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Host and site specificity of chemotactic responses of zoospores of *Pythium* species to roots and root cap cells of *Gossypium Barbadense* and *Gossypium hirsutum*

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HOST AND SITE SPECIFICITY OF CHEMOTACTIC RESPONSES OF ZOOSPORES OF *PYTHIUM* SPECIES TO ROOTS AND ROOT CAP CELLS OF *GOSSYPIUM BARBADENSE* AND *GOSSYPIUM HIRSUTUM*

by

Natalie Pauline Goldberg

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DEPARTMENT OF PLANT PATHOLOGY

In Partial Fulfillment of the Requirements
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Date

This thesis is dedicated to my parents
whose love and support made this work possible.
For this I am forever thankful.

ACKNOWLEDGEMENTS

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ABSTRACT

Root cap cells of two cotton species elicited a specific chemotactic response in zoospores of Pythium dissotocum. When roots of cotton seedlings were placed into a suspension of P. dissotocum zoospores, there was immediate attraction, accumulation and encystment exclusively in the root cap cell region. Furthermore, root cap cells remained attractive when isolated from the root: attraction, accumulation, and encystment on individual root cap cells occurred within seconds after contact. Zoospores penetrated and killed isolated root cap cells within 15-30 minutes, and seedlings died within 24 hours. In contrast, zoospores of P. catenulatum, which exhibited a chemotactic response to roots of Bentgrass, were not attracted to and did not infect seedlings or isolated root cap cells of cotton. Preliminary studies indicate that both Pythium species are capable of infecting cotton seedlings in sand culture, though it is not known if either are pathogens on cotton grown in the field.

INTRODUCTION

The means by which an organism locates a susceptible plant is an important step in the process of disease development. Organisms which produce motile spores would appear to have the advantage of movement toward an attractive plant surface. This movement may be directed by at least two different mechanisms. One example is electrotaxis, which is the movement of an organism toward or away from an electrical charge. Electrotaxis may be an important mechanism for some host-parasite interactions, but it is generally not considered to be a common means of zoospore attraction (Troutman and Willis, 1964). A second mechanism governing attraction is chemotaxis, which is the movement of an organism toward or away from a chemical. The mechanisms governing chemotaxis of fungal zoospores to plant roots are not well understood. Chemotaxis may occur as a response to unidentified components in exudates secreted from a potential host or may be a specific response to chemical compounds and other agents produced in the laboratory (Lange and Olson, 1983). In this study, the chemotactic responses of zoospores of two Pythium species to the roots and root cap cells of two cotton species were observed.

Zoospore behavior

A zoospore is an asexual, unicellular, flagellate, propagating or disseminating body which is capable of limited motility. Zoospores of the Pythiaceous fungi are approximately 10-12 um long and 8-10 um wide. These zoospores are biflagellate. The anterior flagellum is tinsel and the posterior flagellum is nearly smooth. Coordinated movement of the two flagella enable these spores to locate attractive plant surfaces by chemotactic response. Zoospores often move in a helical pattern and are able to change directions by movement of the posterior flagellum. This directional movement allows the zoospore to steer itself toward or away from a chemical source (Carlile, 1983; Hendrix and Campbell, 1983; Hickman and Ho, 1966). Zoospores play a significant role in the life cycle of zoosporic fungi both as a propagative unit and in the spread of the organism from one host to another (Hickman and Ho, 1966).

Zoosporic fungi are dependent on the presence of water for the production and dissemination of the motile spores. The distances traveled by zoospores depend on the water content of the soil, on the soil structure, and on host characteristics, but the overall distances are thought to be relatively short (Lange and Olson, 1983; Schmitthenner 1968). Zoospores can swim effectively in many natural soils, but are transported more rapidly by free water in the soil (Carlile, 1983). Under favorable conditions of humidity and temperature, zoospores can remain motile for several hours.

Biflagellate zoospores swim longer than uniflagellate zoospores; some remain motile for 20 - 30 hours (Lange and Olson, 1983).

Zoospore Chemotaxis

Most of the previous studies of zoospore chemotaxis have been done with species of the Pythiaceous fungi. Pythiaceous fungi include common plant pathogens which readily produce zoospores in vitro. Species in the genus Phytophthora have been studied more frequently than Pythium species, presumably because Phytophthora species have long been considered to be the more important plant pathogens. Inconsistencies in the results of these experiments have made it difficult to ascertain a clear understanding of the chemotaxis phenomenon. However, from these previous studies, two general conclusions have been made regarding the chemotactic response of fungal zoospores: 1) In most cases, attraction is non-specific with respect to hosts and non-hosts; 2) Attraction is usually non-specific in the location of attraction (Dukes and Apple, 1961; Goode, 1956; Royle and Hickman, 1964a).

Host-specificity - Several examples of quantitative variation in chemotaxis to resistant and susceptible plants have been reported. For example, zoospores of Phytophthora cinnamomi Rands. exhibited a strong chemotactic response to roots of susceptible avocado seedlings, with decreased attraction to resistant cultivars of avocado. The zoospores did not accumulate on roots of non-host plants such as tomato, tobacco and mandarin orange, yet some other

non-hosts did attract the zoospores (Zentmyer, 1960). Similarly, blueberry cultivars with varying degrees of susceptibility to Ph. cinnamomi attracted zoospores according to the degree of susceptibility of the cultivar (Erb, et. al., 1986). A similar situation was seen with zoospores of Phytophthora megasperma Drechs. which exhibited a gradient of attraction to 2-day-old alfalfa seedlings (Chi and Sabo, 1978).

In contrast, Phytophthora parasitica (Dast.) var. nicotianae (B. de Haan) Tucker expressed no difference in its attraction to resistant or susceptible tobacco plants (Dukes and Apple, 1961). Zoospores of Pythium aphanidermatum (Edson) Fitsp. were attracted in equal numbers to both susceptible and resistant pea cultivars as well as to non-hosts (Royle and Hickman, 1964a). Similarly, zoospores of Phytophthora fragariae Hickman accumulated on roots of immune, resistant, and susceptible strawberry cultivars as well as on roots of non-hosts (Goode, 1956).

Though some degree of host-specificity was observed in these studies, the attraction of zoospores to plant roots is for the most part not host dependent. (Chi and Sabo, 1978; Dukes and Apple, 1961; Royle and Hickman, 1964a; Zentmyer, 1960). Several reasons for the lack of host-specificity have been suggested. 1) These fungi have broad host ranges which may be reflected in their chemotactic response to many plant species. 2) Tests usually involved cultivated plants which have been developed without regard to rates

or types of root exudation, which can have impact on chemotactic responses. 3) Test plants are usually grown in glasshouses, often in sand, which can cause damage to the roots which can change the rate of root exudation. 4) Subsequent handling of roots, especially prolonged immersion in water can effect the rate of exudation (Mitchell and Deacon, 1986). 5) Nonspecific chemotaxis in these tests may also be due to the fact that metabolic products which attract the zoospores are common to many species of plants and are not linked with disease susceptibility (Cunningham and Hagedorn, 1962). Specificity may occur at a different stage than chemotaxis, for example, a degree of host-parasite specificity regarding adhesion of zoospore cysts to plant roots has been observed. This differential adhesion may reflect differences in the number of or ease of removal of the cysts from receptor molecules that mediate the adhesion of the fungi. This work also emphasized that there was no difference in the taxis of motile spores with regards to any species or host tested (Mitchell and Deacon, 1987).

Tissue specificity - Previous experiments also included studies of the site of attraction of the zoospores on roots. Zoospores of Ph. cinnamomi Rands. exhibited a strong chemotactic response to the area of elongation on avocado seedlings. Zoospores were attracted to root exudates which were produced by the seedlings; no attraction occurred when root exudates were removed by boiling (Zentmyer, 1960; Zentmyer, 1961). Similarly, zoospores of Ph.

megasperma were attracted only to the area of elongation or to wounds of 2-day-old alfalfa seedlings. As with Ph. cinnamomi zoospores, no attraction to boiled roots occurred (Chi and Sabo, 1978). Aphanomyces euteiches Drechs., a zoosporic fungus pathogenic on peas and other legumes, was attracted within seconds to the region of elongation of pea roots, but not to root hairs (Cunningham and Hagedorn, 1962). P. aphanidermatum also exhibited a non-specific attraction for the area of elongation and to wounds of pea cultivars (Royle and Hickman, 1964a). The only study using cotton plants as the attractive plant species showed that both P. aphanidermatum and Pythium debaryanum Hesse were attracted to roots in the area of elongation and the root hair zone, but never to the root tip unless the tip was wounded prior to placement of the root in zoospore suspension. In this study the age of the plant was considered to play a role in the attractive nature of the root (Spencer and Cooper, 1967). In contrast, Ph. fragariae zoospores were attracted in large numbers to the root hair zone and the root tip of susceptible and resistant strawberry cultivars as well as to non-hosts. This study also noted that attraction continued even after roots had been boiled (Goode, 1956).

Little is known about the lack of specificity with regard to the location of attraction of the zoospores. This may be due, in part, to experimental inconsistency of results due to developmental variation in roots. Thus, zoospores may be attracted to a particular

region of the root on one seedling and to a different region on the root of another seedling. In addition, the preparation of the seedlings prior to use in chemotaxis experiments appears to be an important factor in the results of the experiments. Roots grown in a rooting medium or washed prior to use may be altered with respect to cellular make-up which could change the specificity of the attraction site of the root to fungal zoospores.

Function of the Root Cap

The function of the root cap is thought to be many fold. It aids in the protection of the apical meristem, assists the root in moving through the soil, and produces mucilage, a polysaccaride, which is in part responsible for the adherence of soil particles to the root (Barlow, 1975; Esau, 1977; Mollenhauer, 1967). Mucilage is of two types, gelatinous mucilage which arises from the root cap and firm, thick mucilage which arises from the epidermal cells. Histochemical evaluation of the two mucilages show that they are basically similar with just a few distinct differences (Miki, et. al., 1980). Mucilage is primarily made up of simple sugars, such as sucrose, fructose and dextrose (Dukes and Apple, 1961; Longman and Callow, 1987; Miki et. al. 1980; Royle and Hickman, 1964b). The root cap is also regarded as the site of geotropic regulation for the root. Roots which have had the cap removed (decapped) typically grow without respect for gravity. When the root cap regenerates a few

hours after decapping, the roots recover geotropic sensitivity (Esau, 1977; Barlow, 1975).

Peripheral root cap cells - The root cap contains several layers of peripheral cells located at the edge of the root cap. The size and number of cells of the root cap varies among plant species (Esau, 1977; Hawes and Pueppke, 1986; Mollenhauer, 1967; Barlow, 1975). Old cells of the root cap slough off as new ones are produced by a layer of meristematic cells and were believed to die shortly after sloughing from the root. However, in 1919, Knudson made the observation that detached root cap cells remain viable for weeks in nutrient solution. These cells can survive in the absence of the roots for up to three weeks in soil (Vermeer and McCully, 1982) and for several months when placed in culture medium (Hawes and Pueppke, 1986). In addition, differences in surface characteristics of isolated cells result in selectivity in interactions with fungal and bacterial pathogens (Hawes, 1984; Hawes and Pueppke, 1987; Hawes and Wheeler, 1982).

Problems arise when using whole roots in chemotaxis studies. Roots are developmentally variable, with numerous tissue types, each consisting of cells that range from old dying cells to young living cells. This means that three roots all of the same cultivar and all of the same length may provide three distinctly different chemotaxis reactions. Because sloughed peripheral root cap cells are constantly renewed, they constitute a developmentally uniform population.

Isolated nondestructively, so surface characteristics are intact, the use of root cap cells as the attractive plant source in chemotaxis studies can alleviate many of the problems of inconsistency. The root cap cells produce much of the mucilage the primary chemoattractant for the fungus to the root (Cunningham and Hagedorn, 1962; Mitchell and Deacon, 1986; Zentmyer, 1960; and Zentmyer, 1961). Studies of zoospore chemotaxis to a range of simple sugars, amino acids, inorganic salts, organic acids, auxins, vitamins, and mixtures of chemical compounds has revealed differential attraction. Results of these in vitro experiments show that only glutamic acid and combinations of amino acids and sugar mixtures produced effects resembling the attraction of the zoospores for root exudates (Dukes and Apple, 1961; Royle and Hickman 1964b). Little is known about the actual components which govern chemotaxis under natural conditions.

Objectives

In this study, Pythium zoospores and cotton were used as a model system to study host- and tissue-specificity in fungal chemotaxis. The questions addressed included the following: 1. Are zoospores of two Pythium species attracted to the roots and to the peripheral root cap cells of two species of cotton? 2. If attracted, can the spores infect the roots and the isolated peripheral root cap cells? 3. Are the responses specific with respect to host and site of attraction?

MATERIALS AND METHODS

Culture of Pythium species

Isolates of Pythium dissotocum Dreschler and Pythium catenulatum Matthews, respectively, were isolated from spinach in California and from lettuce in Arizona (Stanghellini, personal communication). Cultures were maintained on 1.5% water agar and stored in the dark at 27 C. For experiments the fungi were grown in the dark at 27 C on V-8 agar (2% agar, 10% V-8 juice, .1% $CaCo_3$). Four day old cultures of P. dissotocum and 7-day-old cultures of P. catenulatum were used in experiments.

Preparation of Zoospore Suspensions

Zoospores were produced by placing two 15 mm diameter culture plugs cut from V-8 agar cultures in sterile petri dishes and flooding the plugs with 20 ml of sterile deionized water which had been aerated by shaking. Culture plugs were always taken from the same location on the plates to ensure consistency of mycelium age and zoospore production. The dishes were incubated in growth chambers at 20 C, until at least 5×10^4 zoospores/ml were produced (a minimum of 6 hours). The culture plugs were then removed from the dishes, and the zoospores were used in experiments within 6 hours. A 500 ul sample of suspension containing approximately 5×10^4 zoospores of P. dissotocum and 2.5×10^3 zoospores of P. catenulatum were used in

experiments to evaluate the chemotactic response of zoospores to plant roots and to isolated root cap cells.

Zoospores were encysted by agitation with a vortex mixer for one minute and were then counted using a Spencer microbial cell hemocytometer. Numbers were determined as follows: 12 ul of encysted zoospore suspension was placed on the hemocytometer and the number of spores in five out of nine counting quadrants were counted with a Leitz microscope with 10X magnification. The average number of spores in 1 ml of the zoospore suspension was then calculated. The mean number of zoospores produced per plate was calculated from six different 12 ul samples.

To determine duration of P. dissotocum zoospore motility, eight dishes with two 15 mm diameter culture plugs were set up to release zoospores as described above. Because high populations of zoospores, 110,000 to 160,000 per ml, were consistently produced after 12 hours, plates were routinely incubated for this length of time for all experiments. After incubation, the mycelial plugs were removed and one plate was counted for zoospore population. This number of zoospores was used as the beginning motile population. Several plates set up at the same time were periodically counted and the numbers were consistent within a range of less than 10% difference in spore numbers per replication plate. The remaining seven dishes were left undisturbed at room temperature until each plate was counted at 1 1/2 hour time intervals. Thus, the number of

zoospores in one plate was counted at 7, 8.5, 10, 11.5, 13, 14.5, and 16 hr after the original population had been determined. The population number in each dish was calculated as the percentage of zoospores that remained motile.

Germination of Cotton Seedlings

Two cotton species, Gossypium barbadense (Pima cotton) and Gossypium hirsutum (Upland cotton), were used in chemotaxis experiments. Seeds were surface sterilized for 5 min in 95% ethyl alcohol followed by 5 min in 50% sodium hypochlorite, and were then rinsed three times in 20 ml sterile distilled water. All of the seeds were germinated at 27 C for 48 hours on sterile filter paper placed on .5% water agar in 9 cm petri dishes. Two-day-old seedlings with root lengths between 1 and 2 cm were used in experiments.

Isolation of Root Cap Cells

Root cap cells of both cotton species were isolated from two-day-old seedlings germinated as described above. Intact root tips were placed into 100 ul sterile deionized water for 2 min, after which the water was gently agitated to loosen the cells (Hawes and Pueppke, 1986). The cells were then either counted for the number of cells yielded per root, were checked for viability, or were used in chemotaxis experiments.

The yield of root cap cells per root for both species was calculated by using a hemacytometer. For each root, 12 ul of isolated

cells were placed on the hemacytometer and all of the cells in the nine counting grids were counted, including those cells which bordered the top and the right side only. The average number was calculated from 6 to 8 replications.

Cell viability was determined using fluorescein diacetate staining of isolated cells (Widholm, 1972). The percentage of viable cells was calculated at the time of isolation, and at 24 and 48 hours after isolation. For each test, 2 ul of the stain was placed on a clean microscope slide and 10 ul of isolated cells were mixed with the stain. The cells were observed using fluorescent microscopy on a Zeiss compound microscope IIIRS with 100X or 160X magnification. Because cells begin to be killed by the stain after several minutes the viability of the cells was observed within 1 - 2 min (unpublished). The percentage of viable cells was calculated from 3 different samples of 100 cells.

Chemotactic Response of Zoospores to Roots

The chemotactic response of zoospores to roots was observed in 9 cm quadrate petri dishes as follows: 500 ul of the suspension containing *P. dissotocum* (5×10^4) or *P. catenulatum* (2.5×10^3) zoospores was placed in a petri dish. A root of either *G. barbadense* or *G. hirsutum* seedlings, with root length between 1 and 2 cm, was then inserted into the suspension drop. The drop was then covered with a No. 2 coverslip. The reaction was observed under a Zeiss inverted microscope using 25X, 200X PH 1, or 400X PH 2 magnification.

Observations of the length of time required for attraction to begin, and of the site of attraction were recorded. In addition, the reaction of the zoospores to the roots was recorded using a video camera attached to the microscope. This set-up enabled the reaction to be timed from beginning to end.

Infection of Roots

The ability of the zoospores to infect roots was determined. The same roots which had been used in chemotaxis experiments were subsequently placed in moist growth pouches (Northrup King, Minneapolis, Minnesota) and were incubated overnight at 20 C. Root tip positions (Fig. 1) were marked on the outside of the pouch at the time the seedlings were inoculated and again after overnight incubation. After the change in root length of the seedlings in the pouches was recorded, the roots were placed on 1.5% water agar containing 200 ppm streptomycin sulfate. The plates were incubated at room temperature for 48 hours and then visually observed for evidence of fungal growth. If hyphal growth occurred, the fungus was transferred by subculture to V-8 agar and was incubated for 7 to 10 days at room temperature to confirm species identity (Van der Plaats-Niterink, 1981). Controls included seedlings that were incubated for 2 to 5 minutes in sterile deionized water, and placed into growth pouches. Root growth was recorded and excised roots were transferred to water agar and observed for fungal growth.

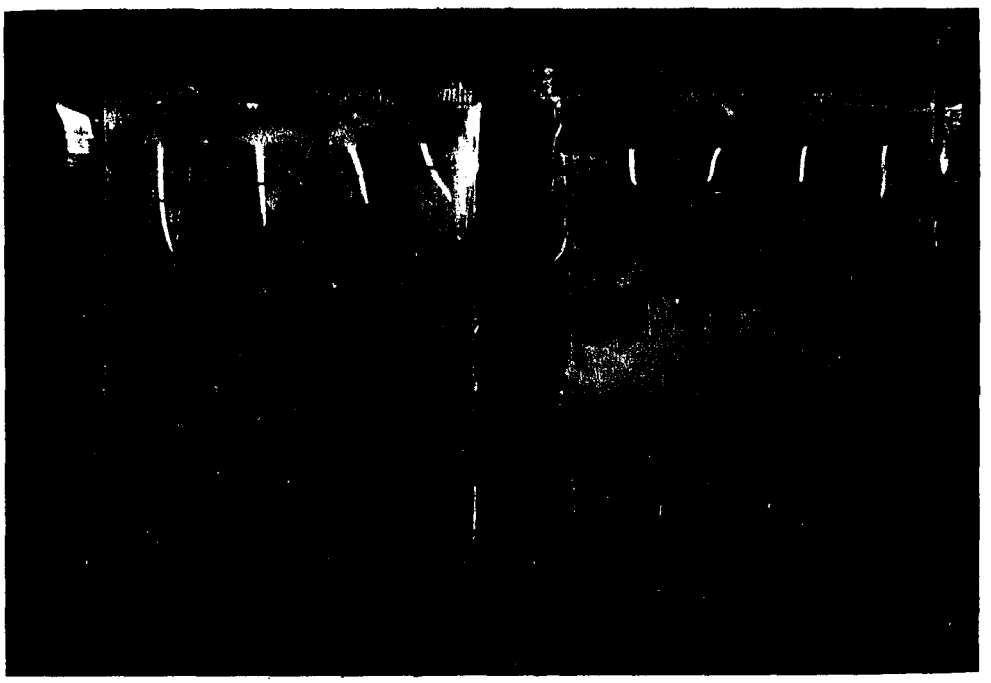

Figure 1. Seedling root growth experiment. Growth pouches marked for root length at time of placement of seedlings in pouches. A). Non-inoculated control seedlings. B). Seedlings inoculated with P. dissotocum.

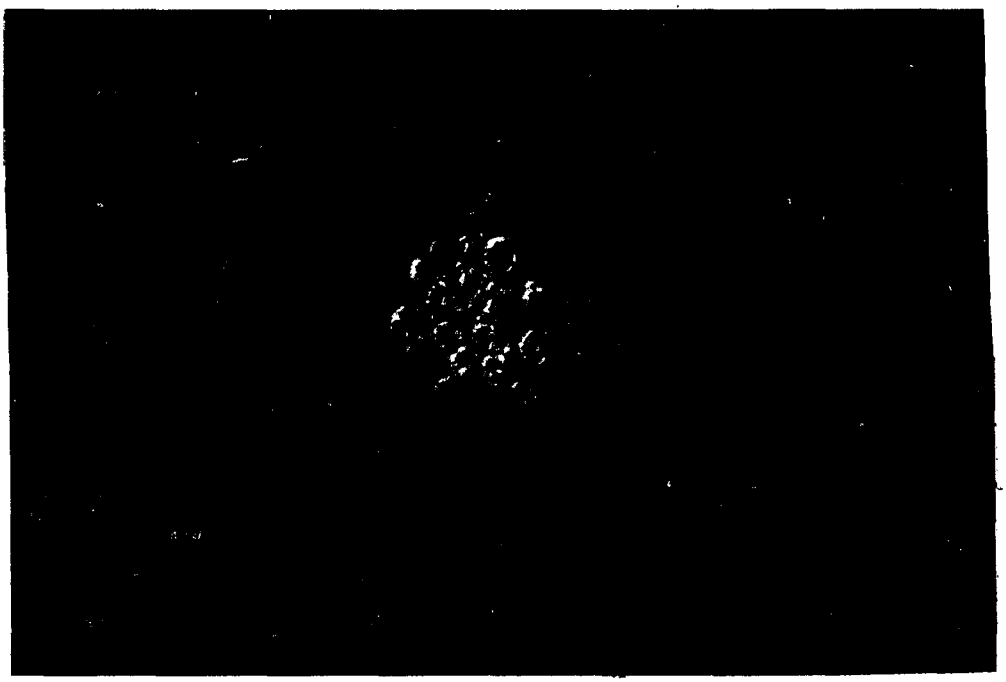

Figure 2. Accumulation and encystment of P. dissotocum zoospores around an isolated peripheral root cap cell of G. barbadense.

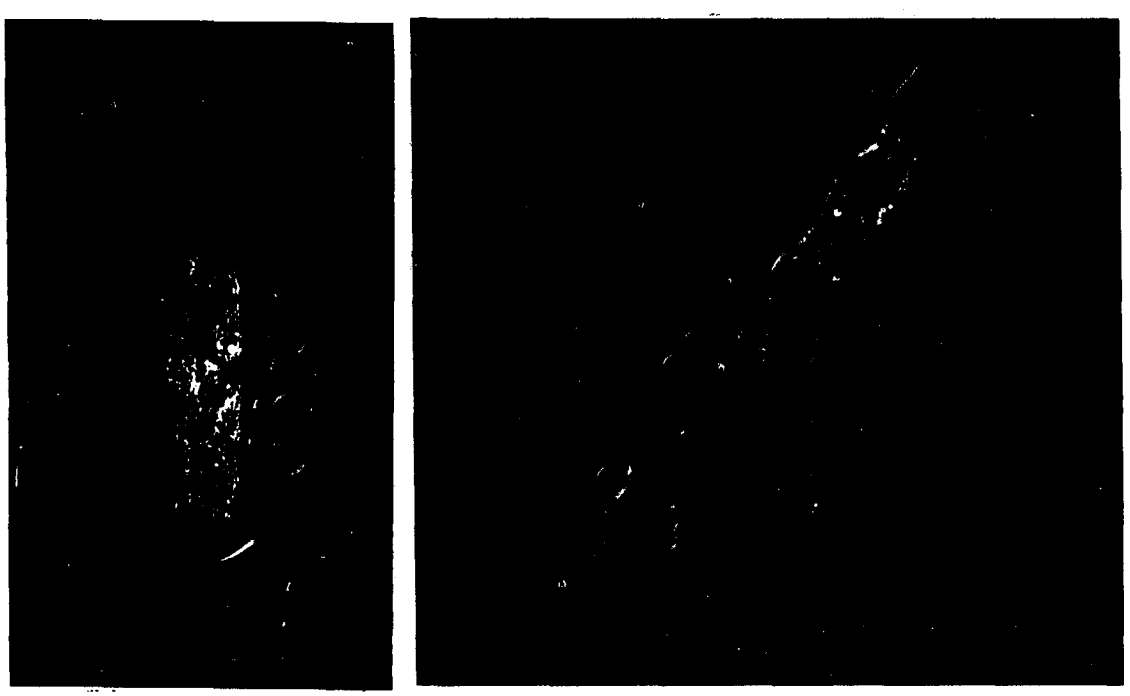

Figure 3. Germination and penetration of P. dissotocum zoospores around an isolated peripheral root cap cells of G. barbadense.

Chemotactic Response of Zoospores to Root Cap Cells

Root cap cells were isolated from the root by placing the intact root tip (10 to 15 mm) in 100 ul sterile deionized water for 2 minutes. The root cap cells were isolated from the root by gently agitating the water with a micropipette. A 50 ul sample of isolated cells was placed into 500 ul of zoospore suspension P. dissotocum (5 X 10^4) or P. catenulatum (2.5 X 10^3). The following aspects of the zoospore-root cap cell interaction were observed and recorded over the next 24 hrs: attraction, accumulation (Fig. 2), encystment (Fig. 2), germination (Fig. 3) and penetration (Fig. 3) of individual cells. Attraction was regarded as directed movement of zoospores toward individual cells. When several zoospores were attracted to the same cell, accumulation was considered to have occurred. Encystment was determined to have occurred when the zoospores became rounded and nonmotile. This event was then followed by the subsequent formation of a cell wall around the spore. Germination began as a germ tube emerged from the encysted zoospore and penetration was confirmed by the visual observation of hyphae within individual cells. Fluorescein diacetate stain was used to determine the viability of colonized cells 1 to 2 hours after the cells were placed in suspension and again after 24 hours. The number of individual cells directly colonized by encysted zoospores was counted after 30 min and again after 24 hours. Colonization percentage was determined by random counts of 100 cells, replicated five times.

An experiment was set up to determine if killed cells could elicit a chemotactic response in the zoospores of P. dissotocum. Root cap cells from G. barbadense seedlings were killed by one of two methods. First, seedlings were allowed to air dry for 2 hours and then root cap cells were isolated into sterile deionized water. Second, root cap cells were isolated in sterile deionized water and frozen at 0 C for 2 hours and then thawed at room temperature. The viability of the cells was then checked using fluorescein diacetate stain. The killed cells were used in chemotaxis experiments. The responses of zoospores to killed cells were observed and recorded using the same procedure described above for living cells.

Preliminary test of Pathogenicity of Pythium sp. on cotton in sand

Pathogenicity tests were carried out using both P. dissotocum and P. catenulatum with G. barbadense. Seeds were soaked in distilled water for 1 hr to allow them to imbibe water and thus accelerate the germination process. Five seeds were planted 1/2" below the surface of 4" pots containing #20 silica sand. Motile zoospores or encysted zoospores were used as inoculum. Zoospores were produced using cultures and techniques described previously. Inoculum concentrations included 1×10^7, 5×10^6, and 2.5×10^5 zoospores/ml of P. dissotocum and 1×10^6, 5×10^5, 2.5×10^4 zoospores/ml of P. catenulatum. The pots were saturated with water and allowed to drain prior to inoculation. For treatments using motile zoospores, a 20 ml sample of zoospore suspension was poured on

the surface of the pots. For pathogenicity experiments using encysted zoospores, the zoospores were agitated on a vortex mixer for 1 min, and 20 ml of inoculum was then poured on the surface of the sand. Pots watered with 20 ml sterile deionized water were used as controls. Each treatment was repeated three times (fifteen seedlings per treatment). The pots were incubated at 28 C and were watered once daily to maintain adequate moisture levels until the seedlings were 2 or 4 days old. The seedlings were then harvested and washed for 1 min in running tap water. The roots were examined for symptoms of Pythium root rot; numbers of leaves present, length of root and lesions present on the roots were recorded. The roots were then plated on water agar containing 200 ppm streptomycin sulfate and incubated at room temperature for two days. Any fungus which grew from the roots was transferred to V-8 agar and was incubated for 7 to 10 days at room temperature before being evaluated under a light microscope with 40X magnification for characteristic fungal structures of the two Pythium species.

RESULTS

Number of Zoospores Produced

Under similar experimental conditions, *Pythium dissotocum* produced more zoospores than *Pythium catenulatum*. Two 15 mm plugs from 4 1/2 day old cultures of *P. dissotocum* consistently produced approximately 1×10^5 zoospores/ml in 20 ml of water after 12 to 13 hours. Two 15 mm plugs from 7-day-old cultures of *P. catenulatum* produced a minimum of 5×10^4 zoospores/ml in 20 ml after 6 hours. Because *P. catenulatum* does not grow as quickly in culture as *P. dissotocum*, different age cultures were required to produce zoospores. Both *P. dissotocum* and *P. catenulatum* began to release zoospores after a 3-hour incubation period. However, *P. catenulatum* produced all of its zoospores within a shorter period of time - 6-hours as opposed to 12-hours for *P. dissotocum*. In addition, *P. catenulatum* zoospores began to encyst after only three hours. In contrast, *P. dissotocum* released zoospores over a longer period of time and remained motile considerably longer. In order to insure that there were adequate numbers of zoospores to use in chemotaxis experiments different release times were required for the two species.

Duration of Zoospore Motility

In order to insure that P. dissotocum zoospore numbers remained the same over the course of chemotaxis experiments, the duration of zoospore motility was determined (Fig. 4). Zoospore release began approximately three hours after the water was added to the plugs and no zoospores had stopped swimming by the time the plugs were removed after 12-hours. The number of motile zoospores did not start to decrease until 7-hours after the culture plugs had been removed from the petri dishes. The percentage of the zoospore population that remained motile then dropped in a linear fashion over the next 9-hours. Approximately 1×10^4 zoospores/ml remained motile 16-hours after the plugs had been removed. Thus, zoospores swam for at least 22-hours before they began to encyst. For the duration of the chemotaxis studies (6-hr) zoospore numbers remained constant.

Yield of Root Cap Cells

The number of root cap cells isolated from individual roots varied among plant species as well as between seedlings of the same species, as observed in previous studies (Hawes and Pueppke, 1986). Gossypium barbadense consistently produced more root cap cells per root than Gossypium hirsutum (Figs. 5 & 6). The number of cells isolated from sixteen G. barbadense seedlings with root lengths between .8 and 2.9 cm were counted using techniques described in Materials and Methods. These seedlings yielded from 5,000 to 12,500 cells per root. The greater number of cells were isolated from

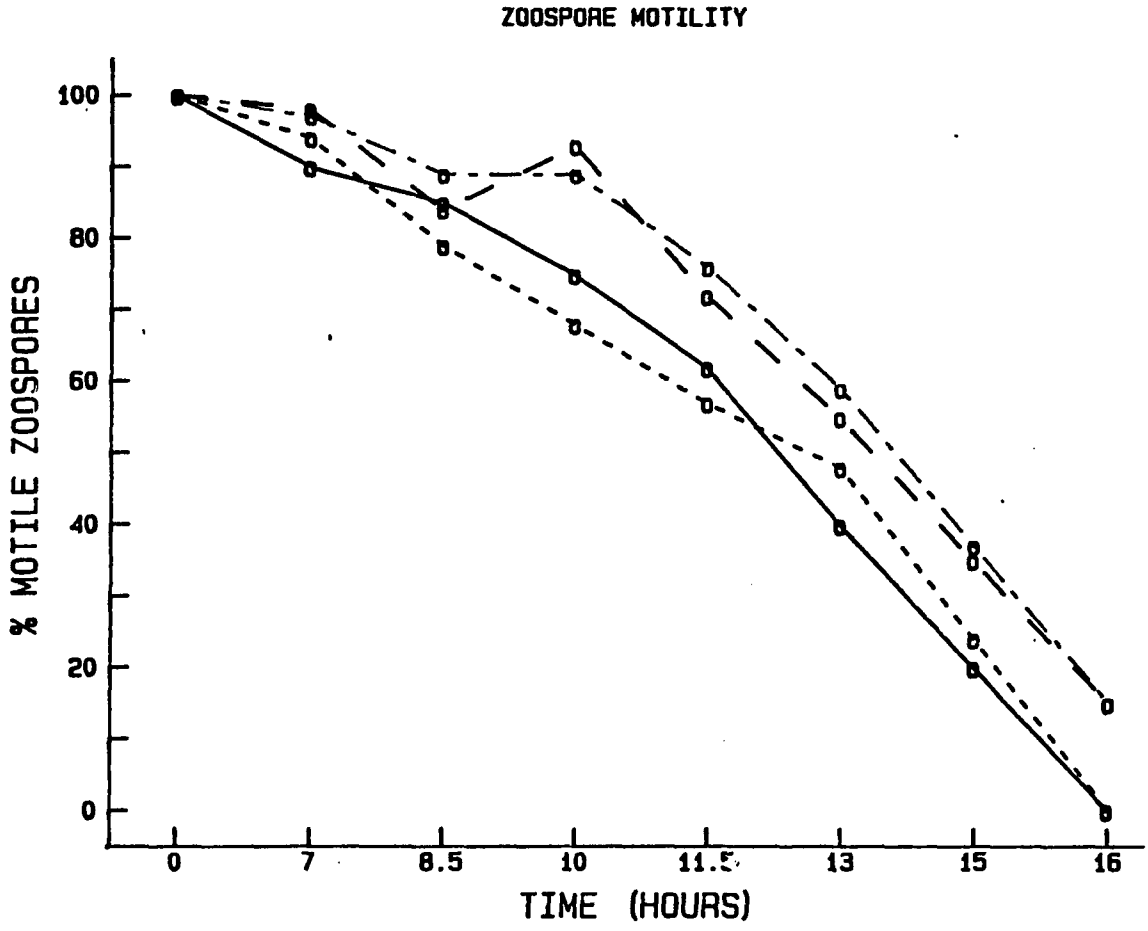

Figure 4. Duration of motility of P. dissotocum zoospores.
——— ; - - - - ; — — ; — · — = replication of experiments.
% motile zoospores represents the number of zoospores remaining motile in the petri dish at the times listed. Time represents hours after culture plugs had been removed from the petri dishes.

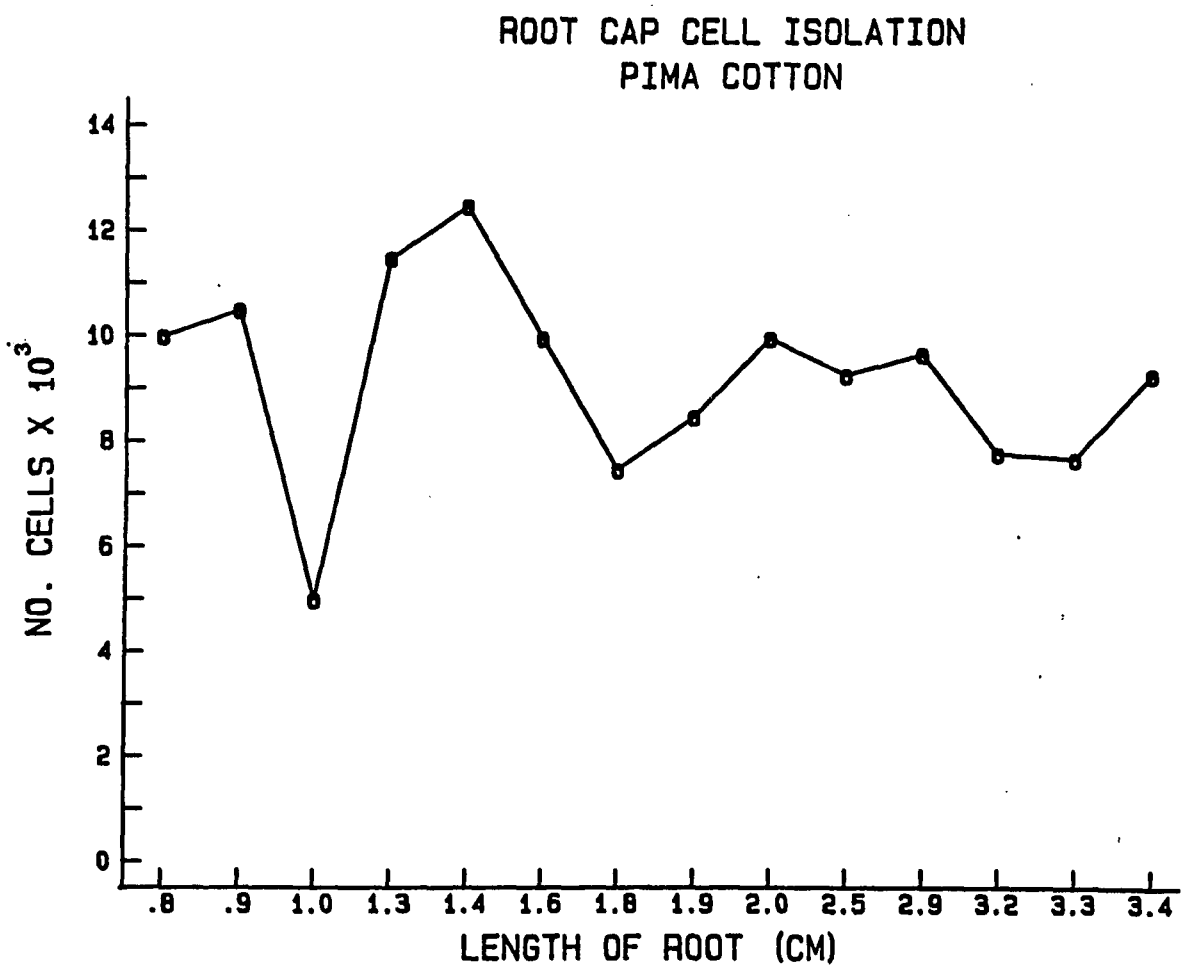

Figure 5. Number of peripheral root cap cells isolated from G. barbadense seedlings with various root lengths.

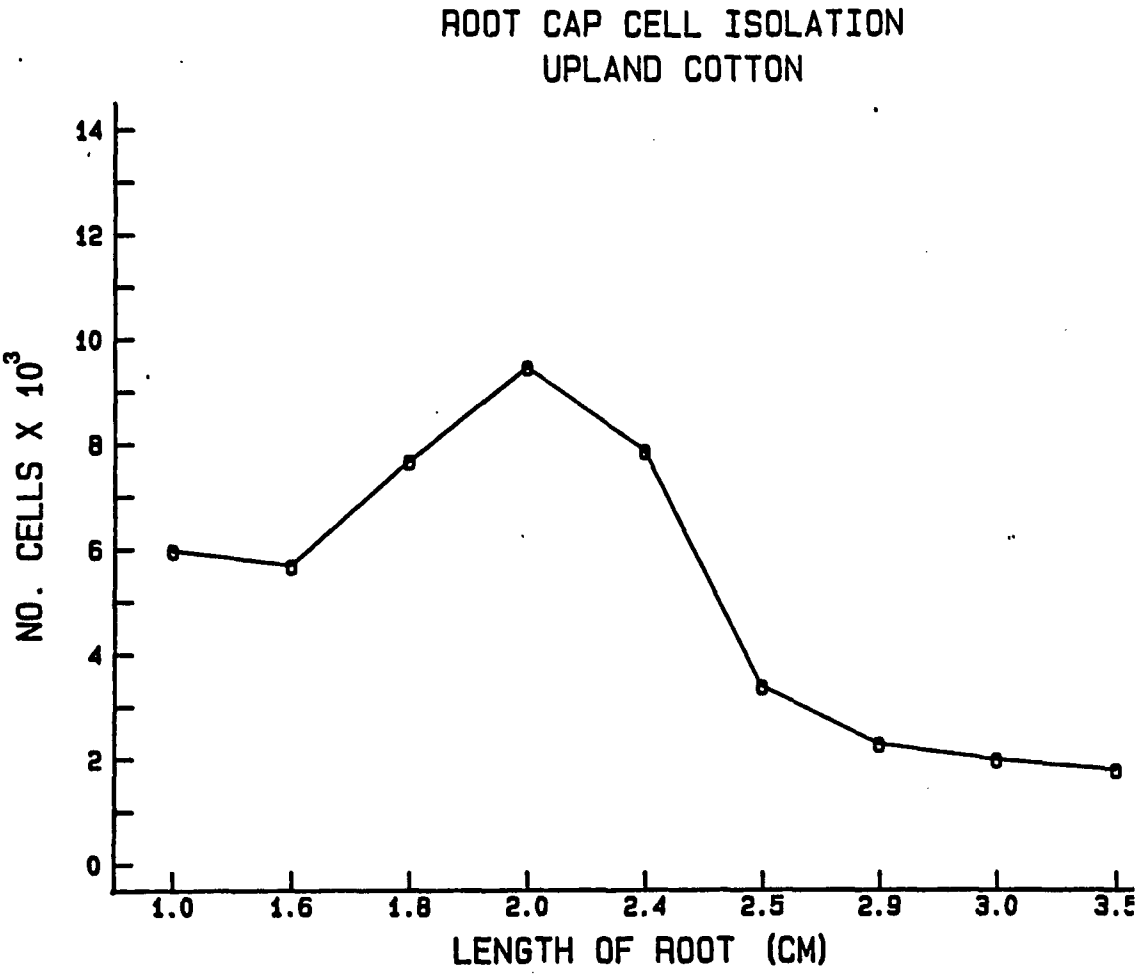

Figure 6. Number of peripheral root cap cells isolated form G. hirsutum seedlings with various root lengths.

seedlings with root lengths between 1.0 and 1.5 cm (Fig. 5). Yields from fourteen G. hirsutum seedlings with root lengths between 1.0 and 3.5 cm ranged from 1,300 to 10,500 cells per root. For G. hirsutum, the greatest yield was from roots that were between 1.9 and 2.4 cm (Fig. 6).

Viability of Root Cap Cells

Root cap cells from several species are viable at the time of isolation and will remain viable in culture medium for several months (Hawes and Pueppke, 1986). Using fluorescein diacetate stain, cotton root cap cell viability was studied at the time of isolation from the root and after 24 and 48-hours (Fig 7). Using techniques described by Widholm, 1972, cells which glowed fluorescent green with fluorescent microscopy were considered to be viable (Fig. 8). Cells were isolated from G. barbadense seedlings with root lengths between 1 and 2.5 cm and were incubated in sterile deionized water. A minimum of 80% of the cells were viable at the time of isolation regardless of root length. The percentage of viable cells in the population dropped by approximately 10% in the first 24 hours after isolation. After 48 hours, viability among seedlings ranged from 60 to 5%.

Chemotactic Response of Zoospores to Roots

Zoospores of the two Pythium species exhibited different responses to the roots of the two cotton species. P. dissotocum zoospores were immediately attracted to cotton roots of both species

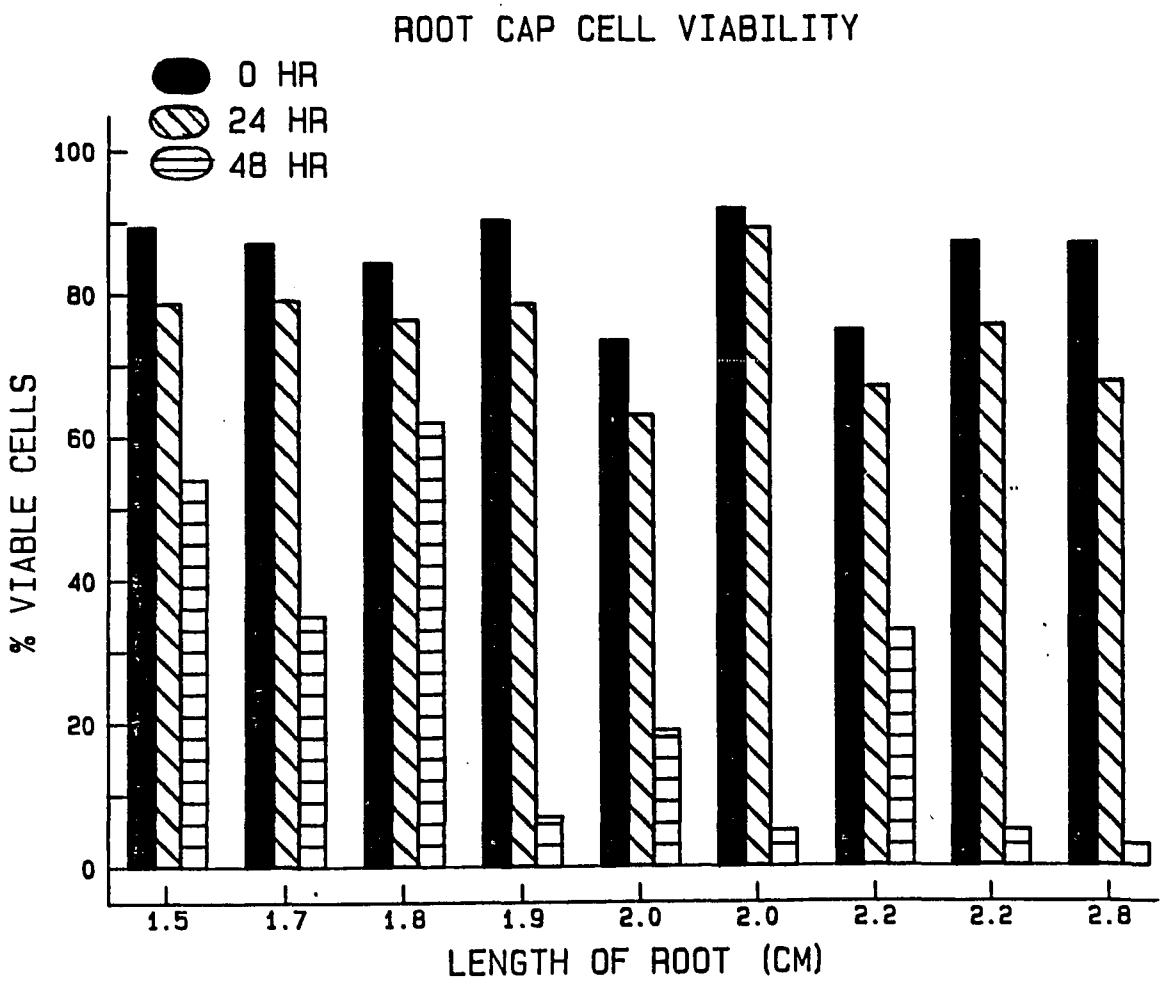

Figure 7. Viability of root cap cells of G. barbadense at the time of isolation, 24-hours and 48-hours after isolation.

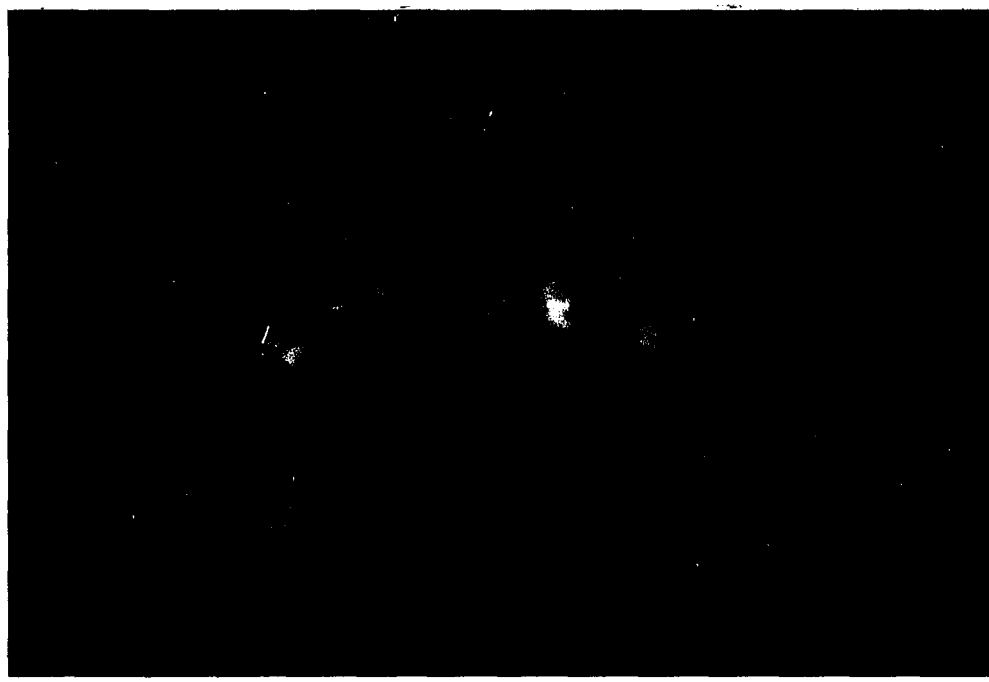

Figure 8. Viable isolated peripheral root cap cells of G. barbadense using fluorescein diacetate stain.

in the root cap cell region. The zoospores were attracted to, accumulated around, and encysted on the root tip. On roots with intact root caps, zoospore accumulation was so great around the root cap that the normal sloughing of root cap cells was inhibited (Fig. 9B). On roots from which peripheral root cap cells had been removed by agitation in water, zoospores were attached to the root tip, where encystment of the zoospores occurred (Fig. 9C). Zoospores of this species were never attracted to any other part of the root including the area of elongation, the root hair region, or to wounds made with a pin prick. In contrast, P. catenulatum zoospores were not attracted to the root cap region or to any other part of the root or to wounds of either species of cotton.

The chemotactic responses of zoospores of both Pythium species to other plant species was also evaluated (Table 1). Although P. dissotocum zoospores exhibited a chemotactic response to seedlings of all plant species tested, the location of the attraction varied among species and between seedlings. Zoospore attraction was not restricted to the root cap region of any of the other plant species. Zoospores of P. catenulatum were attracted in large numbers to six other species, with especially strong responses to Agrostris pulustris (bentgrass), Latuca sativa (lettuce) and Salicornia species. The zoospores were attracted to and encysted on these species either in the area of elongation, the root hair zone or

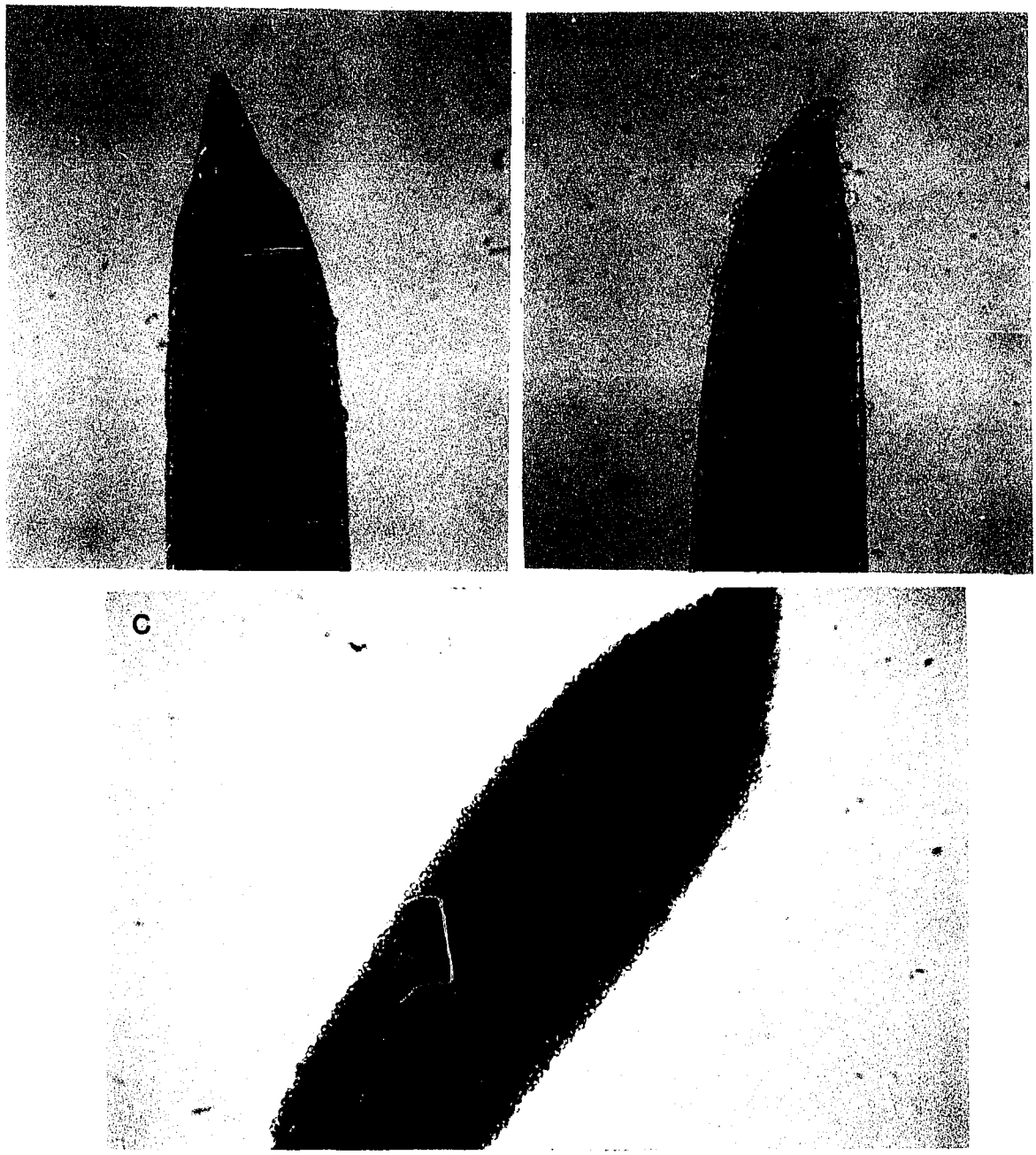

Figure 9. Accumulation and encystment of Pythium zoospores to a plant root. A). Healthy root with no zoospores. B). Root with zoospore beginning to encyst on the root tip of a seedling with the peripheral root cap cells removed. C). Root with zoospores attachment to the root cap region on a seedling with intact root cap.

Table 1. Responses of *Pythium dissotocum* and *Pythium catenulatum* zoospores to various plant species.

Plant Species	*Pythium dissotocum* Attraction[a]	*Pythium dissotocum* Infection[b]	*Pythium catenulatum* Attraction	*Pythium catenulatum* Infection
Agrostis palustris Bentgrass	++++ ER	+	++++ ER	+
Latuca sativa Lettuce	+++ ER	+	+++ ER	+
Lycopersicon esculentum Tomato	+ AE	+	+ AE	+
Zea mays Corn	++ RH,AE	+	+/- RH,AE	+/-
Spinacia oleracea Spinach	++ AE,RC	+	++ AE	+
Salicornia sp.	+++ RH,ER	+	+++ RH,ER	+
Gossypium barbadense Pima Cotton	++++ RC	+	-	-
Gossypium hirsutum Upland Cotton	+++ RC	+	-	-

a Accumulation of number of zoospores.
b Infection verified by inhibition of root growth and by recovery of the fungus from inoculated roots.
- = 0 zoospores
+ = 1-10 zoospores
++ = 11-50 zoospores
+++ = 51-100 zoospores
++++ = 101+ zoospores
ER = Entire Root, RH = Root Hair Region, AE = Area of Elongation, RC = Root Cap Region

both, but there was no attraction or encystment of P. catenulatum zoospores in the root cap region with any of the species tested.

Infection of Roots

The ability of zoospores to infect seedlings after incubation of the roots in zoospore suspension was studied (Table 1). The inoculated seedlings were grown in moist growth pouches, and after 24-hours in the pouches, the amount of root growth was recorded. In all cases when a seedling attracted zoospores, the root ceased growth in the pouches. Control seedlings treated with sterile deionized water continued to grow during this 24-hour period of time. The mean root growth of control seedlings was 2.0 cm to 3.0 cm in 24-hr in comparison with inoculated seedlings which had mean root growth of 0 mm.

After 24-hour in growth pouches, roots were excised and the roots were then placed on water agar containing 200 ppm streptomycin sulfate and incubated 48-hours at room temperature. Streptomycin sulfate was added to the water agar to eliminate any bacterial growth. The fungus was visible within 36-hrs and covered the plate with 72-hrs. After 48-hrs, the fungus was recovered from the water agar and transferred to V-8 agar for species identification. Cotton seedlings which attracted P. dissotocum zoospores exhibited fungal growth from the root tip as well as other places along the root. No fungus was recovered from cotton seedlings incubated in zoospore suspension of P. catenulatum or from control seedlings.

After a 7 to 10 day incubation of cultures on V-8 agar, the fungus was examined under the light microscope for characteristic fungal structures of the two Pythium species. P. dissotocum was recovered from seedlings of all plant species which had attracted zoospores of this species. In addition, P. catenulatum was recovered from all plant species which had attracted zoospores of this species (Table 1).

Chemotactic Response of Zoospores to Root Cap Cells

The responses of the two Pythium species to isolated root cap cells of cotton were consistent with their responses to intact roots. P. dissotocum zoospores were immediately attracted to some individual root cap cells of both cotton species. The zoospores located, accumulated and encysted upon individual cells within 5 seconds after the cells were placed in zoospore suspension (Fig. 2). In some cases, zoospores could be seen germinating and penetrating the isolated cells within 1-2 minutes (Fig. 3). In contrast, P. catenulatum zoospores were not attracted to and did not encyst upon any individual isolated cells of the two cotton species.

Colonization of Isolated Root Cap Cells

The percentage of individual root cap cells directly colonized by at least one encysted zoospore was determined 30 min and 24-hours after initial placement of the cells in suspension (Table 2). The number of zoospores encysted on each attractive cell varied

Table 2. Colonization of isolated living and killed root cap cells of Gossypium barbadense by zoospores of Pythium dissotocum.

	% of cells colonized[a]		Location of attraction[b]	
	30 min	24 hr	nucleus	other
Living cells[c]				
Sample 1[d]	22.4±2.1	23.8±2.6	56%	44%
Sample 2	24.6±6.7	23.2±6.6	46%	54%
Sample 3	28.8±1.5	26.9±2.6	52%	48%
Sample 4	18.8±1.9	20.2±2.0	48%	52%
Sample 5	22.6±4.2	21.4±4.6	50%	50%
Sample 6	24.4±4.1	28.7±3.2	54%	46%
Average	24.4±4.1	24.0±3.2	51%	49%
Killed cells[e]				
Sample 1	8.24±1.9	9.0±1.4	42%	58%
Sample 2	9.8±2.4	8.9±2.6	44%	56%
Sample 3	11.4±1.8	11.7±2.0	52%	48%
Sample 4	12.0±3.2	11.9±3.1	54%	46%
Sample 5	9.7±3.0	10.5±2.7	56%	44%
Sample 6	10.7±2.2	11.1±1.9	46%	54%
Average	10.3±1.4	10.5±1.3	49%	51%

a Percentage of cells in suspension directly colonized by at least one zoospore.
b The location where the zoospores were attached to the cell.
c A 10 ul sample of the cell suspension was checked for viability using fluorescein diacetate stain prior to incubation of 50 ul of the cell suspension with zoospores.
d Number is the average of 5 counts of 100 cells.
e Cells were killed by either drying or freezing and then checked for viability using fluorescein diacetate stain.

from one to several hundred. Some cells were colonized by zoospore hyphae which traveled some distance from an encysted zoospore, but these cells were not counted in the total number of cells directly colonized by the zoospores. Approximately 25% of the cells were directly colonized after 30 min and the percentage of direct colonization did not increase with time. However, all cells which were not individually attractive were colonized by zoospore hyphae within the first 24-hours (Fig. 10). Interestingly, zoospores accumulated around the nucleus of 50% of the colonized cells, while the remaining colonized cells were attacked at various sites along the cell (Table 2).

The viability of the cells in zoospore suspension was tested using fluorescein diacetate. After 1 to 2 hours incubation in zoospore suspension, 20 ul of cells which attracted zoospores were placed on a slide with 2 ul of the stain. Cells which had one or more zoospores attached were no longer viable while cells which had not been attacked by the fungus were still living (Fig. 11). After 24-hours no viable cells were found in the suspension. Control cells incubated in sterile deionized water were still at least 70% viable after the same time period (Fig. 7). Cells which were attacked by zoospores were killed and thoroughly colonized by fungal hyphae within 1-hour after contact (Fig. 3 & 10).

To determine if zoospores were also attracted to killed cells, cells killed by drying or freezing were incubated with

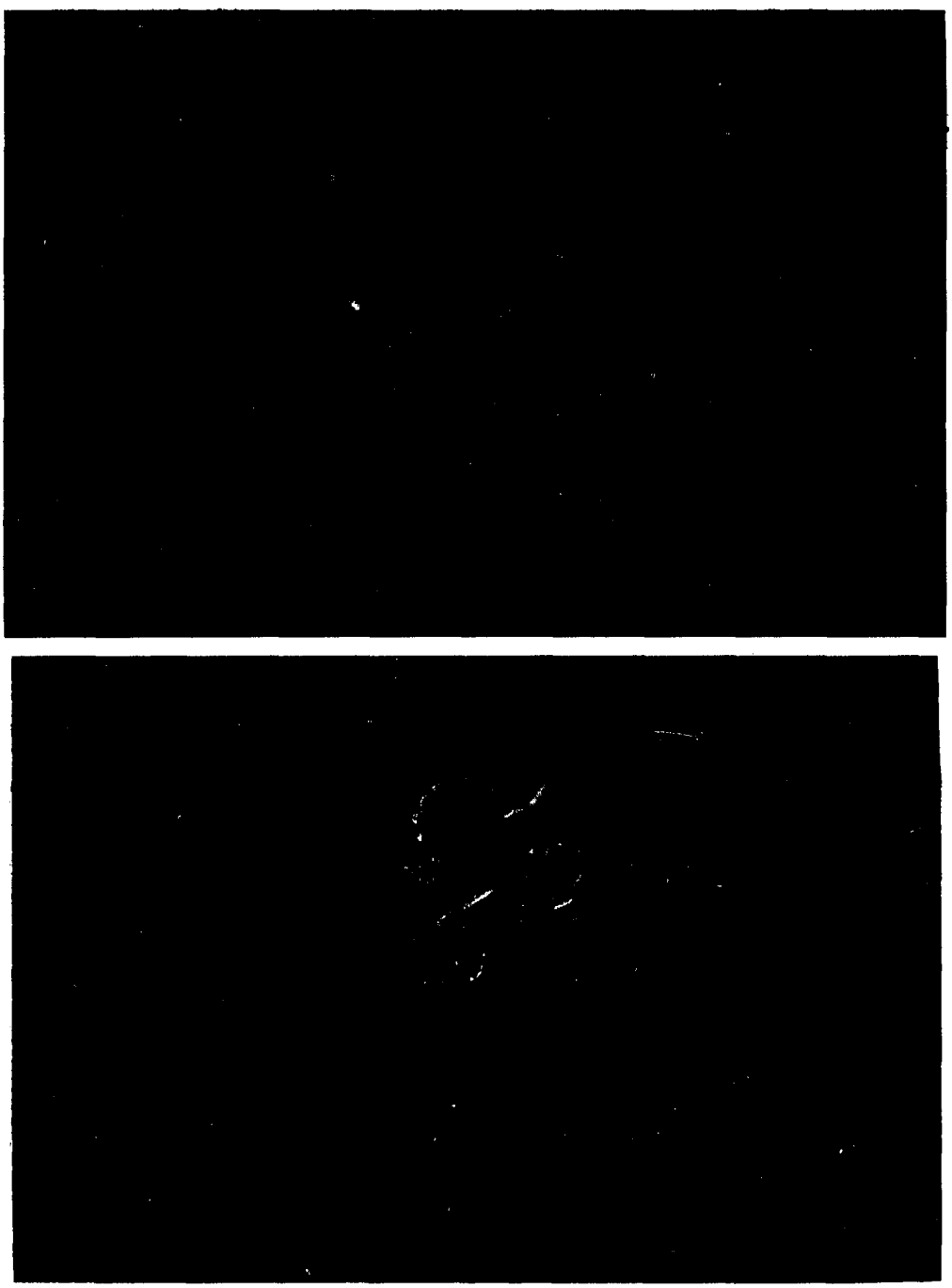

Figure 10. Colonization of root cap cells of G. barbadense after 24 hours incubation in suspension of P. dissotocum zoospores. A). Colonization of all cells in suspension. B). Colonization of an individual cell.

Figure 11. Viability of isolated peripheral root cap cells of G. barbadense colonized by zoospores of P. dissotocum using fluorescein diacetate stain.

zoospores of P. dissotocum. Whereas 25% of the living cells were attractive only 10% of the killed cells were individually attractive (Table 2).

Preliminary test of Pathogenicity of Pythium sp. on cotton in sand

The results of preliminary pathogenicity tests for the two Pythium sp. are recorded in tables 3 and 4. The root length, number of leaves and presence of brown or black lesions on the roots were recorded for each seedling.

Root length of cotton seedlings grown in sand culture after 2-days or 4-days was variable in all treatments. However, the length of roots infected with P. dissotocum tended to be shorter and contain more brown lesions than roots infected with P. catenulatum. Similarly, P. catenulatum infected roots were generally shorter than roots which had not been inoculated with either fungus. There was no difference in the number of leaves produced by any of the seedlings whether inoculated with one of the fungal species or not.

In addition, the fungus was routinely recovered from all seedlings inoculated with the two Pythium species as well as from the control seedlings which were not inoculated with either fungus.

Table 3. Pathogenicity of zoospores of *Pythium dissotocum* on *Gossypium barbadense*[a] seedlings in sand culture.

Conc. of zoospores[b]	Root Length (Avg cm)		% roots with lesions	
	2-d[c]	4-d	2-d	4-d
2×10^7	N/A	1.77±2.9	N/A	10
1×10^6	3.5±.7	3.1±6.4	0	50
5×10^5	4.53±2.9	4.13±2.7	33	33
Control SDW	9.95±1.8	11.0±4.1	0	0

a 5 partially imbibed seeds were planted in 4" pots containing #20 silica sand.
b 20 ml of motile zoospores at concentrations listed were used as inoculum.
c 2-d = seedlings harvested 2 days after germination;
4-d = seedlings harvested 4 days after germination.
N/A Not applicable.
SDW Sterile deionized water.

Table 4. Pathogenicity of zoospores of *Pythium catenulatum* on *Gossypium barbadense*[a] seedlings in sand culture.

Conc. of zoospores[b]	Root length (Avg cm)		% roots with lesions	
	2-d[c]	4-d	2-d	4-d
2×10^6	4.43±2.7	8.18±5.2	50	50
1×10^5	4.23±1.2	4.9±1.3	25	0
5×10^4	7.73±1.3	6.67±2.4	0	33
Control SDW	9.0±.94	11.0±4.1	20	40

a 5 partially imbibed seeds were planted in 4" pots containing #20 silica sand.
b 20 ml of motile zoospores at concentrations listed were used as inoculum.
c 2-d = seedlings harvested 2 days after germination;
4-d = seedlings harvested 4 days after germination.
SDW Sterile deionized water.

DISCUSSION

Both Pythium species used in this research produced a high number of zoospores in vitro. Zoospores of P. dissotocum are able to swim in undisturbed sterile deionized water for over 22 hours. The availability of large populations of synchronized zoospores facilitates analysis of the chemotactic responses.

The chemotactic response of fungal zoospores to plant roots has long been thought to be a non-specific reaction (Chi and Sabo, 1978; Cunningham and Hagedorn, 1962; Dukes and Apple, 1961; Goode, 1956; Royle and Hickman, 1964a; Spencer and Cooper, 1967; Zentmyer, 1960; Zentmyer, 1961). However, it is clear from this research that in some cases the response is specific both to host and to site of attraction. Root exudation has been established as an important component in zoospore attraction to plant roots (Dukes and Apple, 1961; Hinch and Weste, 1979; Longman and Callow, 1987; Miki et. al., 1980; Royle and Hickman, 1964a and 1964b; Zentmyer, 1960; Zentmyer, 1961). Massive attraction of zoospores to the root tip may reflect a greater amount of exudate arising from the root cap (Hinch and Weste, 1979). The reaction between P. dissotocum zoospores and the root cap region or root tip of G. barbadense is evidence of the importance of this region in zoospore attraction. The ability to isolate and

utilize peripheral root cap cells in chemotaxis studies is a key discovery which may aid in determining the genetic basis for specificity in host-pathogen relations.

Zoospores of P. dissotocum were attracted to peripheral root cap cells of cotton. Since zoospores of P. dissotocum were attracted to the root cap region and to the root tip of cotton seedlings, the attraction to the peripheral root cap cells was not surprising. Not all of the individual isolated cells were attractive to the zoospores. This may be due to a number of factors: 1. Individual peripheral root cap cells may vary in the amount and rate of root exudation. 2. The age of the peripheral root cap cells will vary thus altering their attractive nature. 3. Attraction may be mediated by genetic factors found in some but not all of the isolated cells.

In this study, zoospores were not only attracted to plant roots and peripheral root cap cells, but the fungus was able to penetrate these roots and individual cells. Roots which had attracted zoospores would not continue to grow in an environment conducive for plant growth and colonized cells were no longer viable using viability tests, thus the roots and the cells which were attacked by zoospores were killed. In addition, the recovery of the fungus from those roots, both from the root tip - the attractive region in the case of P. dissotocum and G. barbadense - and from other parts of the roots, indicated that the fungus had thoroughly

colonized the root. This clearly showed that if attracted, zoospores are capable of germinating, penetrating and infecting those roots. Infection of a single zoospores of P. aphanidermatum to cotton was recorded at 2-hours. The amount of time required for attraction and encystment to occur were not stated (Spencer and Cooper, 1967). The shortest time previously reported for zoospore attraction to plant roots is 15 minutes (Gold and Stanghellini, 1984). Attraction time of P. dissotocum zoospores to G. barbadense as was shown in this report is 5 seconds. The microscope set up used in this study made it possible to watch zoospores from the moment a root or isolated root cap cells were placed into the zoospore suspension. The difference noted here is primarily due to the fact that 15 minutes was the shortest time assayed in the previous study (Stanghellini, personal communication). Thus, zoospores may actually have been attracted to the roots in a much shorter time period than was seen by these researchers.

The colonization of isolated root cap cells by the fungus is an important finding in regards to what is known about the pathogenesis of soil-inhabiting root-infecting fungi. This study clearly showed the attractive nature of detached root cap cells for at least one species of fungi. A certain portion of isolated cells, 25% for living cells and 10% for dead cells, are immediately attractive to the fungus. Results of experiments presented here also indicated that although the percentage of attractive cells does not

increase over time, all cells in a suspension can be utilized as a food base for the fungus to increase its biomass. Free-living root cap cells have been shown to survive in soil for up to three weeks (Vermeer and McCully, 1982). It is important to understand that although root cap cells can remain viable in soil for some time, results from experiments presented here indicate that the likely situation is that the majority of the cells die shortly after sloughing from the root (Fig. 7). In this case, it would seem that the zoospores can still utilize the cells for food.

Pythium aphanidermatum, Pythium ultimum trow, Pythium debaryanum Hesse and unidentified Pythium species have been isolated from diseased cotton seedlings in California and other cotton growing areas of the world (Spencer and Cooper, 1967). However, neither P. dissotocum or P. catenulatum have been identified as a pathogen on cotton. Preliminary studies have shown that both species are capable of infecting cotton in sand culture. However, it would be premature to state that either one or both of the organisms is a pathogen on this host. The results of the pathogenicity test did not give an indication on seedling death, reduced growth of the test plants, or loss of yield. Further pathogenicity tests may be able to establish if there is a negative effect of the fungus on the host. In the case of P. catenulatum, it is not clear the motile zoospores were the infective propagule. It is possible that mycelium from germinating encysted zoospores penetrated the plant roots. In contrast, it seems

clear that motile zoospores of P. dissotocum are an infective propagule. This is evident in the fact that control seedlings became infected with only P. dissotocum and not P. catenulatum. The pots which had been set up as controls, were placed in the same trays as both P. dissotocum and P. catenulatum treated pots. Motile zoospores of P. dissotocum were able to locate roots of seedlings grown in non-inoculated pots. Interestingly, P. dissotocum was also recovered from several of the control seedlings. The control pots were placed in the same trays as pots treated with both Pythium species. This indicated that P. dissotocum was able to locate by chemotaxis the roots of the control seedlings even through they were a great distance away from the roots. Zoospores of P. catenulatum did not locate these roots. These results are the same as the results obtained from in vitro studies of the two species to the roots of cotton.

It is yet unknown how wide spread the organism is in its association with cotton. However, since the organism is capable of causing death to cotton seedlings in vitro, the possibility for disease losses to occur in the field can not be ignored. Other factors such as temperature, moisture, and microbe competition are very important in the disease process and these may be limiting in regards to the pathogenicity of P. dissotocum on cotton.

In a natural soil environment, the first plant part encountered by a fungal zoospore are the peripheral root cap cells.

These cells have tremendous potential significance in host-parasite interactions. Peripheral root cap cells of Gossypium barbadense and Gossypium hirsutum are viable at the time of isolation as was shown using fluorescent microscopy. The majority of these cells remain viable for a minimum of two days in sterile deionized water. Since these are living cells, it is possible to use these cells as the attractive plant surface for the zoospores in the model system utilized in this study. Thus, many of the problems encountered by previous researchers can be avoided.

The chemotactic responses of fungal zoospores to plant roots has been documented by previous researchers (Chi and Sabo, 1978; Cunningham and Hagedorn, 1962; Dukes and Apple, 1961; Erb, et. al., 1986; Goode, 1956; Hinch and Weste, 1979; Miller and Maxwell, 1984; Mitchell and Deacon, 1986; Royal and Hickman, 1964a; Spencer and Cooper, 1967; and Zentmyer, 1960). However, this is the first report of a specific response to hosts and to a distinctive site on the host root. This is also the first report of attraction of a fungus to individual plant root cap cells. These findings open many new doors into the studies of fungal chemotaxis and plant cell-fungus interactions.

These findings may differ from the results obtained by previous researchers for two key reasons. First, this study used two species of Pythium which produce long swimming synchronized zoospores. Most of the previous studies used Phytophthora species

which are not known to have long swimming zoospores. Second, a model system using intact root caps as well as isolated root cap cells was utilized. In previous work, the root cap was often removed prior to chemotaxis studies or was inadvertently removed by the germination process. Since the specificity of the response of zoospores was to the root cap region, these results would not be obtained in studies in which the root cap had been removed.

The root cap region of plant roots has mostly been ignored by researchers in plant pathology. It is apparent now, however, that this region and these cells play an important role in the attraction of some plant pathogens to plant roots. Indeed, these cells may be a key to pathogenesis in some host-parasite interactions. The need to use intact, undisturbed seedlings is apparent. By germinating seedlings in a manner which disrupts the root cap region as little as possible, it may be possible to demonstrate that these cells provide more functions for the plant and the pathogen than were previously known. The factors which mediate the reaction of the zoospores to the specific cells is as yet unknown. Using isolated peripheral root cap cells and zoospores produced in vitro in a model system, it is possible to obtain a known number of cells and zoospores and thus, it is possible to quantify chemotaxis responses. In addition, it is possible to observe the interaction between one individual cells and one fungal zoospore. The process of attraction and infection can be evaluated and timed step by step. As more plants species and fungi

are tested it is possible that more specific responses will be discovered. These new discoveries along with further research may provide new insight to enable us to more fully comprehend the factors which govern the attraction of pathogens to plants.

APPENDIX

PRELIMINARY STUDIES ON THE ROLE OF TOXINS IN PLANT DISEASE

Introduction

Many plant pathogenic fungi and bacteria produce chemical toxins which are capable of causing disease symptoms in plants. Some toxins are host selective while others cause damage to any plant species (Misaghi, 1982). The significance of pathogen produced toxins in pathogenesis is currently being studied by many researchers (Gowda and Rai, 1978; Husain and Kelman, 1958; Misaghi, 1982; Monod et. al., 1986; Sher and Ussery, 1940; Steinberg, 1947ab; Whatley et. al., 1980). While information regarding several host-parasite interactions and the role of pathogen produced toxins is available, there are many more diseases where toxins have merely been suggested as a factor in the cause of the disease (Misaghi, 1982).

Phytotoxic metabolites produced by Macrophomina phaseolina (Tassi) Goid. have been implicated in frenching disease of tobacco (Monod et. al., 1986), and Pseudomonas solanacearum E. F. Sm. causing bacterial wilt of solanaceous plants has been thought to produce a toxin which is capable of causing disease symptoms in susceptible hosts (Husain and Kelman,1958). To date, little work has been completed regarding toxins produced by these two organisms. Prior to

the work presented in this thesis, experiments were carried out to check the ability of these two organisms to produce toxic metabolites, and to see if these metabolites were capable of causing disease in susceptible plants.

Involvement of toxins in frenching disease of tobacco

Though no one organism has been proven to be the causal agent for frenching disease of tobacco, both M. phaseolina and Bacillus cereus have been associated with the disease. Disease symptoms can be induced by using both living and killed cells of the non-pathogenic, soil-inhabiting strain of B. cereus (Steinberg, 1947b). In addition, disease symptoms can be induced by plant pathogenic strains of the fungus M. phaseolina. Many researchers have suggested that the symptoms are caused by absorption by the roots of microbially produced toxins from the soil (Monod et al, 1986; Shear and Ussery, 1940; Steinberg, 1947a).

Experiments using the same strains of M. phaseolina and B. cereus as were used by Monod et. al. 1986 were set up in the spring of 1986. Attempts to induce symptoms of frenching disease in tobacco (Nicotiana tabacum L.) var. Ottawa were made using five strains of the fungus and one strain of the bacterium. The fungus was grown on Potato Dextrose Agar (PDA) (Difco) for 7-days at room temperature. Seedlings were inoculated by placing 4 agar plugs 5 mm in diameter from the PDA cultures around the roots of 3-wk, 6-wk or 10-wk old tobacco seedlings growing in a 1:1:1 mixture of non-sterile peat,

vermiculite and sand. Plants inoculated with culture plugs from sterile PDA culture served as controls. The plants were then incubated in a greenhouse at 24-26 C and observed for disease symptoms over the next 4 weeks. The experiment was repeated twice.

In addition, liquid cultures containing a 5 mm diameter culture plug of one each of the five strains of M. phaseolina were grown on a shaker table at room temperature for 10-days. After 10-days, the fungus cultures were filtered aseptically to obtain cell-free culture filtrates of the fungus. The cell-free extract was then used as inoculum to attempt to induce frenching-like symptoms in tobacco. Tobacco seedlings planted as above were inoculated with 20 ml of the cell-free extract, 10 ml extract + 10 ml sterile distilled water SDW, 5 ml extract + 15 ml SDW, or 20 ml SDW. Plants inoculated with 20 ml of filtrate from non-inoculated liquid medium served as controls. Seedlings were incubated in growth chambers at 25 C in 12-hours of light and 12-hours of dark. Each treatment was replicated 5 times and the experiment was repeated twice.

Bacillus cereus cultures were grown on PDA at room temperature for 3-days. Ten ml of SDW was placed on top of the culture and the cells were loosened from the media with a sterile inoculating loop. For experiments, 10^8 ml of bacterial cell suspension containing 10 cells/ml were poured on the surface of 3-wk, 6-wk or 10-wk old tobacco seedlings grown as above. The roots were injured slightly with a sterile needle prior to inoculation with

the bacterium. Plants inoculated with 10 ml SDW served as controls. The plants were incubated in a greenhouse at 24-26 C and observed for disease symptoms over the next 4 weeks. Each treatment was replicated 5 times and the experiment was repeated twice.

Liquid cultures of B. cereus were grown on a shaker table at room temperature for 3-days and then filtered aseptically to obtain a cell-free extract. Plants were then inoculated in the same manner as with M. phaseolina cell-free extract and were incubated in growth chambers. Each treatment was replicated 5 times and the experiment was carried out twice.

In addition to the above experiments, a set of plants were inoculated with both M. phaseolina and B. cereus. Plants were grown and inoculated as above for both the fungus and the bacterium. In vitro, B. cereus inhibited the growth of M. phaseolina on solid PDA medium. This experiment was set up in order to see if B. cereus could stop M. phaseolina from inducing frenching-like symptoms should the fungus cause disease symptoms in the tobacco plants.

The experiments were carried out in an effort to establish an association between the disease and the organisms. However, at no time did typical frenching disease symptoms appear on any of the inoculated seedlings. Because no association between the fungus or the bacterium and the disease could be made, further experiments were not carried out.

Role of bacterial toxins in wilt of tomato

Bacterial wilt, caused by P. solanacearum, is a serious disease of many agronomically important crops in many tropical areas in the world. This bacterial pathogen has been well studied and several researchers have suggested that the organism may produce a metabolite which is, in whole or in part, responsible for the disease symptoms which occur in susceptible plants (Gowda and Rai, 1978; Husain and Kelman, 1958; Whatley et. al., 1980). Preliminary studies by M. Olsen (personal communication) indicated that virulent colonies of the bacterium were capable of inhibiting the growth of tomato (Lycopersicon esculentum) seedlings in vitro. This preliminary observation led to subsequent studies to determine if the bacterium was producing a toxic metabolite in the medium which caused the seedlings to stop growing.

Virulent colonies of P. solanacearum Ps-80 pathogenic to tomatoes were selected from tetrazolium medium (Kelman, 1954) and used in these subsequent studies. The bacterium was streaked along the bottom third of 15 cm media plates containing one of several different types of media, such as PDA, water agar, Kings medium-B, nutrient agar, tetrazolium chloride, medium A (1.5% water agar, 1/2 strength hoagland's solution, and .1% KNO_3), medium B (1.5% water agar, 1/2 strength hoagland's solution with Iron, and .1% KNO_3), or medium C (1.5% water agar and .1% KNO_3), which had been poured on a slant. Aseptically germinated two-day-old tomato seedlings where

placed at the top of the slanted media, radicle pointed down toward the bacterial streak. Control plates were set up by streaking plates with a sterile inoculating loop without bacteria. The plates were placed on their sides and incubated in growth chambers in the dark at 32 C for several days while observations of growth and heath of seedlings were observed. Although in some cases, seedlings did not grow down beyond the bacterial colony of P. solanacearum, in other cases they were able to continue strong growth through the colony. This phenomenon was seen with all types of media tested. In all cases, the seedlings grew all the way to the bottom of control dishes. Because of the inconsistent results of these experiments, no conclusions could be made regarding the ability of the bacterium to produce a phytotoxic metabolite.

These experiments regarding pathogen produced phytotoxic metabolites did not provide support for the role of toxins in pathogenesis for the two diseases studied. At the same time, they did not eliminate the possibility that the organisms may produce phytotoxic chemicals. Because a multitude of variables may be involved in the production of these metabolites, inconsistency of results occurs in toxin related experiments.

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