STUDIES IN ANTIBIOSIS BETWEEN BACTERIA AND FUNGI.

II. Species of Actinomyces Inhibiting the Growth of Colletotrichum gloeosporioides Penz. in Culture

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In the first paper of this series[1] the writer and his associates reported that *Actinomyces albus* Krainsky was found to inhibit the growth of all ten of the species of fungi against which it was tested in culture. The inhibitory effects were shown to be due to some chemical substance toxic to the fungi which the inhibiting organism probably manufactured in the medium. It was stated there, that during the past two years the writer "has observed that the colonies of organisms belonging to this genus (Actinomyces), occurring as contaminations in Petri dishes in the routine laboratory work with fungi, frequently inhibited the growth of many fungi." These observations led to experiments with a large number of species of Actinomyces in order to determine how universally such toxic substances are produced by members of this genus.

The fungus employed in these experiments was a monoconidial isolation of *Colletotrichum gloeosporioides* Penz. isolated from diseased twigs of *Citrus sinensis* Osb. from Gargalianoi, Greece. This was grown together with 80 different Actinomycetes obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, as follows:

A. albidoflavus (R. D.) Gasp.
A. albidus Duché.
A. alboflavus W. et C.
A. albosporeus Krainsky.
A. albus (R. D.) Gasp.
A. albus (R. D.) Gasp. var. *alpha* Cif.
A. albus (R. D.) Gasp. var. *cretaceous* (Krüger) Wr.
A. albus (R. D.) Gasp. var. *ochroleucus* (Neuk.) Wr.
A. almquisti Duché
A. annulatus Beij.
A. asteroides (Ep.) Gasp.
A. aureus W. et C.
A. baarnensis Duché.
A. bobili W. et C.
A. bovis Harz.

[1]Ohio Jr. Sci., 38: 221-235, 1938.

A. buccalis (Wins.) West.
A. cacaoi Waks.
A. californicus W. et C.
A. canis (R. D.) West.
A. caprae (Sil.) Lieske.
A. carnosus Mill. et Burr.
A. cellulosae Krainsky.
A. chromogenus Gasp.
A. citreus Gasp.
A. clavifer Mill. et Burr.
A. craterifer Mill et Burr.
A. dassonvillei (B. R.) West.
A. deri (R. D.) West.
A. diastaticus Krainsky.
A. epidermidis (Rosenb.) West.
A. farcinicus (De T. et Trev.) West.
A. fimicarius Duché.
A. flavogriseus Duché.
A. flavovirens Waks.
A. flavus Krainsky.
A. flavus Mill. et Burr.
A. fradii W. et C.
A. gougeroti Duché.
A. griseus Krainsky.
A. halstedii W. et C.
A. heimi Duché.
A. intermedius Wr.
A. keratolytica Act. et McG.
A. krainskii Duché.
A. langeroni Salvanet-Duval.
A. lipmanii W. et C.
A. luteus (Ch. et Ar.) West.
A. maculatus Mill. et Burr.
A. madurae (Vinc.) Lehm. et Neum.
A. microflavus Krainsky.
A. nigrificans (Krüger) Wr.
A. odorifer (Rull.) Lieske.
A. oligocarbophilus Beij.
A. olivaceus Waks.
A. orangicus (R. D.) West.
A. pelletieri (Lav.) West.
A. pheochromogenus Conn.
A. praecox Mill. et Burr.
A. purpeochromogenus W. et C.
A. reticuli W. et C.
A. roseodiastaticus Duché.
A. roseus Krainsky.
A. ruber Krainsky.
A. rutgersensis W. et C.
A. salmonicolor Mill. et Burr.
A. sampsonii Mill. et Burr.
A. sanfelicei (Red.) West.
A. sanninii (Cif.) West.
A. scabies (Thaxt.) Güssow.
A. setonii Mill. et Burr.
A. sulphureus Gasp.
A. tenuis A. Cast.
A. tumuli Mill. et Beeley.
A. tyrosinaticus Krainsky.
A. verne W. et C.
A. violaceus-caesari W. et C.
A. violaceus-ruber W. et C.
A. viridis Mill. et Burr.
A. viridochromogenus Krainsky.
A. xanthostroma Wr.

No attempt was made to verify the identity of the various species, the names used being those supplied with the cultures by the Centraalbureau voor Schimmelcultures. All specific names regardless of their derivation are spelled in lower case letters.

Each species of Actinomyces was grown together with *Colletotrichum gloeosporioides* in the same Petri dish. The fungous inoculum was placed in the center of the Petri dish while the Actinomyces was inoculated two centimeters away from the center on opposite sides. The fungus was inoculated five days after the Actinomyces so as to give the latter an opportunity to become well established. The fungus was inoculated by means of a small loopful of a water suspension of conidia. The Actinomyces was inoculated by using a small loopful of a heavy, water suspension of the organism and dividing it between the two points of inoculation in the same Petri dish.

The medium employed was prepared according to the following formula:

Maltose	30.00	gr.
$MgSO_4$	.50	gr.
K_2HPO_4	1.00	gr.
KCl	.50	gr.
$FeSO_4$	.016	gr.
$NaNO_3$	2.00	gr.
Agar	20.00	gr.
Water	1000.00	cc.

Sufficient N/1 NaOH was added to the medium so that after sterilization the pH was approximately 7.4 .15 cc. of agar were used to the Petri dish. All tests were carried out in triplicate. Ten Petri dishes inoculated with the fungus alone served as controls. Observations on growth were made at intervals of 24 hours, and the inhibitory distances[2] recorded as in previous experiments.

According to the results obtained in these experiments, the 80 forms of Actinomyces studied can be classified into three groups with reference to their effect upon the growth of *C. gloeosporioides* in culture, as follows:

1. *Strong inhibitors.* Forms which inhibited the growth of the fungus at an inhibitory distance of 10 or more millimeters.
2. *Weak inhibitors.* Forms which inhibited the growth of the fungus at an inhibitory distance of less than 10 mm.
3. *Non-inhibitors.* Forms which had no effect upon the growth of the fungus.

The Actinomycetes are classified accordingly in Table I. It will be seen from this table that 45 (56.25%) of the cultures of Actinomyces tested, inhibited the growth of *C. gloeosporioides* while 35 (43.75%) had no effect upon the growth of the fungus. Of the inhibiting organisms 14 (31%) were strong inhibitors while 31 (69%) were weak inhibitors.

It now seemed advisable to test the ability of filtrates from liquid cultures of a few species of Actinomyces belonging to each of the above three groups, to retard the growth of the fungus. Accordingly, a liquid medium of the following formula was prepared:

Glucose	30.0	gr.
Peptone	5.0	gr.
KH_2PO_4	1.0	gr.
Water	1000.0	cc.

Sufficient NaOH was added to impart a pH 7.4 to the sterilized medium. No effort was made, however, to regulate the pH very accurately because previous experiments had shown that even rather large variations in pH did not seem to have an effect on inhibitory phenomena. 20 cc. of the medium were used to each 200 cc. flask.

From each Actinomycete group, three species were selected for this experiment. From the strong inhibitors, *A. ruber*, *A. flavus* Kr. and

[2]Ibid, p. 223.

A. oligocarbophilus were used; from the weak inhibitors, *A. bobili*, *A. flavovirens*, and *A. chromogenus*, while from the non-inhibitors, *A. albosporeus*, *A. sulphureus*, and *A. canis* were selected.

Three flasks of medium were inoculated for each of the above species. Four flasks were also inoculated with *C. gloeosporioides*, and four others

TABLE I

ACTINOMYCES SPECIES CLASSIFIED ACCORDING TO THEIR ABILITY TO INHIBIT THE GROWTH OF *Colletotrichum gloeosporioides* IN CULTURE ON MALTOSE AGAR

Strong Inhibitors	Weak Inhibitors	Non-inhibitors
A. albidoflavus	*A. albidus*	*A. albosporeus*
A. alboflavus	*A. albus* var. *alpha*	*A. albus* (R. D.) Gasp.
A. annulatus	*A. albus* var. *cretaceous*	*A. almquisti*
A. clavifer	*A. albus* var. *ochroleucus*	*A. asteroides*
A. farcinicus	*A. aureus*	*A. baarnensis*
A. flavus Kr.	*A. bobili*	*A. canis*
A. flavus Mill. et Burr.	*A. buccalis*	*A. carnosus*
A. krainskii	*A. bovis*	*A. epidermidis*
A. madurae	*A. cacaoi*	*A. fimicarius*
A. microflavus	*A. californicus*	*A. fradii*
A. oligocarbophilus	*A. caprae*	*A. griseus*
A. ruber	*A. cellulosae*	*A. halstedii*
A. sanninii	*A. chromogenus*	*A. heimi*
A. scabies	*A. citreus*	*A. keratolytico*
	A. craterifer	*A. luteus*
	A. dassonvillei	*A. nigrificans*
	A. deri	*A. olivaceus*
	A. diastaticus	*A. orangicus*
	A. flavogriseus	*A. pelletieri*
	A. flavovirens	*A. phaeochromogenus*
	A. gougeroti	*A. roseodiastaticus*
	A. intermedius	*A. roseus*
	A. langeroni	*A. rutgersensis*
	A. lipmanii	*A. salmonicolor*
	A. maculatus	*A. sampsonii*
	A. odorifer	*A. sanfelicei*
	A. praecox	*A. setonii*
	A. purpeochromogenus	*A. sulphureus*
	A. reticuli	*A. tenuis*
	A. verne	*A. tumuli*
	A. xanthostroma	*A. tyrosinaticus*
		A. violaceus-caesari
		A. violaceus-ruber
		A. viridis
		A. viridochromogenus

were left uninoculated to serve as controls. Sixteen days after inoculation, the organisms were filtered off by means of filter paper and the filtrates were used in preparing nutrient agar which after sterilization at 15 lbs. pressure for 15 minutes, was poured into Petri dishes and inoculated with a loopful of a spore suspension of *C. gloeosporioides*. Measurements of the largest and smallest diameter of each colony were made and recorded for seven days. The results are summarized in

Table II. The figures given are the averages of six diameters, one large and one small for each of three colonies. The flasks inoculated with *A. sulphureus* became heavily contaminated with a species of Penicillium and no results were, therefore, obtained for this filtrate.

TABLE II

SEVEN DAYS GROWTH OF *Colletotrichum gloeosporioides* IN AGAR PREPARED FROM VARIOUS FILTRATES, EXPRESSED IN AVERAGE DIAMETERS IN MILLIMETERS, OF THREE CULTURES

Organism from which Filtrate was Prepared	Diameter in mm.
Strong Inhibitors:	
A. oligocarbophilus	18.0
A. flavus Kr	30.5
A. ruber	34.0
Weak Inhibitors:	
A. flavovirens	57.0
A. bobili	60.5
A. chromogenus	63.0
Non-inhibitors:	
A. canis	58.0
A. albosporeus	59.5
A. sulphureus	Contaminated
Controls:	
C. gloeosporioides	49.0
Uninoculated	62.5

The results of these experiments indicate that the growth of *Colletotrichum gloeosporioides* is greatly inhibited by the filtrates taken from strong inhibitors, but not at all by the filtrates from the week inhibitors or non-inhibitors.

If inhibition of fungous growth is due to a toxic substance manufactured in the medium by the Actinomycetes—and there is little question as to that—several theories can be proposed to explain the facts observed.

1. It may be reasonably assumed that the difference between inhibitors and non-inhibitors is the ability of some Actinomycetes to produce a substance toxic to the fungus and the inability of others to manufacture such a substance.

2. The difference between strong and weak inhibitors may be explained either on a quantitative or a qualitative basis or both. If the toxic substance produced by the inhibiting Actinomycetes is assumed to be identical or similar in all species, then the degree of inhibition caused by different species may be a measure of the amount of toxin formed in a given period of time, the strong inhibitors differing from the weak in the speed with which they manufacture the toxin.

If the inhibiting substance is different in each case, however, it may be that that produced by the strong inhibitors is thermostable while that produced by the weak inhibitors is thermolabile. This would explain why the filtrates from weak inhibitors produced no inhibitory effects on the growth of *C. gloeosporioides*.

Furthermore, it is possible that a combination of factors are responsible for the discrepancy shown in the behavior of the weak inhibitors directly through their colonies on agar and indirectly through their filtrates. If, for instance, we assume that these Actinomycetes manufacture a relatively small amount of toxin which is partially or totally thermolabile we would expect results similar to those obtained. The amount of toxin which emanated from the colonies growing on agar may have been sufficient to cause a small amount of inhibition on the neighboring fungous colony, but enough of the toxin may have been destroyed in the filtrates of the liquid cultures when these were subjected to autoclaving so that no inhibitory effects could be produced on the fungus growing on media prepared from such filtrates.

3. Growth of the fungus on its own filtrate agar was much slower as compared to that on the uninoculated controls, as well as that on the weak-inhibitor and non-inhibitor filtrate agars. This seeming autoinhibition may be explained partly on the basis of an accumulation of waste products of metabolism in the liquid culture, and partly on the basis of a partial food depletion of the medium. Previous experiments have shown that food depletion of the medium is not responsible for the inhibitory effects caused by *Actinomyces albus* Kr. and it is reasonable to suppose that this would hold true to a large extent for other Actinomycetes. *Colletotrichum gloeosporioides*, however, produces a much greater growth than any of the Actinomycetes tested and consequently probably uses much more food.

An autotoxic substance similar to the toxin produced by the Actinomycete inhibitors cannot be postulated for *C. gloeosporioides* since two colonies of the fungus growing in the same Petri dish do not inhibit each other, but merge.

In view of the results reported herein, it would be of interest to determine if the production of a fungus-inhibiting toxin by certain Actinomycetes is in any way indicative of the relationship of such species.

This work was carried on in 1939 while the writer, on leave of absence from Kent State University, Kent, Ohio, was associated with the Institut de Chimie et d'Agriculture "Nicolaos Canellopoulos" as plant pathologist, at Piraeus, Greece.

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