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A study of organisms in soil samples from southern Indiana which inhibit the growth of Escherichia coli and Staphylococcus aureus

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During the years of the journal's publication, the Butler University Botany Department had an active program of research and student training. 201 bachelor's degrees and 75 master's degrees in Botany were conferred during this period. Thirty-five of these graduates went on to earn doctorates at other institutions.

The Botany Department attracted many notable faculty members and students. Distinguished faculty, in addition to Cain and Friesner , included John E. Potzger, a forest ecologist and palynologist, Willard Nelson Clute, co-founder of the American Fern Society, Marion T. Hall, former director of the Morton Arboretum, C. Mervin Palmer, Rex Webster, and John Pelton. Some of the former undergraduate and master's students who made active contributions to the fields of botany and ecology include Dwight. W. Billings, Fay Kenoyer Daily, William A. Daily, Rexford Daudenmire, Francis Hueber, Frank McCormick, Scott McCoy, Robert Petty, Potzger, Helene Starcs, and Theodore Sperry. Cain, Daubenmire, Potzger, and Billings served as Presidents of the Ecological Society of America.

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A STUDY OF ORGANISMS IN SOIL SAMPLES FROM SOUTHERN INDIANA WHICH INHIBIT THE GROWTH OF ESCHERICHIA COLI AND STAPHYLOCOCCUS AUREUS[1]

By DORIS COLLIGAN

The purpose of this investigation was to determine the numbers and kinds of organisms—bacteria, actinomycetes, or fungi—found in certain soils which inhibit the growth of two test organisms. Soil samples collected from beneath different species of trees in wooded areas were used to ascertain what correlation there might be between the number and kind of inhibitors found and the kind of soil from which they were isolated.

This ecological aspect of the study of soil organisms which produce antibiotic substances has not been emphasized up to the present time by any of the workers studying antibiotics and the organisms which produce them, but it would seem to be of some value to have an idea where the largest number of most active inhibitors are found.

Certainly any study of soil organisms showing inhibition to other micro-organisms is valuable since notations like the following in a paper by Hoogerheide (5) are common in botanical literature:

"Waksman and Woodruff isolated from the soil in 1940 a new chromogenic species of Actinomyces which showed strong antagonistic properties toward all bacteria belonging to both the Gram-positive and Gram-negative types. This new species was later described as *Actinomyces antibioticus*."

Much work remains to be done in the search for new antibiotic substances to combat plant and animal diseases.

PROCEDURE

Soil samples were collected in October, 1947, from wooded areas in southern Indiana in regions of unglaciated clay soil: at Cornus Ridge, in Brown County, and at Stoney Lonesome, in Bartholomew County. The samples of soil were collected under seven different

[1] A portion of a thesis submitted in partial fulfillment of the requirements for the graduation honor magna cum laude, Department of Botany, Butler University.

kinds of trees. From the Cornus Ridge area the trees used were *Pinus strobus, Liriodendron tulipifera,* and *Quercus montana;* while at Stoney Lonesome soil was collected under *Acer saccharum, Carya ovata, Liriodendron tulipifera, Juglans nigra,* and *Quercus alba.*

Two samples were taken from beneath each tree, one from the surface soil and one at a depth of two or three inches, so that a comparison might be obtained between the number of inhibitors at the two depths. In collecting the surface soil, care was taken to avoid getting only the humus material on top of the ground. When the subsurface sample was taken, the surface earth was turned back first and then the soil taken from two or three inches down. An alcohol lamp was used to flame the trowel used in taking samples so that organisms would not be carried from one area to another, and the soil was put into paper bags, numbered according to location and lettered "A" for surface soil or "B" for subsurface soil.

Two methods of proceeding to find organisms in the soil which would inhibit the growth of the test organisms, *Escherichia coli* and *Staphylococcus aureus,* were tried. Up to a certain point both methods were parallel: one gram of soil was allowed to stand overnight in 10 cc. of sterile distilled water. One cc. of this was again diluted in 10 cc. of sterile distilled water, making a dilution of 1-100. One cc. of the 1-100 dilution of soil was plated with sterile pipettes into each of 14 sterile petri dishes. Seven of these petri dishes contained agar at at suitable pH for the growth of bacteria and actinomycetes and the other seven were poured with agar suitable for the growth of fungi. Following are the formulae for the media used:

Medium I (for bacteria and actinomycetes):

Dextrose	10 grams
Beef Extract	5 grams
NaCl	5 grams
Peptone	5 grams
Agar (2%)	20 grams
Water	1 liter

Medium II (for fungi, pH lowered to about 4.5 by the addition of corn steep liquor):

Dextrose	30 grams
$NaNo_3$	3 grams
K_2HPO_4	0.5 grams

$MgSO_4$	0.5	grams
$ZnSO_4$	0.01	grams
Agar (2%)	20	grams
Water	1	liter
Corn Steep Liquor	2.6	cc.

These plates were incuhated at 37° C. for from two to five days, the growth of the fungi being somewhat slower than that of the bacteria and actinomycetes.

At this point the two methods diverge. The first method used was that of inoculating tubes of agar with *E. coli* and *S. aureus* and pouring this on top of the soil sample cultures after there was sufficient growth on the plates. Zones of inhihition to *E. coli* and *S. aureus* would then he visible around the colonies from the soil which produced a substance inhibitory to the test organisms. This method proved unsatisfactory, however, because it was not always easy to ascertain whether a colony was inhibiting the test organisms. Also it was extremely difficult to obtain a pure culture of the soil colony doing the inhibiting due to the fact that it was covered with the layer of agar containing *E. coli* or *S. aureus.*

These difficulties led to the use of the second technique, the isolation from each soil plate of all colonies differing in appearance. The first method was used on six samples, the second technique on the other ten. The number of plates poured for each soil sample was cut from 14 to 10 with this change in technique. Five plates were poured with each of the two types of agar.

Two hundred twenty-one colonies were isolated from the 16 soil samples used. Each organism was numbered as it was isolated and notation was made of which numbers came from each soil sample. When colonies were isolated they were cultured on the same type of agar on which they originally appeared except in two or three cases where growth seemed to be very poor and a switch to the other type of agar brought about a better growth response.

To test for the antibiotic production from each type of colony isolated, one streak of the organism being tested was made across an agar plate and allowed to grow for from two to four days at 37° C. This growth period was allowed so that the organism might have sufficient time to produce any substance which would inhibit the

growth of *E. coli* or *S. aureus*. When this incubation time had elapsed the plates were inoculated with the two test organisms, one line of inoculation being made at a right angle to the line of growth of the organism being tested. The inoculation was done with the sterile loop from the line of growth to the edge of the petri dish.

Other workers (5) have allowed time for the "antagonist" to develop into a colony and excrete sufficient antibiotic substances before the test organism develops by seeding the agar plate first with a slow-growing test organism, such as *Mycobacterium phlei,* and afterwards inoculating the plate with the organism being tested.

Readings of inhibition were made after 48 to 72 hours, the amount of inhibition being recorded in millimeters or, if the growth was not completely inhibited but only seemed to be retarded, the amount was recorded as slight inhibition. This second method used is similar to one described by Helner and Norton (4):

"Our procedures were adapted from those described by Waksman, Bugie, and Schatz (1944) and in general resembled those described by Emerson et al. (1946). The actinomycetes were isolated from greenhouse soil (a rich and convenient source) by plating the soil in nutrient agar and selecting colonies of actinomycetes for isolation. The selection of colonies of actinomycetes was made entirely at random, no attempt being made to pick colonies differing from one another in appearance, nor to favor colonies which were inhibiting other soil organisms growing in the same plate. We assumed that different strains of the same species of *Actinomyces* differed in antibiotic potentialities and that colonies apparently inactive on the primary isolation plate might be active producers of antibiotics after prolonged incubation. . . . The preliminary screening of the isolates for antibiotic activity was made by growing them on plain nutrient agar for 4 to 8 days at room temperature, after which their action against *E. coli* and sometimes *Mycobacterium smegmatis,* was determined by streaking the bacteria on the same plate and observing zones of inhibition."

Those organisms found to produce an inhibitory substance were stained and examined with the microscope to determine which were bacteria and which actinomyces and also to determine the Gram stain reaction of the bacteria.

RESULTS

Twenty-seven organisms which inhibited the growth of *E. coli* and/or *S. aureus* were isolated from the 16 soil samples.

In four of the soils tested the number of inhibitors found below the surface of the soil was greater than the number found at the sur-

face (table I). These four cases were the samples from under *Liriodendron tulipifera* (Cornus Ridge), *Quercus montana, Carya ovata,* and *Juglans nigra.* In the samples representing the soil under *Pinus strobus, Acer saccharum, Liriodendron tulipifera* (Stoney Lonesome), and *Quercus alba* the number of organisms inhibiting *E. coli* and/or *S. aureus* at the surface was either the same or greater than those found at a depth of two to three inches.

The greatest number of inhibitors, both at the surface and below the surface, was found in the soil samples from beneath *Quercus montana* and *Juglans nigra,* both contributing six organisms to the study. *Liriodendron tulipifera* (Cornus Ridge) was next with 5 organisms, *Liriodendron tulipifera* (Stoney Lonesome) and *Quercus alba,* 3 each; *Pinus strobus,* 2, and *Acer saccharum* and *Carya ovata,* 1 each (table I).

The largest zone of inhibition, 30 mm., was produced against *E. coli* by an actinomycete from the subsurface soil beneath *Juglans nigra* (table II). The surface soil from *Quercus alba* and *Acer saccharum* each gave a bacterium which produced a zone of inhibition of 22 mm., in the former case against *E. coli* and in the latter case against *S. aureus.* Eleven other organisms gave inhibition zones of 10 mm. or more against *E. coli* and/or *S. aureus.*

TABLE I

Number of inhibitors of *E. coli* and/or *S. aureus* found in soil samples from beneath eight trees.

	Number of Inhibitors in Surface Soil	Number of Inhibitors in Subsurface Soil	Total
Pinus strobus	1	1	2
Liriodendron tulipifera (Cornus Ridge)	2	3	5
Quercus montana	1	5	6
Acer saccharum	1	None	1
Carya ovata	None	1	1
Liriodendron tulipifera (Stoney Lonesome)	2	1	3
Juglans nigra	2	4	6
Quercus alba	2	1	3

TABLE II

Width of zone of inhibition produced by soil organisms.

		E. coli	*S. aureus*
Pinus strobus	Below Surface	3 mm. (actinomycete)	
Liriodendron tulipifera (Cornus Ridge)	Surface	13 mm. (actinomycete)	10 mm. (fungus)
			2.5 mm. (fungus)
			4 mm. (fungus)
	Below Surface	20 mm. (bacterium)[1]	15 mm. (bacterium)[1]
		11 mm. (bacterium)[1]	14 mm. (bacterium)[1]
Quercus montana	Surface	slight[2] (fungus)	
	Below Surface	20 mm. (actinomycete)	12 mm. (actinomycete)
		2 mm. (fungus)	2 mm. (fungus)
		3 mm. (fungus)	
		7 mm. (fungus)	
		12 mm. (actinomyeete)	
Acer saccharum	Snrface	15 mm. (baeterium)	22 mm. (bacterium)
Carya ovata	Below Surface		slight (actinomycete)
Liriodendron tulipifera (Stoney Lonesome)	Surface		slight (actinomycete)
			5 mm. (actinomycete)
	Below Surface	13 mm. (bacterium)	slight (bacterium)

TABLE II—(Continued)

Width of zone of inhibition produced by soil organisms.

		E. coli	*S. aureus*
Juglans nigra	Surface	slight (actinomycete)	slight (bacterium)
		15 mm. (actinomycete)	13 mm. (actinomycete)
		slight[2] (actinomycete)	
	Below Surface	10 mm. (actinomycete)	
		30 mm. (actinomycete)	
Quercus alba	Surface	17 mm. (bacteria)	6 mm. (bacteria)
		22 mm. (bacterium)	12 mm. (bacterium)
	Below Surface	13 mm. (bacterium)	

E. coli proved to be more susceptible to the antibiotic substances produced by the inhibitors found in these soil samples than did *S. aureus,* as shown in table III. The actinomycetes not only made up the greatest number of inhibitors, but the total amount of inhibition produced by these organisms was greater than that of the fungi or bacteria (tables III and IV). Although the number of inhibitors which were fungi was almost the same as the number of bacterial inhibitors (7 and 6 respectively), the total amount of inhibition produced by the bacteria was more than three times that produced by the fungi. The Gram-positive bacteria proved to be more active than the Gram-negative, causing a larger total amount of inhibition, and the Gram-positive organisms were more active against *E. coli* than against *S. aureus.*

[1] Possibly same organism.

[2] No complete inhibition; growth slightly retarded.

TABLE III

Total amount of inhibition by soil organisms in 16 soil samples representing 8 trees.

Classification of organism	Number of millimeters of inhibition		Total
	E. coli	S. aureus	
Fungi	12	28.5	40.5
Actinomycetes	111	30	141
Bacteria			
Gram +	50.5	26.5	77
Gram —	28	22	50
Total	201.5	107.0	308.5

TABLE IV

Total number of soil organisms showing inhibition in 16 soil samples representing 8 trees.

Classification of organism	Number of organisms showing inhibition			Total
	E. coli	*S. aureus*	*E. coli* and *S. aureus*	
Fungi	3	3	1	7
Actinomycetes	8	3	2	13
Bacteria				
Gram +	1	–	2	3
Gram —	–	1	2	3
Total	12	7	7	26

DISCUSSION

When more than one inhibitor was isolated from a given soil sample, care was taken to be sure all the organisms were different species, but some of the same species may have been isolated from different samples of soil. Since this study was a comparison between various soils, it was not necessary to know whether a given organism had been isolated previously from another sample.

Eleven of the 27 organisms isolated were taken from the fungus medium and 18 from the bacteria-actinomycetes medium. In some cases, however, colonies of fungi which produced inhibition to the test organisms were isolated from the bacteria-actinomycetes medium.

On the whole the greater number of inhibitors was found in the subsurface soil, 16 being found in this study in soil samples from below the surface as compared with 11 inhibiting organisms found in the surface soil samples. Table I shows that there is a much greater variation from tree to tree in the number of inhibitors found below the surface than in the number found at the surface.

Table II indicates in millimeters the size of the zone of inhibition produced by each inhibiting organism, which type of tree was represented by the soil from which the organism was isolated, whether the sample was taken at the surface or below the surface, and whether the organism was fungus, bacterium, or actinomycete. In one case in this table, the subsurface soil sample taken from beneath the *Liriodendron tulipifera* at Cornus Ridge, two inhibitors are listed separately which were later thought to be of the same species. These organisms, from two petri plate colonies differing in appearance, were isolated, but growth characteristics in the agar slants and morphological appearance when the organisms were stained led to the conclusion that the two organisms were the same. However, since they were Gram-positive short spore-formers it is possible that they are two separate species in spite of their morphological similarity. The amount of inhibition which the two organisms produced against *S. aureus* was almost the same, 14 and 15 millimeters, but there was a difference of 9 mm. (20 mm. for one and 11 mm. for the other) in the size of the zones of inhibition against *E. coli*. In computing the figures on amount of inhibition for table III these two organisms were considered as being the same species and an average of the two different amounts of inhibition was used. Where the amount of inhibition is recorded as slight in table II there was no completely clear zone where the test organisms had been entirely kept from growing, but there was a zone where growth had been retarded.

One colony which had inhibited the growth of both *E. coli* and *S. aureus* is omitted from these tables because when it was stained it was found to be a mixed culture of a Gram-positive coccus and a Gram-negative rod. Efforts to obtain a pure culture of both these organisms were defeated because the growth of the organisms was so exceptionally poor on both kinds of media and at 37° C. or at room temperature.

Since a different method of isolation was used on ten of the soil samples a comparison is possible between the number of organisms isolated and the number which proved to inhibit the growth of either or both of the test organisms (table V). Of the 184 organisms isolated from the ten soil samples, eleven showed inhibition to *E. coli* alone, six showed inhibition to *S. aureus* alone, and five were found which affected the growth of both organisms. A percentage of from 5.5 to 23.8 of the total number of organisms isolated from each soil sample showed inhibition to the test organisms. Coincidentally, these two extremes were found in the surface and subsurface soil respectively, from the same tree, *Quercus montana*. The soil samples from three of the five trees showed a greater percentage of inhibitors in the subsurface soil than in the surface soil. In one case, *Pinus strobus*, both percentages were the same, while in the soil from beneath *Liriodendron tulipifera* from Stoney Lonesome the percentage of inhibitors in the surface soil was twice that of the soil from two or three inches down.

TABLE V

Comparison of number of organisms isolated and number showing inhibition to *E. coli* only, *S. aureus* only, and both organisms in the 10 soil samples on which the second technique was used.

Soil Sample from		Total No. Organisms Isolated	Number Organisms Showing Inhibition to			Percentage of Organisms Showing Inhibition
			E. coli	*S. aureus*	*E. coli* and *S. aureus*	
L. tulipifera (Stoney Lonesome)	Surface	13		2		15
	Below Surface	14			1	7.1
Juglans nigra	Surface	22	1	1		9
	Below Surface	19	3		1	21
Pinus strobus	Surface	21	1			9.5
	Below Surface	21	1			9.5
L. tulipifera (Cornus Ridge)	Surface	19	1	1		10.5
	Below Surface	16		2	1	18.7
	Surface	18	1			5.5

TABLE V—(Continued)

Soil Sample from		Total No. Organisms Isolated	Number Organisms Showing Inhibition to			Percentage of Organisms Showing Inhibition
			E. coli	*S. aureus*	*E. coli* and *S. aureus*	
Quercus montana	Below Surface	21	3		2	23.8
Total		184	11	6	5	11.9

CONCLUSIONS

1. In the sixteen samples of soil studied for this investigation it was found that a greater number of organisms producing a substance inhibitory to *E. coli* and/or *S. aureus* were found in the samples taken at a depth of two to three inches than were found in the samples taken from the surface.

2. The soils from beneath *Quercus montana* and *Juglans nigra* were found to be the richest source of inhibitors.

3. There is a greater variation from tree to tree in the number of inhibiting organisms found in the subsurface soil than there is in the number of organisms found in the surface soils.

4. The actinomycetes isolated gave a larger total amount of inhibition than did the bacteria and fungi; the bacteria gave larger zones of inhibition than did the fungi.

5. The organism producing the largest single zone of inhibition was an actinomycete isolated from the subsurface soil beneath *Juglans nigra*.

6. *E. coli* was found to he more susceptible to antibiotic substances produced by the inhibitors than was *S. aureus*.

7. Of the 10 samples on which the second technique of isolating soil colonies was used, a percentage of from 5.5 to 23.8 of the total number of organisms isolated from each soil sample showed inhibition to the test organisms.

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