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DISSERTATION

CONTROL OF FUSARIUM WILT OF CUCUMBER BY CHEMICAL AND BIOLOGICAL AGENTS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HASSAN HASSAN NASSAR ENTITLED CONTROL OF FUSARIUM WILT OF CUCUMBER BY CHEMICAL AND BIOLOGICAL AGENTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

CONTROL OF FUSARIUM WILT OF CUCUMBER BY CHEMICAL AND BIOLOGICAL AGENTS

The main goal of the study was to search for potential pesticide alternatives for the control of Fusarium wilt of cucumber. Biological control and induction of systemic acquired resistance by chemical and biological agents were the logical choices. The results of the investigation are presented in four separate chapters. Each chapter approached the same problem from a different angle. In the first chapter, the nature of the potential of pesticide pollution was emphasized in the future of disease control. A thorough literature review concerning various aspects of modern control methods is presented.

In chapter two, the induction of systemic acquired resistance in cucumber by various agents was investigated. Isonicotinic acid as a chemical agent and *Pseudomonas cepacia* as a biological agent proved to be the most effective inducers among the five tested. *P. cepacia* had the greatest effect on hyphal growth and fungal sporulation among all the agents. *P. cepacia* was able to cause severe deformities in four test pathogens. Isonicotinic acid as a chemical inducer, did not have a similar effect but was able to significantly suppress infection of cucumber seedlings by *Fusarium oxysporum* f. *sp. cucumerinum*. Both isonicotinic acid and *P. cepacia* were able to delay the initial onset of wilt disease and to significantly reduce disease incidence in all test cucumber cultivars either as a stem injection or as a root dip treatment.

The third chapter focuses on various possible mechanisms for control of Fusarium wilt in cucumber by nonpathogenic isolates of *F. oxysporum* f. *sp. cucumerenium*. Isolate N1 was able to delay the appearance of wilt symptoms. When the time between introduction of N1 into root system and challenge to the system with wilt pathogen was increased the plants had a better opportunity to build up their resistance and reduce infection. Seventy-two days after inoculation of plants previously treated with N1 or C14 (a nonpathogenic isolate) the plants had a mortality ratio of 30, and 55, respectively compared to the 94% of unprotected plants.

Various etiological and pathological methods were evaluated in chapter four. A protocol for screening *Trichoderma* and *Gliocladium* isolates to select the most efficient one is proposed. Twenty-seven *Trichoderma* spp. and nine *Gliocladium* spp. isolates obtained from commercial Egyptian cucumber fields and greenhouses were subjected to the study. When isolate Eg7 of T. *harzianum* from Egypt was compared with other T. *harzianum* isolates the level of protection was equal to or greater than the established biological control agents.

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Dedicated to

the memory of Dr. Ralph (Tex) Baker

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Continuous growth of human populations creates a need for more food and a conversion of increasing amounts of land to crop production. Intense, continuous cultivation of cropland has resulted in an increase of plant disease pressure. Although historically the application of synthetic fertilizers and pesticides have resulted in high crop yields, environmental concerns occur and considerable crop damage by insects and pathogens still persists. By recent estimates, plant diseases of economic crops alone cause a worldwide annual loss in production of 13-20%, representing a 500 million dollar loss (James, 1981). Even in the United States, with one of the most advanced crop disease management technologies in the world, the extent of loss is quite similar. More than 50% of the crop loss in the United States due to diseases is caused by microbes inhabiting the soil (Lewis and Papavizas, 1991), and 90% of the 2000 major diseases of the 31 principal crops are caused by soilborne pathogens (Wilson, 1968). Most of these pathogens are categorized in 50 genera of fungi, of which 10 have a major role in the root disease complex that includes wilt diseases (Cook and Baker, 1983).

Fusarium wilts

Formae speciales of *Fusarium oxysporum* Schlectend. ex Fr.

predominantly contain soilborne pathogens that collectively cause vascular wilt diseases affecting a large number of economic hosts (Farr et al. 1989). These pathogens are known to persist through long intervals between susceptible crops (Burgess, 1981; Garret, 1970). Persistence of the fungi in the absence of a susceptible host has been attributed to the production of long-lived chlamydospores (Garrett, 1970), the infection of a non-susceptible plant as a symptomless carrier (Smith and Snyder, 1975), and the colonization of wounded tissue and crop residues (Banihashemi and deZeeuw, 1973). These modes have been documented for *F. oxysporum* Sclecht emend. Sny. et. Hans. f. sp. *cucumerinum* Own which causes Fusarium wilt of cucumber (*Cucumis sativus L.*). Numerous researchers have shown that formae speciales of *F. oxysporum* can colonize crop residue; however, for saprophytic growth to be important in the long term persistence of a pathogen, the pathogen must be able to compete effectively with other soilborne fungi (Gordon et al. 1989).

Typical wilt symptoms in cucurbits, resulting in mortality are manifested in both young and mature plants and may be severe especially when infection occurs at the seedling stage (Latin and Snell, 1986). Many difficulties are encountered in controlling Fusarium wilt pathogens by conventional methods. This makes the use of biological control an attractive and useful addition to the plant disease control measures already available (Postma and Luttikholt, 1993).

Etiology of *Fusarium oxysporum* f. sp. *cucumerinum*

In a field experiment, Ogura (1992) reported that *F. o. cucumerinum* in plant debris survived more than 1 year in soil while it survived only 4 months

without the debris. Wilt damage to cucumber was most severe in the first year and decreased during the subsequent 2 years, although the population of the pathogen gradually increased. After three years, the pathogen population remained stable and damage to the crop remains constant. The pathogen can survive unfavorable conditions in soil in the form of chlamydospores. Ogura et al. (1990 b) reported that chlamydospores of *F. o. cucumerinum* can survive in soil up to 40 C, whereas conidiospores die at this temperature.

Soil conditions have been reported to have a significant influence on the growth, sporulation and survival of the pathogen. Lida et al. (1982) studied the effect of soil condition on population and chlamydospore formation of *F. o. cucumerinum*. They concluded that higher populations of the fungus were maintained, and more chlamydospores were formed in sterilized (autoclaved) rather than in nonsterilized soil. Chlamydospores were formed abundantly at 20-25 C in sterilized soil and at 15-20 C in nonsterilized soil while the rate of vegetative growth reached its maximum at lower temperatures.

The most favorable moisture content of the soil for maintaining high fungal populations was 20-50% in sterilized soil and 20% in nonsterilized soil. Many chlamydospores were formed at a moisture content of 20-50% in sterilized soil. In nonsterilized soil, however, there was no difference in the number of chlamydospores formed in soils of different moisture content. In both sterilized and nonsterilized soils, alkaline conditions were favorable for maintaining high populations of the fungus, and the optimum pH for chlamydospore formation was pH 7. During a study on the influence of soil conditions on disease development,

Costache et al. (1979) found that cucumber plants were most susceptible to *F. o. cucumerinum* when grown at a soil temperature of 30 C where Fusarium wilt infection reached 29.9%. The infection percentage was 35% and 15% at 40% and 80% soil moisture, respectively. Prolonged daylight (12 h) decreased infection to 18%, whereas infection increased to 40.3% with only 4 h of daylight. In a similar study, Costache et al. (1978) reported that cucumber plants grown at 25-30 C and at low relative humidity are more susceptible to Fusarium wilt infection that those at 15-20 C and moderate or high RH. In a more detailed in vitro study on fungal growth and spore germination, Huang et al. (1988) found that the optimum temperature range for spore germination and hyphal growth of *F. o. cucumerinum* was 24-27 C; no growth occurred above 37 C. Chlamydospores, the persistent structure of the fungus, survived exposure to 50 C for 24 h.

The pH range for the mycelial growth was 3-10. Growth and germination could be inhibited by light. Also in cultural studies, Costache et al. (1981) found that growth and sporulation of *F. o. cucumerinum* were best on media containing the nitrogen sources asparagine, arginine, proline, glutamic acid, or serine. The most favorable concentration of the nitrogen source in culture media was 0.8 g/L.

In a scanning electron microscopy study, Huang et al. (1988) found that, after the pathogen penetrated cucumber plants, hyphae were found in vessel cavities, parenchyma cells as well as between parenchyma cells. Hyphae grew upward along the vessel wall and penetrated from one vessel to another. The water transport in infected plants was inhibited by blockage of the vessels,

resulting in wilting.

Soil nutrients and amendments affected the survival of, and the colonization of cucumber roots by the wilt pathogen. In a study on the population dynamics of *F. o. cucumerinum* in the rhizosphere of four different crops including cucumber, Ogura et al. (1990 a) found that in nutrient rich soil, the pathogen population was higher and more roots became infected. Adachi et al. (1987) added lignin and/or chitin in pot soil at a concentration of 2,000 and 1,000 µg/g, respectively. Chitin application decreased the population of *F. o. cucumerinum* when the fungus was introduced into the soil at a level of 10^9 propagules per pot. In contrast, lignin application inhibited multiplication of antagonistic fungi, induced the growth of a fungal flora dominated by Phycomycetes, promoted the survival of the pathogen in the soil, and masked the beneficial effect of chitin application. Seo (1986) found that adding organic matter such as mushroom compost and chicken manure, under glasshouse conditions, suppressed cucumber wilt by 30-35% and delayed disease development. He also found that the numbers of actinomycetes, fungi, and bacteria were greater 30 days after the addition of organic compost, while the pathogen population decreased.

In order to explain the ability of the cucumber wilt pathogen to survive for years in the absence of a susceptible host, and also to understand the mechanism, by which the fungus spreads in the soil, many experiments were performed over multiple seasons. Experiments were performed in the presence of various crops that usually exist together in the field. Most recent work in this

area, by Ogura et al. Ogura and Ma (1992), found that continuous growth of the same crop over years in a monoculture system had a dramatic effect on the population of *F. o. cucumerinum*, depending upon the type of crop. The characteristic saprophytic or parasitic ability of the pathogen on the roots of a crop determined the dominance of the fungus in the fusarial flora of each continuously cropped field. When infested fields were cropped with cucumber, soybean, or sorghum continuously for 6 yr, *F. o. cucumerinum* survived in large numbers in cucumber and soybean fields, but populations decreased with the sorghum crop. The fungus colonized cucumber roots readily at any stage, but colonization of soybean roots decreased as the plants grew (Farias and Griffin, 1990). In general, favorable or unfavorable conditions in the rhizosphere of different crops determined the colonization ability of the pathogen.

In a similar experiment on the dispersal of the cucumber wilt pathogen in tomato, soybean, and cucumber fields, Ogura (1992) reported that cucumber and soybean crops were more favorable for dispersal of the pathogen than tomato crops. The fungus spread further in soybean fields than in those cropped with cucumbers, but the fungus population was greater in cucumber fields. Also, Ogura et al. (1990a) tested pathogenicity of the fungus on cucumbers, soybeans, and sorghum. The fungus was parasitic on cucumber and saprophytic on the other crops. These results indicated that *F. o. cucumerinum* colonizes roots of both host and non-host plants in order to survive over seasons.

A close relationship was found between severity of different cucumber root diseases, including Fusarium wilt, and soil pH. Most of the soil suppressive to

cucumber Fusarium wilt had a higher pH than the non suppressive soils and the disease was almost completely suppressed at pH 8.0 (Kobayashi and Komada, 1995). Soil type was also found to affect the germination of *F. o. cucumerinum* spores. Shim and Lee (1984) noted that, out of 102 soil samples, spores of the fungus germinated best in 21 soil samples at pH 5.1-6, and in four samples with a moisture content of 41-60%.

Disease complexes involving root-knot nematodes (*Meloidogyne* spp.) and vascular wilt *Fusarium* spp. have been recognized for many years. As early as 1892, Atkinson observed that severity of Fusarium wilt of cotton was greater when plants were infected by *Meloidogyne incognita* (reviewed by Starr et al. 1989). In cucumber, a similar relationship was found to enhance fungal population in soil, fungal infection, and severity of wilt disease. Choo et al. (1990) found that cucumber wilt was much more severe in plants inoculated simultaneously with the nematode plus the pathogen than with the pathogen alone. Plant growth was retarded by infection with the fungus, nematode, or both. Plant height and shoot weight were reduced more by the fungus plus nematode than by either alone. Also, the number of propagules of *F. o. cucumerinum* detected from the root and stem was greater in plots with fungus plus nematode than with fungus alone. Similar results were obtained by Pelcz et al. (1983) where it was concluded that the resistant cucumber variety, Polo, had the same disease index when infected by both *F. o. cucumerinum* and the nematode, *M. incognita*, as when the susceptible variety, Trix, was infected by the pathogen alone. No wilt symptoms induced by the pathogen were observed

in Polo in the absence of infestation by *M. incognita*.

Biological control as an alternative choice

It became apparent in the 1960's that certain agricultural chemicals were one source of environmental pollution, were present in the food chain, and were capable of inducing pest resistance. Pesticides also became very expensive to produce and register for use. It has been estimated that the cost of development of each new pesticide is equal to $ 30,000,000 (Chet, 1990). For these and other reasons, researchers in academic institutions and private companies all over the world have increased their efforts to develop nonchemical approaches to plant disease control. Biocontrol represents an alternative for future use because of the many political, ecological, and economical concerns that have arisen regarding pesticide use.

Biocontrol of plant disease occurs naturally and is the main reason why crops are not entirely destroyed during cultivation. However, our knowledge of how biological control operates is still insufficient to allow exploitation in agriculture. Biocontrol in plant pathology can be defined in various ways, but perhaps the most inclusive is a reduction in pathogen inoculum or its disease producing capacity by the action of one or more organisms other than man (Cook and Baker, 1983). Control of soilborne pathogens can be achieved through cultural practices that create an environment favorable to antagonists, development of host resistance, stimulation of resident microflora, or through the mass introduction of different antagonists into the soil. The approach most often investigated in the implementation of biocontrol has been, and continues to be,

the introduction of an antagonist to the crop ecosystem (Deacon, 1988). We used this approach as a biocontrol measure against Fusarium wilt in cucumber, in addition to exploring the induction of systemic acquired resistance (SAR) by chemical inducers in the present investigation.

The concept of suppressive soil

It is generally accepted that a plant disease results from the intimate interaction between the plant and a pathogen, with disease severity influenced by environmental factors that affect the plant, the pathogen or both. The existence of soils that suppress diseases caused by soilborne plant pathogens has received the attention of many researchers over the last three decades. As early as 1892, Atkinson recognized that different soils have different potentials for disease development. Based on their potentials, he considered soils to be conducive or suppressive. According to Baker and Cook (1974), in suppressive soil, a pathogen fails to establish or establishes but fails to cause disease or establishes and causes disease at the beginning, but eventually diminishes. Alabouvette (1990) suggested that in suppressive soils, disease incidence remains low despite the presence of the pathogen, a susceptible host plant, and climatic conditions favorable for disease development. In conducive soils, disease develops and causes severe damage to the crop.

Studies on suppressive soils led to the basic concept of "soil receptivity" to disease (Alabouvette, 1986). To benefit from such phenomenan in reducing the damage caused by wilt fungi, an understanding of the role of various biotic and abiotic soil components must be acquired. Soil is not a neutral medium where

pathogenic microorganisms interact freely with the host plant. On the contrary, the soil interferes in several ways with the relationships between and among microorganisms, pathogens, and plants. It can modify interactions among microorganisms themselves. In other words, every soil possesses some ability to limit disease development. This soil receptivity, therefore, is involved in inoculum potential, which as defined by Garrett (1970) is the energy available for infection of a host at the surface of the infection court.

Soils suppressive to Fusarium wilt

Soils suppressive to some of the most important soilborne plant pathogens have been described from different parts of the world (Amir et al. 1996; Yuan et al. 1995; Kobayashi and Komada, 1995). Among the best known are soils suppressive to Fusarium wilt pathogens. These soils limit the incidence or severity of wilt in cucumber, radish, clover, banana, carnation, cotton, flax, muskmelon, and tomato (Kobayashi and Komada, 1995; Toyota et al. 1994; Sarathchandra, 1993; Alabouvette, 1990). Despite the diversity of crops, this suppressiveness can be described as "specific" to *Fusarium*, because it is expressed against all formae speciales of *F. oxysporum*, but is ineffective against diseases caused by other soilborne pathogens and nonvascular *Fusarium* species (Smith and Snyder, 1971). Therefore, the mechanisms of this suppressiveness can be studied with any plant susceptible to Fusarium wilt.

Generally, suppressiveness to Fusarium wilt appears to be stable over time and has been described as constitutive in contrast to "acquired" suppressiveness, which is dependent on the cropping system. For example,

during monoculture of susceptible wheat, soils became suppressive to the take-all pathogen (*Gaeumannomyces graminis*) (Cook, 1985). Nevertheless, the apparent stability of suppressiveness to Fusarium wilts points to pre-existing properties of the soil and associated microbiota, which explains why early studies focused on the relationship between disease incidence and soil type (Stotzky and Martin, 1963).

All results to date point to a microbiological basis for the suppressiveness of soil to Fusarium wilts. These more or less complex microbial interactions between the pathogen and all or a part of the saprophytic microflora are responsible for suppressiveness (Alabouvette, 1990). The role of microflora was first demonstrated indirectly by establishing that soils suppressive to Fusarium wilts disappears upon destruction of organisms by treatments such as steam, methyl bromide, or alpha rays (Scher and Baker, 1980 b). Moreover, the suppressive effect can be restored by mixing a small amount of suppressive soil into a previously heat-treated conducive soil (Schneider, 1984), but only microorganisms able to multiply are likely to confer a high level of suppressiveness to a conducive soil.

Microorganisms involved in suppression of Fusarium wilt in cucumber

After the role of microflora in the suppressiveness of Fusarium wilt was indirectly demonstrated, several attempts were made to identify the specific microorganisms involved. The aim of these attempts was to establish a relationship between certain modifications in the microbial balance and associated changes in the level of soil suppressiveness to Fusarium wilt. This

method became a kind of "Koch's postulates" for those who work with biological control. The results obtained by different researchers through adopting this method led to a belief that the suppressive phenomenon could be attributed to certain groups of microorganisms, primarily nonpathogenic fusaria, plant growth promoting rhizobacteria (PGPR, mainly flourescent pseudomonads), and plant growth promoting fungi (PGPF, mainly *Trichoderma* spp. and, to a lesser extent, *Gliocladium* spp.). Other microbial groups, including *Streptomyces*, *Bacillus*, *Arthrobacter*, and many members of the family Enterobacteriaceae also were found to be antagonistic to various plant pathogens, but they have not been intensively studied.

Nonpathogenic *Fusarium Spp*

The first evidence for a possible role for nonpathogenic *Fusarium* spp. in suppression was provided on sweet potato by Smith and Snyder (1971) for soils in California. The first effort to control Fusarium wilt of cucumber by nonpathogenic isolates of *F. o. cucumerinum* was made by Paulitz et al. (1987). The antagonistic isolates were obtained from symptomless cucumber roots grown in natural raw soil, and were screened and tested for their biocontrol ability. One of the nonpathogenic isolates, coded C5, was noted at that time for its ability to reduce the infection rate at all inoculum densities of the pathogen. It was used later as a reference isolate by many researchers. Park et al. (1988) were able to induce suppressiveness against cucumber wilt disease in near neutral (pH 6.7) to alkaline soils (pH 8.1) by adding a mixture of *Pseudomonas putida* and nonpathogenic *F. o. cucumerinum* isolates. It was suggested that the

activity of fluorescent pseudomonads and their siderophore production were enhanced by increased root exudates induced by relatively high population densities of nonpathogenic isolates of *F. o. cucumerinum.* The increased competition for iron, which is essential for successful germination of the pathogen and penetration of the host roots, was attributed as the control mechanism.

The first attempt to use cross protection with another *F. oxysporum* pathogen against *F. o. cucumerinum* was made by Michail et al. (1989). By prior inoculation of cucumber plants with of *F. o. niveum*, the wilt pathogen of watermelon, no apparent wilt symptoms were detected. In an in vitro study, *F. o. niveum* was found to be antagonistic to *F. o. cucumerinum*, and was shown to be externally and internally seedborne in cucumber.

A three-phase mechanism was found to be associated with biological control of cucumber Fusarium wilt by nonpathogenic *Fusarium* isolates. Mandeel and Baker (1991) suggested that the suppression mechanism involved significant reduction in germination of chlamydospores of the pathogen in the rhizosphere of cucumber roots, competition for infection sites, and induction of enhanced systemic resistance in the host. Through these three phases of suppression, nonpathogenic isolates were able to significantly decrease the slope values on inoculum density-disease incidence curves generated for Fusarium wilt of cucumber. Different patterns of penetration and colonization of living intact cucumber root tissues by pathogenic and nonpathogenic *F. o. cucumerinum* isolates were investigated using scanning electron, light, and fluorescence microscopy by Mandeel et al. (1994). They found that the nonpathogenic isolate

C5, which requires root wounds to induce resistance, penetrated through the wounds but rarely through an intact epidermis. Isolate C14, which was superior to C5 in induction of resistance, was similar to the pathogenic *F. o. cucumerinum* in the formation of penetration-like structures on intact roots. Hyphae were found at discrete locations through the entire depth of the cortex, but not in the vascular bundles. Only hyphae of the pathogen were found exclusively in the vascular bundles, deep within the cortex near the stele and completely surrounding the xylem vessels. A relationship between the ability to penetrate and colonize intact living roots and biocontrol efficiency of nonpathogenic isolates was established. Nassar et al. (1995) reported that the nonpathogenic *F. oxysporum* isolates C14 and N1 were able to delay the appearance of cucumber wilt for 3 weeks, and to significantly reduce disease incidence and severity under simulated field conditions and levels of pathogen inoculum. Eight weeks after planting, 80% of the plants treated with N1 survived the infection, whereas only 45% survival was recorded with C14.

Plant growth promoting rhizobacteria (PGPR)

The term "rhizobacteria " was coined by Suslow et al. (1979) to describe bacteria that aggressively colonize roots, and to differentiate them from those whose natural habitats occur elsewhere. Rhizobacteria, when applied to seeds or roots of plants, can alter both the bacterial and fungal composition of the microflora throughout the season (Yuen and Schroth, 1986). The term "plant growth promoting rhizobacteria" (PGPR) was coined by Kloepper et al. (1982) to describe bacteria that colonized roots and caused plant growth promotion without

making any inference about the mechanisms, which were undetermined. However, PGPR are thought to improve plant growth by colonizing the root system and preempting the establishment of or suppressing the deleterious rhizosphere microorganisms (DRMO) on the roots (Schroth and Hancock, 1982). Some members of the PGPR have been implicated as potential biocontrol agents of soilborne fungi. Current emphasis is on *Pseudomonas* spp. as a result of the ease of use compared to other bacterial groups (Weller, 1988).

Worldwide interest in this group of bacteria was sparked by studies initiated at the University of California, Berkeley, during the 1970s. Pseudomonads that are PGPR and those that suppress major plant pathogens should not be considered as functionally separate groups of bacteria, because many strains acquire suppressive ability of the pathogen and provide a growth promoting effect on the host. In the Salinas Valley of California, Fusarium wilt-suppressive soil was identified (Smith and Snyder, 1971) in which disease did not develop after continuous cropping of susceptible cultivars. Previous work indicated that antagonistic *Pseudomonas* spp., isolated from this soil (Scher and Baker, 1980 a and 1982) or other soils (Vidhyasekaran and Muthamilan, 1995), can suppress Fusarium wilt incidence when added to a conducive soil infested with the pathogen. Various mechanisms have been suggested for biological control of Fusarium wilt by several PGPR. A major mechanism is substrate competition, especially for nutrients exudated by roots and seeds that affect most interactions between bacteria and pathogens (Elad and Chet, 1987). Substrate competition reduces the amount of carbon and nitrogen available to stimulate

spore germination of fusarial pathogens or for subsequent colonization of the roots (Jones et al. 1976). Another mechanism depends on niche exclusion, by reducing or preventing the establishment of different pathogens in the favorite sites of infection like cell junction and points of emergence of lateral roots (Suslow and Schroth, 1982). A third mechanism is based on siderophore production, which allows PGPR under low-iron conditions to acquire the majority of the available iron in soil and subsequently cause suppression of formae speciales of *F. oxysporum* (Raaijmakers et al. 1995). A fourth mechanism is antibiosis, with the most well-known example being the antibiotics phenazine and pyoluteorin produced by some fluorescent pseudomonads suppressive to different soilborne plant pathogens (Weller and Cook, 1983; Maurhofer et al. 1995), and lastly, the fifth mechanism is induction of systemic acquired resistance (SAR).

In the present investigation, SAR is of principal interest in using PGPR for biological control of cucumber Fusarium wilt. Reports are available on induction of SAR by plant-growth-promoting, root-colonizing *Pseudomonas* spp. in several plant-pathogen combinations (Alstrom, 1991 ; Van Peer et al. 1991; Wei et al. 1991). In this context, the term "induced systemic resistance" suggested by Kloepper et al. (1992) commonly replaced the original term, SAR, as it is assumed that the plants already possess some level of resistance against the pathogen involved and that the inducing agents activate this resistance. Liu et al. (1995) tested some PGPR strains for their ability to induce systemic resistance in cucumber plants against Fusarium wilt with a split-root assay.

These strains were able to induce systemic resistance against *F. o. cucumerinum* as expressed by delayed disease symptom development and reduced number of dead plants, compared with the PGPR-free, pathogen-inoculated controls 5 weeks after inoculation. Isolation tests proved that the spread of the pathogen was limited in vascular bundles of PGPR- treated plants. Similar success with induction of systemic resistance in cucumber plants was obtained by Wei et al. (1991) against cucumber anthracnose caused by *Colletotrichum orbiculare* with select strains of plant growth-promoting rhizobacteria.

During the 1990s, a very interesting work was carried out in Utrecht University, the Netherlands, on induction of systemic resistance in radish against Fusarium wilt caused by *F. o. raphani* with different agents of PGPR. The results were published in a series of papers in 1995 (Hoffland et al. 1995; Leeman et al. 1995 a, b; Raaijmakers, et al. 1995). It was concluded that suppression of Fusarium wilt of radish (caused by *F. oxysporum* f. sp. *raphani*) was due to *P. putida* strain WCS358 and *Pseudomonas fluorescens* strain WCS374 by induction of systemic resistance. Induced resistance was highly dependent on the level of disease incidence and was expressed as a reduction in the percentage of diseased plants, but not in the disease severity. A threshold bacterial rhizosphere population density of approximately 10^5 colony forming units (cfu)/g root was required for a significant disease suppression. Increasing the rhizosphere population density to levels higher than the threshold level did not result in a significant increase in disease suppression. In commercial

greenhouse trials over four consecutive years, disease reduction by bacterial treatments (compared with the film-coating control treatments) reached an average of 43%, whereas the relative yield increase reached an average of 45%.

Plant growth promoting fungi (PGPF)

The term "plant growth promoting fungi" was used first by Hyakumachi (1994) as an equivalent to "plant growth promoting rhizobacteria" (PGPR). It describes a group of fungi isolated from the plant rhizosphere that is able to induce growth promotion resulting in increased plant height and weight. PGPF also are able to provide potential protection to hosts from infection by soilborne pathogens, with stable and remarkable suppressive effects to the pathogens (Schroth and Becker, 1990). According to this definition, most fungi currently used in biological control of soilborne pathogens fit the discribtion of PGPF, especially *Trichoderma* spp.

Biological control of seedling diseases with nonpathogenic fungi has been receiving increased attention. When PGPF organisms are applied to seeds before planting, they colonize the rhizosphere of seedlings and, thus, are present at or near the infection court. They act by producing antifungal compounds, through hyperparasitism, or by competitively colonizing spermosphere and rhizosphere substrates (Dandurand and Knudsen, 1993). The mechanism of plant growth promotion by fungi might involve one or more of the following: a) control of different pathogens in the rhizosphere (Elad et al. 1982); b) plant hormone production (Chang et al. 1986); c) vitamin production, conversion of materials to a form useful to the plant, and nutrient release from soil or organic

matter; and d) increase the uptake and translocation of the minerals (Barber and Lynch, 1977 for the points b-d).

Many studies have shown the potential of *Trichoderma* spp. as biocontrol agents of soilborne plant pathogens in different parts of the world (Xue et al. 1995; Ahmad et al. 1995; Scodellaro, 1995). *Trichoderma* spp. also have been shown to be able to increase plant growth (Hyakumachi, 1994). Responses to the application of *Trichoderma* spp. are characterized by increased germination, plant height, and dry weight, and a shorter germination time in vegetables (Chang et al. 1986; Paulitz et al. 1986). Earlier flowering and an increased number of blooms in periwinkles (*Vinca minor* L.) and petunia (*Petunia hybrida* Vilm) (Baker et al. 1984) have been reported in response to the treatment with different *Trichoderma* spp. Different mechanisms were found to explain the antagonistic activity of *Trichoderma* spp., including direct parasitism by *Trichoderma* on hyphae of other fungi (Sivan and Chet, 1986); excretion of extracellular lytic enzymes such as cellulases and phenol oxidases (Asiegbu et al. 1996; Tsuneda and Thorn, 1995), alpha-L-arabinofuranosidase (Roche et al. 1995), endoglucanase (Vincken et al. 1995), and chitinase (Limon et al. 1995); excretion of antibiotic compounds (Velikanov et al. 1994) like isonitrile compounds (Faull et al. 1994) and 6-n-pentyl pyrone (Graeme Cook and Faull, 1991); as well as competition (Ahmad and Baker, 1982; Marchetti et al. 1992).

Trichoderma spp. have been used in biological control of cucumber wilt (*F. o. cucumerinum*) (Borda and Arbelaez, 1993; Elias et al. 1993). Hyakumachi (1994) reported that *Trichoderma* spp., among other PGPF, afforded potential

protection to cucumber plants against the wilt pathogen in sterilized and nonsterilized soil. *Trichoderma* spp. showed stable and noticeable suppressive effects compared with other agents. Other reports confirmed the ability of different *Trichoderma* isolates to suppress the hyphal growth of the wilt pathogen in vitro (Xu et al. 1993) and to reduce the incidence of cucumber wilt in the greenhouse (Cho et al. 1989).

Another member in the PGPF group is the fungus *Gliocladium*. Not much work has been done on biological control of Fusarium wilt by this fungus. However, Shahida et al. (1992) showed that *Gliocladium virens* enhanced the ability of a nonpathogenic *F. oxysporum* isolate to suppress infection of okra roots by *Macrophomina phaseolina*. The same isolate reduced incidence of Fusarium wilt disease in both tomato and okra. Lacicowa and Picta (1992) reported that the best control of wilt diseases of runner bean (*Phaseolus coccineus* L.), caused by *F. o.* f. sp. *phaseoli*, was achieved with *Trichoderma koningii*, followed by *T. viride*, *G. catenulatum*, and *G. roseum*. Dohroo (1995) found that *T. harzianum*, *T. hamatum*, and *G. virens* were able to control wilt disease of ginger caused by *Fusarium o.* f. sp. *zingiberi* on ginger (*Zingiber officinale*), and an increase in ginger yield also was recorded. Shahida et al. (1991) stated that seed treatment with four biocontrol agents, including *G. virens,* completely controled of *F. oxysporum* on 30- and 120-day-old tomato plants. All treatments resulted in higher seed germination rates, and increased fresh weight, shoot length and plant length compared with an untreated control.

Cho et al. (1989) tested the antagonistic activities of 56 isolates of *G.*

virens from cucumber against *F. o. cucumerinum* in vitro. Isolate GC27 of *G. virens* strongly inhibited hyphal growth by means of nonvolatile antibiotics. In greenhouse tests, incorporation of a GC27 culture on wheat bran medium into sterilized soil infested with the pathogen was much more effective in reducing the incidence of cucumber wilt than was the application of conidial suspension without an organic food base. A wheat bran culture of GC27 decreased wilt disease incidence by 54-59% compared with the control.

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CHAPTER II

CHEMICAL AND BIOLOGICAL INDUCTION OF SYSTEMIC ACQUIRED RESISTANCE IN CUCUMBER AGAINST FUSARIUM WILT

INTRODUCTION

Plants resist disease development through a wide variety of mechanisms that can be either local or systemic, constitutive or inducible. In most cases, the extent to which these various mechanisms affect disease progression is poorly understood, and is an area of extensive biochemical and genetic research. One particular inducible systemic response, known as systemic acquired resistance (SAR), has become a subject of increasing inquiry. SAR can be induced by infection with a necrogenic pathogen, some natural or synthetic chemical compounds also can trigger similar plant responses (Kessmann et al. 1994).

Several definitions of the SAR have been suggested by researchers, depending upon their point of view. In a simple way, SAR is an inducible defense mechanism that mediates increased resistance against a broad spectrum of pathogens (Neuenscwander et al. 1995). In more detail, SAR is a nonspecific defense response in plants that is associated with an increase in the level of expression of the endogenesis-related genes (Bowlling et al. 1995). Induction of SAR usually follows the death of plant cells as a result of the plant

hypersensitive response (Strobel et al. 1996). Prior to SAR induction, metabolic changes in plant tissue take place, including an accumulation in salicylic acid in plant tissue, high level expression of SAR-related genes which results in high constitutive production of pathogenesis-related proteins (PRP), thus, resistance to a spectrum of pathogens is observed (Alexander et al. 1993; Delaney et al. 1995). SAR results in both a decrease in visible disease symptoms and a reduction in the growth of the pathogen (Ukens et al. 1993).

Biological induction

The natural phenomenon of resistance development in response to pathogen infection was first recognized by Ray (1901) who worked with *Botrytis cineria* on begonia. Ray reported that injecting the plant with an attenuated strain of the fungus induced resistance in the plant to subsequent infections with highly virulent strains of the same fungus.

For over 90 years, researchers and naturalists have observed that when different plants survive a pathogen infection, they develop an increased resistance to subsequent infections (Ryals et al. 1994). In 1933, Chester reviewed 200 publications describing a phenomenon he termed "physiological acquired immunity." In the early 1930's, scientists believed they were investigating a new phenomenon analogous to the immune response in mammals (Kessmann et al. 1994). At least three different processes were generally included under the term "acquired immunity": 1. Viral cross-protection; 2. Antagonism or biological control; and 3. What most researchers refer to now as SAR. During years after Chester's review, many papers were published on

the subject but mostly in a very descriptive way, extending and explaining the original observations (Ryals et al. 1994). In 1961, Ross presented the first systematic study of the SAR (Ross, 1961 a,b). Using tobacco masaic virus (TMV) on local lesion hosts, Ross demonstrated that infections of the virus were restricted and suppressed by a prior infection. This type of resistance was effective against not only TMV, but also tobacco necrosis virus and certain bacterial pathogens. Ross coined the term "systemic acquired resistance" to refer to the inducible systemic resistance (Ross, 1961 b) and the term "localized acquired resistance" to describe resistance induced in inoculated leaves (Ross, 1961 a). So far, it is still unclear whether SAR and localized acquired resistance are aspects of the same response or two distinct processes. Since Ross coined these two terms, SAR has been demonstrated in many plant species. The spectrum of SAR has now been broadened to include not only viruses and bacteria, but also many agronomically important phytopathogenic fungi.

Well-characterized examples of biologically induced SAR are recorded for cucumber (Kessmann et al. 1994). Cucumber was considered as a suitable plant for SAR studies, because it offers a clear and fast response to various treatments. Cucumber plants expressing SAR induced by non-pathogenic fungi and flourescent bacteria were protected against more than 13 viral, bacterial, and fungal pathogens in different studies (Kuc and Richmond, 1977; Marry et al. 1995; and Stroble et al. 1996). Most of the molecular-level studies investigating the SAR process were applied to different varieties of cucumber (Métraux and Boller, 1986; Métraux et al. 1991; Métraux et al. 1988; Xuei et al. 1988).

Pseudomonas cepacia

This plant growth promoting rhizobacterium (PGPR) has attracted considerable interest as a potential biocontrol agent of several plant pathogens. It recently was reported that *P. cepacia* was able to reduce the symptom development of Rhizoctonia stem rot of poinsettia (Cartwright and Benson, 1995 a,b), Fusarium root rot of beans (Sanchez et al. 1994), Fusarium wilt of radish (Toyota et al. 1994), Phytophthora root rot of chickpea (Myatt et al. 1993), Aphanomyces root rot and Pythium damping-off of pea (King and Parke, 1993), and Fusarium root rot and seedling blight of maize (Hebbar et al. 1992).

Several researchers correlated the antifungal activity of *P. cepacia* with production of certain antifungal antibiotic compounds such as phenazine (Cartwright et al. 1995), pyrrolnitrin (Burkhead et al. 1994), aminopyrrolnitrin and monochloroamino-pyrrolnitrin (McLoughlin et al. 1992), 2-keto-D-gluconic acid (Aoki et al. 1991) and a group of phenylpyrrol compounds (Roitman et al. 1990). In vitro studies proved that *P. cepacia* strains able to produce one or more of these compounds were capable of restricting mycelial growth and "conidiation" (rate of conidiospore production), by inducing frequent deformities in the hyphae and the conidiophore of the test fungi (Upadhyay and Jayaswal, 1992).

For many years, the biocontrol mechanism of *P. cepacia* was linked to antibiosis, rapid growth rates, high capacity to utilize a wide range of carbon sources exuded by plant roots (nutrient competition, Sanchez et al. 1994), and production of fungal cell and cell wall degrading extracellular enzymes as beta-1,3 glucanase (Fridlender et al. 1993), and pectinase and lipase (Hebbar et al.

1992 a).

Most researchers accepted the correlation between the in vivo antibiosis ability of *P. cepacia* and its biocontrol capabilities, but only a few researchers tested the hypothesis. Cartwright and Benson (1995 b) found that antagonist free preparations (sterilized culture filtrate) containing the antifungal antibiotic compounds failed to produce any level of biocontrol against Rhizoctonia stem rot of poinsettia (100% mortality). A significant difference in mortality ratio was obtained with preparations containing the antagonist. In a comparative study of different wild strains and antibiosis-negative mutants, McLoughlin et al. (1992) were able to obtain the same level of protection against sunflower wilt disease caused by *Sclerotinia sclerotiorum*. They used both the parent and the antibiosis-defective mutant strains. These investigators suggested that, in addition to various reported types of mechanisms, an important missing type was taking place in the biocontrol process of *P. cepacia* against different plant pathogens. In the present study, the object was to determine if systemic acquired resistance (SAR) is a possible answer to this question.

Chemical induction

Several disease control options are available, including resistant cultivars, biological control, different cultural practices, and chemical pesticides. Many compounds are available as pesticides, but nearly all are based on a direct antibiotic principle. Another option in chemical disease control could be based on compounds that induce the SAR response in plants . These compounds can have a long-lasting, broad spectrum, stable effect. They would, undoubtedly,

have an enormous positive impact on food production. Kessmann et al. (1994) considered a chemical to be an SAR inducer or "activator" if it repeatedly induced resistance to the same spectrum of pathogens and induced expression of the same resistance genes as the biological model. Furthermore, such a chemical should have no direct antimicrobial activity. In the event of antimicrobial activity, an SAR-inducing activity could be established by demonstrating resistance to pathogen isolates that are genetically resistant to the antibiotic effects of the chemical.

Salicylic acid (SA)

SA plays a central role in SAR-signal transduction upon pathogen infection, and has been known as an exogenous inducer of pathogen-related protein accumulation and resistance for long time (White, 1979). Application of SA induces resistance to the same spectrum of pathogens as seen with TMV-induced SAR in tobacco. Also, the same set of genes are induced in treated tissue to levels comparable to those observed with TMV-induced SAR (Ross, 1961a; and Friedrich et al. 1995). Furthermore, neither SA nor its metabolites had significant antibiotic activity (Kessmann et al. 1994). Thus SA fulfills all of the criteria of an SAR activator.

There are, however, problems with SA as an SAR inducer. Although SA is clearly involved in the induction of SAR, but it is not the vascular-mobile signal that moves throughout the plant triggering the resistance process (Vernooij et al. 1994). The exact nature of the systemic signal has not yet been determined. In a recent experiment by Lawton and coworkers (1994), ethylene was identified as

the acquired resistance signal transducer in *Arabidopsis*. More importantly, reports of SA-mediated resistance is restricted to effects in the treated tissue, indicating that SA does not translocate efficiently when applied exogenously. Raskin (1992) showed that exogenous SA rapidly becomes conjugated to other chemical compounds that lack the phloem mobility of free salicylate. This inability to deliver significant amounts of free SA to systemic tissue after exogenous application seriously detracts from its usefulness as an SAR activator. Finally, only a narrow safety margin separates the rates at which the compound is efficacious and the rates at which it is strongly phytotoxic (Kessmann et al. 1994).

Acetylsalicylic acid (ASA)

Aspirin, or ASA, was considered for some time as an inducer of SAR, but the required studies to fulfill all of the criteria of an activator were only partially completed. Mills and Wood (1984) reported that incorporating ASA into liquid shaking medium did not affect the growth of *Colletotrichum lagenarium*. When cucumber plants were treated with it in a split- root-system test, ASA was able to reduce anthracnose disease symptoms. Aspirin also was reported as an inducer of resistance against TMV in tobacco by Antoniw and White (1980). As a mechanism of inhibition, Larque-Saavedra (1979) suggested that ASA was shown to have an antitranspirant effect, closing the stomata in *Phaseolus vulgaris* and *Commelina communis.* White (1979) reported that ASA inhibited the multiplication of some animal viruses in tissue culture. ASA seems to be promising as an SAR activator, but still requires further investigation to establish

its role.

2,6-dichloroisonicotinic acid (INA)

2,6-dichloroisonicotinic acid, or INA, (CGA 41396) and its methylester derivative (CGA 41397; both referred to as INA) were discovered as agents able to induce systemic resistance in plants in the Agriculture Biotechnology Lab of Ciba Geigy Co. (Kessmann et al. 1993; Métraux et al. 1991). These compounds were found to provide good protection against fungal and bacterial pathogens on cucumber, rice, and other crops in greenhouse as well as under field conditions. Staub and coworkers (1992) carried out a detailed study on cucumber. They found that the activity spectrum of resistance induced by INA was identical to the activity spectrum of resistance observed after local inoculation with biological inducers. Also, Métraux et al. (1991) reported that the compound had no in vitro activity and was not converted in vivo into antimicrobial metabolites. Further, it was able to induce the expression of the same genes systematically involved in SAR-induced by microbial infection in cucumber. Thus, INA also fulfilled all criteria of an SAR inducer. At the molecular level, INA induces a group of enzymes involved in the systemic defense response, e.g., b-1,3-glucanase, chitinase, and 6-phosphogluconate-dehydrogenase (Linthorst, 1991). Also in some cases, INA caused single cell necrosis at the site of attempted penetration. Thus, the pathogen hyphae that still penetrate become surrounded by a cluster of necrotic cells and hyphal growth ceases, which is similar to the hypersensitive response of some hosts to incompatible genotypes of the pathogen. In addition to the induction of the SAR genes and enzymes cited above, INA compounds are

able to sensitize plant tissue to respond with additional defense reactions faster than untreated plants (Kauss et al. 1992).

Other potential SAR inducers

Different chemical compounds have been suggested as possible SAR activators but, so far, none were subjected to the kind of intensive pathological, biochemical, molecular, and histological studies that were available for the above mentioned inducers to fulfill the criteria previously established by Kessmann et al. (1994). The most important chemicals in this group are fosethyl-Al (Ward, 1984); triazoles (Hauthal, 1993); 2,2-dichloro-3,3-dimethylcyclopropane carboxylic (DCP) (Langcake and Wickins, 1975); ethephon, the ethylene-releasing agent and jasmonic acid (Dean and Kuc, 1988).

The aim of the current investigation was to explore the ability of SA, ASA, and INA to function as chemical inducers of SAR to suppress cucumber wilt and root rot fungi in vitro and to reduce disease incidence in vivo. Such expected activities could be then compared to the biological induction of SAR by of *P. cepacia,* the fluorescent rhizobacterium.

Materials And Methods

Effect of various inducers on hyphal growth and conidial formation fungi pathogenic on cucumber.

Fungal cultures.

The fungal cultures of cucumber pathogens used were: *Fusarium oxysporum* f. sp. *cucumerinum*, the causal organism of cucumber wilt; *F. moniliforme;* and *F. solani*, the latter two organisms cause cucumber root rot. The

fungi were maintained on corn meal agar.

Biological inducer.

The rhizobacterium *Pseudomonas cepacia,* was obtained from Stine Microbial Products, IA, USA (EPA reg. no. 63950-1).

Chemical inducers.

Acetylsalicylic acid (ASA or aspirin, Bayer, Germany) was used as tablets containing 325 mg of active ingredient with wetable powder carrier; salicylic acid (SA or 2-hydroxybenzoic acid) was purchased from Aldrich, WI, USA; and 2,6-dichloroisonicotinic acid (INA or CGA 41396) formulated as 25% active ingredient with a wetable powder carrier was obtained from CIBA-GEIGY AG, Basel, Switzerland.

a. Effect of biological inducers

The test was applied according to Upadhyay and Jayaswal (1992) with some modifications. The term conidiation will be used for the rate of conidiospore formation and the term chlamydoconidia will be used to describe the unusual transformation of one or more of *F. oxysporum* macroconidial cells into chlamydospores. In vitro inhibition of various fungal pathogens was tested by spotting four of 20µl aliquots of 1 day-old nutrient broth (Difco) culture of the bacterium (10^9 bacteria ml^{-1}) at the edge of corn meal agar (CMA) plates; five replicates (plates) were used per treatment. After 24 h at 28 C, fungal mycelial discs (3 mm in diameter) from the margin of 5 day-old actively growing culture on potato dextrose agar (PDA) were placed at the center of the plates. Plates were incubated at 28 C for 5 days when fungal growth towards the bacterium was

examined. The antagonistic effect of *P. cepacia* was recorded as the presence or absence of an inhibition zone between the colonies. The magnitude of inhibition ranged from none to weak inhibition, where the fungal mycelium did not grow over the bacterial spot, to strong inhibition, where a 20 to 25mm zone free of spore germination or hyphal growth occurred. Inhibition of conidiation was also noted. Small blocks (3 mm in diam.) were cut from the margins of the fungal colonies growing towards the bacterium, stained with lactophenol cotton blue, and observed under a dissecting microscope for morphological abnormalities. The hyphae growing towards the opposite side (i.e., not under the influence of *P. cepacia*) also was stained and observed. The same test was applied with an antagonist-free preparation (sterilized culture filtrate). Study of the morphological changes in the fungal growth followed a similar study by Updhyay and Jayaswal (1992). The average number of conidiospores in a microscopic field was estimated. 100x magnification of the same microscope was used for different estimations.

b. Effect of chemical inducers

In soild culture. SA, ASA and INA were prepared in phosphate buffer (0.01M pH 6.8) at the concentration of 0.2% (w/v). Preparations were sterilized by filtration and added to CMA for a final concentration of 0.02% before the medium solidified (Mills and Wood, 1984). This concentration was used to test different chemical inducers in vivo and in vitro because it was reported as the minimum concentration required for the induction of SAR in plants (Kessmann et al. 1994; Métraux et al. 1991; Mills and Woods, 1984). Five plates from each

preparation were inoculated in the center with 3mm in diam. discs of 5 day-old fungal culture. Inhibition index was calculated as the difference in mm in the average diameter of the fungus colony in treated and untreated plates free from any inducer. In addition, chemical stocks were diluted to 0.02%, and 20 µl of was spotted at the edges of five CMA plates to carry out the test described above.

In liquid culture. The Effect of the chemical inducers on the fungal growth in liquid medium was carried out in potato dextrose broth. Different chemicals were added to the medium at the concentration of 0.02%. Three 250-ml Erlenmeyer flasks were inoculated with 3mm discs of 5 day-old CMA cultures of test fungi. Mycelial dry weight of the different fungi in presence and absence of the inducers was recorded at 3 day- intervals for up to two weeks.

Induction of SAR in split root system by different inducers

Plant material.

Different varieties of cucumber (*C. sativus*) with varied susceptibilities to Fusarium wilt, i.e., Marketer Long, a susceptible cultivar; Straight Eight, a moderately susceptible cultivar; and White Wonder, a moderately resistant cultivar were used. Seeds were planted in 10-cm^2 pots filled with soil mix (peat moss, sand, loam, and vermiculite; 1:1:2:0.5) and grown at 28 C in a growth chamber with a 14 h photoperiod day and 65% relative humidity (RH).

a. Split root test

This technique was applied according to Liu et al. (1995) with modifications and is illustrated in Fig. 1. Roots of 3-week-old cucumber seedlings were uprooted, washed with tap water, and carefully split into two

halves with a dissecting knife. One half was dipped in 50 ml of a spore suspension of *F. oxysporum cucumerinum* (10^3 spores/ml), and the other half dipped in 50 ml of chemical inducer. For chemical induction, preparations of different chemical inducers in phosphate buffer (0.01M, pH 6.8) at a concentration of 0.02% were used. Bacterial suspension in phosphate buffer from a 48 h old culture (10^9 bacteria ml^{-1}) and an antagonist-free preparation (sterilized culture filtrate) were used as biological inducers. Each half of the root system was transplanted into a separate 10-cm^2 plastic pots. The pots were attached together with tape. Transplants were placed in a high humidity chamber (85% RH) at 25 C for 1 wk before being moved to the growth chamber. Relative humidity was maintained at 65% during the entire experiment with a humidifier and humidistat.

SAR treatments included *P. cepacia* and its culture filtrate, ASA, SA, and INA. For the control treatment, one half of the split root was inoculated with the pathogen, and the other half was treated with phosphate buffer (0.01M, pH 6.8). For the healthy control, cucumber roots were split and transplanted as described above but without any treatment on the roots. All treatments also were applied by stem injections. A 0.2ml sample of each preparation was injected with a sterile needle above the first internode four days before root exposure to the pathogen. For the control, 0.2 ml of distilled water replaced the inducer preparation. Ten double pots served for each treatment. Five served for the current study, and the other five were used to study the spread of the pathogen and antagonist in the plant. Each pot was placed in a plastic bag to prevent the

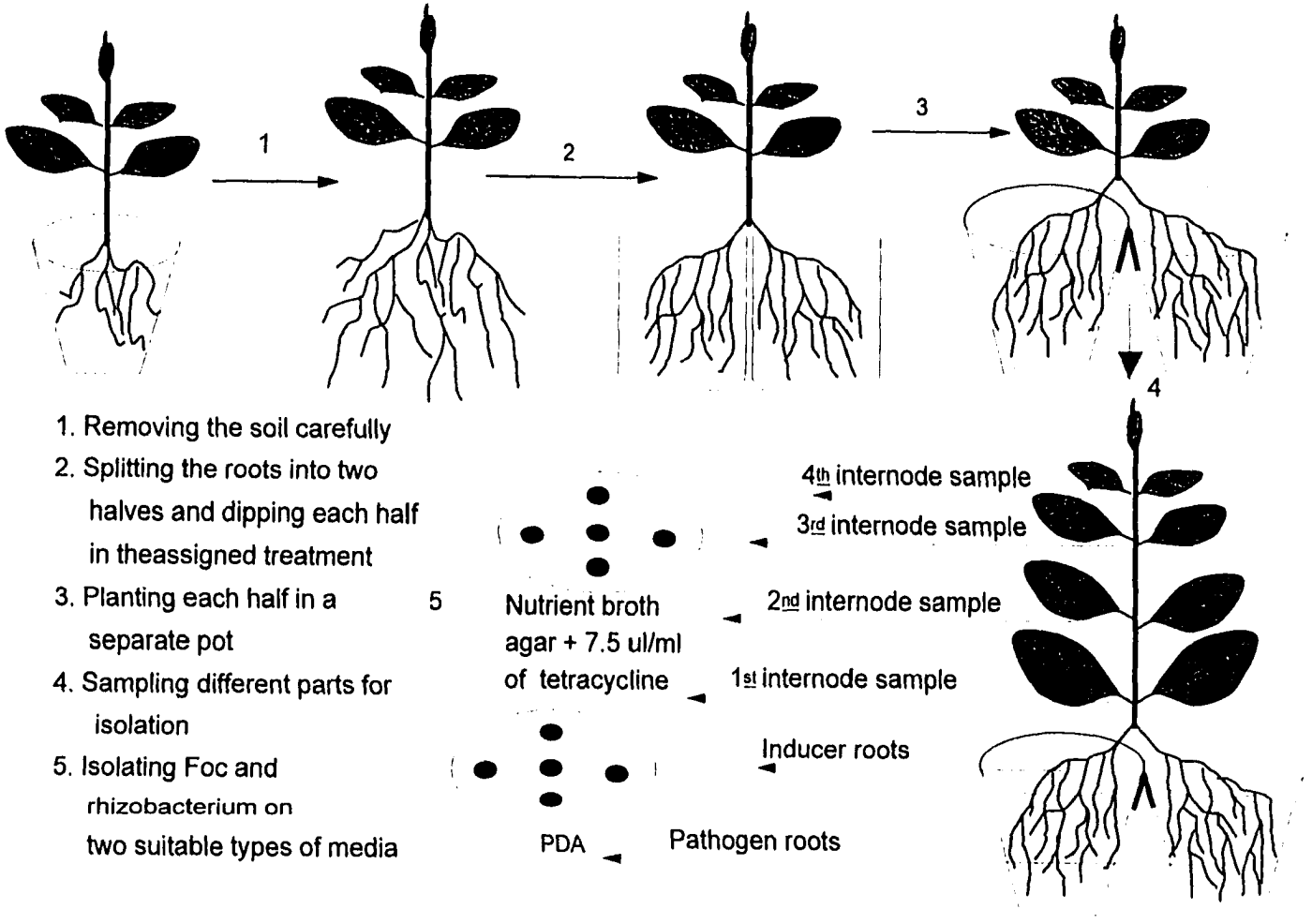

Figure 2.1. The use of split root system to test induction of SAR and internal spread of different microorganisms.

release of the pathogen, the bacterium, or the chemicals from drain holes and the possible subsequent microbial or chemical contamination of other pots. After applying treatments, the number of symptomatic leaves and dead plants were recorded weekly for one month.

Disease development on each plant was rated according to Liu et al. (1995) using the following scale: 5 = plant dead; 4 = 76 to 100% of leaves with symptoms; 3 = 51 to 75% of leaves with symptoms; 2 = 26 to 50% of leaves with symptoms, 1 = < 25% of leaves with symptoms; and 0 = no symptoms. A disease index was calculated from the disease rating by the formula:

Disease Index (D.I.) = Σ (rating no. x no. of plants in the rating) / total no. of plants x highest rating)

b. Spread of *P. cepacia* and *F. o. cucumerinum* within plants

The spread of *F. o. cucumerinum* and *P. cepacia* throughout the plants was determined according to Liu et al. (1995). Samples (5mm long segments) were taken from the roots in both pots of the split system, from the first through the fourth internodes of the stem and from the first through the fourth petioles. Samples were obtained following the growth of plants from all treatments 1 to 5 wk after inoculation. One plant was sampled from each treatment at each sample time. Segments of the different organs were surface disinfested with 1% sodium hypochlorite for 1 minute and rinsed thoroughly with sterile water then split into two parts with a sterile scalpel. Isolation was carried out on PDA for *F. o.* f. sp. *cucumerinum* with one half of the segment, whereas attempts to isolate *P. cepacia* were carried out with the other half by incubating specimens on nutrient

agar with tetracycline at 7.5 µl/ml (as a fungistatic) at 28 C for 48-72 h.

Statistical analysis

All experiments included at least five replicates per treatment and were repeated twice in two successive years. Data were subjected to one-way analysis of variance and when significance among treatments was indicated by the *F* test, means were separated by Duncan's multiple range test at P = 0.05. Data from repeated experiments were combined and analyzed according to Thomas and Hills (1978) when variances between trials were homogeneous. For in vivo experiments, a randomized complete block design was used while completely randomized design was used for in vitro studies. All data were analyzed using the general linear models procedure (GLM Proc.) in PC-SAS (SAS Institute, Cary, NC, USA).

RESULTS

Effect of inducers on hyphal growth and conidial formation of cucumber pathogenic fungi

a. Effect on hyphal growth

Pseudomonas cepacia was antagonistic against all test pathogens (Table 2.1). Growth of the fungi towards the bacterium was restricted, with an inhibition index ranging from 2.25 to 4.0. Maximum inhibition was recorded against the California (I) and Colorado (II) isolates of *F. o. cucumerinum* and *F. solani* (4.0, 3.75, and 3.75, respectively) followed by *F. moniliforme* (2.25). Sterile culture filtrate of the bacterium also was able to suppress the growth of the different fungi, but to a lesser extent. Maximum inhibition was recorded against *F. solani*

and *F. o. cucumerinum* I (2.75 and 2.5 respectively), followed by *F. moniliforme* (2.0), and *F. oxysporum* f. sp. *cucumerinum* II (1.25). None of the test chemical inducers had a significant effect on the growth of the various pathogens.

b. Effect on fungal conidiation

P. cepacia was able to inhibit conidiation of the test fungi to varying degrees. Maximum inhibition was recorded against *F. moniliforme* and *F. o. cucumerinum* I, 2 and 3 spores per microscopic field respectively; followed by *F . o. cucumerinum* II , 5; and *F. solani*, 7. The effect of the antagonist-free culture filtrate on inhibition of conidiation was significantly less than the bacterial effect. The filtrate inhibited conidiation of all of the fungi equally. Although chemical inducers tested negative on fungal growth, some affected conidiation of certain pathogens. Salicylic acid significantly increased conidiation of *F. moniliforme* and *F. o. cucumerinum* II compared with the control, whereas conidiation of *F. solani* was significantly reduced by either SA and ASA. Only INA had no influence on conidiation.

c. Mycelial deformities

Examination of 10 microscopic fields obtained from the edge of the colony of each fungus was carried out to determine both conidiation and mycelial deformities. Morphological changes brought about in the fungi by the inducers are summarized in Table 2.2. *P. cepacia* and its culture filtrate were able to cause morphological deformities in different test fungi, whereas the only chemical inducer able to cause deformities was aspirin. deformities included frequent formation of chlamydia, terminal or intercalary swelling of the hyphae, spiralling

Table 2.1 Inhibition of cucumber wilt and root rot fungi by various inducers *in vitro*

Inducers	Concentration	F.o.c. I [2]		F.o.c. II		F.m.		F.s.	
		I.G.[3]	#[4]	I.G.	#	I.G.	#	I.G.	#
P. Cepacia	10^9 cell/ml	4.00a[1]	3a	3.75a	5a	2.25a	2a	3.75a	7a
P.c filterate	of 10^9 cell/ml	2.50b	8b	1.50b	11b	2.25a	9b	2.75a	10b
ASA	0.02%	0.25c	13c	0.50c	14c	0.75b	13c	0.50b	14c
SA	0.02%	0.25c	15d	0.75c	20e	0.50b	18e	0.75b	17d
INA	0.02%	0.25c	17e	0.25c	18d	0.25b	15d	0.50b	18de
Control	no inducer	0.50c	16de	0.25c	17d	0.75b	16d	0.25b	19e

1. Values followed by different letters in the same column are significantly different (P = 0.05).
2. Fungal strains F.o.c I, California isolate of *F. oxysporum* f sp. *cucumerinum*; F.o.c. II, Colorado isolate of the same fungus; F.m, *F. monilifome*; F.s., *F. solani.*
3. Grading of the inhibition zones: 0, no inhibition; 1, 1-5 mm; 2, 6-10 mm; 3, 11-15 mm; 4,16-20 mm; 5, 21-25 mm.
4. # = Average number of conidia in 10 microscopic fields using 10x objective lens.

of the tip of the hyphae, swelling and other abnormalities in the conidiophores, general change in the thickness of the hyphae, lysis of conidia and hyphae, and the frequent presence of vacuoles of different sizes in the mycelium. Only *P. cepacia*, its culture filtrate and ASA were able to cause such deformities.

All test fungi exhibited various kinds of deformities. In general, *P. cepacia* had the strongest effect followed by the culture filtrate and ASA. The highest level of deformities was noticed in *F. moniliforme* in the presence of *P. cepacia* where more than 50% of the conidiophores were swollen and produced fewer conidia than the control. Clear lysis of the hyphae and the conidiospores occurred in the area next to the bacterial colony. Vacuolation was common and more frequent in hyphae treated with *P. cepacia* than in the control. In the cultural filtrate treatment, swelling of the conidiophore was the most common deformity, but the hyphae were normal. Lysis in the conidiospores was found in a narrow area at the edge of the fungal growth. Intercalary and terminal swelling of hyphae were detected with ASA, creating chlamydoconidia-like bodies. *F. o. cucumerinum* I followed *F. moniliforme* in the level of deformity. These deformities can be summarized as the formation of chlamydoconidia, swelling and coiling of hyphae, and lysis of hyphae and spores. Similar deformities were noticed when fungi were treated with *P. Cepacia* culture filtrate and ASA but to a lesser extent. No effect was found on *F. oxysporum* f. sp. *cucumerinum* II or *F. solani* when ASA was added to the medium, but *P. cepacia* and its cultural filtrate sometimes were able to infrequently produce hyphal abnormality in the region closest to the edge of the colony growth. *F. solani* was affected the least by the

Table 2.2 Morphological changes caused in cucumber fungal pathogens by various inducers

Inducers[1]	Concentration	F.o.c. I[2]	F.o.c. II	F.m.	F.s.
P. cepacia	10^9 cell/ml	Chlamydoconidia, terminal and intercalary swelling, spiraling, conidia and mycelium lysis	Terminal and intercalary swelling, abnormal conidiophores, frequent vacuolation of hyphae	Frequent swelling of conidiophores, vacuolation, lysis of hyphae and conidia, spiraling	Very thin hyphae, few terminal swellings, few spiralings
P.c filtrate	10^9 cell/ml	Terminal swelling, spiraling, and low number of conidia lysis	General swelling, abnormal conidiophores, rare spiraling	Frequent swelling of conidiophores, lysis occurred only in conidia	Few terminal swellings, few spiralings
ASA	0.02%	Terminal and intercalary swelling, terminal spiraling	No effect	Terminal and intercalary swelling of the hyphae	No effect
Control	no inducers	Rare intercalary swelling, few vacuolations in hyphae	Rare intercalary swelling, few vacuolations in hyphae	Frequent intercalary and terminal swelling	Rare spiraling in the hyphae

[1] Only the results of the inducers with positive effect on the fungal growth are shown.
[2] Fungal strains: F.o.c. I, California isolate of *F. oxysporum* f. sp. *cucumerinum*; F.o.c. II, Colorado isolate of the same fungus; F.m, *F. monilifome*; F.s., *F. solani*

inducers. *F. solani* developed smaller diameter hyphae in the presence of *P. cepacia*, a unique symptom.

c. Effect of chemical inducers on fungal growth in liquid medium

Potato-dextrose broth was used in this study, because different fungi had similar growth rates in PDA compared with other liquid media in a preliminary trial. Data shown in Fig. 2.2 summarize the effect of different inducers. None of the chemicals added to the medium at a concentration of 0.02% were able to cause a significant change in hyphal growth of the pathogenic fungi compared to the control.

Induction of SAR in cucumber plants

Most of the cucumber cultivars have different degrees of susceptibility to Fusarium wilt caused by *F. o. cucumerinum*, exhibiting in some cases systemic spread of the fungus and various types of wilt symptoms. In a preliminary test, it was found that cv Marketer Long is a susceptible variety, whereas cv Straight Eight and cv White Wonder are less susceptible. In our study, such variability was used to explore the ability of this group of cultivars to respond to chemical and biological SAR activators. Observations were recorded weekly for five weeks after inoculation. Because no significant change occurred during the first week and no more disease development was noticed after the fourth week, in Table 2.3, the summarized only data obtained at the second and fourth week is presented. These data present the general trend of the SAR induction process.

Analysis of data from two experiments of the split-root system indicted that both SA and INA, and *P. cepacia* were able to delay the initial onset disease by

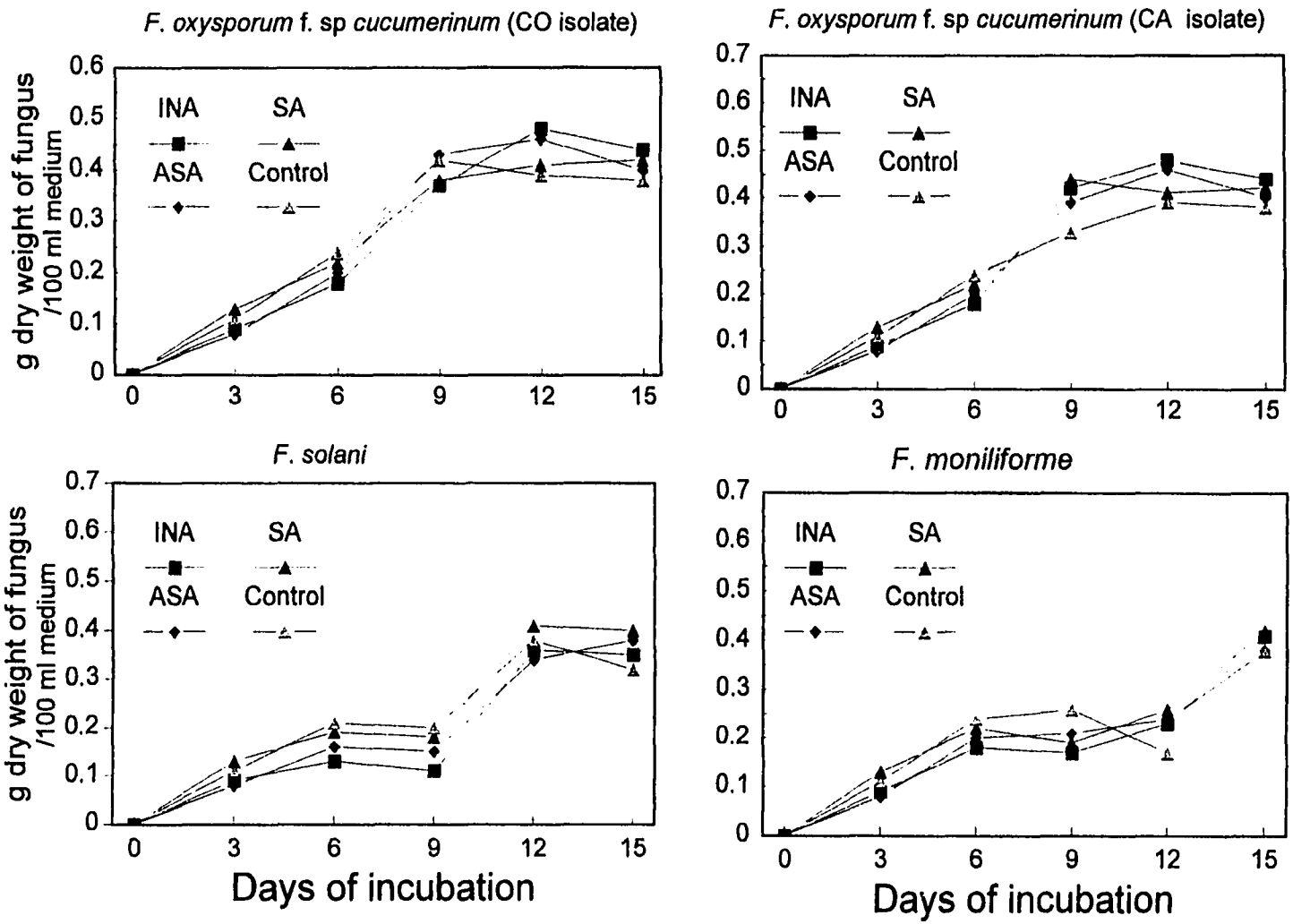

Figure 2.2. Effect of different chemical inducers on growth of cucumber wilt and root rot fungi in liquid medium.

the cultivars. Moreover, the final disease incidence was significantly reduced in cv Straight Eight and White Wonder treated with SA, whereas it was significantly reduced in all cultivars when INA was applied either as a stem injection or as a root dip. None of the other inducers were able to reduce disease incidence the same way except *P. cepacia* cultural filtrate on cv White Wonder, which basically is less susceptible, as a root dip treatment. The failure of the ASA to produce a significant level of protection for different cultivars of cucumber was noted. One hundred percent death of an entire treatment occurred in the control infected with the pathogen 4 wk after inoculation for all cultivars and with cultivars Marketer Long and Straight Eight plants treated with ASA as root dip treatment 4 weeks after treatment. No wilt symptoms were recorded in the noninoculates control plants except 4 weeks after Marketer Long and Straight Eight plants were dipped in sterilized water. *F. oxysporum* was not isolated from these plants. Other root rot fungi were found to be responsible for the symptoms as we used natural, untreated soil in all experiments. First symptoms on inducer-free cucumber plants were visible 7-10 days after root dip inoculation with the pathogen. However, symptoms did not develop simultaneously on every inoculated plant.

Movement of *P. cepacia* and *F. oxysporum* inside plants

P. cepacia was recovered up to 5 weeks after inoculation only from the parts of cucumber roots treated with the bacteria. The bacterium never was recovered from the pathogen-treated roots or from other parts of the plant, such as internodes or petioles. Attempts to isolate *P. cepacia* were carried out only in plants treated with the rhizobacterium. Data summarizing the pathogens internal

Table 2.3 Induction of SAR in cucumber plants against Fusarium wilt

Inducers	Concentrations	Time	Marketer Long		Straight Eight		White Wonder	
			Stem injection	Root dipping	Stem injection	Root dipping	Stem injection	Root dipping
P. cepacia	10^9 cell/ml	2 wk	0.2[1]	0.2	0.2	0.1	0.2	0.1
		4 wk	0.4	0.3	0.3	0.2	0.4	0.2
P.c. filtrate	10^9 cell/ml	2 wk	0.4	0.4	0.4	0.4	0.3	0.4
		4 wk	0.8	0.7	0.6	0.7	0.6	0.5
ASA	0.02%	2 wk	0.4	0.5	0.4	0.5	0.6	0.5
		4 wk	0.8	1.0	0.8	1.0	0.6	0.6
SA	0.02%	2 wk	0.2	0.3	0.2	0.1	0.2	0.1
		4 wk	0.5	0.4	0.3	0.3	0.3	0.2
INA	0.02%	2 wk	0.1	0.0	0.2	0.1	0.2	0.0
		4 wk	0.3	0.2	0.2	0.3	0.3	0.3
Pathogen only	10^3 spore/ml	2 wk	0.5	0.5	0.4	0.5	0.6	0.5
		4 wk	1.0	1.0	1.0	1.0	1.0	1.0
Healthy control	No treatment	2 wk	0.0	0.0	0.0	0.0	0.0	0.0
		4 wk	0.1	0.1	0.0	0.1	0.0	0.0

[1] Disease Index (DI) was calculated as DI = S (rating no. x no. of plants in the rating) / (total no. of plants x highest rating).

movement are presented in Table 2.4. *F. oxysporum* f. sp. *cucumerinum* was recovered from the internal tissue of cucumber plants in all treatments, either from the half-root initially treated with the pathogen, or from the stem and the petioles in other cases. Suppression of *Fusarium* spread inside the plant was noticed with INA, as the fungus was never recovered from internal tissue below the first internode in all sites and sampling times. Conversely, *F. oxysporum* was easily recovered from the first to the third internode in plants treated with *P. cepacia* cells or filtrate, and plants treated with INA, 4 wk after inoculation. Also, the fungus was able to reach the first and the second petioles after 4 wk in the infected control plants, the ASA-treated plants, and the culture filtrate-treated plants.

DISCUSSION

Effect of inducers on hyphal growth and conidia formation of cucumber pathogenic fungi

The investigation demonstrated that *P. cepacia*, when spotted on the edge of PDA plates, suppressed both hyphal growth and conidiation of various *Fusarium* spp. isolated from infected cucumber roots. These results are in agreement with Hebbar et al. (1992 b), who reported that *P. cepacia* suppressed both hyphal growth and conidiation of *F. moniliforme* isolated from maize roots on both PDA and Kings' B media. In addition, Upadhyay and Jayaswal (1992) reported that *P. cepacia* showed strong antagonism against several soilborne fungal plant pathogens, including *Fusarium roseum*, *Sclerotium rolfisii*, *Phytophthora megasperma*, and *Sclerotinia sclerotiorum* in dual cultures, with

Table 2.4 Isolation of cucumber wilt pathogen form different organs of treated Marketer Long plants

Inducers	Concentrations	Time	Internodes				Petioles				Pathogen roots	Inducer roots
			1st	2nd	3rd	4th	1st	2nd	3rd	4th		
P. *Cepacia*	10^9 cell/ml	2 wk.	+[1]	-	-	-	-	-	-	-	+	-
		4 wk.	+	-	-	-	+	-	-	-	+	-
P.c. filtrate	10^9 cell/ml	2 wk.	+	-	-	-	+	-	-	-	+	-
		4 wk.	+	+	+	+	+	+	-	-	+	-
ASA	0.02%	2 wk.	+	+	-	-	+	-	-	-	+	-
		4 wk.	+	+	+	-	+	+	-	-	+	-
SA	0.02%	2 wk.	-	-	-	-	-	-	-	-	+	-
		4 wk.	+	+	-	-	+	-	-	-	+	-
INA	0.02%	2 wk.	-	-	-	-	-	-	-	-	+	-
		4 wk.	+	-	-	-	-	-	-	-	+	-
Pathogen only	10^3 spore/ml	2 wk.	+	+	-	-	+	+	-	-	+	-
		4 wk.	+	+	+	+	+	+	-	-	+	-

[1] One plant from each treatment was used for isolation at sampling time. + indicates presence of the pathogen, - indicates its absence

inhibition of radial growth varying from 10 to 60 %. In the same study, inhibition of conidiation also was noticed for several other fungi, including *F. roseum*.

Morphological changes brought about in *Fusarium* spp. in dual culture with the bacterium or its filtrate was additional evidences for suppression by *P. cepacia*. Most of these changes, such as formation of chlamydoconidia, terminal or intercalary swellings, and stunting of hyphae were reported by Upadhyay and Jayaswal (1992). They attributed these changes to the secretion of antifungal compounds in the medium. One compound was isolated and identified as pyrrolnitrin (Burkhead et al. 1994). Crude filtrate in the present study and crude antifungal extract in the study of Upadhyay and Jayaswal (1992), when placed on medium inoculated with the fungi, were able to cause deformities and similar inhibition of hyphal growth and conidiation. These results were supported by the work of other researchers (Burkhead et al. 1994; Mishagi et al. 1995; Perdomo et al. 1995; Sanchez et al. 1994).

None of the chemical inducers were able to suppress fungal growth when added to the medium directly or by spotting samples on the edge of the plates. Such negative in vitro influences were reported for both SA and INA by Kessmann et al. (1994). Mills and Woods (1984) reported that incorporating ASA into liquid shaking medium did not affect the growth of *Colletotrichum lagenarium*. Furthermore, it was noticed that neither of the compounds were converted in vivo into antimicrobial metabolites. The same conclusion was reached by other researchers (Métraux et al. 1991). None of the studies reported an effect of any of the three compounds used in the present

investigation on conidiation and deformities of different fungi. Although the rate of growth of various fusaria was not affected by the presence of SA, ASA, or INA in solid medium, both SA and ASA were able to significantly suppress or enhance conidiation of all the test fungi. Moreover, ASA was found to cause slight mycelial deformities in *F. moniliforme* at a concentration of 0.02%. To confirm this observation, a single treatment trial was applied with 0.04 and 0.08% of ASA in CMA. More mycelial deformities were observed but at the same level for both concentrations. Thus, ASA may have a slight antifungal property that requires further investigation.

Induction of SAR in cucumber plants

The split-root system has been used to study resistance induced in plants against soilborne pathogens by nonpathogenic fusaria (Kroon et al. 1991; Mandeel and Baker, 1991), and by fluorescent *Pseudomonas* (Zhou and Paulitz, 1994). Maintenance of spatial separation of the inducer and the pathogen in this system offered a suitable method to study the induction of SAR. Results from SAR in vivo and isolation experiments were compatible and complementary to each other. INA and SA did not affect the growth of different cucumber-root pathogenic fungi, including *F. o. cucumerinum*, when they were added to the solid medium or when they were present in liquid medium in a shake culture. Further, INA and SA were not able to affect fungal conidiation or cause abnormalities in fungal growth at the concentrations used in this experiment. However, they delayed the initial onset of wilt and significantly decreased disease incidence. The spread of the pathogen within inoculated plants treated

with different agents also was studied. Reduction in disease development appeared to be related to delayed movement of the pathogen within INA- and SA- treated plants compared with nontreated plants or plants treated with the other agents. Similar conclusions were reached by Liu et al. (1995) in their attempt to explain the success of *Pseudomonas putida* strain 89 B-27 in protecting cucumber plants against Fusarium wilt. Ukens et al. (1992) reported a dramatic response was obtained at the attempted penetration sites in *Arabidopsis* when tissue was treated with INA to induce resistance against different fungal and bacterial pathogens. Single-cell necrosis at the sites was similar to the hypersensitive reaction that occurs in genetically incompatible plant-pathogen interactions (Koch and Slusarenko, 1990). Two kinds of evidence suggest that the fungicidal response observed with INA and SA treatment results not from direct effects on the pathogen, but from a reaction of the plant tissue to the chemical. First, neither INA nor SA had direct antifungal in vivo activity in the present investigation or in previous studies (Kessmann et al. 1994; Metraux et al. 1991). Second, both compounds have been shown to cause dramatic changes in plant gene expression which are similar to the changes observed during pathogen-induced immunization in animals (Friedrich et al. 1995; Ross, 1961a; Uknes et al. 1992; Ward et al. 1991). Thus, such compounds may induce resistance by mimicking some aspects of pathogen attack, possibly accelerating the normal responses of the plant to further infection (Uknes et al. 1992).

Some success was reached with ASA or aspirin to induce resistance.

White (1979) applied both injection and soil-treatment techniques with 0.02% ASA and was able to induce resistance against Tobacco Mosaic Virus in tobacco. A decrease of over 90% in number of local lesions was recorded, and lesion size was minimized. Mills and Wood (1984) reported that ASA in a split-root system was able to protect cucumber plants against *Colletotrichum lagenarium*, the causal organism of cucumber anthracnose. In this study, ASA was the only chemical agent able to affect the growth of the fungi in vitro, but it failed to provide significant protection against Fusarium wilt of cucumber either with stem injection or as a root treatment. In most cases, no significant differences were found between ASA treatments and the infected controls. This contradiction might be explained by the mode of action of this compound. Larque-Saavedra (1979) suggested that ASA might have an antitranspirant effect, closing the stomata in *Phaseoulus vulgaris* and other plants. This way, ASA would mainly work against the establishment of infection of foliar pathogens, but not in the case of soilborne pathogens.

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CHAPTER III

FACTORS AFFECTING BIOCONTROL CAPABILITY OF ANTAGONISTIC STRAINS OF *FUSARIUM OXYSPORUM* AGAINST FUSARIUM WILT OF CUCUMBER

INTRODUCTION

The fungal genus *Fusarium* predominantly contains soilborne pathogens collectively causing root diseases in a number of agricultural crops. Wilt diseases caused by various formae speciales of *F. oxysporum* are of major economic importance throughout the world. *F. oxysporum* is an extremely diverse species. Strains of the fungus are capable of surviving in soil, usually saprophytically on plant debris, and some can be pathogenic to animals, including insects and humans. The fungus infects over 150 species of plants including many of the world's most important export/cash crops produced in developing countries as staple sources of food (Rutherford, 1996) or foreign exchange.

In cucurbits, typical wilt symptoms resulting in mortality are manifested in young and mature plants and may be especially severe when infection occurs at the seedling stage (Freeman et al. 1993). Chemical control of *F. oxysporum* can be highly effective but has a number of major drawbacks, particularly in developing countries where cost and availability are limiting factors. Additionally, many political, ecological, and economical concerns

about pesticide use have motivated researchers in different parts of the world to increase their efforts to develop nonchemical approaches to plant disease control. Biological control of Fusarium wilt has been accomplished by introducing nonpathogenic or avirulent *Fusarium* spp. in soil or infection courts (Biles and Martyn, 1989; Alabouvette et al. 1993) and it consistently has provided a satisfactory level of control (Rutherford, 1996). Three different mechanisms have been suggested to explain the suppression of pathogenic *Fusarium* species in the presence of nonpathogenic *Fusarium* isolates: 1. nutrient competition between pathogenic and saprophytic *Fusarium* spp.; 2. competition for infection sites at the root surface; and 3. induction of systemic resistance against pathogenic strains (Schneider, 1984; Baker et al. 1978; Mandeel and Baker, 1991).

Studies of the possible factors which may influence the interaction between the pathogenic and the nonpathogenic isolates of *F. oxysporum* were initiated in order to provide greater understanding of the biological control process. The present study was initiated to characterize the ability of various nonpathogenic isolates of *F. oxysporum* to compete for infection sites, induce systemic resistance against *F. o. cucumerinum,* and reduce the incidence of cucumber wilt (Nassar and Hill, 1995).

MATERIALS AND METHODS

Fungal isolates.

Pathogenic isolates of *F. o. cucumerinum* and the nonpathogenic isolate C14, were obtained from Dr. Ralph Baker, Colorado State University, Fort Collins, CO,

USA. Avirulent N1 was isolated from Nunn sandy loam soil suppressive to cucumber Fusarium wilt. Isolation was carried out according to Paulitz et al. (1987), with modifications, as follows; Soil was air dried, sieved through a 4-mm-mesh sieve, and stored for 3 months. Inoculum of pathogenic *isolate F. o. cucumerinum* was added to the soil at 3.5×10^3 colony forming units (cfu)/g of soil. Seeds of susceptible cucumber cultivar (Marketer Long) were planted in ten 46 x 25 x 10 cm flats (40 seeds per flat). One month later most of the seedlings were dead and only those that remained without apparent wilt symptoms were used for isolation. Roots of each surviving plant were removed separately, washed, cut into small fragments, surface disinfested in 0.5% sodium hypochlorite (10% chlorox) + 5% ethanol (v/v) for 3-5 min., rinsed thoroughly in sterile distilled water (SDW), and plated on *Fusarium*-selective medium (Nash and Snyder, 1962). Along with the root isolation technique, a 0.5-cm segment of the stem over each internode was washed, surface disinfested, and plated the same way as the root segments. When *F. oxysporum* isolates were obtained from more than one internode of the same plant, only the isolate obtained from the highest point on the stem was selected for further investigation. Resultant colonies were transferred onto half-strength PDA plates and single spore cultures were made. Pure cultures were grown on PDA and carnation leaf agar for identification (Nelson et al. 1983). Cultures were stored in small vials containing twice-autoclaved sandy loam soil supplemented with a small amount of fine oatmeal and ground bean leaves. Although the N1 isolate is a typical avirulent strain of *F. o. cucumerinum*, for convenience, the term "nonpathogenic" will be

used to refer to both the N1 and C14 isolates.

Screening for nonpathogenic *F. oxysporum* isolates

A rapid, continuous-dip inoculation technique for assessing pathogenicity of *F. oxysporum* isolates was applied (Freeman et al. 1993). Cucumber seedlings of the susceptible cv, Marketer Long, were germinated in a vermiculite rooting medium. After 1 wk, seedlings were removed and roots washed under running water. Seedlings with intact roots and others with excised roots were placed in 100-ml beakers containing 50 ml of conidial suspension (10^5 conidia/ml) of various *F. oxysporum* isolates. Beakers were replenished with SDW amended with 0.05% agar whenever necessary. Plants were maintained in a phytotron at 28 C, >90% RH, and a 12h diurnal artificial white light cycle. Both intact-root plants and cuttings were observed daily for disease symptoms, such as cotyledon yellowing and wilting, sometimes accompanied by stem lesions. Control treatments were similar to the test-isolate treatments, except that no conidiospore suspension was added. Only the isolates that produced no symptoms and were reisolated from vascular tissue were considered as potential biological control agents. These were evaluated in separate tests for their protective abilities.

Induction of systemic resistance against *F. oxysporum* f. sp. *cucumerinum*

Cucumber seeds of moderately susceptible cv, Straight Eight were planted in a vermiculite rooting- pot mix, grown in a growth chamber for 3 wk under the above described conditions, and then removed from the medium and washed carefully under running water. For one half of the plants, root tips were

excised, whereas for the other half the roots were left intact. Roots were immersed in a microconidial suspension (10^5 conidia/ml) of C14 or N1 or only SDW for 20 min. Zero, 3, and 5 days after replanting in soil mix (clay soil and peat moss; 1:0.5), a shallow longitudinal wound in the stem, 5 cm above the soil, was made in each plant with a sharp sterile blade. A 0.5cm diameter section of the pathogen culture, grown on water agar, was placed in each wounded covered with moistened filter. Masking tape was wrapped over the wound. Pathogen free water agar discs were used for the control treatment. Plants were grown in a growth chamber under 80% RH, 26 C, and a 2 h diurnal artificial white light cycle for 2 wk before disease assessment. Disease development on each plant was rated according to Liu et al. (1995).

Effect of inoculum density of the pathogen and the antagonists on disease incidence

To study an inoculum density-disease incidence relationship, an organic-mix inoculum rich in chlamydospores from each isolate was prepared (see Appendix). Air-dried Nunn sandy loam soil was mixed with 0, 0.3 x 10^4, 1.2 x 10^4, or 3x10^4 cfu/g of either isolate C14 or N1, mixed in a twin-shell soil blender, distributed in small pots (4 cm in diam.), and planted with one cucumber cultivar Straight Eight seed. One week later, five seedlings with the soil intact were moved into larger pots, 15-cm in diam, containing 0, 500, 1000, or 2000 cfu/g soil of the pathogen. Each treatment consisted of five replicates (pots). Plants were kept under greenhouse conditions (25 ± 3 C per day, 18 ± 3 C per night, 70% RH), irrigated with tap water as needed, and fertilized with 20 ml of 1:200 of 10-

10-10 (gm/ml) fertilizer concentrate once weekly. Disease ratings were recorded 45 days after planting.

Effect of nonpathogenic isolates on disease incidence over time

Isolates C14 and N1 were tested for their effects on disease development over time. One inoculum level was applied for each isolate, 1000 cfu/g of soil for the pathogen and 3 x 10^5 cfu/g of soil of either C14 or N1. Three replicates, each containing ten 15-cm-diam pots, served for each treatment. Two seeds of cucumber cv Straight Eight were planted in each pot, but only the largest seedling was left to grow 1 wk after emergence. Plants were kept in the growth chamber, and disease incidence was recorded as percentage of dead plants every 2-3 days after Fusarium wilt symptoms first appeared, up to 72. Control plants were inoculated with the pathogenic isolate alone.

Effect of the interval between introduction of the biocontrol agent and challenge with the pathogen on disease development

As a result of the success obtained in the systemic resistance experiment, a growth chamber experiment was designed to test the ability of nonpathogenic isolates to induce a higher level of resistance in plants if they were allowed more time challening with the pathogen. Two levels of inoculum were applied for each antagonist, 1.2 x 10^4 and 3 x 10^4 cfu/g of soil. The pathogen was introduced into the soil as a 10 ml of conidiospore suspension (10^4 spores/ml) on a daily interval in separate treatments up to 10 days. Disease incidence was recorded in all treatments as percentage of dead plants 45 days after planting. Three replicates, each containing five pots were used per treatment.

Effect of presence and absence of root wounds on the protection by antagonists against soil infection

In this study, the ability of the antagonists to compete with the pathogen in soil, to penetrate plant roots directly and to protect cucumber seedlings from wilt disease were evaluated. An organic inoculum preparation rich in chlamydospores of each isolate was used. The pathogen was introduced into the soil at 10^4 cfu/g in combination with either the C14 or N1 isolate at a concentration of 3 x 10^4 cfu/g of soil. Three-week-old cucumber cv Straight Eight seedlings with intact or excised roots were transplanted into soils of different treatments. Five replicates, each containing five pots, were used per each treatment. Results were recorded as percentage of dead plants at 10, 20, 30, and 45 days after transplanting.

Statistical analysis

All experiments were repeated twice. Treatments were arranged in a complete randomized block design. Data were subjected to a one-way analysis of variance. When significance was obtained among treatments in the *F* test, means were separated by use of Duncan's multiple range test at $P = 0.05$. Data from repeated experiments were pooled according to Tomas and Hill (1978) when variances between trials were homogeneous. Angular transformations of the percentages were analyzed if the range of percentages was greater than 40. Data were analyzed by the "general linear models procedure" (GLM Proc.) in PC-SAS (SAS Institute, Cary, NC, USA).

RESULTS

Induction of systemic resistance against *F. o. cucumerinum*

Cucumber cv Straight Eight seedlings, with intact or excised roots, were exposed to N1, C14 or water and were stem-inoculated with *F. o. cucumerinum*. There were no significant differences in the degree of induced resistance in the presence or the absence of root wounds with either N1 or with C14 (Table 3.1). The number of infected plants in the N1 and C14 treatments was significantly lower compared with the inoculated control. No significant difference was found between N1 and C14 when seedlings were inoculated simultaneously with the pathogen and the antagonist. However, when the pathogen was inoculated 3 or 5 days after introduction of the antagonist, N1 treatments significantly reduced wilt disease compared with C14 treatments. When the pathogen was introduced after 5 days, no significant differences were detected in either root or stem infection between N1 plants and control plants (treated only with water). Increasing the time between introduction of the antagonist and inoculation with the pathogen resulted in significant increase in the level of induced systemic resistance. In antagonist-free control treatments inoculated with the pathogen, root-wounded plants had a slightly lower infection compared with unwounded plants. This suggests a mechanism in disease suppression induced only by mechanical injury of the roots.

Effect of inoculum density of the pathogen and the antagonists on disease incidence

Increases in inoculum density of the pathogen significantly altered the

Table 3.1 Induction of systemic acquired resistance against *F. o. cucumerinum* by nonpathogenic *F. oxysporum* isolates

Root treatment	Concentration	Days before inoculation	Wounded Roots		Unwounded Roots	
			Foc†	WA†	Foc†	WA†
C14	10^5 spore/ml	0	0.567	0.033	0.600	0.067
		3	0.467	0.067	0.500	0.033
		5	0.267	0.033	0.233	0.067
N1	10^5 spore/ml	0	0.567	0.067	0.533	0.033
		3	0.333	0.033	0.367	0.067
		5	0.067	0.067	0.067	0.033
Water	-	0	0.967	0.033	0.833	0.067
		3	1.000	0.033	0.867	0.033
		5	0.933	0.067	0.800	0.033

† Stem inoculation with: Foc, water agar discs of pathogenic *F. oxysporum* f. Sp. *cucumerinum*; or WA, pathogen-free water agar discs

infection level of the wilt disease in plants protected with different concentrations of either N1 or C14. Addition of either antagonist at different inoculum densities in treatments containing 500, 1000, and 2000 cfu/g of the pathogen significantly reduced disease incidence except where 3×10^3 cfu/g of C14 were added. Regression analysis of disease severity data showed that isolate N1 affected the incubation period of the wilt disease and delayed symptom appearance as evidenced by an increase in the value of the regression coefficient *a* (the y-intercept) with increasing inoculum concentrations of the pathogen (data are not shown).

Both isolates C14 and N1 gave consistent results in both trials of this experiment. At the lowest concentration (3×10^3), however, N1 resulted in lower disease incidence in the first than in the second trial. In the second trial, there was no significant difference between N1 at a density of $3x10^3$ and the inoculated control 1 wk after inoculation, whereas in the first trial, a reduction in disease severity was obtained under the same circumstances. After 2 wk, no significant differences in treatments were recorded between both trials. Isolate N1 significantly reduced disease incidence compared with isolate C14 at different concentrations as shown in Figure 3.1.

Effect of nonpathogenic isolates on disease progression over time

When 3×10^5 cfu/g of either C14 or N1 were added to soil containing 1000 cfu/g of the pathogen, both antagonists delayed the first appearance of wilt symptoms by 8 and 14 days, respectively, when compared with the control treatment inoculated only with the pathogen (Figure 3.2). Development of 20%

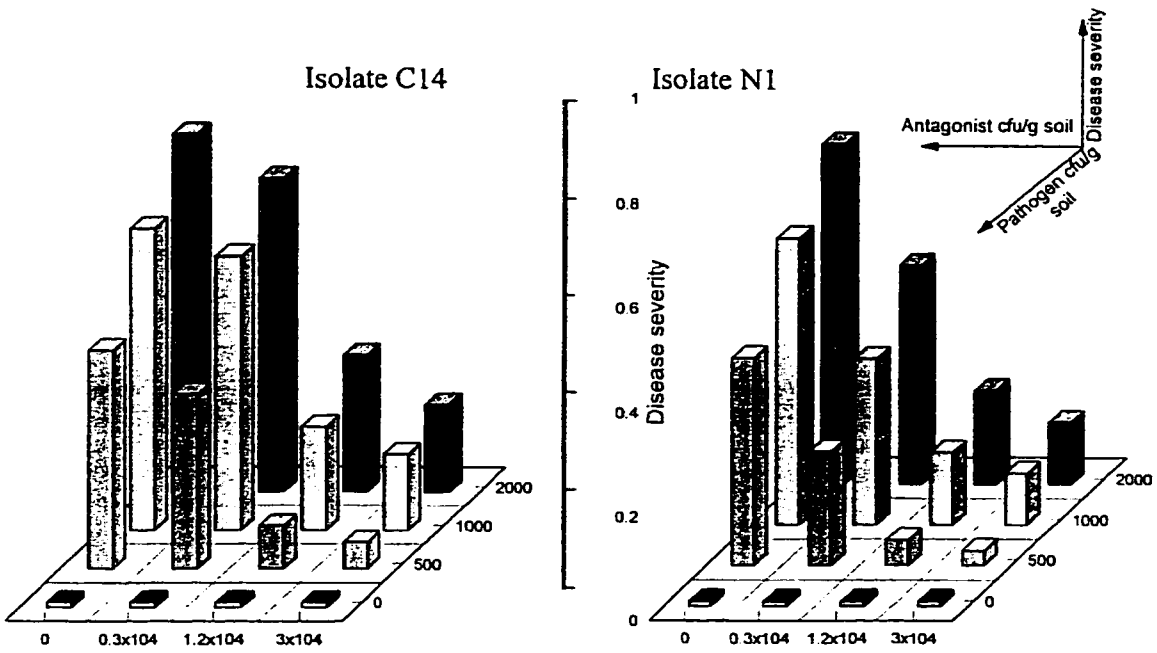


Figure 3.1. Effect of different levels of antagonists on disease occurrence.

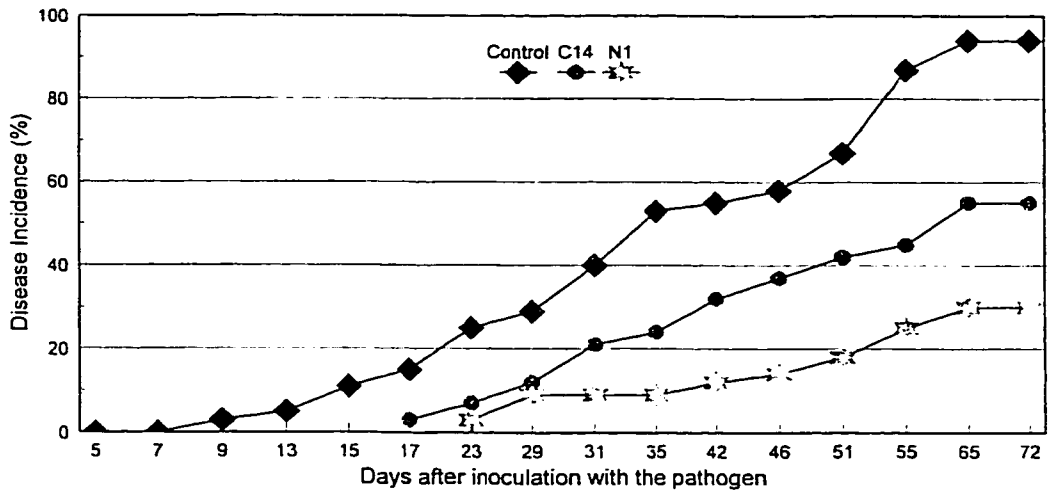
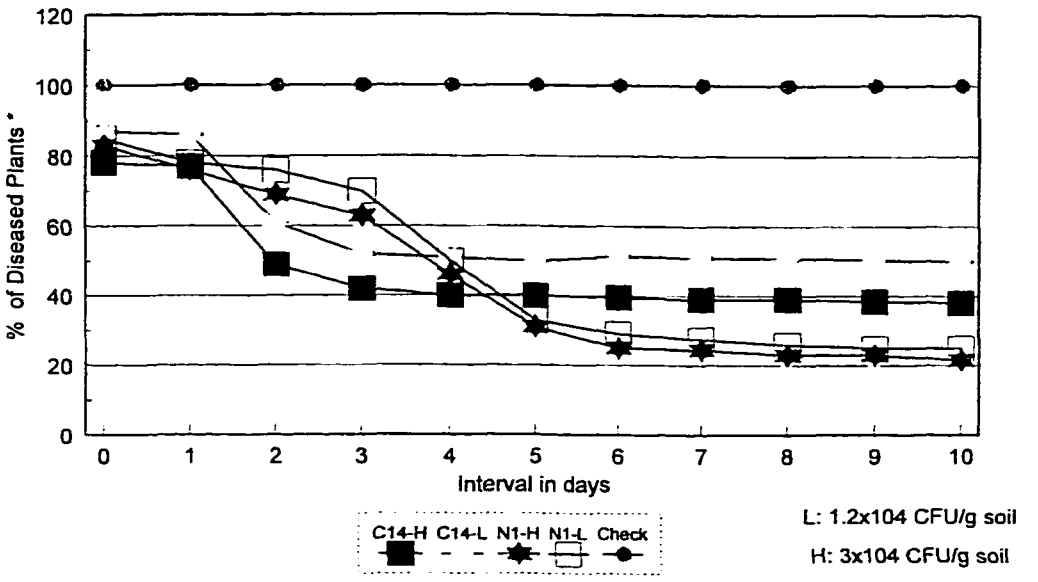


Figure 3.2. Disease progression after inoculation of treated and untreated plants.

disease incidence in control plants was obtained 21 days after inoculation, whereas the same level was reached after 31 and 52 days in the soils treated with isolates C14 and N1, respectively. Both antagonists significantly reduced the rate of increase in disease incidence. Regression analysis showed that slope values were significantly less in the antagonist treatments compared with the inoculated control. Inhibition of disease incidence and rate of disease development were significantly higher in the N1 treatment than in the C14 treatment. Seventy-two days after inoculation with different isolates, the mortality percentages were 30, 55, and 94% for N1, C14, and pathogen treatments, respectively.

Effect of the interval between introduction of the biocontrol agent and challenge with the pathogen on disease development

No significant differences were detected between C14 and N1 in protecting cucumber seedlings from wilt disease when the pathogen was added at the same time as the antagonists, or 1 day later (Fig. 3.3). However, when the pathogen was added between 2-3 days after the antagonist, C14 was significantly able to provide better protection for the seedlings than N1. With 5-10 days difference between the introduction of the antagonist and the pathogen, N1 was significantly more capable of inducing a higher level of resistance in treated plants than C14. At the 5-day interval or more, no significant differences were found between the lower (1.2×10^4 cfu/g of soil) and the higher (3.0×10^4 cfu/g of soil) density of antagonist N1, whereas such differences were found between these densities in C14 treatments.



* The percentage of plants with wilt symptoms recorded 45 days after inoculation

Figure 3.3. Effect of intervals between introduction of the antagonist and inoculation with the pathogenic Fusarium.

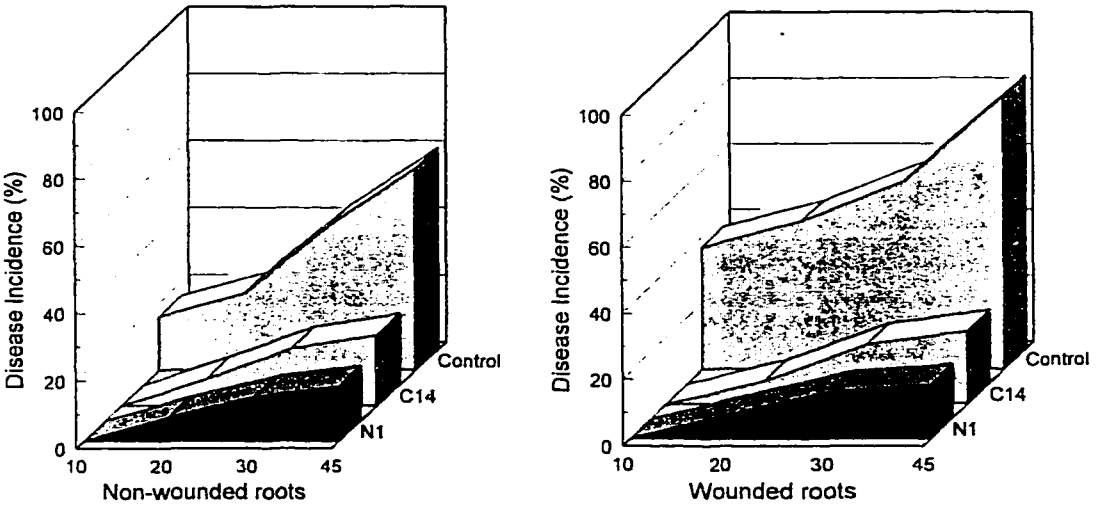


Figure 3.4. Fusarium development in presence and absence of root wounds.

Effect of root wounds on protection of cucumber seedlings from soil infection by *F. o. cucumerinum* in soil

The general ability of both antagonists to compete with the soilborne wilt pathogen for nutrients and infection sites was tested. Data presented in Figure 4.4 are similar to previous experiments when the pathogen was inoculated through stem wounds; i.e., no significant differences were obtained for the same antagonist in the presence or absence of root wounds. Only in the case of the control treatment did root wound enhance the capability of the pathogen to cause wilt disease. Isolate C14 was significantly better than isolate N1 in protecting cucumber roots from the disease.

DISCUSSION

Protection of different vegetable crops against Fusarium wilts by the use of nonpathogenic isolates of *F. oxysporum* is a well-studied phenomenon. Remarkable success was obtained with tomato and cucumber (Nelson et al. 1992; Rattink, 1993), carnation (Rattink, 1992), watermelon (Li and Zhang, 1990), and sweet potato (Ogawa and Komada, 1984). One of the most important criticisms of the techniques used to study biological control phenomenon was made by Paulitz et al. (1987). They noticed that in most of the studies, cut, wounded, or bare roots were dipped in or treated with suspensions containing high concentrations of propagules of the potential biocontrol agents, and that application of agents by use of such artificial methods could bypass resistance mechanisms that might function in intact, noninjured roots. Also, it was concluded that conidiospores, the type of propagules most frequently used in

such experiments, usually are not the propagules involved in pathogen survival under field conditions. Finally, in most of the experiments, plants were grown under adjusted conditions or in steamed greenhouse mixes, which might eliminate typical microbial interactions found in raw soil. Thus, several techniques were used in the current investigation.

A conidiospore suspension was the source of inoculum in the studies of pathogenicity and the induction of resistance studies. An organic preparation containing high concentrations of chlamydospores of the fungus was used for inoculum density and disease development-over-time studies. Both types of inoculum sources were used in the studies on interval introduction of antagonists and the effect of root wounding. Intact and excised bare-root techniques were applied in pathogenicity, induction of resistance, and effect of root wound studies, whereas an intact uninjured root treatment was applied in inoculum density, development-over-time, and antagonist introduction interval studies. Additionally, sterilized medium and raw soils were used in different experiments according to the suitability and convenience of root treatment in each case. In spite of such diversity in methods and techniques, results obtained from the various studies showed a high degree of consistency.

The techniques were used to screen various antagonists obtained from Nunn soil. A rapid inoculation technique, by which excised and intact bare roots were dipped in a conidiospore preparation and a classical technique in which intact uninjured roots were inoculated with chlamydospores from organic compost preparation were used. Both techniques yielded similar results with no

significant differences, therefore, only the data from the new rapid-dip technique are presented here. This conclusion should encourage researchers interested in biological control to explore available techniques that require less time, effort, and space. Some of these techniques can offer accurate inferences of the interaction between antagonists and biological control agents similar to the inferences that can be obtained by the costly, time consuming classical greenhouse or field tests.

Although there are occasional contradictory reports in the literature (Shimotsuma, et al. 1972), most studies show antagonists closely related to the pathogen (i.e., avirulent races and formae speciales) are better inducers of disease resistance than nonpathogens of unrelated hosts (Martyn et al. 1991). Moreover, Bills and Martyn (1989) found that were avirulent races of *F. o. niveum* were better inducers of systemic resistance than related formae speciales with additional differences between the avirulent races. Similar results were reported by Mas et al. (1981) with Fusarium wilt of muskmelon. In their study, avirulent races of *F. o.* f. sp. *melonis* (race 0 or 1) were effective inducers of systemic resistance against virulent races. Results of a recent field study (Martyn et al. 1991) support these general observations. An avirulent race of *F. o. niveum* (race 1) induced a high level of resistance in watermelon against the virulent race 2, whereas a different cucurbit forma speciales, *F. o. cucumerinum*, did not induce significant resistance, although it delayed symptom development.

In the current investigation, two *F. oxysporum* isolates were used to induce systemic resistance against cucumber Fusarium wilt caused by *F. o.*

cucumerinum. The first, C14, is a nonpathogenic isolate originally was obtained by Paulitz et al. (1987) from surface-disinfested, symptomless cucumber roots grown in Nunn sandy loam soil. No systemic relationship between the isolate, C14, and cucumber plants was shown. Also, no attempt to isolate the fungus in other parts of the plant was made by other researchers who tested it as a biological control agent against Fusarium wilt diseases (Paulitz et al. 1987). The fungus isolation was carried out in a preliminary test in the current investigation. One month after introducing C14 into the root system of cucumber cv. Marketer Long seedlings, the isolate was only found in the stem above the ground level below the first internode. The second isolate, N1, was also obtained from Nunn soil during this investigation. It was originally isolated from the vascular system of cucumber cv Marketer Long seedlings, 15 cm above the ground over the fourth internode. This ability to penetrate the vascular bundle of the plant and to move upwards through the stem indicates a compatible systemic relationship between the isolate and the plant.

Induction of systemic resistance by biocontrol agents does not require the spread of the agent to different parts of the plant because a chemical signal is thought to be responsible for the initiation of this type of induction. In the current study, during screening of numerous potential biological control *F. oxysporum* isolates, it was noticed that a higher degree of compatibility between the agent and the host usually indicated a higher degree of induced resistance. Avirulent isolate N1 was superior to nonpathogenic isolate C14 in most cases. It offered a more extensive level of protection to cucumber plants against Fusarium wilt

disease. This resulted in a significant reduction in disease incidence and in overall disease severity.

Martyn et al. (1991) mentioned that it is common in vine crops, such as watermelon and muskmelon, for one or two runners to wilt while the remaining two or three runners appear healthy and bear fruit. Therefore, a further reduction in the number of wilted runners attributable to induced resistance could result in better yields even in the presence of a certain degree of wilt disease. This phenomenon could be demonstrated by comparing the disease incidence data, in which all plants with symptoms were included, with the disease severity data. In the present research (Fig. 3.5), the response of cucumber plants was quite similar to that of watermelon and muskmelon. Development of cucumber wilt in a moderately susceptible cv, Straight Eight, in the presence and absence of the antagonists N1 and C14 was investigated over 72 days. By the end of the experiment, 94% of the control plants were dead, whereas 46.7 and 66.7% survival was recorded for C14 and N1 treatments, respectively. Not all the plants surviving the infection were disease-free. Some of them showed partial wilt symptoms in one or more runners (20 and 15% of the survivors for C14 and N1 treatments, respectively). These plants were able to produce flowers around 50 days after emergence along with the totally healthy plants, and the resultant fruits developed normally until the termination of the experiment. Moreover, when the second and third true leaves of induced and noninduced plants were inoculated with the fungus *Cladosporium* sp. (isolated from greenhouse cucumbers), the severity of leaf infection was less in plants induced by either antagonist than in

Figure 3.5. Cucumber fruits carried on healthy (A) and wilted (B) runners, with enlargement of infected fruits.

noninduced plants (Fig. 3.6). Also, the distribution and the type of leaf spot infection on the leaves was different. On induced plants (Fig. 3.6A), many small colonies developed but, because of the hypersensitive reaction, infected tissue became necrotic 1 wk after the first appearance of symptoms and the leaves survived the infection. On the non-induced plants (Fig. 3.6B), the infection was local and more aggressive. During the first week, different spots, especially on the tip of the leaves, expanded and enlarged in size. Two weeks later, spots fused and most of the leaf was covered by fungal growth and spores before leaf death. These results support those of Martyn et al. (1991) in which no difference in disease incidence was found between induced and noninduced treatments. There were, however, differences in disease severity resulting from different abilities of plants inoculated with different antagonists to bear fruits on the healthy watermelon runners. To explain these results, a thorough isolation from different parts of C14 and N1 healthy survivors resulted in isolation of the pathogen from the vascular bundle between the third and the fourth internodes. Identification of the pathogen isolates was based on microscopical and cultural characteristics of the fungus in addition to a pathogenicity test that was carried out on susceptible Marketer Long plants. According to Martyn et al. (1991), these findings suggest that induction of resistance by the antagonists could not prevent initial infection by the pathogen, but may have limited its colonization and subsequent damage of the plant.

Figure 3.6. Cladosporium leaf spot on cucumber leaves induced (A) and not induced (B) by antagonist.

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CHAPTER IV

THE USE OF PLANT GROWTH PROMOTING FUNGI IN BIOLOGICAL CONTROL OF CUCUMBER FUSARIUM WILT

INTRODUCTION

Trichoderma and *Gliocladium* species are widely distributed all over the world (Domsch et al. 1980) and occur in nearly all soils and other natural habitats, especially those containing or consisting of organic matter (Papavizas et al. 1985). *Trichoderma* species seem to be secondary colonizers, as its frequent isolation from well-decomposed organic matter indicates (Danielson and Davey, 1973). *Trichoderma* is also found on plant root surfaces (Parkinson et al. 1963) on decaying bark, especially damaged by other fungi (Danielson and Davey, 1973) and on the sclerotia and propagules of other fungi (Wells et al. 1972). Although there have been numerous attempts to study the occurrence and distribution of *Trichoderma* and *Gliocladium* species in their natural habitats, few workers have attempted quantitative studies of their population dynamics and survival in soil (Papavizas, 1985). The lack of information on the survival of the antagonists in soil is due, in part, to the lack of precise techniques and the appropriate culture media for isolation and enumeration (Papavizas, 1981). Papavizas (1985) reported that the abundance of *Trichoderma* and *Gliocladium* species in various soils, coupled with their ability to degrade various organic substances in soil, their metabolic versatility, and their resistance to microbial

inhibitors, suggest that they may possess the ability to survive in many ecological niches depending on prevailing conditions and the species or strain involved. He mentioned that this assumption might clarify the distinction between passive survival and active competitive saprophytic ability (CSA), including the dynamic colonization of the plant rhizosphere. Such considerations can be elucidated more readily with introduced isolates than with static, natural populations of *Trichoderma* and *Gliocladium* species.

The fungistasis nature of soil (Lockwood, 1977) and the ability to enhance it (Papavizas and Lumsden, 1980) may have considerable impact on the survival and population dynamics of natural and introduced species of *Trichoderma* and *Gliocladium* (Papavizas, 1985). *Trichoderma* conidia have been found to be either very sensitive to fungistasis (Lockwood, 1977) or to be relatively insensitive (Mitchell, and Dix, 1975). The sensitivity to fungistasis was more pronounced in neutral or alkaline than in acid soils (Danielson and Davey, 1973). Schippers et al. (1982) advanced an intriguing aspect of soil fungistasis in relation to *Trichoderma* and *Gliocladium* conidia that involved volatile substances. They present evidence that ammonia was fungistatic to conidia of the two genera in alkaline soils at levels as low as 1mg/g of soil air. Not much is known of the effect of volatile substances on *Trichoderma* and *Gliocladium* chlamydospores (Papavizas, 1985).

Selective passive or active possession and exploitation of substrates by soil microorganisms may be determined by various factors (Bruehl, 1975), and in many instances, these factors may not be favorable for *Trichoderma* and

Gliocladium species (Papavisas, 1985). Several approaches have been proposed to overcome this difficulty. Lewis and Papavisas (1984) showed that *Trichoderma*, *Gliocladium* and other potential biocontrol fungi proliferate abundantly in various natural soils when added as young mycelia in intimate contact with food base, but not as conidia mixed with or without organic source of food. They suggested that proliferation and subsequent establishment in soil depended on inoculum age and how it was added to the food base. The thorough colonization of the food base by mycelia enables *Trichoderma* or *Gliocladium* to grow to grow relatively unimpeded through the soil. Papavizas (1985) noticed that thorough primary colonization of a substrate prior to addition to soil might not be the only approach to overcoming secondary colonization of the food base by soil microorganisms and of enhancing the chances for antagonist establishment and proliferation.

One of the most critical obstacles to biocontrol by direct massive soil augmentation or seed treatment with biocontrol fungi such as *Trichoderma* and *Gliocladium* has been the lack of methods for mass culturing these fungi. Considerable progress in fungal technology for the large-scale production of metabolites and industrial products has already been accomplished (Kenny and Couch, 1981; Kristiansen and Bu'Lock, 1980). Consequently, it is possible that this technology can be adapted for mass production of biocontrol agents. Solid media frequently have been used in laboratory, greenhouse, and field studies for experimental production of *Trichoderma* and *Gliocladium* (Elad et al. 1980; Elad et al. 1981a), various substances including bark pellets (Sundheim, 1977), wheat

bran (Yildiz, 1993), wheat-bran plus peat(Sivan et al. 1984), barley grain (Abd-El Moity and Shatla, 1981), composted hardwood bark (Nelson and Hoitink, 1983), and pellet formulation have been used as inoculum base for *Trichoderma* species.

Application of *Trichoderma* and *Gliocladium* to seed was suggested as an alternative approach to introducing them into soil (Harman et al. 1981; Taylor et al. 1994). Seed treatment requires smaller amounts of biological material than in-furrow or broadcast applications. The advances in seed treatments with antagonists provide a clear picture of the promises and difficulties inherent in this approach. Control of damping-off of peas caused by *Rhizoctonia solani* was achieved by coating seeds with conidia of *T. harzianum* (Chet and Baker, 1981) isolated from Rhizoctonia suppressive soil (Harman et al. 1980; Harman et al. 1981). Improved yields were obtained in a *Rhizoctonia* infested field by planting soybean seeds treated with *T. pseudokoninigii* (Wu, 1982) and seed of corn and soybean treated with *T. harzianum* (Kommedahl et al. 1981). *T. harzianum* seed treatment also was able to increase seed germination and decrease incidence of *Phoma beta* in sugarbeet seedlings (*D'Ecorole* et al. 1992) and enhance germination in asparagus (Nipoti et al. 1990).

It is still too early to predict the full extent seed treatment with antagonists will develop as a viable biocontrol technology. Selecting the right isolate (Hadar et al. 1984), age of the inoculated seed (Kommedahl et al. 1981), soil reaction (Harman et al. 1980; Harman et al. 1981), microbiota in soil (Hadar et al. 1984), nutritional status of inoculant (Harman et al. 1981), inoculant density on the seed

(Hadar et al. 1984; Papavizas et al. 1982), inoculum potential of pathogen in soil (Wu et al. 1982), and timing of planting (Kommedahl et al. 1981) are all involved in the success of the process.

For success beyond suppression of seed rots and damping-off of young seedlings, *Trichoderma* or *Gliocladium* applied to seed must be able to multiply in the soil or rhizosphere to inhibit a given pathogen by competition, mycoparasitism, or antibiosis (Papavizas, 1985). Evaluating a large number of isolates made it necessary to employ laboratory based tests as a first stage in selecting biocontrol candidates (Schoeman et al. 1994). According to Garrett (1970) " the share of a substrate obtained by any particular fungal species will be determined by its intrinsic competitive saprophytic ability (CSA) and partly by the balance between its inoculum potential and that of competing species." For that reason, to obtain an unbiased estimate of CSA fungi, researchers applied them in the same population density into the soil (Ahmad and Baker, 1985; Ahmad and Baker, 1987a). The CSA could then be linked directly to rhizosphere competence or (RC) the ability to colonize plant root surfaces. Garrette did not list enzymes for cellulose degradation as necessary attribute of rhizosphere fungi, presumably, because amino acids and reducing sugars commonly are thought to be the predominant root exudates (Ahmad and Baker, 1987b). However, Foster et al. (1983) demonstrated that the mucilage overlying the root surface in the region of elongation constitutes the remains of the outer primary wall of root epidermal cells of which cellulose is a major component.

The purpose of this study was to apply a series of in vitro tests to determine the potential biocontrol of fungal isolates, including their ability to utilize cellulose, and to test the selected isolates in the greenhouse for their biocontrol activities.

Materials And Methods

Experimental organisms.

1. *Trichoderma harzianum* (T-95): a benomyl-tolerant mutant with high CSA, derived by Chang et al. (1986) from a wild type originally isolated by Chet and Baker (1981) from a suppressive soil in Colombia, SA.
2. *T. harzianum* (BT12B): a rhizosphere-competent, benomyl-tolerant mutant, derived by Ahmad and Baker (1987a) from a wild type T12 which was originally isolated by Harman et al. (1980) from a soil in NY.
3. *T. harzianum* (Eg-7): isolated from *Fusarium oxysporum* suppressive soil obtained from Giza, Egypt.
4. *T. viride* (T-S-1): was provided by M.T. Dunn (Mycogen Corporation, San Diego, CA).
5. *Gliocladium virens*: recovered from soil by Park et al. (1992).
6. *Fusarium oxysporum. o.* f. sp. *cucumerinum*: was isolated from cucumber wilted field in Nunn, CO, by Scher and Baker (1982).

Single-spore cultures were developed for all isolates, and maintained in sterilized soil cultures supplemented with 1% wheat bran. Soil cultures served the source of isolates at the beginning of each experiment to avoid changes in isolate characteristics that may occur with repeated subculturing.

Screening for potential biocontrol agents

Effect of antagonist metabolites on mycelial dry weight (DW) of the pathogen

The study was carried out according to Lewis and Lumsden (1995). Five ml of water homogenate of each of the test antagonists cultures on corn meal agar were aseptically added to a 100 ml of nutrient-limiting bran extract prepared according to Dennis and Webster (1971). Cultures were incubated and shaken at 25 C for 10 days after which the liquid in each flask was separated from the biomass by centrifugation. The supernatant was sterilized by filtration (0.45 m). Fifty-ml portions were aseptically placed in sterile 250-ml Erlenmeyer flasks with 50 ml of double strength potato-dextrose broth. The metabolite extract was replaced in control treatments with distilled water. A 5 ml conidial suspension ($1x10^3$ coidia/ml) of *F. o. cucumerinum* was added to the flasks and incubated static in the dark at 25 C for 10 days. The biomass from each of three flasks per treatment was separated from the spent medium by filtration onto filter paper. The paper was dried at 60 C for 2 days and the biomass DW was calculated as mg dry mycelium per 100 ml of media.

Effect of antagonist metabolites on radial growth and sporulation of the pathogen

The cellophane membrane technique was applied in this study according to Lewis and Lumsden (1995). Cellophane membrane (Bio-Rad Laboratories, Richmond, CA) was cut to fit the 9-cm-diameter petri dishes and sterilized by autoclaving for 20 min in distilled, deionized water. Petri dishes containing PDA were overlaid with sterilized membranes and inoculated in the center with 5-mm plugs of an antagonist. Plates were incubated in the dark at 25 C until the

colonies approached, but did not overgrow, the edges of the cellophane disks. The cellophane disks covered with antagonist mycelia were removed aseptically from the surface. A 5-mm PDA plug of *F.o. cucumerinum* was placed in the center of each plate and incubated in the dark at 25 C. The area of developing colonies was recorded periodically until colonies were assessed. Five ml of distilled water was added to each plate and conidia were harvested by brushing the culture surface gently with a fine brush. The number of spores per ml was calculated using a hemocytometer.

Effect of antagonist metabolites on germination of pathogen conidia

This effect was evaluated by aseptically placing 1 ml of suspension ($1x10^4$/ml) of pathogen conidia in 1 ml of filter sterilized, nutrient limiting bran extract containing the antagonist metabolites. Mixtures were incubated over night at 23 C in the dark and percent germination of conidia was recorded.

Cellulose decomposing ability assay

The antagonistic fungi were cultured in 250-ml Erlenmeyer flasks containing 50 ml of cellulose-decomposition basic medium (Kok et al. 1992) adjusted with 1 M-HCl or 1 M-NaOH to pH 5.5 or 7.5. The composition of the medium consisted of (gm/l): NH4NO, 2.5; KH2PO4, 2.0; Na2HPO4, 3.0; KCl, 0.5; MgSO4.7H2O, 0.5; FeSO4. 7H2O, 0.01; yeast extract, 0.05; and potassium hydrogen phthalate, 10.0. The medium was prepared without a carbon source. Shredded Whatman no.1 filter paper, wood cellulose, and fine cotton linters were added as carbon sources at 3.0% (w/v) to the media before autoclaving (Ahmad and Baker, 1988). The combinations of KH2PO4, Na2HPO4, and potassium

hydrogen phthalate in the basic medium provided the required buffering capacity to keep the pH at the initial level during the experiment. A preliminary test revealed that the fungi used in this study were unable to utilize phthalic acid as a sole carbon source.

A set of four flasks was seeded with 4-mm diameter disks obtained from the edge of a 4-day old PDA fungus culture. Two weeks after "seeding", hyphal mats were removed aseptically, washed in SDW, dried for 2 days at 60 C, and their weights were recorded. The broth in each flask was filtered and the carbon source was collected and dried to obtain the DW of residual carbon source. To estimate cellulose decomposing ability of different fungi, an efficiency index was calculated according to Ahmad and Baker (1988) as follows:

Efficiency Index = (Biomass carbon / Total cellulose metabolized) x 100

Fifty percent of fungal biomass was assumed to be carbon according to Alexander (1977). To determine whether the insoluble substrates (carbon sources) incorporated directly into the mycelial mats during incubation contributed to the DW of the fungi, the mats were stained with iodine-potassium iodide and sulphuric acid as a test for cellulose (Rawlins and Takahashi, 1952). A blue stain indicates the presence of cellulose. The same set of antagonists used to test the effect of metabolites on pathogen growth were used in this test.

Competitive saprophytic ability assay (CSA)

Cambridge method (Garret, 1970) as modified by Ahmad and Baker (1987b) was used to test CSA of *Trichoderma* and *Gliocladium* species. Isolates were cultured on PDA for 1 wk at 25 C, flooded with sterile distilled water (SDW),

and conidia were gently harvested with a fine brush. The conidial suspension was sieved through cheesecloth, centrifuged at 2,500 g for 15 min, and resuspended in SDW. This was performed three times. Conidia were added to moistened field soil at the rate of 10, 10^2, 10^3, 10^4, and 10^5 conidia per gram of soil. No conidia were added in controls. Soils were distributed in five plastic trays. Clean, mature, polished winter wheat straw was cut in 1 cm segments and 100 sections were buried randomly in each tray. Trays were covered with plastic to retain moisture and arranged in a completely randomized design in growth chamber at 25 C with no light. Twenty wheat segments from each treatment were removed at 3, 6, and 9 days, washed thoroughly in tap water, and surface disinfested in a mixture of 1.1% calcium hypochlorite solution and 5% ethanol for 5 min. The segments were placed on a *Trichoderma* (Elad et al. 1981b) or *Gliocladium* (Park et al.1992) selective medium and incubated at 25 C for 1 wk. Frequency of colonization of wheat segments by each fungus at a given time was recorded. Three replicates were used per treatment and all experiments were repeated twice.

CSA for each fungus was calculated according to Baker (1991) as:

$$\text{CSA index} = \sum_{n}^{I=1} [\ln (1/1-C_i) / (t_i) (\log p_i)] / n,$$

where C = frequency of isolation of specific isolate from the segments, t = time of incubation, p = level of conidia added to soil, and n = number of treatments.

Study of the host-antagonist interaction

Soil (1-kg volumes) was steamed for 1 hr, which nearly eliminated resident microbes, especially fungi, and was necessary to help distinguish hyphae of

Trichoderma in soil (Knudsen and Bin, 1990). Soil was allowed to air dry under a transfer hood and moisture was adjusted according to a previously described soil moisture release curve based on estimation of soil electrical conductivity. Soil pH was approximately 6.5.

Rhizosphere competence assay

RC was studied for the *Trichoderma* and *Gliocladium* isolates according to the method developed by Scher et al. (1984) as modified by Ahmad and Baker (1987 a) and Elad and Chet (1987). Cucumber seeds of cv Straight Eight were placed at the upper part of two longitudinal halves of 50ml polyproplyne tubes measuring 11.5 x 3.2 cm filled with alluvial loam soil with 20% (w/w) moisture content. Antagonists were applied as seed treatments, where *F. o. cucumerinum* was introduced to soil as a conidial suspension (1.5×10^3 per gram of soil). One cucumber seed was placed on the half tube, 1 cm below the rim. The unseeded half tube was carefully placed on the first half and secured with rubber bands. Tubes of all treatments were completely randomized and placed in plastic trays containing the prepared soil with similar water content. Trays were covered with transparent polyethylene sheets to maintain constant matric potential leaving enough space above the tubes for the plants to grow. Trays were placed in growth chamber at 25 C under constant illumination supplied by 20 white, 40 W, 120-cm long fluorescent lamps (approximately 5,000 lx). After 10 days of incubation, tubes were removed from trays and their halves were carefully separated. The roots with adhering soil were cut, starting at the crown, into 1-cm segments. After loosely adhering soil was shaken off, root segments with their

adhering rhizosphere soil were air dried under 100 W lamp for 1 hour. Each segment was weighed and transferred into a 20ml glass vial containing 1 ml SDW. Contents of the vial were stirred vigorously for 1 minute. Colony forming units (cfu) of *Trichoderma* or *Gliocladium* isolates contained in the rhizosphere soil at each centimeter of root were determined by plating series of 10-fold dilutions from each vial on *Trichoderma* or *Gliocladium* selective media. All experiments were repeated twice.

The RC for each isolate at each root depth was calculated after Baker (1991) as:

$$\text{RC index} = \frac{\sum_{I=1}^{n} [\log (pi+1) \ln (d+1)]}{n}$$

where P = population density per milligram of rhizosphere soil, d = root depth of the segment, and n = total root depth.

Effect of seed exudates on the growth of antagonists in the spermosphere.

Treatments were designed to determine radial growth and density of antagonistic hyphae originating from the seed pellets in soil as described by Bin et al. (1991) and modified by Dandurand and Knudsen (1993) under 15, 20, 25 C and soil matric potentials of -0.05, -0.1, or -0.5 MPa. Petri dishes (15cm diam.) were half filled with sandy-loam soil and a single cucumber seed, coated with antagonist conidia in 1% supergel ($1x10^4$ conidia/ml), was placed on the soil surface in the center of a dish. The seed and the soil surface were overlaid with two layers of $1mm^2$ pore-size nylon mesh, and additional soil was added to fill the dish. Soil was gently compressed and each of the four dishes of the same

treatment (antagonist) were placed in a sealed plastic bag to maintain moisture. Dishes were incubated at 25 C in the dark.

Radial growth of the antagonists was measured after 7, 14, 21, and 28 days. There were four replicates per treatment per sample time. At each sample time, the upper layer of the nylon mesh (with the soil above it) was removed and saved and the lower layer served as a reference scale for measuring radial growth of the hyphae. The radial growth pattern of each colony originating from the pellet was observed under a binocular microscope and mapped on graph paper. Maps were digitized using Lasico digitizer, (model 1280-18) to estimate the area of each map. Mean colony radii were calculated from area values (radius =[area/3.1415] 0.5). The experimental data were analyzed as a split-plot design with sample time, temperature, and soil matric potential as main plot effects and radial growth and density of hyphae as dependent variables.

Hyphal density was estimated at 1mm increments in each of four directions from the pellet by using a visual assessment generated by a computer simulation according to Knudsen and Bin (1990). Sterilized glass beads coated with various antagonists were used as a control to clear out the roll of seed exudates in the enhancement of fungal growth.

Effect of plant exudates on pathogen-antagonist interaction.

This technique was applied after Sivan and Chet (1989) with modifications. Cucumber seeds of moderately resistant cv, Straight Eight, moderately susceptible cv, White Wonder, and highly susceptible cv, Marketer Long, were disinfested with 4% sodium hypochlorite for 10 min. Efficacy of

disinfestation was tested by placing samples of the treated seeds on PDA or nutrient agar plates. One hundred seeds of each cultivar were placed in a sterile glass column (15 x 5 cm) containing 200 ml of sterile tap water. The lower part of each column was connected to an air compressor, and air was forced through a sterile fiberglass filter with enough pressure to thoroughly agitated the suspended seeds. A timer ran the air compressor for 30 minutes every 2 hours. An air outlet containing an additional sterile fiberglass filter was mounted at the upper part of each column. Columns with germinated seeds were incubated in a growth chamber at 27 C with 12 hr light period per day for 1 wk. After incubation, seed exudates were collected, the filter sterilized, and placed in sterile flasks under aseptic conditions. Sterilized filtrates were used fresh for interaction tests.

Greenhouse experiments

In greenhouse experiments, in addition to Eg7, T95, and BT12B, two more isolates were involved. One was *T. viride* (TV62), a highly competitive cellulose decomposer isolate, and the other *G. virens,* an isolate with a high level of antibiotic secretion. Experiments were carried out in sandy loam soil. Soil was artificially infested with enriched chlamydospore inoculum of *F. o. cucumerinum* at 1.5 x 10^3 cfu/g soil (Appendix, Fig. 2). Treatments, comprising four replicates, were set up in 25 cm diameter plastic pots each containing 1.0 kg of soil sown with five seeds of each test cultivar. Antagonists were added as wheat-bran-peat conidial preparation or through coating seeds with 0.05% supergel containing 3 x 105 conidiospore per ml of gel as illustrated in Fig. 4.1. For seed coating, a conidial suspension (3x10^{10} conidiospore /ml SDW) of each antagonist was

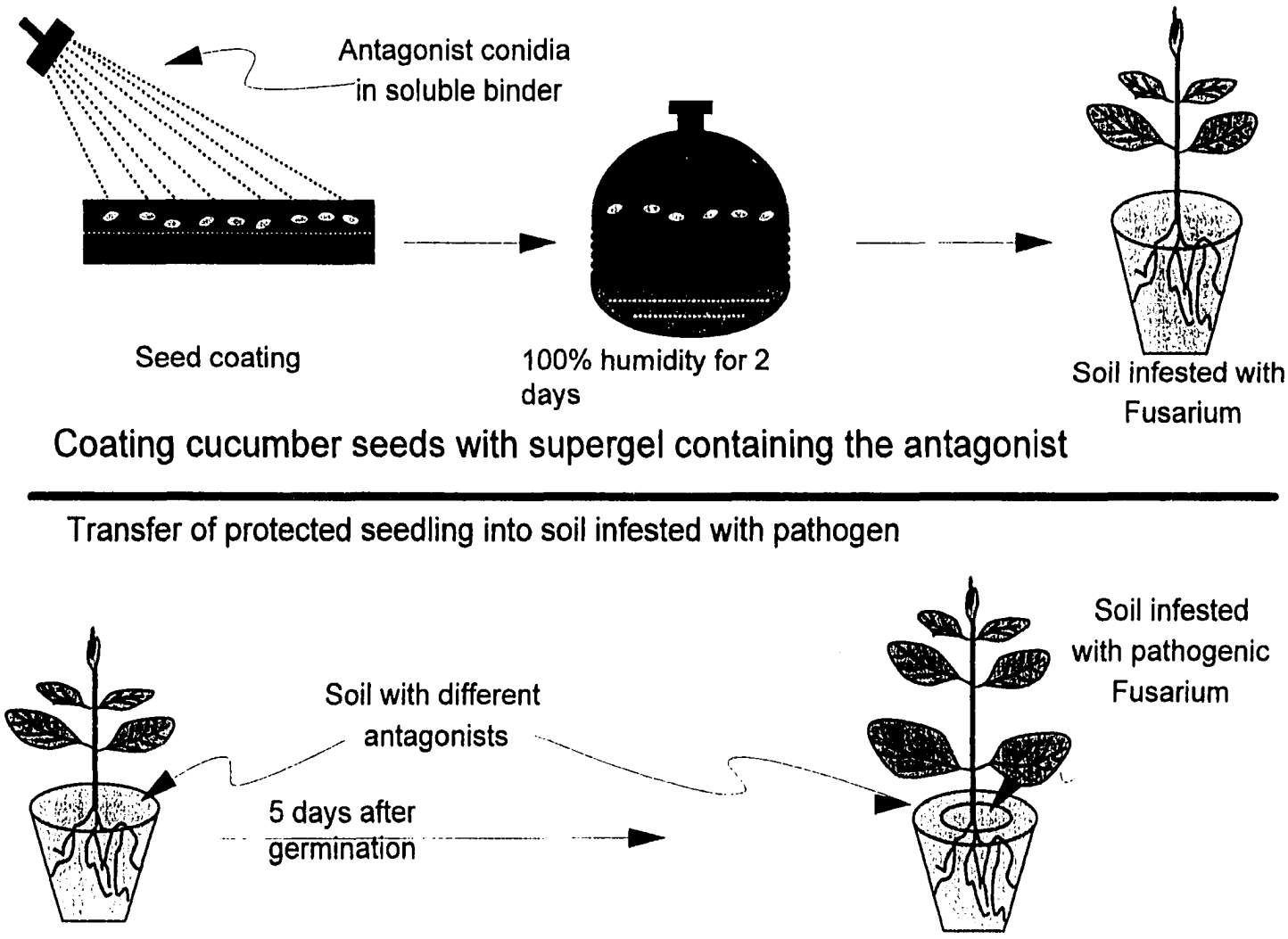

Figure 4.1. Techniques used for antagonist delivery.

prepared from conidia collected from 1 wk-old PDA cultures as previously described. Seeds were sprayed thoroughly with supergel preparation. Data were collected as percent of pre- and post-emergence damping off.

RESULTS

Screening for potential biocontrol agents

Effect of antagonist metabolites on of pathogen growth and sporulation

Nine *T. harzianum* and *T. viride* isolatess isolated from Egyptian cucumber fields and greenhouses were used. One Egyptian isolate of *G. virens* and *G. roseum* originally obtained from roots of symptomless cucumber plants of cv Marketer Long were also used. When pathogen growth rate was measured in the presence of metabolites secreted by various antagonists, the most significant reduction occurred from *T. harzianum* (Eg-7) as shown in Table 4.1. Also, based on visual estimate, a remarkable reduction in growth density was noted when the pathogen grew on media containing metabolites of *T. harzianum* (Eg-7), *G. virens*, and *T. viride* (Eg-6). Similar results were found for sporulation, spore germination, and mycelial DW. *T. harzianum* (Eg-7), *G. virens*, and *T. viride* (Eg-18) caused significant reduction in the amount of fungal DW and conidia germination while significant reduction in conidia formation was recorded only with *T. harzianum* (Eg7) and G. *virens* compared to other isolates.

Cellulose decomposing ability assay.

When the biomass of the antagonists were tested for direct incorporation of three different cellulose substrates in fungal hyphae, all were positive. Data presented in Table 4.2 show that all *Trichoderma* and *Gliocladium* isolates were

Table. 4.1 Effect of antagonist metabolites on pathogen growth and sporulation

	Fusarium oxysporum f. sp. *cucumerinum*				
Antagonist	Growth rate (cm^2/day)	Growth density[b]	Dry weight (mg/100ml)	Conidia formation[c]	Conidia germination (%)
T. harzianum (Eg 1)[a]	3.6 A[d]	1	430 A	2.7 A	92 A
T. harzianum (Eg 4)	3.4 A	1	380 B	2.3 A	90 A
T. harzianum (Eg 7)	1.1 D	3	210 C	0.5 D	45 C
T. harzianum (Eg 19)	2.5 B	2	385 B	1.8 B	63 B
T. harzianum (Eg 23)	3.5 A	1	410 B	2.2 A	58 B
T. viride (Eg 6)	1.7 C	3	356 B	1.8 B	74 A
T. viride (Eg 13)	3.7 A	1	410 B	2.0 A	68 B
T. viride (Eg 17)	3.0 B	1	335 B	1.6 C	76 A
T. viride (Eg 18)	2.6 B	1	318 C	1.6 C	59 C
G. virnes	1.6 C	3	245 C	0.9 D	48 C
G. roseum	2.7 B	2	400 A	2.2 A	66 B
Control	3.6 A	1	445 A	2.6 A	95 A

[a] Eg = Egyptian isolates.
[b] Visual estimate of fungal growth density compared to control: 1, similar to control; 2, slight reduction; 3, large.
[c] 10^3 / cm^2 of fungal growth on PDA plates containing antagonist metabolites.
[d] Numbers in each column followed different letter are significantly different Duncan's multiple range test (P= 0.05).

Table. 4.2 Mycelial dry weight of antagonists grown for 2 weeks on three insoluble cellulose sources

Antagonist	Filter paper		Wood cellulose		Cotton linters	
	5.5 [b]	7.5	5.5	7.5	5.5	7.5
T. harzianum (Eg 1)[a]	0.81 C	0.55 C	1.21 B	0.96 B	0.78 C	0.53 D
T. harzianum (Eg 4)	0.32 E	0.13 F	0.37 E	0.15 F	0.37 E	0.13 F
T. harzianum (Eg 7)	1.20 B	1.30 B	2.30 A	2.20 A	1.10 B	0.97 B
T. harzianum (Eg 19)	0.75 C	0.54 D	0.89 C	0.71C	0.31 E	0.15 F
T. harzianum (Eg 23)	0.87 C	0.46 D	1.30 B	0.96 B	0.75 C	0.55 D
T. viride (Eg 6)	0.30 E	0.16 F	0.56 D	0.36 E	0.46 D	0.23 E
T. viride (Eg 13)	1.20 B	0.89 C	1.90 A	1.57 B	0.98 B	0.78 C
T. viride (Eg 17)	0.32 E	0.15 F	0.33 E	0.29 E	0.31 E	0.17 F
T. viride (Eg 18)	0.67 C	0.56 D	0.79 C	0.54 D	0.45 D	0.32 E
G. virnes	1.30 B	1.10 B	1.80 A	1.20 B	1.10 B	0.94 B
G. roseum	0.51 D	0.48 D	0.43 D	0.43 D	0.39 E	0.41 D

[a] Eg = Egyptian isolates.

[b] pH levels

[c] Numbers followed by the same letter in the table do not significantly differ from each other with Duncan's multiple range test (P= 0.05).

able to grow, differentially, on the three cellulose carbon sources at either pH 5.5 and 7.5. *T. harzianum* (Eg7) and *G. virens* produced significantly higher DW at 7.5 pH on filter paper. At pH 5.5, they were similar to *T. viride* (Eg13). Eg7 and Eg13 of *T. harzianum* and *G. virens*, were significantly greater on both wood cellulose and cotton linters at pH 5.5. While at pH 7.5, only Eg7 was significantly greater on wood cellulose. Both Eg7 and *G. virens* had their highest DW on cotton linters on compared to the other isolates. At pH 5.5 *T. viride* (Eg13) produced amounts of DW on filter paper, wood cellulose, and cotton linters similar to *T. harzianum* (Eg7) and *G. virens*. With few exceptions, *Trichoderma* isolates had better growth at pH.7.5 than at pH 5.5. When the efficiency index of cellulose decomposing ability was estimated according to Ahmad and Baker (1988), Eg7 had the highest value followed by *G. virens* and Eg13 on the cellulose sources at both pH 5.5 and 7.5 (Fig. 4.2).

Competitive saprophytic ability

Data presented in Fig. 4.3 show that Eg7 had the highest CSA of the eleven test antagonists with an index of 2.87, followed by Eg13 and *G. virens*. The CSA index of the remaining isolates decreased gradually.

Analysis of cellulose decomposing ability and CSA shows a positive significant correlation ($r = 0.98$). Based on previous screening studies, isolate Eg7 was selected for comparison with isolates T95 and BT12B, which are highly competitive *T. harzianum* isolates.

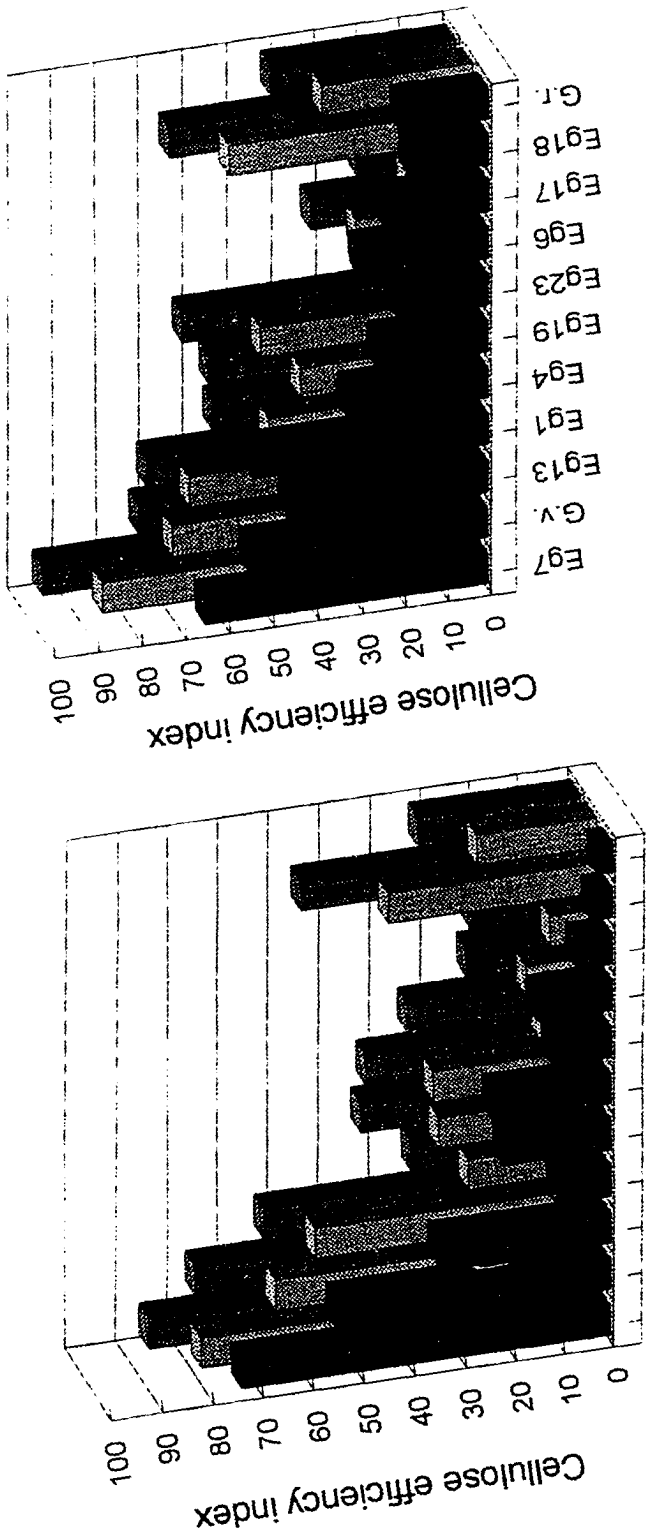

Figure 4.2. Efficiency index of different antagonists on three cellulose sources.

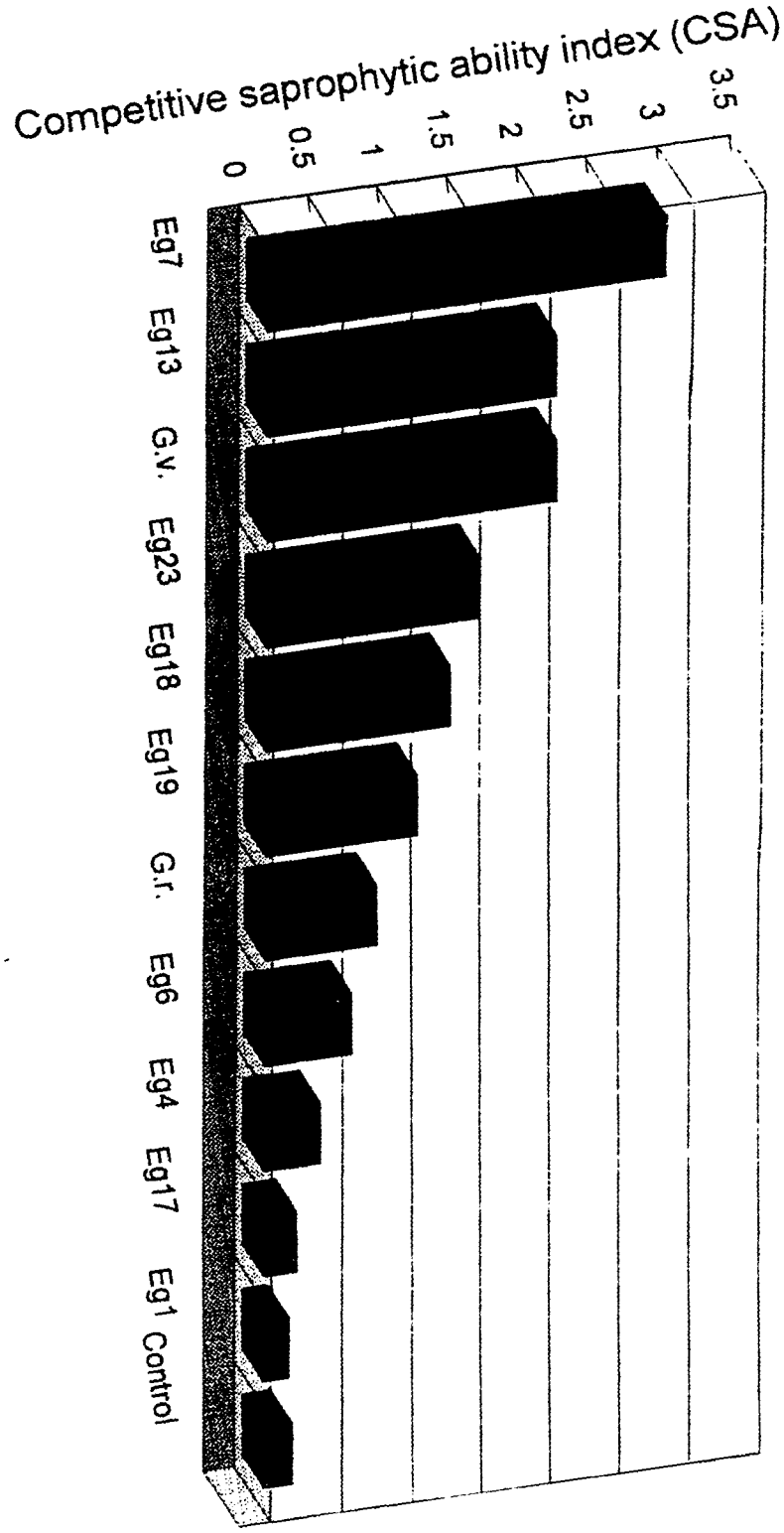

Figure 4.3. Competitive saprophytic ability index of different *Trichoderma* and *Gliocladium* strains.

Rhizosphere competence assay.

The assay was carried out under both 24 and 30 C to study the influence of "normal" and higher temperatures on isolate activity. Data presented in Figure 4.4 show that under 24 C, the population of all test isolates decreased dramatically along the root system. Only T95 was able to rebuild its population after 4 days and to continue growing along the entire roots system (260 cfu/g soil). Isolate Eg7 was able to grow to segment number 6 (12 cfu/g of soil). Isolate BT12B could not be isolated further than 2 cm from the seed. At 30 C, Isolate T95 maintained its superiority and was able to grow along the entire root system (200 cfu/g soil). Isolates Eg7 and BT12B stopped growing after the sixth isolation, with 50 and 160 cfu/g soil, respectively, as shown in Figure 4.5.

The RC index, calculated on the data of the third segment, shows isolate T95 had the highest value 2.6 (24 C) and 2.5 (30 C). The RC index for isolate Eg7 was 1.9 under both temperatures. BT12B had the lowest index value with 1.3 (24 C) and 1.2 (30 C) at 24. Correlation between the RC index data and the cellulose decomposing ability data was not significant.

Effect of seed exudates on the growth of different antagonists in the spermosphere.

In a preliminary test to determine the influence of soil matric potential on growth density, a remarkable degree of similarity was found in growth type among the three isolates. A clear fluctuation in hyphal density was found between -0.1 and 1.0 Mpa. At 0.15 MPa, the isolates grew well with a density level of 65-70 linear mm of hyphae/mm^3 of soil. With an increase in soil matric

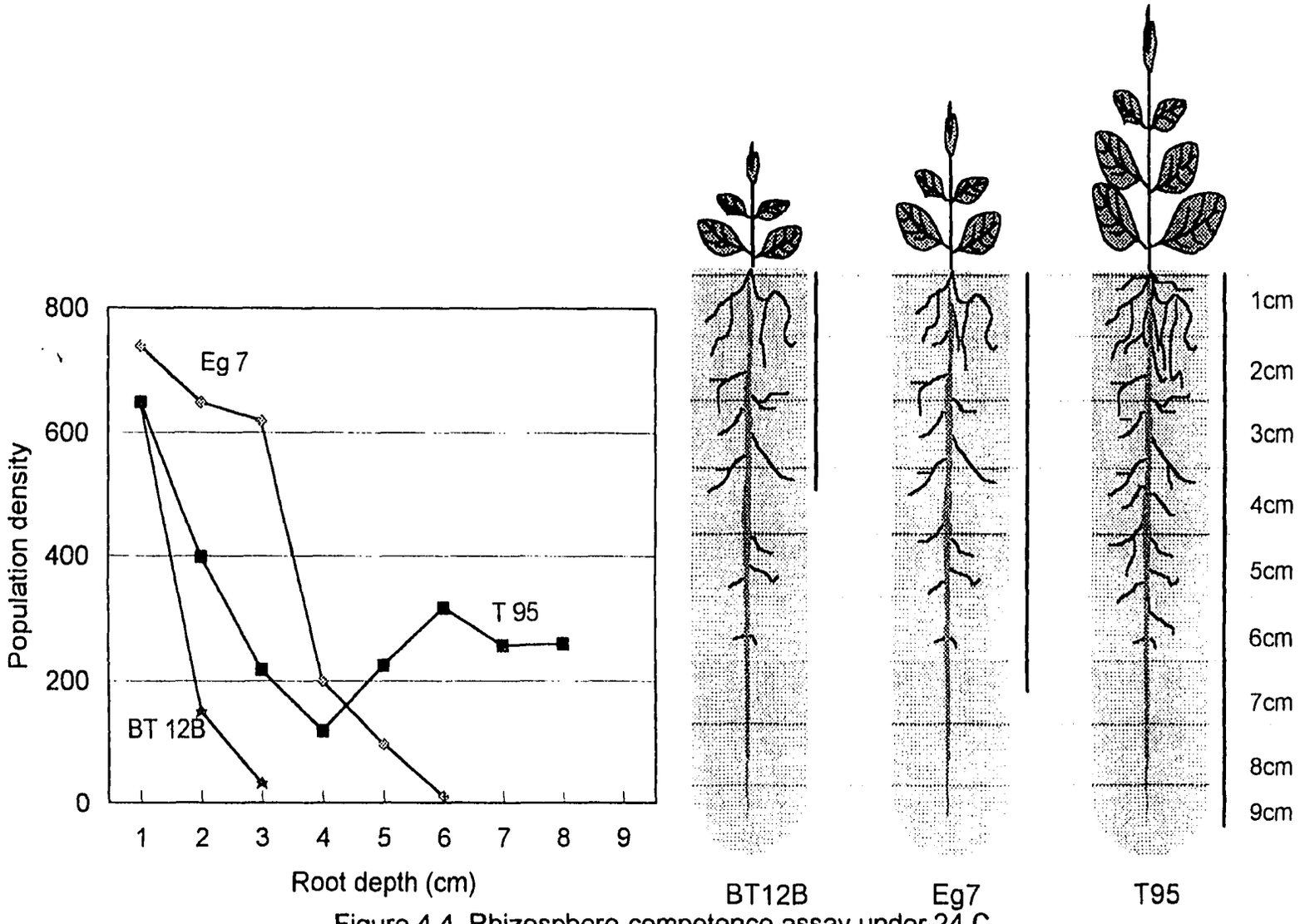

Figure 4.4. Rhizosphere-competence assay under 24 C.

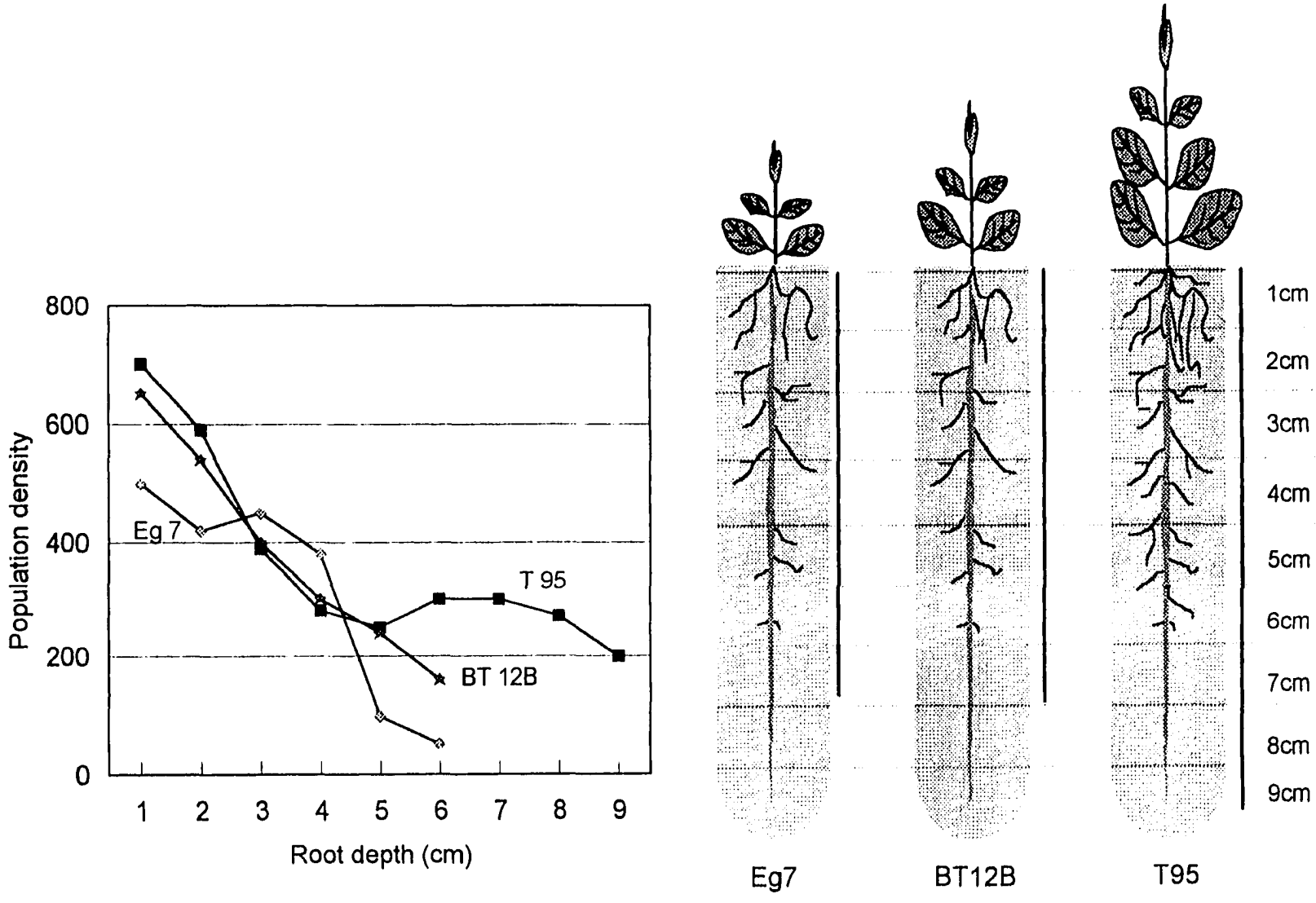

Figure 4.5. Rhizosphere-competence assay under 30 C.

potential, the density of fungal growth decreased dramatically reaching its lowest level at -0.35 MPa (Figure 4.6). Density then increased until it reached 80-90 linear mm of hyphae/mm^3 of soil at -0.5 MPa. After this point density decreased with the increase in matric potential. A second experiment was designed to incorporate the effect of temperature on both hyphal density and radial growth when delivered as a seed coating system. Only matric potential -0.5 MPa was used in this experiment.

Data presented in Figure 4.7 summarizes the effects of time, temperature, and presence of seed exudates on fungal growth. Analysis of variance showed that each of the three test factors had a highly significant positive effect on radial growth and hyphal density ($P < 0.01$). No significant differences were detected in radial growth among the three isolates. The growth of isolate Eg7 and T95 was more dense in the cucumber spermosphere then the growth of BT12B.

Effect of plant exudates on pathogen-antagonist interaction.

Seed exudates of susceptible, moderately susceptible, and resistant cucumber cultivars were tested for their effect on the inhibition of antagonists to the pathogen chlamydospore germination and incidence disease (Sivan and Chet 1989). Significant positive results were obtained in all cases. No significant differences were obtained among exudates of different cultivars. In general, a low concentration of plant exudates (2 ml/ 25 g of soil) was enough to enhance the inhibition effect of Eg7 and T95 on chlamydospore germination and disease incidence. A higher concentration (5 ml/25g of soil) was required for BT12B to

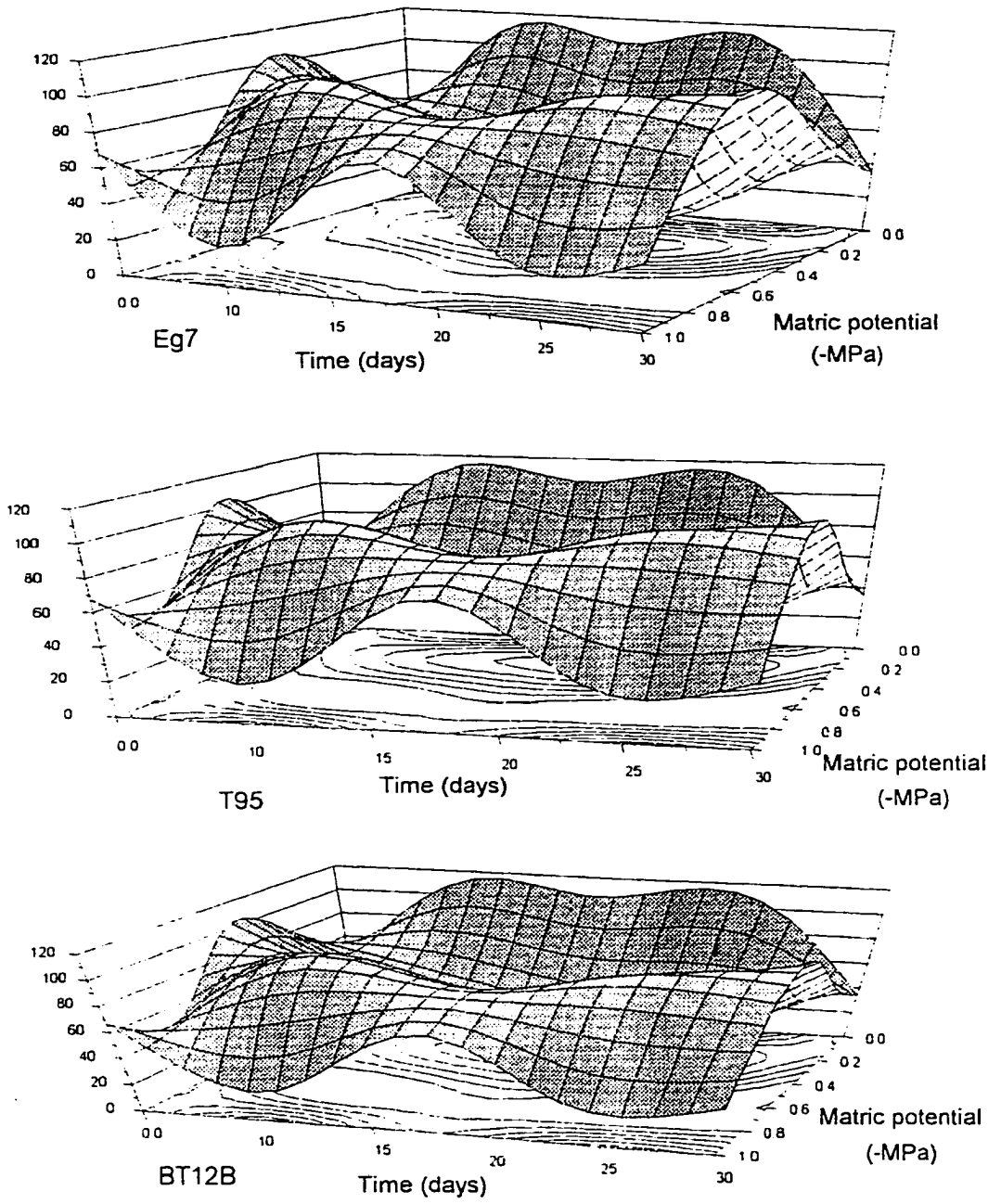

Figure 4.6. Contours-plot graph of the effect of different soil matric potentials on antagonist growth density in the spermosphere.

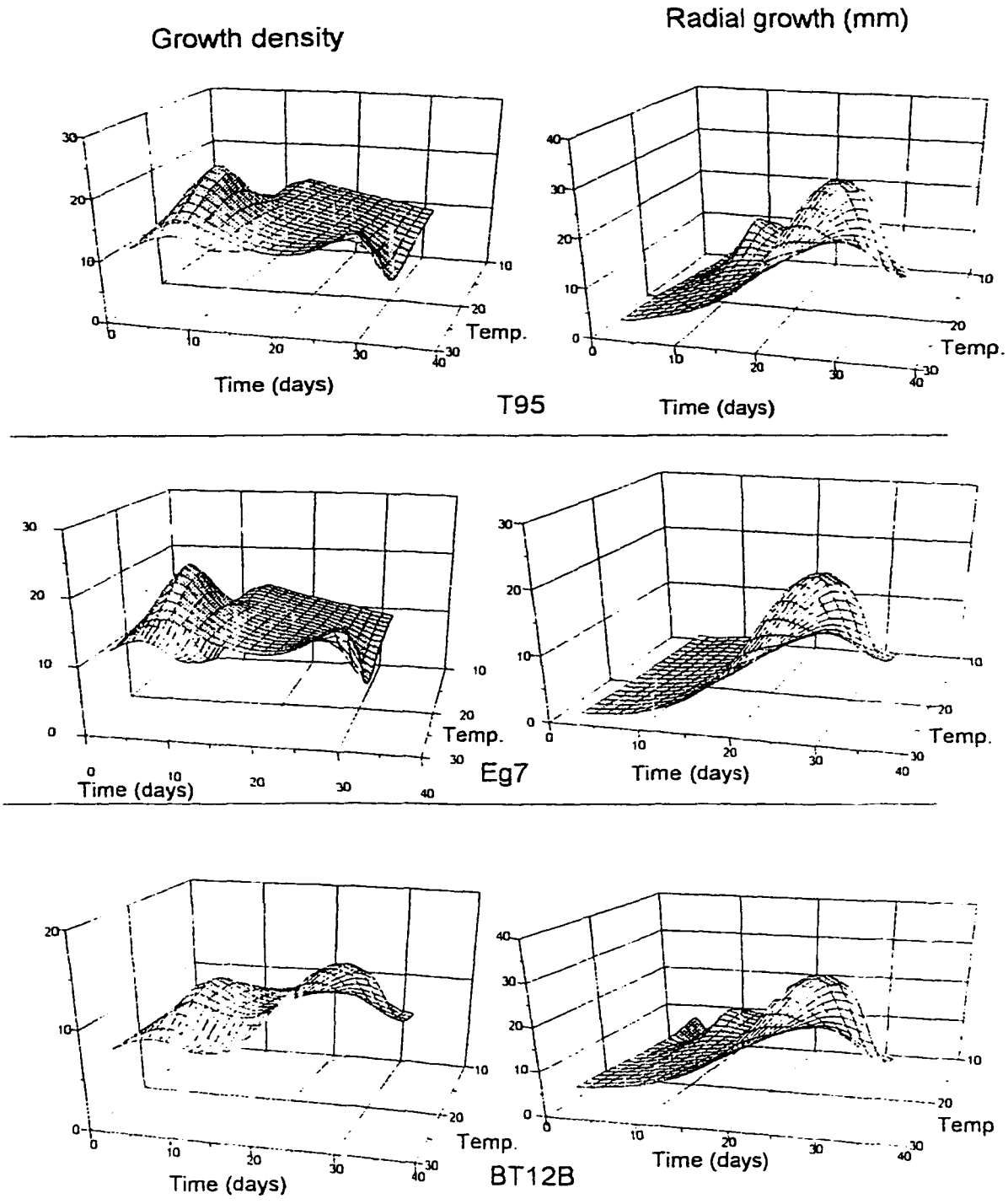

Figure 4.7. Plot figures of continuous effect of temperature, time, and seed exudates on the antagonist growth characteristics over time.

demonstrate similar effects. The higher concentration did not improve the inhibition ability of Eg7 and T95.

Greenhouse experiments.

Data presented in Table 4.3 showed that biocontrol activity of BT12B and *G. virens* were significantly affected by temperature. Their ability to reduce pre- and post-emergence damping off was significantly lower at 24 C than at 30 C. No significant differences were detected for other isolates between the two temperature levels. In general, Eg7 was superior in its biocontrol activity over the rest of the antagonists associated with BT12B at 30 C and by T95 at 24 C. Only with the transplanting delivery system at 30 C, was Bt12B the best inhibitor of post-emergence damping off.

The total number of wilted plants was recorded after 2 months (Table 4.4). Isolate Eg7 was able to maintain high efficiency with no significant differences among treatments (17-23% wilted plants). *G. virens* was not able to maintain its protection and no significant differences were detected between its treatments and the control. More than 50% of TV62 plants were infected with *F. o.* f. sp. *cucumerinum* by the end of the experiment.

DISCUSSION

Although Lewis and Lumsden (1995) stated that generally, antagonist-pathogen interaction under in vitro conditions was not considered of ecological importance, in vitro studies may provide some insight with regard to potential interaction in a natural ecological situation. In this investigation, the impact of antagonist metabolites on various pathogen activities suggested that antagonists

Table. 4.3 Effect of two delivery systems on inhibition of cucumber Fusarium wilt by various antagonists after 3 weeks

Delivery system	Seed Coating				Transplanting			
	Pre-emergence		Post-emergence		Pre-emergence		Post-emergence	
Antagonists	24 C	30 C	24 C	30 C	24 C	30 C	24 C	30 C
TV62	15.8	14.3	16.8	14.6	15.2	13.8	11.3	9.8
T95	6.7	6.7	6.3	7.0	4.7	5.8	6.7	7.2
G. virens	12.2	6.4	13.5	6.1	10.6	5.8	10.4	5.2
BT12B	8.9	5.2	7.8	4.3	7.6	3.9	6.3	3.4
Eg7	6.7	5.2	5.8	5.3	5.1	4.1	5.6	4.9
Control	23.2	22.9	31.8	28.5	39.6	39.0	38.2	41.0

Table 4.4 Total Percent of Fusarium wilt of cucumber two months after planting in the presence of different antagonists

Delivery system	Seed Coating		Transplanting	
Antagonists	24 C	30 C	24 C	30 C
TV62	58 [a]	59	61	68
T95	32	46	28	27
G. virens	98	94	96	96
BT12B	33	38	26	31
Eg7	17	17	23	19
Control	96.5	94.8	100	100

[a] Total Percent of Fusarium wilt.

differ in ability to affect pathogen growth and sporulation. In general, *T. harzianum* (Eg-7) and *G.* virens had the most significant inhibitory effect on the pathogen. Variation in antagonist effectiveness is a well-documented phenomenon (Vajna, 1985; Lewis and Papavizas, 1987; Whipps, 1987). Such variation can probably be explained by production of specific inhibitory metabolites in the media (Lewis and Lumsden, 1995) and by different levels of production. Whipps (1987) emphasized the importance of media in any interaction study. A medium low in nutrients would create enough stress on the fungal growth to simulate the ecological situation (Bu'Lock, 1975; Kuter, 1984). This was demonstrated to some extent by Lewis and Lumsden (1995) in their study of the influence of pathogen metabolites on the growth of biological control agents. It was also demonstrated during this study by using a nutrient-limiting bran extract agar to produce antagonist metabolites and to estimate the impact of these metabolites on pathogen growth.

Cellulose decomposing ability.

Decomposition of plant remains is mainly considered as a microbiological process since the contribution of microbes to the loss of mass in plant material is about 60-70% (Kok et al. 1992). The specific role of fungi and bacteria in the decomposition process is not clear, but some authors believe that fungi are more important than bacteria (Kaushik and Hynes, 1971; Padgett et al. 1985). In this investigation, we were not merely interested in the ability of fungi to decompose plant material, especially cellulose, because of the important ecological role, but were more interested in this ability as a mechanism in competition with

pathogenic fungi. Our results showed that pH is an important factor regulating the growth and ability for competition. Even though all the isolates could develop on media containing various cellulose sources under a range of pH levels, some of the carbon sources were more preferable than the others. The ability to degrade the preferable type was significantly reduced under low pH levels. This is in agreement with data by Kok et al. (1992). Only Eg7 and *G. virens* were able to grow well on both low and high pH. The inhibition of cellulose decomposition under low pH might be due in part to the fact that low pH is not optimum for fungal exoenzyme activity responsible for degradation of crystalline and amorphous cellulose (Chamier, 1985). Also, some cellulose decomposition inhibitors are more active under acid conditions (Kok et al. 1992). Data obtained from this study gave an important indication about what type of soil the test antagonists could compete for plant remains and could be positively involved in biological control. When competitive saprophytic ability was estimated and correlation analysis was carried out, a positive correlation was found between pH and cellulose decomposition ability.

Study of the host-parasite interaction.

Rhizosphere competence assay.

The term rhizosphere competence was first employed by Schmidt (1979) to describe an attribute of rhizobium bacteria characterized by their consistent association with legume root nodules. Thereafter, it was used to describe the ability of a microorganism to grow and function in the developing rhizosphere and to colonize the growing root (Ahmad and Baker, 1987a). This attribute is

valuable in the enhancement of a biocontrol agent since the seed and developing roots are protected by seed coating. Our investigation showed that an isolate, like Eg7, might be less competitive in the rhizosphere than other isolates, but at the same time able to offer a stronger and more durable protection for the plant than the more competitive isolates. Direct colonization of a root system by a biocontrol agent is not the only possible mechanism for the interaction between the agent and the host. A biochemical interaction might enhance disease resistance in plants against soilborne pathogens. Many examples have been recently published about induction of systemic acquired resistance in plants by chemical and biological agents but no complete explanation of the mechanism has been developed (Alstrom, 1991; Van Peer et al. 1991; Wei et al. 1991). Two indirect approaches to test competition as a mechanism in biological control of soilborne plant pathogens are available. The first is based on the hypothesis that biological control results from general competition between antagonist and pathogen for organic matter in the soil. Research has shown that this has little or no effect on the viability of the pathogen (Baker, 1981). The second mechanism is based on a concept that microorganisms may compete for a limiting factor essential for one or more of their vital activities (Sivan and Chet, 1989). In our investigation, rhizosphere colonization was part of a multiple phase study to explore the potential of the antagonists. Although Eg7 was not able to grow completely along the developing root system as isolate T95, it was able to maintain a reasonable level of protection for cucumber plants against the Fusarium wilt pathogen under high inoculum level condition. Isolate T95 was

able to maintain its protectin during the first month, after which, the pathogen infection rate increased dramatically.

Effect of seed exudates on the growth of different antagonists in the spermosphere.

One of the most sensitive stages for nutrient competition in the life cycle of *Fusarium oxysporum* is chlamydospore germination (Baker, 1981). Germination rate in nature ranges between 20-30% depending upon the amount of nutrient reserved in the spores themselves or the residual organic matter in raw soil. However, the major source of nutrients for soil microorganisms are obtained from seeds or roots (Cook and Baker, 1983). The role of plant seed exudates was investigated as an important nutritional factor for rhizosphere microflora and in the general equation of biological control. For control of soilborne pathogens, the ability of the biocontrol agent to explore the three-dimensional soil space via hyphal growth may be more important than proliferation via spatially localized sporulation (Knudsen and Li Bin, 1990). Based on this concept, estimation of the influence of temperature and soil matric potential on radial growth and hyphal density of antagonists was essential. All test isolates were significantly affected by level of soil matric potential and temperature contrary to the data obtained by Knudsen and Li Bin (1990). They reputed that neither soil matric potential nor soil temperature significantly affected radial growth or hyphal density of *T. harzianum*. Significant differences among isolates in most cases were not detected, but in general, radial growth and hyphal density were significantly

affected with the change in soil parameters.

Effect of plant exudates on pathogen-antagonist interaction.

Most of the root exudates present in plant rhizosphere are excreted from the tips (Sivan and Chet, 1989). Thus colonization of this region in the rhizosphere might reduce infection by soilborne pathogens that penetrate the vascular system of their hosts through the undifferentiated xylem of root tip (Cook and Baker, 1983). Compatibility between the antagonist and the exudates of certain root systems is an important aspect in the biological control process. "Compatibility" in this investigation means the ability of the antagonist to utilize nutrients released by the host root and to be able to suppress soil pathogens from attacking the root system. Results showed that plant exudates of susceptible, moderately susceptible, and resistant cultivar had the same effect on the antagonist-pathogen interaction. This leads to a search for alternate reasons for plant susceptibility to soilborne pathogens other than the difference in chemical composition of root exudates. Neutralization of the power of antagonists over the pathogen when an excess amount of plant exudates were present indicates that competition between pathogen and antagonist for nutrients may occur. At low level of exudates, nutrients are limiting and competitive effects are evident, resulting in significant reduction in chlamydospore germination. A similar phenomenon was reported by Sivan and Chet (1989). Rovira et al. (1974) suggested that plant root exudates contain lower amounts of carbon than those required by rhizosphere microflora. A similar inhibition of chlamydospore germination of

F. oxysporum f. sp. *cucumerinum* by several fluorescent pseudomonads has also been reported (Elad and Baker, 1985). Although the method used after Knudsen and Li Bin (1990) was sophisticated, two main sources of experimental error may occur. First, visual estimates may be biased by the investigator. Second, this was the only experiment where sterilized soil was used and as it was impossible to detect the radial growth of test antagonist under the microscope except when there is absolutely no interference from soil microbes. However, in this case, natural interaction between antagonist and soil microflora had to be eliminated.

Greenhouse experiments.

This experiment was carried out using group of selected isolates, which had a well-known record for biological control of plant pathogens or high competitive ability with other microbes. Only Eg7 was selected during the course of our study from among more than 36 antagonists obtained from soil and root system of Egyptian cucumber open field and greenhouse cultivation. We excluded isolates with low biocontrol potential. The result of adapting such protocol was promising, as isolate Eg7 was able to provide consistent and durable protection for cucumber plants in greenhouse throughout the season. We presented data for only the first two months was presented because it was highly representative of the whole season and gave an accurate prediction of the performance of the isolates. Collecting the data for biocontrol work shortly after applying treatments could be misleading as plants need enough time to interact with both antagonist and pathogen and to

reveal the real outcome of their "struggle". The ability of the new isolate to protect cucumber plants from Fusarium wilt for more than two months with equal results from both delivery (seed coating and transplanting,) was remarkable. Some isolates used in this study have one important potential isolate EG7 lacks. T95 was able to colonize plant rhizosphere along the whole root system while EG7 could not. A fusion between more isolates to obtain a superior antagonist is planned for the future.

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APPENDIX

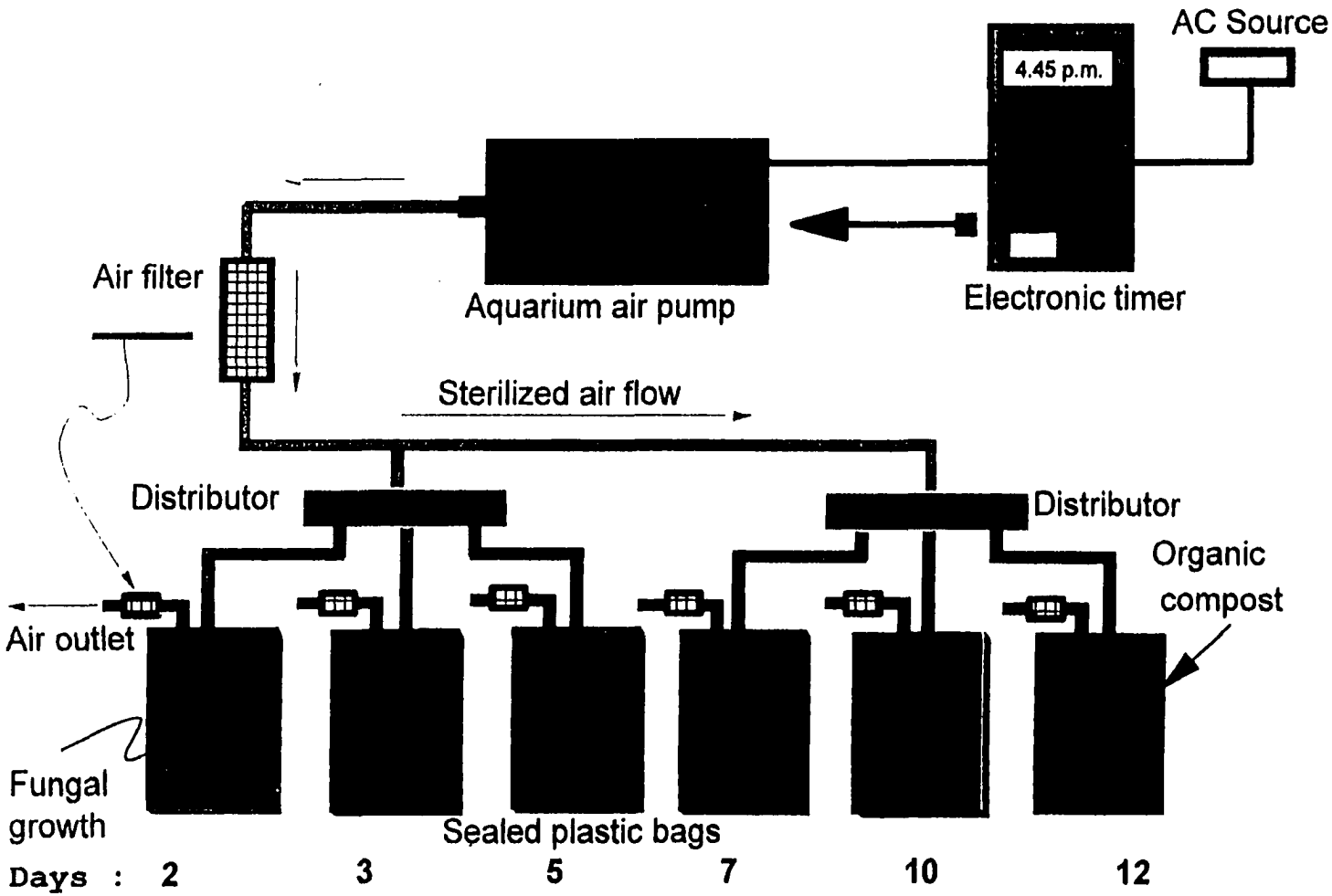

Figure A.1. Diagram for fast preparation of high concentration of the inoculum.

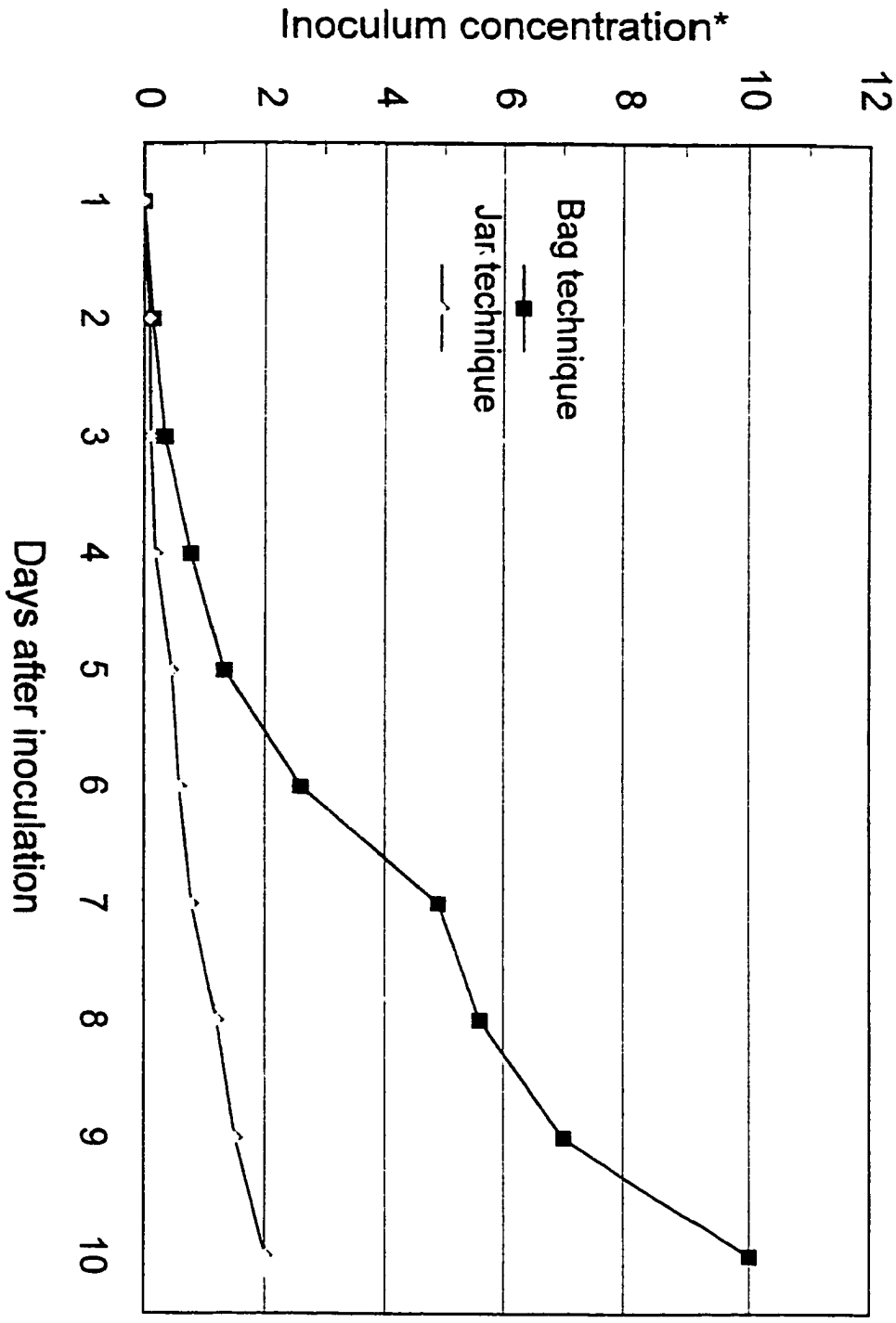

Figure A. 2. Comparison between suggested and common technique for mass inoculum production.