THE INTERACTION OF *RHIZOBIUM MELILOTI* AND TWO GENERA OF FUNGI ASSOCIATED WITH ROOT AND CROWN ROTS OF ALFALFA

By

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CHAPTER I

INTRODUCTION

Root and crown diseases are a major problem with alfalfa (Medicago sativa L.) production in the United States. The diseases cause stand depletion two to three years after establishment. Determination of the pathogens responsible for root and crown rots on alfalfa has been difficult. Species of Fusarium are most commonly isolated from diseased roots, but these isolates exhibit a wide range of virulence. In addition, fungi having virulent isolates causing such diseases are often found associated with healthy plants (Leath et al., 1971), and constitute a part of the normal soil microflora. There may be regional differences in the prevalence of pathogenic species (Couch, 1958; Schmitthenner, 1964; Aube and Deschenes, 1967; Leath et al., 1971). Rhizoctonia solani and Fusarium spp. are commonly isolated from alfalfa exhibiting root and crown rot in Oklahoma (Barnes, 1973). One or several physical and biological factors, such as freezing, drought, insect damage, excessive clipping, very late clipping, or unbalanced fertilization, may create stress in the plants, predisposing them to infection by the causal organisms. Because of the significance of these stress factors in incidence of the disease, it is often referred to as a complex (Leath et al., 1971).

General symptoms include wilting, stunting, and root lesions. Both Fusarium spp. and Rhizoctonia solani Kühn cause necrosis of root

cortical tissues (Chi and Childers, 1964; Leath et al., 1971) and Fusarium causes vascular discoloration upon colonization of the stele (Leath et al., 1971).

No alfalfa varieties resistant to Fusarium or Rhizoctonia root and crown rots are presently available. This method of control does not seem probable because of the extreme variability in virulence of the causal organisms (Leath et al., 1971), so that many control measures relate to cultural and management practices aimed at producing vigorous plants and minimizing stress.

Members of the bacterial genus Rhizobium may supply part of the nitrogen required by leguminous plants through symbiotic association with a host plant. The process results in the formation of nodules in the root system. Rhizobia can survive in sterile soil in the absence of the host for periods exceeding 50 years (Jensen, 1961); however, in the presence of root exudate from a specific host, each Rhizobium species is stimulated to undergo rapid cell division, causing high populations in the rhizosphere (Nutman, 1948). R. meliloti Dangeard is stimulated to the greatest degree by alfalfa root exudate (Rovira, 1961). Species of Rhizobium have been observed to form a sheath on the root system of seedlings of their specific host. This is believed to be in response to certain components in the root exudate (Voegeli and Howard, 1970). Nodulation follows the period of rapid growth.

Complex interactions between soil microorganisms occur in the rhizosphere. The success of any one organism depends on its ability to maintain growth using the nutrients and space available. Nodule-forming R. meliloti and pathogenic Fusarium and Rhizoctonia species

must compete for these resources in the process of their respective entrances into the host plant.

Seedling survival tests by Johnston (1967) indicate that the presence of nodule-forming R. meliloti in the rhizosphere of alfalfa prior to infection results in effective reduction in cortical rot caused by Fusarium oxysporum Schlect. emend. Snyd. and Hans., F. solani (Mart.) App. and Wr. emend. Snyd. and Hans., and F. roseum (Lk.) emend. Snyd. and Hans. R. meliloti antagonisms against these Fusarium species was also shown in vitro. He believed competition for nutrients to be responsible for the antagonism and reduction in disease severity. There have been few reports dealing with interaction between R. meliloti and pathogenic fungi. The purpose of this investigation was to determine if R. meliloti could exhibit antagonism in combination with Rhizoctonia solani in vitro, and to compare the Rhizobium - Rhizoctonia and Rhizobium - Fusarium interactions in the rhizosphere of alfalfa seedlings. Recent evidence published by Drapeau et al. (1973), in which the Rhizobium - Fusarium antagonism was shown to be antibiotic in nature, thus conflicting with the findings of Johnston (1967), prompted a final experiment to re-examine the nature of the antagonism observed between F. oxysporum and R. meliloti.

CHAPTER II

REVIEW OF LITERATURE

A. Alfalfa Root and Crown Disease

Fusarium oxysporum was first reported as a pathogen of alfalfa in 1928 by Wiemer (1928). He designated the isolate *F. oxysporum* f. *medicaginis*, and since that time it has ranked high among the species of *Fusarium* most commonly isolated from diseased alfalfa roots (Leath et al., 1971). *F. roseum* and *F. solani* are also frequently found associated with alfalfa root and crown rots (Leath et al., 1971). *Rhizoctonia solani* is quite often isolated from diseased cortical tissue of alfalfa roots, but is best known as a cause of damping-off of alfalfa seedlings (Shephard and Wood, 1963). Numerous accounts of the pathogenicity of *R. solani* to alfalfa have been published; examples are: Erwin (1954), Khan (1950), and Chi and Childers (1964).

Two differing opinions are presently held on the cause of alfalfa root and crown rots. Fungal pathogens are indicated as the primary agents in one approach. In the other assessment of the disease, root rot is not caused by pathogenic fungi alone, but rather results from fungal infection after inital stress caused by environmental, nutritional, and entomological factors. The latter conclusion is the one most widely accepted at this time (Leath et al., 1971).

Fusarium spp. commonly enter the root through wounds, and histological evidence has also shown invasion by direct penetration of

epidermal cells (Chi et al., 1964). R. solani may penetrate epidermal cells following formation of infection cushions (Chi and Childers, 1964). Colonization of cortical tissues proceeds from the initial invasion area, causing necrotic areas on the surface of the root. Fusarium spp. may extend into the vascular cylinder, resulting in discoloration of the central core (Leath et al., 1971), while R. solani also invades the stele, but has no apparent effect on these cells (Chi and Childers, 1964). When sufficient tissues has been damaged, plant vigor declines, followed by wilting and stunting of growth (Leath et al., 1971). Both Fusarium and Rhizoctonia cause damping-off of alfalfa seedlings (Leath et al., 1971; Erwin, 1954) in which the hypocotyl or upper taproot are infected, followed by massive disruption of the young, unlignified tissues, resulting in rapid death (Leath et al., 1971).

B. Alfalfa Root Nodulation

The nodulation of legumes by rhizobia is host specific. Each species of Rhizobium has particular hosts which it alone can effectively nodulate. It may not enter into a symbiotic relationship (fixing nitrogen) with any other host (Vincent, 1967). Nutman (1956) postulated that nodulating rhizobia enter the host legume by causing invagination of the root hair cell wall at the point of entry, and histological evidence now shows this (Sahlman and Fåhraeus, 1963). This entry mechanism is almost always preceded by characteristic curling of the root hair tip, accompanied by deformation, making the hairs appear twisted and bent, with the point of entry being most commonly in the region of the curled tips. Only a small number of curled root hairs are actually "infected", and less than thirty percent of these result

in nodule formation (Fåhraeus, 1957). The mechanism whereby the host cell wall and the homologous Rhizobium interact to cause invagination has not been completely determined, but it has been generally accepted that the bacteria convert root-exudated tryptophan to indol-3-acetic acid (Georgi and Beguin, 1939). IAA has been shown to effect deformation of root hairs (Valera and Alexander, 1965), as well as the distribution of a specific pectic enzyme along the root hair cell wall (Fåhraeus and Ljunggren, 1959; Ljunggren and Fåhraeus, 1961). Present hypotheses center around a specific pectic enzyme inducer believed to be present in the extracellular polysaccharide layer of homologous Rhizobium strains (Fåhraeus and Ljunggren, 1967; Ljunggren, 1969; Munns, 1969; Hubbell, 1970). However, research results have been inconclusive (Lillich and Elkan, 1968; Macmillan and Cooke, 1969).

Following penetration of the bacteria into the root hair, an "infection thread" is formed continuous with the host cell wall at the point of invagination. This thread traverses the length of the root hair, guided by the host cell nucleus (Fåhraeus, 1957), and enters the cortical tissue. Tetraploid cells of the cortex are the sites of bacterial multiplication, and this thread functions in dispersing the bacteria throughout the dividing cells in the developing nodule (Jordan et al., 1963).

C. Biological Control

As no overall tolerance to root rot has been found in any variety of alfalfa, breeding for resistance does not seem probable (Leath et al., 1971). Most control methods have been limited to cultural and

management practices and efforts to make R. meliloti more effective in nodulation.

Biological control has also been employed as a method to control this disease. Biological control may be defined as the use of living organisms to control the disease-producing capabilities of other living organisms. Antibiosis and competition are two mechanisms by which biocontrol is effected. In antibiosis, metabolic products of organisms may inhibit or kill other organisms. Competition is the detrimental effect of one organism on another by use of or removal of some necessary resource in the environment. Several extensive reviews of the biocontrol of soil-borne pathogens exist (Wood and Tveit, 1955; Brian, 1960; Baker and Snyder, 1965; Baker, 1968; Baker and Cook, 1974).

Various reports of antagonism between soil bacteria and Fusarium spp. and Rhizoctonia have been published. Excellent coverage of these cases may be found in Baker and Cook (1974). A few publications have dealt with Rhizobium - fungal interactions. Aube and Gagnon (1970) reported that the ability of Gliocladium roseum (Link) Bainier and Trichoderma viride Pers. ex Fries to reduce the nitrogen content of alfalfa shoots was inhibited when in combined inoculation with R. meliloti. Chou and Schmitthenner (1974) noted a reduction in the number of alfalfa plants killed by Phytophthora megasperma Drechs. var. sojae A. A. Hildeb. when grown in soil inoculated with R. japonicum (Kirchner) Buchanan. Harris (1953) observed a detrimental effect of Fusarium (species not identified) on the nodulation of clover by R. trifolii. His investigations did not consider antagonism between the two organisms. Chi and Hanson (1961) have found the presence of R. trifolii can reduce disease development as much as one unit, based on

an index of zero to five. Red clover plants and F. oxysporum, F. roseum, and F. solani were used in their experiments. Johnston (1967) observed in vitro antagonism between R. meliloti and F. oxysporum and F. roseum in two component cultures. Based on these results and those obtained in vivo, he concluded that competition for available carbon and possibly certain amino acid root exudates was the mechanism responsible. However, if R. meliloti was present in the alfalfa rhizosphere prior to inoculation of the fungus, Fusarium was repelled at a distance from the root surface. These observations were made in hydroponic glass slide cultures, and Johnston did not test his R. meliloti isolates for antibiotic properties. In these experiments the bacteria also enhanced resistance to cortical rot in alfalfa seedlings. Mew and Howard (1969) studied the effect of hydrogen ion concentration on an observed antagonism between R. japonicum and F. oxysporum (form species unspecified). Rhizobial nodulation was good and root rot was reduced at pH 7.0 and 7.6. At pH 5.2, however, seedlings exhibited severe root rot symptoms, and little or no nodulation occurred. Because of the sensitivity of the genus Rhizobium to acidity and its consequent effects on the observed antagonism, the authors concluded an acid-tolerant strain of R. japonicum could be effective in reduction of soybean root rot. During the progress of this research, Drapeau et al. (1973) published results contradicting the nature of the R. meliloti-Fusarium antagonism as concluded by Johnston (1967). Cell-free extract of the medium (unspecified) on which R. meliloti had grown at 21 C produced inhibition zones when applied to plate cultures of F. melanchlorum (Casp.) Sacc. If the bacterium was incubated at temperatures above 25 C, however, the presence of this substance in the extract

was drastically reduced. The authors judged the Rhizobium antagonism to be antibiotic in nature. F. oxysporum was not tested in these experiments. This report also contained the first published accounts of the Rhizobium-Rhizoctonia combination. R. solani was not inhibited by living cells or cell-free extract of R. meliloti growth medium. Delayed inoculation tests were not performed.

CHAPTER III

MATERIALS AND METHODS

The organisms used in these experiments are listed in Table I. Fusarium species are described according to Snyder and Hansen (1940). Fusarium and Rhizoctonia spp. were maintained on plate cultures of Proteose Peptone (Difco)-dextrose agar (Appendix), and transferred periodically. Rhizobium strains were maintained on slant and plate cultures of Rhizobium X Medium (Appendix) and transferred periodically.

A. Fåhraeus Culture of Alfalfa Seedlings

The Fåhraeus method, published in 1957, consists of the aseptic cultivation of alfalfa seedlings in the presence of Rhizobium on microscope slides which are partially immersed in a nitrogen-deficient mineral salts solution (Figure 1). The seedlings thus obtain their nitrogen supply from symbiotic association with the bacteria. Other microorganisms may be introduced into the tubes.

Seeds of Cherokee variety alfalfa were surface decontaminated by successive immersion in 70% ethanol and a 9:1 dilution of 5.25% sodium-hypochlorite solution for five minutes. This was followed by three changes of sterile water. Several seeds were transferred to sterile plates of Proteose Peptone (Difco)-dextrose agar and allowed to germinate to test for contamination. The remaining seeds were

TABLE I

IDENTITY AND SOURCE OF THE TEST ORGANISMS

Organism	Strain Number	Source
Fusarium oxysporum Snyd. and Hans.	A72-6-SLES-20	Isolations from alfalfa roots
...do...	72-226-S4	...do...
...do...	73-414-1	...do...
...do...	73-365-2	...do...
...do...	A72-6-SLES-5	...do...
...do...	73-365-10	...do...
Rhizoctonia solani Kühn	A72-6-SLES-4	...do...
...do...	73-236-R7	...do...
...do...	A72-6-SLES-23	...do...
...do...	None	Diseased peanut seeds, Perkins, Oklahoma
Rhizobium meliloti Dangeard	ATCC 10312	American Type Culture Collection, Beltsville, MD
...do...	ATCC 10311	...do...
...do...	ATCC 9930	...do...

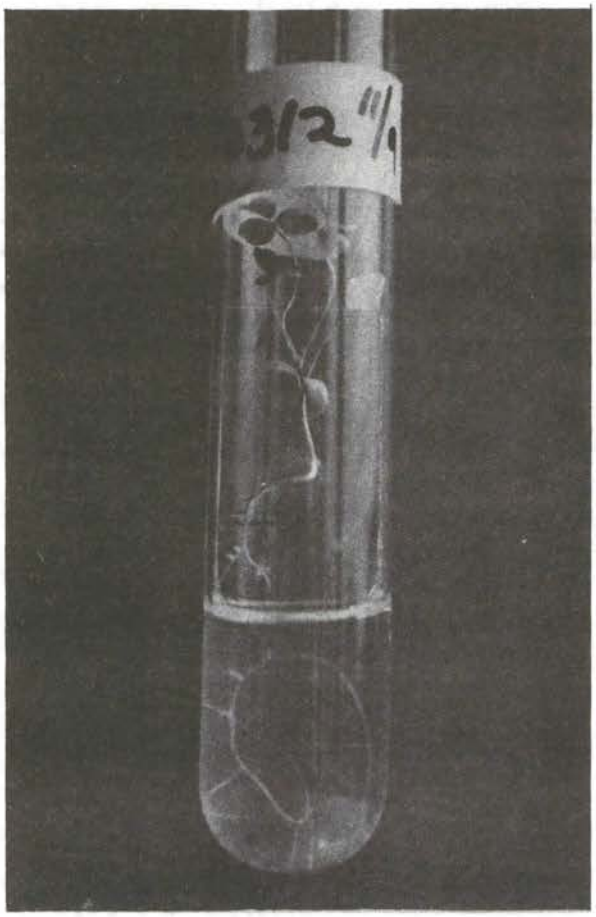


Figure 1. Example Showing Fåhraeus Culture of Alfalfa Seedlings

germinated in sterile plates at room temperature until the roots were approximately two cm long.

Twenty-five ml aliquots of the nitrogen-free mineral solution (Appendix) were dispensed into 30 x 200 mm test tubes, plugged with polyurethane Dispo plugs (Scientific Products), and autoclaved for twenty minutes at 120 C and 15 psi. Microscope slides and cover slips were sterilized in petri plates -- one slide and one cover slip per plate. Approximately 0.2 ml of the nitrogen-free medium containing 0.7% agar was pipetted onto one-half of a slide. A seedling was immediately transferred with sterile forceps to the slide so that its root tip rested in the agar. A cover slip was placed over the agar and root tip with a sterile forceps. The seed coat was removed if it still adhered to the cotyledons, and the slides were then transferred to tubes containing the mineral solution.

Rhizobial inoculation was made by adding two drops of a heavy suspension to the solution in the tubes. In the Fåhraeus experiment comparing Fusarium and Rhizoctonia, rhizobial inoculum was prepared in the following manner: a seven-day old agar plate culture was flooded with sterile distilled water, and the colony was gently scraped with a sterile rubber policeman. The suspension was collected and sterile water added to make 25 ml. Two ml aliquots of this preparation were added to the solution in each tube requiring bacterial treatment. Inoculation of Rhizoctonia was made by transferring sclerotia from ten-day old cultures to the liquid medium in the tubes. Inoculation was made at two rates of one and three sclerotia per tube in the Fåhraeus cultures containing the peanut isolate of Rhizoctonia, and one sclerotium per tube in the remaining cultures containing Rhizoctonia.

Fusarium inoculum was prepared by flooding the surface of a ten-day old plate culture with distilled water, and completing the scraping and diluting as outlined above. The suspension was transferred to a sterile Waring Blendor microcup and macerated for 50 seconds. Two ml aliquots of this preparation were then added to each tube requiring treatment with Fusarium. If only one organism was inoculated in the form of a liquid suspension, two ml of sterile water were added. In those instances where sclerotia only or no inoculum was introduced, four ml of water were added. Thus, each tube was diluted with four ml of water.

The plugged tubes were arranged in cardboard racks at a 30 to 40 degree angle from the vertical, and illuminated for 25 days with a 12 hour diurnal photoperiod at 1500 foot candles. Day and night temperatures were 26.7 C and 21.1 C, respectively. At the end of the incubation period, the slides were taken from the tubes, the excess solution drained off with filter paper, and examined under an American Optical Co. phase microscope. The condition of the rhizosphere and the number of nodules per plant was noted, as well as the outward appearance of the seedlings.

Treatments were made in the following manner: pathogen alone, Rhizobium alone, pathogen plus Rhizobium, and noninoculated controls. In preliminary experiments, the peanut isolate of Rhizoctonia was the most virulent isolate of that species on alfalfa. It was selected for the initial test to determine the antagonistic ability of Rhizobium against this organism, and was inoculated at the rate of three sclerotia per tube. Five replicates of each treatment were used in this experiment. In a following test, the inoculum rate was lowered to one sclerotium, and one tube of each treatment was harvested at 3, 8, and

13 days to observe the development of the pathogen in the rhizosphere. Rhizobium-Rhizoctonia and Rhizobium-Fusarium interactions were compared in a third experiment consisting of three replications which were all examined after 25 days of incubation.

B. Antagonistic Response in Agar Culture

Antagonistic response was measured on Rhizobium X Medium (Appendix) plates poured to a depth of approximately 6 mm. Test methods 1a and 1b as outlined by Johnson et al. (1959), were used for Rhizoctonia. A Rhizobium streak and one sclerotium were placed opposite each other at the periphery of the agar plate. The test pathogen and antagonist were applied the same day, and a delayed inoculation was made in which the test organism was applied two days after the antagonist. Test method 2a as outlined by Johnson et al. (1959), was employed for Fusarium. The Rhizobium was applied as a streak down the center of the plate, and a 4 mm plug of Fusarium was placed approximately 4 cm on either side of the bacterial application. Both organisms were applied the same day. In preliminary tests, morphological changes were observed in the Fusarium mycelia at the perimeter of the bacterial colony. An additional treatment was therefore devised to insure that the bacterial colony was not causing morphological changes in the Fusarium mycelia merely as a physical barrier. Agar was dispensed into bi-petri plates so that the central divider was exposed approximately 2 mm. The Fusarium isolates were placed approximately 4 cm on either side of the protruding divider. All cultures were incubated in a controlled-temperature growth room at 25 C. Lighting conditions consisted of about

eight hours of flourescent illumination per day followed by 16 hours of very low intensity ultraviolet light.

The criteria for antagonism were those of Johnson et al. (1959):

1) zone of inhibition

2) flattening of normally aerial mycelium, or scanty growth on the sides nearest the antagonist

3) die-back or disintegration of hyphae on the fungal test organism

4) parasitism

5) distinct general stunting of test organism colony as compared with control plates; morphological aberrations of test thalli.

Presence or absence of an inhibition zone, and length of persistence of such zones was noted upon periodic examination of two replicates. Appearance of the mycelium was viewed with dissecting and light microscopes at high and low power.

Antibiotic activity of one Rhizobium strain on three F. oxysporum isolates was determined. Three flasks containing 100 ml of Rhizobium X Medium without agar were inoculated with the bacterium, and incubated in the dark at 21, 25, and 27 C in temperature-controlled growth chambers. The flasks were rotated periodically to aerate the cultures. After five days the bacterial broth from each temperature was passed through a Morton fritted-glass filter to remove all contaminating organisms. The agar medium employed in the tests was that of Drapeau et al. (1973), (Appendix).

The surface of all agar plates was seeded with 0.5 ml of a diluted mycelial maceration of the test fungus. The inoculum was prepared in the manner described for Fusarium inoculation of Fåhraeus cultures. Two sterile 12.7 mm filter paper discs were placed on the surface of each

seeded agar plate, and a 0.2 ml sample of undiluted rhizobial culture filtrate was pipetted onto each disc.

Treatments were made in the following manner: fungus alone, fungus plus cell-free filtrate of Rhizobium cultivated at 21 C, fungus plus 25 C filtrate, and fungus plus 28 C filtrate. On those plates containing the fungus alone, 0.2 ml of sterile water was added to the filter paper discs. All plates were incubated in the dark at 21 C, and observations made for three replicates in the manner described for the previous experiments.

CHAPTER IV

RESULTS

A. Fåhraeus Cultures

Rhizobium-Rhizoctonia

Alfalfa plants treated with Rhizobium alone nodulated and appeared healthy 25 days after inoculation. R. meliloti strain ATCC 10311 produced an average of 3.3 nodules per seedling, strain ATCC 9930 produced 2.4 nodules per seedling, and strain ATCC 10312 produced 1.4 nodules per seedling. All plants inoculated with Rhizoctonia were dead after 25 days incubation, and those treated with the pathogen only did not live beyond the cotyledonary stage. Alfalfa seedlings inoculated with Rhizobium in addition to the fungus all produced epicotyls, with spade and first leaves produced on all. There seemed to be no correlation between nodule production of a rhizobial strain alone and its ability to nodulate in the presence of Rhizoctonia. In Fåhraeus tubes inoculated with the pathogen, the average number of nodules ranged from 0.6 to 0.8 per plant. Symptoms induced by Rhizoctonia were: a general browning of the roots and crowns; and dry, brittle leaves and stems above the crown which were covered by mycelial strands of the fungus.

Where Fåhraeus cultures were harvested at intervals to observe colonization of the rhizosphere by Rhizoctonia, mycelia could be

observed traversing the length of the root on fungus-alone treated plants three days after inoculation. Many hyphae aggregated about the root tip; no penetration could be seen, but slight browning of the tip was observed. The same symptoms were seen where the pathogen had colonized the rhizosphere of the Rhizoctonia-R. meliloti strain ATCC 10311 treatment. The pathogen had not reached the rhizospheres of the other fungal-bacterial treatments. Bacterial multiplication was indicated in all tubes inoculated with Rhizobium by characteristic curling of the root hairs. Following eight days incubation there was profuse mycelial growth, penetration of epidermal cells, and distinct browning of the primary root tip where Rhizoctonia alone was used. The fungus had colonized the rhizospheres in tubes of bacteria plus fungus cultures, although not as abundantly as in the previous treatment. Primary root tips appeared slightly necrotic. Root hairs were characteristically curled in all cultures containing Rhizobium, but no nodules were apparent in cultures containing the pathogen. Thirteen days after inoculation the fungus-alone treatment exhibited symptoms much like the dead plants in the previous experiment. All plants in cultures with bacteria plus fungus were living, and had produced spade leaves. Spotted areas of necrosis could be seen along the length of the primary root in all. With the exception of the culture containing Rhizoctonia and R. meliloti strain ATCC 9930, all plants treated with the bacteria possessed at least two nodules. The pathogen grew profusely in all of these rhizospheres.

Rhizobium-Fusarium and Rhizobium-Rhizoctonia

A comparison of the individual effect of both pathogens on longevity, survival, and nodulation is shown in Table II. The effect of both pathogens was approximately the same. Nodulation was reduced in bacteria plus fungus treatments when compared to Rhizobium-only cultures. Plants inoculated with bacteria plus the pathogen lived longer than those treated with the pathogen alone.

B. In Vitro Interactions

The growth of Rhizoctonia isolates 72-236-R7, A72-G-SLES-4, and A72-G-SLES-23 was not affected by the presence of any of the three Rhizobium strains used, both in simultaneous and delayed inoculation. All plates were covered with the fungal mycelium seven days after inoculation.

It was found that the growth of F. oxysporum was inhibited in the presence of R. meliloti, and that the amount of antagonism shown depended on the strain of bacterium present and the fungal isolate used (Table III). Two F. oxysporum isolates had unilateral effects on all three rhizobial strains, but Rhizobium strain ATCC 9930 exhibited little or no antagonistic ability in all but one incident. In several combinations where an inhibition zone was formed, mycelial strands closely appressed to the surface advanced into the open zone after some period of time. Organization of the advancing hyphae took on a wild, unorganized appearance, and some physical bending of individual strands could be seen at the interface of the bacterial colony (Figures 2 and 3). Advancing hyphae of the control plates appeared typically straight

TABLE II

EFFECT OF FUSARIUM OXYSPORUM AND RHIZOCTONIA SOLANI ON LONGEVITY, SURVIVAL, AND NODULATION OF ROOTS OF ALFALFA SEEDLINGS

	Number of Plants* Producing Epicotyls	Number of Plants* Dead After 25 Days	Average Number Nodules/ Plant
Rhizobium meliloti ATCC 10312	3	0	2.0
R. meliloti ATCC 10311	3	0	2.0
Fusarium oxysporum A72-6-SLES-20	1	3	-
Rhizoctonia solani A72-6-SLES-4	0	3	-
F. oxysporum A72-6-SLES-20 + R. meliloti ATCC 10312	3	2	0.6
F. oxysporum A72-6-SLES-20 + R. meliloti ATCC 10311	2	3	1.6
R. solani A72-6-SLES-4 + R. meliloti ATCC 10312	3	1	1.6
R. solani A72-6-SLES-4 + R. meliloti ATCC 10311	3	3	0.3

*From three replicates.

TABLE III

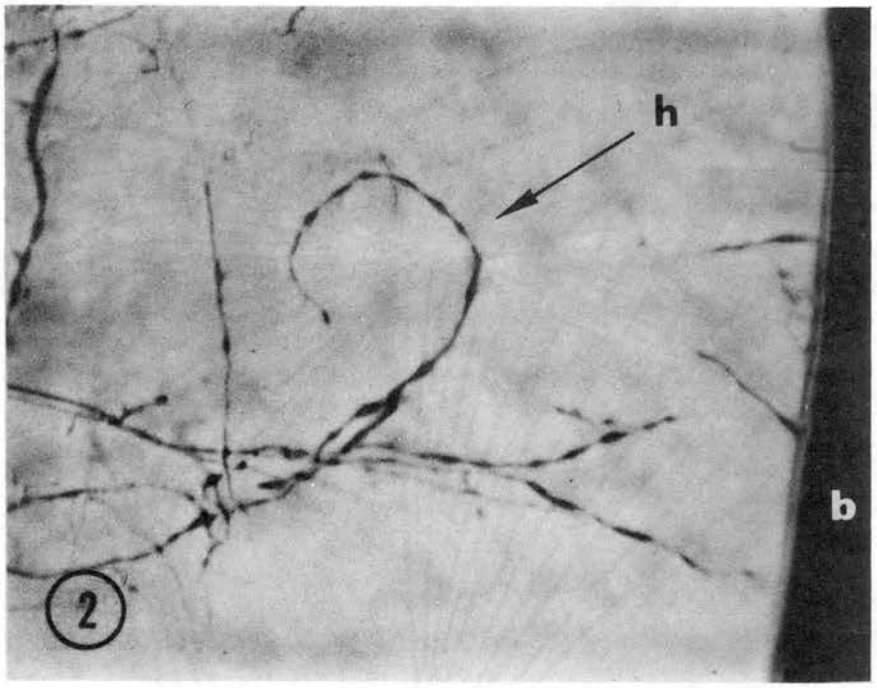
COMPARATIVE ANTAGONISTIC ACTIVITY OF THREE STRAINS OF *R. MELILOTI* AGAINST SIX ISOLATES OF *F. OXYSPORUM*

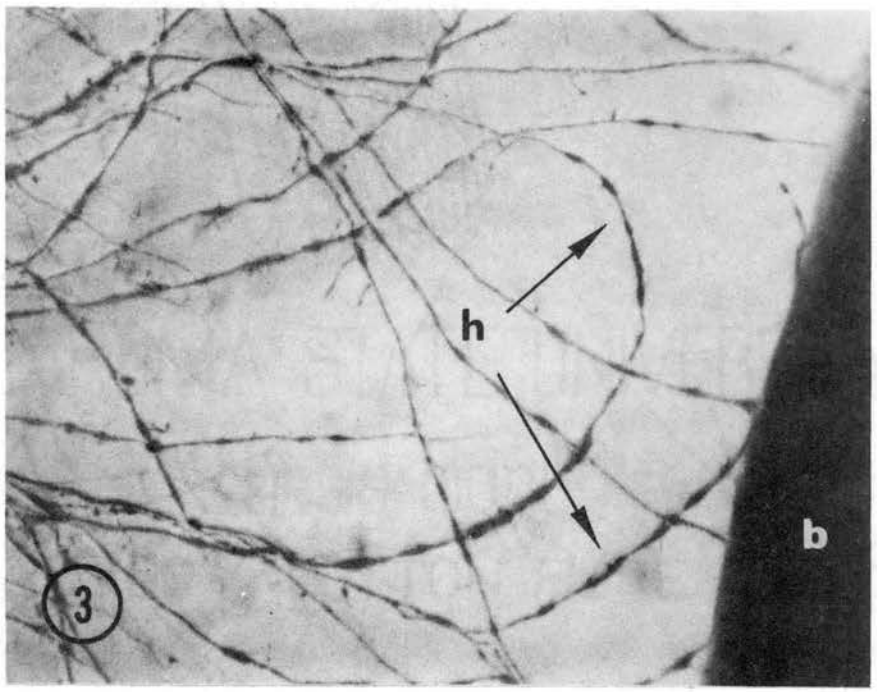
F. oxysporum strain	Antagonistic Activity of *Rhizobium* Strains*		
	ATCC 10311	ATCC 10312	ATCC 9930
72-226-S4	+++	++	-
73-365-2	+++	+++	+++
73-414-1	+	+	+
73-365-10	++	-	-
A72-6-SLES-5	-	-	-
A72-6-SLES-20	+++	++	+

* +++ Inhibition zone remaining wide after 14 days; ++ inhibition zone forming, but colonized by the fungus after a few days; + scanty growth of mycelium on sides nearest bacterial colony but no clear inhibition zone formed; - no antagonism shown.

Figure 2. Photomicrograph of F. oxysporum Isolate 73-365-2 at Interface of Bacterial Colony (b) of R. meliloti Strain ATCC 10312 Showing Hyphal Bending (h). Magnification: 200X.

Figure 3. Photomicrograph of F. oxysporum Isolate A72-G-SLES-20 at Interface of Bacterial Colony (b) of R. meliloti Strain ATCC 10311 Showing Hyphal Bending (h). Magnification: 200X.


h
b
2


h
b
3

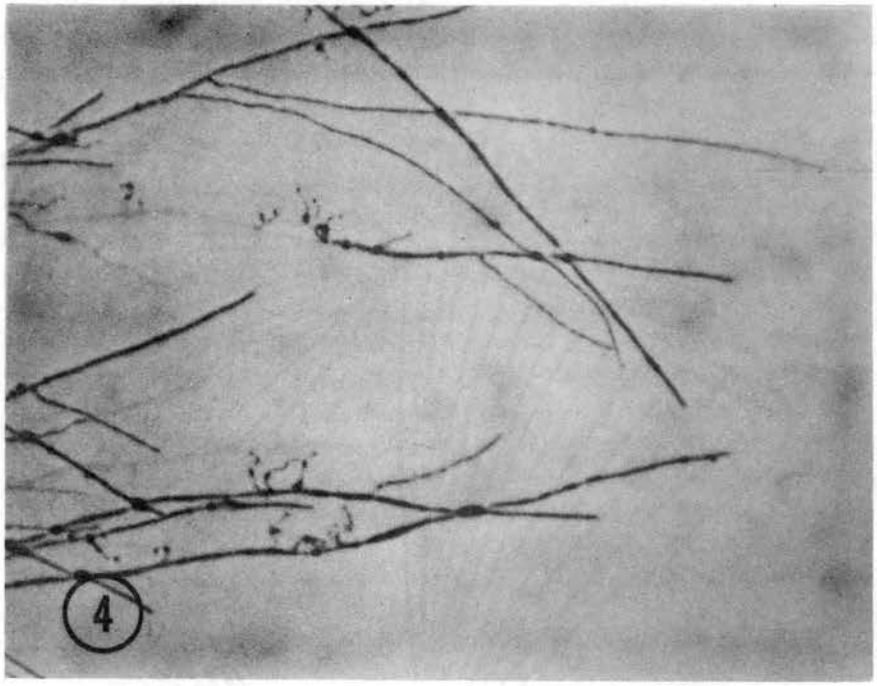
(Figures 4 and 5), and no bending of advancing hyphae could be seen on bi-petri plates in the region of the central divider. F. oxysporum isolate 73-365-2 was inhibited to the greatest extent, and inhibition zones 1.0 to 1.8 cm in diameter could be seen 18 days after inoculation in all rhizobial treatments when compared with the controls (Figures 6, 7, 8, 9, and 10).

The filtrate of cultures of R. meliloti ATCC 10311 had no antibiotic activity against F. oxysporum isolates A72-G-SLES-20, A72-G-SLES-5, and A72-226-S4, regardless of the temperature at which the bacteria were incubated. In the previous experiment, inhibition zones appeared between the colonies of this rhizobial strain and two of the fungi used (Table II). No inhibition zones were observed around the filter paper discs wetted with the filtrate, and all plates were completely covered with mycelium after 14 days of incubation.

The results of a preliminary study to investigate the ability of R. meliloti to quantitatively reduce growth of F. oxysporum in a semi-synthetic liquid medium are summarized in Figure 11. The amount of fungal growth from 7 to 14 days after inoculation was decreased in all of the fungus plus bacteria combinations, while growth increased in all fungus alone controls. F. oxysporum isolate A72-226-S4 alone produced the greatest yield and the lowest yield when grown in combination with Rhizobium during the 7 to 14 day period. No patterns could be seen relating pH concentration of cultures at 14 and 21 days to type of treatment.

Figure 4. Photomicrograph of Advancing Hyphae of F. oxysporum Isolate 73-365-2 on Control Plates. Magnification: 100X.

Figure 5. Photomicrograph of Advancing Hyphae of F. oxysporum Isolate A72-G-SLES-20 on Control Plates. Magnification: 100X.


4

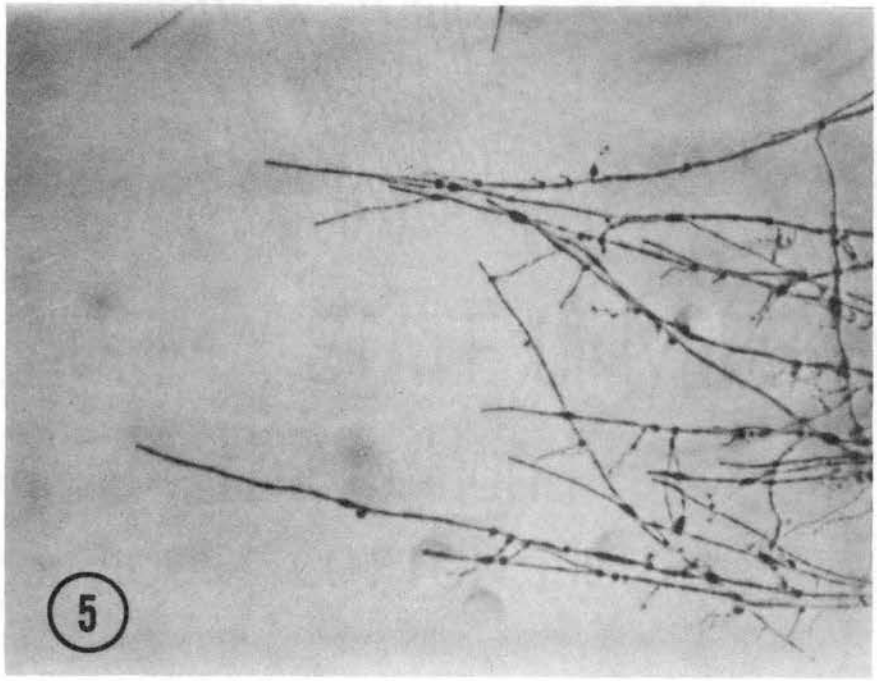

5

Figure 6. Inhibition of Growth of F. oxysporum Isolate 73-365-2 by R. meliloti ATCC 10312

Figure 7. Growth of F. oxysporum Isolate 73-365-2 on Control Plates

Figure 8. Inhibition of Growth of F. oxysporum Isolate 73-365-2 by R. meliloti ATCC 10311

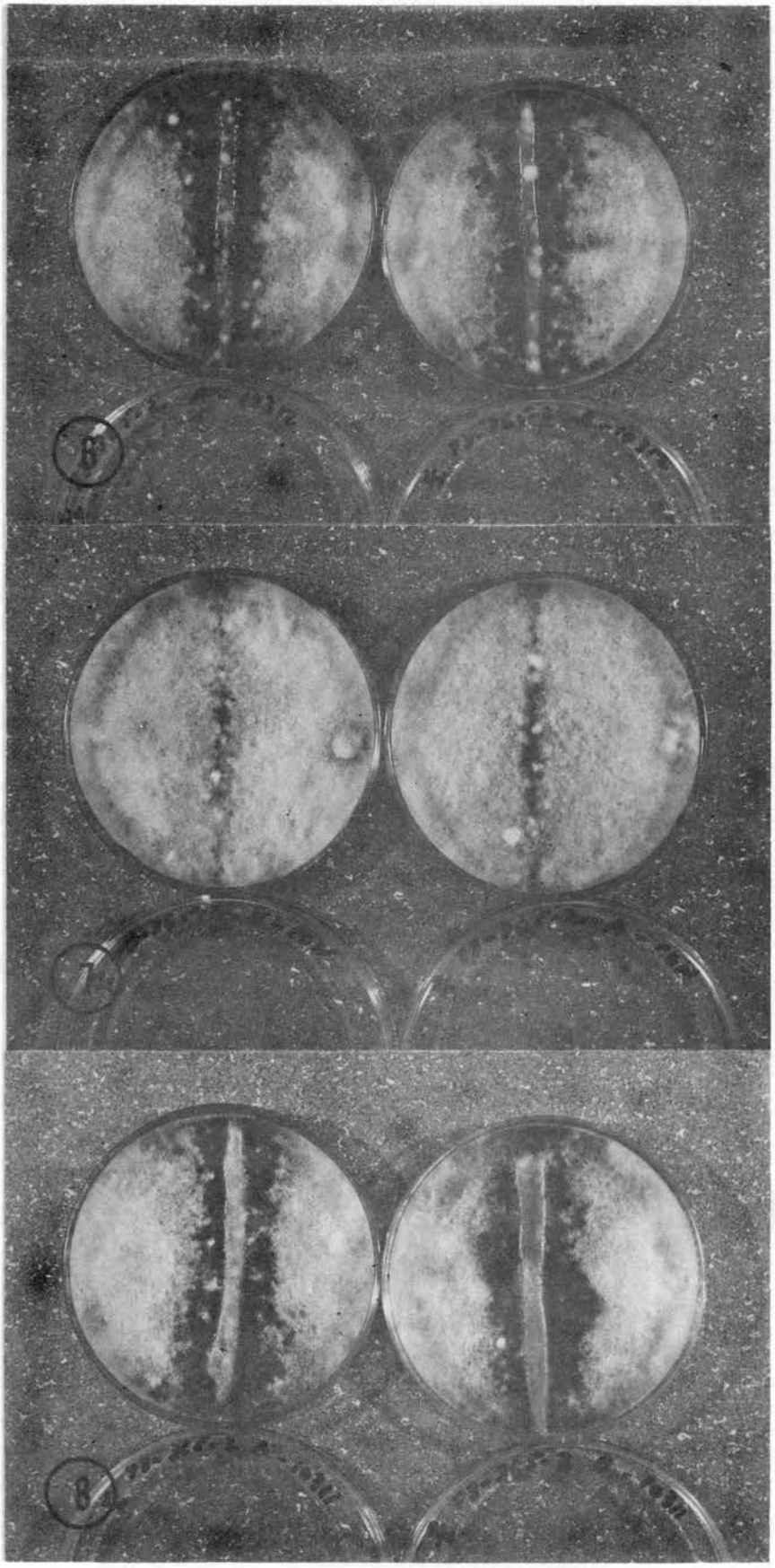
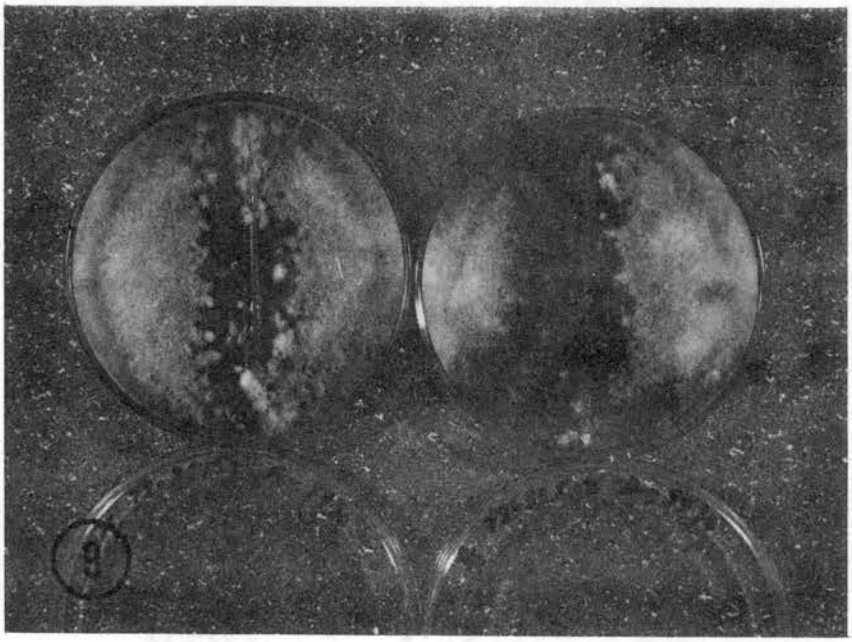


Figure 9. Inhibition of Growth of *F. oxysporum* Isolate 73-365-2 by *R. meliloti* ATCC 9930

Figure 10. Growth of *F. oxysporum* Isolate 73-365-2 on Bi-Petri "Barrier" Plate


9

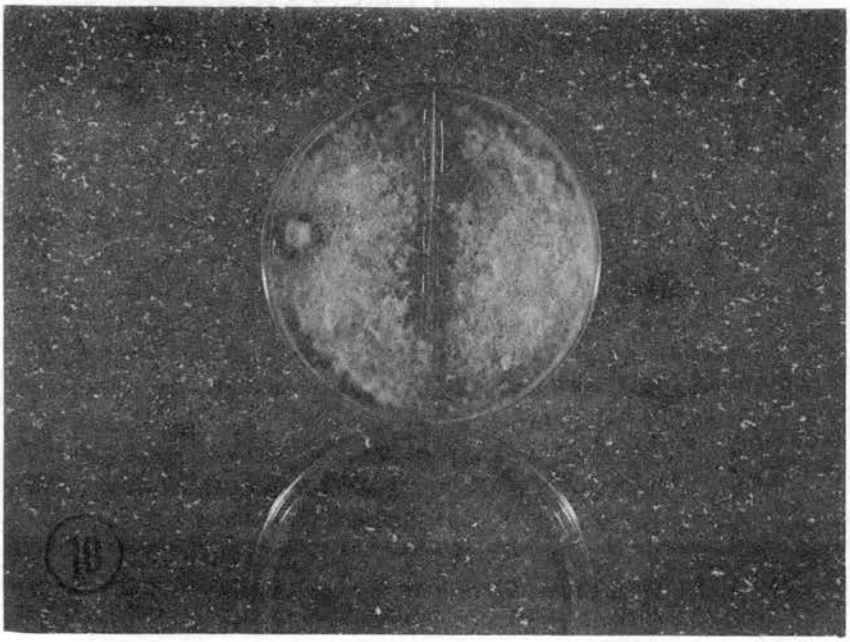
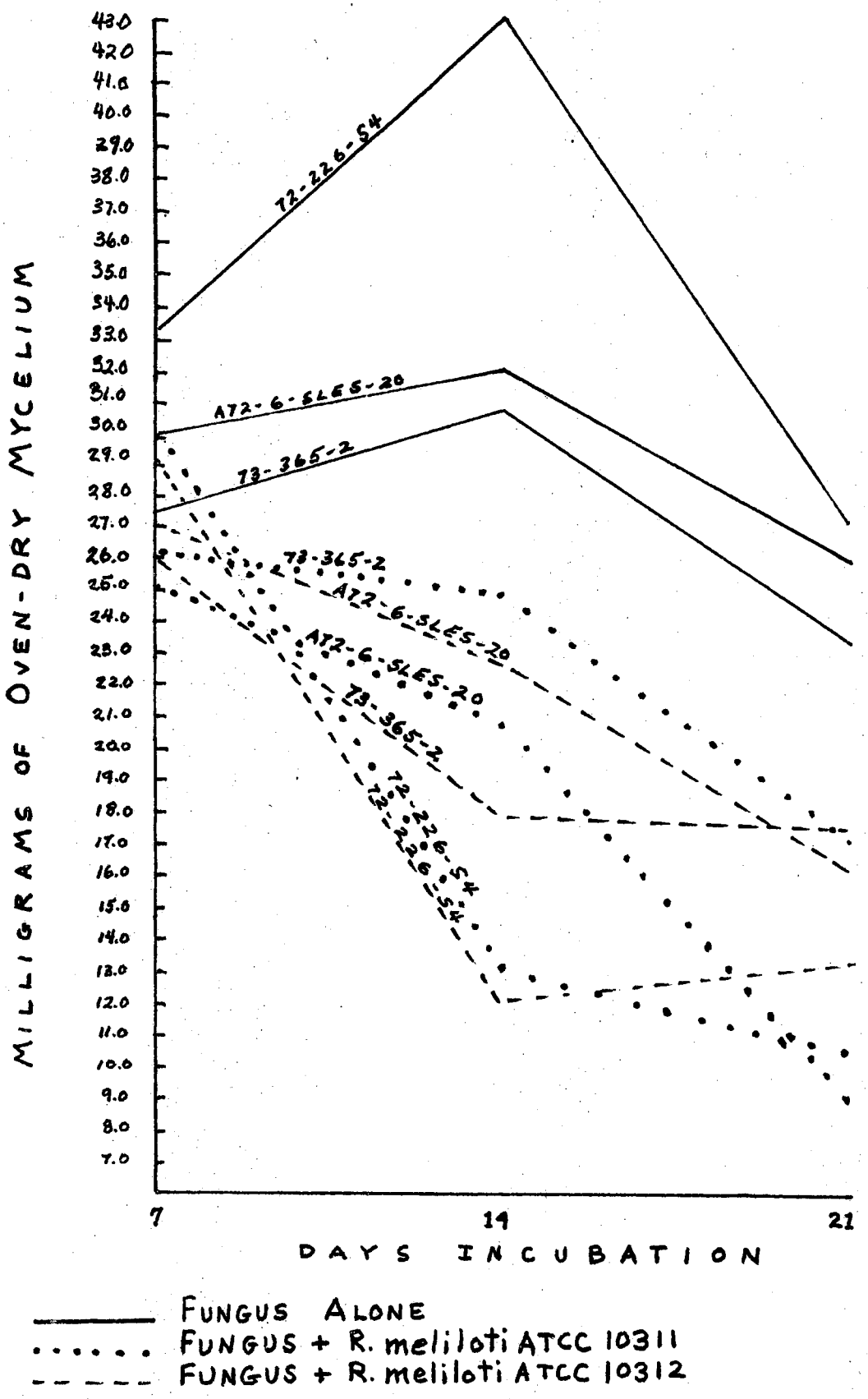

10

Figure 11. Growth of Three Isolates of F. oxysporum Alone and in Combination with Two Strains of R. meliloti in an Unaerated Liquid Medium at an Initial pH of 6.9


MILLIGRAMS OF OVEN-DRY MYCELIUM
43.0
42.0
41.0
40.0
39.0
38.0
37.0
36.0
35.0
34.0
33.0
32.0
31.0
30.0
29.0
28.0
27.0
26.0
25.0
24.0
23.0
22.0
21.0
20.0
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
72-226-54
AT2-6-5L65-20
73-365-2
73-365-2
AT2-6-5L65-20
AT2-6-5L65-20
73-365-2
72-226-54
72-226-54
7
14
21
DAYS INCUBATION
FUNGUS ALONE
FUNGUS + R. meliloti ATCC 10311
FUNGUS + R. meliloti ATCC 10312

CHAPTER V

DISCUSSION

Rhizobium meliloti exhibited some ability to retard development of Rhizoctonia solani in Fåhraeus culture. Plants inoculated with R. meliloti in addition to Rhizoctonia consistently lived longer than those plants treated with the fungus alone, and colonization of the rhizosphere of pathogen plus bacteria treatments was slower than in those cultures containing the fungus alone. Because the arrival of Rhizoctonia mycelium at the rhizosphere was delayed in these treatments, it seems to indicate the bacteria interfere in growth of the pathogen through the medium itself, rather than by formation of the root sheath and consequent colonization of the infection court. Perhaps with higher inoculum levels of Rhizobium this effect could be lengthened. However, if R. meliloti may retard disease development of Rhizoctonia root rot, it cannot afford the degree of protection against Fusarium that was demonstrated by Johnston (1967), due to the growth potential of Rhizoctonia. Tests on agar media tend to select mainly for antibiosis. Here competition for resources is minimal (Baker and Cook, 1974), and the fast-growing, saprophytic properties of Rhizoctonia would obviously benefit the organism -- even though the medium highly favored the bacterium. The same situation may be present in natural soils, and further experiments investigating this interaction in soil would be beneficial. The failure to produce some indication of this "delaying"

capability on agar media points to both the discrepancies so often found in the use of this technique as the sole determiner of antagonism and the need for liberal use of different techniques in biocontrol studies.

Nodulation of alfalfa seedlings was reduced in the presence of Rhizoctonia. The same effect has been demonstrated for Fusarium spp. by Johnston (1967). The main influence of these pathogens on this process is the necrotization of epidermal cells and the consequent loss of "infection" sites for the bacteria. The failure to show a stronger protective effect in Fåhraeus cultures with F. oxysporum and R. meliloti was most likely caused by severely high levels of pathogen inoculum. Johnston (1967) observed surface lesions beginning to form on fungal controls ten days after inoculation, while plants in my experiments necrosed severely and were dead after six days.

The results presented here for antagonistic activity in vitro of three R. meliloti strains in combination with F. oxysporum do not support the findings of Johnston (1967). Using two bacterial strains, he concluded that interactions between F. oxysporum and R. meliloti were uniform and not dependent on the strain of bacterium present. However, it was found that different strains of R. meliloti showed varying degrees of antagonistic activity toward the same fungal species. There also appears to be considerable variation within different isolates of the same fungal species regarding sensitivity to rhizobial presence. This should make the search for an effective rhizobial strain to utilize in reduction of disease caused by F. oxysporum more difficult.

The negative results obtained with cell-free filtrate of R. meliloti growth medium on F. oxysporum apparently discounts antibiosis

as the mechanism responsible for this antagonism. The incubation time allowed for production of any antibiotic substances was as long as the time required to establish a zone of inhibition on plate culture. The view that inhibition of Fusarium is mainly a competitive process is prominent in the literature. Finstein and Alexander (1962) obtained effective suppression of F. oxysporum f. cubense in sterile soil with 19 non-antibiotic Bacillus spp. isolates. Competition for carbon was more intense than competition for nitrogen. Johnston (1967) reported that higher levels of glucose lessened antagonism between F. oxysporum and R. meliloti in vitro. The experiments reported by Drapeau et al. (1973), previously discussed here, do not include any of the common pathogenic species of Fusarium. However, little research has been done on antibiotic production by Rhizobium. Such activity between Rhizobium species (Schwinghamer and Bilkengren, 1968) and by Rhizobium on other bacteria (Roslycky, 1967) has been reported, but the investigation by Drapeau et al. (1973), was the first publication dealing with antibiotic inhibition by these bacteria on fungi. Different strains of bacterial species vary in their antibiotic capabilities, and possibly the use of other R. meliloti strains would result in the discovery of an antibiotic strain effective against F. oxysporum. R. meliloti may effect antagonism by both mechanisms, but only slightly so by antibiosis. This slight antibiotic effect could have been the cause of the morphological aberrations observed in advancing fusarial hyphae at the interface of some rhizobial colonies. Johnston (1967) found that advancing F. roseum hyphae ceased growth completely at the surface of R. meliloti colonies.

Utilizing liquid medium cultures to determine differences in growth between fungi grown alone and in combination with Rhizobium seems to be a good method of quantitatively analyzing the interaction. This kind of analysis cannot be done in soil, sand, or agar culture. Following preliminary presumptive tests for antagonism and ability to grow in liquid culture, further experiments could be done with varying medium component concentrations, etc., using this method.

CHAPTER VI

SUMMARY

The results of investigations on the interaction of Rhizobium meliloti strains and several isolates of two genera of fungi associated with root and crown rots of alfalfa indicate the following:

1. Rhizobium meliloti exhibited an ability to retard development of Rhizoctonia in hydroponic slide culture.

2. Nodulation of alfalfa seedlings was reduced in the presence of Rhizoctonia.

3. The growth of three Rhizoctonia isolates on agar culture was not affected by the presence of any R. meliloti strain used.

4. The growth of Fusarium was inhibited by the presence of R. meliloti in vitro, and different strains of R. meliloti showed varying degrees of antagonistic activity toward F. oxysporum.

5. Different isolates of a Fusarium species may have diverse reactions to rhizobial presence, ranging from no inhibition to severe inhibition.

6. Cell-free filtrate of R. meliloti growth medium produced no inhibition on three F. oxysporum isolates, regardless of the temperature at which the bacteria were incubated.

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APPENDIX

MEDIA USED

Proteose Peptone-Dextrose Agar

Proteose-Peptone #2 (Difco)	10g
Dextrose	20g
Agar	17g
Distilled H_2O to make	1000ml
Final pH	6.7-6.9

Nitrogen-free Mineral Salts Solution[a]

$CaCl_2 \cdot 2H_2O$	0.132g
$MgSO_4 \cdot 7H_2O$	0.120g
KH_2PO_4	0.10g
$Na_2HPO_4 \cdot 7H_2O$	0.150g
Ferric citrate	5.0mg
$MnSO_4 \cdot 4H_2O$	3.50mg
$CuSO_4 \cdot 5H_2O$	0.03mg
$ZnSO_4 \cdot 7H_2O$	1.42mg
Boric acid	0.50mg
Molybdic anhydride	0.0001mg
$CoCl_2$	0.05mg
$CaCO_3$	0.5g
Distilled H_2O to make	1000ml
pH after autoclaving	6.5-6.9

Rhizobium X Medium[b]

Yeast extract	1.0g
Soil extract	200ml
Mannitol	10g
Agar	15g
Distilled H_2O	800ml
Final pH	7.2

Drapeau et al. (1973) Agar Medium[c]

Dextrose	10g
K_2HPO_4	0.5g
$MgSO_4 \cdot 7H_2O$	0.2g
NaCl	0.2g
$CaCO_3$	0.2g
Yeast extract	5.0g
Agar	15g
Distilled H_2O to make	1000ml
inal pH	6.5

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[a]Preparation directions may be found in Fåhraeus (1957).

[b]Preparation directions may be found in ATCC Catalogue of Strains, Ninth Ed., 1970, page 167 (Medium 111).

[c]Preparation directions may be found in Drapeau et al. (1973).

VITA

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