may be due to one or a combination of several factors, such as, antibiosis (Dennis and Webster, 1971), competition, enzymatic degradation (ß-1, 3-glucanases, chitinases, and proteases), and mycoparasitism (Chet et al., 1981; Elad, et al., 1982). *Trichoderma* spp. have been tested as potential biocontrol agents on a number of different crops, including fruits and vegetables, flowers, wheat, and nursery trees as well as to protect wooden products from decay, (Chet, 1987; Cook, 1993; Duffy et al., 1996; Hagle, et al., 1991; Sutton and Peng, 1993; Bruce and Highley, 1991; Bhai et al., 1994. The use of *Trichoderma* spp. in controlling disease in various crops including forest trees suggests a potential use in controlling chestnut blight. Various species of *Trichoderma* have been shown to be able to control *C. parasitica in vitro* (Turchetti and Gemignani, 1981; Arisan-Atac et al., 1995).

1.4 Objectives

The specific objectives of this study are:

1. To evaluate the ability of *Trichoderma* to suppress growth and pathogenicity of *C. parasitica.*
2. To determine the best carrier medium which would enhance the longevity and efficacy of *T. atroviride*.
3. To characterise the mode of suppressive action of *T. atroviride* on *C. parasitica.*

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CHAPTER 2

IN VITRO AND *IN VIVO* STUDIES OF THE INTERACTION BETWEEN *TRICHODERMA ATROVIRIDE* AND *CRYPHONECTRIA PARASITICA*

2.1 Introduction

The most effective control of chestnut blight has been attained with strains of *C. parasitica* which are less virulent or hypovirulent and result in very little damage to the cambium of the affected trees. Hypovirulent transformation of the parasite in affected trees allows the trees to form callous and close the wounded area (Anagnostakis, 1990). Hypovirulent isolates of *C. parasitica* were first described by Grente and Sauret (1969a and b) from samples collected in Italy. They subsequently used the hypovirulent isolates to control the disease in orchards in France. Hypovirulent *C. parasitica* isolates differ from virulent isolates in containing double stranded RNA (dsRNA). The dsRNA appears to affect not only the virulence of *C. parasitica* isolates, but also hyphal colour, sporulation, culture morphology (sometimes reduced growth and lobate margins), and enzyme production (Chung et al., 1994). After anastomising with virulent isolates, hypovirulent isolates transfer dsRNA to the virulent isolates thus converting them to hypovirulent isolates. This phenomenon occurs among closely related isolates such as among the European strains or among the North American (NA) strains of *C. parasitica*. (Anagnostakis and Jaynes, 1973). However, European strains were shown to be unrelated to NA strains and transferal of dsRNA from the European strains to NA strains was restricted (Bradley, H. I. et al., 1992). Although. Anagnostakis and Jaynes (1973) showed

that they were able to successfully treat cankers on American chestnut using a French hypovirulent strain from France.

Since the discovery of the naturally occurring hypovirulent (H) strains the disease is much less of a problem in Italy and France where H strains have been successfully spread by artificial means (Anagnostakis, 1987). The wide use of H strains has been hampered in NA because the transmission of hypovirulence is severely restricted by the vegetative incompatibility within *C. parasitica* populations (Lee et al., 1992). Since its introduction to NA, *C. parasitica* has diversified into a large number of vegetative compatibility groups (Milgroom et al., 1991). In contrast, countries in Europe have only four or five compatibility groups (Anagnostakis and Waggoner, 1981; D'Aulerio et al., 1987). Therefore, the search for and the development of other biological agents antagonistic to *C. parasitica* would be an enormous benefit for the successful control of chestnut blight on this continent.

Many saprophytic fungi colonise and coinhabit the bark with *C. parasitica* (Gloer, 1995). It is therefore possible, that there are competitive interactions between *C. parasitica* and other organisms (Gloer, 1995). Such interactions could reduce the quantity of inoculum on the trees and thus reduce the occurrence of the pathogen (Gloer, 1995). Currently there is a great interest in developing microorganisms as commercial biological control agents and random screening has resulted in the isolation of various antifungal agents that inhibit other organisms (Gloer, 1995; Askew and Laing, 1994). At this time, two commercial biological fungicides are registered for use. F-stop®, (Kodak), contains *T. harzianum* and is used on various crops (Cook, 1993; Harman et al., 1991). The second product is BINAB T®, (Butts International), which is formulated as a pellet

and contains *T. polysporum* and *T. harzianum* and is used to control Dutch elm disease (Ricard, 1981 and 1983).

In vitro experiments are used to initially screen isolates which are possible antagonists against *C. parasitica*. Subsequent greenhouse and field experiments with American chestnut seedlings inoculated with *C. parasitica* and *T. atroviride* , can demonstrate protection from canker formation. The purpose of the following experiments was to determine whether the isolate *T. atroviride*, found on American chestnut bark tissue would be an effective antagonist of *C. parasitica*. The studies included *in vitro* tests and *in planta* studies on potted American chestnut seedlings.

2.2 Materials and Methods

2.2.1 Sources and Maintenance of the Antagonist and Pathogen

Samples of outer bark tissues were previously taken from each of ten large, apparently healthy American chestnut trees located in Western Massachusetts. The tissue samples from each tree were placed onto 2% potato dextrose agar (PDA, Difco), and incubated at 25 °C in the dark. After approximately 10 days of growth, individual isolates were transferred to new PDA plates. One isolate, besides several isolates of *Fusarium* and *Rhizopus* which overgrew *C. parasitica*, was kept and identified as *Trichoderma*. The isolate was identified by the American Type Culture Collection (ATCC) as *T. atroviride* (Fig. 2.1). Isolates were maintained on 2% malt extract agar (MEA) and PDA with or without finely ground American chestnut bark tissue (5 g/ litre of distilled water). Isolates were also stored in 15% glycerol solution at –70 °C. At the start of each experiment, isolates stored in glycerol were revived by plating onto MEA and PDA.

The *C. parasitica* isolate used in these experiments was previously collected in Cadwell Forest, Pelham, Massachusetts. The sample was taken from the advancing margin of an active canker on an American chestnut tree by using a cork borer. *C. parasitica* was isolated by placing the bark samples onto PDA, cambial side facing downwards onto the agar. Mycelium growing from the bark onto the agar was transferred to new PDA and incubated at 25 °C in the dark. The isolate was maintained on 2% MEA and PDA with or without chestnut bark (5 g/litre) and transferred every seven days. Stock cultures were kept in 15% glycerol at –70 °C. Every four months the isolate was used to inoculate American chestnut stems or seedlings and then reisolated from the active cankers.

2.2.2 *In vitro* Interaction between *C. parasitica* and *T. atroviride*

The experiment was conducted to observe interactions between *C. parasitica* and the possible antagonist, an isolate of *T. atroviride*. Modified methods of Dennis and Webster (1971) were used. An agar plug 3 mm in diameter was taken from the edge of a five day-old culture with a sterile cork borer and placed on the edge of a 9.0 cm petri dish of sterile PDA or MEA. The plates were incubated at 25 °C in the dark. Two days later 3 mm diameter agar plugs of *T. atroviride* were cut from the edge of a four day-old culture and placed on the PDA and MEA approximately 5.0 cm from the plug of *C. parasitica*. The plates were then incubated at 25 °C in the dark. To determine whether growth of the antagonist or pathogen was stimulated or inhibited, the mycelial growth of the *T. atroviride* isolate and *C.parasitica* was measured daily as the mean of two measurements at right angles. The control plates contained only *T. atroviride* or *C. parasitica*. The

interactions were observed macroscopically to determine if there was cessation of growth of the fungi, and or overgrowth of one by the other. There were 10 replicates of the pathogen/antagonist combination and 10 replicates of each of the control plates on both PDA and MEA.

2.2.3 American Chestnut Bark Wood Test

The bark/wood pathogenicity test on American chestnut developed by Lee et al., (1992) was modified to determine antagonism of the *T. atroviride* isolate against *C. parasitica*. American chestnut stems previously harvested and stored in a 4 °C cold room with the top ends wrapped with parafilm and the bottom ends in water, were used. They were approximately 1.5 - 2.5 cm in diameter and were cut into 3.0 cm lengths and washed twice in a 10% dilute bleach solution and rinsed three times in sterile double distilled water. Each stem piece was then split radially in the longitudinal direction under sterile conditions and the bark removed. Three pieces of wood and 3 pieces of bark were placed onto a 9.0 cm sterile Whatman No. 4 filter paper (Whatman Inc.) in sterile glass petri dishes. The dishes were incubated in the dark at 25 °C . One of the following seven treatments was applied to bark/wood pieces in each petri dish:

1) A 3 mm diameter PDA plug of *C. parasitica* **(C)** followed 2 days later by sterile water **(H_2O)**

2) **C** followed 2 days later by *T. atroviride* spore suspension **(T)**

3) **H_2O** followed 2 days later by **C**

4) **T** followed 2 days later by **C**

5) **T** followed 2 days later by **H_2O**

6) **H_2O** followed 2 days later by **T**

7) **H_2O** followed 2 days later by a 3 mm diameter sterile plug of PDA.

The *T. atroviride* suspension was made by washing spores from a seven day-old culture and filtering through a sterile piece of miracloth to remove mycelial pieces. The suspension contained an average of 2×10^9 spores/ml sterile water. The 3 mm agar plugs of *C. parasitica* were taken from the leading edges of five day-old cultures maintained on 2% PDA. The sterile plugs of PDA were similarly prepared. The agar plugs were placed onto the inner surface of the bark pieces and on the cut surface of the wood pieces. Two hundred and fifty milliliters of the spore suspension was painted onto the entire inner surface of the bark tissue and the cut surface of the wood pieces, with a small paint brush. The growth of *C. parasitica* and *T. atroviride* on the tissues was observed for 10 days at which time the length of the cankers was measured. Each treatment had 3 replications with 3 pairs of bark/wood pieces per plate making a total of 9 bark/wood pieces used for each treatment. The experiment was repeated another two times, however, the method of inoculating the wood pieces was revised so the cambial surface instead of the cut surface, was inoculated.

2.2.4 Treatment of Chestnut Seedlings

Two year old American chestnut seedlings obtained from the Bear Creek Nursery (Northport, WA) were stored for eighteen months at 4 °C in a cold room. Eighty plants were planted on 14 May, 1994 in 7.57 L plastic containers (Nursery Suppliers Inc.) containing Metromix 510, (a mixture of vermiculite, sphagnum peat moss, processed bark ash, and composted pine bark) (Scotts-Sierra Horticultural Products Company). The

chestnut seedlings were placed out-side in a sheltered area at the University of Massachusetts-Amherst. The trees were supplemented with 75 ml of 5-10-10 formula fertilizer (Sierra General Purpose Green-House Mix, Scotts-Sierra Horticultural Products Company) on 31 May, 1994. Two weeks later after completely leafing out, 24 June, 1994, twenty-one of the seedlings were randomly divided by drawing lots, into groups of seven, and assigned one of seven treatments. The experiment was repeated on 16 July, 1994 and 30 July, 1994. The treatments were as follows:

1) *T. atroviride* spore suspension (**T**) followed two days after with *C. parasitica* (**C**)

2) **C** followed two days later by **T**

3) **C** followed by sterile ($\mathbf{H_2O}$) two days after wounding

4) $\mathbf{H_2O}$ and PDA plug of **C** two days after wounding

5) **T** followed two days later by $\mathbf{H_2O}$

6) $\mathbf{H_2O}$ and **T** two days after wounding

7) $\mathbf{H_2O}$ only

The wounds on the seedlings were made with a 3 mm diameter cork borer approximately mid-way on the main stem. Potato dextrose agar plugs of *C. parasitica* were cut from the margins of five day-old cultures with a 3 mm diameter cork borer. *Trichoderma atroviride* spore suspensions were made by washing spores from seven day-old cultures with sterile water and then filtered through a sterile piece of miracloth to remove mycelial pieces. The suspensions were adjusted to 2.5×10^9 spores/ml. For each treatment of *T. atroviride*, 0.3 ml of the spore suspension was painted onto the wound site through various sequences of drying (paint-dry, paint-dry etc.). The treatment areas were wrapped

with Saran® and sealed at both ends with identification tape (TimeMed Labeling Sytems). Each treatment was replicated 3 times and the treatments randomised in three experimental blocks. Pots in each block were arranged in completely randomised block design (7 pots x 3 pots). Canker lengths and widths were measured at 12 days (8 July and 30 July, 1994), 21 days (17 July and 8 August, 1994), and 39 days (4 August and 26 August, 1994) after all treatments had been applied. Canker areas were calculated using the formula for the ellipse (½ length x ½ width x π). Repeated measured analysis of the variance of the log transformed canker areas of treatments 1-4 from two blocks was calculated using the General Linear Method (GLM) (SAS Institute, Inc., Car, NC). Orthogonal polynomial mean comparison using the GLM was used to further analyse the treatment means.

2.2.5 Treatment of Potted Chestnut Seedlings in the Greenhouse

Two year old American chestnut seedlings obtained from the Bear Creek Nursery (Northport, WA) were stored for ten weeks in a 4 °C cold room. In 1995 fifty chestnut seedlings on 4 separate occasions, (19 January, 24 February, 17 March, and 15 April), were planted in 7.57 L plastic containers (Nursery Suppliers Inc.) containing Metromix 510 (Scotts-Sierra Horticultural Products Company) and supplemented with 75 ml 5-10-10 formula fertiliser (Sierra General Purpose Greenhouse Mix, Scotts-Sierra Horticultural Products Company) once a month. Three weeks after the trees had completely leafed out, (7 April, 14 April, 13 May, and 5 June), a 3 mm diameter cork borer was used to wound the main stem mid way up each one. The seedlings were then randomly assigned a treatment by drawing lots and placing the pots in a 7 x 5 arrangement. The seedlings were

identified with nursery tags. One of seven treatments was applied randomly to each wound. The treatments were as follows:

1) *T. atroviride* (**T**) followed two days later by *C. parasitica* (**C**)

2) **C** followed two days later by **T**

3) **C** followed two days later by sterile water (**H_2O**)

4) **H_2O** followed two days later by **C**

5) **T** followed two days later by **H_2O**

6) **H_2O** followed two days later by **T**

7) **H_2O** only.

PDA plugs (3 mm diameter) cut from the margins of actively growing colonies of *C. parasitica* were used in treatments 1 - 4. The *T. atroviride* inoculum used in treatments 1, 2, 5, and 6 was a suspension of 3.5×10^9 spores per ml sterile water, made by washing the spores from a seven day-old culture of the antagonist and filtering through a sterile piece of miracloth. For each treatment 0.2 ml of the spore suspension was painted onto the wound site. The inoculation site was wrapped with Saran® and secured at both ends with identification tape (TimeMed Labeling Systems). Inoculations were carried out on four occasions three weeks after trees had leafed out in April, May, and June, (7 April, 14 April, 13 May, and 5 June). Canker lengths and widths were measured at 8, 14, 29, and 44 days after inoculations were completed. The experiment contained four blocks with 5 single-plant replicates per treatment. The first of the four blocks (first treated on 7 April) was destroyed by spider mites two weeks after the experiment had begun (terminated 24 April) and data from that block was discarded. Pots in each block were arranged on the greenhouse benches in a completely randomised complete block design (7 x 5). Using the

canker lengths and widths, the formula for the area of an ellipse (½ length x ½ width x π), was used to calculate the areas of the cankers. The calloused wound areas were included in the calculations because they too had elliptical shapes. Repeated measured analysis of variance of the log transformed canker areas of treatments 1-4 from three blocks was calculated using the General Linear Method (SAS Institute, Inc., Cary, NC). Orthogonal polynomial mean comparison using the GLM was used to further analyse the treatment means.

2.2.6 Field Studies in the Quabbin

One hundred and one two year old American chestnut seedlings obtained from Bear Creek Nursery (Northport, WA) were stored at 4 °C in the cold room. Fifty of these trees were soaked for twenty-two hours in water prior to planting and another fifty-one trees were soaked for twenty-two hours in a bath of water containing a 1 L solution of *T. atroviride* spores at a concentration of 2.8×10^9 spores/ml. The seedlings were planted in a recent clear cut area in the north section of the Prescott Penninsula adjacent to the Quabbin Reservoir. The site is located in the town New Salem, south-east of the 304.8 m high area on the Penninsula alongside the unpaved road leading to gate 20. The fifty-one *T. atroviride* treated seedlings were planted on 27 July, 1994 in the northern portion of the site and the untreated seedlings were planted on the same date in the southern portion of the site. The trees were planted approximately 3 m apart and the two plots were planted with approximately 28 m spacing between them. Tubes were placed around each tree to protect them from deer and small mammal predation. Five days later on 1 August, leaf buds and bark pieces were collected from the upper and lower portions of each tree. The

leaf buds and bark pieces were placed onto acidified PDA (APDA) and incubated in the dark at 25 °C, to determine if the the tissues were colonized by *Trichoderma*. When all the trees had completely leafed out in early September, leaf and stem samples were collected (6 September). A 1 x1 square cm sample was cut from each of the leaves as well as a 0.5 cm from each stem sample. The tissues were placed onto APDA to attempt to reisolate *T. atroviride*. The *T. atroviride* treated plants were sprayed (approximately 20 ml/tree) on 6 September, with an aqueous solution of *T. atroviride* spores (3.5×10^9 spore/ml). The control seedlings were sprayed with tap water. The treated trees were again sprayed (approximately 20 ml/tree) on the 24 October with 3.57×10^9 spores/ml spore suspension and the control trees with tap water, after stem and leaf samples were collected.

On 30 May, 1995 when the seedlings had leafed out, stem and leaf samples were collected from all the trees and placed onto APDA as described above. The winter survival rate of the seedlings was also determined. The treated trees were again sprayed (approximately 20 ml/tree) with an aqueous suspension of *T. atroviride* spores (3.54×10^9 spores/ml) and the untreated trees with tap water. Stem and leaf samples taken from all the trees again on 14 October were plated as previously and the treated trees sprayed again with 3.0×10^9 spores/ml *T. atroviride* suspension and the untreated trees with tap water. The surival of transplants was determined

Sampling was repeated the following year at the end of May (1996) and tree viability ascertained.

2.3 Results

2.3.1 *In vitro* Interaction between *C. parasitica* and *T. atroviride*

The *T. atroviride* isolate grew faster than the *C. parasitica* isolate on both media (Figs. 2.2 and 2.3). The antagonist grew faster on PDA than on MEA. *Cryphonectria parasitica* grew slightly faster on PDA than on MEA. The growth of *T. atroviride* on MEA plates was slower on the interaction plates than when it was grown alone on control MEA plates. However, the antagonist grew faster on PDA interaction plates than on PDA plates where it was alone. The *C. parasitica* isolate grew slower in the interaction plate on PDA than on MEA. There was no distinct zone of inhibition which would have indicated that the antagonist had produced antibiotics. The *T. atroviride* isolate completely overgrew *C. parasitica* (Fig. 2.4). There were no distinguising effects on the colony morphology of *C. parasitica.*

Analysis of variance showed that the difference between the media was highly significant. On separating the data from the two different media, analysis of variance revealed that there was a highly significant difference among the treatments. Mean comparison of the treatments showed there was a significant difference between treatments 2 and 3 on both PDA and MEA every day that measurements were taken (data not shown). Only on the final day of measurements was there a significant difference between treatments 1 and 4 on both PDA and MEA. In the presence of *T. atroviride*, the growth of *C. parasitica* slowed dramatically between the second and third day of measurements. Measurements taken on the fourth day indicated that there was no further increase in growth of the pathogen. On the day of the third measurement, *T. atroviride* had overgrown the pathogen by an average of 1.6 cm. The anatagonist continued to

overgrow the pathogen. On 88% of the plates containing both the antagonist and the pathogen, the antagonist began producing green spores approximately three days after *T. atroviride* was placed on the plates. Spores appeared on the other 12% after the fourth day. The antagonist when grown alone did not produce spores until its mycelia had reached the edge of the plate (after five days). The pathogen, which had stopped growing at approximately the fifth day after it was placed on the media, was not reisolated from the interaction plates.

2.3.2 American Chestnut Bark Wood Test

Bark and wood pieces treated with only *C. parasitica* followed by water developed extensive necrotic areas (Fig. 2.5a); trees treated first with sterile water and then *C. parasitica* also had large necrotic area on them (2.5c); those treated first with *C. parasitica* and later with *T. atroviride* had limited necrotic areas (Fig. 2.5b); samples treated first with *T. atroviride* and later with *C. parasitica* had minor necrotic areas extending 0.45 cm (bark) or 0.47 cm (wood) from the plugs of *C. parasitica* (Fig. 2.5d); there were no necrotic areas on samples treated with only the antagonist and or water and PDA plug(Figs. 2.5e and f). The canker lengths on bark pieces varied little from those of the wood pieces (Fig. 2.6). Analysis of variance of the means indicated that there was a significant difference among treatments on both bark and wood pieces (Fig. 2.6). Separation of the means further showed that wood and bark pieces treated with *T. atroviride* had significantly smaller cankers than those which had not been treated with the antagonist. Prophylactic treatment of the antagonist produced the most significant reduction in canker length. The antagonist was isolated on APDA from those wood and

bark pieces that were treated with *T. atroviride*. It was also isolated from samples treated with both the antagonist and the pathogen. *Cryphonectria parasitica* was isolated from 5% of the wood and 7% of the bark pieces which were treated with both fungi. The pathogen was isolated from all samples treated with only *C. parasitica*.

2.3.3 Treatment of Potted Chestnut Seedlings

Canker development began on trees with treatments 2, 3, and 4, within the first week of placing *C. parasitica* on the wound sites (Fig. 2.7). Canker development on trees treated with *T. atroviride* after inoculation with *C. parasitica* (Figs. 2.7 and 2.8a), was significantly smaller than on trees which were not treated with the antagonist after inoculation with *C.parasitica* (Figs. 2.7 and 2.8b). Compared to the control (C-T) (Fig. 2.8c), canker development was also significantly reduced with the prophylactic treatment of *T. atroviride* (Figs. 2.7and 2.8d). Those seedlings not treated with *C. parasitica* did not develop cankers (Fig. 2.8e). Cankers began to develop twenty-one days after the pathogen was placed on the wound site of approximately 1% of the trees with treatment 1 (T-C) (Fig. 2.8d). The pathogen was isolated from those trees. The antagonist was isolated from all seedlings treated with both *T. atroviride* and *C. parasitica* or only *T. atroviride*, whereas in control treatments in which seedlings were treated with only *C. parasitica*, only *C. parasitica* was isolated. *C. parasitica* was also isolated from 91% of the trees treated first with *C. parasitica* and then *T. atroviride*.

Analysis of variance showed that there was no significant difference between the blocks but there was very significant difference among treatments on every day of measurement (2.7). Mean comparison showed that there was significant difference

between treatments 1 and 4 and 2 and 3 on every day of measurement (Fig. 2.7). At the thirty-ninth day 65%, 43%, and 18% of trees with treatments 3, 4, and 2 respectively, had wilt symptoms. Those trees with treatment 1 and the other controls exhibited no wilt symptoms.

2.3.4 Treatment of Potted Chestnut Seedlings in the Greenhouse

Canker development began on trees with treatments 1, 2, 3, and 4 within the first week of placing *C. parasitica* on the wound site. Cankers on seedlings with treatments 2, 3, and 4 developed very rapidly (Fig. 2.9). Analysis of variance revealed that there was significant difference among treatments on each day of measurement. Comparison of means showed that canker areas on trees with treatment 4 were significantly greater than those on trees with treatment 1, on each day of measurements. Canker areas on trees with treatments 2 and 3 were found to be significantly different on the first day of measurement (Fig. 2.9).

Trees with prophylactic treatments of *T. atroviride* (Fig 2.10a), compared to their controls which had sterile water (Fig 2.10b), had limited canker development. Post treatment with the antagonist did not limit canker development as it had done in previous experiments. Trees which had first been treated with *C. parasitica* and secondly with *T. atroviride* had large necrotic areas comparable to those on control trees, first *C. parasitica* and then sterile water, (Figs. 2.10c and d).

The antagonist was isolated from seedlings which had been inoculated with *T. atroviride* and *C. parasitica* or only *T. atroviride* *Cryphonectria parasitica* was isolated from seedlings with treatments 1, 2, 3, and 4. At the forty-fourth day, 84%, 70%, 93%,

and 12% of trees with treatments 3, 4, 2, and 1 respectively, had wilt symptoms. Cankers which developed on trees in the greenhouse were smaller than cankers which had developed on trees outside the greenhouse the previous summer (Figs. 2.7 and 2.9).

2.3.5 Field Studies in the Quabbin

The antagonist was isolated from all samples collected from the *T. atroviride* treated trees on 1 August, 1994, five days after the trees were planted. Only one of those trees not treated was found to have *Trichoderma*. All the trees had produced leaves. Sampling in September showed that *Trichoderma* was also isolated from all the trees in the treated section and three from the untreated site.

The trees were not sampled again until 30 May, 1995. The tops of the trees had died back but leaf buds along the main stems had opened. All trees survived the winter. Plating of samples from the trees showed that *Trichoderma* was on 37 of the treated trees and on 2 of the untreated trees. *Trichoderma* was found more often on stem samples (82%) than on leaf samples (34%). In October, 1995 two trees in the treated section were removed because of death due to root rot or damage by a small mammal. Growth of the branches on many trees was restricted because of the tubes. Forty-seven trees in the treated section and 26 trees in the untreated section were found to have *Trichoderma*. Eighty-seven percent of the stem sections and 91% of the leaves checked had *Trichoderma*. It was not determined in this study which *Trichoderma* sp. was isolated from the trees.

On 24 May, 1996 when the trees were first visited after the winter, 30 trees in the treated section and 23 trees in the untreated section were still alive. *Trichoderma* was

recovered on APDA from 26 of the treated and 18 of the untreated trees. Seventy-three percent of the stem sections and 41% of the leaves checked had *Trichoderma.*

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Fig. 2.1. *Trichoderma atroviride*. (A) Hyaline regularly branched phialidic conidiophores. (B) Conidia smooth and subglobose. Characterization by American Type Culture Collection closely matches that of *Trichoderma atroviride* Karsten, illustrated *in* Bissett, J. 1991. A revision of the genus *Trichoderma*, II: Infrageneric classification. Can. J. Bot. 69: 2357-2372.

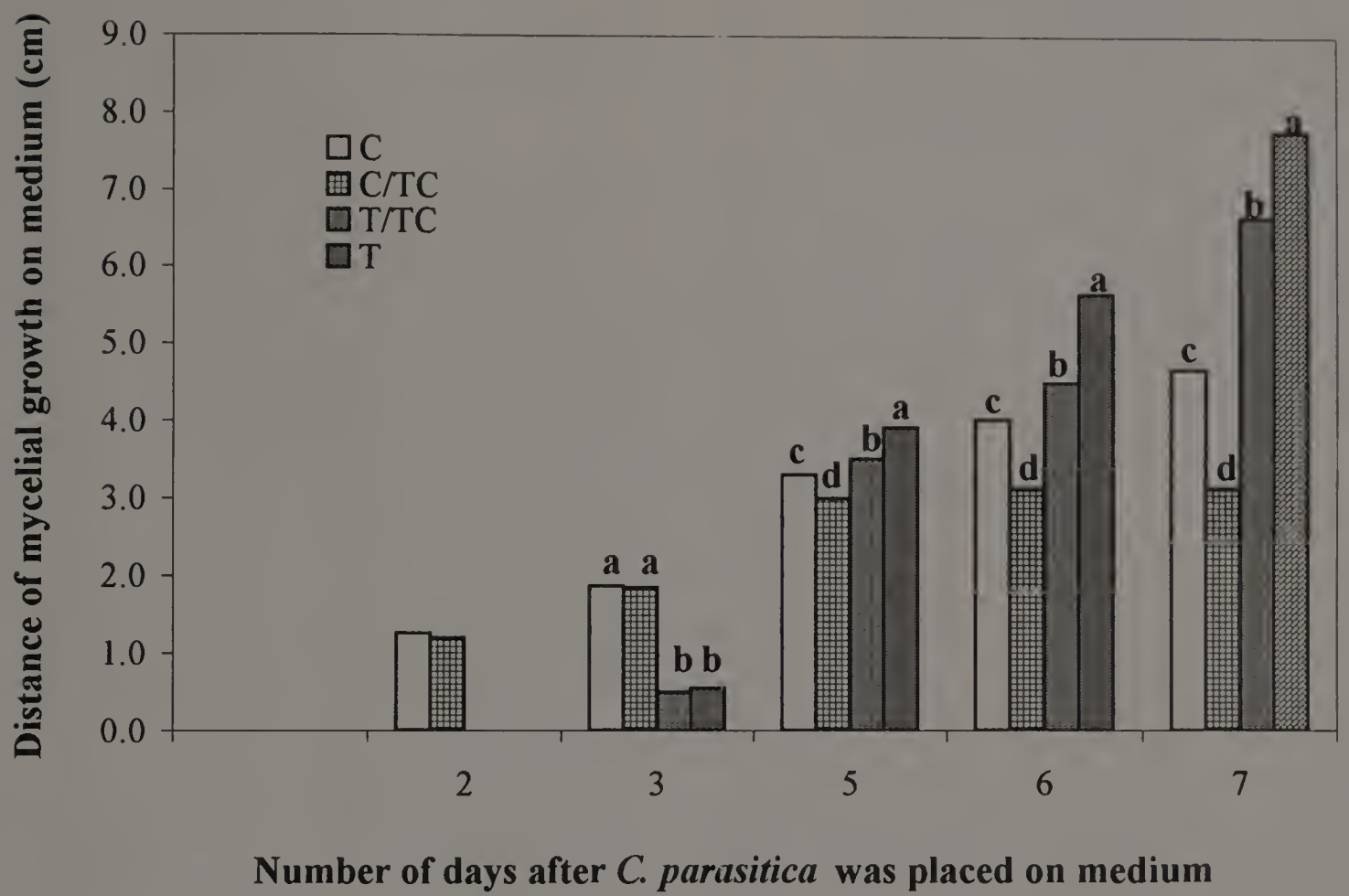


Figure 2.2. *In vitro* interaction between *Cryphonectria parasitica* and *Trichoderma atroviride* on malt extract agar (MEA). C = *C. parasitica* only; C/TC = *C. parasitica* in a petri dish with *T. atroviride*; T = *T. atroviride* only; T/TC = *T. atroviride* in a petri dish with *C. parasitica*. Bars with the same letter within each day grouping are not significantly different at p = 0.05.

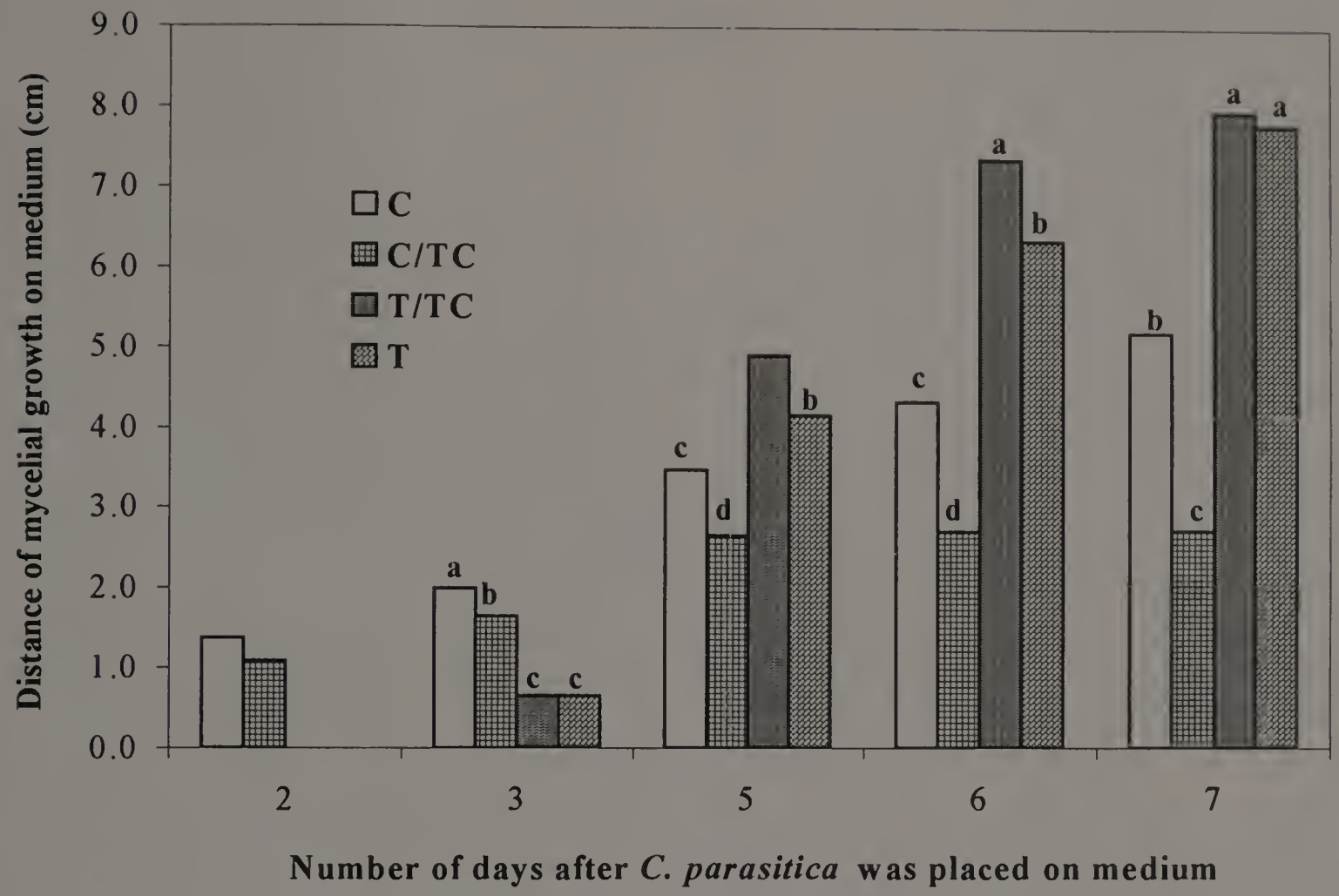


Figure 2.3. *In vitro* interaction between *Cryphonectria parasitica* and *Trichoderma atroviride* on potato dextrose agar (PDA). **C** = *C. parasitica* only; **C/TC** = *C. parasitica* in a petri dish with *T. atroviride*; **T** = *T. atroviride* only; **T/TC** = *T. atroviride* in a petri dish with *C. parasitica*. Bars with the same letter within each day grouping are not significantly different at p = 0.05.

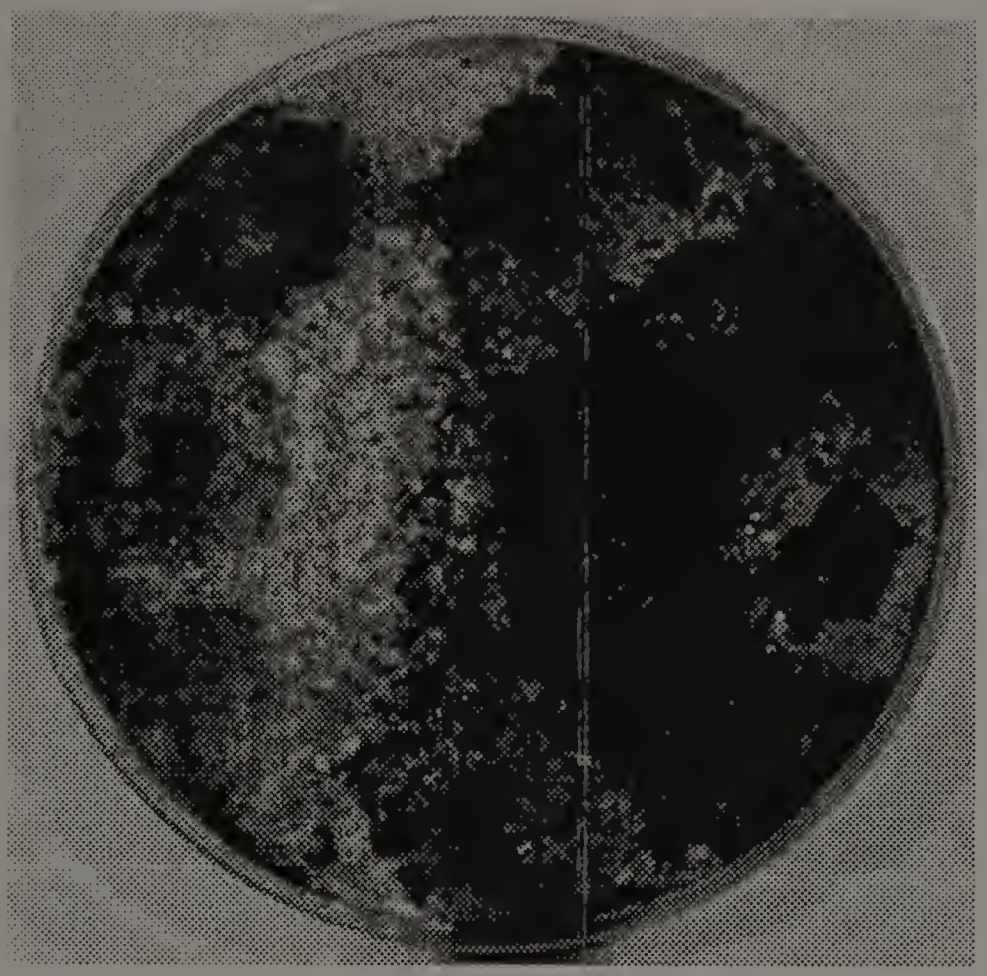


Figure 2.4. *Cryphonectria parasitica* (on left) being overgrown by *Trichoderma atroviride* (on right), fourteen days after the plug of *T. atroviride* was transferred onto the PDA containing a two day old plug of the pathogen. The green and yellow spores produced by *T. atroviride* cover *C. parasitica*.

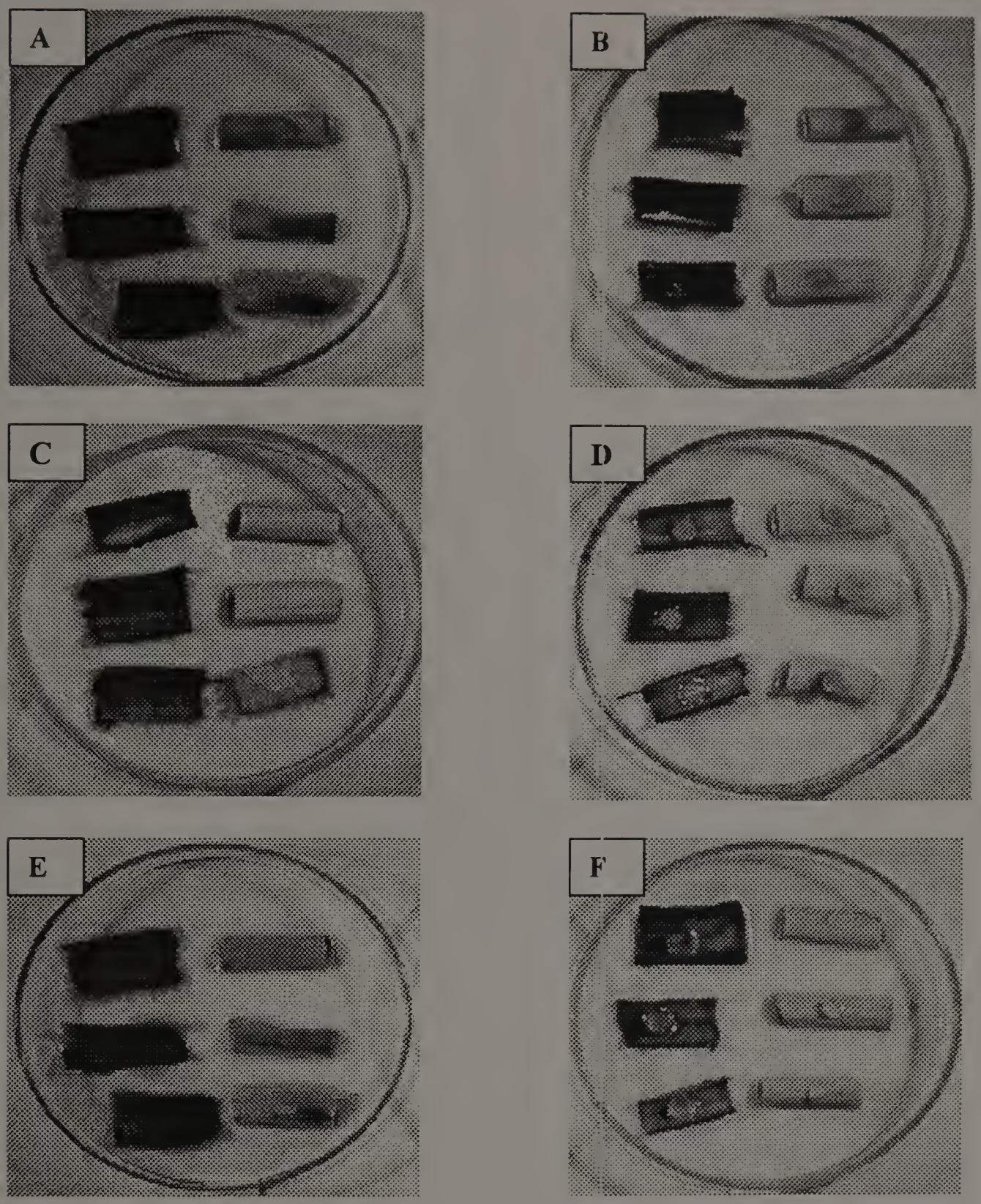


Figure 2.5. Bark and wood tissue pieces from American chestnut trees (*Castanea dentata*) with (A) A 3 mm diameter plug of *Cryphonectria parasitica* followed by sterile water; (B) Plug of *C. parasitica* placed on tissue and followed by *Trichoderma atroviride* spore suspension; (C) Sterile water followed by *C. parasitica*; (D) *T. atroviride* spore suspension followed by a plug of *C. parasitica*; (E) *T. atroviride* spores followed by sterile water (water followed by *T. atroviride* was equal in appearance, hence not shown); (F) Water followed by a plug of potato dextrose (PDA). Secondary inoculations were made two days after the primary inoculations.

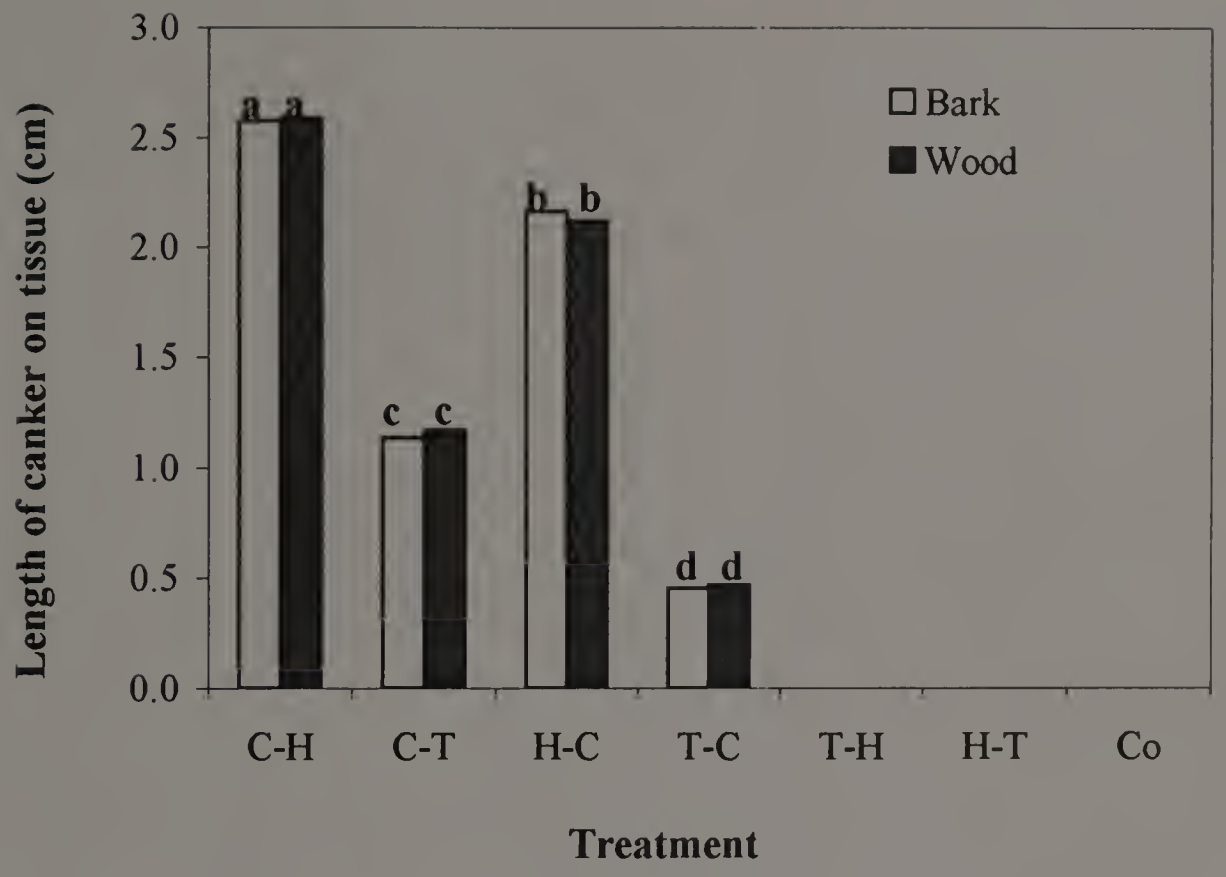


Figure 2.6. American chestnut bark wood test. C-H = *Cryphonectria parasitica* plug followed by sterile water. C-T = *C. parasitica* followed by *Trichoderma atroviride* spore suspension. H-C = Sterile water followed by *C. parasitica*. T-C = *T. atroviride* followed by *C. parasitica*. T-H = *T. atroviride* followed by sterile water. H-T = Sterile water followed by *T. atroviride*. Co = Sterile water followed by a plug of potato dextrose agar. Secondary inoculations were made two days after the primary inoculations. Bars with the same letter are not significantly different at p = 0.05.

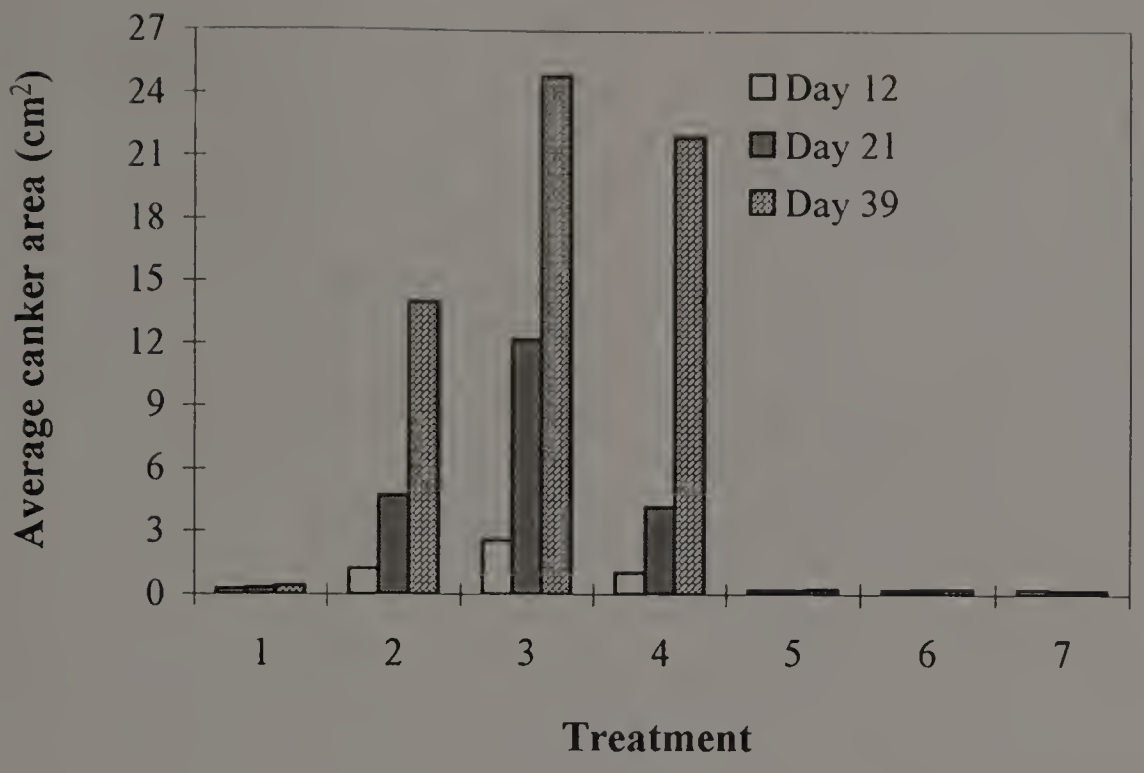


Figure 2.7. Treatment of potted American chestnut seedlings. (1) *Trichoderma atroviride* spore suspension followed by *Cryphonectria parasitica*; (2) *C. parasitica* plug followed by *T. atroviride*; (3) *C. parasitica* followed by sterile water; (4) Sterile water followed by *C. parasitica*; (5) *T. atroviride* followed by sterile water; (6) Sterile water followed by *T. atroviride*; (7) Sterile water followed by sterile water. Secondary inoculations were made two days after the primary inoculations. Orthogonal polynomial mean comparison: Trt. 1 vs trt. 4, significantly different on day 12 and highly significantly different on days 21 and 39; Trt. 2 vs trt 3, significantly different on day 12, highly significantly different on day 21 and significantly different on day 39. Trt. = treatment, vs = versus.

A B C D

E

Figure 2.8. Treated potted American chestnut seedlings thirty-nine days after inoculations were completed. (A) A small necrotic canker on seedling first treated with *Cryphonectria parasitica* followed by a spore suspension of *Trichoderma atroviride*. The green spores of the antagonist are also visible. (B) Necrotic canker on seedling first treated with *Cryphonectria parasitica* and then with sterile water. (C) Large canker on seedling treated first with sterile water and then with *C. parasitica*. (D) Seedling treated first with *T. atroviride* and then with *C. parsitica*. (E) Seedling was treated only with sterile water. Seedlings treated with *T. atroviride* and then sterile water were similar in appearance. Secondary inoculations were made two days after the primary inoculations.

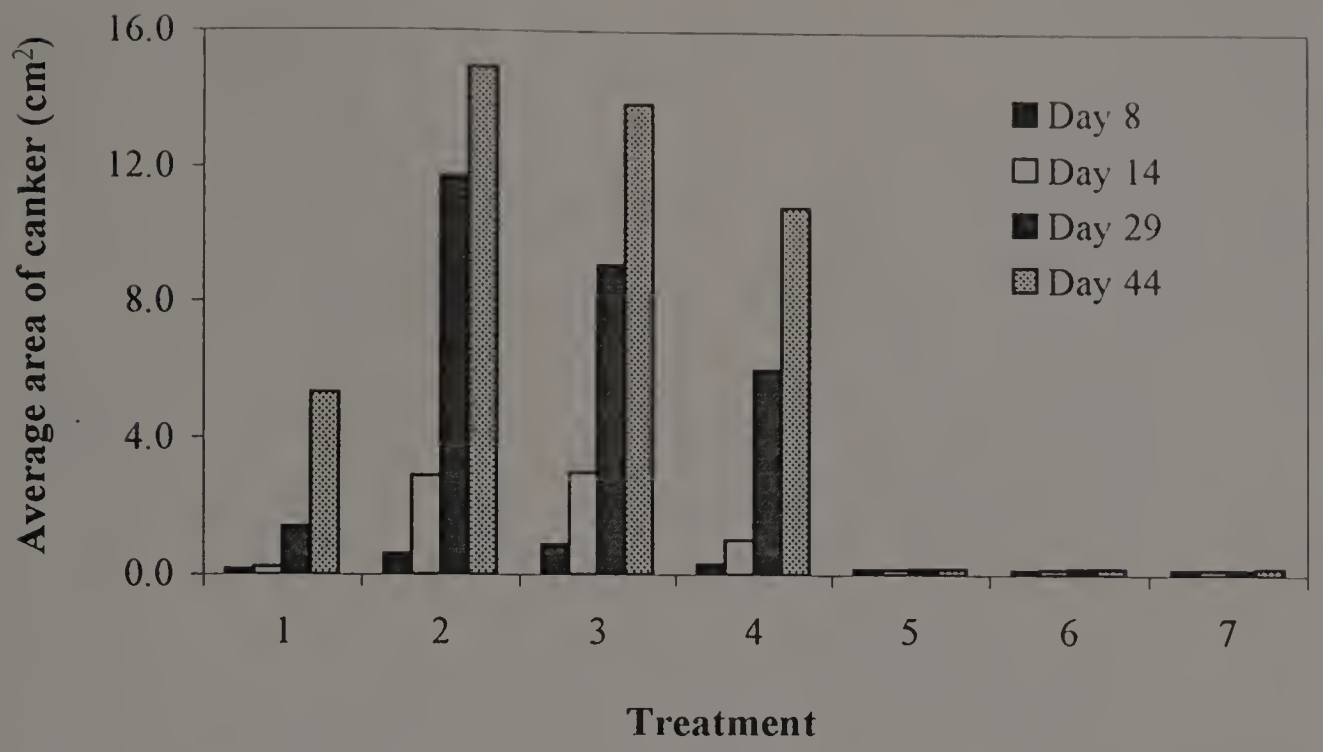
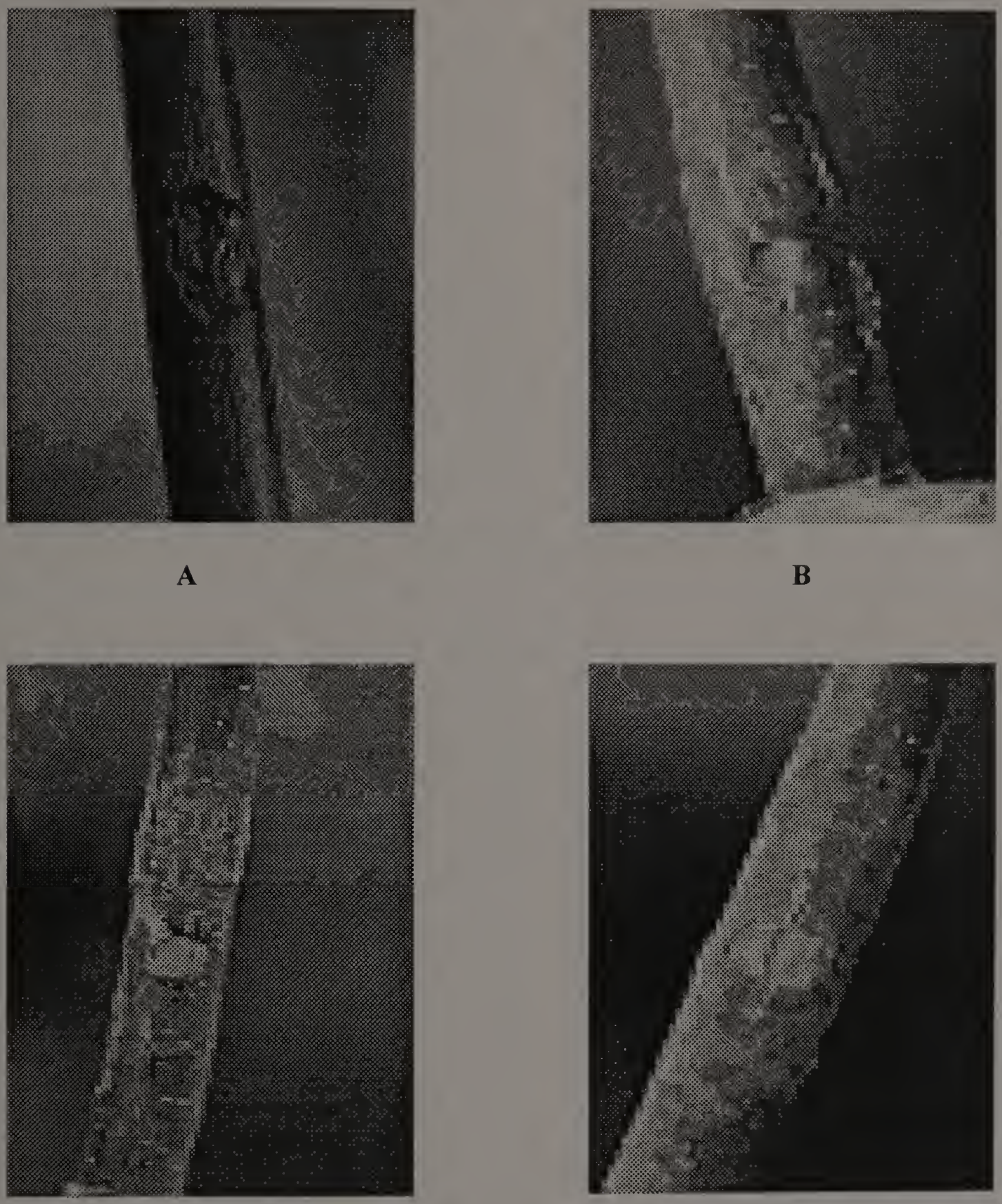


Figure 2.9. Treatment of potted American chestnut seedlings in the greenhouse. (1) *Trichoderma atroviride* spore suspension followed by *Cryphonectria parasitica*, (2) *C. parasitica* plug followed by *T. atroviride*; (3) *C. parasitica* followed by sterile water; (4) Sterile water followed by *C. parasitica*; (5) *T. atroviride* followed by sterile water; (6) Sterile water followed by *T. atroviride*; (7) Sterile water followed by sterile water. Secondary inoculations were made two days after the primary inoculations. Orthogonal polynomial mean comparison: Trt. 1 vs trt. 4, significantly different on day 12 and highly significantly different on all days; Trt. 2 vs trt 3, no significant difference. Trt. = treatment, vs = versus.



A B C D

Figure 2.10. Treated potted American chestnut seedlings in the greenhouse fourteen days after inoculations were completed. (A) Seedling treated first with *Trichoderma atroviride* spore suspension then with *Cryphonectria parasitica*. (B) Seedling treated first with sterile water and then with *C. parasitica*. (C) Seedling treated first with *C. parasitica* followed by *T. atroviride*. (D) Seedling treated with *C. parasitica* and then with sterile water. Secondary inoculations were made two days after the primary inoculations.

CHAPTER 3

EVALUATION OF CARRIER MEDIA FOR THE APPLICATION OF *TRICHODERMA ATROVIRIDE* TO AMERICAN CHESTNUT TREES

3.1 Introduction

In the pursuit to decrease the use of pesticides, microbial antagonists have become an attractive option for the control of plant diseases, particularly disease caused by soil-borne pathogens (Goldman et al., 1994). Microbial antagonists found to be effective biological control agents in laboratory and greenhouse tests have however been difficult to apply to field situations (Kok et al., 1996). These previous studies have shown that the introduction of antagonistic microorganisms into the soil, results in improved pathogen control if the microorganism is formulated in a carrier. The carrier can serve as a protectant and/or a food base, thus allowing the antagonist to survive in greater quantity through the establishment stage (Kok et al., 1996).

Carriers mixed with microbial antagonists have been used with varying degrees of success under experimental conditions in both greenhouse and field situations. Carriers used include clay powder, alginate pellets, vermiculite-bran mixture (Kok et al., 1996), processed manure pellets, peat (Lo et al., 1996), coffee husk (decomposed and fresh), tea waste and neem cake (Bhai et al., 1994), as well as liquid formulations containing an aqueous binder such as Pelgel (LiphaTech, Milwaukee, WI) (Taylor et al., 1991) to coat seeds. As a part of these formulations, the antagonists were able to establish stable populations (Kok et al., 1996). There are very few reports describing the use of carriers

containing *Trichoderma* for the treatment of pathogens which attack above ground. McCabe (1974) reported that a compost applied as a compress to cankers caused by *Cryphonectria parasitica* on American chestnuts resulted in canker healing after six months. Weidlich (1978) also reported control of the cankers caused by *C. parasitica* after three months using muddy soil compresses. From the compresses used by Weidlich (1978), *Trichoderma* was isolated and was thought to be possibly responsible for the antagonistic effects. Turchetti and Gemignani (1981), using sphagnum moss, found cankers healing as did Magnini (1981) when he applied this media to graft unions and cankers. *Trichoderma viride* was isolated and determined to be the antagonist in the sphagnum peat used by Magnini (1981). Liquid coating formulations used by Taylor et al., (1991) on seeds to protect them from *Pythium* and *Rhizoctonia,* were found to provide a conducive environment for *T. harzianum* and allowed the antagonist to be distributed uniformly on the seed surfaces. A pellet formulation of *T. viride* was tested for a number of years on its ability to control the pathogen (*Chondrostereum purpureum*) which causes silver leaf disease affecting fruit trees (Corke, 1979). Ricard (1983) reported antagonism of *Ceratocystis ulmi* by *Trichoderma* isolates when a commercial formulation, called BINAB T® was used. The study conducted in the field (Sainte Adresse near Le Havre, France) with fifteen elm trees, resulted in limited protection from the Dutch elm pathogen, *Ophistoma ulmi*, because the *Trichoderma* did not grow to new areas once the nutrients of the pellet were used up.

The choice of amendments to be used in formulation of bioprotectants depends on their ability to enhance the activity of the antagonist as well as to provide protection. Ease of application and cost of the carrier media are also important considerations. In the

following described studies, the carrier media used which could possible fulfill the above requirements, included lanolin, wax/lanolin wound treatment cream, mineral oil, and Wilt-Pruf®. The experiments were done on stems of American chestnut trees in the field and stem sections of American chestnuts in the laboratory.

3.2 Materials and Methods

3.2.1 Field Studies in Cadwell

The Cadwell Memorial Forest, the research forest of the University of Massachusetts in Pelham, Massachusetts was chosen in 1995 to conduct field testing of the carriers containing *Trichoderma atroviride*. Five sites were selected near the entrance area to the forest. Many young American chestnut trees were found among oak and beech trees. Fifty-five to sixty American chestnut trees (2.5 to 4.0 cm diameter) were selected on five different dates(6 September, 7 September, 8 September, 22 September, and 25 September) at each site. The main stem of each tree was wounded with a 3 mm diameter cork borer. At each wound site an agar plug (3 mm diameter) of *C. parasitica* growing on PDA was placed and the area wrapped with approximately 7 cm width strips of parafilm. Twelve days after inoculation with *C. parasitica* (cankers were approximately 1 cm in width) the trees were tagged and the second treatments applied. The following nine treatments were used:

1) *C. parasitica* (**C**) only.

2) **C** then painted with sterile water ($\mathbf{H_2O}$).

3) **C** then painted with $\mathbf{H_2O}$ and wrapped with parafilm.

4) **C** then plastered with lanolin (**L**) (Sigma Chemical Company, St. Louis, MO).

5) **C** then plastered with Phyton 50® (**W**) (a commercially available wax/lanolin based cream wound treatment from STB Inc., Minneapolis, MN).

6) **C** then *T. atroviride* spore suspension (**T**).

7) **C** then **T** and then wrapped with parafilm.

8) **C** then **T** spores mixed in **L**.

9) **C** then **T** spores mixed with **W**.

The *T. atroviride* spore suspension was made by dividing a PDA plate of sporulating *T. atroviride* into thirds and washing the spores from each section individually with sterile distilled water into sterile beakers. The spore suspensions were filtered through a sterile piece of miracloth and diluted to 3.0×10^8 spores/ml. For each inoculation of *T. atroviride* mixed with lanolin or Phyton 50®, 2 ml of the spore suspension was mixed with 5 g of sterilized lanolin or Phyton 50®. The lanolin and Phyton 50®, base treatments were applied with spatulas while the spore suspension and sterile water were applied with small paint brushes. Around the wound sites on each tree four holes 3 mm diameter were drilled (approximately 2 mm to 3 mm depth) forming corners of a square with 2.54 cm length sides. The area between the drilled holes including inoculation site was treated with the control agent (Fig. 3.1). Each treatment per block was replicated 5 times and a total of forty-five of the trees inoculated with *C. parasitica*/block were chosen to be used in the experiment. Four of the five blocks initially inoculated with *C. parasitica* were used

because cankers failed to develop on the trees inoculated on 6 September. The canker lengths and widths on all trees were recorded sixty-four days after the second inoculation was applied (22 November, 23 November, 7 December, and 10 December). The canker areas were calculated from the trees of the four blocks with the formula for the area of an ellipse (½ length x ½ width x π). Analysis of variance of the log transformed canker areas was calculated using the General Linear Method (GLM) and mean separation by Duncan's Multiple Range test with a level of significance (α) equal to 0.05.

3.2.2 Stem Section Inoculations

American chestnut trees of 2.5 cm to 3.2 cm in diameter were cut and small branches removed on 10 October, 1995. The logs were then cut into approximately 30.0 cm lengths and one end sealed with parafilm and placed into buckets containing a depth of 5.0 cm of water (unsealed end down). The buckets were filled with stems and covered with black garbage bags and then placed into the cooler (4 °C) until needed. Water levels in the buckets were checked every two weeks. Stems were taken from the cooler and washed in a 10% hypochlorite solution followed by three rinses with sterile deionized water. Both ends of each stem were wrapped with parafilm to prevent desiccation. Each stem was then randomly (by drawing lots) labeled with a treatment type, wounded with a 3 mm diameter cork borer followed by the first inoculations. The inoculated site was wrapped with parafilm and the stem sections placed in the dark in 25 °C ± 0.1 °C incubators (Fig. 3.2). Two days following the primary inoculations the secondary inoculations were applied. The twenty-four treatments were as follows:

1) Sterile water (**H_2O**) then **H_2O**

2) **H_2O**/Mineral Oil (**MO**)

3) **H_2O**/Wilt-Pruf® (**WP**)

4) *C. parasitica* (**C**)/**H_2O**

5) **C/MO**

6) **C/WP**

7) **H_2O/H_2O**

8) **MO/H_2O**

9) **WP/H_2O**

10) **H_2O/C**

11) **MO/C**

12) **WP/C**

13) **H_2O**/*T. atroviride*+**H_2O (TH_2O)**

14) **H_2O**/*T. atroviride*+**MO (TMO)**

15) **H_2O**/*T. atroviride*+**WP (TWP)**

16) **C/TH_2O**

17) **C/TMO**

18) **C/TWP**

19) **TH_2O/H_2O**

20) **TMO/H_2O**

21) **TWP/H_2O**

22) **TH_2O/C**

23) **TMO/C**

24) **TWP/C**

Spore suspensions of *T. atroviride* were made by dividing heavily sporulating, seven day old PDA plates containing heavily sporulating into thirds and lightly scraping the spores from the plate with a sterile spatula into 40 ml of sterilized water, Wilt-Pruf® (Wilt-Pruf Products Inc., Greenwich, CT), or mineral oil (Melville Corp., Woonsocket, RI). The spore concentration used in all three experiments was determined to be an average of 2.3 x 10^8 spores/ml by counting the spores in the water suspensions. Half a milliliter of the liquid inoculum including the sterile water was applied directly to the wounded site with pasteur pipettes. Three milliliter diameter PDA plugs of *C. parasitica* were taken from the edges of 5 day old plates. The inoculated stems were placed onto the plastic surface

of sorbent paper. The stems were incubated for 40 days. The experiment was repeated twice (23 March and 22 May, 1996) with 6 replicates per treatment. The experiment was repeated a third time (23 July) using stems cut on 5 April, 1996. However, in this experiment the stems were placed onto the plastic surface of sorbent paper inside degradable black 113.6 L size, plastic garbage bags (Price Chopper Inc., Schenectady, New York) which had one side cut to allow easy access to the stems. The garbage bags were used to decrease the loss of moisture from the stems in the incubator. The stems were incubated in the dark for thirty days at 25 °C ± 0.1 °C. Water was added daily below the towels to prevent desiccation of the stems; two jars of water were placed on each of the shelves in the incubator. Canker widths and lengths were recorded at the end of each experiment and the areas calculated using the formula of the area of an ellipse (½ length x ½ width x π). Analysis of variance of the log transformed canker areas was calculated using the General Linear Method (GLM) and mean separation by Duncan's Multiple Range test with a level of significance (α) equal to 0.05.

3.3 Results

3.3.1 Field Studies in Cadwell

In the first twelve days canker development was very slow on trees in blocks 1 and 3 (0.5 cm extension from edge of wound) whereas on trees in blocks 2 and 4, canker development was more rapid (1.0 cm extension from edge of wound). In the cool temperatures of fall, any treatments containing Phyton 50® or lanolin became very hard, however on a very warm day they would melt and in a few cases dripped down the side of the tree. Analysis of variance of the means indicated no significant difference among the

treatments but there was a very significant difference among the plots. The interaction between plot and treatment was not significant. Mean separation resulted in the treatments being grouped together (Fig. 3.3). There was no correlation of the ranking of the treatment means among the plots. Spore viability tests were not done due to the difficulty in collecting tissue samples in the cold temperatures prevalent at the time of data collection.

3.3.2 Stem Section Inoculations

Plot 3 was analysed separately from plots 1 and 2 which were analysed together. Significant effects of treatment on canker area in plots 1 and 2, were identified by analysis of variance (Fig. 3.4). Mean separation grouped all the prophylactic *T. atroviride* treatments (22, 23, and 24) separately from their corresponding controls (10, 11, and 12) in plots 1 and 2. Secondary treatments with *T. atroviride* (treatments 16, 17, and 18) were significantly different from stems with prophylactic treatments. Treatment 16 was significantly different from its control (treatment 4) but not significantly different from the controls treatments 17 and 18. Both treatments 17 and 18 were significantly different from their controls (5 and 6 respectively). All controls without *C. parasitica* were not significantly different and were therefore grouped together. The combined spore viability in blocks 1 and 2 on stems with prophylactic treatments **TMO**, **TWP**, and **TH_2O** after forty days were on average 3.2×10^7 spores/cm^2, 3.4×10^7 spores/cm^2, and 2.8×10^6 spores/cm^2 respectively.

Plot 3 also showed significant difference among treatments (Fig. 3.5). Two prophylactic treatments (23 and 24) were grouped together but treatment 23 also shared

grouping with treatments which had not been inoculated with *C. parasitica* and with the prophylatic treatment 22. Treatment 22 was not significantly different from those which had not been inoculated with *C. parasitica*. Treatments 17 and 18 were significantly different from their controls (5 and 6 respectively) and treatment 16. Treatment 16 was was not significantly different from its control (4). The areas recorded for those controls without *C. parasitica* were calculated from measurements taken of callous tissue formed, they did not represent canker areas (Figs. 3.4 and 3.5). In block three the average spore viability after thirty days on stems with prophylactic treatments **TMO**, **TWP**, and **TH_2O** were 4.0×10^6, 3.2×10^6, and 3.0×10^6 spores/cm^2 respectively.

3.4 References

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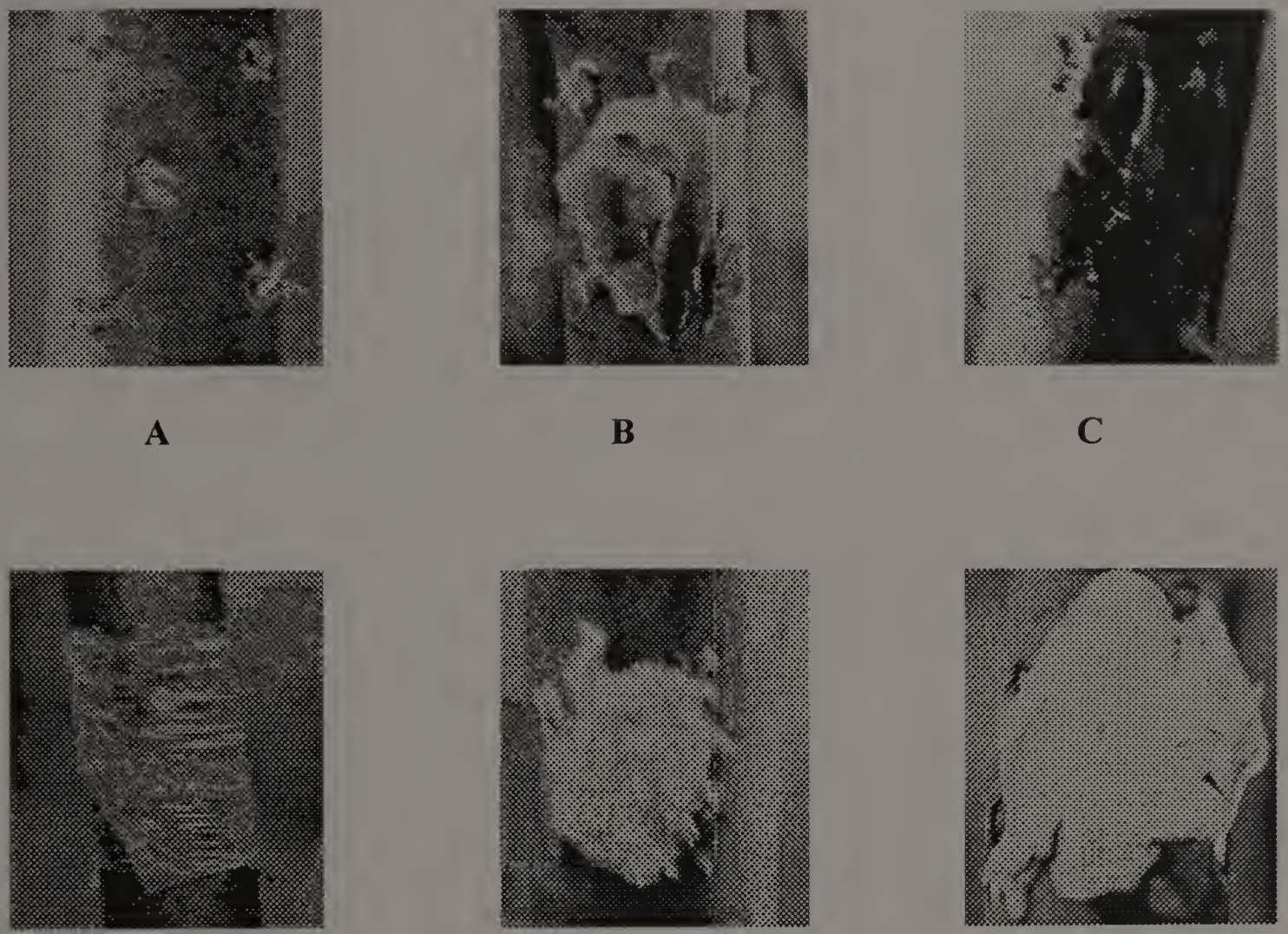

Figure 3.1. Treatments at the Cadwell forest in Pelham, Massachusetts. All wounds were inoculated with a potato dextrose agar plug of *C. parasitica* twelve days before secondary inoculations were made. (A) Treated with only sterile water and left unwrapped. Other trees similar in appearance (not shown) were treated with *T. atroviride* and not wrapped. (B) Treated with Phyton 50® alone. (C) Treated with lanolin alone. (D) Treated with sterile water and wrapped with parafilm. Other trees (not shown) were similarly treated with *T. atroviride*. (E) Treated with a mixture of *T. atroviride* and Phyton 50®. (F) Treated with a mixture of *T. atroviride* and lanolin.

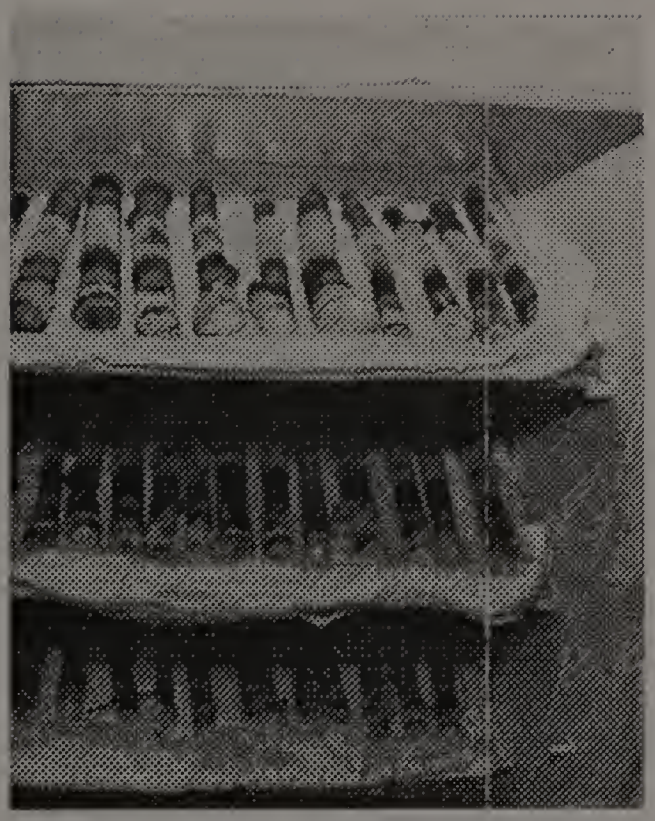

Figure 3.2. Stem sections were placed on sorbent paper and incubated in the dark at 25 °C.

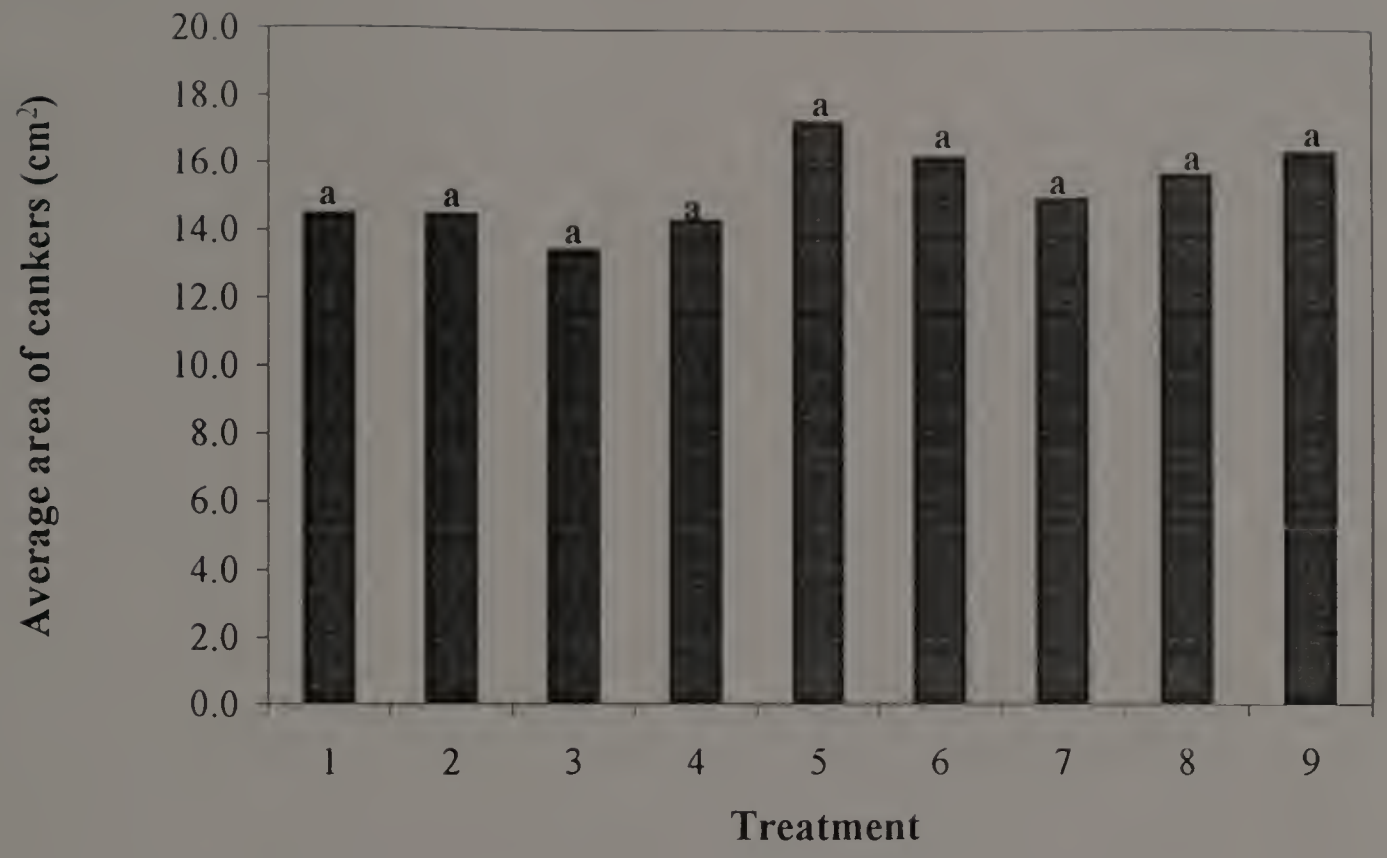

Figure 3.3. Cadwell forest treatments following sixty-four days after the second inoculation. Secondary inoculations were made twelve days after the primary inoculations. (1) *Cryphonectria parasitica* only; (2) *C. parasitica* followed by sterile water; (3) *C. parasitica* followed by sterile water and wrapped with parafilm; (4) *C. parasitica* then lanolin; (5) *C. parasitica* followed by Phyton 50®; (6) *C. parasitica* followed by *Trichoderma atroviride* spore suspension; (7) *C. parasitica* followed by *T. atroviride*; (8) *C. parasitica* followed by *T. atroviride* mixed with lanolin; (9) *C. parasitica* followed by *T. atroviride* mixed with Phyton 50®. Bars with the same letter are not significantly different at p = 0.05.

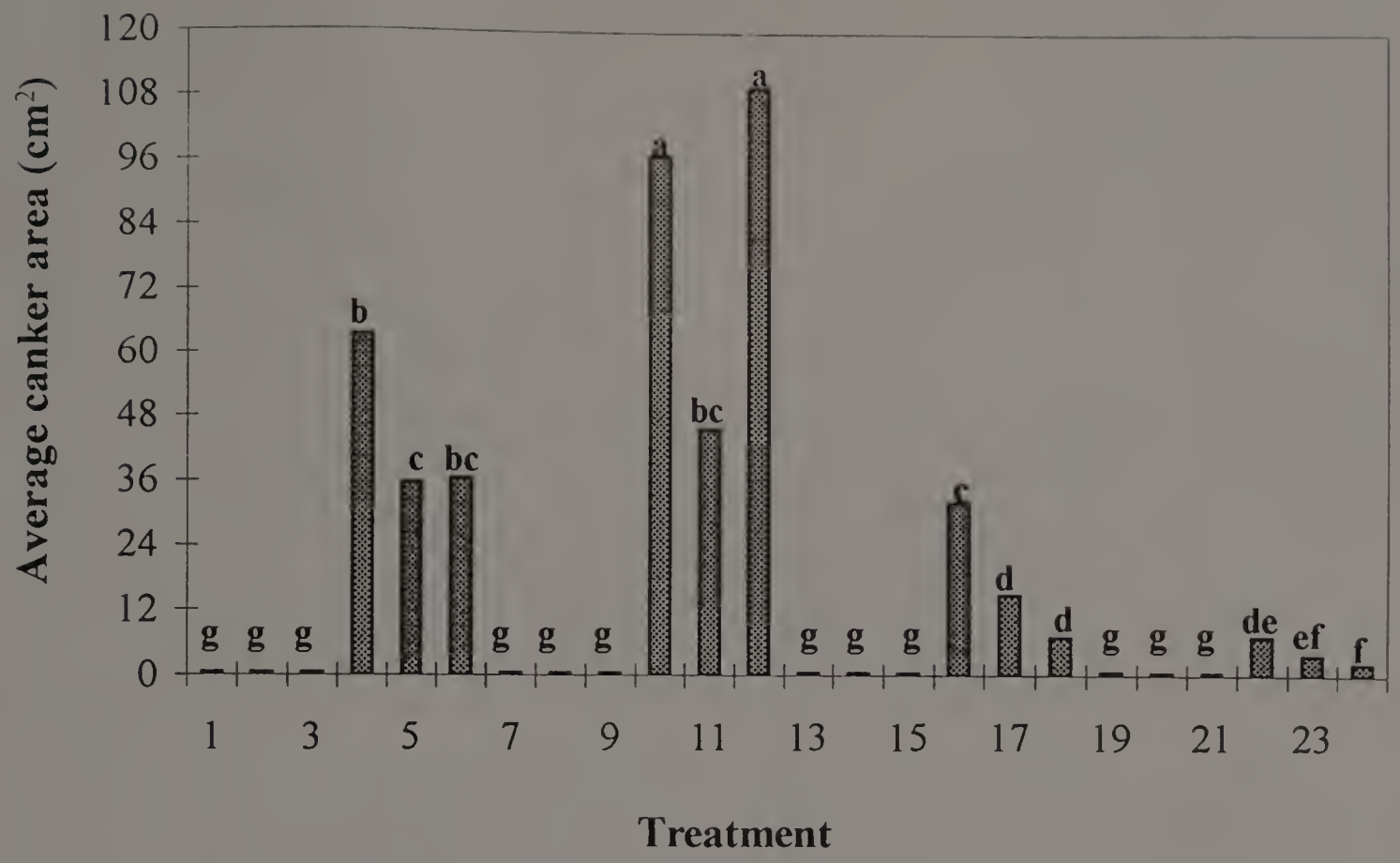

Figure 3.4. Efficacy of three carrier types in the control of canker development on stem sections of American chestnut trees. (1) Sterile water (H_2O) then H_2O; (2) H_2O/Mineral Oil (MO); (3) H_2O/Wilt-Pruf® (WP); (4) *C. parasitica* (C)/H_2O; (5) C/MO; (6) C/WP; (7) H_2O/H_2O; (8) MO/H_2O; (9) WP/H_2O; (10) H_2O/C; (11) MO/C; (12) WP/C (13); H_2O/*T. atroviride*+H_2O (TH_2O); (14) H_2O/*T. atroviride*+MO (TMO); (15) H_2O/*T. atroviride*+WP (TWP); (16) C/TH_2O; (17) C/TMO; (18) C/TWP; (19) TH_2O/H_2O; (20) TMO/H_2O; (21) TWP/H_2O; (22) TH_2O/C; (23) TMO/C; (24) TWP/C. Secondary inoculations were made two days after the primary inoculations. Data was obtained from the combination of observations from blocks 1 and 2, forty days after inoculations were completed. Bars with the same letter are not significantly different at $p = 0.05$.

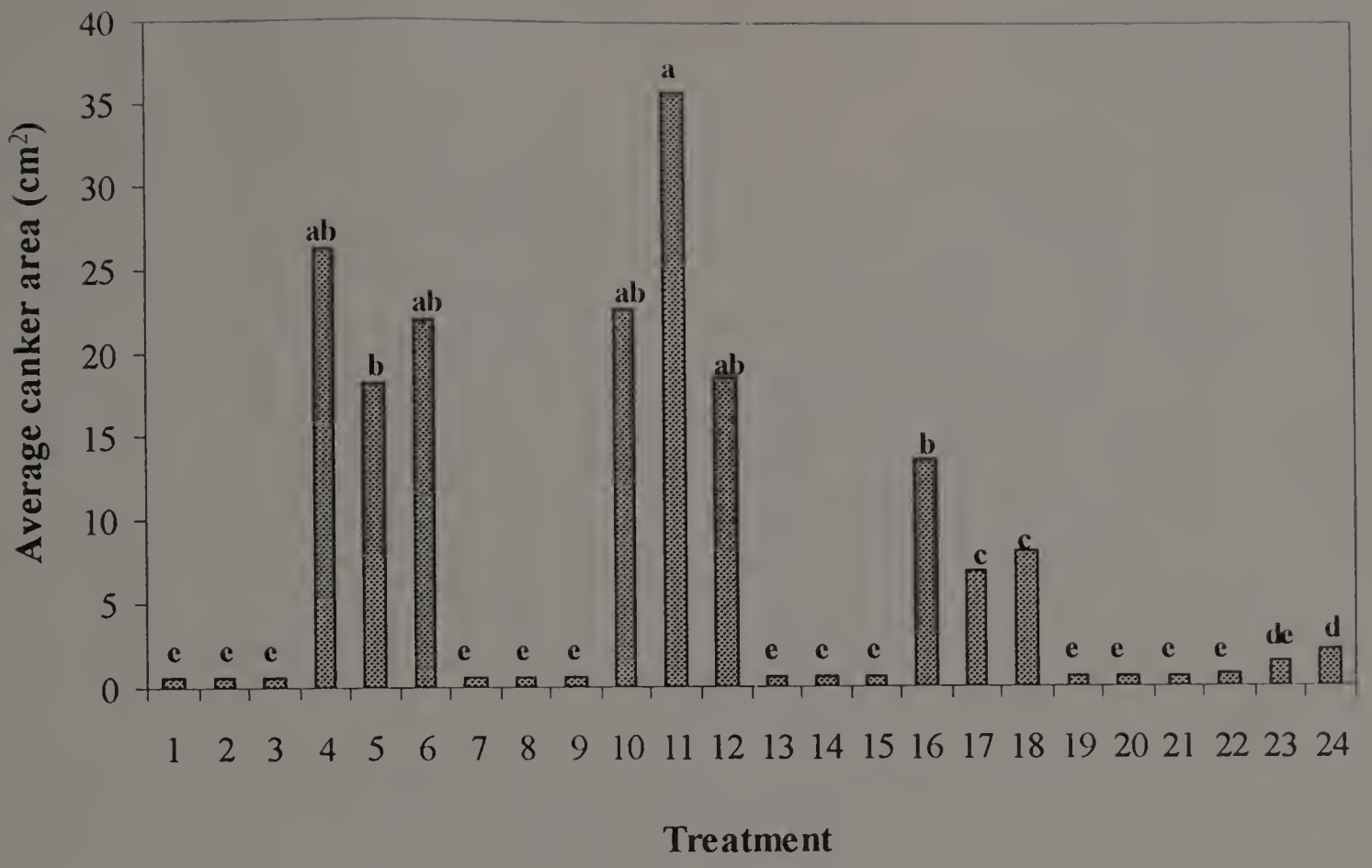

Figure 3.5. Efficacy of three carrier types in the control of canker development on stem sections of American chestnut trees. 1) Sterile water (H_2O) then H_2O; (2) H_2O/Mineral Oil (MO); (3) H_2O/Wilt-Pruf® (WP); (4) *C. parasitica* (C)/H_2O; (5) C/MO; (6) C/WP; (7) H_2O/H_2O; (8) MO/H_2O; (9) WP/H_2O; (10) H_2O/C; (11) MO/C; (12) WP/C (13); H_2O/*T. atroviride*+H_2O (TH_2O); (14) H_2O/*T. atroviride*+MO (TMO); (15) H_2O/*T. atroviride*+WP (TWP); (16) C/TH_2O; (17) C/TMO; (18) C/TWP; (19) TH_2O/H_2O; (20) TMO/H_2O; (21) TWP/H_2O; (22) TH_2O/C; (23) TMO/C; (24) TWP/C. Secondary inoculations were made two days after the primary inoculations. Data was obtained from only block 3, thirty days after inoculations were completed. Bars with the same letter are not significantly different at $p = 0.05$.

CHAPTER 4

ANTAGONISTIC PROPERTIES

4.1 Introduction

Possible mechanisms involved in the pathogen/antagonist interaction include mycoparasitism (Dennis and Webster, 1971c), production of non-volatile antibiotics (Dennis and Webster, 1971a), and the production of volatile antibiotics (Dennis and Webster, 1971b). *In vitro* studies used by researchers (Dennis and Webster, 1971 a, b, and c; Elad et al., 1971; Chambers and Scott, 1995) have been successful in elucidating some mechanisms of biocontrol used by antagonists, particularly, several species of *Trichoderma*.

Dennis and Webster (1971 a, b) showed that *Trichoderma* spp. produce both volatile and water-soluble antibiotics during periods of active growth. Mycelial growth of *Rhizoctonia solani, Pythium ultimum, Fomes annosus, Fusarium oxysporum, Pyronema domesticum* and *Mucor hemalis* were all noted to be affected by the antibiotics in cell free culture filtrates. Spore germination was also noted to be inhibited. Work presented by Bruce et al. (1984) on the control of the wood rotting basidiomycete, *Lentinus lepideus,* by *Trichoderma* spp., indicated that water-soluble antibiotics produced by the antagonists were effective against the pathogen. They however concluded that volatile metabolites alone may have resulted in the inhibition and lysis of the pathogen's mycelium. The antibiotics of this study were unidentified. Characterization of cell free extracts of two strains of *Trichoderma harzianum* by Claydon et al. (1987) showed that

two alkyl pyrones inhibited the growth of *R. solani*. Claydon et al. (1987) also noted finding a patent which described the alkyl pyrone, 6-pentyl-pyrone, as being produced by the spores of *Trichoderma* which together with several biologically active peptides, Trichozianines (also produced by the antagonist) inhibited the growth of the pathogens *Creationistis ulmi* and *Botrytis cinerea*.

Trichoderma spp. also produce various hydrolytic enzymes such as cellulases, chitinases, proteinases, glucanases, and xylanases (Goldman et al., 1994; Lorito et al., 1993). Some species of *Trichoderma* have higher enzyme activity than others and this may depend on what type of substrate they are grown on (Elad et al., 1982). Chitinase and β-1,3-glucanase have been shown to degrade the cell walls of *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pythium aphanidermatum* (Elad et al., 1982). Lipases and proteases from *T. harzianum* are believed to also dissolve certain sections of cell walls of *S. rolfsii* (Elad et al., 1982). Chitinolytic enzymes produced by *T. harzianum* inhibited spore germination of *Fusarium graminearum*, *F. solani*, *Botrytis cinerea*, *Ustilago avenae*, and *Uncinula necator*. These same enzymes did not inhibit sporangia germination nor the mycelial growth of *Pythium ultimum* which as an oomycete contains no chitin in its cell walls (Lorito et al., 1993). Glucanase enzymes and chitinolytic enzymes were also noted to work synergistically (Lorito et al., 1993).

Mycoparasitism appears to play an important role in the control of *S. sclerotiorum* by *T. harzianum* (Inbar et al., 1996). Many researchers (Elad et al., 1982, 1983; Benhamou and Chet, 1993, 1996; Camporota, 1985; Dumas and Boyonoski, 1992; Chambers and Scott, 1995; Askew and Laing, 1994) have described parasitic interactions between *Trichoderma* and soil borne plant pathogens using light and electron

microscopy. Inbar et al. (1996) found *T. harzianum* hyphae growing along the hyphae of *S. sclerotiorum*, forming branches and coiling around the pathogen's hyphae. The *Trichoderma* also penetrated and grew inside the hyphae of *S. sclerotiorum*. Turchetti and Gemignani (1981) after conducting experiments on using soil packs to treat chestnut blight cankers, isolated and showed the mycelium of *T. viride* penetrating and growing inside the mycelium of *C. parasitica*.

The following experiments were therefore conducted to determine if the antagonistic mechanisms found to operate in other *Trichoderma* spp. would also be found to function in the isolate *T. atroviride.*

4.2 Materials and Methods

4.2.1 Volatile Antibiosis Test

One half of each Gray dish containing 2% malt extract agar (MEA, Difco) was inoculated with a 3 mm diameter plug of *C. parasitica* taken from the edge of a five day-old culture. The dishes were incubated in the dark at 25 °C for two days. After two days the other half of the Gray dish was inoculated with a 3 mm diameter plug of *T. atroviride* taken from the edge of a three day-old culture. The dishes were sealed with parafilm and replaced in the incubator. For six days, daily measurements of colony diameters were determined and recorded as the mean of two measurements at right angles. The experiment was repeated 3 times with 10 replicates of the pathogen/antagonist combination and 10 of each control, containing only *C. parasitica* or *T. atroviride* plugs. Repeated measured analysis of the variance of the means of mycelial growth was calculated using the General Linear Method (GLM) and the separation of means was

carried out by Scheffe's test with a level of significance (α) equal to 0.05 (SAS Institute, Inc., Cary, NC).

Volatile antibiosis was also checked using the method of Dennis and Webster (1971). A 3 mm diameter plug of the *T. atroviride* isolate was placed in the centre of a 90 mm diameter petri dish containing 2% MEA (Difco) or PDA (Difco) and incubated in the dark at 25 °C for two days. Plates of 2% MEA and PDA were inoculated similarly with a 3 mm diameter plug of *C. parasitica*. The bases of these newly inoculated plates were inverted and taped to the base of the plates of the 2-day old antagonists. The plates were incubated in the dark at 25 °C and the colony diameters determined daily for five days as the mean of two measurements at right angles. Ten replicates of each pathogen/antagonist combination and 10 replicates of each control which consisted of plates of either *C. parasitica* or *T. atroviride* inverted over plates of non-inoculated 2% MEA and PDA were made. This experiment was only run for a total of seven days, after *T. atroviride* was first placed onto the medium, because spore production by the *T. atroviride* isolate thereafter, would contaminate the plates of *C. parasitica*. Repeated measured analysis of the variance of the means of mycelial growth was calculated using the General Linear Method (GLM) and the separation of means was carried out by Scheffe's test with a level of significance (α) equal to 0.05 (SAS Institute, Inc., Cary, NC).

4.2.2 Preparation of Cell Free Culture Filtrates

Cell free culture filtrates were prepared using a modification of methods used by Bertagnolli et al. (1996). Two hundred millilitre Erlenmeyer flasks containing 50 ml of

one of the following growth media:

1) Potato dextrose broth (PDB, Difco) supplemented with 1 mM 3-[N-morpholino] propanesulfonic acid (MOPS, Sigma Chemical Company, St. Louis, MO) buffer, pH 5.0
2) PDB supplemented with 1 mM MOPS, pH 7.0
3) PDB broth, pH 5.0

were inoculated under sterile conditions with a 3 mm diameter PDA plug of mycelium taken from a four-day old plate of *T. atroviride* grown at 25 °C in the dark. Six flasks of each medium, not containing the antagonist, were prepared. Twelve flasks of each of the three different media containing the antagonist were prepared so that the filtrates from six of the flasks could be used as heat controls. Cultures were shaken in the dark at 110 rpm at room temperature (22 °C) and harvested after 7 days of incubation by vacuum filtering in 115 ml Nalgene Disposable Sterilization Filter Units (Nalge Company, Rochester, NY) (Fig. 4.1). Control cell free filtrates were made similarly, with the same growth media but contained no *T. atroviride*. From each flask, 10 ml of each cell free culture filtrate were transferred under sterile conditions to 50 ml glass tubes. Eighteen tubes of each filtrate type including the control filtrates were prepared. From the remaining filtrates, 20 ml of each filtrate were set aside for agar well-plate-assays.

4.2.3 Interaction of Culture Filtrates with Growth of *C. parasitica*

To each of the glass tubes containing filtrates, 10 ml of new media (PDB/MOPS, pH 5.0, PDB/MOPS, pH 7.0, or PDB, pH 5.0) were added. To all the tubes containing 20 ml of media, a 3 mm diameter PDA plug of *C. parasitica* was added. The plates of *C.*

parasitica were prepared with the same buffers (1, 2, and 3 as stated above) used in the preparation of the filtrates, the plates were poured to 0.5 cm thickness. The heated control filtrates were prepared by autoclaving (temperature of 120 °C and pressure of 1.0 kg/cm^2) the filtrates from six flasks of each of the different cultures containing the antagonist for 15 minutes. The test filtrates were as follows:

1) PDB/MOPS, pH 5.0- no antagonist

2) PDB/MOPS, pH 5.0- antagonist-autoclaved

3) PDB/MOPS, pH 5.0- antagonist

4) PDB, pH 5.0- no antagonist

5) PDB, pH 5.0- antagonist-autoclaved

6) PDB, pH 5.0- antagonist

7) PDB/MOPS, pH 7.0- no antagonist

8) PDB/MOPS, pH 7.0- antagonist-autoclaved

9) PDB/MOPS, pH 7.0- antagonist.

The tubes were placed into test tube racks and shaken at 120 rpm in the dark at room temperature (22 °C). After seven days of incubation each fungal mat of *C. parasitica* was poured out onto a sterile Whatman No. 1 filter paper (Fisher Scientific, Pittsburgh, PA) folded into a cone over a beaker. When most of the filtrate was removed the mat was washed twice with 10 ml sterile water. The mat was then placed onto preweighed Whatman No. 1 filter paper and weighed. The mat was left on the Whatman paper and dried to a constant weight for 18 hours at 80 °C and reweighed. The dry weight of mycelial plugs of *C. parasitica* grown on the three media, which had not been incubated in filtrate, were also recorded. The experiment contained 18 replicates of each filtrate

type, including the heated and the control plugs of *C. parasitica*. The analysis of variance of dry weights was calculated using the General Linear Method (GLM) and mean separation was done by Duncan's Multiple Range test with a level of significance (α) equal to 0.05 (SAS Institute, Inc., Cary, NC).

4.2.4 Agar Well-Plate Assays

One milliliter of each of the filtrates put aside for the agar well-plate assay was pipetted into corresponding 0.5 cm diameter wells in the center of PDA agar plates. The agar plates contained one of the following media

1) PDA with MOPS buffer, pH 7.0

2) PDA with MOPS buffer, pH 5.0

3) PDA, pH 5.0.

A heat treatment was used for this experiment as described in the above experiments. The plates were incubated in the dark for 24 hours at room temperature (22 °C) to allow for diffusion of the compounds. Three millimeter five-day old agar plugs of *C. parasitica* grown on the three PDA media types mentioned above, were placed 3 cm away from the well on either side. The plates were sealed with parafilm and incubated in the dark at 25 °C for a period of eight days. The diameter of mycelial growth was recorded daily as a mean of two measurements at right angles. Twenty plates of each treatment were prepared. The analysis of variance of colony diameter was calculated using the General Linear Method and mean separation was done by Duncan's Multiple Range test with a level of significance (α) equal to 0.05 (SAS Institute, Inc., Cary, NC).

4.2.5 Light and Scanning Electron Microscopy

In order to study the interaction of the two fungi, a 3 mm diameter five day-old plug of *C. parasitica* grown on 2% MEA and 2% PDA, was placed on one side of a 8 cm diameter disk of autoclaved cellophane (Kodak). The cellophane had previously been autoclaved and under sterile conditions placed into 10 petri dishes containing MEA and 10 containing PDA, 24 hours before the first inoculation. The petri dishes were sealed with parafilm and incubated in the dark at 25 °C for 2 days. After two days of incubation, the *T. atroviride* isolate was placed 5 cm away from the *C. parasitica* isolate on the cellophane. The petri dishes were resealed with parafilm and incubated in the dark at 25 °C. Controls were prepared by inoculating MEA and PDA with only *T. atroviride* or *C. parasitica*. After 3 to 5 days when the fungal colonies had grown together, pieces of cellophane (1 x 1 cm) with mycelium from the interaction zone, were cut and mounted on slides and stained with lactophenol blue or simple mounted with sterile water. The samples were observed using phase contrast microscopy at a magnification of 40 with Nikon opti-phot.

To study samples by scanning electron microscopy (SEM), cultures were prepared as above and 2-3 mm^2 pieces of cellophane containing mycelium from the interaction zone or advancing edges of cultures of *T. atroviride* and *C. parasitica* were cut and removed. The samples were vapour fixed with 2% (w/v) osmium tetroxide for 24 hours at room temperature (22 °C). The samples were stored under vacuum and sputter-coated with gold palladium when needed. Other samples were prepared for viewing by freeze etching at –90 °C for forty-five minutes using cryo SEM techniques. All samples were viewed with the JEOL 5400 JSM, located in the Central Microscopy facilities at the

University of Massachusetts-Amherst, with an accelerating voltage of 20 kV. Eight samples per sampling time were examined.

4.3 Results

4.3.1 Volatile Antibiosis Test

Mycelial growth of *C. parasitica* in the Gray dishes was not restricted. Analysis of variance of the mean distances of mycelial growth showed no difference among treatments on each day of measurement (Fig. 4.2). Significant difference was recorded among the experimental blocks on day 5 of measurement (data not shown). However, there was no significant difference recorded in the interaction of block and treatment (data not shown). Mean separation grouped the means of the growth of *C. parasitica* alone and *C. parasitica* with *T. atroviride*, together on each day of measurement (Fig. 4.2).

Mycelial growth of *C. parasitica* on the inverted plates was not restricted. Analysis of variance of the means showed no difference among treatments for each day of measurement (Fig. 4.3). Significant difference among the experimental blocks was recorded on day 2 of measurements. However, there was no significant difference recorded in the interaction of block and treatment. Mean separation grouped the growth of *C. parasitica* alone and with *T. atroviride* (inverted plates), together on each day of measurement (Fig. 4.3).

4.3.2 Interaction of Culture Filtrates with Growth of *C. parasitica*

Fungal mats grew in tubes containing heated filtrates and in media which had not contained the antagonist (Figs. 4.4 and 4.5). There was very little growth if any on plugs incubated in filtrates which had contained the antagonist (Figs. 4.4 and 4.5). Very sparse mycelia grew on 33% of the plugs placed in PDB/MOPS pH 5.0, in which the antagonist had grown. Those plugs in filtrates PDB pH 5.0 and PDB/MOPS pH 7.0 which had contained the antagonist, had no extra mycelial growth. *Cryphonectria parasitica* grew more in PDB/MOPS pH 5.0 than in the other two media, with the growth in PDB/MOPS pH 7.0, being the least (Fig. 4.4). Analysis of variance showed that there was a significant difference among treatments. Mean separation resulted in 5 groupings (Fig. 4.4). The weights of plugs which had been incubated in filtrates 3, 6, and 9 were significantly different the the control plugs which had not been incubated (10, 11, and 12). They were however significantly less than the weights of plugs which were incubated in filtrates which had no antagonist and those that had had the antagonist but had been heat treated. The weights of those plugs in heated filtrates, PDB/MOPS pH 5.0, and PDB ph 5.0 were significantly less than those which had been in filtrates had no antagonist. However, those plugs in PDB/MOPS pH 7.0 were not significantly different from its control PDB/MOPS pH 7.0 - no antagonist.

4.3.3 Agar Well-Plate Assays

There was no inhibition of growth of the pathogen. Analysis of variance showed no significant difference on the first 3 days of measurement but there was a slight

difference on the fourth day of measurement (Fig. 4.6). Mean separation resulted in only treatments 8 and 1 on the third and fourth days being grouped alone (Fig. 4.6).

4.3.4 Light and Scanning Electron Microscopy

Samples of the interaction zone viewed with the light microscope at a magnification of 40, showed the hyphae of *C. parasitica* were not easily distinguished from the hyphae of the antagonist by virtue of hyphal diameter, because they were similar (Fig. 4.7 and 4.8). Only at the time of phialid production by *T. atroviride* did it become easier to distinguish the two fungi (Fig. 4.8). The hyphae of *T. atroviride* grew over and entwined with hyphae of the pathogen (Fig. 4.9). Scanning electron micrographs showed hyphae of the antagonist tightly pressed to *C. parasitica* as it crossed over the pathogen (Fig.10). *T. atroviride* produced many chlamydospores among the hyphae of the pathogen, from which a number of hyphae emerged (Fig. 4.11a and b). The antagonist also produced a large number of spores which sporulated among the hyphae of *C. parasitica* (Fig. 4.12). There was no noticeable mycoparasitism or damage by antibiotics of the pathogen by *T. atroviride*.

4.4 References

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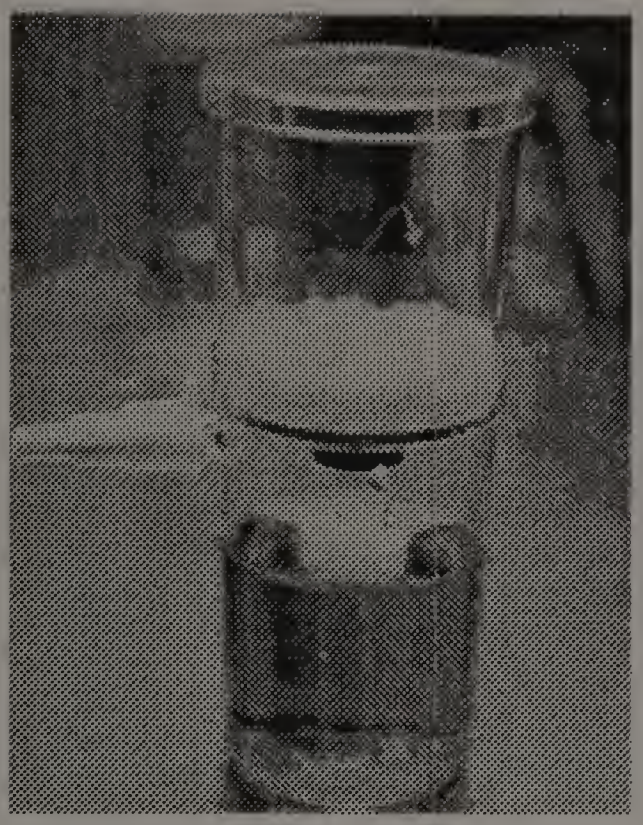


Figure 4.1. Preparation of *Trichoderma atroviride* cell free culture filtrate using a Nalgene disposable sterilization filter.

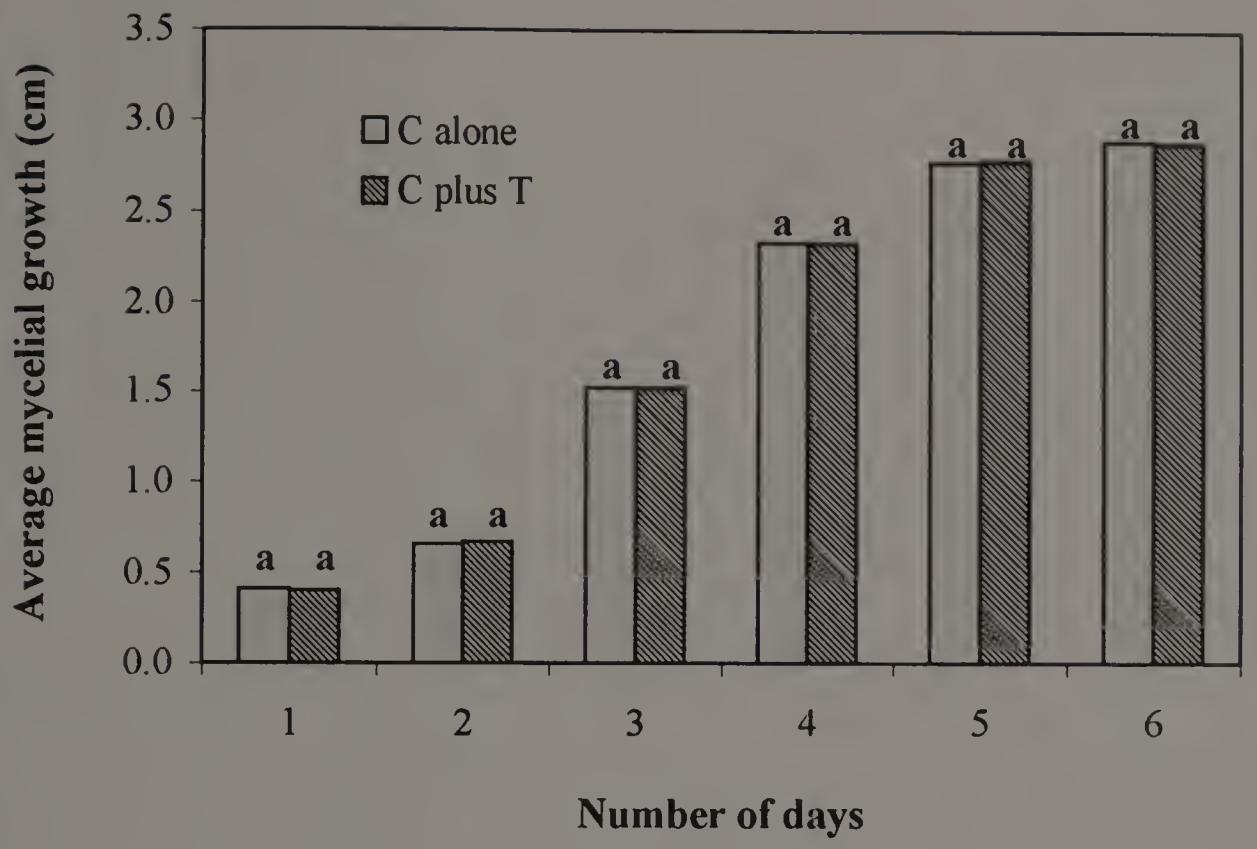


Figure 4.2. Observations following an eight day test of possible effect on mycelial growth of *Cryphonectria parasitica* by volatile metabolites produced by *Trichoderma atroviride* in Gray dishes. Mycelial growth of *C. parasitica* is recorded for six days beginning after the inoculation of the dishes with *T. atroviride*. Bars with the same letter on each day of measurement are not significantly different at $p = 0.05$.

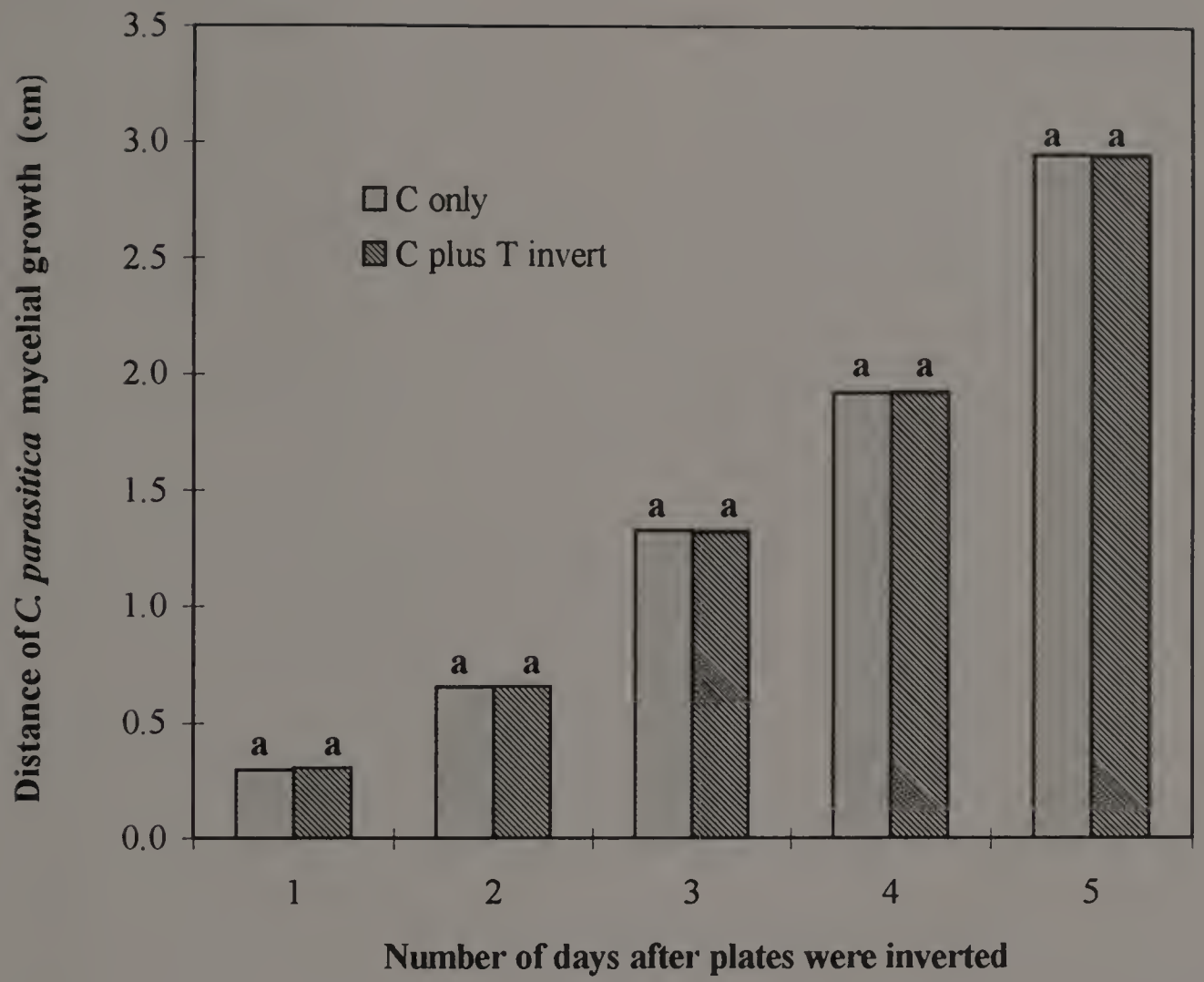


Figure 4.3. The effect on mycelial growth of *Cryphonectria parasitica* by volatile metabolites produced by the antagonist *Trichoderma atroviride*. Colony growth was recorded for five days following the inoculation of *C. parasitica* on potato dextrose agar and the subsequent inversion of the base of the plate onto the base of the dish containing the antagonist. Bars with the same letter on each day of measuement are not significantly different at $p = 0.05$.

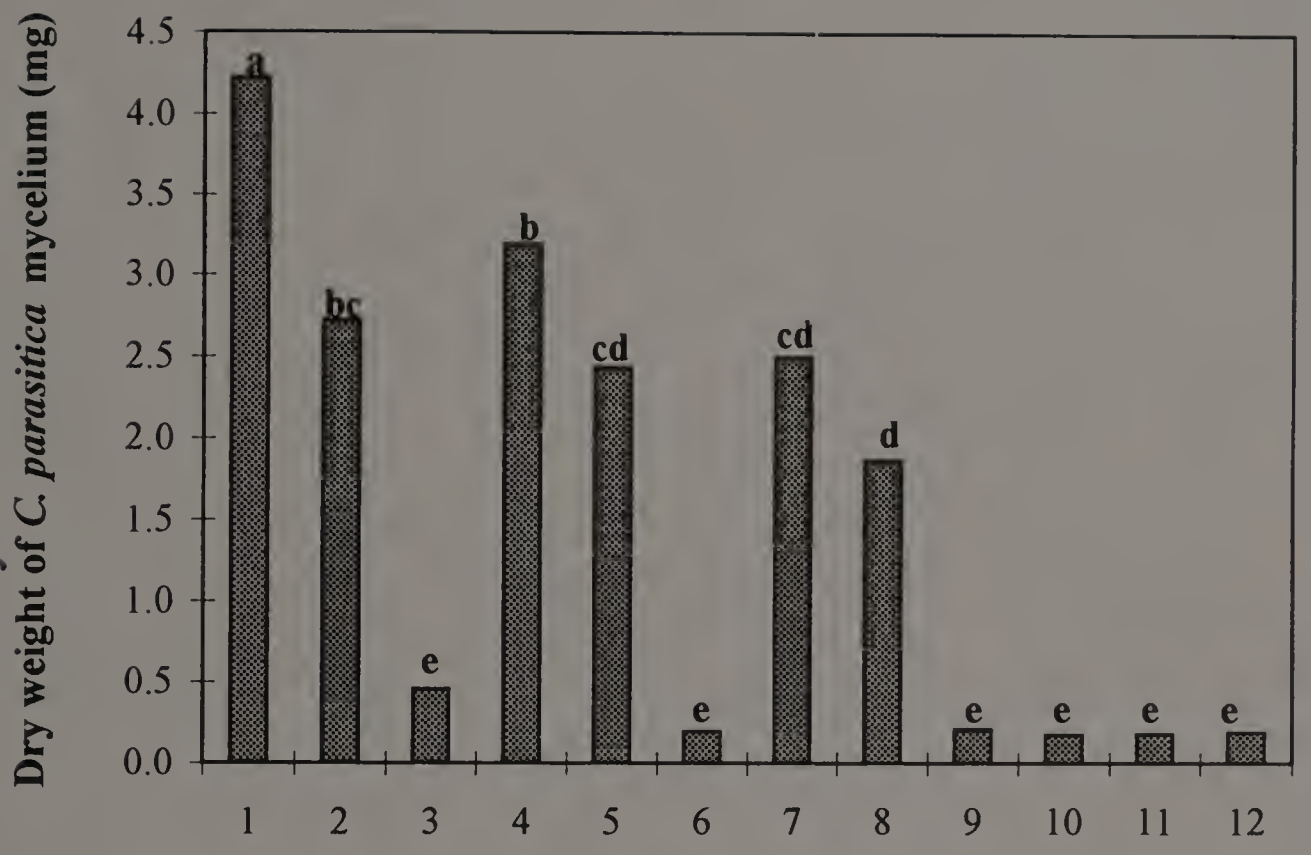


Figure 4.4. The effect of *Trichoderma atroviride* cell free culture filtrate on the mycelial growth of *Cryphonectria parasitica*. Weights were recorded after seven days of incubation in the filtrates. 1) PDB/MOPS, pH 5.0- no antagonist; (2) PDB/MOPS, pH 5.0- antagonist-autoclaved; (3) PDB/MOPS, pH 5.0- antagonist; (4) PDB, pH 5.0- no antagonist; (5) PDB, pH 5.0- antagonist-autoclaved; (6) PDB, pH 5.0- antagonist; (7) PDB/MOPS, pH 7.0- no antagonist; (8) PDB/MOPS, pH 7.0- antagonist-autoclaved; (9) PDB/MOPS, pH 7.0- antagonist; (10) Plug of *C. parasitica* on PDA/MOPS pH 7.0; (11) Plug of *C. parasitica* on PDA pH 5.0; (12) Plug of *C. parasitica* on PDA/MOPS pH 5.0. Bars with the same letter are not significantly different at p = 0.05. PDB = potato dextrose broth. PDA = potato dextrose agar. MOPS = 1 mM 3-[N-morpholino] propanesulfonic acid.

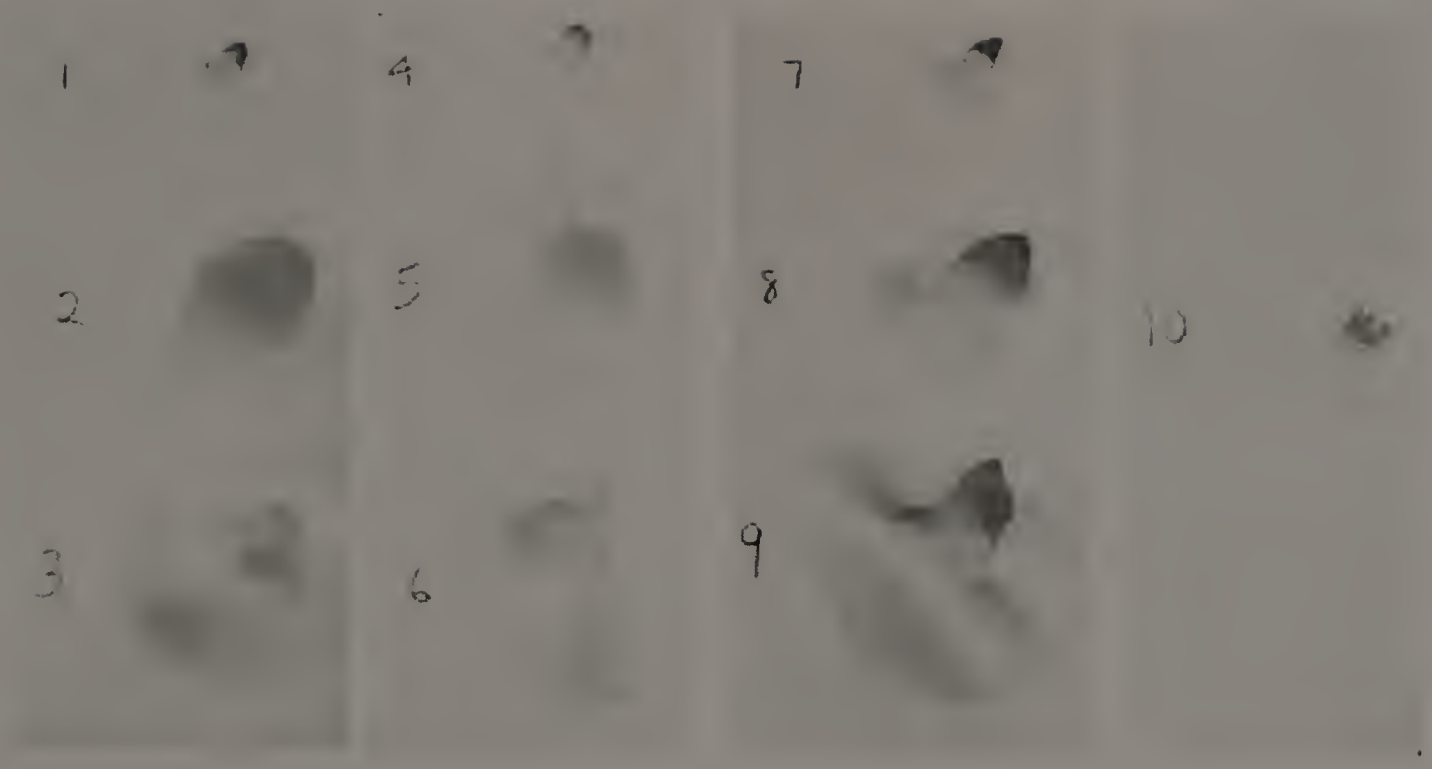
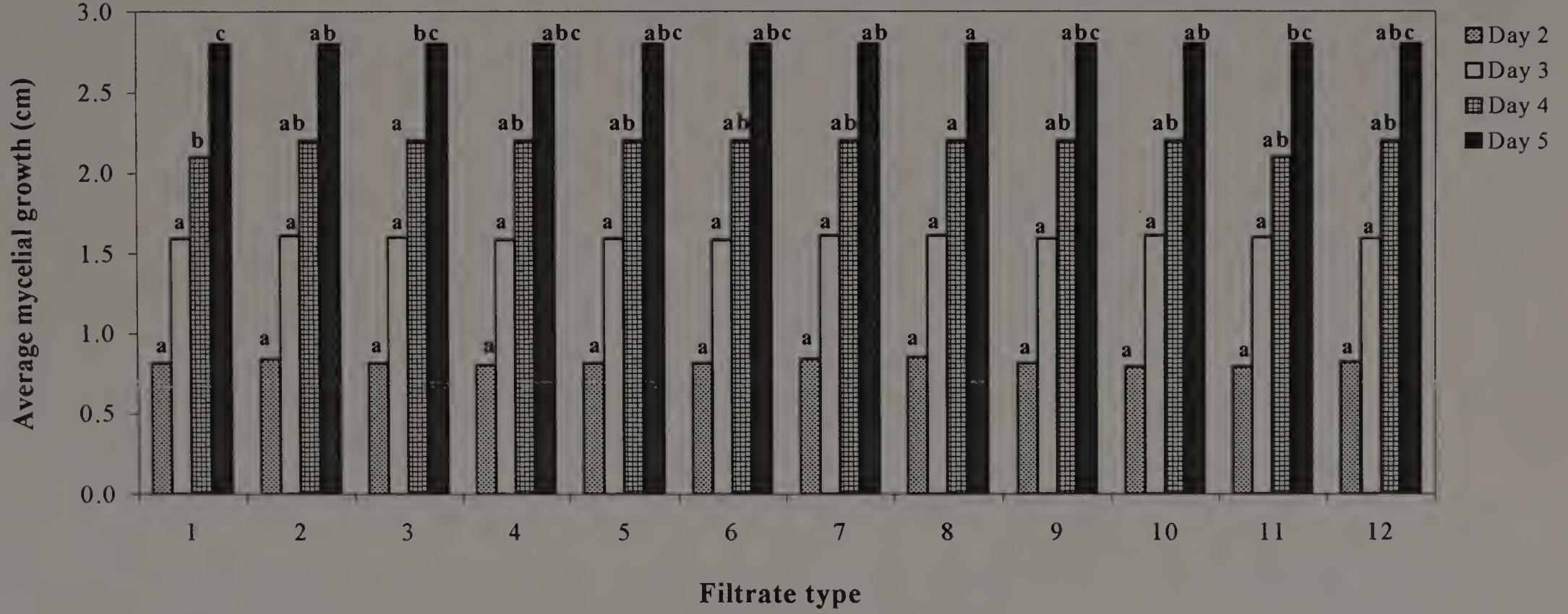


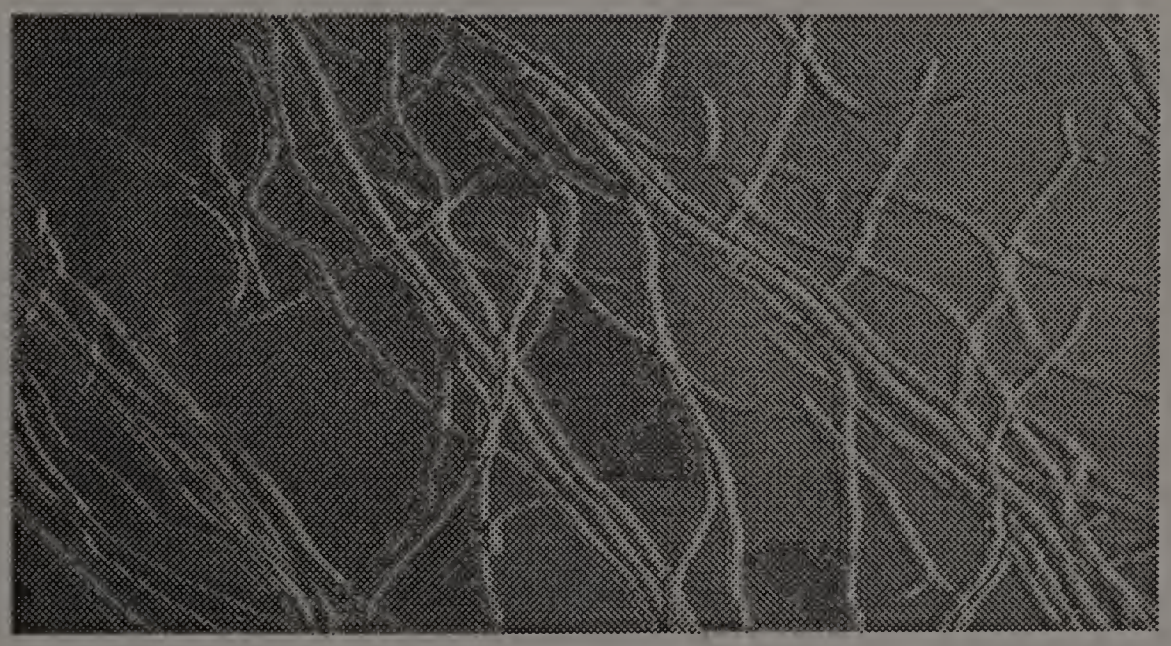
Figure 4.5. The effect of *Trichoderma atroviride* cell free culture filtrate on the mycelial growth of *Cryphonectria parasitica*. Mycelia of *C. parasitica* were removed after seven days from filtrate containing tubes, which were shaken at 120 rpm. The mycelia was dried to constant weight and weighed. 1) PDB/MOPS, pH 5.0- no antagonist; (2) PDB/MOPS, pH 5.0- antagonist-autoclaved; (3) PDB/MOPS, pH 5.0- antagonist; (4) PDB, pH 5.0- no antagonist; (5) PDB, pH 5.0- antagonist-autoclaved; (6) PDB, pH 5.0- antagonist; (7) PDB/MOPS, pH 7.0- no antagonist; (8) PDB/MOPS, pH 7.0- antagonist-autoclaved; (9) PDB/MOPS, pH 7.0- antagonist; (10) Plug of *C. parasitica* on PDA/MOPS pH 7.0. PDB = potato dextrose broth. PDA = potato dextrose agar. MOPS = 1 mM 3-[N-morpholino] propanesulfonic acid.

Figure 4.6. The effect of *Trichoderma atroviride* cell free culture filtrates in well plate agar tests, on radial growth of *Cryphonectria parasitica*. Mycelial growth was recorded from the second day to the fifth day after plugs of *C. parasitica* were placed in the plates. (1) PDB/MOPS, pH 5.0- no antagonist; (2) PDB/MOPS, pH 5.0- antagonist-autoclaved; (3) PDB/MOPS, pH 5.0- antagonist; (4) PDB, pH 5.0- no antagonist; (5) PDB, pH 5.0- antagonist-autoclaved; (6) PDB, pH 5.0- antagonist; (7) PDB/MOPS, pH 7.0- no antagonist; (8) PDB/MOPS, pH 7.0- antagonist-autoclaved; (9) PDB/MOPS, pH 7.0- antagonist; (10) PDB/MOPS pH 7.0; (11) PDB pH 5.0; (12) PDB/MOPS pH 5.0. Bars with the same letter for each day of measurement are not significantly different at $p = 0.05$. PDB = potato dextrose broth. MOPS = 1 mM 3-[N-morpholino] propanesulfonic acid.


Average mycelial growth (cm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Day 2
Day 3
Day 4
Day 5
1 2 3 4 5 6 7 8 9 10 11 12
Filtrate type

A

B



C

Figure 4.7. (A) Hyaline hyphae of *Cryphonectria parasitica* as seen under the light microscope at a magnification x40. (B) Hyaline hyphae and conidiophores of *Trichoderma atroviride* as seen under the light microscope at a magnification x40. (C) The two fungi in an interacting zone, magnification x40. The fungi were grown in dual culture or alone on cellophane on potato dextrose agar. (T) = *T. atroviride*. (C) = *C. parasitica*.

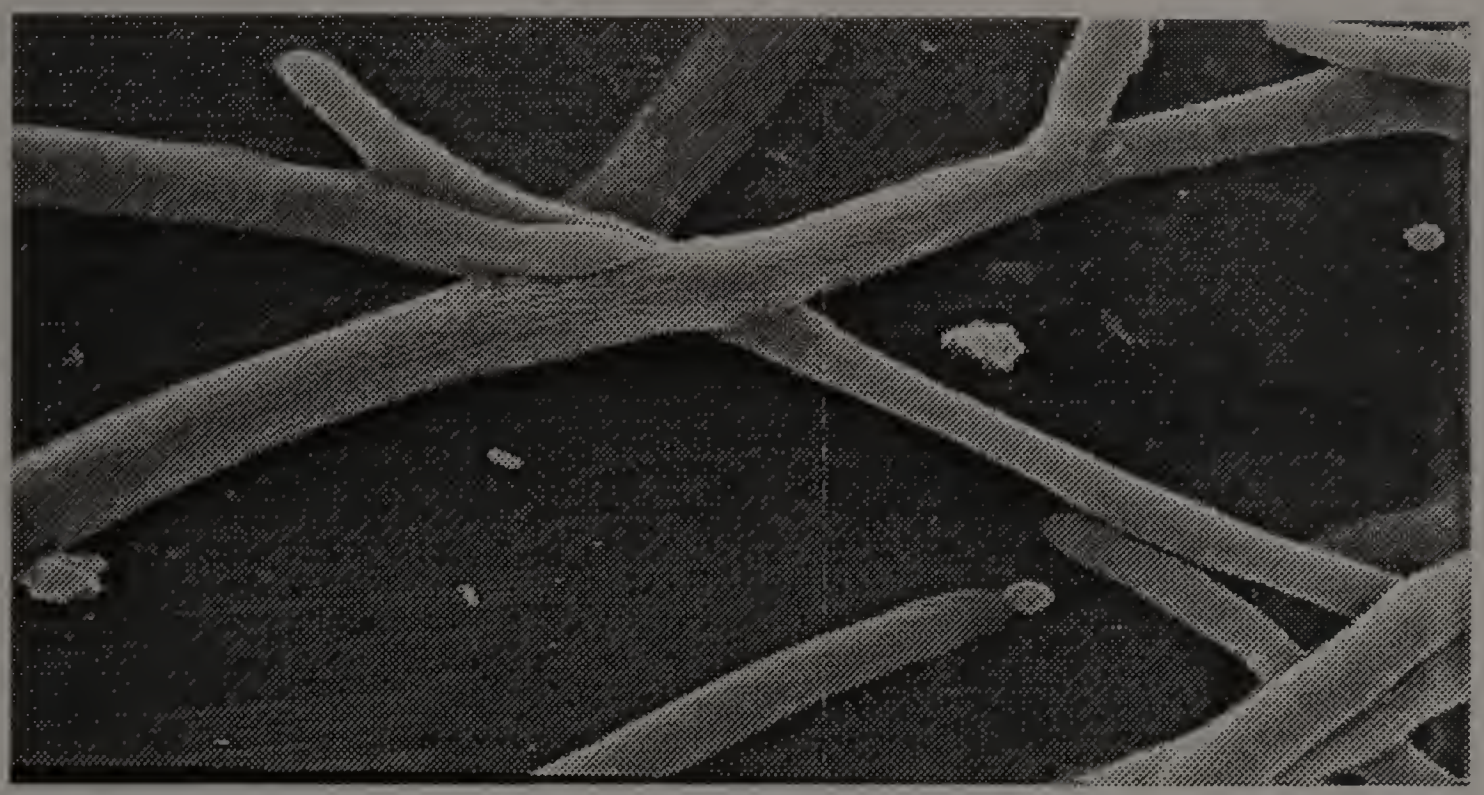


Figure 4.8. Interacting hyphae of *Cryphonectria parasitica* (C) and *Trichoderma atroviride* (T). Fungi were grown on cellophane on potato dextrose agar. A 3 mm^2 piece of cellophane from the interacting zone was vapour fixed with 2% (w/v) osmium teroxide for 24 hr at room temperature (22 °C). The sample was sputter coated with gold palladium before viewing. The sample was viewed with the JEOL 5400 JSM at an accelerating voltage of 20 kV. Magnification x2000. ——— 5 µm.

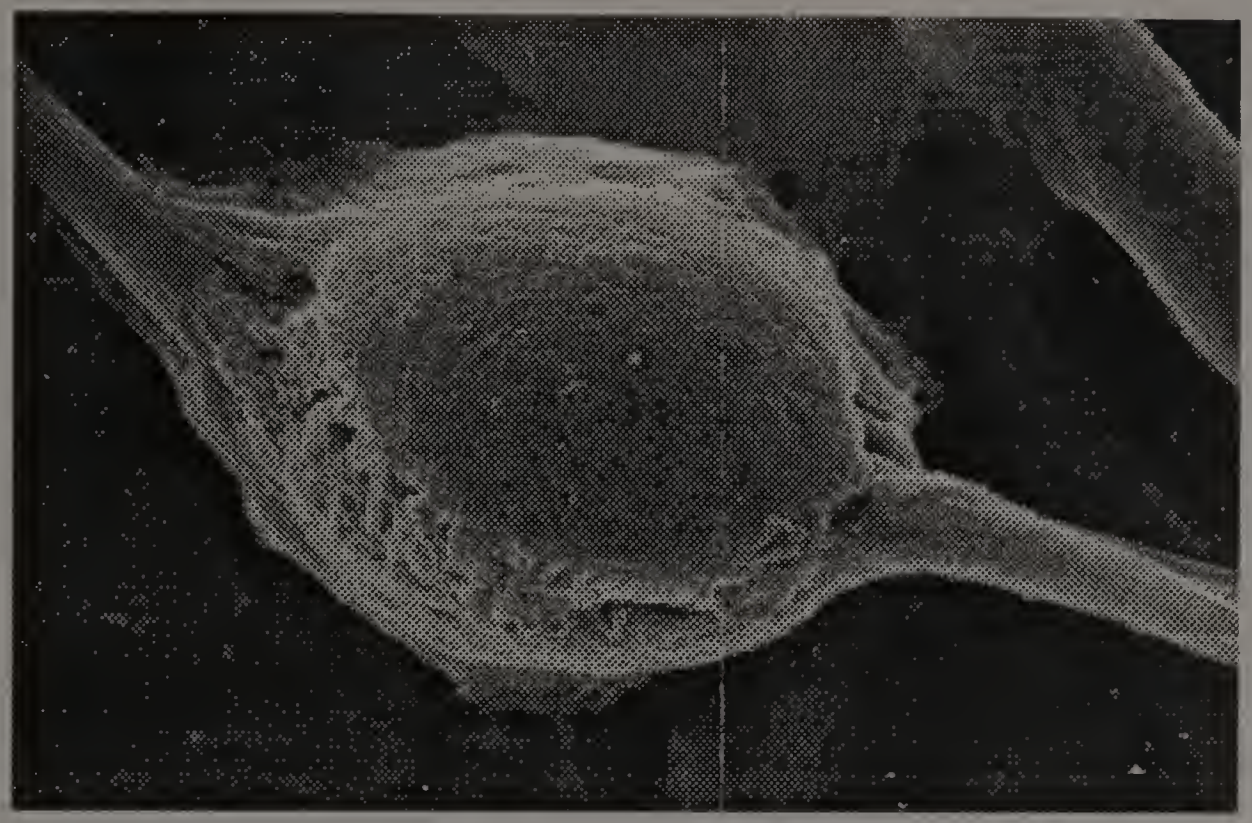


Figure 4.9. Scanning electron micrograph of a possible chlamydospore within a hypha of *Trichoderma atroviride*. The fungus was grown on cellophane on potato dextrose agar. A 3 mm^2 piece of cellophane from the growing edge of *T. atroviride* was prepared by vapour fixing with 2% (w/v) osmium teroxide for 24 hrs at room temperature (22 °C). The sample was sputter coated with gold palladium before viewing. It was viewed with the JEOL 5400 JSM at an accelerating voltage of 20 kV. Magnification x2000. ——— 5 µm.

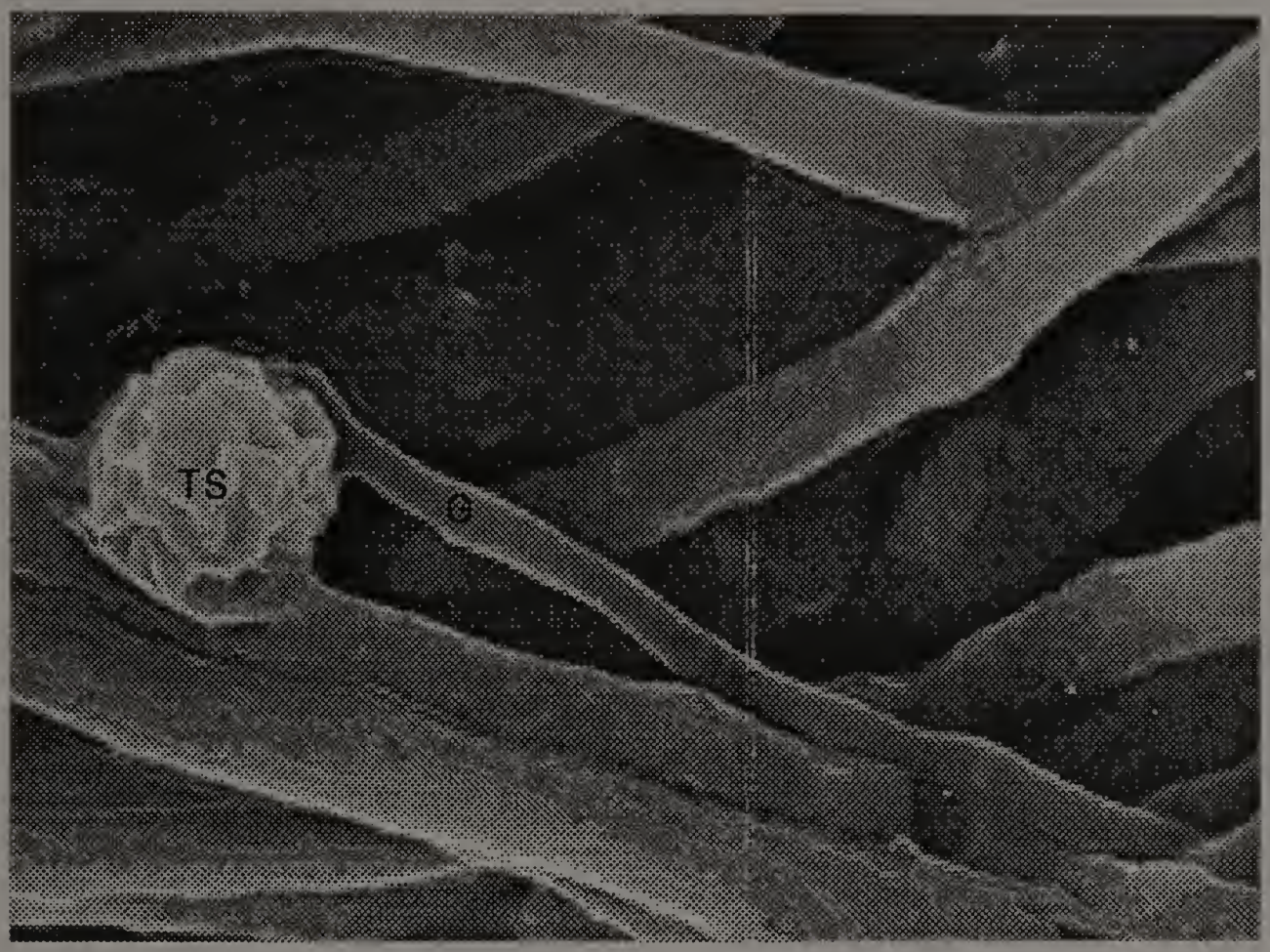


Figure 4.10. Scanning electron micrograph of a germinating *Trichoderma atroviride* spore on a hypha of *Cryphonectria parasitica*. The fungi were grown on cellophane on potato dextrose agar. A 3 mm^2 piece of cellophane from the interacting zone, was prepared by vapour fixing with 2% (w/v) osmium teroxide for 24 hrs at room temperature (22 °C). The sample was sputter coated with gold palladium before viewing. It was viewed with the JEOL 5400 JSM at an accelerating voltage of 20 kV. Magnification x5000. ——— 2 µm. (TS) = *T. atroviride* spore. (G) = Germination tube.

CHAPTER 5

DISCUSSION AND CONCLUSION

Trichoderma spp. can detect a host from a distance and will branch and grow towards it (Goldman et al., 1994). This behaviour is believed to occur because of trophic responses induced by nutrient gradients from the host (Chet et al., 1981). It was not observed that the antagonist on either potato dextrose agar (PDA) or malt extract agar (MEA) produced any atypical branching in interaction plates as opposed to growth alone on the same media. Although *Trichoderma atroviride* was able to overgrow *Cryphonectria parasitica* on MEA interaction plates, both *T. atroviride* and *C. parasitica* grew slower than on PDA. The slowed growth on MEA when compared to its growth on PDA, may be explained by the difference of carbon source and its amount. It has been shown that some pathogenic fungi have varying ability to repel the advancement of an antagonist when cultured on different glucose sources (Tokimoto and Komatsu, 1979; Tokimoto and Ushiyama, 1980; Tokimoto, 1982). Although there was no distinct zone of inhibition, the slowed growth of *C. parasitica* before being overgrown by *T. atroviride* could have been associated to inhibition by small amounts of fungistatic/fungicidal metabolites produced by the antagonist. Nutrient impoverishment of the media may also have slowed its growth .

Dual culture experiments involving *Trichoderma* spp. showed various species capable of completely overgrowing *Phytophthora parasitica* f. sp. *nicotiane* (Bell et al., 1982), *P. cinnamoni* and *P. citricola* (Chambers and Scott, 1995), *Rhizoctonia solani* and

Pythium spp. (Chet et al., 1981), and *C. parasitica* (Turchetti and Gemignani, 1981; Arisan-Atac et al., 1995). Mycoparasitism was usually shown to occur after contact the the *Trichoderma* antagonist. Mycoparasitism was however not seen to occur in the interaction of *T. atroviride* and *C. parasitica*. Light and scanning electron microscopy showed no coiling and penetration of hyphae of the pathogen. There was also no apparent degradation of the pathogen fungus. It appeared therefore that the main mode of antagonistic action was competition. The antagonist grew very fast, produced many conidia which germinated on *C. parasitica* hyphae, thus very quickly outcompeting the pathogen. Although the cell free filtrate tests later showing possible enzyme and antibiotic activity may have been the primary source of inhibition, the microscopy work showed no evidence of direct action of metabolites on the hyphae of *C. parasitica*. Elad et al., (1983) applied fluorescein isothiocyanate conjugated lectins to determine possible locales of *Trichoderma* spp. that produced lytic activity. A similar experiment could also be used to better determine if there is any enzymatic degradation of *C. parasitica* hyphae.

The rapid screening technique using inoculated bark and wood pieces showed that the antagonist was able to significantly reduce canker development by *C. parasitica* in comparison with controls. The results of the bark and wood experiment led to the experiments with the seedlings. Results from these experiments were similar to the rapid screening experiment, showing that prophylactic treatment with the antagonist produced significantly reduced cankers. Application of *T. atroviride* spores after inoculation with the pathogen resulted in reduced canker growth on the bark and wood pieces as well as on seedlings treated outside the greenhouse. However, post treatment of the antagonist did not work in the greenhouse, perhaps for a number of reasons corresponding to high

temperatures existing in the greenhouse throughout the summer of 1995. Widden (1984) showed that temperatures above 30 °C resulted in a significant reduction in the growth rates of various *Trichoderma* spp.. Petri plate tests at 35 °C, (data not presented) showed that *T. atroviride* grew an average of 3.1 cm in 5 days whereas at 25 °C it grew to 8 cm in the same time period. Several authors (Schoeneweiss, 1975; Appel and Stipes, 1984; Madar et al., 1989; Gao and Shain, 1995) have shown that canker expansion on woody plants is greatest when host plants are under water stress. Therefore, water stress may have been a factor in the production of cankers on all the trees treated with *C. parasitica*. The cankers on the trees in the greenhouse were noticeably smaller than those on the seedlings of the previous summer. A possible explanation for this phenomenon is one given by Russin and Shain (1984) and Gao and Shain (1995). They both reported that increased bark moisture hindered the enlargement of chestnut blight cankers. They noted that if the stems were allowed to gain water directly after inoculation, canker expansion would almost cease. Rapid drying of the medium in which the trees were grown, necessitated watering the trees at least twice a day and this may have contributed to the overall decrease in canker sizes as compared to those of the previous summer. The seedlings used the previous summer were neither subjected to very high ambiental temperatures or to very frequent watering.

Experiments using the seedlings showed that after 21 days (outside greenhouse) and 14 days (in greenhouse) cankers began to develop on many of the trees. The marked difference in canker sizes between trees with prophylactic treatment and their controls, indicate that the antagonist was able to provide limited protection from the pathogen. The eventual increase in cankers after the afore mentioned days, suggest that there was a

breakdown in the antagonist's ability to provide this protection. It is possible that there was no longer a nutrient supply for the antagonist on the trees. It is equally possible, that environmental conditions such as heat stress, water stress, and wind may have played a role in reducing its population on the bark. The establishment of antagonists is therefore the main difficulty to overcome in the application of antagonists to soil or plants. The environment to which antagonists are introduced is usually not conducive to their establishment and eventually their population decreases (Cook and Baker, 1983). Several forms of carriers have been used, Papavizas and Lewis (1989) reported using with success clay powder mixed with fermented biomass, Fravel et al. (1985) used alginate pellets, and Lewis et al., (1991) used vermiculite -bran mixture. The carriers allowed the antagonists to establish and multiply in soil environments. Ricard (1983) reported the successful use of the formulated BINAB T® to control Dutch elm disease for 3 years.

The experiments in the Cadwell forest failed to provide evidence as to the enhancement of effectivity of the antagonist with the use of the two carriers. Cankers on trees treated with the antagonist were similar in size to cankers left untreated. The results indicated that the antagonist was not able to control the advancement of *C. parasitica*. The Cadwell experiments were begun at the end of the summer and the temperature in Pelham and the surrounding area dropped early in fall. Widden (1984) reported that at 5 °C the growth rate of several *Trichoderma* spp. was significantly lowered. We can therefore postulate that the low temperatures existing in the Cadwell forest at the time of the experiment hindered the antagonistic activities of *T. atroviride*. Perhaps if the data were collected the following spring we would have been able to make specific conclusions as to the efficacy of the treatments.

The experiment on the stem sections to test possible carriers showed that all the prophylactic treatments containing *T. atroviride* spores significantly hindered the development of cankers. The Wilt Pruf® and the Phyton 50® did not appear to enhance the effectivity of the antagonist compared to the effectivity of the water spore suspension. Spore viability was very similar on stems for each of the three treatments (**TMO**, **TWP**, and **TH_2O**) we can therefore conclude that the three carriers worked equally well with the spores. It must be noted, however, that the physiology of cut stems is very different from the physiology of live seedling stems. It was observed in the 40 day experiment that the ends of some stems had wood decay and in those areas there was a proliferation of *Trichoderma* spores hence perhaps the difference in the number of viable spores between blocks 1 and 2, and block 3. It would have been best to have had environmental conditions similar to those in the previous seedling experiments.

Several species of *Trichoderma* are known to produce volatile antibiotics *in vitro* (Dennis and Webster, 1971b; Hutchinson and Cowan, 1972; Bruce et al., 1984). Results presented by the authors mentioned above, suggested that volatile antifungal metabolites played a role in the antagonism. This was not the case in the antagonism of *C. parasitica*. Tests showed that the organisms, despite any gases that *T. atroviride* may have been producing, grew as well as the controls.

Many more studies have been done on the production of non-volatile antibiotics by *Trichoderma* species (Dennis and Webster, 1971a; Elad et al., 1982; Tokimoto, 1982; Bruce and Highley, 1991; Lorito et al., 1993; Chambers and Scott, 1995). Several different hydrolytic enzymes such as cellulases (Nakari-Setälä and Penttilä, 1995), chitinases, glucanases proteases, and xylanases (Elad et al., 1982), from various

Trichoderma species have been purified and characterised. The well plate experiments produced no significant differences among the treatments but growing the pathogen directly in the cell free culture filtrates produced very different results. The *T. atroviride* filtrates significantly inhibited hyphal extension of *C. parasitica*. Autoclaving significantly reduced the inhibitory effect of the filtrate. The antagonist may have produced a small quantity of degradative enzymes and denaturation of those enzymes may account for the reduced levels of inhibition. Any remaining inhibitory effect may have been a result of toxicity of other more stable metabolites.

Results of dual culture, biocontrol bark/wood and seedling bioassays, and antibiosis experiments, indicated that *T. atroviride* has potential as a biological control agent of chestnut blight. The *in vitro* antibiosis experiments reported here also indicate that the possible mode of action of the antagonist. Characterization of the heat stable and heat sensitive metabolites of the *T. atroviride* filtrate should be conducted. As described by Hankin and Anagnostakis (1975), enzyme production can be detected by various types of solid media. A series of chromatographic analyses as well as mass spectrometry could be used to perhaps determine what other antibiotic compounds may be present in the filtrate.

Further experimentation in the evaluation of *T. atroviride* should be based on a comprehensive study of the various treatment methods on chestnut seedlings planted as in the Quabbin. The experiments should be conducted for an extended period of two to three years.

Such experiments should include extensive data collection pertaining to ambient temperature as temperature effects appeared to play a significant role on the activity of the fungi. Spore population and viability are also important parameters to consider.

Being able to correctly identify the isolate placed on the plants would produce valuable data pertaining to the isolate's longevity and effectivity in protecting the plant from the blight. A method such as DNA-fingerprint analysis could be used to better determine that the test fungus, *T. atroviride*, is indeed the fungus reisolated from the test plants. Gilly et al. (1994) and Arisan-Atac et al. (1995) using the restriction amplified polymorphic DNA (RAPD) technique, and Bowen et al. (1996) and Meyer et al. (1992), using the restriction fragment length polymorphism (RFLP) technique, were able to differentiate specific biological control agents from closely related *Trichoderma* species.

Experiments pertaining to the the testing of carriers could also be done with grafted trees. Application of the isolate with a carrier such as mineral oil, lanolin, or wax/lanolin, may better protect graft areas from infection.

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