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Isolation from Soil Microorganisms That Are Inhibitory to Wheat Seeding Pathogens

Kynita Wilson-Humphrey

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"Isolation from Soil of Microorganisms That Are Inhibitory to Wheat Seedling Pathogens"

Kynita D. Wilson-Humphrey

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ISOLATION FROM SOIL OF MICROORGANISMS THAT ARE INHIBITORY TO WHEAT SEEDLING PATHOGENS

Submitted by

Kynita Deaunce Wilson-Humphrey

Chemistry/Biology Major

Department of Physical Science and Biology

School of Arts and Sciences

Langston University

Langston, Oklahoma

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Thesis Approved:

Thesis Committee Chairperson

Thesis Committee Member

Thesis Committee Member

Director of Honors Program

Vice President of Academic Affairs

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CHAPTER I

INTRODUCTION

Many diseases caused by bacteria, viruses, or fungi are responsible for the ruin of a great number of crops annually. At present, many of these crops are being treated for diseases by synthetic or man-made chemicals. Some of the chemicals have been proved to have adverse effects on the environment, including humans and animals.

Very few non-chemical means are available to farmers at this time to control these pathogens. This is due to the fact that many of the means of control have little effect against the pathogens or diseases.

This research attempts to isolate microorganisms from the soil which in turn will be tested against a particular fungi for inhibition of the fungal growth. It is hoped that these microorganisms will open pathways leading to the discovery of new non-chemical toxins which could be used against plant pathogens. As a result of the use of non-chemical toxins, many crops could be saved from destruction each year. It is also hoped that these non-chemical toxins will be safer for the environment, are target-specific for a particular pathogen, and are easily

biodegradable, thus reducing long-term effects.

Statement of Problem

There are many different types of good and bad microorganisms. The microorganism actinomycete, which is commonly isolated from soil and water, can be pathogenic or indigenous to man and animals or pathogenic or symbiont to plants. By isolating those that are symbiont in plant root nodules and testing them against a particular fungi for inhibition of the fungal growth, one may find a new non-chemical or natural toxin which can be used to treat wheat and other field crops against certain diseases or pathogens.

Purpose of Study

The methodology employed in this research is based on general microbiology techniques. This research attempts to isolate actinomycetes which are inhibitory to wheat seedling pathogens. It also attempts to discover a toxin which can later be identified and used as a basis for other non-chemical means of destroying crop pathogens.

Assumptions

During this research it was assumed that the procedures used were going to work.

Limitations

One limitation which involves the soil samples is that the soil used is limited only to wheat rhizosphere, alfalfa rhizosphere, and non-rhizosphere soil samples. The time factor was the another limitation because it allowed for testing only a small number of samples. Trying to duplicate exact environmental conditions was a limitation. And last, research was limited only to laboratory conditions; therefore, duplicating physical and chemical conditions of the environment was a limitation.

Outline of Thesis

The remainder of the thesis includes the following chapters: Chapter II, Review of the Literature; Chapter III, Methodology; Chapter IV, Presentation of Findings, and Chapter V, Conclusion.

CHAPTER II

REVIEW OF THE LITERATURE

Today few nonchemical plant pathogen control agents with high success rates are available to farmers. Synthetic chemicals, on the other hand, have proved highly effective in controlling diseases and are cost effective. The federal government employs regulatory measures and provides funding for nonchemical plant pathogen controls to minimize the risk associated with chemical fungicides. Nonchemical methods cater to single species of pathogens, making the complete replacement of synthetic chemicals difficult. New products introduced can reduce the level of fungicide use. In CHEMTECH, Leonard Gianessi suggests cross-breeding as an alternative to chemicals (1993).

The Congressional Quarterly Weekly Report states that neither environmentalists nor farmers are satisfied with President Bill Clinton's attempt to secure a compromise policy for pesticides used on food products. The proposal released April 26, 1994, amends the Federal Food, Drug and Cosmetic Act and Federal Insecticide, Fungicide and Rodenticide Act. The Environmental Protection Agency is authorized under the proposal to require hazardous farm chemicals to be phased out but can offer a five-year waiver. The proposal removes the economic analysis requirement from pesticide regulation, basing it instead only on health standards (Benenson 1994).

Humans as well as crops are affected by synthetic chemicals in various ways. For example, synthetic chemicals affect the amount and quality of food people consume and, in some cases, the water they drink. An article in the Journal of Soil and Water Conservation states that government policies on ground water quality should address both the geographic diversity and relative vulnerability of the potential for ground water contamination due to agrichemical leaching (Kellog 1994).

In still other ways chemicals affect the environment. In the book Silent Spring, the author predicts that the extinction of many bird species will come about with the use of certain agricultural chemicals. This prophecy never came to pass, however, because her warnings were heeded (Easterbrook 1994). The indiscriminate use of synthetic chemicals that have endangered wildlife, caused cancer in humans, and developed resistance in pests has been reduced dramatically. A report by the National Academy of Sciences emphasizes the harmful effects on children of pesticide residues on vegetables and fruits. Studies are now in progress to eliminate these problems.

Diseases of many economically important plants are caused by bacteria, viruses, or fungi. These organisms contribute substantially to a great percentage of crops worldwide that are lost to disease. Almost all kinds of plants can be affected by bacterial, viral, or fungal diseases, and many of these diseases are extremely destructive.

Synthetic chemicals such as benomyl, captan, carboxin, and diflolatan are

used today to inhibit the growth and spread of diseases. With their continued use, however, most of the chemicals have harmed, or are believed to have caused harm to, the environment, including animals and humans. Research is currently in progress to find other nonchemical means of destroying these plant pathogens, which in turn would be less harmful to the environment.

Today's pesticides are ninety-nine percent synthetic and are not easily biodegradable; therefore, they accumulate in the environment and ultimately end up leaving their effects for longer periods of time. These synthetic pesticides do not target specific organisms, thus leaving their effects on other organisms. Researchers are trying to get away from synthetic pesticides. They are now looking for target specific and biodegradable pesticides which will kill what they are supposed to without harming the environment. Pesticides should be biodegradable and unharmful to other organisms. They should be natural and developed by the environment, which would possibly allow them to be used in small quantities with the residual effect being almost negligible.

Few nonchemical means of controlling plant pathogens are easily available and effective. This research attempts to isolate microorganisms from the soil and use them as a nonchemical means of controlling plant diseases of wheat and other horticulture and field crops.

The research for this study follows standard and tested procedures for isolation of microorganisms. The purpose is to find microorganisms in the soil, which acts as a fungicidal compound. It is assumed that through following given

techniques and procedures for isolating and culturing these actinomycetes, organisms will be found that are inhibitory to fungicidal growth.

Actinomycetes are bacteria that tend to form branching filaments, which in some families develop into a mycelium. These filaments may be extremely short, as in the Mycobacteriaceae and Actinomycetaceae, or well developed, as in Streptomycetacae (Buchanan & Gibbons 1974). The diameter varies between 0.5-2.0 micrometers, generally less than one micrometer. These filaments are not always observed because in certain families the filaments tend to fragment and can be seen only in some cultural stages of development or in host tissue (Buchanan & Gibbons 1974). They are sometimes rare as in Mycobacterium. Fragmentation of the filaments leads to the formation of coccoid, elongated or diphtheroid elements. In some families the spores are formed on aerial and on substrate hyphae. Spores may be produced singly on hyphae, as pairs or as chains of various numbers of spores. If spores are in sufficient numbers, the chains can be straight, looped, or spiral. Such chains arise singly on hyphae or in a verticillate manner. In the actinoplanceae, spores are born in sporangium and are motile or non-motile, depending on genus (Buchanan & Gibbons 1974).

The actinomycetales are commonly isolated from the soil and less commonly from water (Walksman 1967). Forms pathogenic to man and animal exist in at least four families. Spores can also be allergenic to man. Some species are pathogenic to plants; others are obligate symbiont in plant root nodules and fix nitrogen (Walksman 1967).

Actinomycetes are indigenous to man. The anaerobic form, Actinomyces israelii, are found in the mouth, pharynx, intestine and actinomycotic lesions (Walksman 1967). These are considered parasites of man and animals.

Smooth culture of Actinomyces bovis found in cattle are frequently distinguished from Actinomyces israelii found in man. Actinomycetes are also found in numerous other organs and excreta of human and lower animal body, such as excreta of suckling animals and the intestinal canal of healthy animals (Walksman 1967).

Occurrence of Actinomycetes in organs of certain insects is also of interest to many. A culture of Nocardia rhodnii was isolated from the reduvid bug Rhodinus prolixus by the researcher Erickson (Walksman 1967). The organism is taken up by young nymph from contaminated surface of the egg or transmitted to insects by its dry excreta. When the surface of an egg is sterilized or when the adult is fed with suitable precautions, sterile insects are produced. They grow and moult normally to a point but usually are incapable of reproduction. When the insects are reinoculated with the Actinomycetal cultures, normal growth, molting, and egg production result (Walksman 1967).

Isolation and identification of actinomycetes can be done by various methods. The actinomycetes can be isolated from soils and other natural substrates by first plating out materials in proper dilutions on suitable media followed by incubation at twenty-eight to thirty degrees Celsius for two to seven days. Actinomycetal colonies are picked and transferred to sterile liquid or solid media for further growth. If

obtained cultures are contaminated with other organisms, they may have to be plated again in order to gain pure cultures.

Colonies of actinomycetes can be delineated from fungi and true bacteria. They are compact, often leathery, giving a conical appearance, have a dry surface and are frequently covered with aerial mycelium (International 1966). If the colony is well developed and aerial mycelium is abundant, surface spores can easily be picked with a sterile needle. If growth is limited or the aerial mycelium is not fully developed, sharp razor-like needles are required to transfer a part of the growth to fresh media.

Most actenomycetes are aerobic; few are obligate anaerobic, although some are microaerophilic (Skinner & Sykes 1973). To supply them with proper aeration the organisms are grown on the surface of solid media, in shallow liquid layers, or in a thoroughly aerated submerged condition.

There are many types of media which can be used for growing actinomycetes. Some media contain glucose, glycerol, or starch as a source of carbon; nitrate, casein, N_2; and certain minerals, namely NaCl, K_2HPO_4, $MgSO_4 . 7H_2O$, $CaCO_3$ and $FeSO_4$ are found to give the largest number of actinomycetes and to induce the development of most characteristic properties of the cultures (Skinner & Sykes 1973). Antibiotics such as Nystatin and Cycloheximide with the ability to inhibit the growth of fungi without affecting that of actinomycetes are frequently added to plating medium.

CHAPTER III

METHODOLOGY

Basic general microbiology techniques were employed in doing this study. The methodology for the following procedures was obtained from general microbiology lab manuals and from published research articles. Components for media were obtained from previously recorded data on the topic.

Before beginning the research, it was necessary for the researcher to become acquainted with the laboratory, the equipment, and the required procedures which would be used throughout the research. Initially different techniques were employed in order to determine the best method for procuring the most sterilized or uncontaminated results. Procedures were as follows:

1. *Pipeting- Pipeting was used to transfer solutions during the dilution techniques. This procedure required aspiration of the solution into the pipet, then total evacuation. This allowed the inside of the pipet to become saturated. As a result of the saturation, when it was time to transfer the solution to a different solution, all or most of the proper amount of solution would transfer to the next solution.*
2. *Pouring plates- This procedure was done using a florence flask and*

plastic petri dishes or plates. The plates were used to grow the microorganisms and the fungi. Care was used when pouring the plates to minimize contamination: (1) the neck of the flask was sterilized with seventy to seventy-five percent ethanol, then seared with a flame; (2) the flask always remained tilted while pouring instead of vertical; (3) the lid of the petri dish was opened only slightly to pour the medium into the plate. The medium was allowed to set, then placed in a refrigerator to minimize dry-out.

3. *Dilutions- A variety of bottles and tubes with different amounts of solutions or sterilized water and pipets of different volumes were employed. Pipeting techniques were used to obtain the various amounts of solutions, diluting the soil samples into various soil to solution ratios. Example, ten grams of soil were added to a ninety (90) milliliter Ringer's solution and shaken vigorously by a shaker; then the solution was allowed to settle. One milliliter sample of this solution was placed in another bottle filled with ninety-nine (99) milliliters of sterilized water. The first solution, which was 1:10 fold or 10^{-1}, was diluted to 1:1000 fold or 10^{-3}. The dilution procedure was necessary to accelerate the growth of the actinomycetes.*
4. *Sampling- Target soil for the first dilution was obtained from the wheat rhizosphere. This soil was located in a wheat field on Highway 33 a few miles from Langston University. The soil was shaken from the root*

nodules and dried. After drying, it was sieved to remove any grass, rocks or other unwanted material. The soil was weighed and the pH was measured. The pH was obtained by preparing a paste consisting of five grams of the soil and five milliliters of distilled water and boiled water. The pH rod was placed in the paste for approximately ten to fifteen minutes or until the pH meter became stable.

Ringer's solution was needed for complete suspension of soil. This allowed for an accurate dilution. Preparation of the Ringer's solution was completed before the dilutions took place. The Ringer's solution is an isotonic solution consisting of the components listed in table 3.1:

RINGER'S SOLUTION

NaCl	*0.54g*
KCl	*0.02g*
$CaCl_2$ (anhydrous)	*0.03g*
$Na_2S_2O_3 \cdot 5H_2O$	*0.12g*
Distilled H_2O	*1000ml*

Table 3.1

The listed ingredients were added and allowed to dissolve. In the dilution preparation of the wheat rhizosphere solution (WR), five (5) ninety milliliter bottles of Ringer's solution, five (5) ninety-nine milliliter bottles of distilled water, fifteen (15) nine-milliliter tubes of distilled water, and thirty (30) nine-milliliter tubes of water agar were prepared and sterilized. The water agar was prepared by using

eight grams of agar per liter of water, letting it boil and then allowing it to cool enough to be syringed into the tubes. All containers (bottles and tubes) were sterilized a day prior to dilution in an autoclave at 121 °C at 15 atmospheres pressure for fifteen minutes.

Thirty plates each of Glucose-Asparagine-Phosphate (GAP) and Oatmeal (O) agar were prepared to use for plating the dilutions. Each of the media solutions was treated with Actidone (Cyclohexamide) an antifungal agent and Nystatin (Mycostatin). These antibiotics were used to eliminate fungal and bacterial growth that could develop during the dilution series. The checklist and procedure followed before beginning each dilution series are listed in Table 3.2:

DILUTION SERIES CHECKLIST

1. *Ten grams of soil were placed in ninety milliliters of Ringer's solution.*
2. *Soil mixture was shaken for twenty to thirty minutes.*
3. *Water agar tubes were placed in autoclave to melt (approximately fifteen minutes).*
4. *With lysol, the table was wiped down, then the bunsen burner was lit.*
5. *All agar plates, tubes, and water bottles were carefully labelled correctly and in order.*

6. *When autoclave was finished, water agar tubes were placed in warm water bath at 42-45 °C. To prevent solidification, the water level was monitored during the course of the procedure. (Agar will not solidify if kept at this temperature.)*
7. *Pipets were seared for sterilization.*

Table 3.2

The dilution used for the wheat rhizosphere is listed in Table 3.3:

Dilution Mixture	***Dilution Ratio***
A. 10g soil in 90ml Ringer's solution	*1:10*
B. 1ml of A in 99ml water	*1:1000*
C. 1ml of B in 9ml water	*1:10000*
D. 1ml of C in 9ml water	*1:100000*
E. 1ml of D in 9ml water	*1:1000000*

Table 3.3

Ten grams of soil were placed in the Ringer's solution and then placed on a shaker for twenty to thirty minutes. This mixture was then allowed to settle for two to three minutes. One milliliter of the supernatant was then transferred to a ninety-nine milliliter bottle of distilled and sterilized water and shaken vigorously by hand for one minute. This one hundred-milliliter mixture was a 1:1000-fold dilution mixture. One milliliter of this second mixture was transferred to each of nine

separate tubes containing nine milliliters of distilled water to produce 1:100000 fold and additionally to three (3) separate tubes each containing nine milliliter of water agar 1:10000 diluted. Then the twelve mixtures with each of the water agar tube's contents were then transferred to three different agar plates containing Glucose-Asparagine Phosphate media and to the three plates of Oatmeal media (Table 3.4). The above dilution steps for the 1:10000 fold dilution mixture were repeated to obtain 1:100000 and 1:1000000 fold dilution mixtures.

GLUCOSE-ASPARAGINE-PHOSPHATE MEDIUM

Agar	*15.0g*
K_2PO_4	*0.5g*
Glucose	*10.0g*
Asparagine	*0.5g*
Water	*1L*

OATMEAL AGAR

Oatmeal	*20.0g*
Trace sol'n (1mL/L)	*1mL*
Soil Extract	*100mL*
1N NaOH	*0.5mL*
	plus 12 drops
Water	*up to 1L*

Table 3.4

The prepared Glucose-Asparagine-Phosphate plates were placed in an incubator at twenty-eight to thirty degrees Celsius for seven to twelve days. After the twelfth day of incubation, using the Quebec colony counter, the Glucose-Asparagine-

Phosphate and Oatmeal agar plates were counted. Actinomycetes were distinguished and counted. The second and final count for the actinomycetes was taken on the sixteenth day using the same instrument and the results were recorded.

The above procedure was used for the alfalfa rhizosphere and the non-rhizosphere. The differences for each soil were basically the location from which they were obtained and the dilution beginning amount. The alfalfa rhizosphere (AR) was obtained along Highway 33 west of Langston University from an alfalfa field. The non-rhizosphere was obtained from the same field as the wheat rhizosphere. This soil was not taken from the root nodules of any plant. The dilutions used for the alfalfa rhizosphere are listed in Table 3.5:

Dilution mixture	***Dilution ratio***
A. 5g soil in 95ml Ringer's solution	*1:20*
B. 10ml from 1:20 into 90ml water	*1:200*
C. 2ml from 1:200 into 8ml water	*1:1000*
D. 1ml from 1:1000 in 9ml water	*1:10000*
E. 1ml from 1:10000 in 9ml water	*1:100000*
F. 1ml from 1:100000 in 9ml water	*1:1000000*

Table 3.5

The dilution was the same for the non-rhizosphere as for the wheat rhizosphere.

The first count for the alfalfa rhizosphere was taken on day eight. For the non-rhizosphere it was taken on day eleven. The second count for the alfalfa rhizosphere was taken on day thirteen and for the non-rhizosphere it was taken on

day eighteen.

After the incubation and growth period of 7-12 days, each plate was observed to see if any actinomycetes could be isolated and transferred to other plates for purification. Additional transfers would have been employed if further purification had been needed or if any contamination had occurred. After pure cultures were obtained, the pure actinomycetes were then transferred to new agar plates and also into agar slants (tubes with agar set at a slant). The agar slants would be used for storing actinomycetes for future use.

From the above agar plates, five from each rhizosphere were chosen to test against a selected fungus, Phyphium irregulare. These plates were each prepared in duplicate and were allowed to grow for seven to ten days. After the growth period, samples from each plate were taken and macerated in sterilized tubes, each containing three milliliters of distilled water. Approximately one milliliter of this solution was pipeted onto each of five plates and spread over half of the plate. These plates were incubated for fifteen days. This incubation period allowed for the actinomycete to grow and to produce a possible toxin and allowed time for the toxin to spread throughout the plate. After incubation, P. irregulare fungus was tested against the actinomycete and its toxin if present. Two disc samples were cut from a plate of P. irregulare (using a cork borer) and placed on the unprepared side of the plate where the actinomycete had not spread, with one disc close to the culture and the other disc far from the culture. These plates were incubated for three days. After this incubation period, the plates were observed and rated on a scale of 0-III

for inhibition or non-inhibition: O being complete inhibition, III being no inhibition at all. Plates which contained discs that demonstrated inhibition to the growth of the P. irregulare were further tested.

The discs in which the growth of the P. irregulare were inhibited were removed from the test plate and placed on a new plate to see if the P. irregulare would resume normal growth patterns. These discs were compared with a control disc of P. irregulare which was also placed on an agar plate. These plates were allowed to incubate for approximately seventy-two hours and then the diameter was measured.

Further studies and research are underway to isolate and identify the particular toxins from each successful culture that enabled the fungal growth to be inhibited.

CHAPTER IV

PRESENTATION OF FINDINGS

After the incubation and growth period of seven days, the wheat rhizosphere plates were looked at and observed. It was through this observation that the 10^{-4} dilutions were omitted from use because of excessive growth of bacteria and actinomycetes. In the 10^{-4} dilutions lots of bacteria with gummy secretions were observed. This excessive growth would not allow the actinomycetes in this dilution to be easily isolated or purified. The next reading or count of wheat rhizosphere dilution was taken after an incubation and growth period of ten days. Using the Quebec colony counter, the actinomycetes were observed and counted. The results from the first count can be seen in table 4.1:

First Count for Wheat Rhizosphere

		10^{-4}		10^{-5}		10^{-6}	
		G	O	G	O	G	O
	1.	16	5	4	5	////////	4
WA	2.	14	5	3	9	////////	2
	3.	13	////////	////////	3	////////	1
	1.	////////	3	7	4	////////	1
WB	2.	////////	4	5	6	////////	1
	3.	////////	////////	3	3	1	4
	1.	11	6	6	////////	1	5
WC	2.	9	////////	3	15	2	4
	3.	////////	////////	6	10	1	0

//// too overgrown to count or to contaminated

Table 4.1

Some of the actinomycetes may have misidentified when first count was taken, which may have resulted in some of the actinomycetes not being counted or some microorganisms counted that may have not been actinomycetes.

The final count for the wheat rhizosphere was taken only of the dilution 10^{-5}. This dilution had the best number of actinomycetes and the least contaminated. This dilution would be easier to transfer and to obtain pure cultures of actinomycetes. The results are listed in table 4.2.

Final Count for Wheat Rhizosphere

	A		B		C	
	G	O	G	O	G	O
1.	///////	7	10	7	12	6
2.	///////	9	6	12	11	16
3.	6	8	3	8	12	16
AVG.	6	8	6.3	9	11.6	12.6

[///] too overgrown to count or to contaminated

Table 4.2

On the final count of the wheat rhizosphere, only the 10^{-5} dilution was counted because it was a midpoint between the 10^{-4} which had to many actinomycetes and 10^{-6} which had too few actinomycetes. However, the dilution 10^{-6} would also be used to isolate or purify actinomycetes.

Obtaining the results for the alfalfa rhizosphere and the non-rhizosphere was basically the same as for the wheat rhizosphere. The results of the counts are listed in table 4.3:

FIRST COUNT FOR ALFALFA RHIZOSPHERE

		10^{-4}		10^{-5}		10^{-6}	
		G	O	G	O	G	O
	1.	[///]	[///]	[///]	[///]	5	2
WA	2.	[///]	[///]	[///]	[///]	4	7
	3.	[///]	[///]	[///]	[///]	3	8
	1.	[///]	[///]	[///]	[///]	8	5
WB	2.	[///]	[///]	[///]	[///]	5	8
	3.	[///]	[///]	[///]	[///]	9	8
	1.	[///]	[///]	[///]	[///]	8	6
WC	2.	[///]	[///]	[///]	[///]	6	9
	3.	[///]	[///]	[///]	[///]	8	6

[///] too overgrown to count or to contaminated

FINAL COUNT FOR ALFALFA RHIZOSPHERE

	A		B		C	
	G	O	G	O	G	O
1.	6	2	12	8	12	14
2.	8	5	11	9	9	13
3.	8	13	11	8	12	12
AVG.	7.3	6.6	11.3	8.3	11	13

[///] too overgrown to count or to contaminated

FIRST COUNT FOR NON-RHIZOSPHERE

		10^{-4}		10^{-5}		10^{-6}	
		G	O	G	O	G	O
	1.	[///]	[///]	[///]	[///]	3	5
WA	2.	[///]	[///]	[///]	[///]	4	4
	3.	[///]	[///]	[///]	[///]	4	3
	1.	[///]	[///]	[///]	[///]	5	2
WB	2.	[///]	[///]	[///]	[///]	6	1
	3.	[///]	[///]	[///]	[///]	9	3
	1.	[///]	[///]	[///]	[///]	1	3
WC	2.	[///]	[///]	[///]	[///]	3	5
	3.	[///]	[///]	[///]	[///]	1	3

[///] too overgrown to count or to contaminated

FINAL COUNT FOR NON-RHIZOSPHERE

	A		B		C	
	G	O	G	O	G	O
1.	7	4	8	10	5	3
2.	9	5	7	8	4	7
3.	11	4	10	5	2	3
AVG.	9	4.3	8.3	7.7	3.7	4.3

[///] too overgrown to count or to contaminated

Table 4.3

Isolation of actinomycetes from each rhizosphere to obtain pure, uncontaminated cultures resulted in a total of 126 cultures being isolated. Results of isolation and purification yielded 49 WR (20 used), 32 AR (15 used), and 45 NR (18 used). These were used for further isolation and purification transfers.

After determining which plates seemed to be unique and eliminating identical cultures, the researcher chose fifteen plates or cultures were chosen for inoculation, five from each rhizosphere. Each of these was prepared in duplicate and allowed to incubate for seven to ten days to allow for maximum growth of the actinomycete (Figure 4.1).

Figure 4. 1 Plate completely covered with actinomycete.

Five plates for each of the fifteen isolates were then prepared. These plates were used to test the actinomycetes with the fungus P. irregular. These were allowed fifteen days so that possible toxin could spread throughout the plate (Figure 4.2).

Figure 4.2 Plate half covered by culture. It was assumed that toxin would saturate unprepared side of plate.

After the previous step, the fungal inoculum was prepared for use during testing actinomycetes against pathogens. This was done by a disc (agar) being cut from a plate of already prepared P. irregular being placed on another plate to grow a fresh specimen. The disc was placed on the center of the plate. Six of these plates

were prepared, one to be used as a control. The discs were cut using a cork borer. The fungal inoculum was cut only on the edge or outside, never in center. Cutting on the outside ensures the fungus is alive.

The previously prepared plates (half covered) of actinomycete cultures were then tested for inhibition against the fungal inoculum. This procedure was completed by taking the prepared plates with actinomycete cultures and placing two agar discs containing the P. irregular and inoculating or placing them on the plate with the actinomycete culture. One was placed close to the culture, the other was placed far from the culture (Figure 4.3).

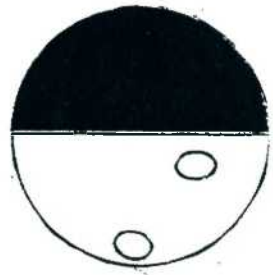

Figure 4.3 Plate half-covered with culture and two discs of P. irregular, one placed close and the other placed far from the culture.

These plates were allowed to incubate for three days. This procedure was repeated for seventy-five plates.

The results of inoculation with P. irregular are listed in Table 4.4:

Rhizosphere type #	Inhibition *(scale O-III) close	far
W1	II	III
W2	III	III
W3	O	I
W4	III	III
W5	III	III
A1	III	III
A2	II	III
A3	II	III
A4	O	II
A5	III	III
N1	O	O
N2	III	III
N3	III	III
N4	O	O
N5	III	III

* *O- No growth; complete inhibition of fungal growth*
I- A few hyphae strands
II- Moderate growth (3-5 mm diameter)
III- No inhibition

Table 4.4

The discs which showed inhibition were taken from samples of tested actinomycete, and placed on clean GAP plates to see if normal growth patterns of the P. irregular would resume. Two disks from P. irregular were also placed on clean GAP plates to use as a control. These were compared to the fungus growth by measuring the diameters. It was assumed that the disc from the actinomycete culture would grow, but not as rapidly as P. irregular after a period of seventy-two hours. The results are listed in Table 4.5:

	Phyphium irregulare discs		
	Close	*Far*	*Control*
W3	*1. 8.5 cm*		*7.3 cm*
	2. 7.7 cm		*6.6 cm*
A4	*1. 8.5 cm*		*8.5 cm*
	2. 8.5 cm		*8.5 cm*
N1	*1. 0.0 cm*	*0.0 cm*	*8.5 cm*
	2. 0.0 cm	*0.0 cm*	*8.5 cm*
N4	*1. 0.0 cm*	*0.0 cm*	*8.5 cm*
	2. 0.0 cm	*0.0 cm*	*8.5 cm*

Table 4.5

The final result from testing the actinomycete cultures against medium inoculated with the pathogen was that in both of the non-rhizosphere showed no growth. The fungus, P. irregular did not resume normal growth patterns. The two non-rhizosphere cultures acted as fungicidal compounds. This was the result which had been hypothesized.

CHAPTER V

SUMMARY AND CONCLUSION

Diseases of many economically important plants are caused by bacteria, viruses, or fungi. These organisms contribute substantially to a large percentage of crops worldwide being lost to disease. Almost all kinds of plants can be affected by bacterial, viral, or fungal diseases, and many of these diseases can be extremely destructive.

Synthetic chemicals used today such as benomyl, captan, carboxin, and diflolatan inhibit the growth and spread of diseases. With continued use, however, most of the chemicals are believed to have caused harmful effects to the environment, animals, and humans. Research is in progress to find nonchemical means of destroying these plant pathogens which would also result in being less harmful to the environment.

The hypothesis of this study was that nonchemical means of controlling plant pathogens which inhibit fungal growth, by way of biocontrol, can be found by

isolating microorganisms from soil and using them in abundance against these diseases. The hypothesis was tested through a controlled research study.

Few nonchemical means of controlling plant pathogens are easily available and effective. This research isolated microorganisms from the soil and used these microorganisms as a nonchemical means of controlling plant diseases of wheat and other horticulture and field crops.

The procedure followed during research consisted of isolating microorganisms from different soil rhizospheres which included wheat, alfalfa and non-rhizosphere. The isolation was completed by way of dilution techniques and soil suspension. The plates were incubated for different intervals of time, allowing the microorganisms to grow. The next process attempted to isolate contamination-free microorganisms. Once the isolates were contamination-free, they were stored in agar slants. Following this procedure, screening of organisms against pathogens on agar plates was done.

After testing the best fifteen isolates of forty-five, this study concludes that one of the five actinomycete cultures from the alfalfa rhizosphere inhibited the growth of the selected fungi placed close to the culture; one of the five from the wheat rhizosphere inhibited the growth of the selected fungi placed close to the culture; and two of the five isolated from the non-rhizosphere inhibited the growth of the selected fungi placed both close and far from the culture. The selected fungi discs which were inhibited were then tested on clean media to see if the fungi would continue to grow. Of the four tested, only the non-rhizosphere had complete

inhibition. No further growth of the fungi was observed.

The hypothesis stated was found to be true. Two cultures were found to act as a fungicidal compound. Recommendations include that further studies need to be conducted in order to isolate and identify the particular toxins from each successful culture that enabled the fungal growth to be inhibited.

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Vita

Kynita Deaunce Wilson-Humphrey

Candidate for the Degree of
Bachelor of Science

and

Completion of
E.P. McCabe Honors Program

Thesis: *Isolation From Soil of Microorganisms That Are Inhibitory to Wheat Seedling Pathogens*

Majors: *Chemistry/Biology*

Biographical Information:

Personal Data: Born in Ardmore, Oklahoma, June 6, 1973, the daughter of Danny Blake Wilson and Karen Deaunce King.

Education: *Graduated from Ardmore High School, in Ardmore, Oklahoma, in May 1991; will complete requirements for the Bachelor of Science degree with majors in Chemistry and Biology at Langston University in May 1996, having also completed all requirements in the E.P. McCabe Honors Program.*

Honors and Activities:

Edwin P. McCabe Honors Program, Edwin P. McCabe Scholarship, Langston University Scholars Club, President's and Dean's List, Tau Beta Sigma National Honorary Band Sorority, Zeta Phi Beta Sorority, Inc., Langston University Pan Hellenic Council, Beta Kappa Chi National Scientific Honor Society, Alpha Chi National Scholarship Society, Health Professions Club, BACCHUS, Who's Who Among American Students in American Colleges and Universities, Who's Who Among American Scholars, Nominated for Barry M. Goldwater Scholarship, Langston University Marching/Concert Band, Langston University Percussion Ensemble, Peer Tutor and Counselor, Intramural Volleyball