TWO ISOLATES OF *STREPTOMYCES ALBUS* ANTAGONISTIC TO *CORTICIUM CENTRIFUGUM* IN UNSTERILE SOIL, AND A NEW ANTIBIOTIC, IMOTICIDIN, PRODUCED BY THEM IN LIQUID AND SOIL CULTURE

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I. INTRODUCTION

Many studies on the control of soil-borne plant diseases by means of antagonistic microorganisms have been reported for many years, but their application has been determined difficult in most cases. It may be concluded from the results of many of the trials for the control of these diseases with antagonistic actinomycete, that some control effects may be expected in sterile and amended soil, but, hardly in unsterile natural soil. The evidences of antibiotic production in unsterilized soil by the antagonistic fungi were given by Wright and his cooperators and other workers, and they showed the possibilities to establish the control measure of soil-borne diseases with the antibiotic producing fungi. On the other hand, many of the antibiotic producing actinomycete were found almost worthless by Gottlieb and his cooperators and other workers.

In this paper, the authors screened and selected two isolates of *Streptomyces albus* effective against *Corticium centrifugum* and other soil-borne plant pathogens. These two isolates were effective in the control of damping off of cowpea caused by *C. centrifugum* in glasshouse experiments using natural unsterilized soil. Furthermore, the authors found that these isolates of Streptomyces produced a new antifungal antibiotic, imoticidin, in the liquid and the soil cultures. The production and the stability of imoticidin in soil and its role in the antagonism in sterile and nonsterile soil are also studied in the present paper.

II. SELECTION OF THE ANTAGONISTS

Cross streak method, agar disk method and other convenient methods were used in the selection of microorganisms that produce antibiotics effective against bacteria or fungi that form spores under culture. These methods were not applicable for such fungi as *Corticium centrifugum* which does not produce any spores when cultured. The authors contrived the available "modified agar disk method" and "soil antagonism method" for the purpose of selecting the antagonists. As a first step in the screening process, the "modified agar disk method" proved comparatively rapid and with less difficulties.

From 58 soil samples collected in Okayama, Osaka, Nara, Northern Kyushu district and other places in Japan in 1954 and 1955, 1083 isolates of actinomycete were isolated on starch nitrate agar. These isolates were purified and cultured on glucose-asparagin-agar or on potato-sucrose-agar.

1. *Screening procedure*

Antagonistic actinomycetes were selected after passing through several steps of "modified agar disk method". The antagonistic effects of antagonists thus selected were further assured by "agar antagonism method" which is the usual method employed to determine antagonism. These selected antagonists were then tested for their antagonistic effects in sterilized soil by "soil antagonism method". The effective isolates for the control of damping off of cowpea in unsterilized natural soil were then selected by experiments in glasshouse.

Modified agar disk method: Actinomycetes were streaked along their diameters on the surface of 15 ml of potato-sucrose-agar (pH 7.0—7.2) in petri-dishes and cultured at 30°C. After 4 days of incubation, 4 pieces of large agar disks, 19.5 mm in diameter, removed from the agar medium adjacent to the colony with a cork borer, were placed into a sterilized petri-dish. Small fungus disks, 5.5 mm in diameter, of 2—4 days culture of *Corticium centrifugum* and *Pythium* sp. were placed on the center of each of the large agar disks mentioned above and cultured at 24°C. Growth of the test fungi were examined after 2 days of incubation. Either *C. centrifugum* or *Pythium* sp. was inhibited in their growth by 213 of the 1083 actinomycete isolates used.

The inhibitory effects of these 213 isolates were reexamined in the same manner, and the inhibition of growth of test fungi on agar disks was recorded daily. Number of isolates which completely prevented the growth of the test fungi in 5 days of culture are shown in Table 1. Either or both of the test fungi were inhibited in their growth respectively by 54 or 10 of the 213 isolates.

Table 1. Modified agar disk method (1)

	Days of culture				
	1	2	3	4	5
Corticium sasakii	63	44	31	26	23
Pythium sp.	80	56	47	42	41
Both of test fungi	35	21	12	10	10

Number of isolates which completely prevented the growth of the test fungi

The effects of these 54 isolates were examined further by using *C. centrifugum*, *C. gramineum*, *Rhizoctonia solani* and *Sclerotinia lebertiana* as the test fungi in the same manner mentioned above. The results are shown in

Table 2. The growth of *C. centrifugum*, *C. gramineum*, *R. solani* and *S. libertiana* was prevented by 7, 17, 11 and 27 actinomycete isolates respectively after 5 days of incubation. All of the test fungi were inhibited by 3 of the 54 isolates. Isolates such as A3-6, B12-2, C1-1, C1-2, C3-2, C10-9, E3-3 and E6-81 prevented strongly the test fungi in all the trials of modified agar disk method.

Table 2. Modified agar disk method (2)

	Days of culture			
	2	3	4	5
Corticium centrifugum	13	8	8	7
C. gramineum	24	22	18	17
Rhizoctonia solani	17	15	12	11
Sclerotinia libertiana	37	33	29	27
All of the test fungi	11	6	4	3

Number of isolates which completely prevented the growth of the test fungi

Agar antagonism method : Fifteen isolates of actinomycete which prevented the growths of 3 or out of four test fungi in the modified agar disk method were streaked a 3 cm chord on the surface of potato-sucrose-agar in petri-dish and cultured at 30°C for 2 days. Small fungus disks of *C. sasakii* and *Pythium* sp. were placed 5 cm from the edge of the actinomycete colony and incubated at 24°C. Zone of inhibition was measured after 6 days. Using *C. sasakii* for *C. centrifugum* and others, because of its rapidity of growth and the similar sensitivity in the antagonism, the results are shown in Table 3. The isolates such as B12-2, C1-1, C1-2, C10-9 and E3-3 were strongly antagonistic to the test fungi.

Table 3. Agar antagonism method

	> 20mm	20 — 10mm	10mm >
Corticium sasakii	11	4	0
Pythium sp.	3	7	5

Number of isolates formed inhibition zone

Soil antagonism method in sterilized soil : Twenty-three isolates, which showed good growths on "wheat-bran soil media" (WS-media[1]), out of 30 isolates selected through the modified agar disk method were examined as to their antagonisms in sterilized soil in petri-dish. Five to six small fungus disks of test fungi were placed on the surface of each petri-dish containing steamed "soil wheat-bran medium" (SW-medium[2]) mixed thoroughly with 1.5 g of WS-culture of the actinomycete. The growth inhibition of the test fungi, *C. centrifugum*, *C. gramineum*, *Pythium* sp., *R. solani* and *S. libertiana*,

1) 100 g garden soil, 500 g wheat-bran, 5 g sucrose, 2 g lime, 350 ml water.
2) 150 g garden soil, 50 g wheat-bran, 10 g sucrose, 10 g lime, 380 ml water.

was recorded after 5 days of incubation at 24°C. All of the isolates covered the entire surface of SW-media with their white or grayish growths after the incubation period, and the test fungi of the control (test fungi only) also grew over the whole surface of SW-media. In some cases where non-effective isolates were used as test fungi, the actinomycete colonies were overwhelmed completely by their mycelium. In the petri-dishes treated with the WS-culture of effective isolates, the growth of test fungi was prevented in various degrees. *C. centrifugum*, *C. gramineum*, *Pythium* sp., *R. solani* and *S. libertiana* were inhibited either strongly or completely in their growth by 7, 6, 2, 2 and 13 isolates of actinomycete. Such isolates as B12-2, C1-1, C1-2, C10-9, D11-3, D11-4, E5-80 and E6-81 were strongly effective upon the test fungi by the soil antagonism method using with sterilized soil.

It is convenient to show here the results of the antagonism tests *in vitro* with the isolates B12-2 and C1-2, which had been decided effective antagonists against *C. centrifugum* in unsterilized natural soil after the experiments in glasshouse, compared to the results with the isolate C10-9 which was effective isolate in sterilized soil but not in natural soil. The results are shown in Table 4.

Table 4. Antagonistic effect of Streptomyces isolate B12-2, C1-2 and C10-9

Agar disk method (Period that the growth of test fungi was prevented—days)

	Cc	*Cg*	*Cs*	*P*	*Rs*	*Sl*
B12-2	2—5	4—5	> 5	> 5	< 2	> 5
C1-2	3—5	> 5	3—5	< 1	> 5	> 5
C10-9	0	> 5	> 5	> 5	3—4	> 5

Agar antagonism method (Inhibition zone)

	Cs	*P*
B12-2	22.5 mm	8.5 mm
C1-2	24.0	11.0
C10-9	31.0	22.0

Soil antagonism method (Degree of growth inhibition of test fungi)

	Cc	*Cg*	*P*	*Rs*	*Sl*
B12-2	⫶⫶	⫶	−	±	⫶⫶
C1-2	⫶⫶	⫶	−	⫶	⫶⫶
C10-9	±	⫶⫶	⫶⫶	⫶	⫶⫶

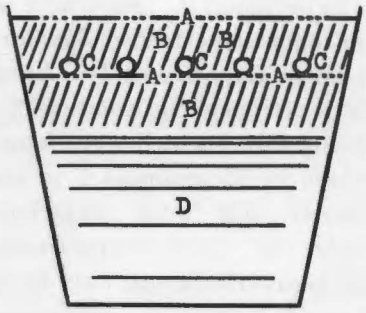
Cc : *Corticium centrifugum* *Cg* : *C. gramineum*
Cs : *C. sasakii* *P* : *Pythium* sp.
Rs : *Rhizoctonia solani* *Sl* : *Sclerotinia libertiana*

The experiments on antagonism under various conditions in soil antagonism method were carried out further. The details of the experiments are described in the next chapter.

2. *Two isolates of Streptomyces effective against Corticium centrifugum in unsterilized soil*

At the final step of screening for the antagonsim against *C. centrifugum*, the experiments were carried out with unsterilized natural soil under glasshouse condition. SW-cultures of 8 antagonistic isolates which had been selected through the several steps of screening *in vitro* were used in the experiments. Seeds of cowpea, using highly susceptible variety, Kurodane-sanjaku, to *C. centrifugum*, were sown in clay pots, 15 cm in diameter, and in 40×50×15 cm wooden boxes. As seen in Figure 1, cowpea seeds were sown into the inoculum layer. The uppermost surface of soil and the level on which the cowpea seeds were sown were covered with thin layer of the antagonists. (The antagonism mixed thoroughly with soil exhibited only little effect.) In every pot and wooden box, 30 and 100 g of the soil culture of the antagonists were applied respectively. As the inoculum, 2 types of soil were used : the soil mixed with WS-culture of *C. centrifugum* and the infested soil which had been mixed previously with the pathogen and had been assured the pathogenicity on cowpea seedling. The glasshouse was kept under favorable conditions for the infection of damping off by providing with high humidity and moderate temperature. Experiments were carried out in summer (Experiment 1 and 2) and in autumn (Experiment 3) with 3 or 4 replications. The results obtaind were examined statistically.

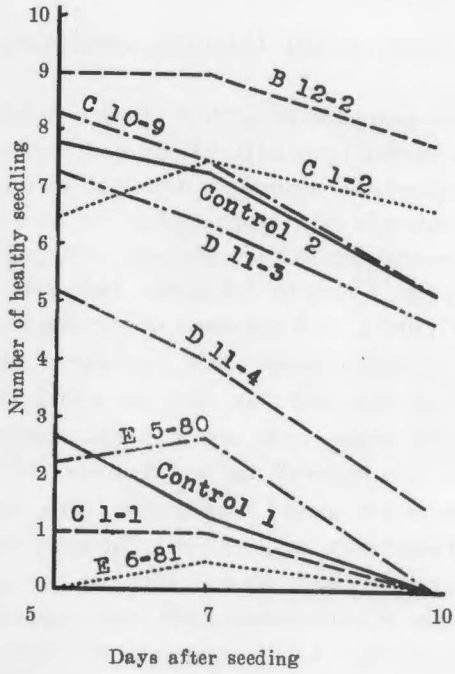
Fig. 1. Application of antagonist for antagonism test in non-sterile soil



A : SW-culture of antagonist
B : Infested soil
C : Cowpea seed
D : Garden soil

Experiment 1. (Selection of effective antagonist) — Eight isolates, namely B12-2, C1-1, C1-2, C10-9, D11-3, D11-4, E5-80 and E6-81, were examined for their antagonistic effects in natural soil. In every clay pot, 10 seeds of cowpea were sown, and the number of healthy seedling were counted after 5, 7 and 10 days from the date of seeding. The results were as summarized in Figure 2. In Figure 2, Control 2 was non-treated natural soil, and Control 1 was natural soil mixed with WS-culture of the pathogen. In the treatments applied with 3 isolates, the number of healthy seedlings of cowpea increased, but not with the rest. The differences among the numbers of

Fig. 2. Effect of antagonistic isolates on daming off of cowpea in non-sterile soil



healthy seedling of Control 1 (diseased soil) and those of B12-2 or C1-2 (diseased soil and antagonist) were significant in 1 % level, and among control 1 and C10-9 or Control 2 (natural soil) were significant in 5 % level. Those cowpea seedlings treated with the isolates C1-1, E5-80 and E6-81 were affected severely, resulting in bad germination and brown necrotic flecks on the cotyledons and the primary leaves; but only slightly with the isolates B12-2 and C1-2, and none with C10-9 and others.

Experiment 2. (Assurance of the antagonistic effects of 3 isolates selected in Experiment 1)—Antagonistic effects of the isolate B12-2, C1-2 and C10-9 which were found effective in Experiment 1 in unsterilized soil were examined further in this experiment. Methods were similar to Experiment 1, but 120 seeds of cowpea were sown in every wooden box, and the infected soil strongly pathogenic to cowpea seedling was used as the inoculum. Numbers of healthy seedling of cowpea were counted 10 days after seeding. As shown in Table 5, isolate B12-2 or

Table 5. Effect of antagonists in unsterilized natural soil (Experiment 2)

		Healthy seedling	
		Total	%
(1)	Non-treated natural soil	207	57.5
(2)	(1) + infested soil	135	37.5
(3)	(1) + B12-2	236	65.5*
(4)	(1) + C1-2	255	70.8**
(5)	(1) + C10-9	183	50.8

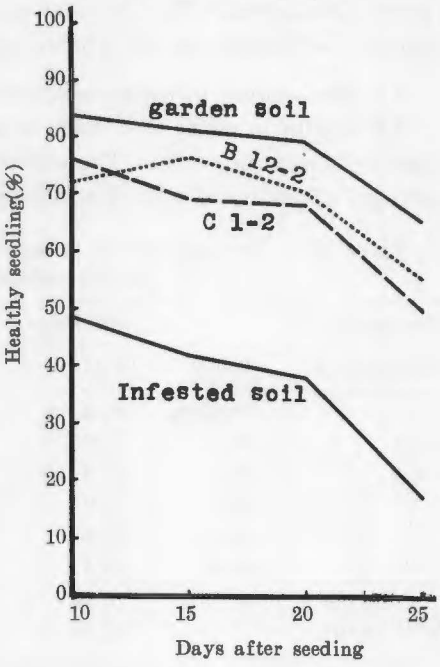
Significant in 5(*) and 1(**) % level

C1-2 was apparently effective in preventing damping off of cowpea, but C10-9 was not so effective. Sixty-five and 70 % of seedlings remained healthy by the treatment with B12-2 and C1-2, but only 37 % with the infested soil. The effect of controlling damping off by these antagonists was decreased gradually at first, but rapidly after 7—10 days as similar as shown in

Figure 1 of Experiment 1. In this experiment since the conditions for the infection of damping off were very favourable, it was decided that the isolate B12-2 and C1-2 were apparently effective against the disease in unsterilized natural soil.

Experiment 3. — Further experiment was made under the conditions which were not so favourable, being rather cool for the infection of damping off. Isolates B12-2 and C1-2 were examined for their antagonistic effects by the similar method employed in Experiment 2 in autumn of 1955. Number of healthy seedling were counted after 10, 15, 20 and 25 days from the date of seeding. In Figure 3, the summarized results are shown. The differences among Control 1 (infested soil) and Control 2 (natural field soil), B12-2 or C1-2 (infested soil and antagonist) were significant in 1% level through the whole period of the experimens. In Control 1, about 80% of test plant were diseased, but 50—60% remained in B12-2, C1-2 and Control 1 even after 25 days from the seeding.

Fig. 3. Effect of the selected isolate B12-2 and C1-2 on damping off of cowpea in non-sterile soil



III. SOIL ANTAGONISM METHOD UNDER VARIOUS CONDITIONS FOR THE ANTAGONISM TEST

Soil antagonism method contrived and applied to the screening for the antagonism in soil is described in previous chapter. Further experiments were carried out to examine the availability of this method, by varying the conditions of experiment as follows: the amount of antagonist applied in soil, the time when the antagonist was introduced in soil, the kind of organic material for the soil amendment, the sort of soil type and the water content of the soil.

The proportions of the component of SW-media were made to differ slightly from those of the medium, which was used in the preceding experiments. SW-media in this experiment consisted of 1 kg garden soil, 30 g wheat-bran, 10 g lime, 10 g sucrose and 250 ml water. Two pieces of fungus disks of *Corticium centrifugum* and *Rhizoctonia solani* were placed 4 cm apart on 30 g of SW-media in petri-dish (87 mm in diameter) which was previously

steamed amd mixed thoroughly with 1g of SW-culture of the antagonist (Streptomyces isolate labeled C1-2, which was identified to a strain of *Streptomyces albus* in the next chapter). The growth of test fungi was measured after regular incubation periods at 26°C. The growth of test fungi was not uniform in all directions from the fungus disk, but the growth was measured along the greatest diameter of the colony. Five petri-dishes were used in every treatment. The production of sclerotia was also observed, and the amount of sclerotia was designated as −, +, ++, ···, +++ in the table.

1. *The amount of antagonist applied in soil*

SW-media in petri-dish were mixed with 0.25, 0.5, 1, 2 and 4 g of SW-culture of the antagonist. The growths of *C. centrifugum* or *R. solani* were examined after 4 and 8 or 4 and 5 days incubation respectively. In Table 6

Table 6. The amount of antagonist and the inhibition of test fungi in soil antagonism method

The amount of antagonist (g)	*C. centrifugum*			*R. solani*		
	4 days	8 days	Growth* (%)	4 days	5 days	Growth* (%)
0	39.6 mm	84.9 mm	100	76.8 mm	87.0 mm	100
1/4	35.7	74.0	84.6	76.8	86.2	96.1
1/2	34.0	63.9	66.0	66.9	79.5	123.5
1	36.2	57.0	45.9	62.5	69.2	65.7
2	32.0	43.4	25.1	52.9	57.1	42.1
4	22.0	26.4	1.0	30.4	30.3	−1.0
LSD (0.05)		10.44			7.41	
LSD (0.01)		13.96			9.91	

* Growth of test fungi within the last 24hr of the experiment is shown as percent.

the results are shown. The growths of *C. centrifugum* and *R. solani* after 8 and 5 days incubation are seen in Figure 4. Stronger inhibition of the growth of test fungi was observed by the application of larger amounts of antagonist. Significant inhibitions of *C. centrifugum* and *R. solani* after 8 and 5 days incubation occurred from the use of more than 0.5g and 1 g of the antagonist respectively. As *R. solani* in the control grew over the entire surface of SW-media in petri-dish after 5 days incubation, it was suggested that the average inhibition value shown in the table appeared rather weak.

Daily production of sclerotia on the surface of soil media appears as shown in Table 7, in which the number of fungus disks that produced sclerotia out of 10 disks are shown from the average of 5 replications. Fewer and the earlier formation of sclerotia resulted by increasing the amount of antagonist applied. Application of 2 g of SW-cultre of the antagonist appeared suitable for the soil antagonism method.

2. *The time when the antagonist was introduced in soil*

To determine the effect of time of application of antagonist in the soil

Fig. 4. Influence of the amount of antagonist on the growth of test fungi in soil antagonism method

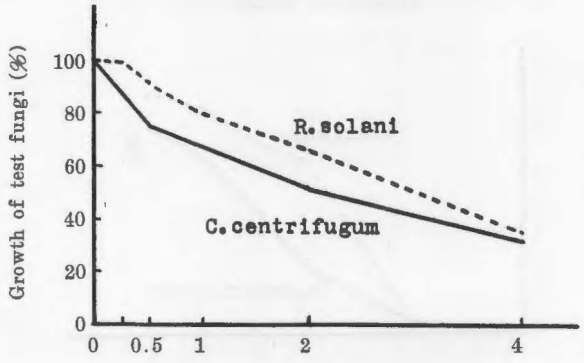


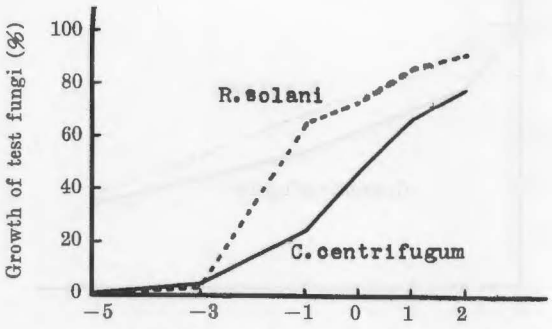
Table 7. The amount of the antagonist applied in soil antagonism method and the production of screlotia by the test fungi

The amount of antagonist (g)	*C. centrifugum*						*R. solani*				
	Days					Amount of sclerotia	Days				Amount of sclerotia
	3	4	5	6	7		3	4	5	6	
0	0*	0	9	10	10	##	0	10	10	10	##
1/4	0	0	10	10	10	##	0	10	10	10	##
1/2	0	0	10	10	10	##	0	10	10	10	#
1	0	0	10	10	10	#	0	7	9	10	#
2	0	1	9	10	10	+	0	4	8	10	#
4	0	1	8	8	9	+	0	4	9	10	+

* Number of fungus colony on which sclerotia were formed
Every 10 fungus colonies was observed

medium contaning test fungi, the SW-culture of the antagonist was mixed with SW-media before, simultaneously or after the establishment of the test fungi on soil media. The growth of test fungi was measured 6 and 3 days after the establishment of *C. centrifugum* and *R. solani* respectively. The results are shown in Figure 5. Growth of both test fungi was inhibited completely on SW-media when the antagonist was introduced 5 days earlier than the test fungi. The respective growth of *C. centrifugum* and *R. solani* introduced in the 3 days SW-culture of the antagonist was only 3.7 and 2.8 % of the non-treated control. Lesser effect on the test fungi was observed when the application of the antagonist was made after the establishment of the test fungi. However, there was about 20 % inhibition in the growth of test fungi even when they were introduced 2 days earlier than the antagonist. When the test fungi grew less by inhibition, there was also less sclerotia produced as was observed in the preceding experiment.

Fig. 5. Influence of time of introduction of the antagonist on the growth of test fungi in soil antagonism method



3. *Effect of organic material as a component of S W-media*

Varying amounts of plant materials were substituted for wheat bran in the SW-media to test the effect upon the antagonism. Test materials were wheat-bran (A), dried bean leaves and stems (B), chopped rice straws (C), rice hulls (D), saw dust (E) and dried soy bean leaves (F) which were applied at the rate of 3 % as organic component in SW-media. The results are summarized in Figure 6. Growth inhibition of test fungi was observed in all of the soil media containing the plant materials. Above all, strong inhibitions of *C. centrifugum* and *R. solani* resulted in the media containing (B) and (C) respectively. Additional amounts of plant materials did not affect the growth inhibition of the test fungi, even when as much as 10 % addition of such materials was made. Wheat-bran was most suitable for the growth of the antagonist under the condition of the experiment.

The amount of sclerotia formed decreased by the addition of plant materials to 1/4—2/3 of the soil media that did not contain such material.

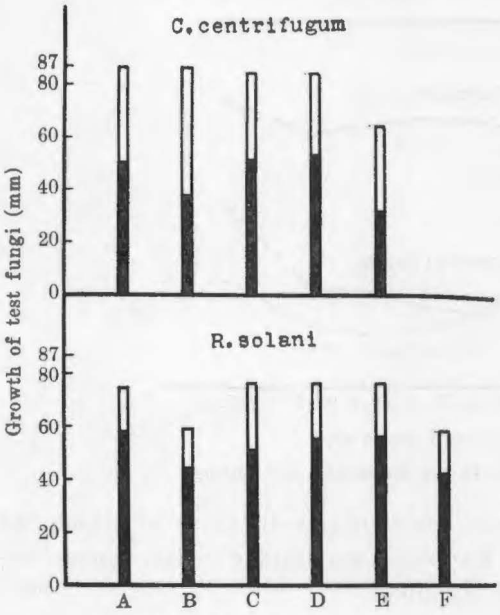
4. *Effect of soil type on the soil antagonism method*

As given in Table 8, air dried garden soil and sandy soil were mixed in various proportions. Soils thus prepared were moistened with water, and the pF value of the soil was adjusted to about 2.0.

The inhibition of growth of test fungi in these soil media by the antagonist appears in Figure 7. In sandy soil, the growth of test fungi was less vigorous than in garden soil; and it was observed that the greater the proportion of sand in the soil, the stronger the growth inhibition. The growth inhibition in sandy soil was about 4 times that of garden soil.

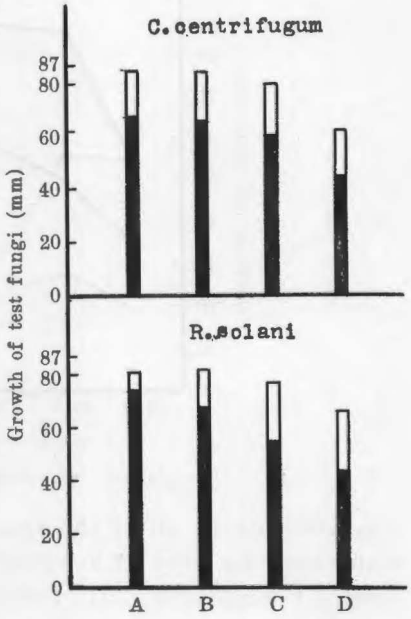
A larger amount of scrclotia was found on the soil media of the control than on the media in which antagonist was present. Here again, the larger the proportion of sand in the soil, the less the production of the sclerotia.

Fig. 6. Effect of plant material on growth inhibition of the test fungi in soil antagonism method



Plant metarial applied in soil media
(cf. p.10)

Fig. 7. Effect of soil type of soil media on growth inhibition of the test fungi in soil antagonism method



Soil type of soil media
(cf. Table 8)

Table 8. Preperation of different soil type and the water content

	water content per 100 g dried soil
(A) Garden soil	25.0 ml
(B) Garden soil 2 : sandy soil 1	20.8
(C) Garden soil 1 : sandy soil 2	16.7
(D) Sandy soil	12.5

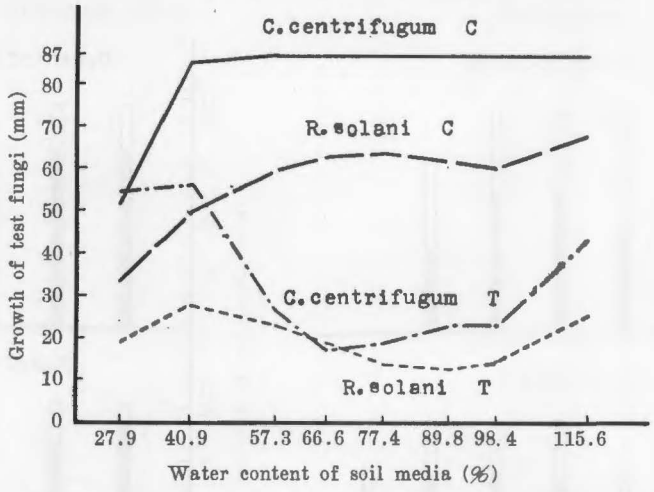
5. *Water content of the soil media*

SW-media were prepared with varying amounts of water content, as shown in Table 9. The growth of *C. centrifugum* and *R. solani* after 7 and 3 days of incubation is shown in Figure 8. The mycelial growth of *C. centrifugum* in all the treatment without No. 1 and No. 2, covered the entire surface of soil media in petri-dishes. Strong inhibition of growth of test fungi

Table 9. SW-media prepared with various water contents

	No. 1	2	3	4	5	6	7	8
The amount of water added in every petri-dish (ml)			2.5	5.0	7.5	10.0	12.5	15.0
Water content of SW-media (%)	27.9	40.9	57.3	66.6	77.4	89.8	98.4	115.6

Fig. 8. Effect of water content of soil media on growth inhibition of the test fungi in soil antagonism method



C : control T : treated with the antagonist soil culture

was observed in all of the treatments, particularly in those of which the water contents were 57.3—98.4%. Excessive amounts of water content decreased the inhibitory effect of the antagonist.

A similar relation to the preceding experiments was observed in these experiments between the production of sclerotia and the growth inhibition as affected by the water content of soil media.

From the results obtained here, following condition were determined important in the soil antagonism test method :

Dosage of the antagonist : about 10 % of the soil media

Vegetative materials for soil amendment: wheat-bran, dried bean leaves and stems, rice hulls.

Time of antagonist application : 2—3 days before the transferrence of the test fungi.

Soil type : garden soil.

Throughout the experiments when the production of sclerotia is small and early, the growth inhibition of test fungi was generally observed to be strong. This suggested that the observation of the process of sclerotia production is useful in determining the antagonism.

IV. A NEW ANTIBIOTIC, IMOTICIDIN, PRODUCED BY TWO ANTAGONISTIC STREPTOMYCES ISOLATES

Antagonistic effects of *Streptomyces albus* B12-2 and C1-2 in sterilized and in unsterilized natural soil against *Corticium centrifugum* and other soil-borne pathgenic fungi were described in the preceding chapters. It seemed very important whether any antibiotic played any part in the antagonism

in soil. Antibiotic activity was detected, in a preliminary experiment, in the water, methanol or acetone extracts of the SW-culture of *Streptomyces albus* B12-2 and C1-2. The antibiotic properties found in these extracts were recognized as similar to those found in the culture luquid of those streptomyces. And then, it was considered that an antibiotic agent should not be disregarded in the antagonism observed in soil. Under these circumstances, the authors tried to extract the antibiotic produced by these two strepto myces. The antibiotic obtained here is a new substance, imoticidin, possessing specific physical, chemical and antimicrobial characters. Both *Streptomyces albus* B12-2 and C1-2 produced the same antibiotic, but the latter showed a higher production of imoticidin in its liquid culture than the former. In the following experiments *Streptomyces albus* C1-2 was used chiefly for the imoticidin production.

1. *Biological natures of Streptomyces albus B12-2 and C1-2*

Streptomyces albus B12-2 and C1-2 were isolated from soil samples collected at Kurashiki and Tamashima of Okayama Prefecture, respectively in 1955. In Table 10, comparative cultural and other characters of these isolates

Fig. 9. Production of imoticidin in various liquid media
1: 3% wheat-bran, 2% sucrose, 0.1% K_2HPO_4
2: 1+0.25% meat extract
3: 1+0.25% peptone
4: 1+0.25% yeast

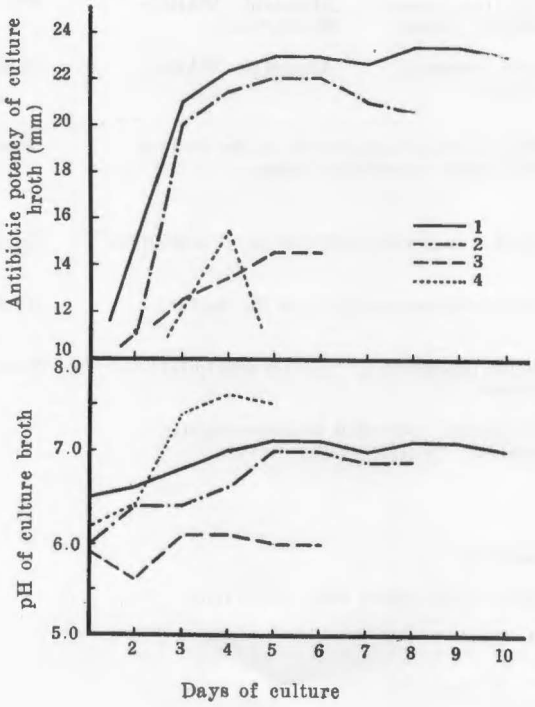


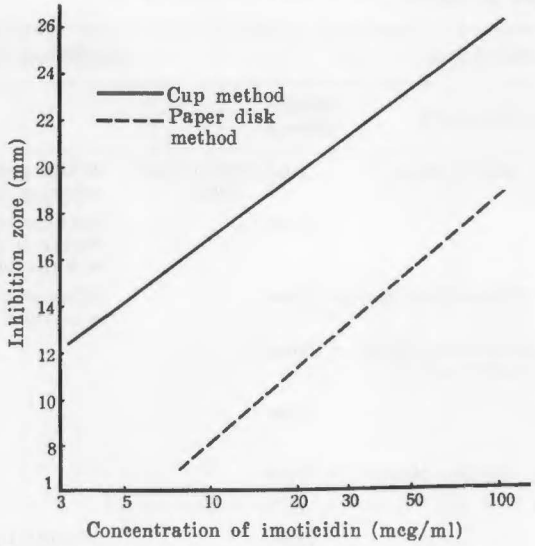
Table 10. Cultural natures of *Streptomyces albus*

	Stredtomyces albus C1-2			
	Growth	Aerial mycelium	Soluble pigment	
Starch agar	Fair, colorless.	Much, Mouse Gray→ Black.	None	Hydrolysis : 8 mm
Czapek's agar	Colorless.	±	None	
Ca-malate agar	Fair, colorless.	Less, White→Pale Smoke Gray.	None	
Glucose asparagin agar	Good, colorless~ cream-colored.	Abundant, thick, White→ Mouse Gray.	±	
Nutrient agar	Fair, rugose, cream-colored.	None	None	
Potato sucrose agar	Good, rugose, colorless.	Abundant, thick, White→ Mouse Gray.	Faint reddish purple.	
Plain agar	Scanty, colorless.	±	None	
Egg alubmen agar	Scanty, colorless.	Less, White→Lilac Gray.	None	
Tyrosin agar	Cream-colored.	Less, White.	None	Tyrosinase : negative.
Potato plug	Excellent, cream-colored, raised.	Abundant. White→ Mouse Gray.	None	
Carrot plug	Good, cream-colored.	Abundant, White.	None	
Glucose broth	Much folded ring growth on the surface with flaky growth on bottom.		None	
Starch solution	Pellicle on the surface with aeral mycelium.		None	
Czapek's solution	Small colorless colonies on the surface.		None	
Gelatin (20°C)	Rapid liquefaction, colonies precipitate on bottom.		None	
Milk	Coagulated. Reaction becomes slightly alkaline. Pellicle on the surface.			
Nitrate reduction	Negative,			
Aerial mycelium	Short closed spiral with 1~3 turns.			
Optimum temperature	30—33°C.			

C1-2, B12-2 and type strain

Streptomyces albus B12-2				*Streptomyces albus* (Type strain)
Growth	Aerial mycelium	Soluble pigment		
Fair, colorless.	Much, Mouse Gray→Black.	None	Hydrolysis : 13mm	White aerial mycelium covering the whole surface.
Colorless.	±	None		Colonies of medium size ; the center only is covered with a white aerial mycelium.
Fair, colorless.	Less, White→Pale Smoke Gray	None		(Glucose agar) Gray aerial mycelium becoming brownish.
Good, colorless~cream-colored,	Abundant, thick, White→Pale Varley's Gray.	None		
Fair, rugose, cream-colored.	None	None		
Fair, colonies not spread.	Much, White→Mouse Gray.	None		
Scanty, colorless.	±	None		No aerial mycelium, but a chalky white deposit forms on old colonies.
Scanty, colorless.	Less, White→Light Drab.	None		
Cream-colored.	Less, White	None	Tyrosinase : negative.	
Good, cream-colored, raised.	Abundant, White→Pale Mouse Gray.	None		White aerial mycelium. Growth folded, cream-colored.
Good, cream-colored, raised.	Abundant, White→Pale Olive Gray.	None		Excellent growth.
Much folded ring growth on the surface with flaky growth on bottom.		None		(Broth) Flaky growth on bottom with surface pellicle in old cultures. White aerial mycelium.
Pellicle on the surface with aerial mycelium.		None		
Good growth with large colonies.		None		
Rapid liquefaction, colonies precipitate on bottom.		None		Rapid liquefaction. Gray colonies. No soluble pigment.
Coaglulated. Reaction becomes slightly alkaline. Pellicle on the surface.				Rapidly peptonized after coagulation. Reaction becomes alkaline. Cream colored surface ring. White aerial mycelium.
Negative.				Positive.
Short closed spiral with 1~3 turns.				
30—33°C				

Fig. 10. Diffusion curve of imoticidin



with type strain of *Streptomyces albus* are shown. Growth of both isolates resembled each other on most culture media, except on potato-sucrose-agar on which certain apparent differences were noted. Antibiotic characters of the two streptomyces by the cross streak method are shown in Table 11. Cultural and molophological natures of these two isolates were almost identical with those of *Steptomyces albus*, except the the reaction to milk and the utilization of nitrate.

2. *Production of imoticidin*

Plant extracts such as wheat-bran, potato, soybean meal were suitable for the production of imoticidin, but glucose broth, Czapek's solution and starch solution were not suitable. Various media consisting of 3% wheat-bran or soybean meal as the basal component, with the following in various combinations were prepared : sucrose, glucose, glycerol, starch, meat extract, peptone, yeast, $(NH_4)_2SO_4$ and K_2HPO_4. Of the above media tested, the medium consisted of 3% wheat-bran, 2% sucrose and 0.1% K_2HPO_4 gave the best result for the production of imoticidin. Additions of 0.25% meat extract, peptone, yeast, NH_4NO_3 and $NaNO_3$ to this medium decreased the antibiotic potencies of broth as shown in Figure 9. Therefore, the medium above mentioned was used for the imoticidin production. The amount of imoticidin in culture broth was raised near to the maximum in 6 or 7 days incubation at about 28°C on shaking machine. It did not decrease even after 10 days culture as seen in Figure 9.

The antibiotic potency was assayed by cup or paper disk method with the use of *Bacillus subtilis* PCI 219 as the test organism on glucose-peptone-agar. Figure 10 shows the diffusion curve of the purified imoticidin. In-

Table 11. Antibiotic characters of *Streptomyces albus* B12-2 and C1-2 with cross streak method

	Streptomyces albus			*Streptomyces albus*	
	B12-2	C1-2		B12-2	C1-2
B. subtilis PCI 219	16.5 mm	8.5 mm	*A. kikuchiana*	19.0 mm	19.0 mm
S. aureus TERAJIMA	15.0	7.0	*A. oryzae*	10.0	7.5
E. coli mommunior	0	0	*B. cinerea*	22.0	24.0
E. aroidae	0	0	*C. lindemuthianum*	10.0	10.5
P. tabaci	0	0	*F. niveum*	14.0	14.0
			O. miyabeanus	23.5	23.0
S. sake	19.0	18.0	*P. oryzae*	24.0	26.0
C. albicans	19.5	18.0	*R. nigricans*	8.0	7.0

Bacteria : 30°C 48 hr. *S. sake*, *C. albicans* : 27°C 48 hr.
Fungi : 27°C 72 hr.

hibition zones were measured within 20—24 hr. incubation at 30°C, because of the bacteriolytic nature of imoticidin. The diameter of inner circle was measured for the assay of the potency of culture broth, of which inhibition zone appeared as a double concentric circle.

3. *Extraction and purification of imoticidin*

Imoticidin was contained in both culture filtrate and mycelium. Imoticidin in culture filtrate was easily adsorbed on activated carbon, activated kaolin, Seitz' filter, but was hardly eluted from them. Imoticidin was extractable with butanol, benzen and other organic solvents from the culture filtrate. The authors extracted and purified imoticidin by the following method as seen in Figure 11.

Culture broth containing mycelium was acidified to pH 2.4 with conc. HCl, and to it diatomaceous earth or celite in the amount 0.5% of the broth was added, stirred and then filtered. Diatomaceous earth (or celite) pad was extracted with methanol. The aqueous residue, after removing the solvent, was extracted with chloroform. And then, it was extracted with benzen, and also with a small amount of anhydrous acetone. A short column of alumina was loaded with the anhydrous acetone extract, and eluted with the same solvent.

Fig 11. Extraction and purification of imoticidin

Culture liquid (containing mycelium)
↓ Acidified to pH 2.4 with conc. HCl. Added 0.5%. diatomaceous earth or celite. Stirred and filtered.
Diatomaceous earth (or celite) pad
↓ Extracted with methanol.
Methanol
↓ Concentrated *in vacuo*. Extracted with chloroform.
Chloroform
↓ Concentrated to dryness *in vacuo*. Extracted with benzen.
Benzen
↓ Concentrated to dryness *in vacuo*. Extracted with anhydrous acetone.
Acetone
↓ Chlomatography on short column of alumina eluting with anhydrous acetone. Concentrated active fractions *in vacuo*.
Crude imotieidin crystals
↓ Recrystallization from anhydrous acetone solution.
Pure imoticidin (m. p. 245—246)

Concentrating the active fractions of the elution *in vacuo*, the crude crystals of imoticidin were obtained. After several recrystallizations from anhydrous acetone solution, fine elongated cubic or prysmoid white crystals of imoticidin (m. p. 245—246°C) were obtained.

4. *Physical and chemical properties of purified imoticidin*

Melting point : 245—246°C (uncorrected). It darkens gradually at near 210°C.

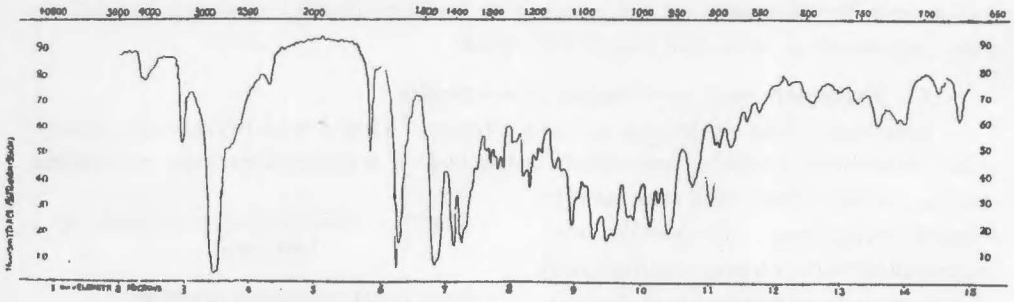
Annalysis of element*: C, 64.71; H, 9.50; N, 0; H_2O, 7.63; ash, 3.16; S, 0; Halogen, 0.

Molecular weight*: 534—553.

Ultraviolet absorption : No absorption at any wave length from 220 to 300 mμ.

Infrared absorption*: Infrared absorption spectra of imoticidin is shown in Figure 12.

Fig. 12. Infrared absorption spectra of imoticidin



Solubilities : Easily soluble in methanol, ethanol, butanol and chloroform. Soluble in ethyl acetate, amyl acetate, acetone, benzen, ether and dioxan. Sparlingly soluble in petroleum ether, and almost insoluble in water.

Color reactions : All of the reactions as follows gave the negative results; Sakaguchi, Ninhydrin, Xanthoprotein, Biuret, Millon and Tollen.

Stabilities : As seen in Table 12, culture filtrate is not decreased in its activity with 10 minutes boil at neutrality and at alkaline side, but is decreased slightly at acidic side. After autoclaving, it decreases slightly at

Table 12. Heat stability of imoticidin

pH	Room temperature	100°C 10 min	Autoclaved
5.0	13.0 mm	12.3 mm	11.0 mm
7.0	13.0	13.5	12.5
9.4	13.3	13.5	13.0

Inhibition zone of culture filtrate by paper disk method

* The annalysis of element and the survey of molecular weight and infrared absorption of imoticidin were made by Sankyo Pharmaceutical Company.

neutrality and at alkaline side also, but considerably at acidified filtrate.

Paper chlomatography : Summarized papergram of imoticidin is shown in Figure 13. Papergram of the culture filtrate of *Streptomyces albus* B12-2 is quite similar in pattern to that of imoticidin.

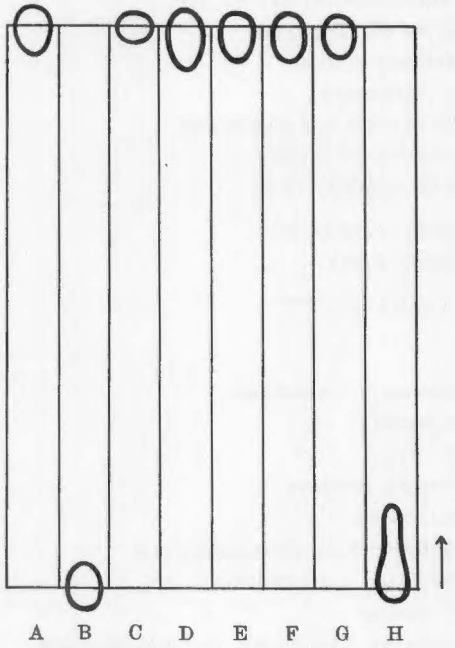


Fig. 13. Summarized papergram of imoticidin

A : Wet Butanol
B : 3% NH_4Cl
C : 80% Phenol
D : 50% Acetone
E : Butanol, Methanol, Water, Methyl orange (40 ml, 10 ml, 20 ml, 1.5 g)
F : Butanol, Methanol, Water, (40, 10, 20)
G : Benzen, Methanol (80, 20)
H : Distilled water

5. *Antimicrobial properties of imoticidin*

Antibiotic properties of pure imoticidin are given in Table 13, in agar dilution streak method. Glucose-meat-peptone-agar, potato-sucrose-agar (pH 7.0—7.2) and potato-sucrose-agar (pH 5.4) were used for bacteria, yeasts or *Candida* and fungi respectively. Pure imoticidin was dissolved in a small amount of 70% acetone and then diluted it in regular concentrations with phosphate buffer (pH 7.2). Gram-positive bacteria such as *Bacillus subtilis* PCI 219, *Staphyllococcus aureus* TERAJIMA and *S. aureus* 209 P were inhibited in their growth at low concentrations of imoticidin (0.1—0.5 mcg/ml). Yeasts were inhibited at 5—10 mcg/ml, and *Botrytis cinerea* and *Helminthosporium sativum* were inhibited at 5—10 mcg/ml. Imoticidin was not effective to gram-negative bacteria. *Candida albicans* and many of the other fungi tested were partly affected at low imoticidin concentrations, but they needed high concentrations for the complete inhibition. *Corticium centrifugum*, whose growth was prevented by *Streptomyces albus* B12-2 and C1-2 in soil, was not completely inhibited in its growth even at 100 mcg/ml, but was affected strongly at lower concentrations.

Imoticidin was bacteriolytic on *B. subtilis*, and also bactericidal. In Table 14, the bactericidal effect of imoticidin is shown. Heavy suspensions

Table 13. Antimicrobial spectrum of imoticidin

Test organism	Minimum inhibition concentration (mcg/ml)	
	24 hr	48 hr
Bacillus subtilis PCI 219	0.1—0.5	0.1—0.5
Staphyllococcus aureus 209P	0.1—0.5	0.1—0.5
S. aureus TERAJIMA	0.1—0.5	0.1—0.5
Erwinia aroidae	>100	>100
E. carotovora	>100	>100
Escherichia coli communior	>100	>100
Pseudomonas tabaci	>100	>100
Xanthomonas oryzae		30—50
Saccharomyces sake	5—10	5—10
Tolura utilis	5—10	5—10
Candida albicans	10—50	50—100
	48 hr	5 days
Alternaria kikuchiana	1—5	20—30
Aspergillus niger	50—100 (20)	>100 (20)
A. oryzae	75—100	>100
Botrytis bassiana	>100 (10)	>100
B. cinerea	5—10 (1)	5—10 (1)
Colletotrichum lindemuthianum	5—10 (5)	50—100 (5)
Corticium centrifugum	>100 (1)	>100 (1)
C. sasakii	75—100 (3)	>100 (3)
Fusarium bulbigenum var. *nelumbicolum*	>100 (1)	>100 (1)
F. caeruleum	>100	>100
F. lini	75—100 (10)	>100 (20)
F. oxysporum f. *lycopersici*	>100	>100
F. niveum	>100 (5)	>100
Gibberella Fujikuroi	>100	>100
G. zeae	30—50 (20)	50—100 (20)
Gloeosporium nelumbii	75—100 (25)	>100
Helminthosporiuu sativum	5—10 (1)	5—10 (1)
Oospora destructor	75—100 (50)	>100
Ophyobolus miyabeanus	30—50 (5)	>100 (20)
Penicillium chrysogenum	50—100 (10)	50—100 (10)
P. glaucum	25—50 (15)	>100 (20)
Piricularia oryzae P2	0.5	0.5—1.0
Rhizoctonia solani	>100 (10)	>100 (10)
Rhizopus nigricans	20—30 (10)	20—30 (10)
Rosellinia necatrix	5—10 (1)	10—15 (3)
Sclerotinia libertiana	10—15 (1)	10—15 (1)
S. minor	25—50 (1)	50—75 (1)
Thielavia basicola	>100 (10)	>100
Trichoderma sp.	>100 (10)	>100 (25)
Trichophyton interdigitale	25—50 (10)	>100

Table 14. Bactericidal effect of imoticidin

Concentration of imoticidin (mcg/ml)	*B. subtilis*					*S. sake*				
	30 min	1 hr	5	10	24	30 min	1 hr	5	10	24
0	++	++	++	++	++	++	++	++	++	++
1	++	++	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++	++	++
10	+	+	+	+	+	++	++	++	++	++
25	+	+	−	−	−	++	++	++	++	++
50	−	−	−	−	−	++	++	++	++	++
100	−	−	−	−	−	++	++	++	++	++

Incubated at 30°C

of *B. subtilis* and *S. sake* were added with imoticidin solutions at various concentrations and incubated at 30 C. After the regular incubation periods, viable cells were examined. Imoticidin did not decrease the number of viable cells of *S. sake*, but, it was bactericidal on *B. subtilis* after 5 hr. incubation with 25 mcg/ml. *B. subtilis* could not survive after 30 min. treatment with the concentration of 50 mcg/ml or more of imoticidin.

6. *Comparisons of imoticidin with other known actinomycete antibiotics*

There are several reports on the classification of antifungal antibiotics. The antifungal antibiotics were classified by means of ultraviolet absorption spectra by Utahara and others (1954), and Yajima (1955) reported the classification by summarized papergram. Sumiki (1955) described his opinion on the classification of actinomycetes producing antifungal antibiotic by means of the ultraviolet absorption spectra and other characteristics. And, he classified the isolates of actinomycete in 9 groups. *Streptomyces albus* B12-2 and C1-2 described here were regarded as the actinomycete belonging to Groupe VIII according to Sumiki's classification. Blasticidin B and C which repoted by Fukunaga and others (1955) have no ultraviolet absorption, but they differed from imoticidin in the pattern of summarized papergram, antimicrobial spectrum and other properties. According to Yajima's report, Hygroscopin (Nakazawa and others, 1954), Mycelin (Aiso, 1952), Trichonin and Ascosin 1st substance presented the similar pattern of summarized papergram with imoticidin. These antibiotics have their own characteristic absorption spectra of ultraviolet ray, and they have no effect on *Bacillus subtilis*. On the contrary, imoticidin showed no absorption of ultraviolet ray at any wave length, but it was effective to *B. subtilis*. It was regarded also that imoticidin differed from any other known antifungal antibiotics, with its pattern of infrared absorption. Therefore, the identification was made that imoticidin reported in this paper was apparently a new antifungal antibiotic.

V. PRODUCTION OF IMOTICIDIN IN SOIL BY *STREPTOMYCES ALBUS C1-2* AND THE EFFECT OF IMOTICIDIN TO *CORTICIUM CENTRIFUGUM* IN SOIL

It is reasonably acceptable that the antagonism in soil may be understood by seperating into 3 phases (Asuyama, 1957). The first phase is the parasitism of the antagonist, and the second phase is the exclusive posession of nutrient by the antagonist, and the third is the antibiotic production by the antagonist. In the preceding chapters, the authors obtained an antibiotic, imoticidin, produced by the antagonist effective to *Corticium centrifugum* in sterilized and unsterilized soil. The antibiotics produced by fungi such as gliotoxin, viridin, griseofulvin were detected from soil, (Jefferys, 1952; Wright, 1952—1956) but, the antibiotics produced by actinomycete were not detected chemically from soil, except the chloromycetin produced by *Streptomyces venezuerae* (Siminoff & Gottlieb, 1951; Gottlieb et al, 1952; Martin & Gottlieb, 1952; 1955).

In the present chapter, production of imoticidin in sterile soil, the role of imoticidin in the preventive effect of *Streptomyces albus* C1-2 against *C. centrifugum*, was studied together with the fate of imoticidin in soil. Though the details of experimental methods should be described case by case, the general methods and materials of the experiments were as follows:

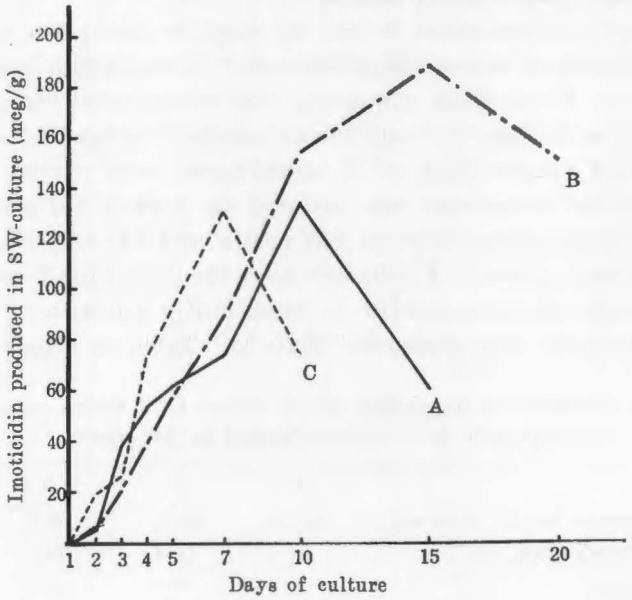
The antagonist, *Streptomyces albus* C1-2, and the test fungi, *Corticium centrifugum*, were cultured and treated in the same way as in the soil antagonism method mentioned previously. Imoticidin crystals dissolved in small amount of methanol was diluted with water in regular concentration and mixed thoroughly with soil. Imoticidin was assayed by the paper disk method, using *Bacillus subtilis* PCI 219 as the test organism on meat-peptone-agar. The summarized papergram of the soil extract, together with the expansion of inhibition zone by the bacteriolysis as the characteristics of imoticidin were observed. Standard diffusion curves of imoticidin were figured at every assay. Imoticidin in soil was determined by the following method. The soils in 5 petri-dishes were dried in 17—18 hr. at 30°C, and 20 g of the soil taken from each petri-dish was extracted with 35 ml methanol in 17—18 hr. at room temperature. They were re-extracted twice within 10 minutes with 15 and 10 ml of methanol. All the extracts were mixed and assayed the concentration of imoticidin. Experiments were repeated 3 times and averages were calculated.

1. *The production of imoticidin in sterilized soil*

As shown in the preceding chapter, imoticidin was detected in water, methanol or acetone extract of SW-culture of *Streptomyces albus* C1-2. The production of imoticidin in sterile soil was confirmed and assayed here in various conditions.

a) The amount of imoticidin produced in SW-culture of *S. albus* C1-2 after various culture periods

Fig. 14. The amount of imoticidin produced in SW-culture of *S. albus* C1-2 after various culture period



S. albus C1-2 were cultured on SW-media and the amount of imoticidin in the soil culture was assayed after 1, 2, 3, 4, 5, 7, 10 and 15 days incubation at 30°C. The results are summarized in Figure 14. The amount of imoticidin in SW-media was raised to the sufficient concentration for paper disk assay after 2 days incubation. The incubation period when the amount of imoticidin was raised to the maximum, and the maximum amount of imoticidin in SW-media differed in every 3 repeated experiment. But, 130 mcg/g (dried soil) or more of imoticidin was detected in these soil media. Imoticidin in SW-culture reduced rapidly after it had been raised to the maximum, but it was detectable for assay even after about one month.

Relation between the amount of imoticidin and the number of viable cells of *S. albus* C1-2 in SW-culture was examined. In Table 15, the number of

Table 15. Alteration of number of viable cells of *Streptomyces albus* C1-2 in SW-culture (cf. Fig. 14)

Days of culture	1	2	3	4	5	7	10	15
Number of viable cells in per g of soil culture ($\times 10^6$)	5.9	11.2	50.8	77.1	181.7	190.2	5400.5	4685.3

viable cells in Experiment A, which had been shown in Figure 14, are shown. The number of cells in soil culture differed in every experiment as similar as the concentrations of imoticidin. These difficulties might be caused by the slight differences of water content of SW-media and other unavoidable causes of cultural condition.

b) Production of imoticidin by *S. albus* C1-2, which prevents the growth of *C. centrifugum* in SW-media

Experiment was conducted to find out whether imoticidin was produced or not in SW-media in which the growth of *C. centrifugum* was prevented by *S. albus* C1-2. Petri-dishes containing SW-media were seperated into 4 sets and treated as follows: (1) only the antagonist *S. albus* C1-2 was cultured (SW-culture), (2) fungus disks of *C. centrifugum* were placed on the SW-media in which the antagonist was cultured for 2 days, (3) both organisms were introduced simultaneously in SW-media and (4) only the test fungi *C. centrifugum* was grown. All the sets were incubated for 7 days at 27°C, and the concentration of imoticidin in these media was assayed. Also, the growth of test fungi was measured (Plate I). Table 16 shows the results.

Table 16. Production imoticidin by *S. albus* C1-2 which preventing the growth of *C. centrifugum* in SW-media

	1	2	3	4
Imoticidin production (mcg/g dried soil)	136.0	113.5	83.7	0
Growth of *C. centrifugum* (%)		14.4	64.3	100.0
Amount of sclerotia		−∼±	±∼+	+∼++

1 : *Streptomyces albus* C1-2 only
2 : *C. centrifugum* was introduced 2 days after the establishment of *S. albus* C1-2
3 : Both of the organisms introduced simultaneously
4 : *C. centrifugum* only

Imoticidin was produced in SW-media, in which only the antagonist or both of the antagonist and the test fungi was grown. The amount of imoticidin produced in SW-media, in which the test fungi and the antagonist had been introduced simultaneously, amounted to about 64% of that of the SW-culture of the antagonist. And, about 82% of imoticidin was detected from the SW-media, in which the antagonist had been introduced 2 days before the test fungi. The amounts of growth of *C. centrifugum* in the treatment (2) and the treatment (3) were 14.4 and 64.3% of that in the treatment (4), respectively.

Gottlieb and Siminoff (1952) reported that less and delayed production of chloromycetin resulted in the soil when *S. venezuerae* and *B. subtilis* were grown together. Similar result as in the case of chloromycetin was obtained in the present experiment.

c) Influence of fertilizer on the production of imoticidin

The production of chloromycetin by *S. venezuerae* was affected by organic materials added in soil, according to Gottlieb and Siminoff (1952), and the actinomycete was not antagonistic to *B. subtilis* in unamended soil.

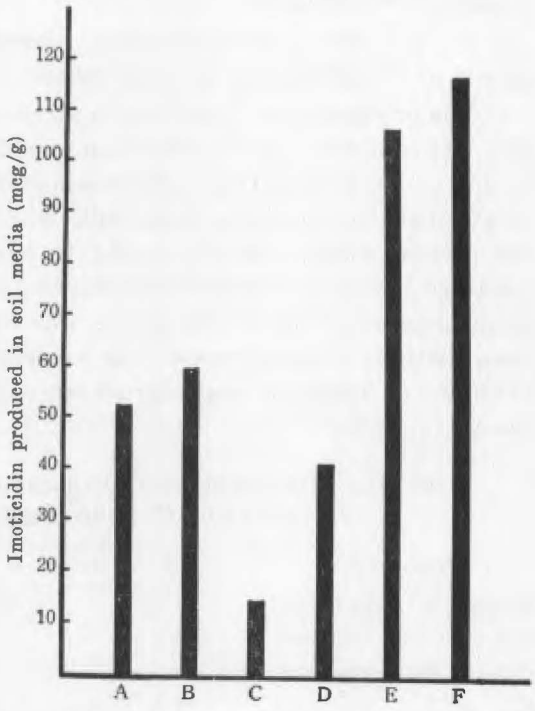
Influence of fertilizer such as ammonium sulfate, potasium nitrate, superphosphate and lime on the production of imoticidin was examined. Each fertilizer in the amount of 1% was added to garden soil. As the control,

SW-media which contained garden soil, wheat-bran, sugar and lime, and the nontreated garden soil were prepared. *S. albus* C1-2 was cultured in these soil media in 7 days at 30°C, and the amount of imoticidin was assayed. The pH of soil media after the autoclaving was as follows:

Fertilizer added	pH	Fertilizer added	pH
Ammonium aulfate	5.5	Lime	7.5
Potasium nitrate	5.6	Lime, sugar (SW-media)	7.5
Superphosphate	5.6	None (garden soil)	5.9

The average of the results of experiments repeated 3 times was obtained as seen in Figure 15. About 50 mcg/g imoticidin was produced even in the non-treated garden soil. The addition of potasium nitrate, and superphosphate checked the production of imoticidin, but ammonium sulfate and lime showed apparent production. Espcially, lime was an important component for the imoticidin production. It proved 91% of imoticidin in the SW-culture of the antagonist and was twice that of the nontreated garden soil.

Fig. 15. Influence of fertilizer on the production of imoticidin in soil media



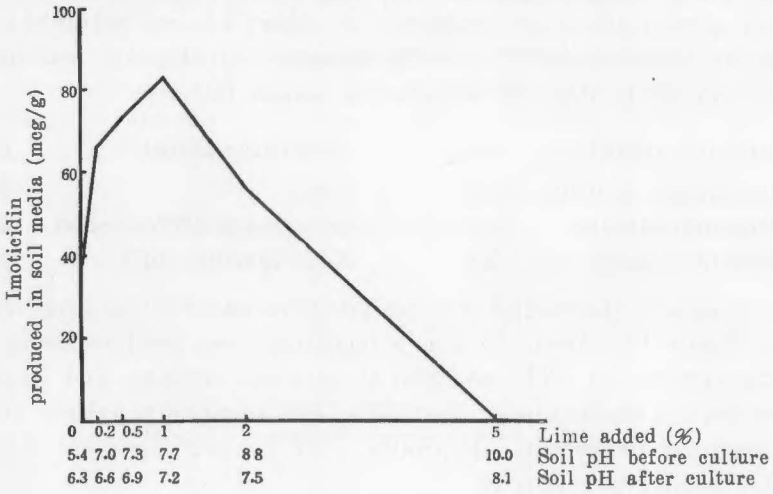
A: None
B: Ammonium sulfate
C: Potasium nitrate
D: Superphosphate
E: Lime
F: Lime and sugar (SW-media)

d) Effect of lime on the production of imoticidin

Wright (1954, 1955) showed the effect of lime on the production of gliotoxin by *Trichoderma viride* and griseofulvin by *Penicillium nigricans.*

The authors crried out the experiment varying the content of lime in soil media. *S. albus* C1-2 was cultured for 7 days at 30°C on soil media, in which lime was added 0.2, 0.5, 1, 2 and 5 % of soil media. The amount of imoticidin produced in the media appears in Figure 16. The greatest production of imoticidin was observed in the soil media, in which 1% lime was added, but no production in the soil that contained 5% lime. The concentration of imoticidin in that soil media was about twice as much as in the soil that con-

Fig. 16. Effect of lime on the production of imoticidin



tained no lime. By culturing the antagonist the pH of soil media was altered towards the neutrality.

2. *The role of imoticidin in the preventive effect of S. albus C1-2 on the growth of C. centrifugum in sterilized soil*

The production of imoticidin in sterilized soil (SW-media) by the antagonist was confirmed in the preceding experiments. This was not a sufficient evidence to understand the antagonism of *S. albus* C1-2 against *C. centrifugum* in sterilized or in unsterilized soil, which was described in former part of the present study. But, it might be agreeable to assume that imoticidin produced in soil by *S. albus* C1-2 played an imortant role on the antagonism, as the amount of imoticidin in soil was considered sufficient to prevent the the growth of *C. centrifugum*. The experiment, containing various treatment as shown in Table 17, was carried out to determine the role of imoticidin in the antagonism.

Table 17. Treatments of experiment for the analysis of preventive effect on *C. centrifugum* in sterile soil

Treatment	A	B	C	D	E	F	G	H
Cultured *S. albus* C1-2					+	+	+	+
Extracted with methanol	+	+			+	+		
Supplied sugar and wheat-bran		+		+		+		+
Soil pH after autoclaving	7.2	7.2	7.2	7.2	6.8	7.0	7.0	6.8

+ : Treated.

A~D : Garden soil containing lime ; E~H : SW-media cultured *S. albus* C1-2

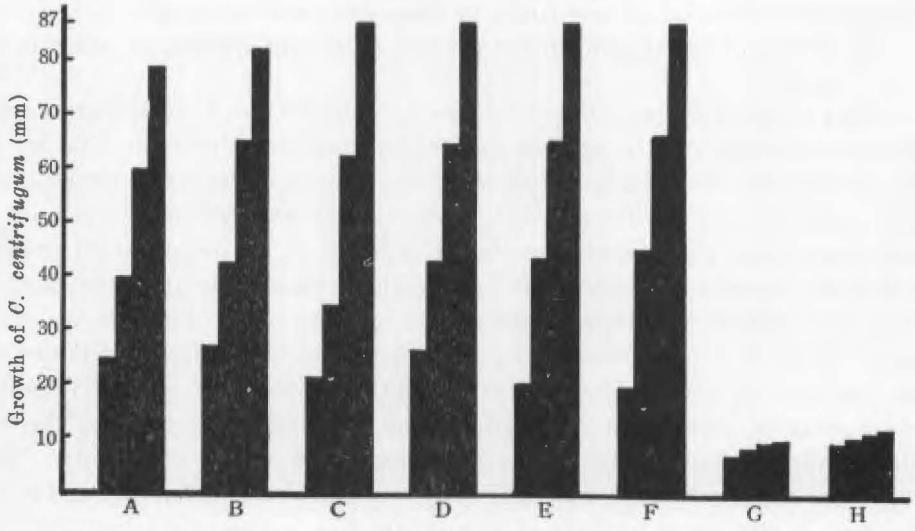
Soil media of A~D or E~H were mixed thoroughly and the halves of them (600 g) were extracted with methanol.

Every treatment contained 10 petri-dishes with every 30 g of soil media.

The SW-cultures of *S. albus* C1-2 in 40 petri-dishes, each containing 30g soil media, cultured for 7 days at 30°C, were dried 17—18 hr. at 30°C. The half of the dried SW-cultures was extracted 17—18 hr. at room temperature with one and a half quantity of methanol. And then, it was reextracted three times with the equal quantity of methanol. SW-culture, after removing methanol soluble substances, was air dried and was seperated into 3 parts for the treatments shown in Table 17. Sterilized soil, in which lime was added, was also seperated into 2 parts, one was extracted with methanol and the other was not, and used for the experiment. Another part of SW-culture not extracted with methanol was supplied with the nutrient sauces (sugar and wheat-bran) and prepared them for the treatments as shown in the table.

Each 300 g sample of soil media contained in 10 petri-dishes had one of the following treatments : had been or had not been cultured with the antagonist, had been or had not been extracted with methanol, or had been or had not been supplied with the nutrients. The fungus disks of *C. centrifugum* were placed on the soil media in these petri-dishes after autoclaving. Growth of the test fungi was measured after 2, 3, 4 and 5 days of incubation. The results are summarized in Figure 17.

Fig. 17. Effect of methanol extractable substance in soil culture of *Streptomyces albus* C1-2 (cf. Table 17)



Diameter of colonies of *C. centrifugum* after 2, 3, 4 and 5 days of culture

Methanol extraction and supply of nutrient in the uncultured soil media had very little effect and good growth of *C. centrifugum* was observed (cf. A vs. B and C vs. D in Figure 17). The effect was strong, however, by the methanol extraction in the soil media that were cultured with the actinomycete. Marked inhibition of growth of test fungi was observed in the soil media

that were either not extracted or supplied with the nutrient (cf. G and H in Fig. 17). On the contrary, the test fungi grew well in the soil media that were extracted with methanol similar in extent as was in the soil media which the actinomycete had not been cultured upon (cf. E, F, and C, D in Figure 17).

The result suggested that methanol extractable substances including imoticidin played an important role in the antagonism in sterilized soil.

3. *Effect of imoticidin on the growth of C. centrifugum in soil*

Antibiotics added to soil are generally adsorbed by soil or decomposed by soil microorganisms, and strongly reduce their activities. Only 10% of actinomycin (Martin and Gottlieb, 1955) was revealed from soil, in which the antibiotic of 500 mcg per g of soil had been added. Terramycein (Martin and Gottlieb, 1952) prevented the growth of *B. polymyxa* at the concentrations of 200 mcg and 500 mcg per g of soil at pH 6.2 and 5.6, respectively. Chloromycetin (Gottlieb and Siminoff, 1952) prevented *B. subtilis* at the concentration of 10 or 15 mcg per g of soil.

The authors suggested in the previous experiment, that imoticidin produced in sterile soil by *S. albus* C1-2 played an important role in the growth inhibition of *C. centrifugum*, and further experiments were carried out, to confirm the effect of imoticidin on the test fungi. In the experiments to follow, sterilzed or unsterilized soil was treated with pure imoticidin, and the growth inihibition of test fungi in these soils was examined.

a) Effect of imoticidin on the growth of *C. centrifugum* in unsterilized soil

Two pieces of fungus disks, 10 mm in diameter, of *C. centrifugum* were placed on each sample of garden soil which contained thorough mixture of 10, 25, 50 and 100 mcg/g (dried soil) of imoticidin prepared respectively with distilled water. These disks were slightly covered with the soil. A large glass plate was placed over the petri-dishes as a cover to avoid growth of various Mucor and Aspergillus fungi, which usually became luxuriant in those petri-dishes containing unsterilized garden soil. Petri-dishes were placed at room temperature. The growth of the test fungi on soil surface was measureable within 3—6 days of incubation (October 18—21, 1957). After 7 days or more incubation, the soil became dry and the growth of the test fungi stopped. The result of the experiment was as in Figure 18. The growth of the test fungi was markedly prevented until 3rd day of the experiment at the concentrations of 50 and 100 mcg/g imoticidin, but not so at the concentrations less than 25 mcg/g. Prevention was not so apparent even at 100 mcg/g after 4 days or more incubation. It was concluded that the concentration of imoticidin effective on *C. centrifugum* in natural gardin soil was 50 mcg/g or more until 3rd day at room temperature.

b) Effect of imoticidin on the growth of *C. centrifugum* in sterilized soil

Steamed garden soil was mixed thoroughly with the solutions containing

various concentrations of imoticidin as was done in the previous experiment. To each of the 5 petri-dishes containing 30 g soil mixed with imoticidin, two pieces of fungus disk of *C. centrifugum* were placed on the surface of the soil and cultured for 11 days at 27°C. The growth of test fungi was measured daily. In Figure 19, the summarized result obtained from the averages of triplicated experiments is shown. Although the mycelium of test fungi completely covered the surface of the soil media, the higher the concentration of imoticidin in soil, the more prevention of growth of the test fungi was observed. According to the result of 4th day of the experiment, the differences among every treatment excepting the difference between 50 and

Fig. 18. Effect of imoticidin on the growth of *C. centrifugum* in unsterile soil

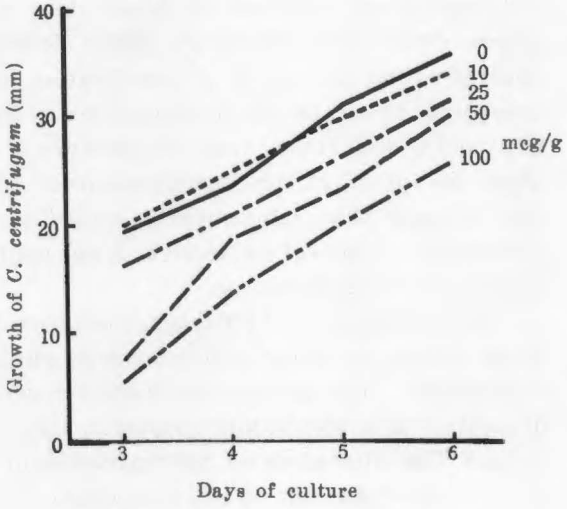
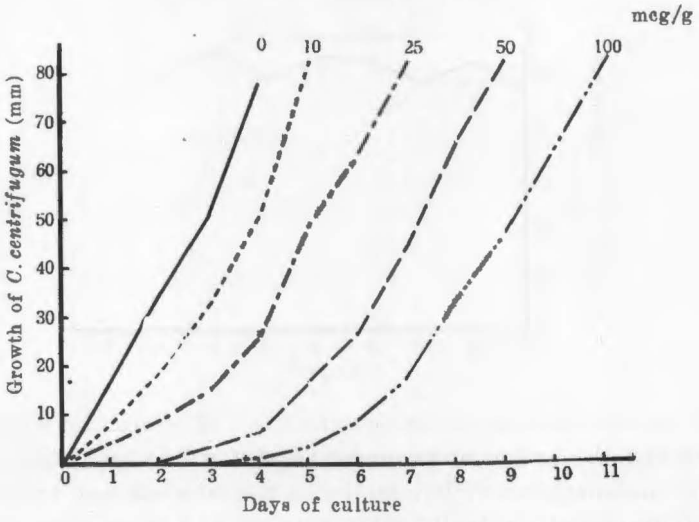


Fig. 19. Effect of imoticidin on the growth of *C. centrifugum* in sterile soil



100 mcg/g imoticidin treatment were significant under 1% level. The growth of *C. centrifugum* was apparently prevented for 2 or 3 days with the treatment of 10 mcg/g imoticidin, 4 days with 25 mcg/g, 6 days with 50 mcg/g and 8 or 9 days with 100 mcg/g, but not prevented after these periods.

4. *The stability of imoticidin in soil*

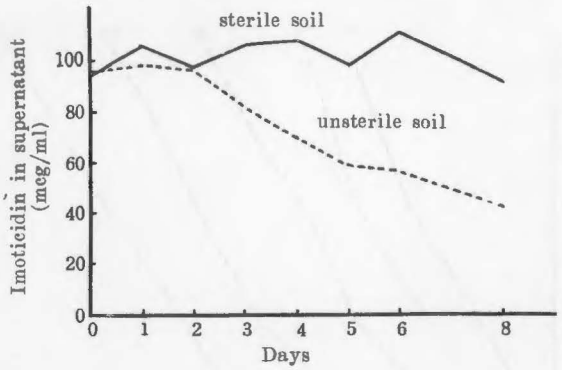
It has been reported that many of the antibiotics were incactivated in soil, and it was difficult to detect these antibiotics form soil (Gottlieb and others, 1951—1955; Gregory, 1952; Jefferys, 1952). It has been considered generally that the cause of inactivation of antibiotic in soil is due to the adsorption by soil or the decomposition by soil microorganisms. Gottlieb and Siminoff (1952) stated that the recovery of chloromycetin in sterile soil was about 60—70% at the concentrations of 100 mcg per g of soil or less. And they showed that chloromycetin added in unsterile soil was rapidly inactivated 2 or 3 days after, though it was maintained about 80% of the activity after 2 weeks in sterile soil.

Imoticidin in soil (100 mcg/g) prevented the growth of *C. centrifugum* in 9 and 3 days in sterile and nonsterile garden soil, as shown in the previous experiment. The authors made some experiments to determine the stability of imoticidin in sterile and nonsterile soil.

a) The alteration of concentration of imoticidin solution, in which a small amount of soil was added

To each 20 ml of 100 mcg/ml imoticidin solution held in test tubes 5 g of either sterile or nonsterile soil was introduced. Two test tubes were used for each treatment. The concentration of imoticidin in supernatant was assayed daily. Figure 20 shows the averages of experiments repeated three

Fig. 20. Antibiotic potency of supernatant of imoticidin solution, in which a small amount of soil was added



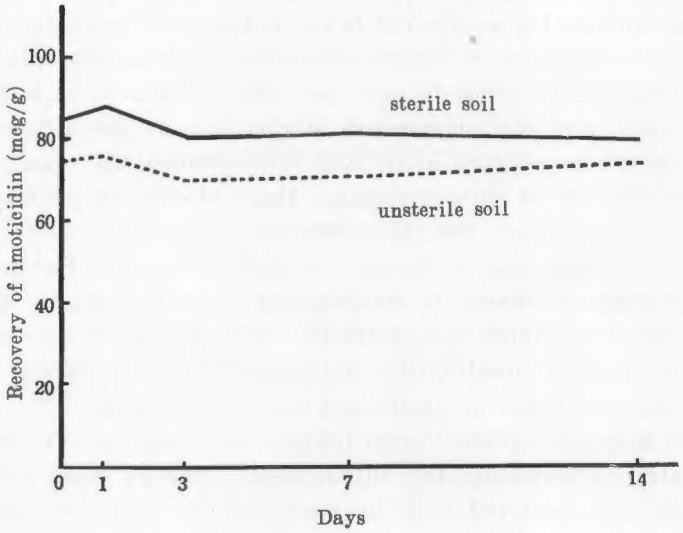
times. Apparent decrease in the concentration of imoticidin was not observed even after 8th day in the supernatant treated with sterile soil. On the other hand, the concentration of imoticidin in the supernatant treated with nonsterile soil decreased gradually after 3rd day, and about 60% imoticidin was inactivated on 8th day. In the supernatant of imoticidin solution treated with nonsterile soil, various soil microorganisms made luxuriant growth. From the result obtained here, it is suggested that only a small amount of imoticidin was adsorbed by sterile soil. And, it seems that the reduced part

of imoticidin concentration in the supernatant treated with unsterilized soil might be caused by the decomposition of imoticidin by some soil microorganisms.

b) The alteration of imoticidin concentration in soil, which was moistened with imoticidin solution

Each 10 g of either sterilized or unsterilized soil was moistened with 5 ml of 200 mcg/ml imoticidin solution (1mg imoticidin per 10 g of soil). Imoticidin was extracted and assayed immediately, and after 1, 3, 7 and 14 days. The recovery of imoticidin was made as follows : Soil (10 g) was extracted 5 times each with 10 ml of methanol in 10 minutes at room temperatuer. Every methanol extract was assayed for imoticidin concentration. The amount of recoverable imoticidin was assumed from the integrating curve obtained from the antibiotic potencies of these successive extract. Nearly all of imoticidin in soil was recovered by these 5 extractions. The results are shown in Figure 21. Imoticidin recovered from the sterilized soil was 85—90 mcg/g after 24 hr. It decreased slightly after 2 days, but no more reduction was observed even after 14 days. Similar pattern of alteration was observed on the amount of imoticidin recovered from the unsterilized soil, but recoverable imoticidin was about 10 mcg/g less than in sterile soil.

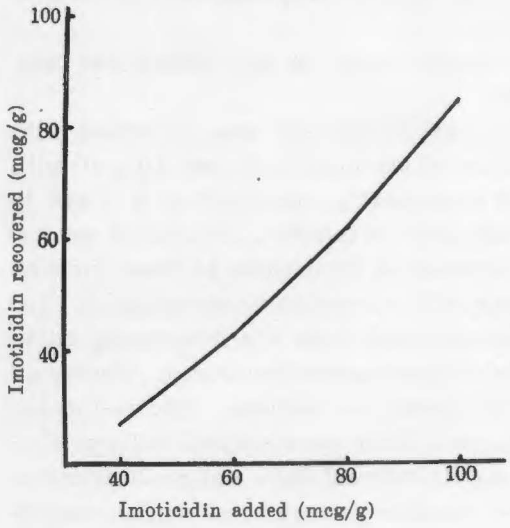
Fig. 21. Alteration of imoticidin concentration in soil



Respectively, about 20 and 25% of imoticidin were inactivated in sterilized and unsterized soil. Though the water content of soil in this experiment differed from the previous experiment, it seemed that the most part of imoticidin inactivated in soil might have been adsorbed by the soil.

c) Recovery of imoticidin from the soil containing antibiotic at various concentrations

Fig. 22. Recovery of imoticidin added in sterile soil



Recovery of imoticidin from the soil that contains antibiotic lower than 100 mcg per g of soil was examined. Imoticidin solutions were mixed with sterile soil at the concentrations of 80 and 100 mcg/g. Recovery of imoticidin was made using the same method as described in the foregoing experiment. Summarized result of experiments repeated 3 times are given in Figure 22. At the concentrations of 40—100mcg/g, about 20—25 % of imoticidin was adsorbed as similar as shown in the previous experiment.

VI. DISCUSSION

Wright and his cooperators studied the antibiotics produced in soil by fungi, in which some of the antagonist to soil pathogenic fungi were included. They showed the production of fungal antibiotics such as gliotoxin, viridin, griseofulvin, frequentin, gladiolic acid and other substances in soil. On the contrary, Gottlieb and his cooperators studied on the production and the fate of actinomycete antibiotics in soil and they obtained the negative results excepting only the case of chloromycetin. Many studies on the antagonistic actinomycete were reported. But, there was no report on antibiotic produced by antagonistic actinomycete so far as the authors knew. The anthors, in this study, obtained 2 isolates of *Streptomyces albus* through the systematic screening test for antagonism and antibiotic, and obtained a new antibiotic, imoticidin, which is produced by the actinomycetes and is effective on some of the soil pathogenic fungi in sterile and in nonsterile soil.

"Modified agar disk method" used for the screening was very convenient at the first step of screening for antagonism. Though some undersirable drying of agar disk occurred from long observation, this method was available, because of the following conveniences; it had: (1) a number of isolates for the 1st screening can be conducted with comparatively a small number of petri-dishes and (2) there is little chance of contamination. The antagonism of isolates was confirmed by "soil antagonism method", which was the 2nd step of screening. The usefulness of this method for screening of antagonist and for study on the interactions between soil fungi was confirmed by varying the conditions of culture.

The isolates selected after the 2nd step of screening were effective antagonists in sterile soil, and it was considered that most of them should produce antibiotic in their cultural agar. As the final step of screening, control effect of the antagonists on damping-off of cowpea by *Corticium centrifugum* was examined by using nonsterile garden soil. Two isolates of Streptomyces exhibited control effects on the disease, and they were decided as the effective antagonist in nonsterile condition. These two isolates were identified as a strain or a variant of *Streptomyces albus*. The growth of these isolates B12-2 and C1-2 apparently differed as to their cultural habits.

The antibiotic production in the liquid cultures of these two antagonists was confirmed, and imoticidin, a new antibiotic, was extracted. The specific antibiotic effect on gram-positive bacteria and some of plant pathogenic fungi such as *Piricularia oryzae* and *Botrytis cinerea*, bacteriolytic and bactericidal activity on *Bacillus subtilis* PCI 219 or non absorption of ultraviolet ray were the marked characteristics of this antibiotic.

The antibiotic detected in water, methanol or acetone extract of the soil culture of *S. albus* B 12-2 or C1-2 was identified as imoticidin; and this led the authors to further study on the role of imoticidin in the antagonism. Imoticidin was produced in sterile soil with a similar pattern as that for chloromycetin of *Streptomyces venezuerae*. Chloromycetin, according to Gottlieb and Siminoff, when produced in heavily amended sterile soil, amounted to 25 mcg/g. But, in the case of imoticidin, more antibiotic, about 130 mcg/g or more was produced in SW-culture of *S. albus* C1-2. Although the minimum concentration for complete inhibition of *Corticium centrifugum* on agar media was much more than 100 mcg/ml, the growth of *C. centrifugum* was partly prevented at 1 mcg/ml. Therefore, the authors believe that the amount of imoticidin produced in soil was sufficient to prevent the growth of *C. centrifugum*. Some of fertilizers or organic materials and lime help the growth of the antagonist and the production of imoticidin. Especially, lime was very effective component among soil amendments tested. Gottlieb and Siminoff showed that chloromycetin was not produced in unamended sterile soil. But in the case of imoticidin, about 50 mcg/g imoticidin was produced even in unamended sterile soil.

The role of imoticidin was suggested by the experiment on the effect of methanol extraction on the growth of *C. centrifugum*. Methanol extractable substances in sterile soil media showed no effect on the antagonism. And, it might be reasonable to consider that imoticidin in methanol extractable substances in the soil media after culturing the antagonist was the main factor which exhibited growth inhibition of *C. centrifugum*. Further, the stability of imoticidin in unsterilized soil suggested the role of imoticidin in the controlling effect on damping off of cowpea as shown in the early part of the present study. From the results obtained in sterile condition, however, it seemed unsufficient to conclude so simply that imoticidin was also the

chief factor of the antagonism in unsterilized soil.

Aknowledgements

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SUMMARY

This study deals with the antogonism of *Streptomyces albus* B12-2 and C1-2 in sterile or in nonsterile soil, a new antibiotic, imoticidin, produced by the antagonistic actinomycetes, and the role of the antibiotic in the antagonism.

1. Two isolates of *Streptomyces albus*, namely B12-2 and C1-2, were selected from 1083 isolates of actinomycete through several steps of screening for antagonism and antibiotic. Modified agar disk method was used for the first screening, and soil antagonism method for the second screening. In the final step of the screening, *S. albus* B12-2 and C1-2 exhibited controlling effect in nonsterile garden soil on damping off of cowpea caused by *Corticium centrifugum*.

2. In order to find the best condition under which the soil antagonism method can be applied to antagonism test, further experiment was carried out in detail varying the conditions of experiment as follows; the amount of antagonist introduced in soil, the time when the antagonist was introduced, the kinds of organic material used as soil amendment, the soil type, and the water content of soil.

3. A new antibiotic, imoticidin, produced by the antagonists in their liquid or soil cultures was extracted and purified. Imoticidin was strongly antibiotic on gram-positive bacteria, yeasts and some plant pathogenic fungi such as *Piricularia oryzae*, *Botrytis cinerea* and others. Bacteriolytic and bactericidal effects were observed on *Bacillus subtilis* PCI 219. Imoticidin was soluble in methanol, ethanol, butanol, chloroform, acetone and other organic solvents, but not in water. Purified crystal of imoticidin was darkened near 210°C and melted at 245—246°C. Annalysis of element was as follows: C, 64.71; H, 9.50; N, 0; H_2O, 7.63; ash, 3.16; S, 0; Halogen, 0. Molecular weight was 534—553. Imoticidin had no property of absorption of ultraviolet ray at any wave length from 220—300 mμ.

4. Imoticidin was produced even in unamended sterile garden soil. About 130 mcg/g or more of imoticidin was produced under favourable condition. Lime was an important component for the production of imoticidin. Imoticidin added in sterile or nonsterile soil prevented the growth of *C. centrifugum*. The most part of the imoticidin added to soil was recoverable from

sterile or nonsterile soil. Methanol extractable substance, including imoticidin, in soil culture of *S. albus* C1-2 played important role in the antagonism. Imoticidin was considered the principal factor in the antagonism in the soil used.

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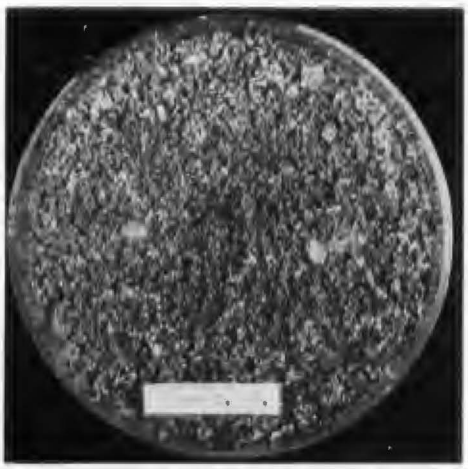
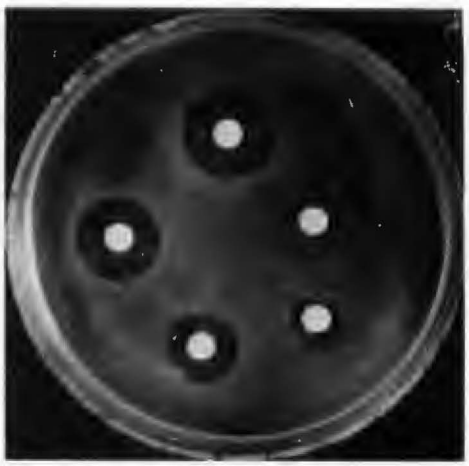
Plate I

Soil antagonism method (4 days culture)-(1)
Corticium centrifugum only.

Soil antagonism method (4 days culture)-(2)
Antagonist and test fungi were introduced simultaneously.



Soil antagonism method (4 days culture)-(3)
C. centrifugum was introduced 2 days after the establishment of *Streptomyces albus* C1-2.



Assay of imoticidin
Concentration of imoticidin: 100, 75, 50, 25 and 10 mcg/ml (anticlockwise from the top)