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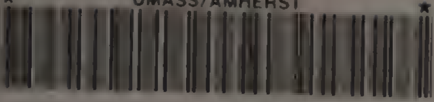
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AN INVESTIGATION OF THE MICROORGANISMS NATURALLY OCCURRING ON THE BARK OF AMERICAN CHESTNUT, *CASTANEA DENTATA*, AND THEIR *IN VITRO* ANTAGONISM TO *CRYPHONECTRIA PARASITICA*

A Thesis Presented

By

PATRICIA C. GROOME

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2000

Department of Plant Pathology

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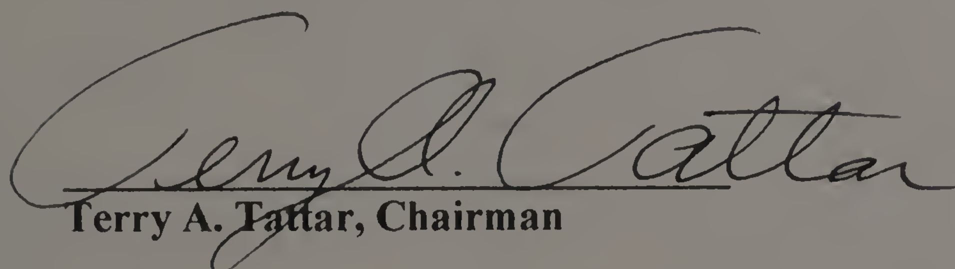

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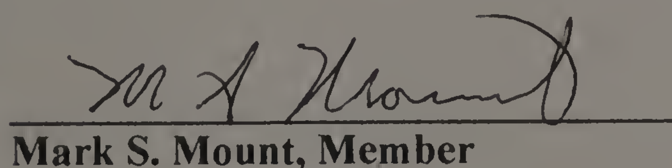

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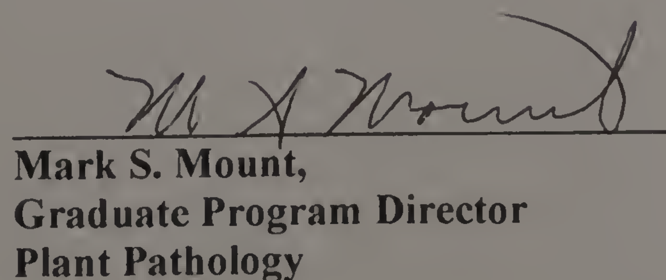

Mark S. Mount,
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Dedicated to my mother, without whose love, friendship and support none of this would have been possible.

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THANK YOU ALL!

ABSTRACT

INVESTIGATIONS OF THE MICROORGANISMS NATURALLY OCCURRING ON THE BARK OF AMERICAN CHESTNUT, *CASTANEA DENTATA*, AND THEIR *IN VITRO* ANTAGONISM TO *CRYPHONECTRIA PARASITICA*

May 2000

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In hopes of finding potential biocontrol organisms against *Cryphonectria parasitica*, the causal agent of chestnut blight, bark samples were taken from large (>20cm dbh) American chestnut (*Castanea dentata*) trees. The purpose of this study was to (1) isolate fungi and bacteria associated with American chestnut bark; (2) determine the biocontrol potential of the collected microorganisms in laboratory studies; (3) identify those organisms showing the highest degrees of *in vitro* antagonism to *C. parasitica* and determine their survivability in the field.

Trichoderma spp. were frequently isolated from American chestnut bark samples, and all showed some degree of *in vitro* antagonism to *C. parasitica*. Three of those showing high degrees of antagonism to *C. parasitica* were identified by the USDA-ARS Systematic Botany and Mycology Laboratory as *Trichoderma harzianum*. Attempts were made to select for and produce auxotrophs resistant to the fungicides propaconizole and

benzimidazole carbamate (DEBC) by culturing onto plates containing increasing concentrations of each of the fungicides. While the isolates were able to build up tolerances to given concentrations of both DEBC and propaconazole, this tolerance was lost upon sub-culturing back to media without fungicide amendments.

Though bacteria were rarely islolated from the bark of American chestnut trees in the forest, bacteria were cultured frequently from three American chestnut trees on a farm in New Hampshire. These bacterial isolates showed high degrees of *in vitro* antagonism to *C. parasitica* and were identified as *Bacillus megaterium*. A clear zone of inhibition occurred between the cultures of *C. parasitica* and *B. megaterium*. Agar removed from this zone of inhibition and placed before advancing hyphae of *C. parasitica* repelled oncoming mycelial growth. These results indicate the production by *B. megaterium* of an extracellular compound(s) inhibitory to *C. parasitica*. These compounds do not, however, appear to inhibit the *Trichoderma harzianum* isolates. In dual cultures, the *T. harzianum* isolates were able to overgrow the *B. megaterium* colonies, and the bacteria could be reisolated from beneath the mycelium.

Field survival studies of the bacteria were conducted. The isolates were screened for naturally occurring antibiotic-resistance. Mutants resistant to both streptomycin and rifampicin were applied to American chestnut trees in three settings: an orchard, forest trees stump-sprouted from previously killed trees, and potted seedlings. At the end of three months, there was 100% recovery of all strains from all sites. After one year, the three *B. megaterium* strains, 2A, 2C, and 3A, were isolated from 24%, 26% and 44% of the trees, respectively, and recovery remained 0% for the control at all sites.

Results of the previous experiments indicate that the *Trichoderma* and *Bacillus megaterium* strains collected from the bark of healthy and surviving American chestnut trees show genuine potential as biocontrol agents against *Cryphonectria parasitica in vitro*. Future experiments are needed to begin studies of *in vivo* antagonism of *C. parasitica* by *Trichoderma* and the *B. megaterium* isolates, both in combination and alone.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The Rise and Fall of the American chestnut

The American chestnut (*Castanea dentata* [Marsh.] Borkh.) was once one of the most significant tree species in the forests of the Northeastern United States. In terms of population, chestnut trees commonly comprised up to 25% of some forests (Holt, 1970). Its natural range stretched from southern Maine down into Mississippi and west into Indiana (Fig. 1.1). In the Appalachian Mountains of North Carolina and Tennessee they could grow up to 100 feet in height and six feet in diameter (Collingwood, 1947). The nuts produced by the tree were an important food source for the indigenous wildlife of the forests, and came to be equally valuable to the people later settling these areas, who used the nut to feed both themselves and their domesticated animals (Kuhlman, 1978). Its fruit aside, the tree itself came to be tremendously useful. The wood proved to be light, durable, and decay resistant, making it an ideal material for the construction of cabins, ships, fencing, and railroad ties. Its easy workability and attractive grain made it suitable for furniture and interior trim. Tannin extracts from the bark were used extensively by the leather industry for tanning (Hepting, 1974). In the 1870s, thirty years before disaster actually struck, the American chestnut was doomed by the inadvertant importation of a fungus on chestnut seedlings from Asia, to which the American chestnut had no resistance (Tattar *et al.*, 1996).

1.2 The Beginning of the End: The Chestnut Blight

In 1904, H. W. Merkel, the chief forester of the New York Zoological Park, discovered that the chestnut trees in the gardens were dying. There were cankers on the bark of the trees, areas where the tissue had died, and when these ringed the tree, in a process called girdling, translocation would be cut off, resulting in the trees death. He urgently reported his findings and within two years, the botanist W. A. Murrill had discovered that the disease was caused by a fungus which he named *Diaporthe parasitica* (Murrill, 1906). Immediate attempts were made to eradicate or control the ravages of the fungus. Merkel endeavored treating the disease by surgically removing the infected tissue and following with a spray of Bordeaux mixture (copper sulfate and lime), the first successful fungicide ever developed, but his efforts proved futile. By 1911 the potential for disaster had been recognized and a Chestnut Blight Commission was formed. Some members believed the situation was already hopeless while others proposed measures they hoped would halt the spread of the disease if not eradicate it. These included the felling and burning of all infected trees as well as clear-cutting chestnut trees in advance of the disease, but none was effective (Metcalf and Collins, 1911). By the 1940s the American chestnut in its natural range was all but gone. Forty years after its introduction, the chestnut blight had reduced an important and magnificent tree species to an insignificant understory shrub. The environmental impact and economic losses are incalculable.

The fungus had also made its way to Europe by 1938 and was expected to produce the same devastation (Gravatt, 1952). This, however, did not happen. The European chestnut (*Castanea sativa*, Mill.), is an important crop plant, and suffers from

infection by *Cryphonectria parasitica*, but is more resistant to the fungus than the American chestnut. Chestnut blight in Europe was still carefully monitored, however, and in the 1950s, an Italian plant pathologist (Biraghi, 1951) reported finding spontaneously healing cankers on some European chestnuts. This discovery afforded some of the first real hope for control of the chestnut blight.

1.3 *Cryphonectria parasitica*, the causative agent of chestnut blight

The fungus that causes chestnut blight, initially termed *Diaporthe parasitica* by its discoverer, has undergone several taxonomic refinements in the ensuing years. In 1913 the fungus was renamed *Endothia parasitica* (Anderson and Babcock, 1913), and in 1978 the genus *Endothia* was further divided into two additional categories based on differences in stromatic tissue, and the causative agent of chestnut blight came to be known as *Cryphonectria parasitica* (Murr. [Barr]), its current taxonomic designation (Roane, 1986).

Cryphonectria parasitica is an Ascomycete of the order Diaporthales (Kendrick, 1992). Its conidia and ascospores, disseminated by rain splash, wind, insects and animals, germinate in bark wounds. The occurrence of a wound is considered to be necessary for the initiation of infection. While a germinating spore can grow saprophytically on the detritous of the wound for a short time, hyphae will soon invade healthy tissue (Griffin and Elkins, 1986). The mycelium penetrates into both the outer and inner bark, using such degradating enzymes as polygalacturonase to macerate the host tissue, and forms mycelial fans beneath the bark. The xylem under the bark is also killed. It has also been suggested that the production by *C. parasitica* of oxalic acid,

which is toxic to chestnut protoplasts, may be an important factor in pathogenesis (McCarroll and Thor, 1978). A few weeks after lesion initiation, stromata begin to break through the bark. The stromata can be up to 3mm long and 0.5mm deep and contain myriad pycnidia which mature throughout the season and extrude sticky conidia in tendrils of orange cirrhi. In the same stromatal layer there are also usually perithecia, which eject the wind disseminated ascospores from early spring until late autumn (Sinclair, Lyon, and Johnson, 1987). With this abundance of inoculum, the disease is spread rapidly, both locally and over long distances.

1.4 Methods of controlling chestnut blight

While *Cryphonectria parasitica* has destroyed virtually all the mature American chestnuts in their natural range, the trees fortunate propensity to stump sprout has preserved the genotype. The species has not been completely lost but currently exists as a short-lived, shrub-like tree. American chestnuts are monoecious, but cannot self-pollinate and so must have another sexually mature tree in close enough proximity for cross pollination to occur (Fulbright et al 1988). Due to the blight, this is an extremely unlikely circumstance. The chances for the chestnut to develop genetic resistance through sexual reproduction in the forest are essentially non-existant.

Since the first report of the chestnut blight, valiant and varied attempts at control have been made, all with little or no success. The wealth of inoculum and the extreme virulence of the fungus outstripped the initial efforts to control the spread of the disease. Even the development of fungicides did not add greatly to the trees chances. Early formulations had little effect and the more recently developed products currently

available are not effective. For example, the systemic fungicide methyl-2-benzimidazolecarbamate (MBC) was found to have some limited use as a sprayed protectant, but is still a far from practical method for control. The large number of spray applications necessary would make the method cost-prohibitive even in an orchard setting, and virtually impossible in the forest (Elkins et al, 1978; Griffin, 1986; Jaynes *et al.*, 1976). As well as these considerations, the use of systemic fungicides carries with it the very serious threat of creating fungicide-resistant strains.

The discovery in Italy of healing cankers on infected European chestnuts provided some of the first real hope for the chestnuts. While it was at first believed that the European chestnut had somehow acquired resistance to the blight fungus (Biraghi, 1953), the *C. parasitica* isolates from these cankers were shown to be atypically white in culture and exhibited reduced growth, sporulation, and virulence (Grente, 1969). These unusual strains of *C. parasitica*, and others morphologically and behaviorally similar, were termed "hypovirulent". They were all found to contain a cytoplasmic agent that could be transmitted via hyphal anastomosis (Van Alfen et al., 1975). Day et al. (1977) found that the cytoplasmically contained author of hypovirulence was a double stranded RNA virus. This dsRNA virus could also be found in conidia, but single ascospore cultures from hypovirulent strains always proved pathogenic.

Hypovirulence as a means of biocontrol of the chestnut blight has been termed successful in Europe though the truth of this is debatable (Milgroom, 1999). Its efficacy on American chestnut in the United States has been severely hampered by several factors (Elliston, 1981). Hypovirulent strains have slowed growth rates as well as significantly reduced production of conidia and ascospores (MacDonald and Fulbright, 1991). An

avirulent culture may also produce large numbers of virulent conidia as well as the invariably pathogenic ascospores (Garrod et al., 1985 ; Melzer et al., 1997).

The greatest problem with hypovirulence as a viable biocontrol strategy in the United States lies in the difficulty of transferring the dsRNA virus from hypovirulent strains to virulent strains. This can only be accomplished through hyphal anastomosis (Milgroom, 1999). Unfortunately, *C. parasitica*, like many Ascomycetes, has a system of vegetative compatibility (v-c) genes, and hyphal anastomosis can occur only between strains of *C. parasitica* whose v-c genes are sufficiently similar (Anagnostakis, 1977; Anagnostakis and Day, 1979; Caten, 1972; Kuhlman et al., 1984). It is believed that this is the basis of the success of hypovirulence as a biocontrol agent in Europe, where there were only a very limited number of v-c groups. Unfortunately, there are vast numbers of v-c groups in the U.S.. In 1977, Anagnostakis had identified 28 different v-c groups. By 1981, the number had increased to 77 (Anagnostakis and Waggoner, 1981). From one small area of Massachusetts alone over 50 v-c types were recovered (Lee et al., 1991). It has since been found that vegetative compatibility is not as stringent as was once believed. While there have been shown to be networks of compatibility groups that allow anastomosis between v-c groups, this has yet to have a significant effect on the ability of hypovirulent isolates to convert virulent strains in the field (Anagnostakis, 1983; Kuhlman and Battacharyya, 1984). In isolated areas outside the natural range of the chestnut, there has been limited success with hypovirulence as a biocontrol strategy (Fulbright *et al.*, 1983), but it has been recently concluded that hypovirulence has little potential for biocontrol of chestnut blight (Milgroom, 1999).

1.5 The search for other potential biocontrol agents

In 1963, W. H. Weidlich (Weidlich, 1978) noticed cankers developing on chestnut roots exposed by a logging road. Ordinarily, though cankers may form at the base of chestnuts, they do not grow more than a centimeter below ground level, and Weidlich wondered if the soil itself might not have some inhibitory effect on *C. parasitica.* He found that muddy soil compresses not only greatly inhibited canker growth, but often healed the canker. As autoclaved soil failed to provide similar results it was deduced that the mechanism was biotic rather than abiotic in nature. McCabe (1974) found that compost applications had the same curative effect on cankers. He postulated that some of the bacteria commonly present in compost and soil might be antagonistic to *C. parasitica.* Weidlich's experiments led to the isolation of an inhibitory fungus. From his soil samples he cultured various fungi, bacteria, and actinomycetes, but only one fungal isolate, a *Trichoderma*, was found to be active against the blight fungus. In 1981, Italian researchers working on European chestnut reported similar results using sphagnum moss and soil on graft unions (Turchetti and Gemignani, 1981). This was ascribed to antifungal compounds and antagonistic organisms, one of which was *Trichoderma viride.*

There has been some limited success using bacteria as a biocontrol agent against *C. parasitica.* Bacteria cultured from the xylem sap of healthy European chestnut were screened *in vitro* for antagonistic activity towards *C. parasitica.* The most aggressive organism, *Bacillus subtilis*, was then injected into chestnut stems that were later challenged with *C. parasitica.* Those stems pre-inoculated with the bacteria were less susceptible to infection and canker growth was greatly reduced, though this may well be in part a systemic induced resistance that would disappear with time (Wilhelm, 1992).

1.6 Objectives

The purposes of this study were:

1. To investigate what microorganisms are associated with the bark of surviving American chestnut trees.
2. To determine the biocontrol potential of the collected microorganisms against *C. parasitica* in laboratory studies.
3. To identify the organisms showing the highest degree of antagonism to *C. parasitica* and determine their longevity in the field.

Figure 1.1 Natural range of the American chestnut (Collingwood, 1947).

CHAPTER 2

INVESTIGATION OF THE FUNGI COMMONLY FOUND ON THE BARK OF AMERICAN CHESTNUT

2.1 Introduction

Cryphonectria parasitica, while well-adapted to parasitism, is a less successful saprophyte. It's introduction into this country upset an ecological balance that had been established for millenia. A large number of saprophytic fungi inhabit the bark of chestnut trees (Gloer, 1995). It is possible that natural control of the blight might someday take the form of those organisms which inhabit the bark of the chestnuts evolving towards higher degrees of antagonism to *C. parasitica*. If this were to be the case, then those organisms must certainly exist now on the phelloderm of the chestnut. The presence of large populations of such antagonists could be a potential explanation for those trees that manage to survive, apparently blight-free despite clear evidence of inoculum of *C. parasitica* within their environments, or those that achieve significant size (>20 cm diameter at 1.4 m aboveground), stubbornly surviving in defiance of infection.

With the increased interest in biocontrol, more work is being done to discover and develop microorganisms as biocontrol agents. Among those that have garnered the most attention are *Trichoderma* spp., many of which have been found to be successful antagonists of many plant pathogens (Chet, 1987). *Gliocladium*, a close relative, is another fungus under development as a biocontrol agent, and bacteria in the genus *Bacillus* and *Pseudomonas* are already being used agriculturally against a number of soil-

borne pathogens. All these biocontol agents, fungal or bacterial, are aggressive, fast-growing, r-strategist saprophytes that may out-compete pathogenic organisms for available resources, some have the ability to produce antibiotic compounds, while others are mycoparasites (Campbell, 1989).

Trichoderma spp. have been demonstrated to inhibit *Sclerotium cepivorum*, which causes white rot of onion (Abd-El Moity, Papavisas, and Shatla, 1982). It actually parasitizes others such as *Rhizoctonia solani* by coiling around its hyphae, and producing toxic metabolites that partially digest the walls, allowing the cells to be penetrated (Askew and Laing 1994; Elad, et al, 1983; Benhamou and Chet, 1996). Researchers have created new biotypes of *Trichoderma harzianum* tolerant to benomyl to make them more useful to integrated pest management programs (Papavisas, et al. 1982).

The purpose of the following experiments was to determine what fungi are commonly found on the bark of large surviving American chestnuts, and which, if any, exhibit antagonism towards *C. parasitica*. This was established using *in vitro* tests.

2.2 Materials and Methods

2.2.1 Sources and Maintenance of the Pathogen

Cultures of virulent isolates of *Cryphonectria parasitica* were obtained from American chestnut trees at a number of different sites in Massachusetts, New Hampshire, and Vermont. Tissue samples of the outer bark from infected chestnut trees were taken from the advancing margin of active cankers using a cork borer. These samples were then placed cambial side down in the center of 9 cm petri plates containing 2% potato dextrose agar (PDA, Difco) and incubated in the dark at

25 °C. After approximately one week, hyphal transfers were then made from the leading edge of growth on to a new PDA plate. The isolates were maintained on PDA, incubated at 25 °C, and transferred every seven days.

2.2.2 Sources and Maintenance of Potential Antagonists

Tissue samples were taken from the outer bark of large, healthy American chestnut trees from sites in Shelborne Falls and Turners Falls, Massachusetts and infected but large, surviving trees in Walpole, New Hampshire. Samples were collected from the base, and at one and two meters using tweezers and a knife to flake away portions of the bark. Great care was taken not to initiate bark wounds. The samples were then taken to the lab and plated on to PDA and incubated at 25 °C in the dark. The plates were monitored daily for microbial growth, and as distinct colonies grew out, transfers were made to new and separate plates and returned to incubation.

Bark samples from a previous study had yielded thirteen *Trichoderma* sp. isolates (Tattar and Mount, 1997. Unpublished data) and these isolates were added to the study at this time.

2.2.3 Determination of Antagonism to *C. parasitica in vitro*

PDA plates were inoculated with an agar plug 3mm in diameter taken from the leading edge of a culture of *C. parasitica* five days old. The plug was placed 1.5cm from the edge of the petri dish. These plates were then incubated at 25 °C in the dark. After three days, the plates were removed from the incubator and inoculated with a 3mm plug of agar taken from the growing edge of the potential antagonist, ie. fungal isolate, and

placed 1.5mm in from the edge of the dish. The plates were returned to the 25 °C incubator and grown in the dark. The plates were monitored daily to look for evidence of antagonism. Antagonism was determined by the occurrence of one of the following reactions: (1) growth of the two cultures until hyphal tips approached one another and produced a barrage line, (2) inhibition of growth of *C. parasitica* to the degree that a clear zone of inhibition developed in advance of the antagonist, or (3) growth of the two cultures until hyphal tips met, and the potential antagonist then grew over and sporulated on the *C. parasitica*. All isolates that showed some degree of antagonism were maintained on PDA plates and stored at 25 °C in the dark. Isolates that showed no antagonism of any kind at this point were excluded from ensuing experiments.

2.2.4 Effects of the *Trichoderma spp.* Isolates on the Growth of *C. parasitica* and Assessment of Antagonism

PDA plates were inoculated with a 3mm plug of taken from the leading edge of a five day-old culture of *C. parasitica* placed 1.5mm in from the edge of the dish. These were then incubated in the dark at 25 °C. Measurements of hyphal growth were taken every 24 hours. After three days, the plates were co-inoculated with a 3mm plug taken from the leading edge of the *Trichoderma* isolate which was then placed 1.5mm in from the edge of the dish exactly across from the plug of *C. parasitica*. The plates were returned to incubation. Measurements of the hyphal growth were taken for both fungi every 24 hours.

Evidence of antagonism as determined by visual observation of the culture of *C. parasitica* and the isolate of antagonistic fungus to look for signs of antibiosis, as well as

a visual estimate of the amount of sporulation of both the antagonist and *C. parasitica*. The interaction was scored using a rating system on a scale of 1-6: 1 = No barrage line produced between the antagonist and *C. parasitica*. The hyphae of the antagonist overgrows the entire medium surface and sporulation of the antagonist covers 100% of the plate, 2 = No barrage line produced between the two fungi, hyphal growth and sporulation of the antagonist covers 75% of the medium surface, 3 = No barrage line produced between the two fungi, visible hyphal growth and sporulation of antagonist limited and random, 4 = An initial barrage line produced between the antagonist and *C. parasitica* is crossed by the antagonist. Hyphal growth and sporulation cover 100% of the medium surface, 5 = The barrage line initially produced between the two cultures is crossed by the antagonist, but hyphal growth is reduced and sporulation of the antagonist covers 75% of the medium surface, and 6 = Initial barrage line produced between the two fungi is not visibly crossed by hyphae of the antagonist. Each of the cultures occupies approximately 50% of the medium surface, and neither organism appears to dominate the other.

2.2.5 Screening and Selecting for Fungicide Tolerance

Resistance to one or more fungicides is a method that has been frequently used to identify a particular fungal strain. Previous researchers have managed to culture fungicide-resistant strains of *Trichoderma* spp., and this was the method chosen for this experiment. (Papavisas, et al, 1982, Abd-El Moity et al 1982).

Both mycelial and spore transfers of the *Trichoderma* isolates were made onto three PDA-based media, one containing 1.0ml/L of a 4.0% propaconizole solution, one

containing 1.0ml/L of a solution containing 1.7% [2-(2-ethoxyethoxy) ethyl-2-benzimidazole carbamate] and 0.3% methyl 2-benzimidazolecarbamate (DEBC), and the last containing 1.0ml/L of each of these fungicide solutions. Both fungicide solutions were obtained from Mauget capsules (J.J. Mauget Co., Arcadia, CA). The fungicides were pipetted into flasks of cooled PDA and the flasks were swirled to completely mix the liquids. All *Trichoderma* isolates were maintained on control plates of PDA alone for comparison.

Mycelial transfers of the *Trichoderma* isolates were made by taking 3mm plugs from the edge of three day-old cultures which were then placed in the center of petri plates containing the fungicide media. Spore transfers were made using cultures which exhibited heavy sporulation. The cover was removed, and the bottom half carefully inverted over one containing the fungicide media. The bottom of the inverted plate was then tapped lightly from the center in a widening spiral to dislodge and evenly distribute spores on to the new media. After transfers were made the cultures were incubated at 25 ^{o}C in the dark. As the DEBC at this concentration completely inhibited mycelial growth and spore germination, the amount of the solution pipetted into the media was reduced to 0.5ml/L. Isolates that were able to grow in these media were then transferred to media containing increased concentrations of the fungicides up to 0.75ml/L of DEBC and 7.5ml/L of propaconazole.

2.3 Results

2.3.1 Isolation and Screening of the Collected Fungi for Antagonism to *C. parasitica in vitro*

Fungi were by far the most numerous microorganisms cultured from the bark samples taken from American chestnut trees. From the samples taken in Shelburne Falls, Massachusetts, twelve of the fungal colonies isolated showed some degree of antagonism to *C. parasitica*. Eleven of the isolates were identified as *Trichoderma* sp. and one was a *Fusarium* sp.. All of the bark samples collected from eight trees in New Hampshire yeilded *Trichoderma* spp.. Several bacteria were also cultured from the bark samples.

The *Fusarium* isolate grew much more slowly than any of the *Trichoderma* isolates and showed a lesser degree of antagonism to *C. parasitica* as well, tending to form barrage lines it never crossed, and sporulating poorly. As it compared unfavorably to any of the *Trichoderma* strains as a potential biocontrol agent, it was dropped from the study at this time. All the *Trichoderma* isolates collected were found to be antagonistic to *C. parasitica* to some extent. The thirteen previously collected *Trichoderma* isolates also all exhibited some measure of antagonism to *C. parasitica*. In total, thirty-seven Trichoderma isolates were obtained from the bark of American chestnuts from locations in Vermont, New Hampshire, and Massachusetts.

2.3.2 Comparative Growth of *C. parasitica* and *Trichoderma* in Culture

When *Trichoderma* and *Cryphonectria parasitica* are grown together on agar culture, the extreme difference in their mycelial growth rates is evident. For example, in

its first 24 hours the mycelia of *C. parasitica* grows an average of 2.0mm, and by the end of a week may be growing up to 8.0mm in a 24 hour period, while mycelia of a typical culture of *Trichoderma* begins by growing approximately 8.0mm in its first 24 hours and doubling that in the next 24 hours. This difference in comparative growth rates requires that the *C. parasitica* be transferred to the plate a minimum of three days before the *Trichoderma* is added, thus giving it sufficient time to establish itself.

2.3.3 Effects of the *Trichoderma spp.* Isolates on the Growth Rate of *C. parasitica in vitro*

The growth rate measurements showed that *in vitro*, no particular strain of the collected *Trichoderma* isolates had an effect on the growth rates of the *Cryphonectria* cultures they challenged. When the *Trichoderma* isolate was transferred in, the growth pattern of the *C. parasitica* remained unchanged until the hyphae of the two cultures actually met on the agar. There appeared to be no flattening of the culture in response to the approach of the *Trichoderma*, and no zone of inhibition developed. However, the differences in barrage line production and sporulation characteristics became the major determining factor in which strains would be chosen for continued research.

2.3.4 Evaluation of Antagonism

Experiments to test the level of antagonism between *C. parasitica* and the collected *Trichoderma* strains allowed for an examination of several aspects of the interaction between the two fungi. Assigning a rating to the interaction between the two fungi (Table 2.1) allows one to track the responses of each individual isolate, and to determine what if any significant relationships exist between the responses of the

Trichoderma and *C. parasitica* isolates. The range of response from each isolate demonstrates with what consistency that particular strain responds when challenged by varying strains of another fungi. As the rating system went from 1-6, the widest range of response would be 5 and the narrowest 0. Using chi square analysis and Fisher's Exact test, a highly significant relationship was found between the breadth of the range and the type of fungi. The *Trichoderma* isolates had a much narrower range, with an average of 1.3. This means that out of a range of 5 possible reactions, a *Trichoderma* isolate rarely varied in its response, regardless of the *C. parasitica* strain with which it was challenged (fig. 2.1) However, the *C. parasitica* isolates gave a much wider range of response, with an average of 3.3 (fig. 2.2). This demonstrates the inconsistency in response of the *C. parasitica* isolates to the *Trichoderma* isolates with which they were challenged. This data would seem to suggest that the result when the hyphae of the two different fungi meet is under control of the *Trichoderma* isolate rather than the *C. parasitica* isolate. This hypothesis is supported by the data analysis of the type of fungus and the production of a barrage line.

Monitoring the *C. parasitica* and *Trichoderma* isolates for consistent production of a barrage line, one finds that only the *Trichoderma spp.* isolates exhibit consistent responses. The *C. parasitica* isolates, as their span of range shows, produced both responses at very close to the expected frequencies. Only one of the *Trichoderma* isolates, 8B2, was inconsistent in its production of a barrage line: after seven trials with different *C. parasitica* isolates, it had produced a barrage line six times, and no barrage line only once. All the other *Trichoderma* isolates were consistent in either always or never producing barrage lines in culture with *C. parasitica*, regardless of the *C.*

parasitica isolate. This would seem to indicate that barrage line production is also under control of the particular strain of *Trichoderma* rather than *Cryphonectria parasitica*. The *Trichoderma* isolates that never allowed barrage production by any of the *C. parasitica* strains were isolates 1AA, 1AB, 1BA, and 1BB, all from the Shelburne Falls, Massachusetts site.

2.3.5 Selection for and Production of Fungicide-tolerant Strains

Two of the strains of *Trichoderma* chosen for fungicide tolerance, 1AA and 1AB, were from among those that never allowed production of a barrage line by *C. parasitica* and typically overgrew it, sporulatoring profusely. The third, though producing an initial barrage line when encounterring *C. parasitica in vitro*, also overgrew the *C. parasitica* and exhibited profuse sporulation. These were sent to the USDA-ARS Systematic Botany and Mycology Laboratory in Beltsville, Md. and were all identified as *Trichoderma harzianum* (fig. 2.3) (Samuels, 1999).

Transfers to media containing continually increasing amounts of fungicide gradually produced *T. harzianum* strains that were able to tolerate up to 7.5ml/L of propaconazole and 0.75ml/L of EBDC. Incubation time necessary to produce mycelial growth progressively lengthened with each increase in concentration of fungicides, but this lag time decreased as the strain became more accustomed to each fungicide. Cultural morphology of the *Trichoderma* strains also changed considerably when grown on fungicide containing medias. Mycelium, instead of growing at an even pace, thereby creating a near perfect circle, grew in convolutions and the culture margin was highly lobate.

To test the stability of this tolerance, the strains were transferred back to media without fungicide. They were maintained on normal PDA through two transfers, and were then placed back on the fungicide media. As a control, transfers were also made from cultures of the *Trichoderma harzianum* strain that had never been exposed to the fungicides on to the fungicide containing media. The growth rates of the previously fungicide-tolerant strains, that had been allowed to regrow on plain media through 2 transfers, were indistinguishable from those of the fungicide-intolerant controls.

2.4 Discussion

From the isolations made from bark samples of chestnuts in Massachusetts, Vermont, and New Hampshire, it appears that isolates of *Trichoderma* sp. are commonly found on the bark of the American chestnut. Its very prevalence indicates with what ease this fungus can colonize this particular niche. While *Trichoderma* is most often thought of as a soilborne organism and, therefore, best suited to the control of root diseases, it has also been used to combat pathogens on other plant tissues. A study that examined the saprophytic population of elm bark and screened for potential biocontrol organisms from amongst them, found both *Gliocladium* spp. and *Trichoderma* spp. that showed *in vitro* antagonism to *Ceratocystis ulmi* (Campbell, 1989).

Since all of the thirty-seven *Trichoderma* isolates collected exhibited some degree of antagonism to *Cryphonectria parasitica*, this would suggest that this fungus might make an effective biocontrol agent against the *C. parasitica*. However, the discovery of numerous *Trichoderma* strains on chestnut tree bark poses some new questions and creates some new problems. *Trichoderma* appears, from this study and from the reports

of other researchers (Tattar, et al., 1996; Gonzalez, 1998; MacDonald, 1998), to be a saprophyte commonly found on chestnut bark. Previous reports of *Trichoderma* strains antagonistic to *C. parasitica* found on chestnut bark were not the oddities that they were initially assumed, but *Trichoderma* strains would appear to be instead frequent members of a large community of bark microorganisms of American chestnut.

If indeed *Trichoderma* spp. are common inhabitants of this environmental niche, does this mean that *Trichoderma*, by its near ubiquitous presence, has already proved itself an ineffective biocontrol agent? Much of the successful work using *Trichoderma* species as biocontrol agents has been against soilbourne pathogens, and thus has taken place in a very different environment. Soil type and pH appear to have a significant impact on the effectiveness of *Trichoderma* as a biocontrol agent (Chet, 1987). Perhaps the presence of *Trichoderma* on the bark of American chestnut is inadequate in terms of biocontrol in that particular environment without some sort of amendation. The success of soil compresses, in which *Trichoderma* sp. have been found, in inhibiting C. parasitica and healing cankers support this idea, but such a method is too labor intensive for practicality on any large scale.

Can a method be found that correlates *in vitro* with *in vivo* antagonism? How significant are the differences in antagonism between the various *Trichoderma* strains *in vivo*? Does *in vivo* antagonism necessarily confer protection from infection, in other words, successful biocontrol? To even begin to address these questions, one needs to get into the field, but the prevalence of strains of *Trichoderma* sampled means that any strains chosen for their antagonism to *C. parasitica* must be marked in such a way that

they can be differentiated upon re-isolation from those already present in the environment.

The experiment to screen for and/or induce tolerance to the fungicides propaconazole and EBDC in the three selected *Trichoderma* strains resulted in the production of cultures that could tolerate up to 7.5 mg/L, but the tolerance was likely due to nongenetic adaptation and was quickly lost upon subculturing back to media without any fungicides, rendering this too unstable a method for practical use in field experiments. Papavisas (1982) had some success in inducing benomyl-resistant mutants by irradiation with UV light. If resistance to propaconizole cannot be created in a similar way, a change of fungicides and a repetition of the experiment might allow for the creation of a biotype that would allow for the investigation of *in vivo* antagonism . Clearly, a stable fungicide-resistant auxotroph will be needed to continue this research.

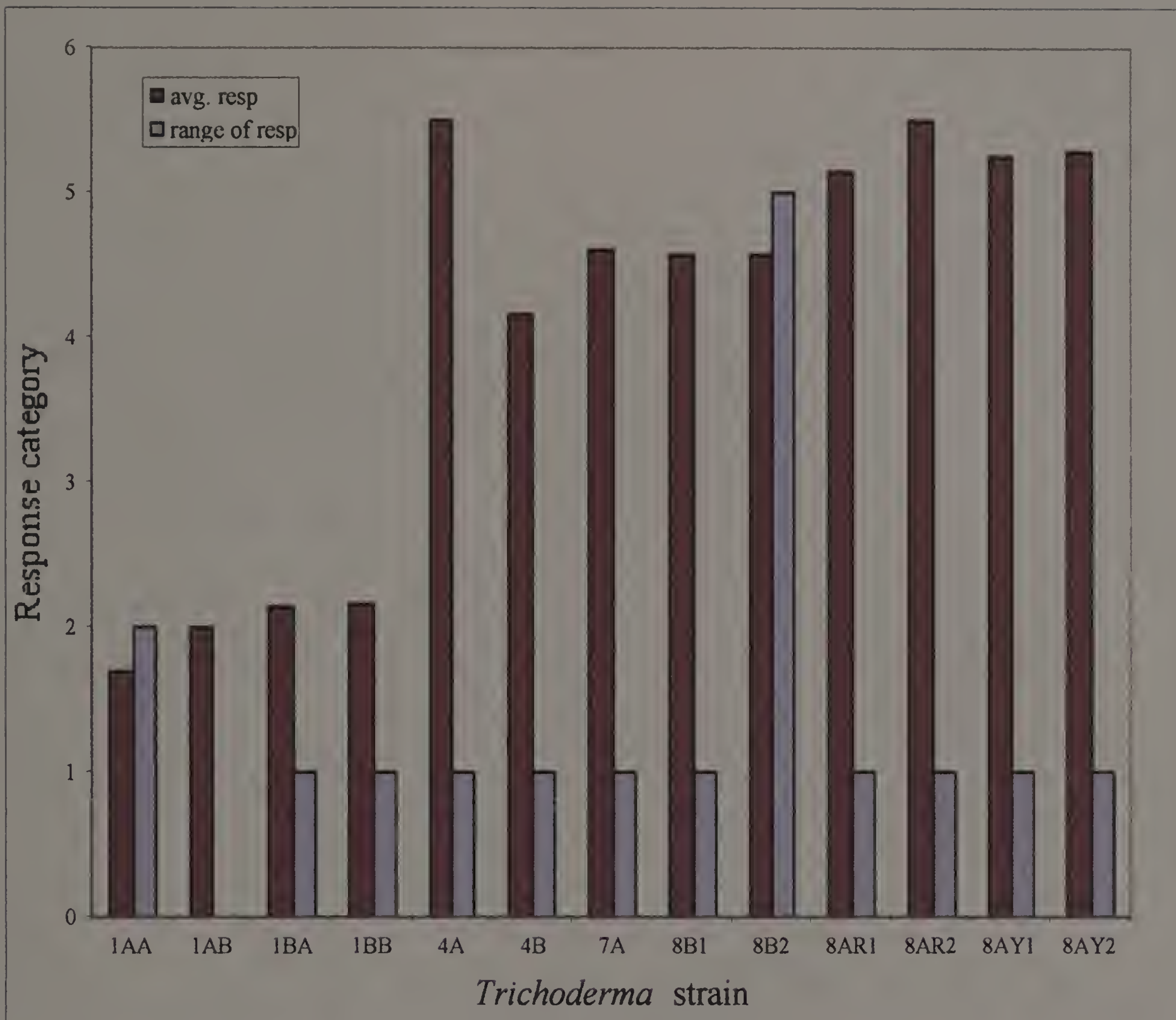


Figure 2.1 Average response and range of response by the *Trichoderma* isolates in response to challenge with *C. parasitica* on potato dextrose agar (PDA). 1= No initial barrage line is produced between the two fungi. Sporulation by *Trichoderma* covers 100% of the plate. 2= No initial barrage line and sporulation by *Trichoderma* covers at least 75% of the plate. 3= No barrage line and sporulation by *Trichoderma* covers <50% of the plate. 4= Initial barrage line produced between *C. parasitica* and *Trichoderma* isolates is crossed by the *Trichoderma*. Sporulation by *Trichoderma* covers 100% of the plate. 5= Barrage line produced between the two fungi is crossed by the *Trichoderma* and sporulation covers 75% of the plate. 6= Barrage line produced between the two fungi. Growth of the *Trichoderma* is limited and sporulation covers 50% of the plate.

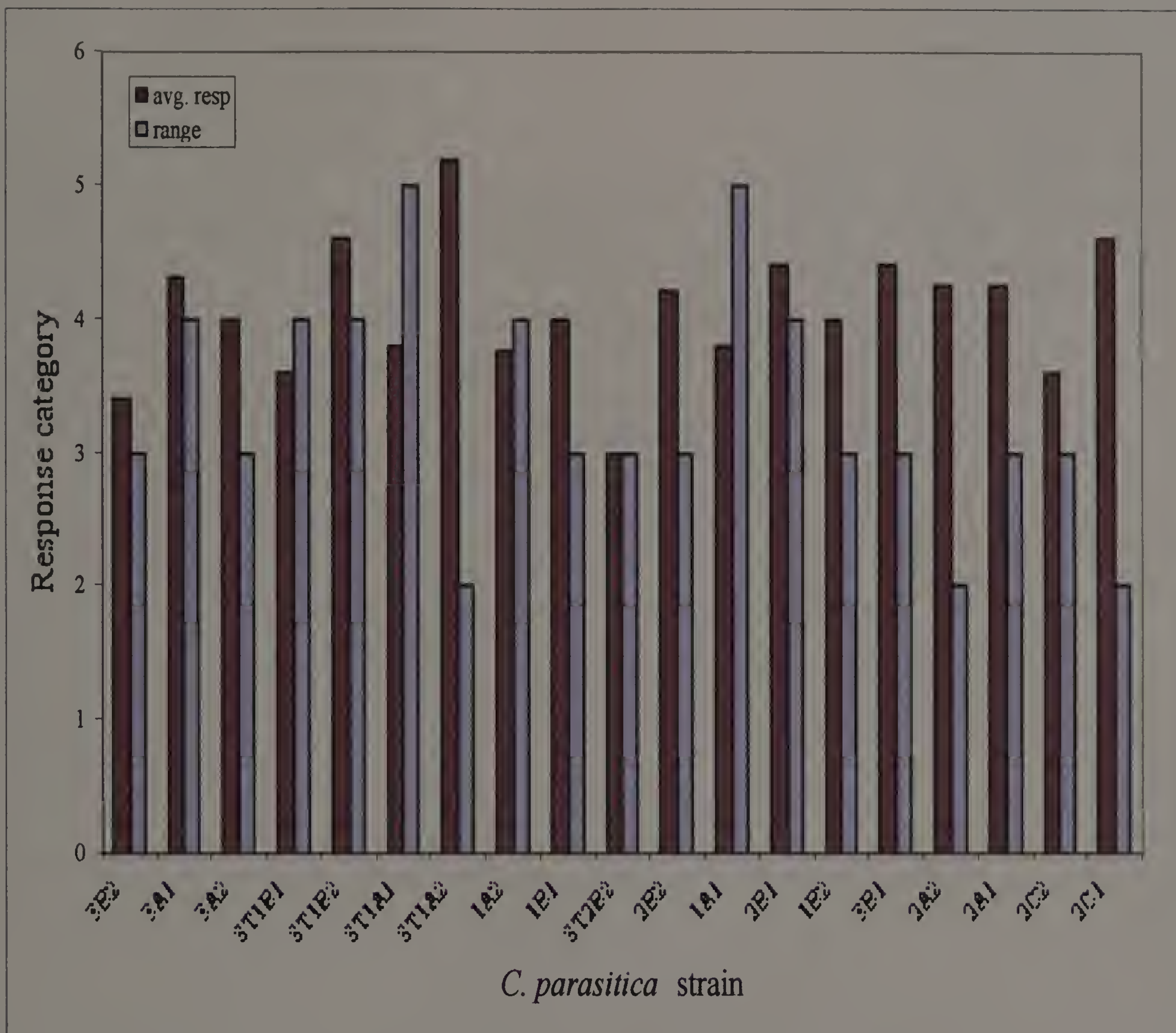


Figure 2.2 Average response and range of response by the *C. parasitica* isolates when challenged with the T*richoderma* isolates (a range of 0 would indicate an identical response for each trial). 1= No initial barrage line is produced between the two fungi. Sporulation by *Trichoderma* covers 100% of the plate. 2= No initial barrage line and sporulation by *Trichoderma* covers at least 75% of the plate. 3= No barrage line and sporulation by *Trichoderma* covers <50% of the plate. 4= Initial barrage line produced between *C. parasitica* and *Trichoderma* isolates is crossed by the *Trichoderma*. Sporulation by *Trichoderma* covers 100% of the plate. 5= Barrage line produced between the two fungi is crossed by the *Trichoderma* and sporulation covers 75% of the plate. 6= Barrage line produced between the two fungi. Growth of the *Trichoderma* is limited and sporulation covers 50% of the plate.

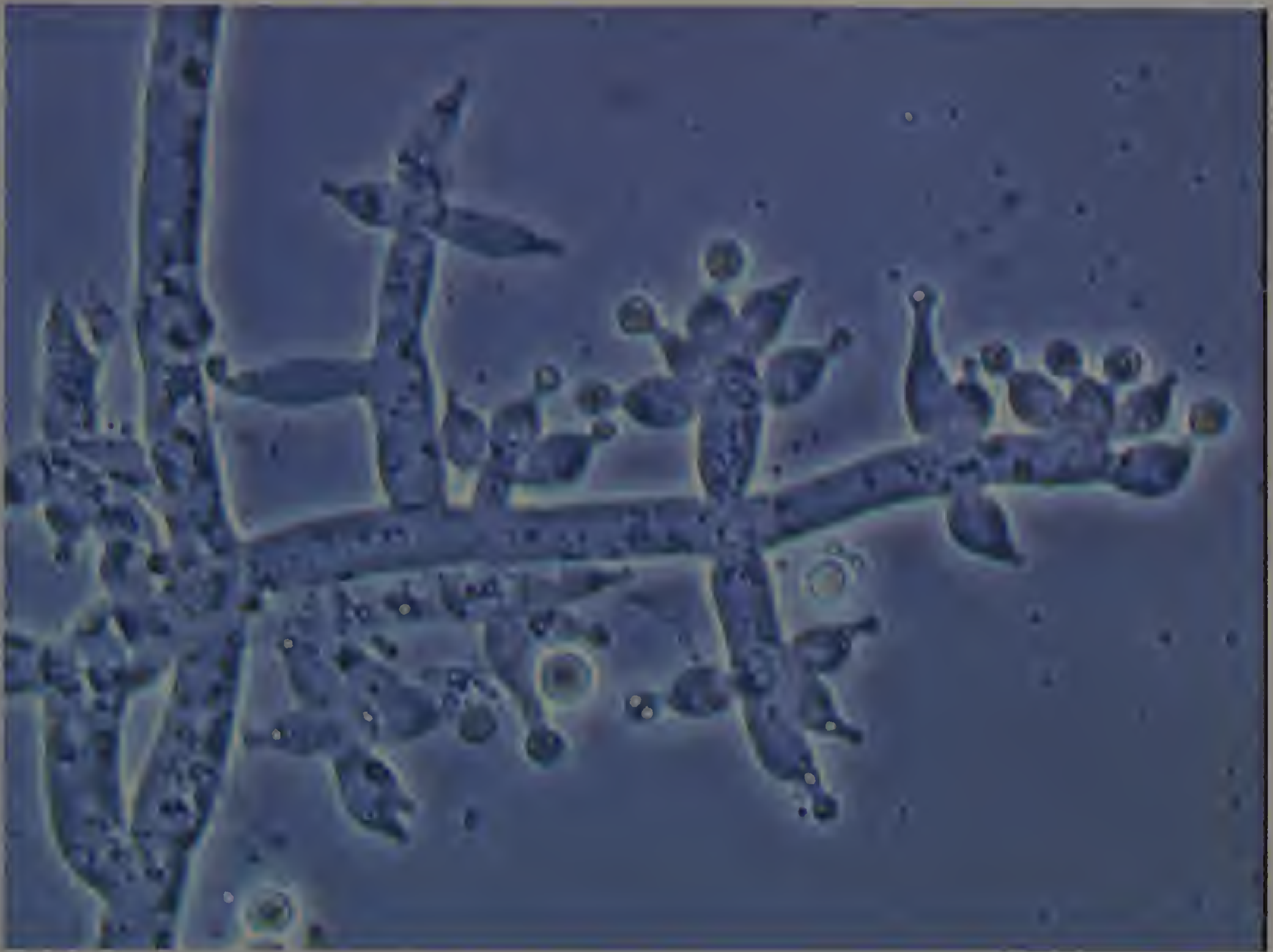


Figure 2.3 Typical *Trichoderma hazianum* hyphae and conidiophore at 100x taken with phase contrast microscopy. The culture was grown on cornmeal dextrose agar (Image courtesy of Gary Samuels, USDA-ARS).

CHAPTER 3

INVESTIGATIONS OF THE BACTERIA FOUND ON AMERICAN CHESNUT BARK

3.1 Introduction

Fungi are the microorganisms most often cultured from samples of American Chestnut bark, and, as a result, have been investigated frequently as potential biocontrol organisms. In the lone case of a bacterium being used in chestnut blight biocontrol, the organism, *Bacillus subtilis*, was cultured from xylem sap. This organism was injected into the xylem of European chestnut, *C. sativa*, as a treatment, but its effectiveness was questionable (Wilhelm, 1992). However, *B. subtilis* is contained in a commercially available wax used in Europe to protect graft unions on European chestnut trees (Heiniger and Rigling, 1994) .

Many soil-borne plant pathogens, including fungi, protoctists, and bacteria have been successfully controlled using bacteria cultured from soil and plant surfaces (Campbell, 1989). *Bacillus* species have proven to be effective biocontrol agents for several reasons. They produce endospores in response to nutritional deprivation, and these survival structures are more resistant to environmental stresses such as heat, drying, and freezing than the vegetative, parental cells (Norris *et al.*, 1981). Biocontrol using *Bacillus* sp. has been effective on a variety of plant tissues against a diverse group of diseases. Successful control of bean rust, caused by *Uromyces phaseoli*, predominantly a leaf disease, was achieved by spraying the leaf surfaces with bacterial suspensions prior

to inoculation with the fungus. Both uredospore germination and germ tube production were greatly reduced. Culture filtrates revealed a heat stable, nondialyzable inhibitory compound that proved to be 95% protein and 5% carbohydrate (Baker *et al.*, 1983). Pusey and Wilson (1984) obtained post-harvest biological control of brown rot of stone fruit, caused by *Monilinia fructicola*. The fruit surface was pre-treated with bacterial suspensions before inoculation with fungal spores. The mechanism of activity again appeared to be a heat stable compound isolated from culture filtrates. Reseachers studying the extracellular enzyme profiles of a strain of *Bacillus megaterium* in connection with biological control of *Rhizoctonia solani*, a causative agent of soybean root rot found that several of the proteins secreted by the bacteria were capable of inactivating enzymes produced by the pathogen as well as attacking cell wall and membrane structures of the fungus (Bertagnolli *et al.*, 1996). Control of crown rot of apple trees was achieved using isolates of *B. subtilis* which produced diffusable antibiotics antagonistic to *Phytophthora cactorum* (Utkhede, 1984). Olive green mold, caused by *Chaetomium olivaceum*, one of the most destructive weed molds of the commercial white mushroom, *Agaricus brunnescens*, can be controlled using *Bacillus subtilis*. Again, control was ascribed to an extremely potent compound secreted by the bacteria (Tautorus and Townsley, 1984).

The following experiments were designed to (1) evaluate the antagonism of any bacteria found on the bark of American chestnuts to *C. parasitica in vitro*, (2) to identify any antagonistic bacteria isolated, and (3) to test their survivability after application in the field.

3.2 Materials and Methods

3.2.1 Sources and Maintenance of Pathogen

Cultures of virulent *C. parasitica* were obtained from American chestnut Trees at a number of different sites in Massachusetts, New Hampshire, and Vermont. Tissue samples of the outer bark from infected chestnut trees were taken from the advancing margin of active cankers using a cork borer. These samples were placed cambial side down in the center of 9 cm petri plates containing 2% PDA and incubated in the dark at 25 °C. After approximately one week, hyphal transfers were made from the leading edge of growth onto new PDA plates. The isolates were maintained on PDA, incubated at 25 °C, and transferred every seven days.

3.2.2 Source and Maintenance of Potential Bacterial Antagonists

Bacterial colonies isolated from the bark samples from healthy and surviving trees were transferred using a loop to petri plates containing 0.8% nutrient agar (NA, Difco), and incubated and maintained at 37 °C in the dark.

3.2.3 Determination of Antagonism Between Isolated Bacteria and *C. parasitica* *in vitro*

In order to find potential antagonists among the collected bacteria, a medium had to be found on which both *C. parasitica* and the bacterial isolates in question would grow equally well. *Cryphonectria parasitica* exhibited stunted growth and lobate colony morphology when cultured on NA, and the bacterial isolates grew less profusely on PDA. Both were found to grow almost equally well on 1.4% of a yeast malt extract

agar (YMEA, ISP Medium 2, Difco), and subsequent experiments were conducted on this medium.

A 3mm plug was taken from the leading edge of a five day-old culture of *C. parasitica*, placed 1.5cm from the edge of the petri plate, and incubated at 25°C for three days. The bacteria was then streaked with a loop in an arc approximately 3-4cm from the leading edge of mycelial growth. The plates were returned to incubation at 25°C as that was the temperature more favorable for fungal growth. The plates were visually monitored every 24 hours for signs of antagonism. An antagonistic reaction would consist of the inhibition of fungal growth, as either an inability of the mycelium to grow beyond the line proscribed by the bacterial culture, or the appearance of a zone of inhibition developing in advance of the bacterial cultures growth.

3.2.4 Determination of Antagonism Between Isolated Bacteria and *Trichoderma spp.* Isolates

Bacterial isolates showing antagonism to *C. parasitica* were screened for antagonism to the *Trichoderma* isolates. A 3mm plug was taken from the leading edge of growth of the *Trichoderma* isolate and placed 1.5 cm from the edge of a plate containing YMEA and incubated at 25°C in the dark for 48 hours. At this time the plate was removed from incubation and inoculated with a loopful of bacteria which were streaked in an arc approximately 3-4cm from the edge of mycelial growth. The plates were returned to incubation at 25°C in the dark. The plates were monitored daily for evidence of antagonism, which again consisted of the inhibition of fungal growth and/or the development of a zone of inhibition between the two microorganisms.

3.2.5 Identification of Antagonistic Bacteria

Bacterial isolates which exhibited antagonism to *C. parasitica* were Gram tested using the KOH method (Wick, R. 19). The isolates were streaked on to YMEA and grown for 24 hours at 37°C in the dark. A loopful of the bacteria from these plates was placed in the center of a glass slide and to this was added a drop of 3%KOH. The bacteria and KOH were then stirred vigorously with the loop for a minute after which time it was examined for evidence of free DNA and then each isolate was given a Gram test rating.

Bacterial isolates were checked for motility using a light microscope and the hanging drop technique. A loop was touched to bacteria from a 24 hour-old culture and added to a drop of distilled water in the center of a glass coverslip. The coverslip was inverted and placed over the center of a well glass slide so that the droplet was suspended and the coverslip secured with a dab of stopcock grease. The bacteria were then observed for motility using a light microscope at 1000x.

Bacterial isolates were given the standard tests for phytopathogenicity; the test for fluorescent *Pseudomonad*s on King's B media and the Levan test on sugar agar to check for the production of polysaccharides. They were checked for evidence of pectolytic activity by streaking onto CVP media and observed for pitting. Cultures were grown in Hugh/Leifson media to determine their ability to utilize glucose anaerobically. The oxidase test for the presence of cytochrome c was administered to isolates cultured on NA slants, to which dimethyl-p-phenylene-diamine and α-napthol were added. The development of a blue color within 2 minutes indicates a positive reaction (Wick, 19).

In order to determine if any of the bacteria were spore-formers, a sporulation medium was prepared containing 0.8% nutrient broth, 0.3% yeast extract, and 1.5% agar. To this was added filter sterilized $MnSO_4.H_2O$ to a final concentration of 25μg/ml. The sporulation media was inoculated and incubated at 37 °C for 48 hours. The cultures were then placed at 80 °C for 30 minutes after which time they were plated out.

Isolates that were Gram positive spore-formers were subjected to tests to determine catalase production by flooding 48hour cultures grown on nutrient agar with hydrogen peroxide and observing macroscopically for the production of gas bubbles. Ability to hydrolyze casein was assessed by streaking the bacteria on to milk agar, incubating for 7 days and then observing for clearing of the agar around and under the bacterial growth. Acetylmethylcarbinol production (the Voges-Proskauer reaction) was determined by inoculating tubes containing Voges-Proskauer broth, incubating for 3 days, and then mixing 3ml of 40% sodium hydroxide to the test tube and adding 0.5-1.0 mg of creatine. A positive reaction is indicated by the production of a red color after 30-60 minutes at room temperature. Each of the strains were also stained to check for the presence of polyhydroxyalcanoate (PHA) inclusion bodies by heat fixing samples on microscope slides. They were then stained for 15 min at 55 °C in 1% (w/v) Nile Blue A, destained for 30s in 8% (v/v) acetic acid, rinsed in water briefly, and allowed to air dry. Inclusion body presence was determined by visualization at 1000x magnification under fluorescent light at 460 nm excitation, 590 nm barrier, and 580 dichroic mirror.

3.2.6 Screening for Antibiotic Resistance

All antagonistic bacterial isolates were screened for naturally occurring antibiotic resistance. Bacterial isolates were inoculated into flasks containing 0.8% nutrient broth (Difco) which were placed on an agitator at 130rpms at 37°C in the dark. YMEA was prepared containing 1.0mg/ml of streptomycin (Sigma), which was dissolved in distilled water and filter sterilized before being added to the cooled autoclaved media. The flask was then swirled to thoroughly mix in the antibiotic before the plates were poured.

After 24 hours, 1ml aliquots of the bacteria in nutrient broth were dispensed to the antibiotic media and spread with a sterilized glass rod. These plates were returned to incubation at 37°C in the dark. The antibiotic plates were monitored daily for growth, and as colonies appeared, these were transferred to fresh plates of streptomycin media for maintenance.

The streptomycin-resistant mutants that were isolated were then screened for additional resistance to rifampcin (Sigma). Petri plates with a gradient of rifampcin concentration from 0μg/ml to 100μg/ml were prepared and streaked with a lawn of the streptomycin-resistant bacteria. Plates were incubated at 37°C in the dark and monitored daily for growth. Isolates were maintained at the rifampicin concentration they were initially isolated.

3.2.7 Survivability of the Antagonistic Bacteria in the Field

In order to test the survivability of the bacteria on the bark of the American chestnut, the isolates of antagonistic bacteria were tested in three different settings: 200 3-5 year old seedlings obtained from Bear Creek Nursery (Northport, WA), maintained in 2 gallon containers in a sheltered courtyard on the campus of the University of Mass., 200 naturally occurring forest trees stump-sprouted from previously killed trees in Cadwell Memorial Forest, MA, and 80 trees 8-10 years of age in an orchard setting at the South Deerfield, MA, research site that had been obtained as seedlings from Bear Creek Nursery and established in the field in 1993. At each site there were four treatments in four plots, one each of the three bacteria, and one control consisting only of uninoculated media.

Bacteria isolates were each grown separately in nutrient broth on an agitator at 130 rpms 37°C in the dark for 36 hours to insure they had reached the stationary phase and approximately 10^{10}CFUs/ml. The cultures were then transferred into sterilized hand-held mist sprayers and applied to the bark of the marked trees. Each tree was sprayed with approximately 15ml, and the bark was visibly damp. At two week intervals, sampling of the bark was done by spraying the previously inoculated portions with sterile distilled water, and taking a swabbing of the bark with a sterile cotton swab, Swube ™ applicator (Becton Dickinson and Co., Cockeysville, Md.) (fig. 3.1), which was returned to its sterile plastic tube for transport back to the laboratory. The cotton swabs were then streaked out on to the streptomycin-containing media, incubated at 37°C in the dark and monitored daily for growth. All bacterial colonies that grew were then transferred to the rifampicin media, returned to incubation and monitored.

3.3 Results

3.3.1 Sources and Maintenance of Potential Antagonistic Bacteria

Bacteria were found much less frequently than fungi at all the forest sites sampled, but were found occasionally at all sites. Those bacterial isolates collected from forest trees exhibited little to no evidence of antagonism to *C. parasitica* and were not identified. However, on a farm in Walpole, New Hampshire, several large American chestnut trees, infected but surviving, yeilded several bacterial isolates in quantity, all of which showed high degrees of antagonism to *C. parasitica*. At the South Deerfield research site, the location of the chestnut orchard, bark swabbings of the young trees were taken. The resulting cultures yielded a large number of bacteria that appeared at least morphologically very similar to the bacterial isolates from the New Hampshire site. Several of these isolates showed antagonism to *C. parasitica*. Isolates of antagonistic bacteria were all large, Gram positive, spore-forming rods.

3.3.2 Antagonism to *C. parasitica in vitro*

When the bacteria were streaked onto plates previously inoculated with virulent strains of *C. parasitica*, fungal growth slowed to a halt and a clear zone of inhibition developed between the two cultures (fig 3.2). Mycelium of *C. parasitica* grew to an average of 18mm from the edge of the bacterial isolates when growth was stopped. When mycelial growth had halted, agar was removed from this zone of inhibition using a dissecting needle, and placed into a well made with a sterilized cork borer (15mm diameter or size 11) in the middle of a plate of YMEA previously inoculated with a virulent strain of *C. parasitica*. As a control, pieces of YMEA from fresh uninoculated

plates were also placed in wells on plates also prepared with *C. parasitica*. The wells filled with agar from the zone of inhibition stopped the hyphae of the advancing *C. parasitica* (fig. 3.3), while the fungus grew unimpeded into the wells filled with the control agar.

3.3.3 Antagonism to Isolated *Trichoderma spp. in vitro*

Each *Trichoderma* isolate antagonistic to *C. parasitica* was grown together with the bacteria antagonistic to *C. parasitica* on YMEA to examine the effect these microorganisms had on each other. Unlike *C. parasitica*, the hyphal growth of the *Trichoderma* isolates was uninhibited by the antagonistic bacterial isolates, and the mycelium grew over the bacteria and sporulated. Bacteria isolates could be re-isolated from beneath the *Trichoderma* mycelium.

3.3.4 Identification of Antagonistic Bacteria

The three antagonistic bacterial strains collected all proved to be gram positive using the KOH method. They were non-motile rods and tested negatively for fluorescence, pectolytic activity, and fermentative ability. They were incapable of polysaccharide production on sugar agar and were oxidase negative. All proved to be spore-formers and were then tested for catalase production, casein hydrolysis, the Voges-Proskauer reaction and polyhydroxyalcanoate (PHA) inclusion bodies. All three strains produced gas bubbles upon addition of H_2O_2, and so tested positive for catalase. All were able to clear the milk agar, a positive for casein hydrolysis, and all gave a negative Voges-Proskauer reaction (table 3.1). These results indicated that the three bacterial

isolates were strains of *Bacillus megaterium*. The three strains were then subjected to staining for polyhydroxyalcanoate inclusion bodies, a phenomena typical of *B. megaterium* (McCool *et al.*,1996). All proved to have PHA inclusions (fig. 3.4a,b,c). This result, in combination with the size of the bacteria, which were all 1 μm in width or more, confirmed the identification of the three isolates as *Bacillus megaterium*.

3.3.5 Selection of Double Antibiotic-resistant Mutants

Naturally occurring streptomycin resistant (1.0mg/ml) mutants were found for each of the three bacterial strains. The streptomycin resistant mutant strains were then screened for resistance to rifampicin. The highest level of rifampicin they were able to tolerate was 0.75mg/ml. When the two antibiotics were combined at these concentrations on one medium, growth was substantially inhibited, so subsequent re-isolations were made first onto the streptomycin media, and the colonies that grew were then transferred on to rifampicin media.

In order to check the stability of the antibiotic resistance, the mutants were transferred to media free from antibiotics and maintained there through at least five transfers, and were then transferred back to the antibiotic media where they were still able to grow. To determine if the level of antagonism to *C. parasitica* of the double antibiotic resistant mutants was different from the non-resistant bacteria isolates, a repetition of the initial antagonism experiment was conducted. Results indicated that while the antagonism still existed, the zone of inhibition had been reduced to just a few millimiters. Therefore, experiments to determine *in vivo* antagonism could not be conducted using these mutants. However, the purpose of this experiment, which was to

determine the ability of the bacteria isolates to survive on chestnut bark, was not compromised.

3.3.6 Field Survival of the Streptomycin/Rifampicin Resistant Strains of Antagonistic *B. megaterium*

The antibiotic resistant bacteria to be field tested were applied on August 15, 1998. The first re-isolation attempt was made two weeks later. The first site visited was the Cadwell Forest in the morning. It was initially believed that the sterile cotton swabs would be sufficient to take samples from the surface of tree bark. When the bark was damp from dew formation, this was true, however, when the bark dried, an unmoistened cotton swab picked up very little of the bark surface, based on visual observations. When the swabs were streaked out onto plates, the results confirmed that the field observations on the recovery technique decreased as the bark dried. The plots that were visited first, while the bark was still wet typically showed the highest recovery rates with an average of 80%, while those visited later in the morning when the bark had visibly dried, had a recovery rate that had dropped to an average of 62%, and the last plot responded with 60% recovery (fig 3.5). Because of the correlation between the drop in recovery rates and the decreasing dampness of the bark, the recovery technique was revised to include a bark wetting with approximately 2-3ml of sterile distilled water which was applied using the same hand-held mist sprayer just prior to swabbing. For the following 12 weeks, recovery rates were 100% for the three bacterial treatments at all sites but remained 0% for the control from all sites.

In July of 1999, to determine the continued survival of the three bacterial strains in the field, the Cadwell Forest site was revisited and swabbings were taken as before. From the control group, the recovery rate remained at 0%, and from the other three treatments, recovery had dropped to 44% for strain 2A, 24% for strain 2C, and 26% for strain 3A. Statistical analysis of the survival rates showed not only highly significant differences in the survivability of the control and the three bacterial strains, but also a highly significant difference between the three bacterial strains themselves.

3.4 Discussion

Bacteria were rarely cultured from the untreated bark of American chestnut trees in the forest, but were cultured frequently from the untreated bark from the trees in the New Hampshire plot. These trees had been planted as shade and nut trees on a farm some 60 or 70 years ago, and were different from all the trees at any of the other sites which were stump sprouts from previously killed trees. The owner of this farm had been nursing these chestnut trees for most of his life, by pruning, spraying, and, after reading the McCabe article in 1974, began applying moist soil compresses to active cankers, which subsequently healed. Twenty-five years later, these trees, while still infected with *C. parasitica*, have survived with little further treatments since that time.

As none of the bark samples from forest trees ever yielded bacteria in this quantity, and none had yielded *B. megaterium*, the data suggest that these *B. megaterium* isolates may have been initially introduced to the bark as a result of the soil compress treatments in 1975. This theory was supported by the results of the field isolation experiments from the South Deerfield research site. It should be noted that the ground

beneath these trees is predominantly bare soil. Bacteria isolated from the bark of untreated trees in S. Deerfield may be a result of rain splash of soil and its accompanying microorganisms onto the bole. This occurrence of bacteria was an additional factor in the need to isolate antibiotic resistant mutants for field work. It was found that the *B. megaterium* isolates can at least survive long term on chestnut bark. *In vivo* antagonism studies could then be undertaken to critically assess the potential of this organism as a biocontrol agent.

The *in vitro* studies of the antagonism between the *B. megaterium* found and virulent *C. parasitica* suggest some modes of action for the antagonistic interaction. Dual cultures of the two organisms resulted in a wide zone of inhibition developing between the bacteria and the fungus. Mycelial growth never advanced to within more than 15mm of the edge of the bacterial culture. Agar removed from this zone of inhibition and placed onto plates previously inoculated with *C. parasitica* remained uncolonized by the mycelium which did grow around, but not on, the agar cubes. This leads one to believe that the *B. megaterium* strains used in this study produced compound(s) inhibitory to the growth of *C. parasitica*. Additional experiments are needed to characterize the inhibitory compound(s) produced by the bacteria and examine the nature of the antagonism.

Bacillus spp. are known to produce a number of extracellular compounds (Priest, 1977). *Bacillus subtilis*, the *Bacillus* species most widely studied for its potential as a biocontrol agent, has been frequently reported to produce antibiotics in culture. Accounts of control by *B. subtilis* attribute much of the phenomenon to the production of extracellular antibiotic compounds (Pusey and Wilson, 1984; Utkhede, 1983). Baker, et

al. (1983) reported contol of *Uromyces phaseoli*, bean rust, by a *Bacillus subtilis* and extracted an inhibitory component from culture filtrates. Disease control using *B. subtilis* has been achieved on such plant surfaces as roots, crown, and leaves, and also against another bark pathogen, *Nectria galligena*, which parasitizes apple (*Malus*) (Campbell, 1989).

Bacillus megaterium have been studied as potential biocontrol agents of soybean root rot, caused by *Rhizoctonia solani*, a soilborne fungus (Zheng and Sinclair, 1996a; Zheng and Sinclair, 1996b). Bertagnolli, et al. (1996) extracted 11 extracellular enzymes from culture filtrates of *B. megaterium.* The strain produced DNase, lipase, β-glucanase, and protease in the greatest quantities and in culture significantly reduced the growth of *R. solani*. *Trichoderma* was also tested together with *B. megaterium* and *R. solani*. It was demonstrated that the culture filtrates of *Trichoderma* and *B. megaterium* did not significantly effect each other, which agrees with the results of this study, which found that though hyphae of the *Trichoderma* overgrew the *B. megaterium* colonies, the bacteria could be re-isolated from beneath the *Trichoderma* and were still viable.

Results of the previous experiments indicate that the *Trichoderma* and *Bacillus megaterium* strains collected from the bark of healthy and surviving American chestnut trees show genuine potential as biocontrol agents against *C. parasitica*. *In vitro*, both have been demonstrated to antagonize the chestnut blight fungus, and the abundance of *Trichoderma spp.* isolates collected from bark samples, as well as the bacterial field survival experiments demonstrate how successfully both these organisms can colonize the bark of American chestnut. Future experiments are needed to begin studies of *in vivo*

antagonism of *C. parasitica* by *Trichoderma* and the *B. megaterium* isolates, both in combination and alone.

	Bacterial Strain			
Diagnostic Test	2A	2C	3A	*B. megaterium*[*]
Gram	+	+	+	+
Motility	–	–	–	–
Fluorescence (King's B)	–	–	–	–
Polysaccharide production (Levan)	–	–	–	–
Fermentation	–	–	–	–
Oxidase	–	–	–	–
Spore formation	+	+	+	+
Catalase production	+	+	+	+
Casein hydrolysis	+	+	+	+
Voges-Proskauer reaction	–	–	–	–
PHA inclusion bodies	+	+	+	+

[*]Results of expected *B. megaterium* taken from Norris, *et al* 1981.

Table 3.1 Results of the diagnostic tests used to identify the three bacterial isolates, 2A, 2C, and 3A, and the responses of a known *B. megaterium*.

Figure 3.1 Bark swabbing of American chestnut tree using a disposable sterile Swube^{TM} applicator.

Figure 3.2 Zone of inhibition between *C. parasitica* (on right) and *B. megaterium* ten days after inoculation with the bacteria onto YMEA containing a three day old plug of *C. parasitica.*

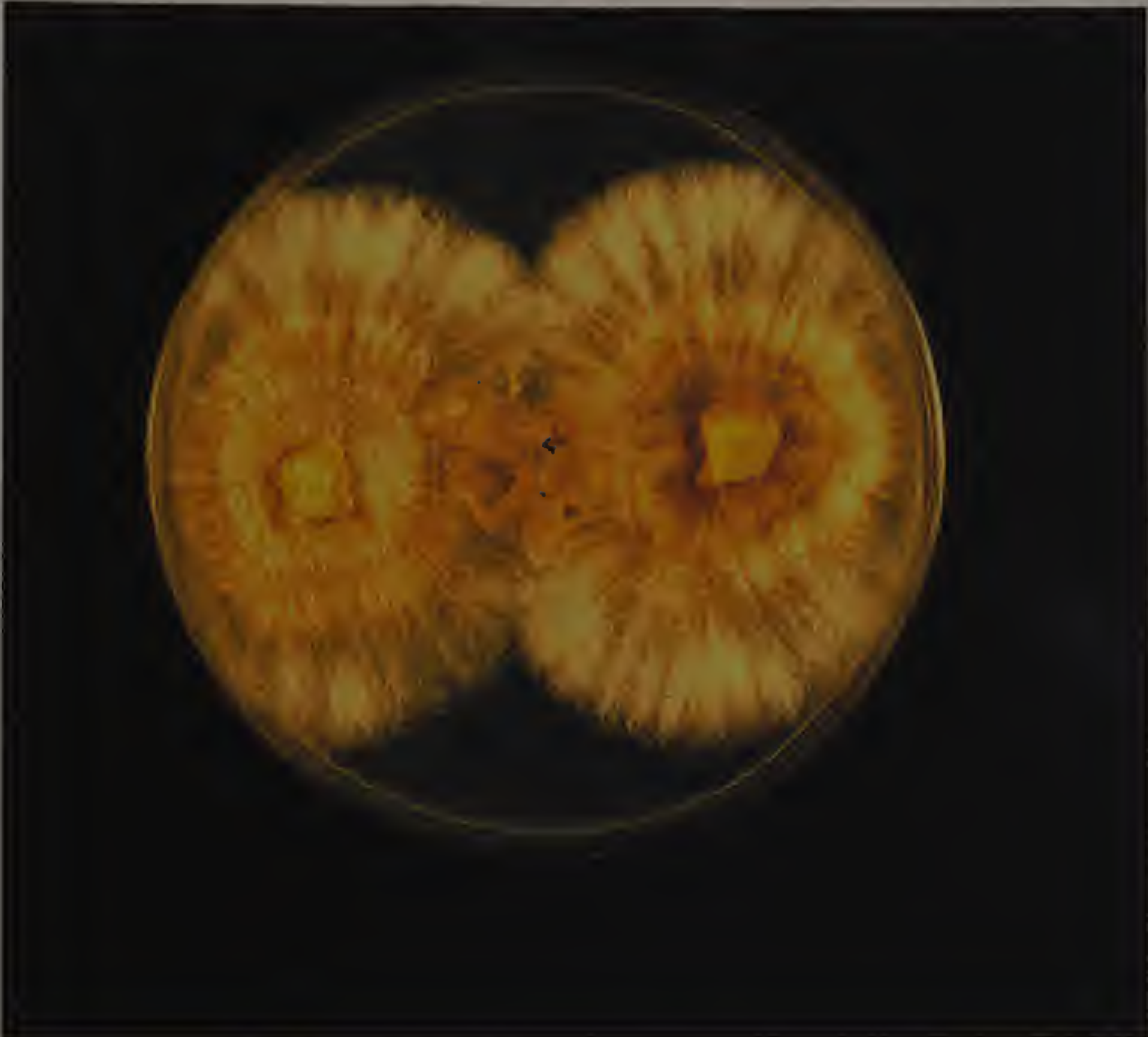
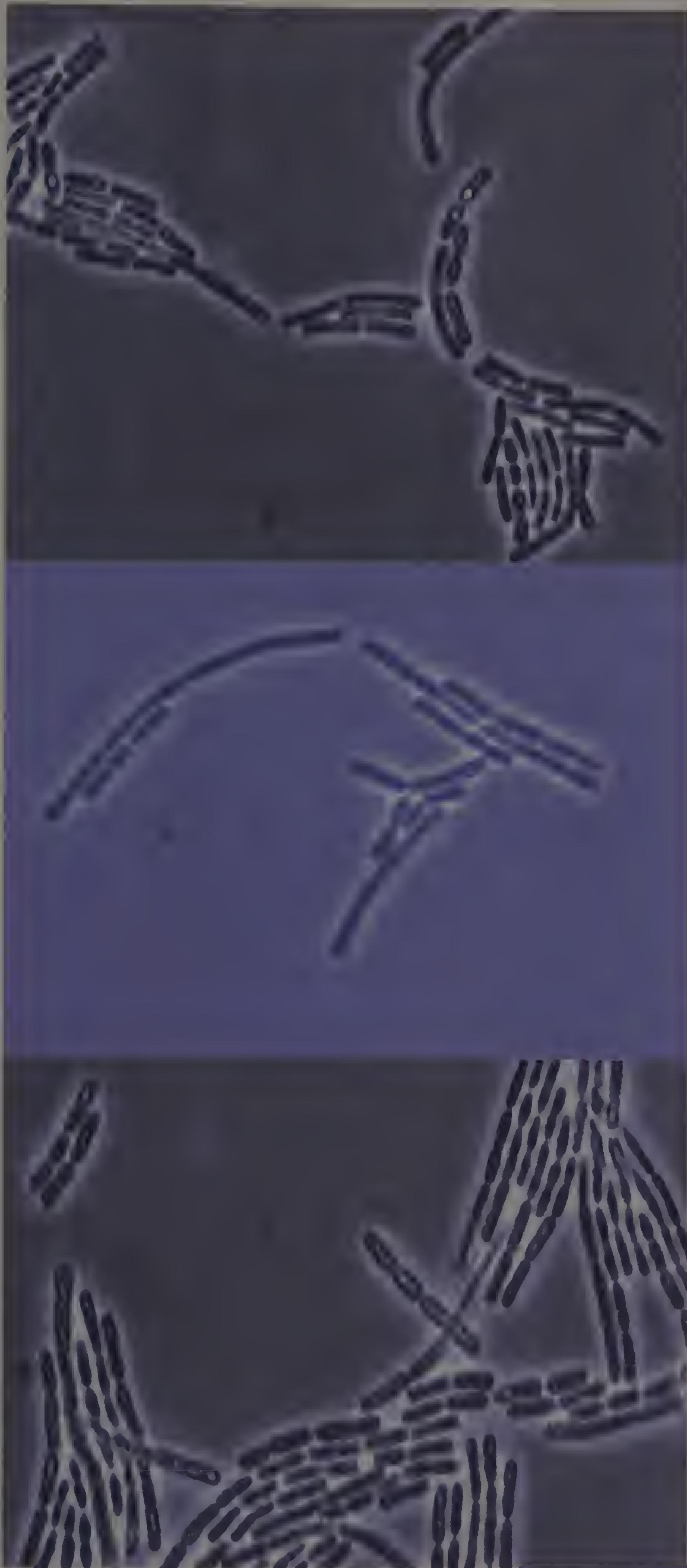


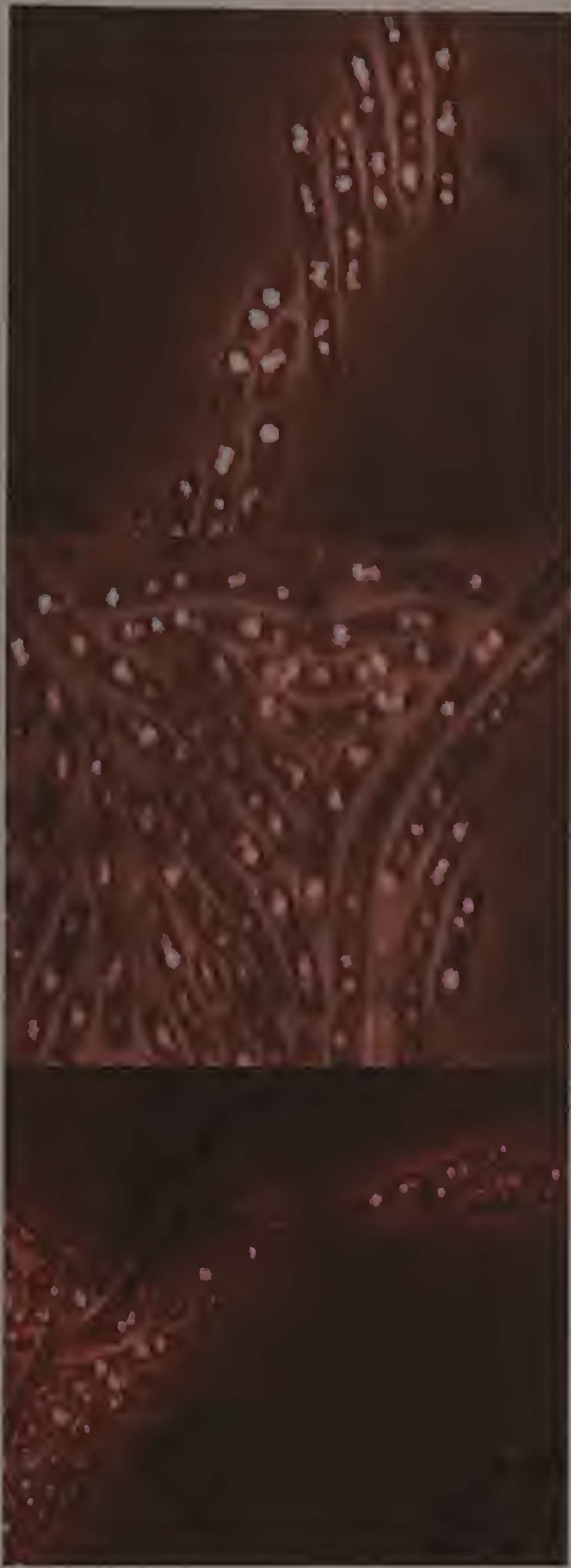
Figure 3.3 YMEA well plates five days after two different but compatible isolates of *C. parasitica* three days old were inoculated with YMEA cubes taken from the zone of inhibition between *C. parasitica* and *B. megaterium* (see fig. 3.2) and placed in the central well.



a.

Figure 3.4 The three *B. megaterium* isolates (2A, 2C, and 3A) as seen under the light microscope at x1000.

Continued next page



b.

Figure 3.4cont. *B. megaterium* isolates 2A, 2C, and 3A stained for visualization of PHA inclusion bodies and viewed at x1000 under fluorescent light.

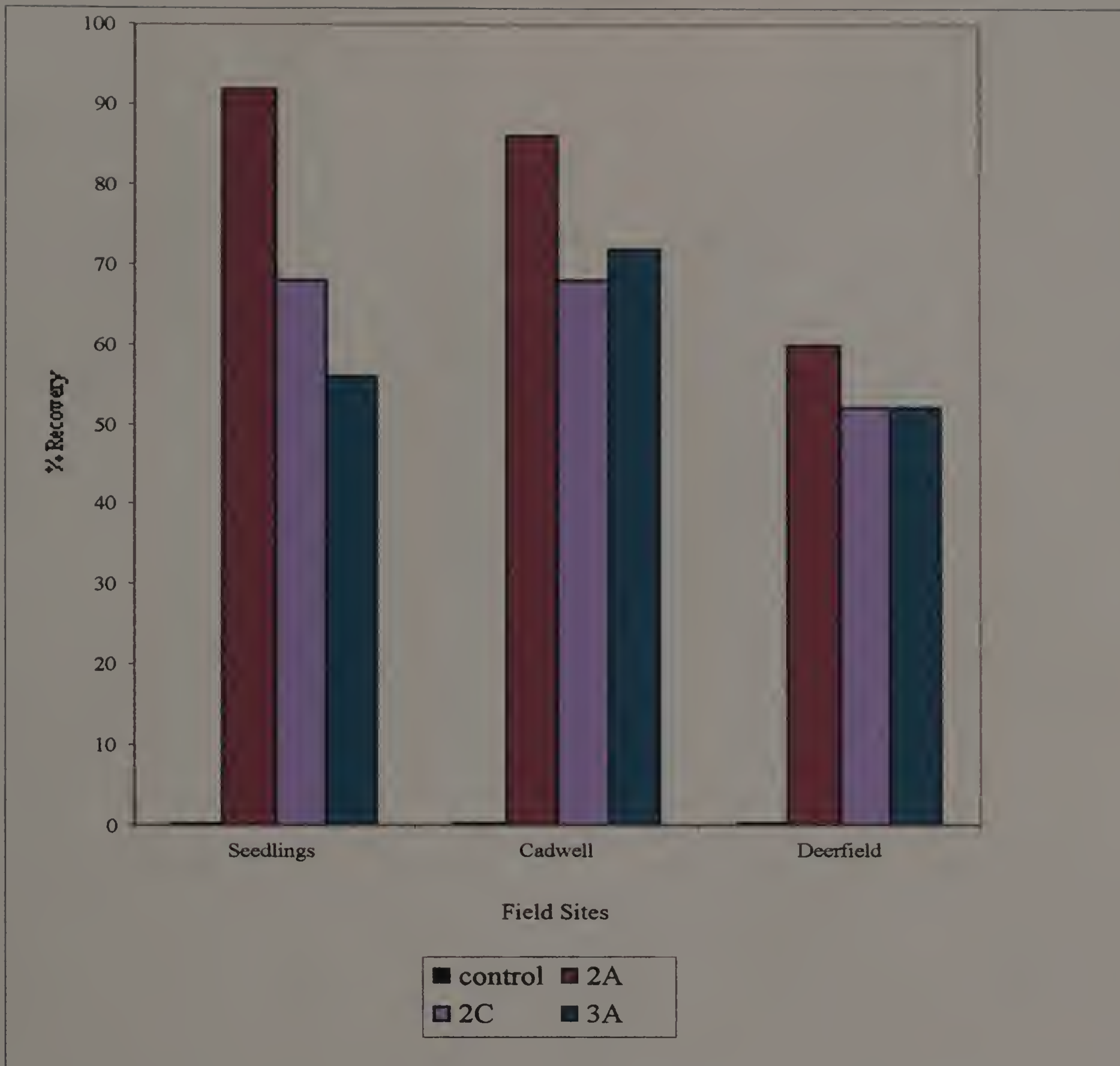


Figure 3.5 Reduction in % recovery of the three *B. megaterium* strains corresponding to the gradual drying of the bark with time.

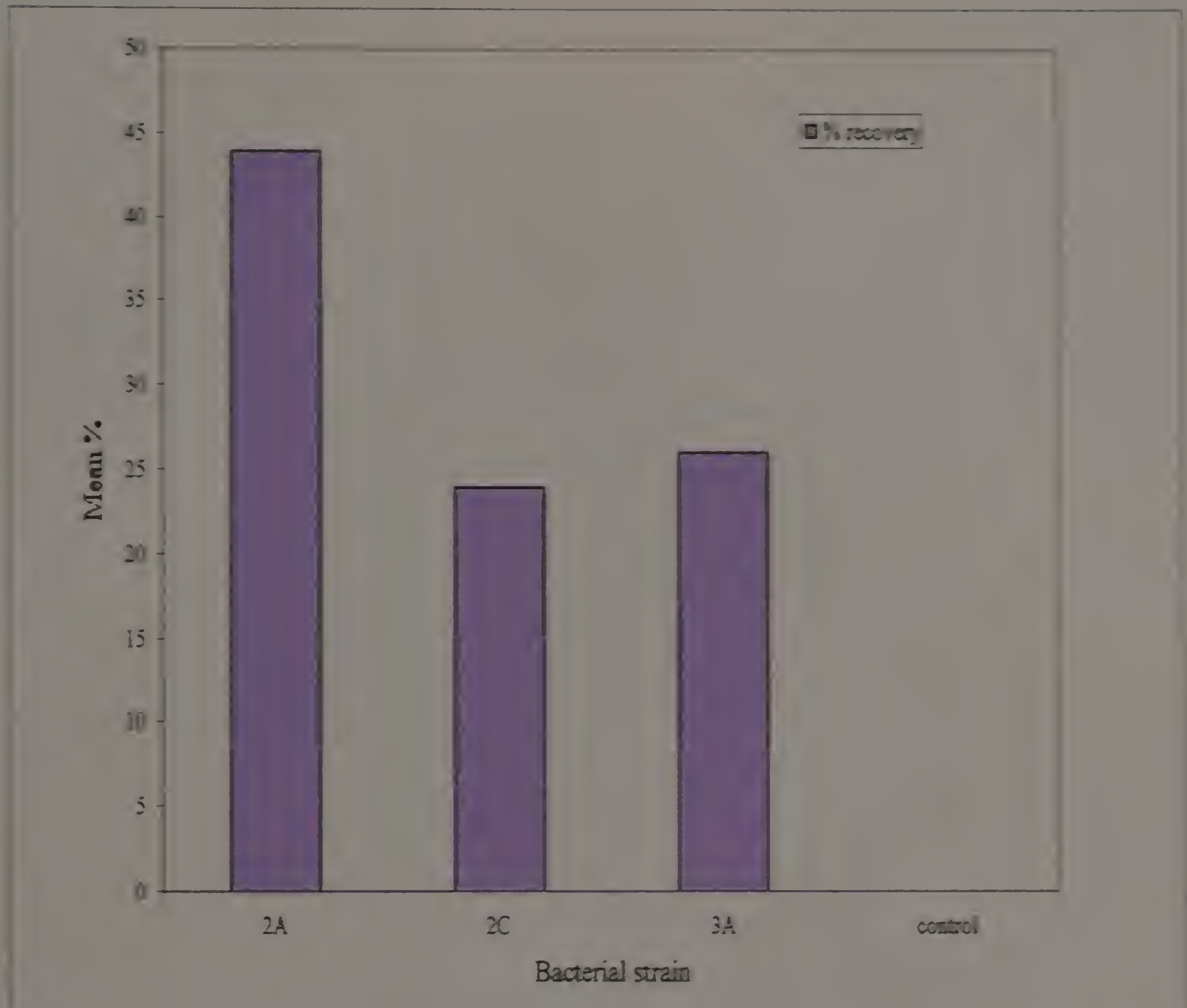


Figure 3.6 Percent recovery of *B. megaterium* strains 2A(a), 2C(b), 3A(b), and the control(c) after one year in the field. Strains followed by the same letter are not significantly different at $p=0.05$. Means based on 50 isolations from each treatment.

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