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Studies on Pseudomonas syringae in relation to American elm, to Ceratocystis ulmi, and to Dutch elm disease.

Daniel F. Plourde
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STUDIES ON *PSEUDOMONAS SYRINGAE* IN RELATION TO AMERICAN ELM, TO *CERATOCYSTIS ULMI*, AND TO DUTCH ELM DISEASE

A Dissertation Presented

By

Daniel Fernand Plourde

Submitted to the Graduate School of the
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D E D I C A T I O N

To my wife, Joanne, for her faithful support
in this endeavor and to my daughter, Jennifer,
for the endless joy she brings to us.

A C K N O W L E D G E M E N T S

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ABSTRACT

STUDIES ON PSEUDOMONAS SYRINGAE IN RELATION TO AMERICAN ELM, TO CERATOCYSTIS ULMI, AND TO DUTCH ELM DISEASE

May, 1985

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Therapeutic treatments of American elms (Ulmus americana) were performed in municipalities of Massachusetts with suspensions of a bacterium that were prepared from a formulation supplied by the Chevron Chemical Company as Pseudomonas syringae strain M27 (PsM27). The results indicate that the bacterium may help to slow symptom development of Dutch elm disease caused by the fungus Ceratocystis ulmi. However, the effects were not carried over to the following season. Our studies on the ability of P. syringae to survive in American elms from season to season show that the bacterium does not colonize elm xylem tissue. This was determined by the failure to isolate the organism in significant numbers from PsM27 treated elms when compared with both water-treated and non-treated elms.

A new injection technique for application of the bacterium was developed and tested. Dye and chemical studies indicate that the method provides for placement of injectable materials into the outer

xylem with good distribution into the crowns. Wounding by this injection process was kept to a minimum and no wetwood fluxing was observed. The placement of bacteria into the outer xylem was not limited by the injection technique.

Several bacteria that were isolated from elm trees were found to inhibit C. ulmi. These included: Klebsiella ozaenae, Pseudomonas spp, Corynebacterium sp, and P. cepacia. The PsM27 isolates were determined by our tests to actually be P. maltophilia and not P. syringae.

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C H A P T E R I

INTRODUCTION

More than 60 years since its first appearance, Dutch elm disease (DED), caused by the fungus Ceratocystis ulmi (Buisman) C. Moreau (C. ulmi), has become a serious problem of elm trees worldwide. It was inadvertantly brought into the United States in the early 1930's. Methods to control the spread of the disease among highly susceptible American elm species have been studied up until the present time but with little or no success.

American elm, Ulmus americana L., has been widely planted in the United States and was popular as a forest species for its lumber qualities. In urban areas its hardiness, fast growth, and tall arching form provided aesthetic qualities to city streets. In open areas, especially in the western U.S., it serves an an extensively planted windbreak species. Its popularity and the consequent overplanting have led to its near demise by providing a host continuum for the pathogen to spread easily and rapidly.

The principal vector of this disease in North America, the smaller European elm bark beetle, Scolytus multistriatus (Marsh.), was found in the U.S.A. as early as 1906, a quarter century before the pathogen was introduced. Its presence further enhanced the spread of the disease by adding to the effect of the North American native elm bark beetle, Hylurgopinus rufipes (Eichhoff). The problem is augmented by the smaller European beetle's capacity

to produce 2 generations per year at Massachusetts' latitude compared to 1 1/2 generations for the native elm bark beetle (3 emergences). In the southern regions of the U.S. both beetles can produce yet more. The complex life cycle of the fungus, with its four spore stages and its ability to use at least two beetle vectors as well as to spread by root grafts, has helped it to attack many native American elm species. Control tactics have been aimed at both beetle populations, at tree populations, and at the destruction of the fungus, but as yet with no long-term benefit. These methods include chemical sprays and systemics for control of beetles, eradication and sanitation strategies, root graft treatment, systemic antifungal chemicals, breeding of resistant elm varieties, and beetle traps and lures. Most success has been obtained with a combination of all of the strategies that were available at any given time.

More recently, Pseudomonas syringae pv syringae strain M27 (PsM27) was recognized as a potential biological control agent by Dr. Gary Strobel of Montana State University, Bozeman, MT. He found that PsM27, when inoculated into elm saplings, could prevent the discoloration of xylem due to C. ulmi inoculated into the same trees 2 weeks later (118). The Ortho Consumer Products Division of the Chevron Chemical Company, San Francisco, CA, entered into an agreement in May 1980 with the Montana State University Endowment Corporation and the Minnesota Freshwater Foundation, of Minneapolis, MN, to develop and market specific strains of P. syringae for DED control. Several strains were distributed to cooperators throughout

the country to test their effectiveness in preventing or eliminating DED.

The Shade Tree Laboratories at the University of Massachusetts, Amherst, was selected as one cooperator in the project. This study is a result of the cooperative grant program with the Chevron Chemical Company.

The objectives of this study were: 1) to determine the effects of treating DED-infected American elms (street trees) with a PsM27 suspension and to compare the degree of disease progression to that in non-treated, infected elms; 2) to determine the ability of PsM27 to survive in elm xylem by comparing the recoverability of the bacterium from PsM27 injected elms to recovery of bacteria from elms injected with sterile distilled water and to non-injected trees over several seasons; and 3) to determine the ability of P. syringae to establish itself into the elm microflora of elm seedlings so that it might provide biocontrol potential throughout the elm's lifespan.

Other studies which relate to the major objectives above included: 1) the development of an improved injection system; 2) the determination of the effects on growth of elm seed treated with PsM27; 3) the determination of charactistics of PsM27 in relation to inhibition of C.ulmi and other fungi, fluorescent pigment production, and growth on elm wood substrates; and 4) the diagnostic identification of unknown bacterial isolates from elm tissue, that also inhibit C. ulmi in vitro.

C H A P T E R I I

LITERATURE REVIEW

Dutch Elm Disease: Biological Control, and Pseudomonas syringae

The limited success afforded by various chemicals for the control of DED, along with the advent of the knowledge of antibiotic compounds, led to the pursuit of possible biological control mechanisms by microorganisms and/or their by-products. More recent concern for the environment, as it is affected by chemical use, has encouraged contination of this pursuit.

As early as 1943 (115), the antibiotics actinomycin and clavacin were found to have high activity *in vitro* against the fungus then known as *Ceratostomella ulmi*. Szkolnik (110) found that a species of *Actinomyces* different from the source of the antibiotics previously tested also showed inhibition to *C. ulmi* and that this was independent of pigment production.

Holmes (40), found during 1950 to 1954, that when 22 commercially produced antibiotics were applied to growing elms and subsequently challenged with *C. ulmi*, none of the treatments prevented DED symptoms. Six of the antibiotics, however, did inhibit *C. ulmi* and *Verticillium* growth *in vitro*.

Another *in vitro* study (93) was done using antibiotics such as mycostatin, endomycin, fradicin, fungichromin, ascosin, and others

as well as plant growth regulators belonging to the phenyl carbamates. Many of these proved to be 75 to 100% effective *in vitro*.

Similarly, Peterson (84) found that eight *C. ulmi* isolates were sensitive to the antibiotic myxin (produced by *Sorangium* sp.). It was a strong *in vitro* inhibitor at 0.5 to 1.0 ug/disc concentrations using a paper disc assay.

Studies of the antagonistic compounds aureofungin (4) and nystatin (15) were unsuccessful at therapeutic treatment of DED. An unknown bacterial antagonist to *C. ulmi*, *in vitro*, was characterized by Asante and Neal (3) and identified as a *Bacillus* species. They found that the bacterium produced acidic compounds that were in themselves inhibitory to the fungus.

In 1958, May (63) reported investigating the possibility that plant compounds may be useful for inhibition of *C. ulmi*. Extracts from 21 different plants inhibited the fungus while extracts from 10 others including *U. americana*, *U. pumila*, and *U. carpinifolia* cv. "Christine Buisman" lacked inhibitory factors.

Arisumi and Higgins (2) found that it was more difficult to establish DED symptoms in young elm seedlings than older ones. Unlike May (63), Schreiber (94) found that extracts from American elm tissue of 1- to 5-month-old plants were fungistatic. When the concentration was increased, the extracts were fungicidal. The juvenile resistence factor reported by Arisumi and Higgins (2) was supported by the loss of inhibition to *C. ulmi* from extracts of

older seedlings (6-7 mo for stems, 10-12 mo for leaves). From American elm seedls, Doskotch et. al. (21) identified an antifungal agent, capric acid, which was active against C. ulmi and other fungi. These findings further substantiate the presence of a juvenile resistance factor to DED.

Several reports have indicated that biological control processes may already be in effect without the influence of any human beings who may apply materials and organisms. Webber (116,117) found that the fungus Phomopsis oblonga retarded beetle larva development in Wych elm (U. glabra). This was considered a possible factor in the observed slower spread of DED in northern England and in Scotland than had been earlier recorded in these areas. In North America, it has been suggested that the spread of DED may be affected by the presence of a double stranded ribonucleic acid (dsRNA) virus (87). The association of the dsRNA particles with non-aggressive isolates found in areas where DED has been present for a long time compared to its absence from isolates in areas more recently affected by DED suggests it may have a role as a hypovirulence factor against C. ulmi. Hoch et al. (89) substantiated that specific dsRNA segments in C. ulmi were correlated to non-aggressiveness but any overall relationship has not been determined.

One strategy for finding a potential biological control agent is to study the microflora associated with healthy growing plants. Many researchers have looked at organisms present in or associated with elm trees (1,10,46,77,78,81, 103). Jewel and Campana (46) extracted

microorganisms from American elm sap and found that bacteria present in the extracts inhibited C. ulmi in vitro. Species of Fusarium, Penicillium, Trichoderma, Bacillus, and Streptomyces were obtained from dead elm roots exhibiting DED vascular discoloration (103). Dead roots older than one year did not sustain C. ulmi growth. These organisisms inhibited C. ulmi in vitro and may, therefore, have hindered its survival in the dead root tissue.

Bacteria that have been associated with injection wounds (1) include species of Enterobacter and Klebsiella. Species of these organisms are the most commonly isolated from wetwood regions of elm wood (72). Evaluation of wetwood capillary liquids by Murdoch and Potaro (77) demonstrated the presence of Pseudomonas fluorescens and Bacillus species as well members of Enterobacteriaceae. However, Murdoch and Campana (72) associated Pseudomonas and Bacillus more frequently with sapwood not affected by the wetwood condition.

Other studies of wetwood in elm (10,71) in white fir (121) have shown the inhibitory nature of wetwood tissue to decay fungi. This inhibition may be attributed to reduced oxygen levels and the formation of methane, neither of which is conducive to growth of wood-inhabiting fungi (71,121).

The efficacy of resident elm microflora in controlling DED has been examined by use of a bacterial species (19) and by use of Klebsiella oxytoca (18). These organisms inhibited C. ulmi

by destroying synnematal heads but the development of C. ulmi in elm tissue was not prevented (18). Streptomyces species were evaluated for antagonism in vitro against C. ulmi (80,81) but, when inoculated into elm saplings, did not prevent development of DED symptoms (81). The actinomycete was still recoverable from elm sapling tissue up to 8 weeks after inoculation.

None of the previously mentioned studies were able to demonstrate any ability to suppress DED in the living tree. In 1981, Strobel and Myers (107) gave evidence that when suspensions of Pseudomonas syringae cells were introduced into 22 diseased elms (78,106,107) the amount of decline in seven of them did not increase over the period of two growing seasons. On the other hand, 21 of 22 control trees died in the same time period. Discoloration of the vascular system was also reduced (78,79,105). Scheffer (89) also tested the efficacy of Pseudomonas species inoculated into elms and found that those treated only with bacteria remained healthy for two seasons while those inoculated with C. ulmi developed severe DED symptoms. Elms injected with bacteria after C. ulmi inoculation also developed severe symptoms while those injected with bacteria prior to C. ulmi inoculation showed fewer symptoms.

Although the P. syringae isolates that were examined for biocontrol potential were not isolated from elm tissue but from other plants such as apple, pea, barley, cucumber, and others (79) this bacterium is a logical selection to be considered because of its

history in biological control, particularly in soil systems (44,61, 91,92).

There are many ways in which pseudomonads appear to affect plant disease severity. Fungistasis is the inhibition of fungal growth. A fluorescent compound produced by Pseudomonas isolates was shown by Misaghi et al. (70) to be inhibitory to growth of several fungal isolates. Also, the antibiotic, tropolone, produced by a Pseudomonas species, was fungicidal to several fungi (60). In at least one case, the antifungal nature of pseudomonads has negated biocontrol effects, such as with Trichoderma hamatum for control of Pythium (44).

Suppressive soils have been the focus of much attention and here again pseudomonads have been implicated as factors in the mechanism of that type of biocontrol (50,90,91,118,119). Moreover, many pseudomonads have been characterized as producing siderophores (9,16,33,67,68,81,111,112). Siderophores bind with iron compounds and many microbes produce them to bind iron and convert it into more readily utilizable forms. This is beneficial for higher plants which cannot produce their own siderophores (24).

Kloepper et al. (50) explained suppressive soils by showing that upon the addition of either the fluorescent Pseudomonas strain B10 or its siderophore, pseudobactin, to both Fusarium-wilt and take-all conducive soils inoculated with the respective fungi, the soils became disease suppressive. This suggests that the suppression is due to siderophore competition for iron(III). The addition of iron(III)

to suppressive soils converted them back to being conducive to disease. Similar relationships were found by Scher and Baker (92) when using P. putida and synthetic iron chelators. They found that flax, radish, and cucumber wilt were significantly reduced when the bacterium was added to Fusarium oxysporum-conducive soil and that the addition of iron chelators produced the same result. Fusarium uses iron for germ tube elongation and the bacterium competes for it. Smidt and Vidaver (101,102) isolated and characterized a bacteriocin (114), named syringacin, from a P. syringae strain indicating that these bacteria can antagonize other bacteria as well as fungi. Recently, Colyer and Mount reported the reduction of the incidence of potato soft rot, caused by Erwinia, when P. putida was applied to tubers.

Gross (33) concluded that the compound produced by P. syringae pv. syringae called syringomycin, a phytotoxin, is a ferric siderophore that may serve to disrupt cell tissues and at the same time bind iron for its own growth. Cody and Gross (9) demonstrated that the fluorescent siderophore, pyoverdine, from P. syringae pv. syringae strain B310D may be essential for iron acquisition by the bacterium. Iron may or may not be a limiting nutrient for these organisms, however, since some P. syringae strains grown in iron deficient media simply do no produce the fluorescent pigment and there is no evidence of fluorescent pigment production in situ on leaf surfaces (62). The relationships between siderophore production, iron utilization, bacteriocin

production, and their requirements for growth, survival, or pathogenicity still remain unclear.

Growth promotion of plants is another phenomenon in which pseudomonads have been shown to play a significant role. Kloepper et al. (53) correlated the presence of two strains of fluorescent Pseudomonas species (isolated from celery and potato) to the increased growth (500%) of potato plants treated with the bacteria compared to untreated controls. These plant growth promoting rhizobacteria (PGPR) colonized the entire rhizosphere of treated plants. Kloepper and Schroth (51) further determined that the growth increases were not due to bacterial products that stimulated growth but suggested that PGPR increase growth by interacting with native microflora. The relationship between microflora and PGPR was further determined to be an antibiosis effect in the root zone (52). Iron deprivation of microflora by the production of siderophores has also been suggested as a mechanism by which growth promotion occurred (49). Suslow and Schroth (108) showed that a similar antagonism by fluorescent Pseudomonas species on sugar beets to various bacteria and fungi was a biostatic effect rather than biocidal and that the establishment of high populations of PGPR on roots is related to plant growth promotion effects. It seems reasonable that an iron limiting situation, as described by Kloepper (49), would be more of a biostatic regulation rather than biocidal.

The overwhelming influence of Pseudomonas species in biological systems indicates that it might be a good choice as a biological

control agent, at least in soil related diseases. The ability of P. syringae to be an effective biocontrol agent of DED remains to be determined. It is not known if it can survive in the xylem vessels of elm trees. Resident pseudomonads have been isolated from above-ground plant parts such as leaf surfaces (58), buds of apple (5), and peach cankers (25), but the survivability potential in xylem tissue is unknown. Studies (72,73,121) indicate that anaerobic regions exist in wetwood-infected elm. It has not been determined whether the level of oxygen in elm xylem is conducive to P. syringae growth. Since P. syringae is a strict aerobe (88), oxygen availability is an important survival factor.

Another consideration in the use of P. syringae is the fact that it is a plant pathogen of many hosts (43). Green (43) and Canfield et al. (7) have reported on increases in symptoms of nursery-grown shade trees such as species of maple, pear, linden, and dogwood due to P. syringae. It is important to consider the ramifications of using a plant pathogen with such a wide host range when developing it for use as a biocontrol agent.

Tree Injection Systems

Injection of trees is not a recent concept. It has been reported that Leonardo da Vinci systemically injected apple trees with arsenic to control apple thieves (38). More recently, tree injections have been used to place systemic materials into, citrus trees (17,35),

eucalyptus (37), peach (47), chestnut (45), oak (48), pear (57), pecan (120), elm (23,28,32,40,54,55,85,86), and others.

When systemicss are used as therapeutic or prophylactic controls for diseases in large trees, the materials must be introduced uniformly into the tissue regions which are inhabited by the organisms that cause those diseases. The method of injecting the systemic material should cause a minimum of injury to the tree.

The U.S. Forest Service was successful in 1971 (32,65) in applying large volumes of solubilized benomyl using pressure injection. The apparatus consisted of a solution reservoir pressurized by nitrogen gas, flexible tubing, and injector heads.

A more simplified method of high pressure injection was used by Himelick (38) in which hollow lag bolts were used as the injector heads enabling them to be screwed into a drilled hole. These required even deeper holes than the Forest Service method (32) and because of their screw heads, the lag bolts caused greater tissue disruption.

Kondo (54) developed a method for introducing water soluble materials into elms through severed roots. This technique was similar to the other methods in that a reservoir and tubing were connected to a pressure source, but connections were made at the ends of cut roots instead of onto the tree trunk. This approach required considerable excavation around the base of the tree, the labor for which is costly and time consuming.

The Elm Research Institute (ERI) of Harrisville, NH has marketed a less complex system since 1972 (99). The low cost and simplicity of this ERI design, and the safety associated with its relatively low pressures, made this an effective system (100). Preliminary models used pipe fittings screwed into holes 12.7 mm wide and approximately 5.0 cm deep (100). Later models used plastic tap-in injector heads which were held in place by friction (99).

Holmes (42) used the ERI system to inject acid fuchsin dye into elms in order to determine patterns of solute movement, since chemical treatments by injection were producing variable effectiveness in controlling DED (41). He found that most of the dye injected into the elms went into the wood vessels of years earlier than the current years' wood (Figure 1). In American elm, however, DED is an infection of the current years' xylem. Since elm is ring-porous and depends on the current-season vessels for transport (122), control materials should be placed into those vessels. The injection spiles used for most techniques tend to block the outer xylem tissue and therefore tend to prevent uptake by the recent xylem. Placing the bulk of materials into older xylem tissue wastes expensive chemicals and also misses the target pathogen. When injection was done by severing roots as described by Kondo (54), dye was taken up mainly by the current xylem.

Besides material distribution efficiency, damage done to trees caused by injection is a major concern. Severe wounding by injection may serve to negate the benefits afforded by disease control.

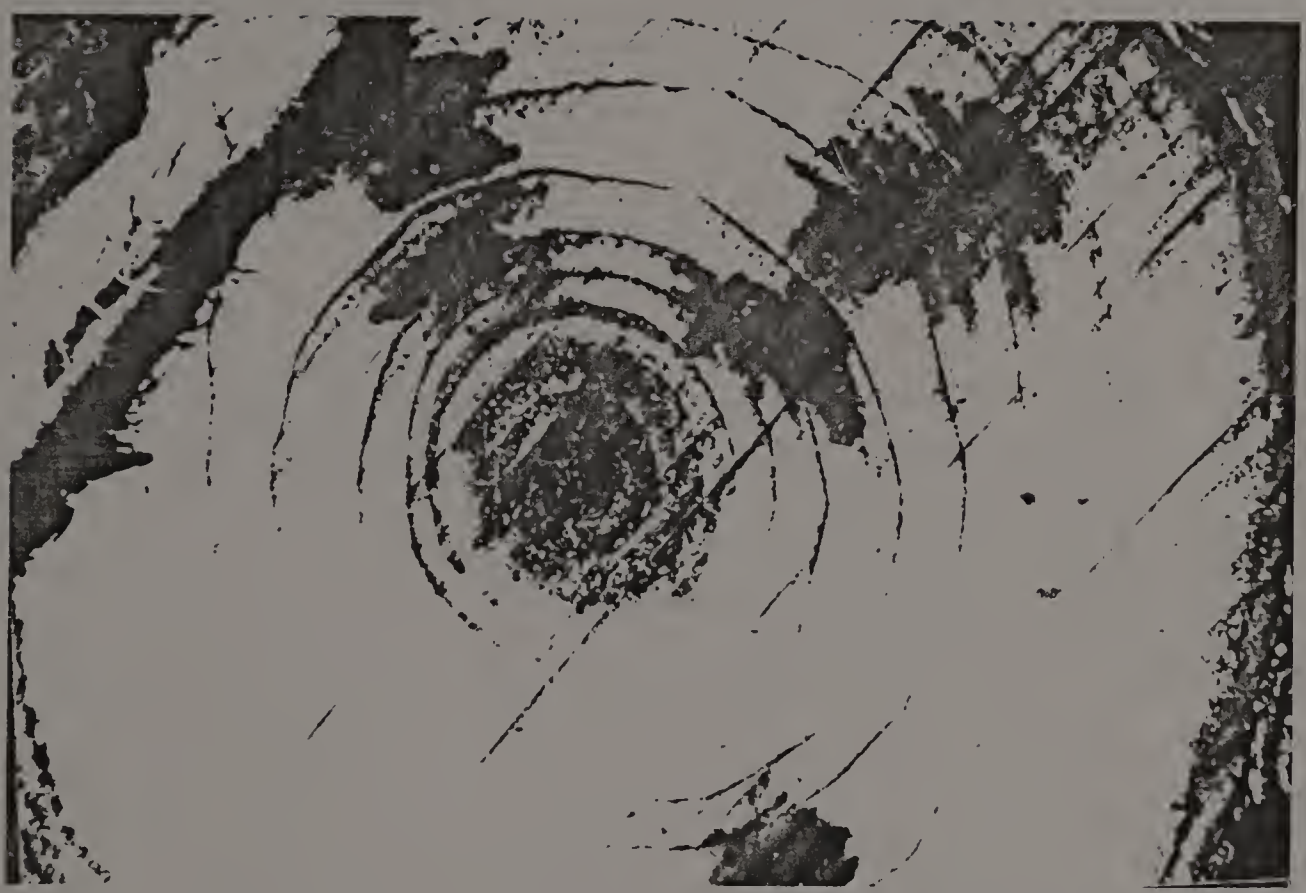

Figure 1 - Cross-section of an elm tree showing the distribution of acid fuchsin dye in the wood after application by use of the ERI injection system.

Murdoch et al. (75) associated the occurrence of vertical bark cracks with stem wounds. Also, the frequency of bleeding or fluxing of wetwood associated with injection wounds was shown to increase with depth of injection wounds (73).

Costonis (14) proposed the term "micro-injection" to represent the use of small injection holes (0.5 cm dia. or less, and to a depth of 1.9 cm or less) as opposed to the currently used practices of "macro-injection" which use larger, deeper injection holes (greater than 1.0 cm dia. and 2.5 cm depth). Techniques developed by the J.J. Mauget Company, Burbank, CA (36) were investigated by Shigo et al. (98) with regard to internal effects and wounding of trees. They found that negligible amounts of discolored wood and cambial dieback were produced with wounds made on red maple, white oak, and shagbark hickory. In another report, Shigo (96) outlined some of the important considerations for minimizing wounding. In addition to reducing injection hole width and depth, wounds should be made cleanly, placed on root flares (not root flare valleys), and care should be taken to avoid areas where other wounds occur (75,96,97). The number of wounds is also considered important and trees should not be wounded annually (96). The use of a living organism such as P. syringae as a biocontrol agent might eliminate the need for repeated wounding if the biocontrol organism can sustain a population within the tree, and if the organism can move annually into the xylem layer,so that it may continually provide protection.

The most recent contribution to injection methods was introduced by Phair and Ellmore (85). Their injector head design incorporated the practical function of placing injection materials into the outer xylem. At the same time it may minimize the damage due to the injection process by using shallow injection holes. This provided the impetus for developing the techniques used in this study to attempt placement of Pseudomonas syringae M27 into nursery-grown American elms.

C H A P T E R III

METHODS AND MATERIALS

Therapeutic Treatment of American Elms Using *Pseudomonas syringae* M27

During the period of June 25, 1981 to July 28, 1981, American elms were selected in eight Massachusetts cities and towns based on the presence of apparent Dutch elm disease symptoms in the crown. The municipalities and their respective cooperating Tree Wardens and/or Superintendents of Insect Pest Control (or City Foresters) were Lexington (Paul Mazzerall), Amherst (Stanley Ziomek), Springfield (Henry Mathiew), W. Springfield (Seth Swift), Northampton (Richard Stone), Hadley (Michael Majewski), Longmeadow (Henry Reynolds), and Agawam (Elmer Cascio).

These trees were located either along public rights of way or in public parks. A total of 37 symptomatic trees were selected of which 26 were later determined, by tissue platings and isolation into pure culture of the pathogenic fungus, to actually have natural infections of DED. The symptoms in the remaining 11 trees must be ascribed to other causes. The height of the trees ranged from 9 to 20 m determined by visual approximation to the nearest meter. The average

tree height was about 14 m. The diameter at breast height (dbh) values ranged from 35.6 to 124.5 cm with an average of 75.3 cm.

The ERI model 104 injection system (Elm Research Institute, Harrisville, NH) was used to inject 22 elms (prior to DED confirmation) with a formulation of Pseudomonas syringae M27 (PsM27) supplied in powder form by the Chevron Chemical Company (Ortho Consumer Products Division, San Francisco, CA). This 1981 formulation is herein referred to as Ps81. The apparatus is shown in Figure 2 and consisted of a large tank (60.5 L capacity), 0.635 cm i.d. Tygon plastic tubing (Norton, Akron, OH), and T-shaped plastic spiles with an i.d. of 0.4 cm. Pressure was applied to the system up to 726 g/cm^2 using a hand bicycle pump. Injection holes were made 3.5 to 5.0 cm deep and 0.8 cm dia. at 15 to 25 cm intervals at the trunk base. Injectors were placed in the region of root flares whenever possible.

The injection material was prepared by mixing one 100 g package of the formulation containing 1.0×10^{10} colony forming units (cfu)/g of Ps81 into 36 L of tap water (2.78×10^7 cfu/ml). Material preparations were done immediately prior to injection. The formation of a reddish color in the suspension indicated bacterial viability. The standard formulation packets supplied by Chevron also contained nutritional materials whose identity was not disclosed. The packages used were identified by the fundamental lot number 7190-34 with thirteen subsidiary lot numbers: -05, to -07, -97, -103, -109 to -115, and -127.

Figure 2 - Elm Research Institute pressurized injection system.

At the time of injection, the percent of wilt symptoms in relation to the total crown volume was recorded. This percent was based on a subjective evaluation by the researcher for all trees to reduce error in the evaluations which might occur if different persons rated the symptoms on different trees. Periodic observations were made during the summer of 1981 and again in early summer of 1982. The amounts of additional wilt and total wilt to date were recorded on the subsequent visits. Initial signs of wilt in the following season (1982) were considered additional to the first season since the branches that had wilted during the first season were assumed to be have been killed. Thus, 10% wilt observed in 1982 is actually 10% of that portion of the crown which had remained healthy after the 1981 damage.

Other parameters recorded include volume of PsM27 uptake, the xumber of injection holes, time period (duration) of injection, dbh, and approximate tree height.

Development of Improved Injection System

Design Features

In personal communication with Mr. William Phair of Harvard, MA the following idea arose for an improved injection technique. He had modified a standard type of injector "T" so as to reduce the size of the injection wound and so as to enable chemical materials to enter

the critical region of the elm wood, namely the outer xylem -- where active transport occurs in ring-porous species such as elm.

For our system, a 4.0 mm i.d. plastic "T", such as those used with the ERI system was modified. The tapered portion which is normally inserted into an injection hole was shortened so that only 3.0 mm remained visible after a wooden spacer and a 3.0 mm thick plastic faucet washer had been placed onto the taper. The washer was 2.0 cm wide with an 8.0 mm dia. center hole which fit tightly on the injector shaft. The dimensions of the wooden spacers were dependent on the stock they were cut from, so the taper was shortened to accommodate the variability in the spacers. Thus, only 3.0 mm of the taper was exposed for each injector. Figure 3 shows a diagram of the actual dimensions for the components of an injector.

The top center portion of the "T", which normally would receive the impact while being hammered into a tree, was drilled with a 2.5 mm bit to allow a 7d duplex type nail to be driven through the "T" and so that the nails' point emerged through the taper end. The inside diameter of the tapered portion of the "T" was increased to 5.0 mm to enhance the flow of materials around the nail. Figure 3 also shows a model injector designed by Phair which is larger in all dimensions. His design does not require the use of the spacers since that feature is incorporated into the body design. Our system has the advantage of using smaller diameter holes as well as the shallow ones. A 1.3 cm length of Tygon tubing (0.635 cm i.d.) was plugged with a small cork and this unit was used to cap the injector at the

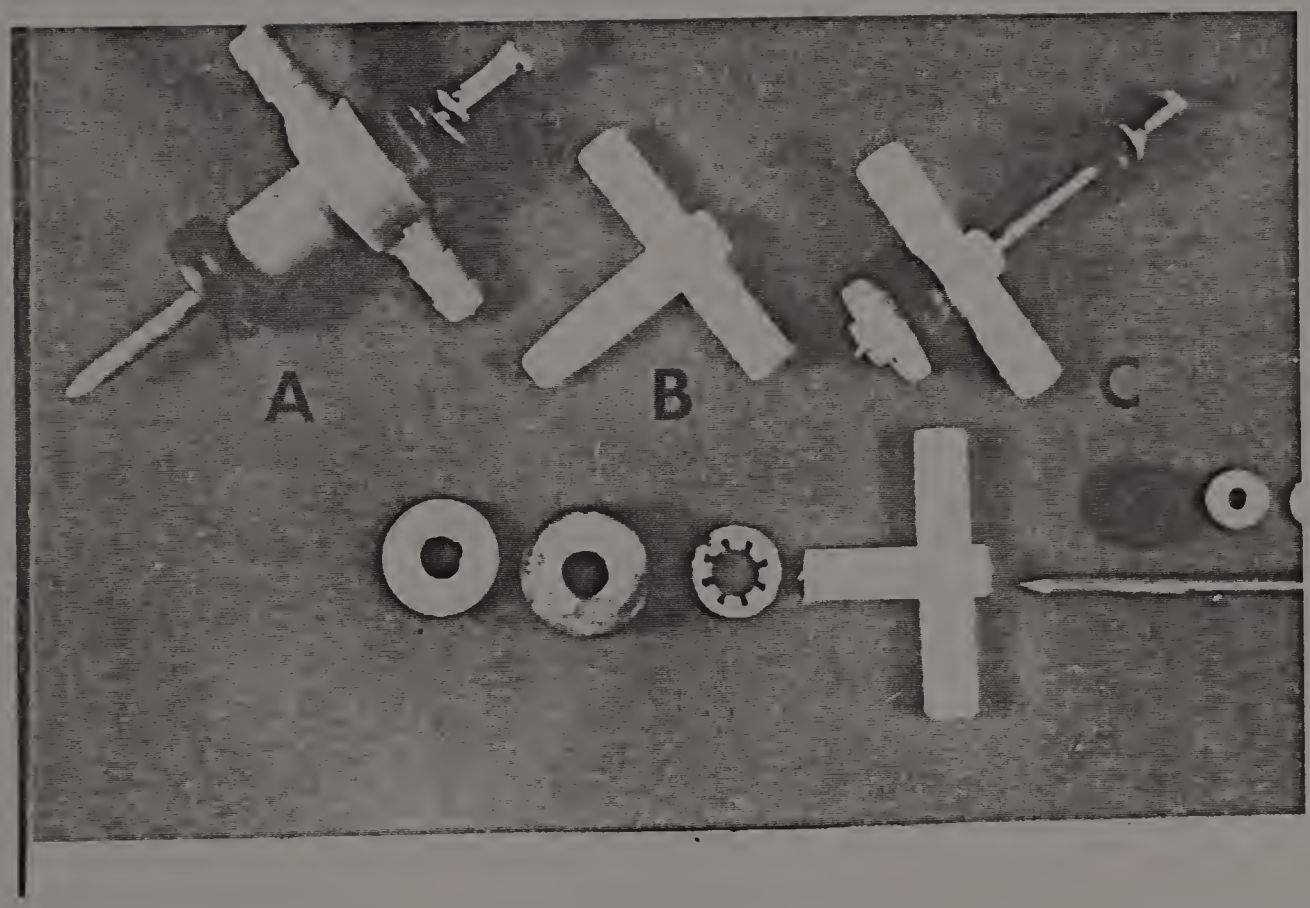

Figure 3 - A) New injector design proposed by W. Phair; B) injection "T" connector before modification into an injector; and C) modified injection "T" used in this study. The lower portion of the figure shows the various components of the injector.

exposed 3.0 mm portion of the taper. These caps enabled the entire injection unit to be filled with fluid prior to injection. A thumb screw clamp minimized the flow when the caps were removed prior to placement of each injector. A small amount of flow was desirable to keep the injection hole moist and to reduce effects due to possible embolisms.

Rubber washers were placed between the duplex nail head and the injector body so that leakage would not occur at that junction. A set of injectors was connected to a pressure tank and into a tree at up to 726 g/cm^2 to determine the ability to hold pressure without leaking.

Efficiency Tests

Several tests were conducted to determine the capabilities of the new injector design to deliver materials where necessary -- particularly the current season xylem with good distribution into the crown.

Dye Injection Acid fuchsin dye (AF) (Sigma Chemical Co., St. Louis, MO) with a 70% dye content was prepared as 1-2% solutions for injection. A gravity system was used in which 1.0 L plastic reservoir bottles marked in 25 ml increments were suspended at 1.5 m height on the tree trunk. Tygon tubing 2.0 m long (0.635 mm i.d.) was attached to the reservoir and the opposite end was attached to four of the new injectors. The injectors formed a ring with 15 cm

sections of tubing between each injector. A non-modified plastic "T" connector (Kimble Products, Toledo, OH) was placed between one pair of injectors for attachment to the reservoir/tubing unit. The reservoir bottle, tubing, and injectors were filled with the dye solution prior to preparing the injection sites. A knife was used to smooth the bark surface where the injectors were to be positioned. A wood boring bit 1.1 cm wide was used to drill holes 1.0 to 1.5 cm deep. The injectors were centered and hammered into place tightly against the bark. The plastic washer formed a seal between the injector and bark while held in place by the duplex nail. Figure 4 shows how the injectors were placed at the base on an elm.

Observations were made on the amount of dye uptake and the rate of uptake. On the same day that the injection was completed, stem cross-sections were made with a chainsaw to determine the extent of distribution.

Dye Injection of Other Trees Similar observations were made on other ring-porous tree species including black ash (*Fraxinus nigra* Marsh.) and northern red oak (*Quercus rubra* L.). One diffuse-porous species, sugar maple (*Acer saccharum* Marsh.), was also compared.

Two red oaks, with dbh measurements of 12.1 and 14.0 cm, were provided with 800 ml each of 1% AF injected by use of the system described. Injections, which were performed during the month of

Figure 4 - Placement of injectors at base of elm tree.

July, began during the morning hours and continued until complete uptake was achieved.

Similarly, two black ash, 13.6 and 15.0 cm in dbh, were injected during the same week (as the oaks) and were provided with 800 ml of 1% AF for a 24 hr period.

The two sugar maples that were injected, 13.3 and 13.6 cm in dbh, were provided with 1.0 L of the dye (1% AF) and more was added during the day as it was taken up by the trees. Dye injection occurred for 24 hr and the total uptake was recorded.

After injection of these three tree species, the bark was removed from the lower stems and the stems were cut in cross-section to observe dye movement. Also noted were the total times for dye uptake of these trees.

Chemical Injection Chemicals injected for control of DED may vary in their properties. Some may well differ from the water-soluble dyes. Because of this, the ability of control chemicals to move through the tree after injection with the new system was investigated. The presence of these chemicals in injected elms was then determined by use of bioassay techniques.

An American elm that was 13.0 cm in dbh was injected with 1.0 L of Arbotect 20S (20% 2-(4-thiazolyl) benzim- idazole hypophosphate; Merck and Company, Inc., Rahway, NJ) using the gravity system already described. Similarly, another 13.0 cm dbh elm was injected with Lignasan BLP (carbendazim phosphate; Dupont of Canada, Toronto,

Ont.). Both materials were applied undiluted over a 24 hr period after which the trees were cut down. A total of 10 leaves were randomly collected from the top portion of each tree crown. A disk of leaf tissue was cut from each leaf using a no. 6 cork borer (dia. = 0.95 cm). These disks were placed on potato dextrose agar (PDA) media plates which had been previously oversprayed with a conidial and hyphal fragment suspension of C. ulmi. [This suspension was prepared by flooding, with 25 ml of sterile distilled water, a plate of 7-14 day old cultures of C. ulmi isolated from a naturally infected elm. The cultures were rubbed gently with a sterile glass rod and the resulting suspension was transferred by sterile pipette to a sterile atomizer and then sprayed over the the surface of the PDA in the plates.]

Twig samples 1.0 to 1.5 cm long were excised from the crowns of these Arbotect- and Lignasan-treated trees. Chips from these twigs were placed on other oversprayed PDA plates in the same manner as the leaf disks.

Yet other PDA plates oversprayed with C. ulmi had single wells cut into the center of the agar by use of a no. 3 cork borer (6.0 mm dia.). These wells were filled with either the Arbotect or Lignasan compounds, at either full strength or half strength. A dilution series from $1/2^0$ to $1/2^{12}$ of Lignasan and one of Arbotect was prepared and placed in similar wells to challenge C. ulmi in culture. The sizes of the inhibition zones were observed to measured an activity curve for each compound.

Leaf disks and twig sections from an untreated elm were also plated on PDA, against oversprayed C. ulmi suspension in the manner described. This would determine inhibitory effects against the fungus, if any, due to compounds in healthy elm tissue.

Greenhouse grown elms in pots were cut at the base of the stems. The cut shoots from these were then placed into reservoirs of Arbotect or Lignasan. Twig and leaf samples from those trees were placed on PDA in plates that had been inoculated similarly with a C. ulmi suspension.

For all these treatments and controls, observations were made as to the presence or absence of inhibition zones of C. ulmi growth around the plant or around the chemical material and the zones were measured. Zone diameters were determined by averaging two perpendicular measurements of a given zone.

Wound Effects Several nursery elms that had been injected by means of the new delivery system were cut down 2 years after being injected. The extent of discoloration around the injection sites was recorded.

Evaluation of Other Methods

Single Injector Preliminary success using the new injection technique prompted the question as to whether a single injector might not suffice for distribution. Such a system then would have the

advantage of decreasing the number of wounds necessary for the injection on any given occasion.

An elm 13.9 cm in dbh was connected from the root flare region to a reservoir containing 750 ml of 1% AF dye and attached to a single injector head. Within 12 hr of complete uptake of the dye, the tree was felled and its trunk and branches were sectioned to permit observation of the dye's distribution.

Mauget Mauget capsules (J.J. Mauget Co., Burbank, CA), as shown in Figure 5, have been promoted as having the advantage that they can place materials in the outer xylem while using only relatively small injection holes (0.5 cm dia.). Clearly, therefore, this technique deserved to be compared to our new system. Four Mauget capsules were filled to their capacity (10 ml) with 1% acid fuchsin and inserted into holes 1.5 cm in depth by 0.5 cm in width which had been drilled in the root flares of an elm of 14.6 cm dbh. Four other, similarly filled Mauget capsules were placed on a second elm measuring 15.2 cm dbh. Both trees were allowed to take up dye for 1.5 hr. At that time the trees were cut and the bark was stripped to observe dye distribution.

Modified Mauget In another trial, the Mauget system was modified to accommodate larger volumes of material by connecting four of the injection nozzles, which are supplied with the capsules, to Tygon tubing (Figure 6). The tubing which had been manifolded by the use of 8.0 mm dia. glass "Y"s, was attached to a 1.0 L plastic reservoir bottle containing 750 ml of 1% acid fuchsin. After an elm

Figure 5 - Mauget capsule on elm root flare.

Figure 6 - Modified Mauget system.

tree had been injected via this system, the tree was cut, the bark peeled, and dye distribution was noted.

Pseudomonas Survival Study: Injection of Nursery Elms

Description of Study Area

The University of Massachusetts Shade Tree Laboratories maintain a nursery, in the town of Hadley, on the west edge of the Amherst campus which contains many small to medium sized elms trees (Figure 7). These are the result of a planting of elms that germinated from locally collected seed that had been irradiated previously with thermal neutrons at the Brookhaven National Laboratory, Long Island, NY. Of five million seed irradiated over a 10 year period, 30,000 lived to be planted out. The available trees had been left from the final year of this program. The vast majority of these survivors -- more than 99% -- seem unaltered in their reaction to Dutch elm disease. It must be noted, however, that any results from studies using these trees may in some undetectable way have been affected by this initial treatment.

The elms selected for the study of the survivability of PsM27 came from 3 blocks of trees within a portion of the Shade Tree Laboratories nursery. Trees were selected on the basis of their form (straight stemmed trees preferred over low forking ones), their dbh, and their proximity to other trees in the experiment (due to the high

Figure 7 - A section of the Shade Tree Laboratories Research nursery containing experimental elm trees.

probability of root grafting). In the 3 blocks considered, a total of 104 trees were so selected. Of these, 52 were designated as treatment trees (PsM27) and the remaining 52 were either of two controls: sterile distilled water injection and non-injected. Trees were further classified into two separate size classes: 7.60 to 13.9 cm dbh (class 1) and 14.0 to 20.2 cm dbh (class 2).

Block A contained 28 trees in class 1 of which 14 were treated and 14 served as controls. Also in block A were 4 more trees of class 2: 2 treated, 2 controls.

Block B contained 20 trees, all belonging to class 1. These were divided into 10 treatments and 10 controls.

Block C contained 28 trees of class 1 and 24 trees of class 2 trees. Each of these groups was divided evenly into treated trees and control trees as above. All trees were assigned to treatment type using a randomization scheme and each tree was tagged with a number designating block, row, position in row, and treatment.

Elm injections were begun on July 2, 1982 using the improved injection system previously described. A new formulation of PsM27 was provided by Chevron Chemical Co. in which the bacterium had been preserved on a wheat bran formulation. The quantity of lyophilized material was thus reduced so that one 2 g package was added per 3.78 L of sterile distilled water. The resulting bacterial concentration was expected to be 1.0×10^{10} cfu/3.78 L. Dilution plating tests determined that the number of living cells arising from the formulation was actually 8×10^{9} to 1.2×10^{10}. This formulation

was designed to decrease the problems of uptake (presumed to have been due to sugars) that had been encountered in the use of the 1981 forumulation. All packages used for injection were obtained from lot no. 477I1 (from here on referred to as Ps82).

Injections were begun in block C as a random choice, followed by block A, then block B. All class 2 trees were done first, since they required 6 injectors whereas class 1 trees required only 4 (arrangement of the work in this order compensated for the attrition rate of injectors due to breakage). Ten trees were injected on any given day, of which the five water-injected trees were done first, and then the five related Ps82 treated trees. This protocol prevented the possibility of contaminating the control equipment with bacteria through contact with hands or tools. As a further precaution, reservoirs, tubing, and injectors that were used for Ps82 injections were never used for any of the water injections.

Preparation of Bacterium

One package containing 2 g of Ps82 was emptied into an Erlenmeyer flask containing most of a 1.0 L aliquot of sterile distilled water. The remaining portion of that distilled water was used to rinse the foil package, in such a manner that these contents were added to the flask, to yield the final 1.0 L of suspension. This suspension was allowed to sit still for at least 1 minute, and then was filtered through a 60 mesh (250 μ) sieve (Central Scientific Co., Chicago, IL) to remove the bran. The filtered solution containing the bacteria

was then placed in a 1.0 L plastic reservoir connected to the injection system. The injection procedure was the same as that used for the dye experiments.

Injections were begun in the morning and the reservoir bottles were refilled as necessary during the day. The beginning volume was recorded as well as any additional volumes added. At the end of the day the reservoirs were filled and left overnight. After a total of 24 hrs, the final level of solution was recorded and the apparatus removed.

Bacterial suspensions that remained in the reservoirs after those 24 hrs were tested in the laboratory for viability by placing 1 ml aliquots onto BCBRVB selective medium (88) and observing growth and fluorescence of the resulting colonies.

A dilution series of 10^{-1} to 10^{-20} was prepared to determine what actual amount of bacteria was present in a representative package. Aliquots of 0.5 ml of the dilutions were plated on King's medium B (88). The colonies in the dilution plates (containing a range of 30 to 300 colonies) were counted to determine the cfu/ml.

1981 Formulation

To compare the relative effectiveness of the 1982 formulation of PsM27 to that of the 1981 formulation in its ability to colonize elms, five elm trees similar in size to the survival study trees (4 of class 1, and 1 of class 2) were selected for treatment with the 1981 formulation. These trees were injected using the same technique

as the experimental trees already described and the isolation results were compared.

Pressure Injection

Five elms (4 of class 1, and 1 of class 2) were selected for pressure injection, with the ERI technique at 726 g/cm^2 and with Ps82. The isolation data was used to compare gravity injection versus pressure injection for their effectiveness in the incorporation of Ps82 into elms.

Biocontrol

All nearby, DED infected elms (both within the injection plots and the remaining nursery) were removed before and during the injection period as symptoms were detected. The number of elms which exhibited DED symptoms both within the experimental group of trees and the nursery as a whole was recorded, to monitor the possible suppression of DED in Ps82 treated trees during a natural infection process. The observations were made over the course of three growing seasons (1982-1984).

Sampling

To determine the survivability of Ps82 in the experimental elms, sampling of stem tissue was begun in January 1983 using the same sequence of trees as had been used for the injection process. Ten trees were sampled on any given date. These included Ps82 treated,

sterile distilled water treated, and non-treated individuals. Care was taken to perform sampling only when weather was conducive, since rain and/or wind might lead to contamination of tissues.

Five samples were taken from each tree starting at the base 1.5 m from the ground, and then measuring upwards at 1.5 m intervals from that point. The lower 4 samples were obtained by using a hammer and a wood chisel, which was surface sterilized with 95% C_2H_5OH, to cut blocks of tissue from the stem. The blocks measured approximately 3 x 5 cm and were deep enough to include the outer bark, cambium, and at least the current and previous years' growth increments of wood. The uppermost sample (7.5 m above ground) was out of reach of the extension ladder that we used, so it was obtained by use of a pole pruner with cutting blades that had been surface sterilized with 95% C_2H_5OH. After this pruner was used to cut a small branch from the main stem, a section from that branch, 13 to 15 cm in length, was saved as the sample for isolation.

Each sample was wrapped at once in aluminum foil to prevent contact with other pieces and then labeled. During warm weather, as the samples were collected, they were placed at once into a foam cooler with a frozen icepack. Once back at the lab, the stem block samples were processed according to the technique described by Kostka (56) to remove aseptically a portion of the most current xylem. This tissue was immediately placed into 10 ml of liquid KB (without agar) for 48 hrs in an incubator set at 21-27^{o}C. The branch specimens were surface sterilized with 95% C_2H_5OH, flamed, and the bark was

aseptically peeled back exposing the non-contaminated wood. A section of the exposed xylem approximately 2.5 x 0.7 cm was removed and also placed in 10 ml of liquid KB for 48 hrs.

At the end of the incubation period, sterile loop transfers of the liquid in the tubes were made to plates of BCBRVB selective medium. After 24-72 hrs, bacterial colonies exhibiting fluorescence when illuminated with light from a long wave UV source were saved and streaked for isolation on either BCBRVB or KB media. Positive fluorescent isolates were further differentiated by means of the oxidase test (88), by the production of arginine dihydrolase in Thornley's medium (27), and by the inhibition of C. ulmi. Fluorescent bacteria that were oxidase negative, arginine dihydrolase negative, and showed inhibition to C. ulmi were considered to be PsM27 or closely related. Ps82 isolated from the 1982 formulation was used as a control. Pseudomonas aeruginosa (Pa) was used as a positive control for the arginine dihydrolase and oxidase tests.

In Vitro Inhibition Test

A C. ulmi isolate obtained from naturally infected elms in the Shade Tree Lab nursery was used to test the antibiotic production of isolated bacteria found to have characteristics similar to PsM27. The known PsM27 isolates were also tested for comparison. One- to two-week-old cultures of C. ulmi were flooded with 25 ml of sterile distilled water. These suspensions were used to overspray petri plates containing Dye's medium and histidine (Dye's) (Appendix

A) in the same manner described in the chemical injection section. The bacterial isolates to be tested had been point-inoculated with a sterile loop onto the Dye's medium 8-12 hrs prior to overspraying with C. ulmi. The plates were incubated at room temperature for several days until hyphal growth was apparent. The presence of inhibition zones was recorded as a positive antibiotic inhibition reaction.

Short-term Survival

A separate study was conducted to determine the survivability of PsM27 over shorter periods of time after injection into elm trees. The improved injection technique described was used to inject three elms with dbh values of 13.6, 13.5, and 14.9 cm. Three other elms with dbh values of 12.7, 13.3, and 12.7 cm were used as untreated controls. A culture of PsM27 with a transposon for resistance to nalidixic acid (PsM27t) was provided by Dr. Gary Strobel of Montana State University, Bozeman, MT. The bacterium was grown on a series of concentrations of nalidixic acid in KB medium so that the test bacterium to be injected was screened for resistance to 300 mg/L of nalidixic acid. A suspension the bacterium in sterile distilled water was prepared using two plates of 4-day-old cultures of PsM27t. The suspension was adjusted to 76% transmittance at 560 nm on a Spectronic 20 spectrophotometer (Bausch and Lomb Inc., Rochester, NY). A dilution series from 10^{-1} to 10^{-10} was prepared and 0.5 ml aliqots of each dilution were placed on duplicate plates of KB.

Plates with colony numbers between 30 and 300 were used to determine bacterial concentration. From the dilution tests, it was determined that between 4.06×10^9 and 4.33×10^9 cells were placed into each treated tree. Each PsM27 treatment tree received between 750 and 800 ml of the suspension.

Sampling was performed 1 day, 21 days, and 42 days after injection for both the treatment and control trees. Samples were analyzed for presence of bacteria with characteristics of PsM27 and the ability to grow on KB medium amended with nalidixic acid.

Treatment of Elm Seed

Soil Drench Test 1

The possibility that PsM27 could colonize young elm seedlings and become established as a permanent part of the vascular microflora was investigated. Such colonization could possibly give the seedlings a means of biological protection against attack by *C. ulmi* in future years. Also, if the bacterium could survive indefinitely, then preventive treatment of elms in subsequent years might be unnecessary.

To study the feasibility of this concept, a preliminary test was performed by planting elm seed collected from street trees in Old Deerfield, MA. They were placed in two 60.0 x 22.5 x 7.5 cm wooden flats. The planting medium was Terra-lite Redi-earth Peat-lite mix

(W.R. Grace and Co., Cambridge, MA). The seed were covered with the soil mix to a depth of approximately 0.7 cm.

The control flat was watered with tap water while the treatment flat was watered using a suspension of Ps81. This suspension was prepared by mixing 100 g of the powder with 3.78 L of tap water. The treatment flat was watered with the 3.78 L of bacterial suspension at the time of planting and this was repeated the following day. After these two treatments, both flats were watered with tap water as necessary for proper germination. When the resulting seedlings reached 10-15 cm in height, five stem samples from each of 10 seedlings per treatment were excised. The 2 cm long samples were split longitudinally The stem sections were placed into liquid KB tubes and the procedure outlined for identification of Ps82 for nursery elm isolations was followed. The absence or presence of fluorescent colonies was recorded after 4 days of incubation of the BCBRVB selective medium.

After sampling was completed, both flats were allowed to continue growth without subsequent treatments except for watering and fertilization with Miracle Gro (Stern's Nurseries, Inc., Geneva, NY). Both flats were exposed to the same environmental and nutritional regime after Ps81 treatment.

Soil Drench Test 2

A growth effect on these seedlings that appeared to be due to Ps81, was further investigated. A completely randomized scheme of

sixteen 10 cm plastic pots was set up in which each pot was assigned to either treatment with Ps81 or distilled water. Each pot contained approximately 80 g of Terra-lite Redi-earth Peat-lite soil mix.

Of 800 elm seed collected from Old Deerfield, MA, 50 were placed into each of the pots. The seed were spread evenly on the mix surface of each pot and covered with 0.7 cm of additional soil mix. The soil in all pots was pre-moistened with water prior to planting. An additional 100 ml of either Ps81 suspension or distilled water was applied to the appropriate pots as soon as the seed had been planted. For every application of Ps81 to the "treatment" pots, an equal amount of distilled water was applied to the control pots. All together, 500 ml of Ps81 was applied to each of the 8 treatment pots over a 14 day period. After 125 days, the differences in growth were noted and the seedlings from a sample number of pots from each group were counted and weighed (fresh wgt).

Soil Drench Test 3

A third experiment was designed to determine whether the growth effects might have been due to other factors in the formulation rather than to Ps81. A randomized experiment was set up, with twenty 10 cm pots (15 elm seed per pot), 5 of which were assigned to each of the following following four treatments: Ps81 formulation, Ps81 culture suspension (i.e. bacteria in absence of formulation), Ps81 formulation minus bacterium, or distilled water. The 1981 formulation was prepared as previosly described.

The Ps81 suspension was prepared by growing the isolated bacterium in pure culture on KB medium in petri plates for 24 hrs. Four such plates were flooded each with 20 ml distilled water and the resulting suspension was added to additional water to make final volume of 250 ml. Transmittance of the suspension was standardized to 19% at 560 nm. A dilution series was performed to determine the actual number of bacteria applied.

The 1981 formulation minus the Ps81 component was prepared by centrifuging 200 ml of the formulation (100 g/3.78 L) at 9000 rpm for 25 minutes. The supernatant was passed through a 0.45 u millipore filter (Millipore Filter Corp., Bedford, MA) to remove any remaining cells. Sample aliquots of this filter-sterilized solution were streaked onto KB medium and any bacterial growth was monitored. For all treatments, 100 ml of material were applied over a 7 day period.

An experiment, conducted simultaneosly with the third soil drench test, was conducted using soybean seed to test for possible growth effects on that species. The test was performed in the same manner as, and concurrently with, the elm seed test. The only exception to the procedure was that 7 seed were used instead of the 15 used for elm because of the larger size of soybean seed.

Characterization of PsM27

Inhibition of C. ulmi on Various Media

The ability of PsM27 to inhibit C. ulmi in vitro may be a function of the type of culture medium used for testing this phenomenon. If this were found to be true, then the ability to produce inhibition within the tree environment would come seriously into question. It is also possible that various isolates of PsM27 may provide variable responses against C. ulmi. To investigate these possibilities, several isolates of PsM27 were obtained and tested on a wide variety of laboratory media.

Ps81 and Ps82 isolates were obtained from the 1981 and 1982 formulations, respectively. PsGS was a PsM27 isolate obtained from the culture collection of Dr. Gary Strobel, Montana State University, Bozeman, MT in addition to the PsKA isolate (a mutant resistant to kanamycin) which he supplied. Each of these isolates was inoculated onto each of the following media: Dye's, KB, PDA, nutrient glucose agar (NGA) (88), King's agar A (KA) (27), malt agar (MA) (20), nutrient agar (NA) (20), and a low iron medium (MASK) (91) with or without 0.1 g/L ferric chloride ($FeCl_3$-$6H_2O$). These media represent types that are conducive to growth of either C.ulmi or PsM27 or both. The inoculated plates were oversprayed with C. ulmi suspensions prepared as previously described for growth inhibition tests. The presence or absence of growth inhibition zones was recorded.

Since PDA medium was determined to give the best visual reponse for *in vitro* inhibition of *C. ulmi* in the above tests (see Results chapter), it was selected for testing inhibition of growth from DED infected twigs. Twigs collected from the Shade Tree Laboratories nursery, exhibiting vascular browning, were cut into 2 cm pieces. The bark was aseptically removed and the pieces were split longitudinally in half to expose the discolored wood. A single split section was placed onto each of 5 PDA plates which had been previosly streaked with Ps81. The bacterial cultures were 48 hr old and well distributed on the plates. Ps82 and PsGS cultures were similarly streaked onto the same number of PDA plated and elm twig samples were placed onto the bacteria in the same manner. The plates were observe for presence of absence of *C. ulmi* growth from the twigs into the areas of bacterial growth.

Inhibition of Other Fungi

Using the same PsM27 isolates and media described above, antibiotic growth inhibition was tested for the following fungal isolates: *Leptographium thundbergii*, *L. teribrantis*, *L. sp.*, *Verticicladiella sp.*, *Ceratocystis ips*, and *Verticillium dahliae*. The presence or absence of inhibition zones was noted.

Fluorescence

Fluorescent siderophore pigments are produced by species of Pseudomonas when iron is limiting. In the presence of iron the production of the fluorescent compound can be quenched. The levels of iron concentration that inhibit fluorescence production may differ among various isolates of Pseudomonas and therefore provide another criterium for differentiation.

The isolates Ps81, Ps82, PsGS, PsKA, and Pa were grown on KB medium amended with $FeCl_3$-$6H_2O$ at concentrations of 0.04, 0.08, 0.12, 0.16, 0.20, and 0.40 g/L of medium. The presence or absence of fluorescence was determined for each isolate at each concentration using a long-wave UV source.

Growth on Elm Components

In order for Pseudomonas to survive in elm trees it must have a nutrient source. The suitability of some elm tissue components as a growth medium was investigated.

A 1.6% water agar (WA) and a an elm agar medium (EM) were prepared. The WA represented a limited nutrient source whereas the EM contained extracts from elm tissue in addition to the agar. EM was prepared by using elm stem sections approximately 1.0 cm in diameter which were cut into short pieces and quartered to expose the xylem and phloem. A total of 100 g of wood tissue was placed into a beaker containing 300 ml of distilled water and boiled for 3 minutes. The solution was filtered through no. 1 Whatman filter paper

(Whatman Ltd., England) and 250 ml of the filtrate was combined with 3.75 g of Bacto Agar (Difco Laboratories, Detroit, MI) and autoclaved.

Each of the isolates Ps81, Ps82, PsKA, PsGS, Pa, and an isolate of C. ulmi were plated onto each of the two media. Four days after the plates were inoculated, they were observed for growth of the bacteria and of C. ulmi on each of the two media.

A second experiment was performed to test whether PsM27 could grow on cellulose as a carbon source. Ps81, Ps82, PsKA, and PsM27t were plated onto either minimal agar (69) containing 1% glycerol (MinA) or onto MinA amended with 2% sodium carboxymethyl cellulose (Nutritional Biochemicals Corp. Cleveland, OH) (83). The growth differences on the two media were determined by making two diameter measurements per colony (90^{o} apart) to obtain an average colony diameter after 4 days of incubation.

Characterization of Unknown Bacteria

Many bacterial colonies were isolated from elm tissue during the attempts to isolate PsM27. Although these bacteria did not exhibit characteristics like those of PsM27, inhibition tests were performed to see whether other bacteria might turn out to be able to inhibit C. ulmi in vitro. Those isolates that did inhibit C. ulmi were then characterized by means of various diagnostic tests.

A Fisher gram stain kit (Fisher Scientific Co, Fair Lawn, NJ) was used to determine the staining properties of the unknown bacteria. The Gram stain results were substantiated by use of the potassium hydroxide (KOH) test described by Suslow et al. (108). Cell shape, colony morphology, and pigmentation were recorded.

Gram-negative, oxidase positive bacilli were inoculated into OXI/FERM tubes (Roche Diagnostic Systems, Nutley, NJ). These were prepared, sterile, multimedia tubes for the differential identification of oxidative-fermentative gram-negative rods. The diagnostic tests performed include: anaerobic and aerobic oxidative fermentation of dextrose; oxidative fermentation of xylose; production of arginine dihydrolase, of N_2 gas, of H_2S, of indole, and of urease; and the utilization of citrate as a sole carbon source.

Gram-negative, oxidase-negative bacilli were inoculated into Enterotube II tubes also obtained from Roche Diagnostic Systems, Nutley, NJ. These, too, are prepared, sterile, multimedia tubes for differentiation of Enterobacteriaceae. The tests performed by these media tubes include: utilization of glucose; production of gas, of lysine decarboxylase, of ornithine decarboxylase, of H_2S, and of indole; fermentation of adonitol, of lactose, of arabinose, of sorbitol, and of dulcitol; production of butylene glycol (Voges-Proskauer test), of phenylalanine deaminase, and of urease; and citrate utilization as a sole carbon source.

Oxidase negative organisms with negative Enterotube dextrose reactions indicate the presence of oxidase negative non-fermenters. These organisms were also inoculated into OXI/FERM tubes.

Isolates of Pa, Bacillus cereus (Bc) (from Kevin Carr, Univ. Massachusetts), Corynebacterium sepidonicum 3311 (Cs) (from Franzine Smith, Univ. of Massachusetts) and Escherichia coli (Ec) (also from F. Smith) were used as quality control test organisms. Final identification of unknowns was obtained by comparing the information obtained on these test media, against the information in a computer-coded identification system that was provided by the manufacturer of the test media.

C H A P T E R I V

RESULTS

Therapeutic Treatment of American Elms Using *Pseudomonas syringae* M27

The average uptake for all elms injected with the *P. syringae* suspension was 20 L/tree. Initially, the recommended concentration of 100 g/3.78 L would not enter preliminary test trees until it was diluted by a factor of ten. Therefore, each treatment tree in the study was injected with a concentration of 100 g/378 L. The average number of cells placed into each tree was 5.29×10^{10} as determined by dilution plating. By analysis of variance (ANOVA), the variation in volume uptake was determined not to be due to differences in tree size (5% significance level).

American elms included in the study were observed periodically during the 1981 growing season. The percent of crown wilt for each tree was determined before it was injected with Ps81 or assigned as a control. Table 1 shows the percent wilt observed for treated and non-treated trees at the start of the experiment and also the percent wilt at the end of the 1981 season. The percent increase was determined by adding the wilt values for the end of 1981 (minus starting percentage) and the amount of increased wilt observed between 1981 and 1982. The latter was adjusted to represent wilt of the portion of the tree crown remaining after 1981. Table 1 includes

Table 1 - Percentages of symptom severity in Ceratocystis ulmi infected American elms treated with Ps81 or untreated. Percentages are recorded for start of treatment, end of 1981, and end of 1982.

	Percent Wilt Symptoms			
Tree No.	1981Start	1981End(1)	1982End(2)	%Increase(3)
Ps81				
01	10	27	64	50
03	10	20	20	0
05	15	20	36	20
12	5	5	100	100
18	20	25	32	10
23	15	40	70	50
24	5	5	5	0
29	20	50	65	30
34	15	37	50	20
35	5	15	53	45
37	20	25	32	10
Average	13	24	50	30
Non-treated				
04	25	70	100	100
06	20	50	100	100
15	25	25	25	0
19	5	75	100	100
21	20	60	88	80
25	15	45	48	5
28	25	40	64	40
31	05	20	28	10
Average	17	49	69	54

(1) Values are averages of several observations during season.

(2) Values represent total % wilt since start of 1981.

(3) Percent of healthy crown remaining after 1981 times the new percent symptom value for 1982 plus the 1981 symptom value i.e. the 1982 observations are based on less than 100% of the original crown if it was infected in 1981.

only those treated and non-treated trees which (a) were confirmed by diagnostic culture tests to have DED infections and (b) also had a starting percent wilt not greater than 25%. The average wilt for treated trees at the start was 13% compared to 17% for control trees. ANOVA indicates that the difference in means between these two groups was not significant at the 5% level.

The average percent of wilt at the end of 1981 season for treated trees was 24% compared to 49% for non-treated trees. These differences were significant at the 1% level. The mean increase in wilt for treated trees (12%) was significantly smaller (5% level) than the average percent wilt increase for non-treated trees. It should be noted that some trees representing both groups did not have any increase in symptoms. There was considerable variability in the progression of DED symptoms in both groups.

ANOVA of treated tree percent wilt increase based on percent wilt at the start indicates that increases in wilt after treatment are a function of the starting percentage. Smaller starting percentages yield smaller increases in percent wilt (5% significance level) when treated with Ps81. By contrast, ANOVA of non-treated trees shows that the smaller amounts of wilt to start with were not necessarily associated with smaller final wilt values.

All trees were observed again for symptom development during the 1982 growing season. Table 1 shows the increase in wilt symptoms for treated and non-treated trees between the 1981 and 1982 observations.

The 100% values for 1982 observations represent trees that died and were cut down.

The results from the first season indicate that treatment with Ps81 has the effect of reducing the progression of symptoms in elm while symptom development in non-treated trees remains unchecked. The observations of wilt in 1982, however, indicate that it is onlyslowed in treated trees since there was no significant difference between treated and non-treated trees at that time. Overall, treated and non-treated trees ultimately reached similar levels of wilt, as indicated by a lack of significant differences in the percent of wilt at the end of 1982.

Improved Injection System

Design Features

Figure 8 shows one of the newly designed injectors mounted on a piece of wood cut in cross-section. It demonstrates the space that is created surrounding the injector tip which enables fluid to surround the tip and reach the outer xylem tissues. An injector hole 1.0 to 1.5 cm deep and 1.1 cm wide can be used with this system. The plastic faucet washer seals the chamber to prevent leaks while the nail holds the unit firmly in place. Figure 9 shows an end view of the injector unit and the space which surrounds the nail allowing for unobstructed flow as demonstrated in Figure 10.

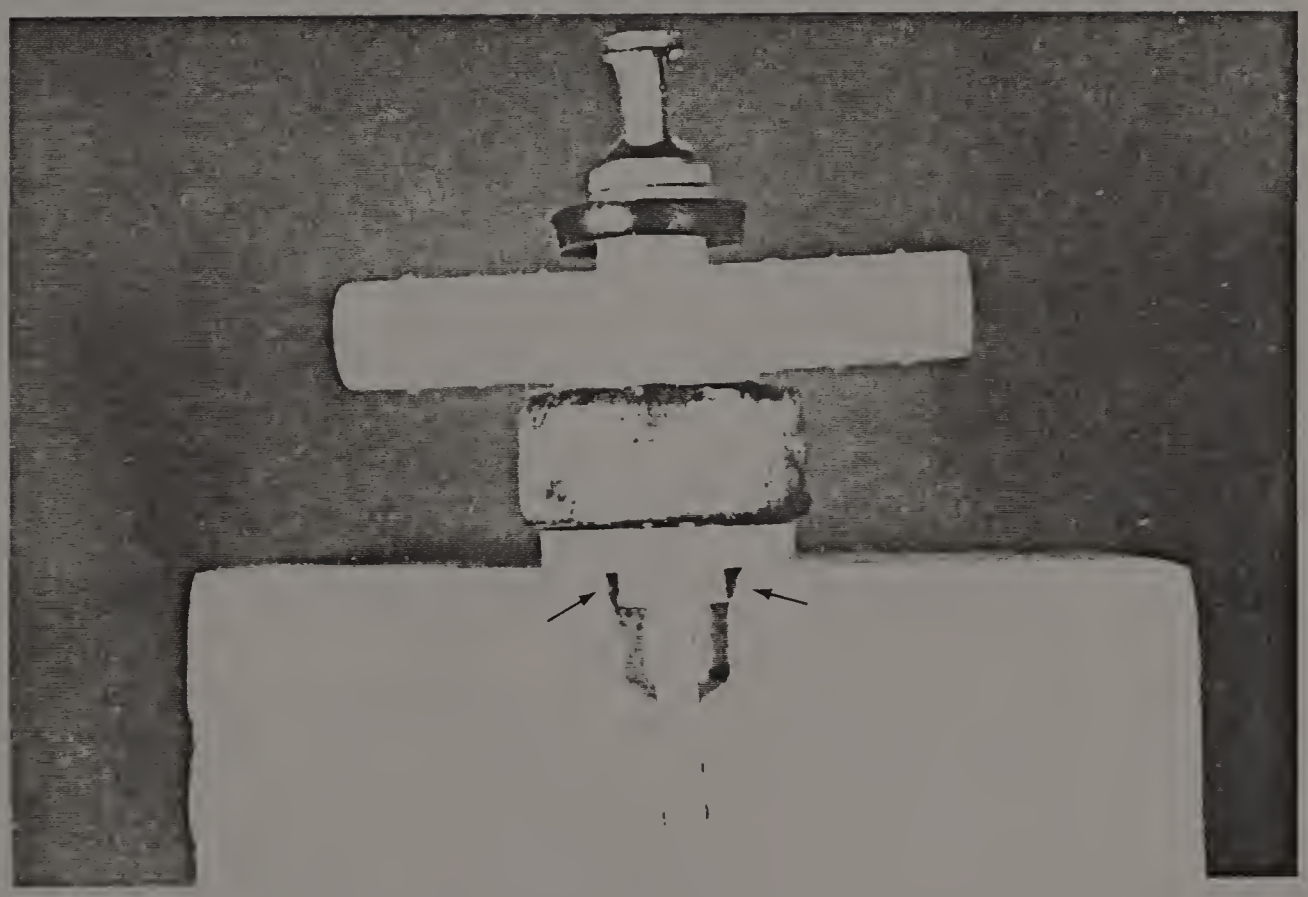

Figure 8 - A new injector mounted onto a cross-sectional piece of wood to show the space surrounding the injector tip.

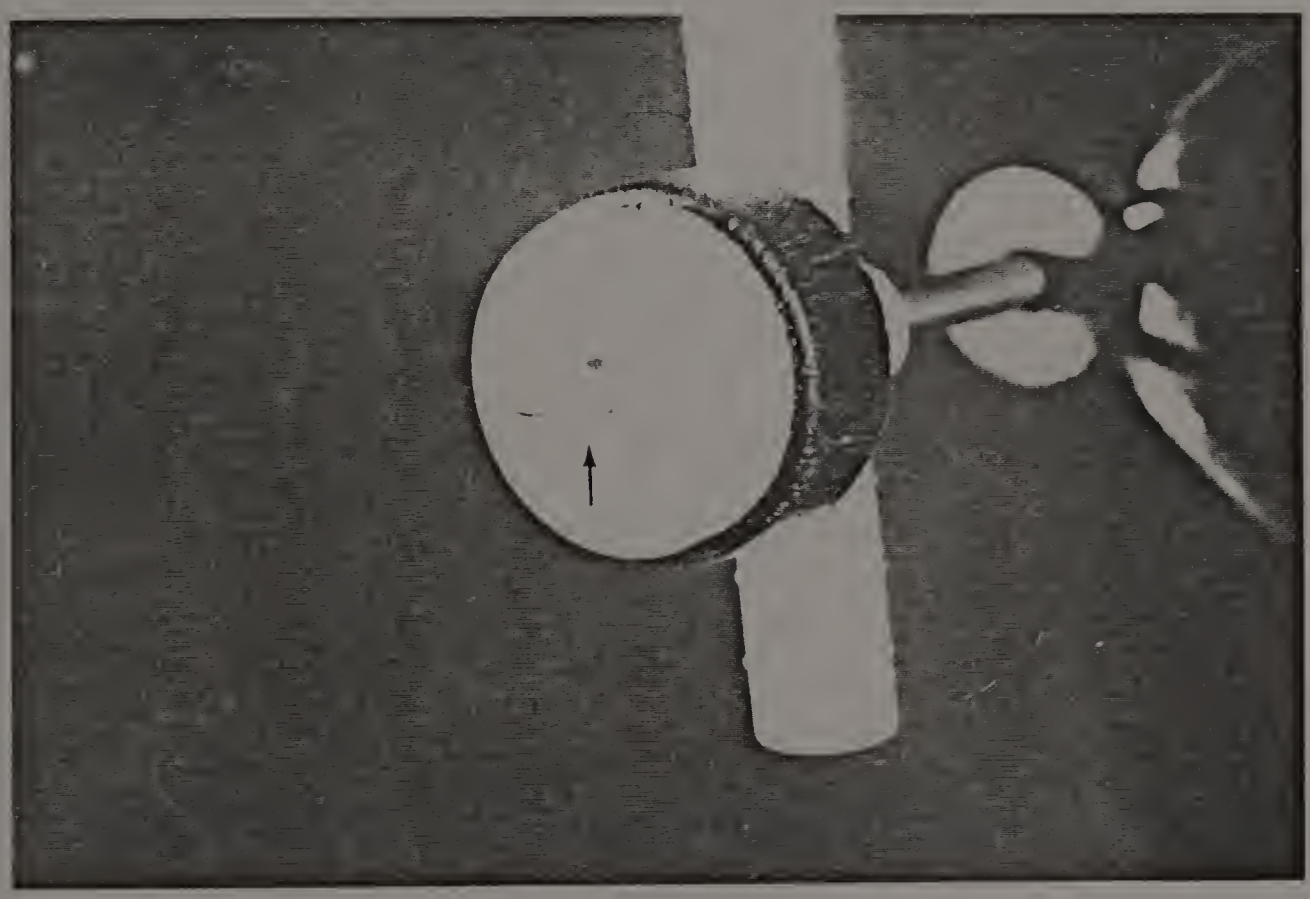

Figure 9 - Endview of injector tip showing space surrounding the nail

Figure 10 - A ring of injectors with water passing through them to demonstrate unobstructed flow.

Dye Injection

For all six American elms injected with the 2% AF during July 1982, the dye distributions were judged to be similar. The range of dye uptake was 1.45 to 4.70 L over a 24 hr period. When the bark was stripped from dye injected elms it was found that the dye moved upward and downward from the injection holes in bands that became wider up the stem until the complete circumference of the wood had dye coloration at 1.5 to 2.0 m above the point of injection. Figure 11 shows the longitudinal distribution of dye in tree number 6 (dbh = 16.5 cm), which was characteristic of other elm injections. A close-up view of the same tree (Figure 12) shows movement of dye into a branch as well as in the main stem. The same tree in cross-section (Figure 13) shows that the dye was confined to the outer xylem tissues and shows that an even ring of distribution occurred toward the top of each tree. These trees ranged in height from 7.6 to 9.1 m.

For all AF injected elms the foliage of the entire crown was changed in color from green to a magenta red due to the presence of dye in the leaf tissues. This indicated that distribution was complete.

One American elm injected in June 1984 was used as a comparison with other dye experiments. It took up 800 ml of 1% AF in 2 hr and had a distribution pattern similar to that previously described for dye injected elms. The reduced concentration of AF did not appear to

Figure 11 - Longitudinal distribution of acid fuchsin dye injected into an elm with the new injection system.

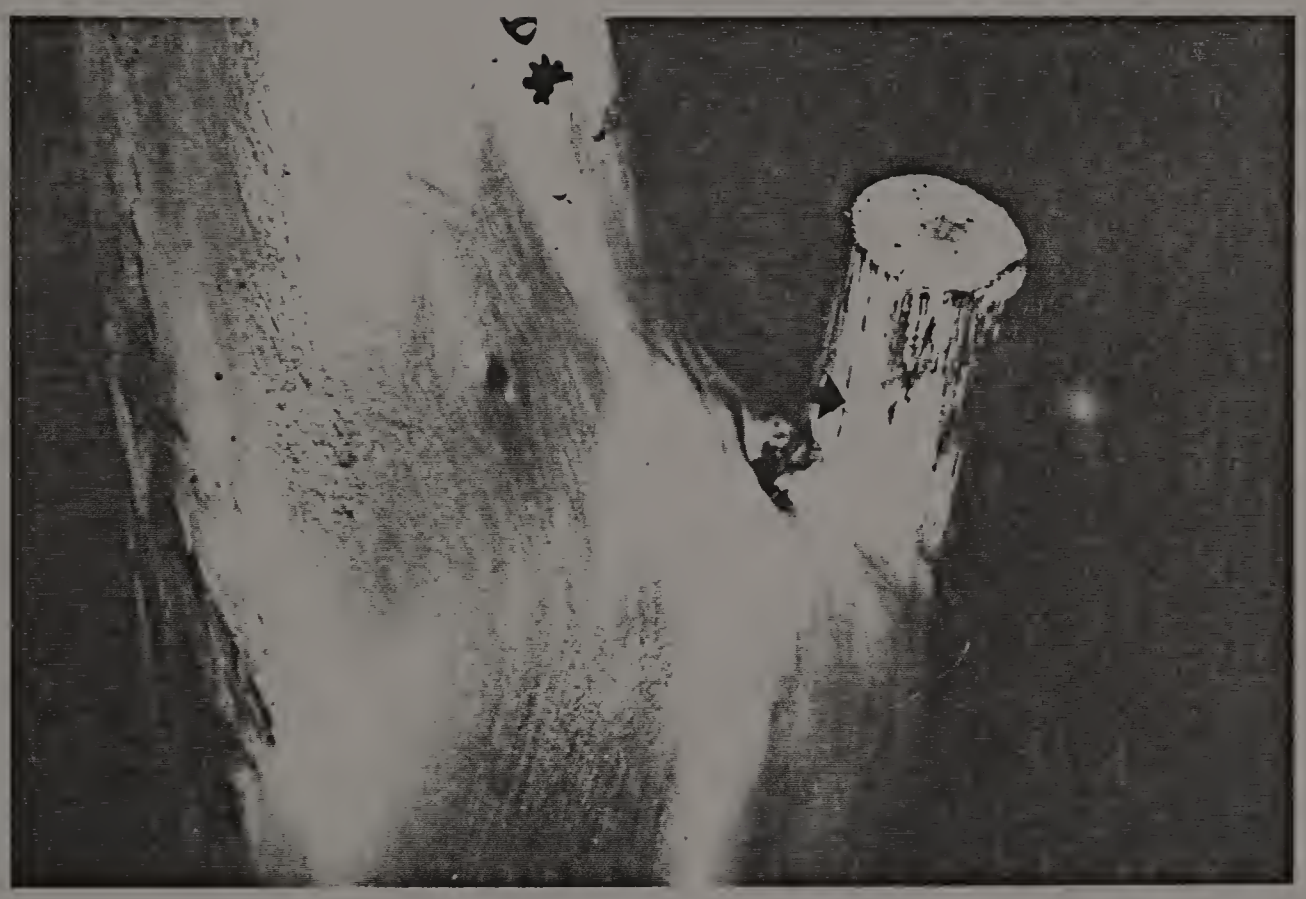

Figure 12 - Close-up of elm injected with acid fuchsin showing presence of dye in a branch.

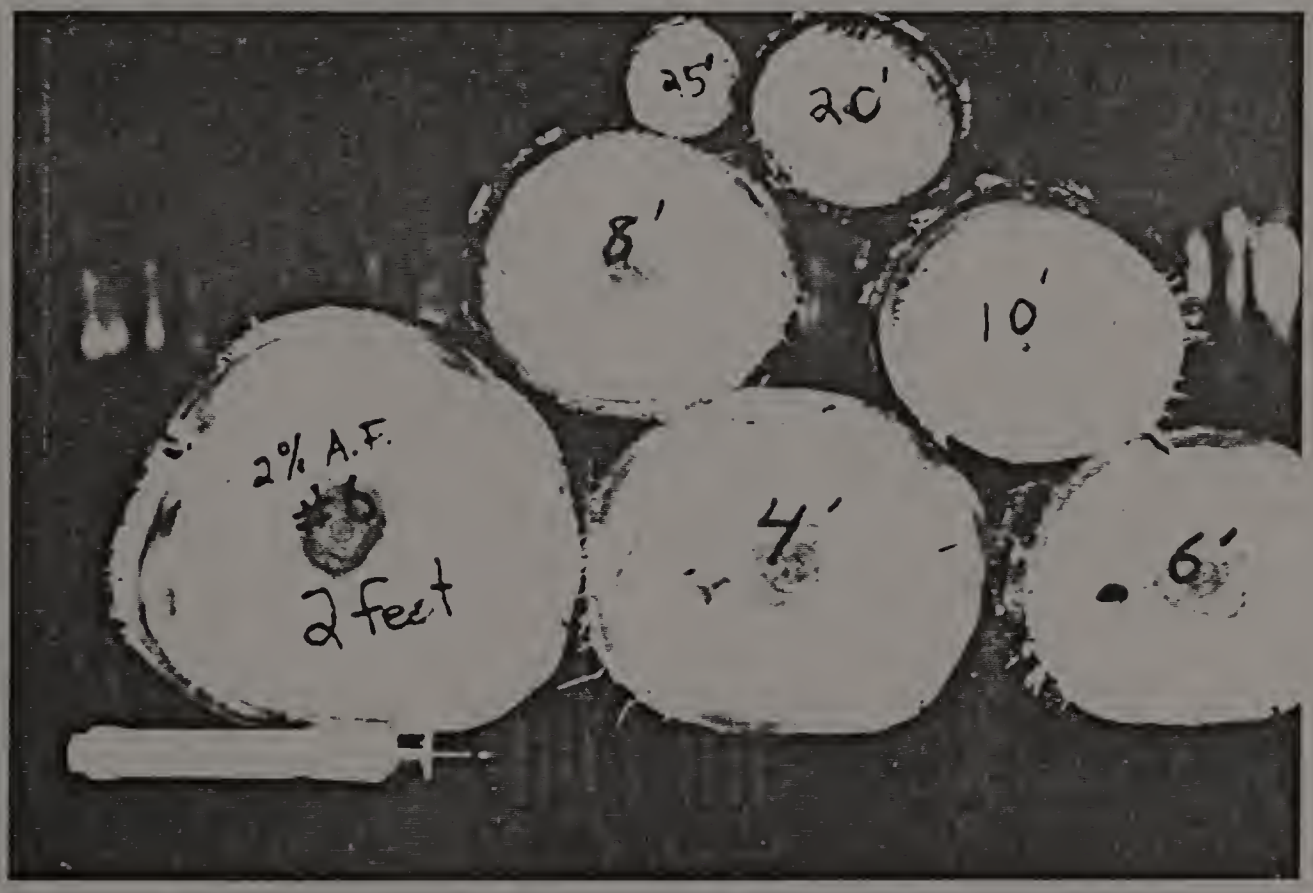

Figure 13 - Cross-section of elm injected with acid fuchsin showing radial distribution of the dye.

affect the ability to observe dye distribution. Crown foliage color was changed throughout despite the smaller volume of the dye.

Dye Injection of Other Trees

The distribution of dye in red oaks was similar to that of elms. The heights at which the total circumference of the stem was colored by the dye was 1.4 m for the 12.7 cm dbh tree and 1.6 m for the 14.0 cm dbh tree. Both trees took up 800 ml of the 1% dye within a 1 hr period. As with the elms, dye was detected at the tops of the stems and the foliage color darkened and became tinted red. Figure 14 shows a red oak twig from one of the injected trees that exhibits vascular coloration by the dye. Twigs approximately 1.0 cm in diameter were longitudinally sectioned and it was observed that the dye was confined to the very narrow region of most recent xylem tissue.

Distribution of dye in black ash was expected to be similar to that of elm and oak since it also is a ring- porous species. The 13.6 cm dbh ash took up only 125 ml in 24 hr while the 15.0 cm dbh ash took up 500 ml in a 2 hr period. When the bark was removed from the lower stems, both trees had only very narrow bands of color (approximately 1.0 to 1.5 x diameter of injection hole), a very limited longitudinal spread from the injection sites (Figure 15). Dye columns in the ash with the smallest volume uptake were undetectable at or above a height of 3 m. The ash with greater uptake also had narrow bands of dye, but these continued to the top

Figure 14 - Red oak twig with acid fuchsin dye detected under the bark.

of the tree. Radial spread of the dye was minimal but when a branch was in line with the dye band, the branch also received dye and the foliage there took on a reddish hue. There was no evidence for complete radial spread of the dye in ash.

Injections of sugar maples, having diffuse-porous vessel distribution, resulted in the least amount of dye movement for all injected species studied. The two maples (13.3 and 13.6 cm dbh) took up 1850 and 800 ml, respectively, in a 24 hr period. When the bark was stripped from the stem up to 2 m from the injection holes, only narrow bands of dye uptake were observed, as shown in Figure 16. This result was similar to that observed in ash but in no case did branches receive dye nor did foliage change color. In cross-section, lateral dye distribution was not observed much beyond the diameter of the injection hole.

In an effort to understand the different results obtained with ash and maple, stem samples from these species and from elm were used and thin slices of the xylem tissue of each were cut with a razor blade. Micro-scopic examination of the vessels revealed that ash had the largest vessel sizes while the smallest vessels occurred in sugar maple. Vessel diameter does not appear to correspond to the observed radial distribution (or lack of it).

Chemical Injection

Table 2 shows the results of bioassay for Lignasan in elm leaf and twig samples from an elm treated with this fungicide using the

Figure 15 - Black ash with bark removed to show narrow dye distribution pattern.

Figure 16 - Sugar maple with bark peeled off to show dye distribution.

Table 2 - Inhibition reactions of C. ulmi, oversprayed onto potato dextrose agar, to twig and leaf samples from nursery and greenhouse elms treated with Lignasan BLP or non-treated.

Source	#Samples	#Inhib. Zones	Mean Dia.(cm)(1)
Nursery			
twig	10	2	0.95
leaf	10	0	0.00
Greenhouse			
twig	5	5	3.48
leaf	5	0	0.00
Non-treated			
twig	15	0	0.00
leaf	15	0	0.00

(1) Based on 2 measurements at an angle of 90^{o} from one another for each inhibition zone.

new injection system. An inhibition zone was the criterion used as an indicator of the chemical. Some of the twig sections (2 out of 10) from the nursery elm inhibited C. ulmi while none of the leaf disks did. The average size of inhibition zones for twig samples was 0.95 cm. Similarly, leaf samples from the greenhouse elm did not exhibit inhibition to the fungus. All of the greenhouse stem sections did inhibit C. ulmi with an average zone diameter on 3.48 cm. None of the twig or leaf tissue samples from a non-treated elm produced any inhibition to C. ulmi, thus, eliminating the possibility that inhibition was due to plant compounds.

Stem samples from a nursery elm treated with Arbotect produced an average inhibition zone of 0.75 cm for 2 out of 10 samples tested. As with the Lignasan test, no leaf samples produced inhibition. Stem sections from a greenhouse grown elm treated with Arbotect all produced inhibition zones greater than or equal to 8.5 cm (plate diameter). In contrast to Lignasan, 3 out of 5 leaf samples from a greenhouse elm produced inhibition zones which averaged 1.77 cm in diameter. Non-treated leaf disks and twig sections did not inhibit C. ulmi. The data are summarized in Table 3.

Table 4 presents the average diameters of C. ulmi inhibition zones for dilutions of Arbotect and Lignasan ranging from 2^{-3} to 2^{-11}. These values were plotted on a graph (Figure 17) to show the dosage response for each chemical. The inhibition zone values were shown to be highly correlated at the 0.1% significance level when paired according to concentration. Calculations with values obtained

Table 3 - Inhibition reactions of C. ulmi, oversprayed onto potato dextrose agar, to twig and leaf samples from nursery and greenhouse elms treated with Arbotect 20S or non-treated.

Source	#Samples	#Inhib. Zones	Mean Dia.(cm)(1)
Nursery			
twig	10	2	0.75
leaf	10	0	0.00
Greenhouse			
twig	5	5	full plate(2)
leaf	5	3	1.77
Non-treated			
twig	15	0	0.00
leaf	15	0	0.00

(1) Based on 2 measurements at an angle of 90^{o} from one another for each inhibition zone.

(2) Inhibition zone greater than or equal to petri plate diameter.

Table 4 - Mean diameters of inhibition zones for Arbotect 20S and Lignasan BLP when placed into wells in potato dextrose agar plates at concentrations of 2^{-3} to 2^{-11} (of original concentration) and oversprayed with C. ulmi.

Concentration	Mean Inhibition Zone Diameter (cm) Arbotect 20S(1)	Lignasan BLP(1)
2^{-3}	na(2)	7.40
2^{-4}	6.60	7.00
2^{-5}	6.20	6.25
2^{-6}	5.20	5.70
2^{-7}	4.80	4.80
2^{-8}	3.70	3.90
2^{-9}	3.10	3.10
2^{-10}	2.20	2.20
2^{-11}	0.00	0.00

(1) Paired values for each chemical at the same concentration were highly correlated at the 1% significance level.

(2) Erratic growth at outside edge of plate prevented the accurate determination of diameter.

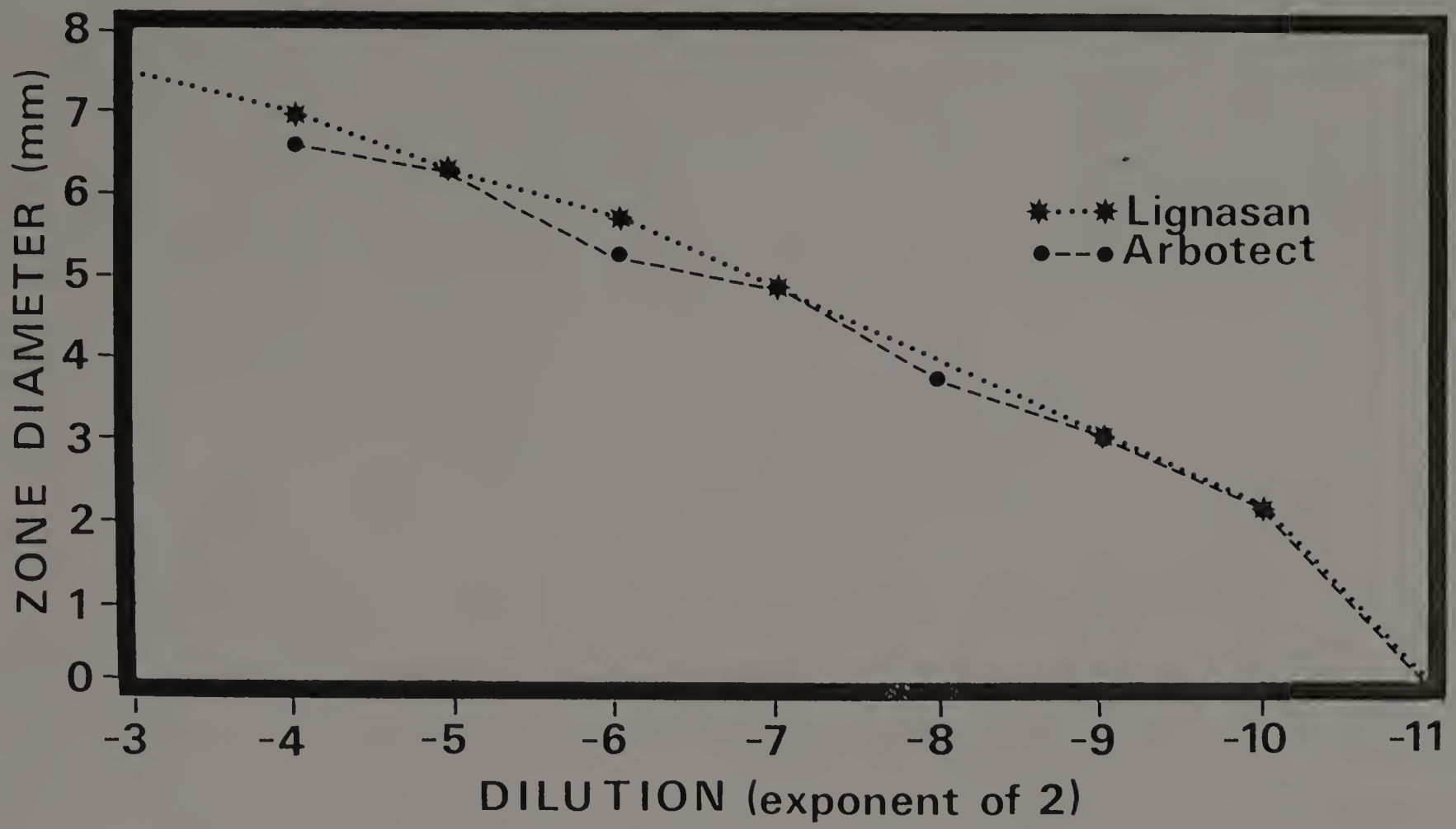

Figure 17 - Graph of inhibition zone diameter of C. ulmi versus Lignasan BLP or Arbotect 20S concentrations.

from the graph indicate that the nursery twig samples contained 0.62 µl of Arbotect per gram of tissue. The average twig weight was 0.07 g compared to leaf disks which were 0.02 g. Leaves from the Arbotect treated greenhouse elm had a concentration of 3.05 µl/g.

Calculations for Lignasan show that nursery twig specimens contained 0.65 µl/g of tissue while samples from the greenhouse elm contained 2.62 µl/g. The results suggest that these fungicides are distributed into at least the twig tissues of nursery elms when the new injection system was used.

Wound Effects

American elms that were cut and examined for effects due to wounds caused by the new injection system appeared to compartmentalize the wounds and subsequent discoloration quite well. All wounds made on each of the injected trees were healed and covered by new wood by 1982. During the course of 2 years, no wounds were observed to exhibit bleeding from wetwood. Figure 18 shows the appearance of wounds and discoloration typical of the four elms cut. The average width of discoloration was 1.53 cm and the average depth of discoloration was 2.47 cm. Figure 19 is a close- up of the typical wound reaction observed. Many of these wound areas had small cracks developing from the original wound tissue radially through the new wood towards the outside of the tree. This could lead to "frost crack" symptoms in the future (111).

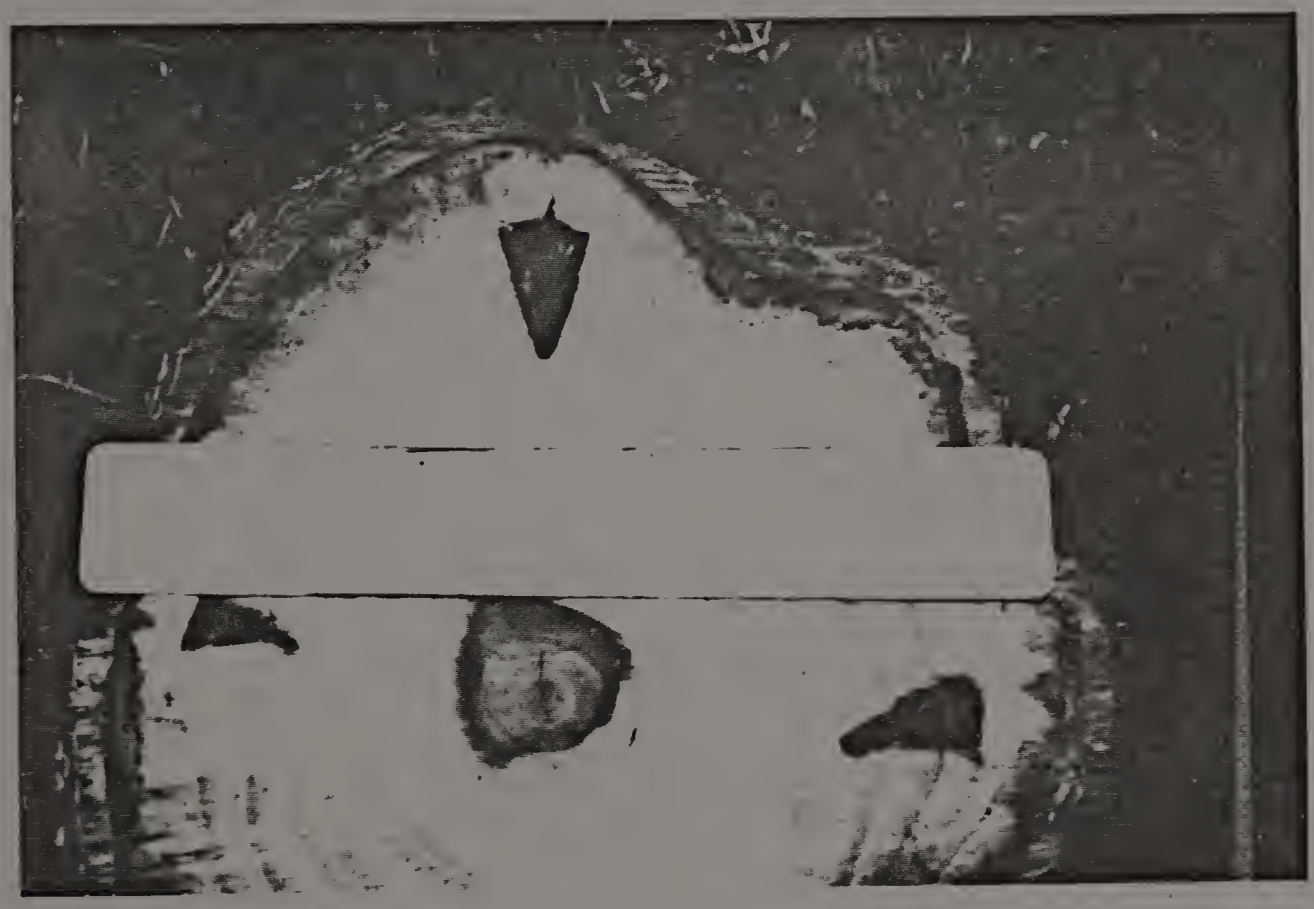

Figure 18 - Appearance of injection wounds made by the new injection system when used on elm.

Figure 19 - Close-up of a typical wound response by elm to the new injection system.

Stem sections were cut longitudinally through the original wound sites to determine the vertical discoloration present in these trees. Figure 20 shows that discoloration moved up and down much more readily than it did in the radial direction. The extent of discoloration was determined to be at least 10.5 cm up from the injection hole. Accurate determinations were not obtainable since the height of discoloration varied with the depth at which the longitudinal cut was made. The extent of downward discoloration was not determined since the injection holes were very low on the stem.

Evaluation of Other Methods

Single Injector When a single injector was used to apply AF to an elm, 750 ml of the dye were taken up within 2 hr. When the tree was cut and sectioned it was found that dye did not completely surround the entire circumference until it reached a height of 7.0 m compared to 1.5 to 2.0 m for the other dye injected elms. Also, only a portion of the crown foliage (approximatly 1/3) exhibited any color change compared to the 4 injector system which provided total crown coverage.

Mauget The two elms injected with 1% AF using 4 Mauget capsules took up the 40 ml of dye per tree (10 ml/capsule) within 1.5 hr. When the bark was removed the same day, a very narrow band of dye uptake was observed that was very faint. It was not possible to record the distribution on a photograph because of the weakness of

Figure 20 - Longitudinal discoloration of elm wood due to wounding by the new injection system.

the coloration. The dye was not detectable more than 1 m above the injection holes; and in cross-section the dye was located only immediately around the injection wounds.

Modified Mauget Figure 21 shows the longitudinal dye distribution obtained when 550 ml were taken up in 24 hr using the modified Mauget system. The tree was provided with 750 ml of dye at the start of the experiment but apparently uptake was much slower than in the other experiments using 4 new type injectors and similar volumes. Figure 22 shows that circumferential distribution occurred in the outer xylem but at 4.3 m which is higher than for the new injector system. Dye was distributed into each of half of the branched main stem (Figure 22) and approximately 50% of the crown exhibited color change. These observations suggest that fairly good distribution is achieved higher up in the stem.

Pseudomonas Survival: Injection of Nursery Elms

Fluorescent Pseudomonads

The number of fluorescent pseudomonads isolated from elm tissue samples was determined by growing isolates on KB medium and checking for fluorescence. For all 102 trees, including those treated with Ps82, sterile distilled water, or left un-treated, a total of 137 fluorescent isolates were obtained from 1405 samples (9.8%) for three sampling periods. Of these, 34 were isolated from 340 samples of water treated trees (10%) and 24 from 355 samples of non-treated

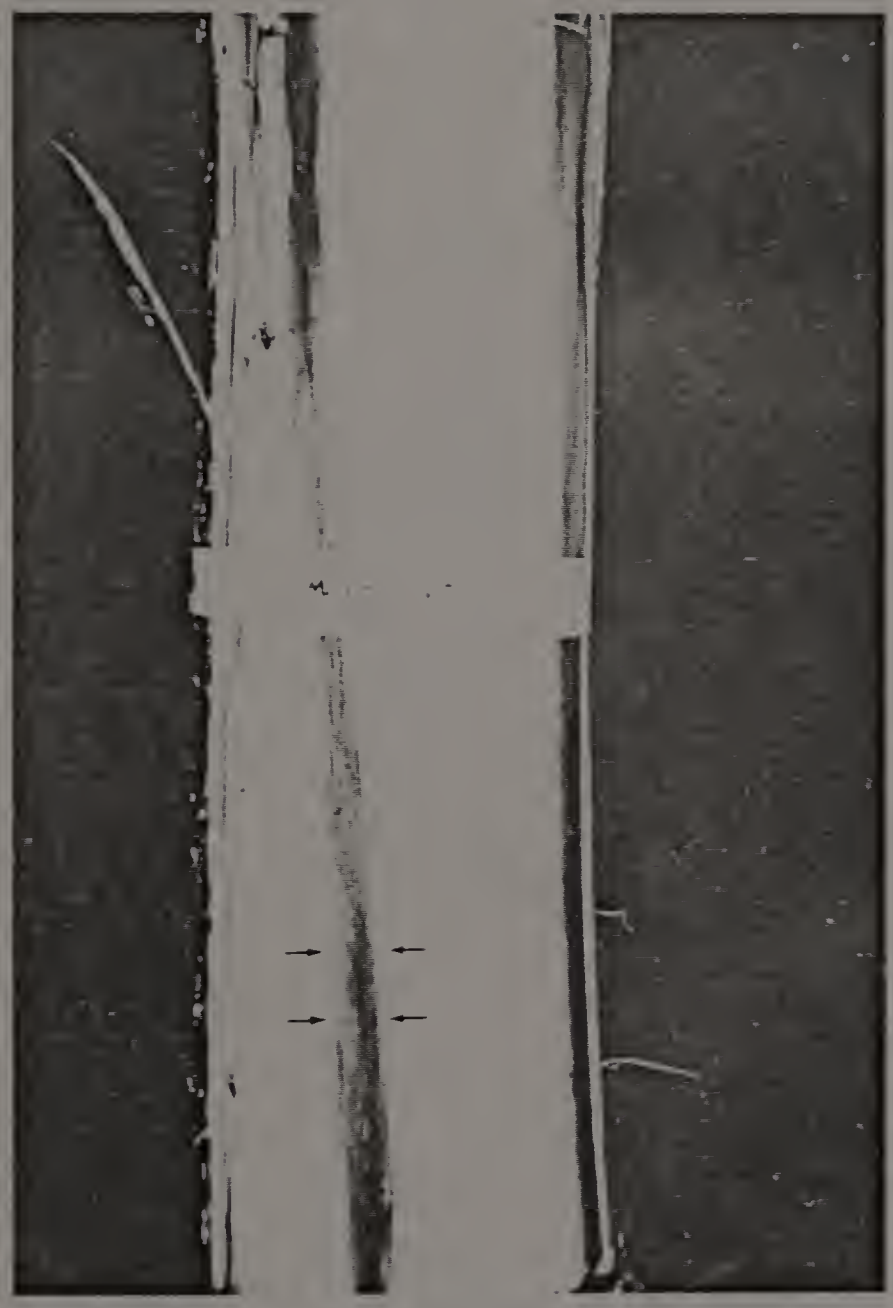

Figure 21 - Acid fuchsin dye distribution in elm injected by use of the modified Mauget system.

Figure 22 - Circumferential distribution of acid fuchsin in elm after injection by use of the modified Mauget system.

trees (6.8%). A total of 710 samples for Ps82 treated trees yielded 79 isolates that fluoresced on KB (11.1%). ANOVA revealed that the differences in number of fluorescent pseudomonads isolated between the three groups of trees was not significant at the 5% level.

A breakdown of the data into separate sampling periods was done to determine if more fluorescent pdeudomanads were present at a given period from the time of injection. Sample period 1 occurred between 5 and 9 months after injection. Sample period 2 occurred between 10 and 12 months after injection and sample period 3 began 13 months after injection and ended 26 months after the treatments.

During the first sampling, the mean number of fluorescent pseudomonads isolated was 0.6, 0.5, and 0.3 for Ps82 treated, water treated, and non-treated, respectively. These differences were not significant at the 5% level.

Similarly, there were no detectable differences between the populataion levels for sample periods 2 and 3. There was a significant difference between the fluorescent pseudomonad population means of sample period 2 (5% significance level) but the significance was between the water-treated population and the non-treated population. There were no differences between the other comparisons (water vs Ps82, non-treated vs Ps82).

Pseudomonas syringae

All fluorescent isolates were further examined as described in Chapter III and those that were fluorescent on KB and BCBRVB media,

were oxidase negative, and also were arginine dihydrolase negative, were then considered to belong to the P. syringae group of bacteria. Therefore, the isolates identified were a sub-population of the total fluorescent pseudomonads isolated. For all trees in the study, 26 of the 137 fluorescent pseudomonads exhibited these characteristics of P. syringae. Of the 26 isolates, 17 were from Ps82 treated elms while 2 were from non-treated and 7 were from water-treated elms. Comparisons of the mean number of P. syringae-like isolates for each of the treatment groups by ANOVA showed no significant difference (5% level) between them as a whole and between the different sampling periods.

Inhibitory P. syringae

Only 6 of the 26 P. syringae-like bacterial isolates were found to inhibit C. ulmi in vitro. Three of these inhibitory isolates were obtained from Ps82 treated trees, 2 from water treated trees, and 1 from a non-treated tree. The distribution of these isolates among the three groups was also not significant at the 5% level.

Inhibitory Bacteria

The total number of bacteria which were isolated and produced inhibitory reactions to C. ulmi in vitro was 25. Besides the 6 P. syringae-like isolates, 13 other fluorescent pseudomonads and 6 non-fluorescent bacteria also inhibited C. ulmi.

Inhibition by these unknown bacteria indicated that resident populations may also serve as protective agents against the DED fungus. Attempts therefore were made to identify some of the unknown bacteria; theseare described in the "Characterization of Unknown Bacteria" section.

Effects Due to Tree Size

Comparison of the total number of fluorescent isolates with tree sizes showed that the larger sized trees yielded significantly more (1% level) fluorescent pseudomonads (mean = 0.96) than smaller size class trees (mean = 0.33). The relationship between the overall number of total fluorescent pseudomonads and size class was similar (significance at 1% level) to total results of just the first sampling period, but not within the second and third. However, there were significantly more (5% level) P. syringae-like isolates associated with class 2 trees than class 1 trees during the second sampling period. The results suggest that fluorescent pseudomonads are more likely to be found in larger trees than in smaller ones.

Effects Due to Treatment Volume

The average of uptake by water-treated trees was 1.38 L which included a minimum of 0.25 L and a maximum of 3.20 L. The average uptake for Ps82 treated elms was 0.62 L with a minimum of 0.15 L and a maximum of 2.50 L. Water uptake was much greater than Ps82 with the differences significant at the 0.1% level. It was determined

that the rate of application was between 4.2×10^9 and 6.3×10^9 cells per liter. Therefore, a minimum number of 6.3×10^8 cells was applied to each Ps82 treated tree.

When the Ps82 volumes were considered alone and correlated with the total number of fluorescent pseudomonads isolated from Ps82 treated trees, the correlation was found to be significant at the 5% level. However, the volume of water also correlated to the presence of fluorescent pseudomonads at the same level of significance. Uptake volumes for non-treated trees could not be correlated since the value was zero for all individuals.

1981 Formulation

Five elms that were treated with the 1981 Chevron PsM27 (Ps81) formulation in the same manner as those with the 1982 Chevron PsM27 (Ps82) formulation were sampled for the presence of fluorescent pseudomonads, P. syringae-like pseudomonads, and inhibitory bacteria. Only one fluorescent bacterium was isolated from 25 samples and it was oxidase positive, unlike P. syringae. That one isolate did not inhibit C. ulmi. Since the number of isolates was not significantly different from that found for trees treated with Ps82 (first sampling) the sampling of these trees was discontinued.

Pressure Treatment

Five elms that were treated with Ps82 which was applied by use of 726 g/cm^3 pressure were sampled for the presence of fluorescent and P. syringae-like pseudomonads. From 25 total samples, no fluorescent isolates were obtained. Sampling of these trees was discontinued since differences between the number of isolated pseudomomads from them compared to the main experimental trees could not be detected (5% level).

Biocontrol

The number of American elms attacked by DED in the Shade Tree Laboratories nursery, in the form of natural infections, was recorded over the 1982 to 1984 growing seasons. Previously infected trees had been removed from the study area prior to the experiments. The killing of 258 elm trees by DED during this time period indicated that a natural infection process was actively occurring. Of the trees that died during this time span, 75 died during the summer of 1982, 61 in 1983, and 122 in 1984. The possibility that treatment with Ps82 may affect the rate of disease progression was determined by monitoring the number of experimental trees affected by DED. The number of Ps82 treated trees that died was 13 while the number of water treated and non-treated trees that died was 5 and 8, respectively. The water treated and the non-treated figures can be added together since neither treatment would be expected to decrease the disease rate. The disease rate of 25% was the same for both the

Ps82 treated group and the combined water and non-treated groups indicating that DED disease progression was not affected in trees treated with the bacterium.

Short-term Survival

The number of fluorescent and P. syringae-like pseudomonads isolated from trees treated with a nalidixic acid resistant mutant of P. syringae M27 (PsM27n) was used to compare the ability of P. syringae to survive in elm for shorter periods of time after injection than in the previous studies mentioned above.

Fifteen samples obtained from the PsM27n treated trees 24 hr after injection yielded 7 bacterial isolates, but none of these 7 isolates fluoresced on KB medium. Similarly, the 15 samples from 3 non-treated trees yielded 12 bacterial isolates, of which none were fluorescent. When sampled 21 days after injection, the PsM27n treated trees yielded 13 bacterial isolates, again none fluorescent. Twelve bacteria were obtained from non-treated trees and 1 did fluoresce. This fluorescent isolate also grew and fluoresced on BCBRVB selective medium, but was oxidase positive and arginine dihydrolase positive indicating that it was not of the P. syringae group. It also did not grow on KB agar amended with 300 mg/L of nalidixic acid.

The third sampling of these trees occurred 42 days after injection. Out of 14 bacterial isolates from PsM27n-treated trees,one fluoresced. A total of 15 bacterial isolates were obtained

from non-treated trees and 2 were fluorescent. All the fluorescent isolates from both tree groups grew and fluoresced on BCBRVB medium, were oxidase positive, and were arginine dihydrolase negative. None of the 3 isolates grew on KB with nalidixic acid. The results of further identification of these bacteria appear in the Characterization of Unknown Bacteria section.

The results indicate that the PsM27n bacterium put into the trees could not be re-isolated during the span of 42 days after injection. The differences in the number of fluorescent pseudomonad isolates between the treated and non- treated trees from a total of 45 samples per group were not significant at the 5% level.

Treatment of Elm Seed

Soil Drench Test 1

For both the Ps81 treated seed and the water treated seed, the samples obtained from the resulting seedlings yielded no fluorescent pseudomonads. However, 60 days later the Ps81-treated seeds produced seedlings which were strikingly larger and darker green than the water-treated elms. Figure 23 shows the dramatic differences between the two treatments. The following results were obtained while investigating the apparent growth enhancement phenomenon.

Soil Drench Test 2

The number of germinated plants per pot was monitored over a 2 week period. The average number of plants per pot at the end of 2

Figure 23 - Growth differences observed in elm seedlings resulting from seed that were treated with Ps81 (A) or water (B).

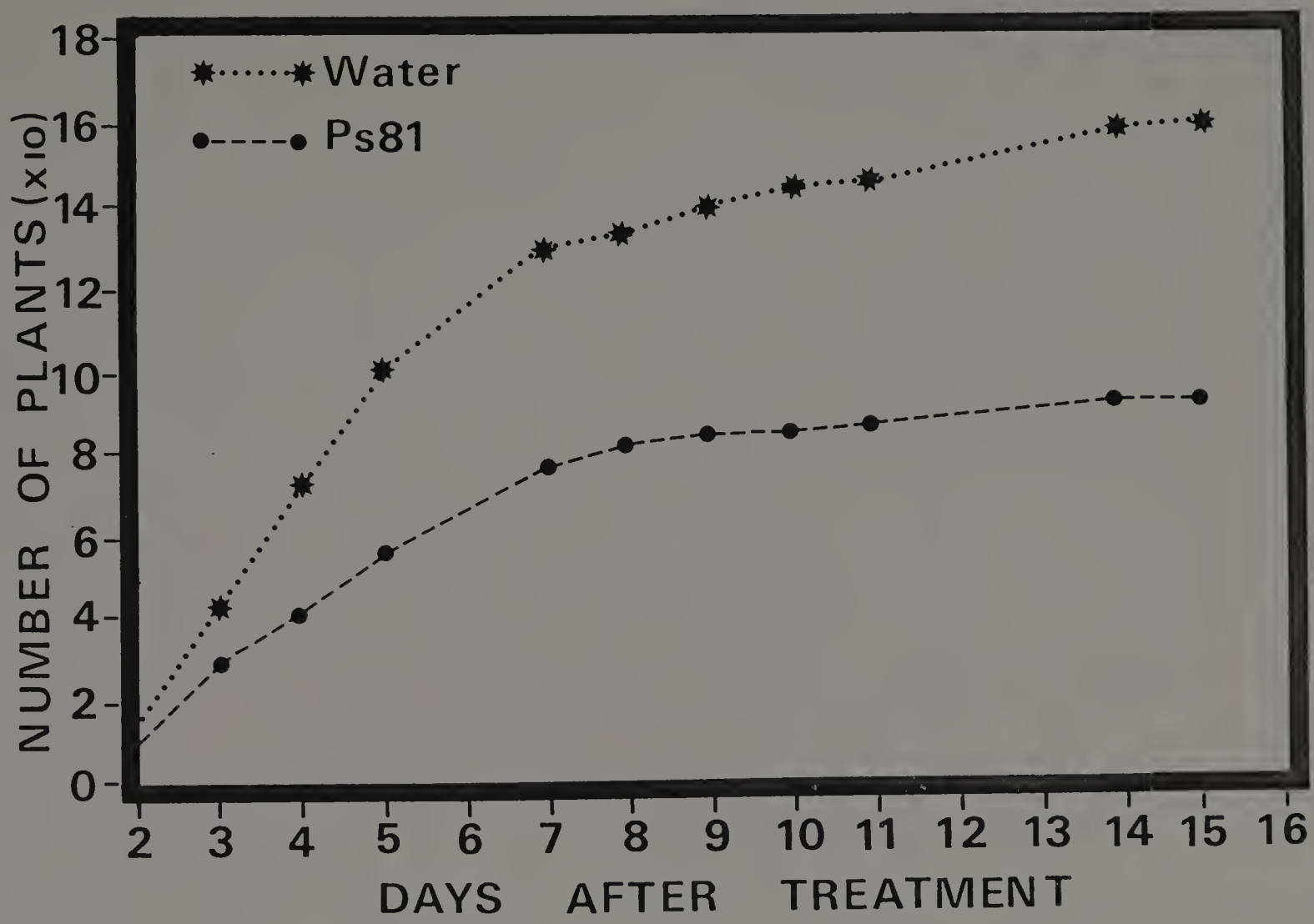

Figure 24 - Graph of the number of elm seedlings produced by germinating elm seed that were treated with either Ps81 or water during a 15 day period.

weeks, for Ps81-treated pots, was 11.9. The average number of water treated plants was 20.4 at the end of 2 weeks. The difference between these treatment means is significant at the 1% level. The number of plants per pot was observed daily and Figure 24 shows the relationship between the number of plants and the number of days after each treatment. The graph indicates that for all observation dates, the water treated pots contained more plants than the Ps81 treated pots. Differences in the numbers became significant on the third observation date (5 days after treatment) at the 5% level. The significance increased to the 1% level by the fourth observation date (6 days after treatment) and was maintained for the rest of the 2 week period.

Plant shoot fresh weight was determined for a random selection of Ps81 treated pots and water treated pots. The plants associated with Ps81 treatment appeared much larger than those associated with water treatment. The average plant weight for the Ps81 treatment was 2.20 g compared to 0.49 g for the water treatment. These differences were significant at the 0.1% level. Visual differences in leaf color were detected as the Ps81 treatment produced darker green leaves than the water treated plants which appeared chlorotic and nutrient deficient.

Soil Drench Test 3

Three replications of this experiment to separate the factors which may be involved in the observed growth promotion effect were performed. No significant differences were found to occur between

the treatments of Ps81 formulation, Ps81 culture suspension, Ps81 formulation minus bacterium, and distilled water. Similarly, growth promotion effects were not observed when the same treatments were applied to soybean seed.

Characterization of PsM27

Inhibition of C. ulmi

Table 5 shows the results of growing the 4 P. syringae isolates on 9 different media in the presence of the C. ulmi isolate from Chevron (C. ulmi C). Inhibition of C. ulmi C by Ps81 occurred on Dye's, PDA, MASK, MASKFe, and MA. Inhibition of the same fungus by Ps82 occurred on the same media. PsGS and PsKA isolates did not inhibit C.ulmi C in vitro. The best observable reaction of inhibition for both Ps81 and Ps82 was on PDA because the fungus grew better on the areas of medium outside the inhibition zone, thus making the zone appear more defined.

Figure 25 shows the degree of inhibition by Ps81, Ps82, and PsGS. PsGS actually appears to encourage the growth of C. ulmi C on Dye's medium. Likewise, the growth of C. ulmi C on the Ps81/Ps82 plate appeared to be more dense in the regions closest to the boundaries of the inhibition zones. Dense areas of growth of the fungus near the inhibition zone boundaries were observed on other media where inhibition occured. In addition, isolate PsGS appeared

Table 5 - Results of inhibition test on 9 different media using Ps81, Ps82, PsGS, and PsKA isolates of P. syringae on plates oversprayed with C. ulmi C (Chevron isolate).

	Isolate			
Medium	Ps81	Ps82	PsGS	PsKA
Dye's	+ (1)	+	-	-
KB	- (2)	-	-	-
PDA	+	+	-	-
NGA	-	-	-	-
KA	-	-	-	-
MASK	+	+	-	-
MASKFe	+	+	-	-
MA	+	+	-	-
NA	-	-	-	-

(1) + = inhibition

(2) - = no inhibition

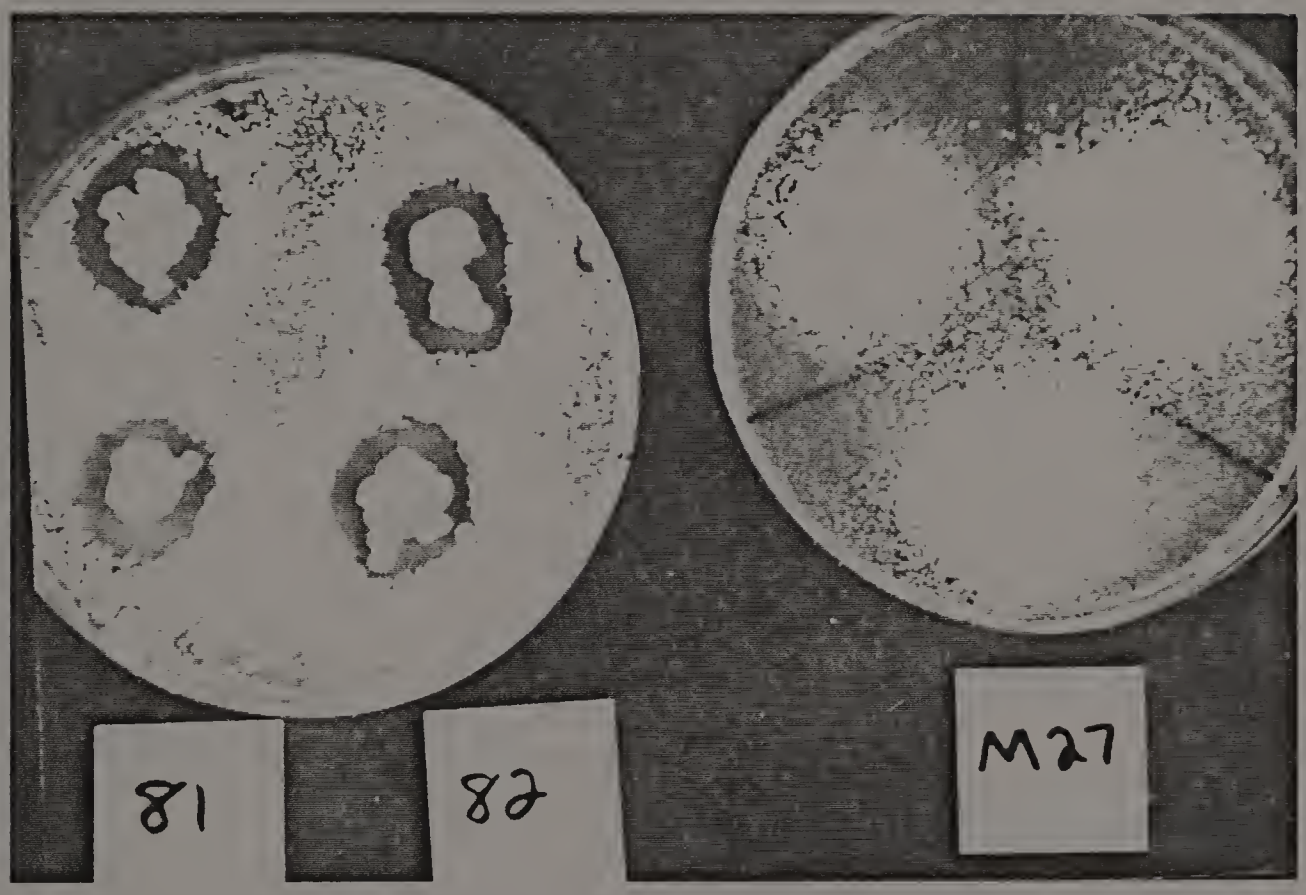

Figure 25 - Inhibition responses of C. ulmi S oversprayed onto the surface of potato dextrose agar previously inoculated with Ps81, Ps82, or PsGS (designated M27 in figure).

to enhance the formation of fungal coremia on the bacterial colonies (Figure 26) on MA.

Table 6 presents the results of testing the same bacteria on the same 9 media using an isolate of C. ulmi obtained locally from the Shade Tree Laboratories nursery (C. ulmi S). Inhibition of the fungus was observed to occur only for isolate Ps81 growing on MASK medium. Interestingly, Ps81 seemed to enhance coremia formation on NGA and MA. Ps82, PsGS, and PsKA also enhanced coremia formation on MA. Split twig samples infected with C. ulmi placed on plates containing the 4 bacterial isolates showed no fungal growth emanating from the twigs when Ps81, Ps82, and PsKA were in the plate. Figure 27 shows the occurrence of enhanced coremia production when PsGS was placed in association with the C. ulmi S isolate. There appears to be considerable variation among isolates of P. syringae M27 in their ability to cause inhibition of C. ulmi. It seems to be dependent on the growth medium used and the particular fungal isolate.

Inhibition of Other Fungi

Table 7 shows the results of growing various fungi in the presence of Ps81 inoculated onto the 9 test media. Leptographium sp., L. terebrantis, L. thundbergii, Ceratocystis ips, and Verticicladiella sp. were all inhibited by Ps81 on PDA. Verticicladiella was also inhibited on Dye's medium and Leptographium sp. was inhibited on MA. Figure 28 shows the

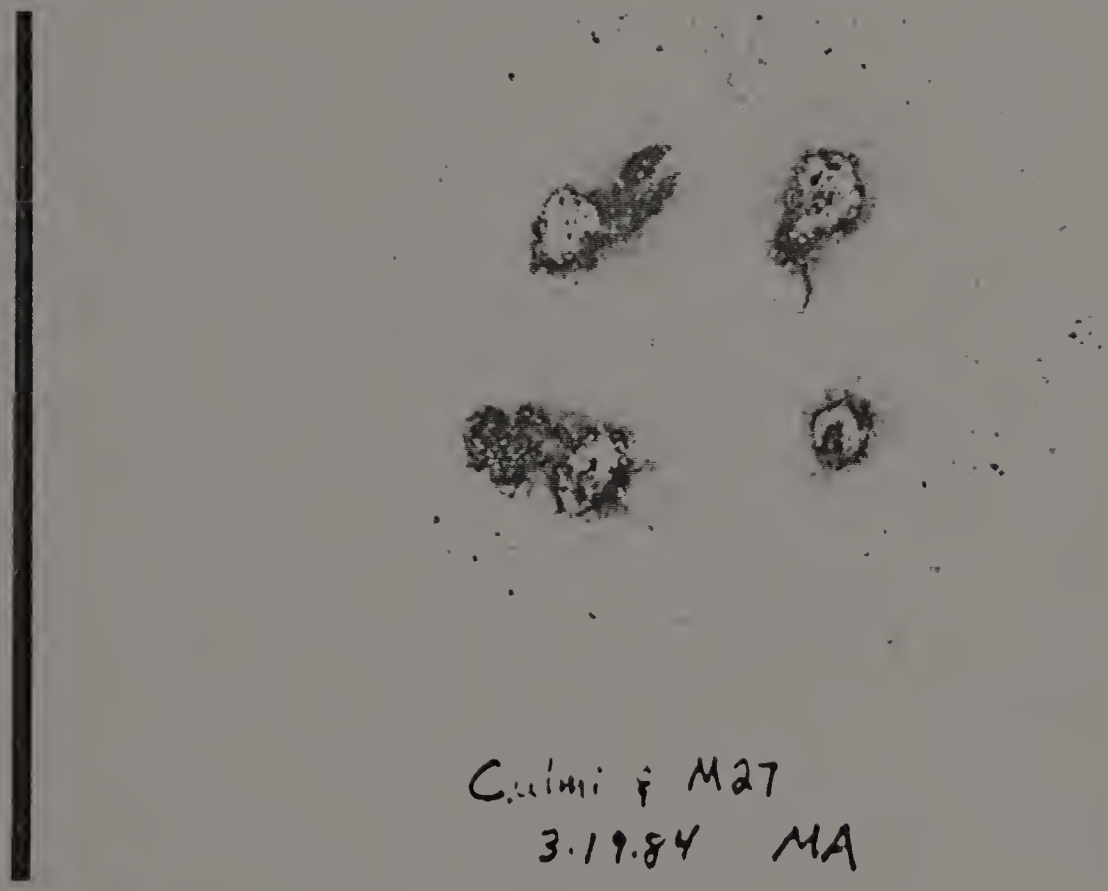

Figure 26 - Enhancement of coremia formation of C. ulmi S on the bacterial colonies of PsGS (M27) when grown on malt agar.

Table 6 - Results of inhibition test on 9 different media using Ps81, Ps82, PsGS, and PsKA isolates of P. syringae on plates oversprayed with C. ulmi S (Shade Tree Lab isolate).

Medium	Ps81	Ps82	PsGS	PsKA
Dye's	-(1)	-	-	-
KB	-	-	-	-
PDA	-	-	-	-
NGA	-(2)	-	-	-
KA	-	-	-	-
MASK	+(3)	-	-	-
MASKFe	-	-	-	-
MA	-(2)	-(2)	-(2)	-(2)
NA	-	-	-	-

(1) - = no inhibition

(2) coremia formation

(3) + = inhibition

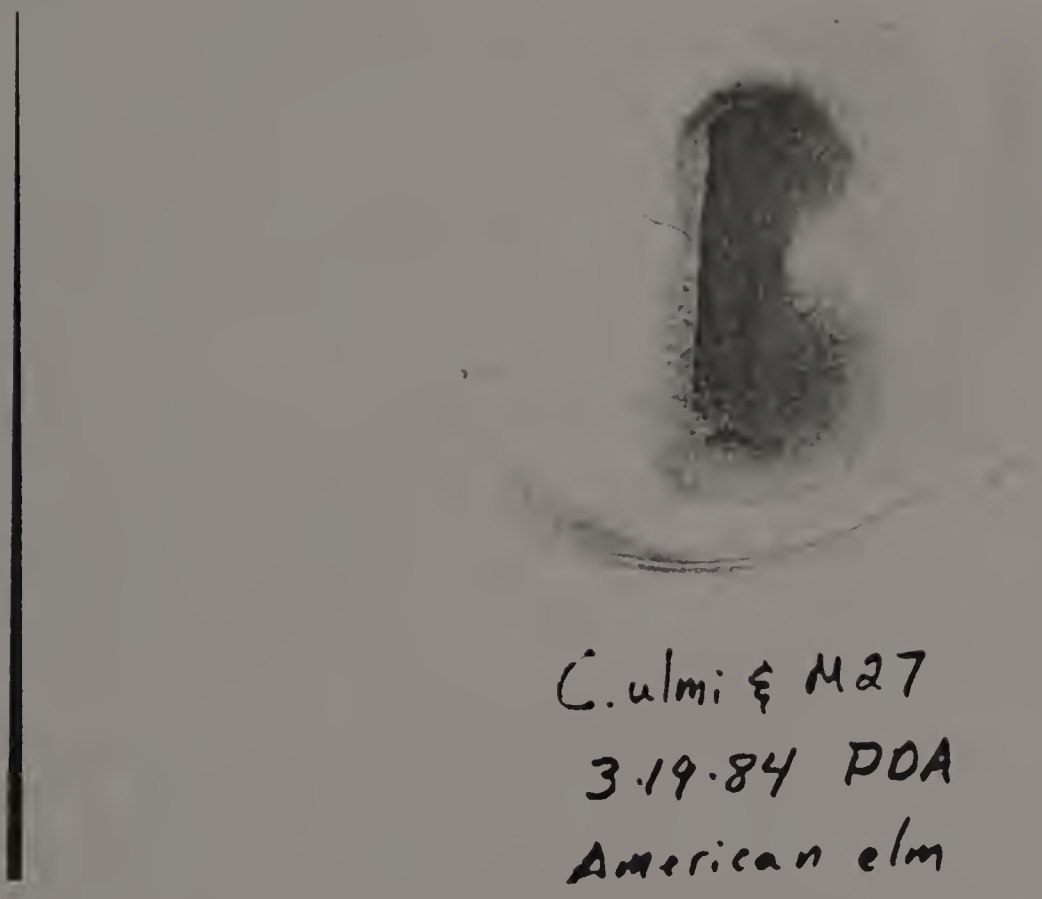

Figure 27 - Enhancement of coremia production from C. ulmi S mycelium emanating from a DED infected elm twig placed on a culture plate with PsGS.

Table 7 - Inhibition with Ps81 on 9 different media oversprayed with 6 fungal isolates.

	Fungal Isolate (1)					
Medium	Lsp	Lte	Lth	Ci	Vsp	Vd
Dye's	-(2)	-	-	-	+	-
KB	-	-	-	-	-	-
PDA	+(3)	+	+	+	+	-
NGA	-	-	-	-	-	-
KA	-	-	-	-	-	-
MASK	-	-	-	-	-	-
MASKFe	-	-	-	-	-	-
MA	+	-	-	-	-	-
NA	-	-	-	-	-	-

(1) Lsp = Leptographium sp.; Ci = Ceratocystis ips;
Lte = L. terebrantis; Vsp = Verticicladiella sp.;
Lth = L. thundbergii; Vd = Verticillium dahliae

(2) - = no inhibition

(3) + = inhibition

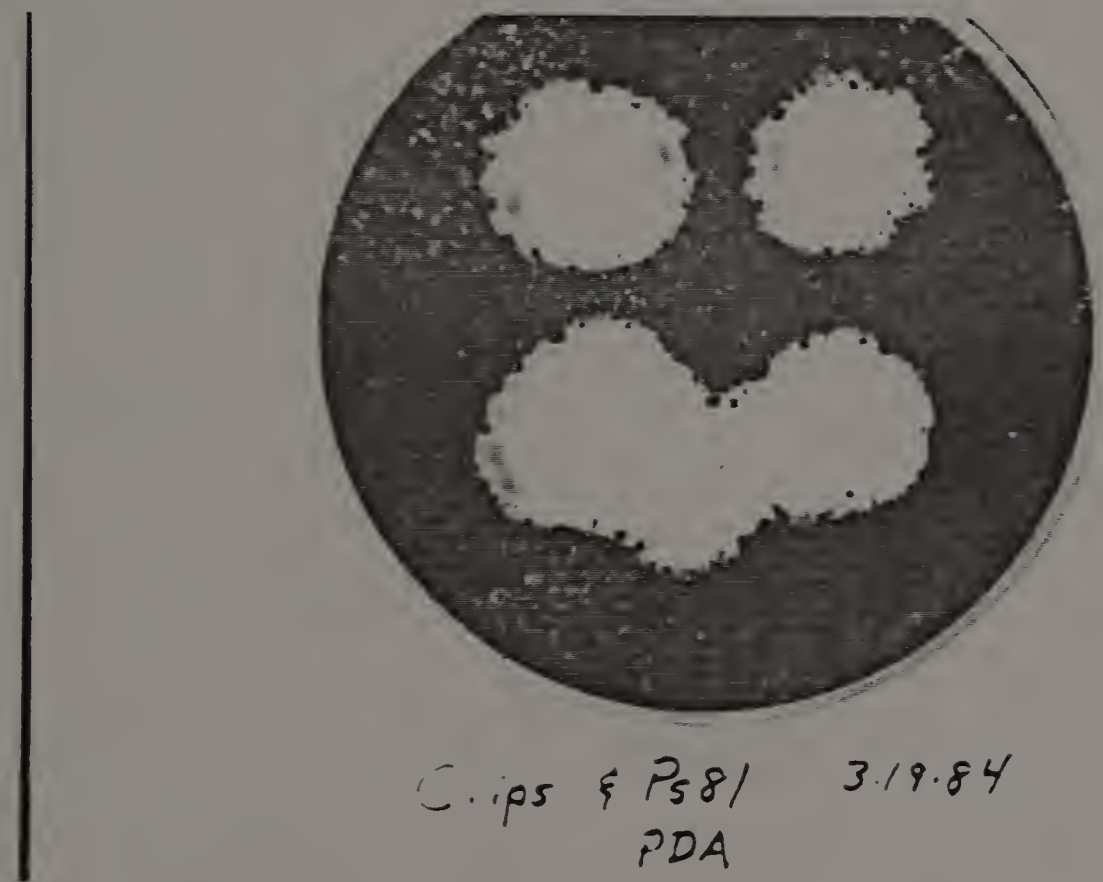

Figure 28 - Inhibition of C. ips by Ps81.

inhibition observed when Ps81 was oversprayed with C. ips on PDA. It represents the type of reactions that were observed for the other inhibited fungi.

Verticillium dahliae was not inhibited on any of the media. PsGS was tested in the same manner and was found to not inhibit any of the fungi.

Fluorescence

Table 8 summarizes the results of growing Ps81, Ps82, PsKA, PsGS, and Pa on KB medium amended with concentrations of ferric chloride ranging from 0.04 to 0.40 g/L. Pa fluoresced at the lowest concentration of 0.04 g/L of ferric chloride and not at concentrations above that. Ps81, Ps82, PsKA, and PsGS all fluoresced at 0.04, 0.08, 0.12, and 0.16 g/L of ferric chloride. Fluorescence was quenched in all of them when they were grown on 0.20 and 0.40 g/L concentrations. Thus, no differences were observed between the four P. syringae isolates in terms of iron and its effect on fluorescence, but there were differences between these isolates and Pa.

Growth on Elm Components

When Ps81, Ps82, PsKA, PsGS, and Pa were streaked onto WA, no growth was observed after 4 days. All the isolates grew when streaked onto EM (Table 9). The amount of growth appeared no

Table 8 - Fluorescent compound production by 4 P. syringae isolates and P. aeruginosa growing on 6 different concentration levels of ferric chloride in King's medium B.

	Concentration of Ferric Chloride (g/l)					
Isolate	0.04	0.08	0.12	0.16	0.20	0.40
Pa	+(1)	-(2)	-	-	-	-
PsKA	+	+	+	+	-	-
PsGS	+	+	+	+	-	-
Ps81	+	+	+	+	-	-
Ps82	+	+	+	+	-	-

(1) + = Fluorescent

(2) - = Non-fluorescent

Table 9 - Evaluation of growth responses of 4 P. syringae isolates and P. aeruginosa on water agar (WA) and elm medium (EM).

Isolate	Growth Medium WA	EM
Pa	-(1)	+(2)
PsKA	-	+
PsGS	-	+
Ps81	-	+
Ps82	-	+

(1) - = No growth

(2) + = Growth

different from that observed on other bacteriological media. Fluorescence did not occur on this medium.

When Ps82 was grown on either MinA or MinA amended with 2% sodium carboxymethyl cellulose, no differences were observed in rate or type of growth. Table 10 shows the average diameters of colonies 72 hr after point inoculation. ANOVA for 5 colonies per plate and 4 replicates per treatment showed no significant difference between colony sizes at the 5% level.

Characterization of Unknown Bacteria

During the 1984 season, six unknown bacteria that inhibited *C. ulmi* (nursery isolate) were isolated. Five of these isolates, designated as 57C3, 78A3, 89D3-1, 85A3, and 91A3 were determined to be Gram negative bacilli. One Gram positive bacillus, 71D3, was isolated. All of these were isolated from different trees and 3 were isolated from treated trees. The other 3 were from water treated and non-treated trees (1 and 2, respectively). Gram reactions were confirmed by the KOH test (199). Figure 29 shows the type of inhibition typically observed, here represented by isolates 85A3 and 89D3-1. Three non-inhibitory fluorescent isolates from the short-term survival study were also evaluated for similarities to Ps82.

Table 11 summarizes some diagnostic characteristics of inhibitory bacterial isolates, that separate them for purposes of further

Table 10 - Mean colony diameters for Ps82 on minimal agar or minimal agar amended with 2% sodium carboxymethyl cellulose.

Medium		Mean Colony Diameter (cm)(1) Repl	Rep2	Rep3	Rep4
MinA+CMC					
colony	1	0.80	0.75	0.92	0.75
	2	0.75	0.70	0.80	0.75
	3	0.75	0.70	0.95	0.78
	4	0.78	0.62	0.85	0.70
	5	0.75	0.70	0.82	0.65
MinA					
colony	1	0.70	0.70	0.78	0.65
	2	0.85	0.75	0.70	0.68
	3	0.75	0.70	0.78	0.88
	4	0.80	0.65	0.78	0.88
	5	0.80	0.70	0.80	0.75

(1) 2 measurements per colony taken 90° from each other.

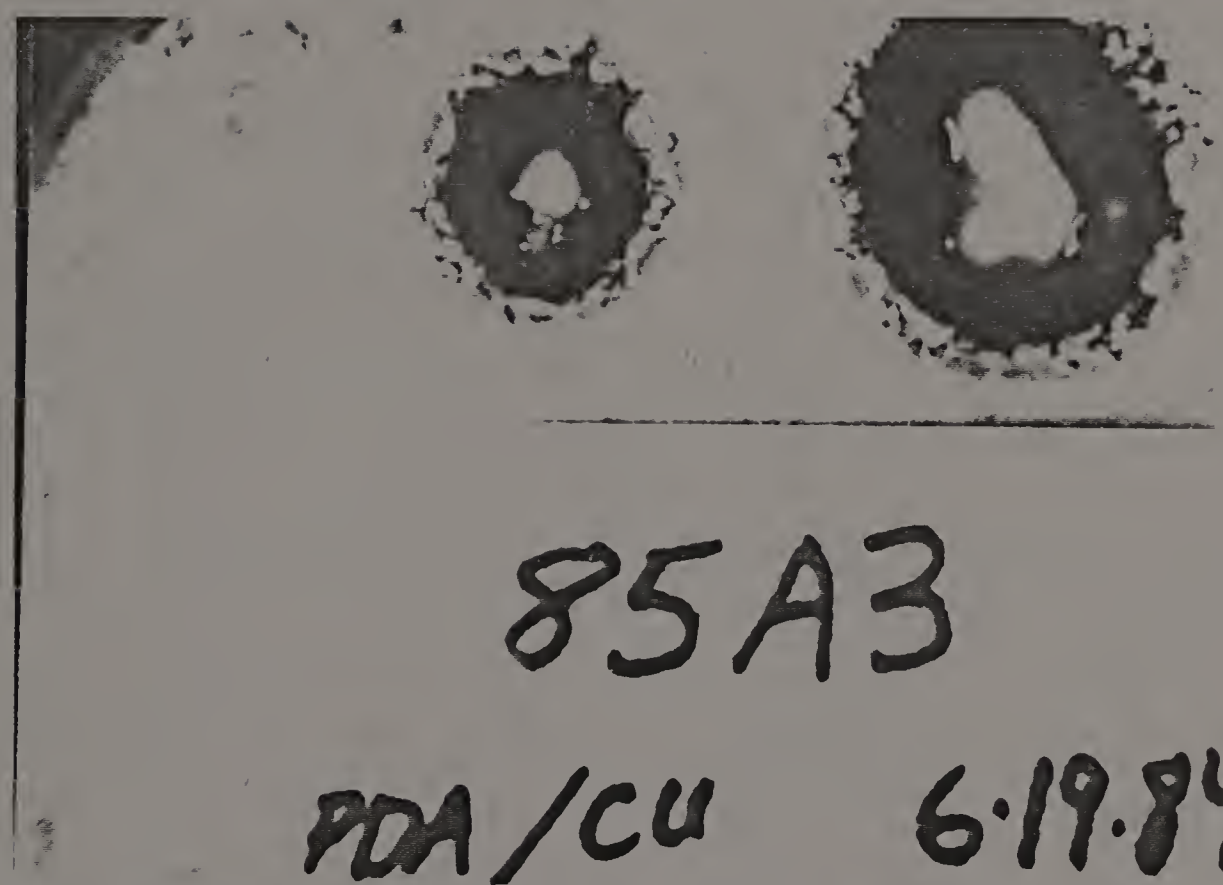

Figure 29 - Inhibition of C. ulmi S by unknown bacterial isolates 85A3 and 89D3-1 (from elm) grown on potato dextrose agar.

Table 11 - Characteristics of 9 unknown bacteria and 2 *P. syringae* isolates for determining the additional tests required for identification.

Isolate	Morphology	Gram	KOH(1)	Oxidase	Inhibition(2)
Unknowns					
57C3	rod	-	+	+	+
71D3	rod	+	-	-	+
78C3	rod	-	+	+	+
85A3	rod	-	+	-	+
89D3-1	rod	-	+	-	+
91A3	rod	-	+	-	+
Ps3D	rod	-	+	+	-
C3A	rod	-	+	+	-
C3C	rod	-	+	+	-
Knowns					
Ps82	rod	-	+	-	+
PsM27t	rod	-	+	-	+

(1) KOH test (107) confirms Gram stain procedure. A negative KOH = positive Gram stain and vice versa.

(2) Inhibition of *C. ulmi* S (nursery isolate).

evaluation. The oxidase positive organisms (57C3 and 78A3) were tested using the OXI/FERM tubes. Isolates 59D3, 85A3, and 91A3 were tested with Enterotube II media since they were oxidase negative. Known isolates of P. syringae, Ps82 and PsM27t (supplied by Dr. Gary Strobel) were used for comparison in both types of tests, even though they are oxidase negative, since some Pseudomonas species can react in both types of tubes. The Ec and Pa isolates were tested as quality control organisms on Enterotube II while only Pa was used for OXI/FERM tests.

Table 12 shows the results of inoculation of the diagnostic media with the oxidase negative unknowns. These results indicate that the 3 organisms 85A3, 89D3-1, and 91A3 (from different elm trees) are similar based on the diagnostic tests. All of them showed positive reactions for oxidative fermentation of glucose, fermentation of adonitol, of lactose, of arabinose, and of sorbitol, and a positive Vogues-Proskauer test for acetoin. The latter test indicates a butylene glycol fermentative pathway for the utilization of glucose. When these characteristics were compared to the diagnostic coding system from Roche Diagnostics (Nutley, NJ), the organisms were identified as Klebsiella ozaenae. Isolates Ps82 and PsM27t had negative glucose reactions as well negative results for the other tests so they had to be tested using the OXI/FERM tubes. This identification system correctly identified the Pa and Ec control isolates as Pseudomonas aeruginosa and Escherichia coli, respectively.

Table 12 - Diagnostic characteristics of 3 unknowns, 2 *P. syringae* isolates, *P. aeruginosa*, and *E. coli* using Enterotube II tests.

	Diagnostic Tests (1)														
Isolate	GL	GA	LY	OR	HS	IN	AD	LA	AR	SO	VP	DU	PA	UR	CI
Unknowns															
85A3	+	–	–	–	–	–	+	+	+	+	+	–	–	–	–
89D3-1	+	–	–	–	–	–	+	+	+	+	+	–	–	–	–
91A3	+	–	–	–	–	–	+	+	+	+	+	–	–	–	–
Knowns															
Ps82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PsM27t	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pa	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Ec	+	+	+	–	–	+	–	–	+	+	–	–	–	–	–

(1) GL = oxidative fermentation of glucose
GA = gas production
LY = lysine decarboxylase production
OR = ornithine decarboxylase production
HS = hydrogen sulfide gas production
IN = indole formation
AS = adonitol formation
LA = fermentation of lactose
AR = fermentation of arabinose
SO = fermentation of sorbitol
VP = Voges-Proskauer test for acetoin (Butylene glycol pathway intermediate)
DU = fermentation of dulcitol
PA = phenylalanine deaminase production
UR = urease production
CI = citrate utilization as sole carbon source

Table 13 shows the results of diagnostic tests for the unknown, oxidase positive isolates, 78A3 and 57C3. Results of testing glucose negative organisms mentioned above are also included as well as results for the control organism Pa and the non-inhibitory isolates Ps3D, C3A, and C3C (obtained from the short-term survival study).

Isolate 57C3 was determined to be negative with all tests except for utilization of citrate as a sole carbon source. The coding scheme identified this organism as a Pseudomonas sp. Isolate 78A3 had a positive test for anaerobic oxidative fermentation of dextrose as well as a positive test for citrate utilization. The organism was identified as Pseudomonas cepacia.

Surprisingly, Ps82 and PsM27t were both identified by this procedure as Pseudomonas maltophilia. The only difference between the two was a positive reaction from PsM27t for the aerobic oxidative fermentation of dextrose. This variability is a characteristic of P. maltophilia. The Pa isolate again proved to be correctly identified by the system.

Results of this diagnosis of the 3 non-inhibitory bacteria further indicate that they are not the same as the bacterium placed into the trees that they were isolated from. Isolate Ps3D utilized citrate while C3A and C3C utilized citrate and produced urease. Citrate utilization and urease production were not found to be positive reactions for Ps82 or PsM27t. C3A, C3C, and Ps3D were identified as Pseudomonas sp.

Table 13 - Diagnostic characteristics of 5 unknowns, 2 *P. syringae* isolates, and *P. aeruginosa* determined by OXI/FERM tests.

	Diagnostic Tests (1)									
Isolate	OX	anDE	AD	NG	HS	IN	XY	aeDE	UR	CI
Unknowns										
57C3	+	-	-	-	-	-	-	-	-	+
78A3	+	+	-	-	-	-	-	-	-	+
Ps3D	+	-	-	-	-	-	-	-	-	+
C3A	+	-	-	-	-	-	-	-	+	+
C3C	+	-	-	-	-	-	-	-	+	+
Knowns										
Ps82	-	-	-	-	-	-	-	-	-	-
PsM27t	-	-	-	-	-	-	-	v(2)	-	-
Pa	+	-	+	-	-	-	+	+	+	+

(1) OX = oxidase production
anDE = anaerobic oxidative fermentation of dextrose
AD = arginine dihydrolase production
NG = nitrogen gas production
HS = hydrogen sulfide gas production
IN = indole formation
XY = oxidative fermentation of xylose
aeDE = aerobic oxidative fermentation of dextrose
UR = urease production
CI = citrate utilization as sole carbon source

(2) variable reaction

Finally, characterizations were determined for the one Gram positive organism, 71D3. Colony morphology, growth pattern, and color were compared to the sample isolate Corynebacterium sepidonicum 3311, a plant pathogenic, Gram positve bacterium. When grown on nutrient yeast extract broth medium (88), the unknown isolate and the Corynebacterium specimen appeared identical. The whitish color of C. sepidonicum is a characteristic of that species when compared with other Corynebacteria which appear more yellow (88). The unkown organism had the same colorless appearance as Corynebacterium, had the same short rods, and had the same spreading characteristic of colony growth. They were concluded to be sufficiently similar to be presumed to be the same species.

C H A P T E R V

DISCUSSION

Therapeutic Treatment of American Elms Using *Pseudomonas syringae* M27

The results of treating large American elms with Ps81 indicate that control of DED by the bacterium can possibly be achieved when used as a preventative treatment rather than as a therapeutic one. This is indicated by the findings that smaller increases in disease symptoms resulted from treatment of trees with smaller percentages of initial wilt. In non-treated elms, small initial wilt percentages were not associated with small increases in the disease progression.

The observations of symptom development in treated trees versus non-treated trees in the second season revealed that the rate of disease progression was not hampered in treated trees as it was initially. Apparently, the effect of slowing the disease was not maintained after the first season of treatment. Initial studies by Myers and Strobel (79) and Strobel and Myers (106) had shown inhibition of the vascular discoloration in small elm trees. In larger trees, symptom development was slower in treated trees compared to non-treated trees. Scheffer (89) found similar results when using a 'gouge- pistol' to inoculate another isolate of the bacterium. He showed that when *P. syringae* was applied after *C. ulmi* inoculation, severe DED symptoms developed but when

bacteria were applied first, symptom development was significantly slower.

Our inability to control DED successfully by treatment with Ps81 as a therapeutic agent prompted us to further investigate the ability of the bacterium to survive in elms. The results of those investigations are also presented.

Improved Injection System

Dye Injection

Observations of dye distribution within trees when using the new injector heads do indicate that materials are placed primarily into the outer xylem. This type of distribution prevents the wasting of materials and allows for placement of materials at the required target sites. The presence of dye in the whole crown foliage indicates the potential; to achieve total distribution by this method of injection. It should be remembered, however, that dye distribution studies only indicate what the translocation potential of the dye is. Other chemicals or biological organisms used for disease control may not move the same way.

A drawback encountered with this system was the high rate of attrition due to injector breakage. Improvements in the basic design of these prototype injectors could eliminate or reduce such losses. The wooden spacer could be incorporated into the body design to increase strength and reduce the number of parts. The use of stainless steel nails would eliminate rust formation and facilitate

cleaning, especially if corrosive disinfectants should be used. With improvements made on the basic design, the new injector heads show promise for general use in the shallow injection hole technique which is essential to attain good distribution with minimal damage to the tree.

Dye Injection of Other Trees

The effectiveness of the trunk injection technique, in general, appears to be limited to particular tree species. When trunks of sugar maples were injected, only very narrow distribution bands could be observed. Sugar maple is diffuse-porous (26), that is, all the vessels are more or less equal in diameter and all are functional, so the outer xylem is less important for transport than it is in ring-porous species. Older vessels in diffuse-porous species remain functional for a longer time than in ring-porous ones (26). A network is formed between vessels of a growth ring and apparently the secondary xylem (26). Perhaps, in diffuse-porous species, these interconnections of functional vessels cause a dilution of the dye or whatever material is injected, thus affecting the total distribution.

Ring-porous elms and oaks, on the other hand, depend on outer xylem vessels for transport, so the dye becomes concentrated in these tissues. The interconnections would only be between the more recently developed vessels. This provides for radial spread but not inward spread.

Diffuse-porous species such as black ash showed an intermediate type of distribution, in which dye moved into the crown but only into branches directly in line with vessels above the injection holes. It is possible that the radial connections of the outer xylem vessels may be less abundant than those found in elm or oak. Ash vessels being larger than than those of either maple or elm, one might suppose that faster uptake by such large vessels could decrease the chance for lateral spread. However, it was observed that the rate of uptake was no faster than that observed in elm and probably slower. It is probable that fewer lateral connections accounts for the incomplete distribution in black ash.

One can conclude that effective trunk application of materials to trees by use of the new type of injector, depends on the vessel distribution patterns of the tree species and the associated connections between those vessel systems (among other factors). Use of this injection system is primarily intended for ring-porous species. Preliminary tests must be performed on any given species to determine its compatability.

Chemical Injection

The applicability of the new injection system for use in chemical treatments was shown by bioassay for the presence of both the Lignasan and Arbotect compounds in nursery twig specimens. Not all twig samples inhibited *C. ulmi*, but the occurence of inhibition reactions from at least some of them shows promise for distribution

of the chemicals. McClain (64) achieved only 34% coverage in twigs and 73% of leaves for trunk injected carbendazim. His root flare injection provided only 27% twig coverage and 62% leaf distribution. With thiabendazole trunk injections, he obtained only 62% coverage of twigs and found no material in leaves (64).

Bioassay of our greenhouse grown elms showed that Arbotect did enter 3 out of 5 leaves sampled while both Lignasan and Arbotect was recovered from all twig samples. The absence of these chemicals from leaves of the larger nursery trees is not surprising, given the less-than-100% recovery from much smaller greenhouse seedlings which were treated with greater amount of the chemical on a weight/ volume basis.

The inhibition zones obtained by placing nursery elm twig samples on plates oversprayed with C. ulmi appear to be similar to those obtained in a study by Clifford et al. (8) in which the zones averaged 10.3 mm for Lignasan compared to our of 9.5 and 7.5 mm for Lignasan and Arbotect, respectively. They also found that distribution was incomplete. The treatments by McClain (64) in which distribution was incomplete, involved the use of up to 29 L of chemical solution for a single tree applied through 5 cm deep holes. Most of the chemicals may well not have reached the target sites.

The results provided indicate that the injection system could be used for chemical applications. Although leaf samples from nursery trees did not indicate the presence of chemical, it is not necessary to pervade into those tissues for DED control. An important

consequence of this technique is that considerably less volume of material need be used.

Wound Effects

The initial indications from the sampling of elms injected with the new type of injector are that the resulting small wounds became e well compartmentalized. Discoloration occurs only slightly beyond the boundaries of the injection hole in the radial direction and only to the depth penetrated by the injector nail. The longitudinal discoloration was the least compartmentalized, which is reasonable since it is known that vertically associated regions are generally the weakest areas for compartmentalization of decay (95). Future injection of these trees would have to be confined to areas of wood between the vertical axes of former injection holes and not directly above or below them...at least not for the next few years.

The presence of small cracks which appeared to arise from the wound origins could be of concern later on. The penetration of wetwood tissues was not observed since the wounds were relatively shallow. Unfortunately, the nail does increase the depth of the wound but it was observed that discoloration of the wood due to the nail wounds was at a minimum and it is felt that the elasticity of the wood would help close the wound once the nail was withdrawn.

Evaluation of Other Methods

Single Injector Use of a single injector on an elm of size class 1 did not give radial distribution as complete as when 4 injectors were used. The distribution at the top of the tree is the most important when the smaller European elm bark beetle is a concern since it feeds in the upper crown twig crotches and inoculates the tree with the fungus there (103). If the native beetle is predominant in a DED infested area, this kind of distribution pattern would not be adequate, since this beetle feeds lower on the stem.

If the materials to be injected are to serve as eradicants, then fast and complete distribution would be desirable. For preventative treatments, more lead time is available, so that complete distribution may be obtained by injecting for longer periods. In such situations, the single injector method may suffice. The advantage to using one injector obviously lies in having to make only one wound.

When using living organisms for biological control strategies, two possibilities come to mind. Either the organism distributes itself within the tree as it multiplies, or it multiplies in a given region and the toxic materials, which it produces, do the moving. In both these cases, one might expect that the amount of the organism initally applied would not be critical since it can reproduce rapidly. Such assumptions could justify use of a single inoculation site.

Mauget and Modified Mauget Distribution of dye in elms injected with Mauget capsules was not detectable This was probably a function of volume since the Mauget system did provide for detectable distribution when modified so that larger volumes could be used. The dye was visible to the top of the tree (observed in cross-section), but the lowest point in which the dye surrounded the outer xylem higher than was the case for new system. This was probably due to Mauget's use of smaller injection holes than are used in our new system. Also, the Mauget injector tips are designed so that part of the tapered end blocks some of the current layer of xylem. Thus, as much as half the diameter of the hole is non-functional for uptake of dye. This is particularly true if the Mauget injectors are placed on areas of root flares having thin bark so that the wood holds the injector in place and not the bark tissue.

Our observations lead us to conclude that the new injector system provides for more efficient use of the injection hole and thereby leads to better distribution. The Mauget system with increased capacity comes close in distribution potential to the new system but it is much slower. The Mauget injectors have the advantage of using even smaller injection wounds than our new system. Both of these systems should be further investigated for use in applying biocontrol agents.

Pseudomonas Survival: Injection of Nursery Elms

Recovery of PsM27

One of the main purposes of our study was to determine whether PsM27 had the ability to survive in elms from season to season so that it might provide continuous protection from DED without the need to re-inject it. Although it is difficult to distinquish between closely related pseudomonads, it was felt that by sampling a large number of trees that either were treated with the bacterium or were non-treated, survival of the injected bacterium could be determined by detection of significantly larger populations of *P. syringae*-like organisms within the treated trees. If population differences were not significant, then we might conlude that the bacterium did not survive or did not translocate to the tissues being sampled.

Our findings are that the numbers of fluorescent pseudomonads and *P. syringae*-like pseudomonads were not significantly different between the treatment and control trees. This indicates that injection of Ps82 had little or no effect on the microflora population of elms. Of the *P. syringae*-like organisms that were isolated, few proved to be inhibitory to *C. ulmi in vitro*, and these were isolated from water treated and non-treated trees as well as from PsM27 treated trees. Our inability to detect Ps82 during sampling performed shortly after injection further

substantiates the lack of distribution and/or survival of the bacterium.

A recent study by Harrison-Lavoie et al. (34), using PsM27 and two other strains of the bacterium, confirmed our findings when they were not able to recover the bacteria at any greater frequency than from untreated control trees. Despite an elaborate identification procedure, based on reactions to 16 carbohydrates, and to 24 enzymes, and resistence to antibiotics, they could not re-isolate the bacterium. Tattar and Kostka (unpublished data, 1982) used a pressure apparatus to apply Ps82 and also could not re-isolate the organism to any significant degree.

Campana et al. (6) reported isolating PsM27 from elm twigs up to 112 days after injection, but at a rate no greater than 4%. They did show, however, that recovery of P. syringae was possible up to 2 years after injection even if only from around the injection holes. It appears from their work that distribution was primarily limited to injection sites.

Phytopathogenic pseudomonads range in size from 0.5 to 1.0 μ in diameter and 1.5 to 4.0 μ in length (88). The inability of Ps82 to translocate within the tree may be a function of the size of the perforations in vessel pit membranes which are likely to be less than 0.3 μ in diameter in vessels (13). American elm has simple perforation plates (12) with vessel diameters greater than 25 μ (66), but mostly 40 to 65 μ (22). It would be expected that upward movement through the vessels would not be hindered due to the large

vessel size compared to the bacteria. As previously discussed, radial movement depends upon the interconnections between vessels, which are the pits and pit membranes. If the bacteria cannot pass through the pits then lateral spread will be hindered.

Our findings that the uptake of the bacterial suspension was significantly less than for water alone suggests that the bacteria may have occluded the vascular system and slowed the uptake. The large numbers of bacterial cells would have to enter a limited number of vessel elements and the extent of movement would be limited by vessel length. As expected, injected dye did not have any trouble traversing the pit membranes.

Observations by Strobel (105) that discoloration of the vascular system was prevented above points of bacterial inoculation, but less so between them, would tend to support the idea of upward movement with little radial spread. Vessel-to-vessel spread would have to occur at the perforation plate junctions, at which point joined vessels may go in different directions. Otherwise, bacteria could pass through pits with disrupted membranes, although slowly, since pit diameters are small, too (e.g. 4-5 μ in Tilia americana (122)). It is possible that larger trees have a greater potential for spread within the vascular system, as we found a significantly greater number of fluorescent pseudomonads in large size class elms than in smaller ones.

Biocontrol

Since no measurable differences in the percentage of experimental trees infected by DED exist between the treatments, it might be assumed that no biological control was achieved. It should be considered that lack of colonization may have preempted the control. The use of a resident microflora that could inhibit C. ulmi would probably be more beneficial, since these bacteria then might be better adapted to the vascular system environment. Recently, Murdoch et al. (74) used P. fluorescens, a commonly isolated saprophyte, and showed in vitro inhibition to C. ulmi accompanied by initial effectiveness in controlling DED in elms. Gregory et al. (31) have found Bacillus and Trichoderma species to be highly inhibitory to C. ulmi in vitro. After being introduced into elms, they were periodically re-isolated from leaf petioles, an indication of their potential as colonizers.

As discussed earlier, colonization of a tree by a biocontrol agent does not necessarily mean that the agent has to move within the tree. An organism that establishes itself at a site but produces copious amounts of toxin, or other effective by-product, could be just as effective as one that is located throughout the tree. PsM27 probably does not produce enough toxic materials, as evidenced by the variability in its achieving any control of the disease. If the siderophore is the toxic factor, it is possible that trace iron compounds in the sapstream may be quenching the production of the siderophore. The level of iron in the sap would probably be related

to a particular growing site. Several PsM27 isolates did not fluoresce when grown on EM derived from preparation of elm tissue. The presence of iron in elm tissues or sap could account for the lack of fluorescence.

Similar relationships might also exist between trace elements and other compounds produced. Toxin production is a secondary metabolic function and is affected by the growth medium and environmental regimes (114). Evidence of this occurred when P. syringae isolates were grown on the 9 different media with C. ulmi. Variation also exists between isolates of the same organism in their ability to produce inhibitory substances. As presented earlier, some appear even to stimulate fungal growth. Likewise, the particular fungal isolates being used will respond to the inhibition factors in varying degrees.

Treatment of Elm Seed

The inital purpose of treating elm seed with the bacterium was to determine whether P. syringae could colonize the stems of young elm seedlings upon germination of the seed. The inability to recover the bacterium from seedlings arising from bacteria treated seed shows that colonization does not occur.

The dramatic enhancement of elm seedling growth was demonstrated in the first two experiments using Ps81 as a soil drench. Related work of other researchers (90,91,113) has dealt primarily with growth promotion of plants as resulting from suppression of other soil

antagonists by bacteria. The elm seed, however, were not subject to any identifiable pathological antagonists. It appeared that the growth enhancement was directly related to the presence of Ps81 acting on its own.

It was considered that perhaps Ps81 enhanced iron utilization by the plant since leaf color was much darker than the chlorotic water treated ones. The significantly greater plant numbers for water treated versus Ps81 treated seed, however, indicate that the affects observed may be due more to plant-versus-plant competition. Somehow, Ps81 reduces the number of plants that survive. Lower plant numbers could be associated with changes in the soil environment initiated by the bacterium. Such changes might include the increase in moisture retention which could lead to pre-emergence damping-off situations. Also, pH and nutrient availability could be altered and, finally, the bacterium could produce substances inhibitory to germination.

The inability to reproduce the growth enhancement effects when the various factors of the formulation were separated leaves many quentions unanswered. It was beyond the scope of this study to investigate further this multitude of factors. The initial results of improved growth warrant a closer evaluation in future studies.

Characterization of PsM27

Inhibition of C. ulmi

Some of the variation in disease control observed when using PsM27 can be attributed to variation of the bacterium itself. When

placed *in vitro* against *C. ulmi* C, the isolate PsGS actually seemed to enhance mycelial growth and coremia formation of the fungus. The other isolates inhibited it. None of the isolates provided inhibition against *C. ulmi* S from the nursury.

Attempts to find characteristics that might aid in the diagnostic separation of these isolates and to separate them from other pseudomonads were partially successful. This is necessary to distinquish the treatment bacterium from other isolated pseudomonads.

The possibility that fluorescent pseudomonads might cease fluorescing at differing levels of ferric iron was tested and it was found that Pa could be separated from the *P. syringae* isolates. However, the *P. syringae* isolates could not be separated between themselves based on that characteristic.

When grown on the various media, no isolate grew better than the others on a given medium, but there were differences in the ability to inhibit *C. ulmi*. Similar inhibition responses of the bacteria on MASK and MASKFe show that the siderophore compound may not be the fungal inhibition factor, since inhibition occurred on MASKFe which quenched fluorescence.

We were able to separate some of the isolates based on their reaction to the fungus but not for any other characteristics. The inhibitory response is closely related to the medium. Therefore, *in vitro* determinations of inhibitory organisms for biological control provide no real information as to the responses that will occur *in vivo*.

Inhibition of Other Fungi

The inhibition of other fungi including some related to C. ulmi, indicates that the inhibition response is not a specific one. Again, the inhibition of the fungi was dependent on the growth medium involved. PDA and MA gave the most dramatic inhibition results. Also, the Ps81 isolate showed inhibition and the PsGS isolate did not. The reasons for the variations in response between fungus and bacterium have not been determined.

An evaluation of whether structural and nutritional components of elm are factors in Ps82 survival indicate that Ps82 does not utilize cellulose in its metabolism. Goto (29) found similar results for another P. syringae while examining bacteria for cellulolytic activity. We found enhanced growth on the EM medium, indicating that some other material might provide nutrition. The total solute content of xylem sap is very low and the actual contents are difficult to determine (122). The presence of amino acids and various minerals has been demonstrated (122) but the EM we prepared undoubtedly contained phloem components as well.

Because Ps82 does not utilize cellulose, it appears that it is incapable of digesting cell walls (whereby it might better disperse throughout the tree) nor can it obtain phloem materials directly. Its survival inside elm trees, then, would have to depend on whatever nutrients were available through the sapstream.

Characterization of Unknown Bacteria

The isolation from elms of at least three different bacterial species that inhibit C. ulmi in vitro again indicates that the inhibition phenomenon is a fairly general one. The three isolates identified as Klebsiella ozaenae belong to the Enterobacteriaceae. The metabolism of bacteria in this group involves the ability to grow anaerobically. One of the OX/FERM bacteria, 78A3, was identified as Pseudomonas cepacia and brought about anaerobic oxidative fermentation of glucose. These results give an indication of the type of environment in the xylem vessels. Ps82 could not be expected to survive the anaerobic conditions suitable for these other bacteria, since it is an obligately aerobic organism.

Klebsiella has been isolated by other researchers from elm in association with bacterial wetwood (18) and wetwood has been shown to have regions that are anaerobic (26,125). The isolation of Pseudomonas species as identified by the OXI/FERM tubes may compare to the previous isolation of P. fluorescens from sapwood (72). Murdoch and Leach (76) recently isolated 187 non-pathogenic bacteria representing several genera from potato stems. They suggest that these non-pathogenic, stem inhabiting bacteria might be modified and applied as control agents for vascular wilts or for production of growth promoting substances. One could make a similar suggestion for the bacteria isolated from elm.

Further characterization of PsM27 isolates Ps82 and PsM27t, using the OXI/FERM diagnostic system, indicates that this bacterium is not really P. syringae! Its identity as P. maltophilia does not necessarily detract from the information presented in these studies, but it does serve to point out the difficulties of identifying the bacteria in such studies.

Summary

The bacterium contained in the Chevron product that was labeled as containing Pseudomonas syringae M27 does not appear to colonize the stem tissues of American elm. However, distribution of the bacterium does not appear to be limited by the method of injection specifically designed for this study. Instead, limited distribution is probably due to the anatomy of the elm and/or the growth requirements of the bacterium.

Within the elm vascular system are numerous other bacteria which also inhibit C. ulmi in vitro. Although in vitro analysis of inhibition is questionable, these naturally occurring stem inhabitants warrant consideration as possible biocontrol agents since they may be better adapted to survival in the trees from which they were isolated. The diagnostic tests indicate that the actual identity of PsM27 is in question.

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APPENDIX

APPENDIX A

DYE'S MEDIUM + HISTIDINE

Stock A	$(NH_4)_2$	40 g/400 ml H_2O	1.30 g/liter
Stock B	KCl	8 g/400 ml H_2O	0.26 g/liter
Stock C	$MgSO_4-7H_2O$	8 g/400 ml H_2O	0.26 g/liter

ADD (per liter of medium):

1. Stock A - 13 ml
2. Stock B - 13 ml
3. Stock C - 13 ml
4. glucose - 10 g
5. L-histidine (free base) - 2.33 g
6. Bacto agar - 15 g

AUTOCLAVE

Provided by J.J. Butler, Chevron Chemical Company, San Francisco, CA