A SELECTIVE MEDIUM FOR THE ISOLATION OF *Agrobacterium radiobacter* FROM SOIL

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ABSTRACT

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Smith and Dawson's and Martin's observation that at times large, soft, raised, glistening bacterial colonies developed on the glucose-nitrate-soil-extract media used by them was confirmed. The morphology and physiology of representative isolates from these media indicated that 65% belonged to Agrobacterium radiobacter or were closely related to it. Since a larger proportion of bacterial colonies on the medium containing streptomycin (Martin's) was "radiobacter-like", further studies were confined to this medium.

In an attempt to develop a selective medium, actidione was added at three concentrations to Martin's medium. Most bacteria and many fungi failed to grow in the presence of actidione and those fungi that developed showed less tendency to spread. The effect was most marked at the highest concentration.

Actidione was tested at varying pH levels. Fewer fungi developed at pH 4.7 and at 5.3, and "radiobacter-like" colonies grew well. Eighty-five percent of the bacterial colonies proved to be Agrobacterium radiobacter or were closely related to it.

Rose bengal, streptomycin and actidione, each singly at various concentrations, showed no marked effect. Rose bengal (1 part in 15,000), streptomycin (30 µg./ml.),

and actidione (up to 800 µg./ml.), in combination, showed no marked effect on Agrobacterium radiobacter, whereas the higher concentrations of streptomycin in the same medium inhibited this species. Rose bengal (1 part in 15,000) singly or combined with streptomycin or with actidione inhibited Agrobacterium tumefaciens. Neither streptomycin nor actidione had an effect on this species.

Potassium sorbate at 0.5% (w/v) and nystatin at 2400 units/ml. practically inhibited fungi in Martin's medium.

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INTRODUCTION

INTRODUCTION

Agrobacterium radiobacter (Beijerinck and van Delden) Conn has been under extensive study for many years by various workers--Hendrickson *et al.* (1934), Sagen *et al.* (1934), Smith and Brown (1935), and Hofer (1933, 1935, 1941, 1943)--chiefly because of the difficulty in distinguishing it from species of *Rhizobium* and from certain plant pathogens.

The species, according to Bergey *et al.* (1957), consists of small Gram negative non-spore-forming short rods, occurring singly, in pairs and, under certain conditions, in star-shaped clusters, and typically motile with one to four peritrichous flagella. Distinctive characteristics include: no organic acid or visible gas from carbohydrates; browning of mannitol-calcium-glycerophosphate agar; inability to cause plant disease or to produce nodules on roots of legumes; and, complete utilization of inorganic nitrogen.

Notwithstanding the fact that Kennedy (1950) stated that "neither the nitrate-glycerol agar proposed by Smith (1928) nor Hofer's medium (1943) can be considered selective for *Agrobacterium radiobacter*", the present investigation, using glucose-nitrate-soil-extract agar as a selective medium, evolved from the observations made by Smith and Dawson (1944) and Martin (1950) that in a few

cases with the medium used by them primarily for fungal counts large, soft, raised, glistening bacterial colonies developed.

The studies are presented in five sections each dealing with a stage in the development of a satisfactory medium.

HISTORICAL

HISTORICAL

The medium, glucose-nitrate-soil-extract agar, has been used for many years for studying the numbers of soil microorganisms. Waksman (1922a) and Smith and Worden (1925) observed that higher and more uniform counts of soil microorganisms were obtained on a soil-extract agar when compared with a mannite-salts medium and an egg albumin medium.

In the past, a great deal of extensive work has been carried out by many investigators in developing a fungal medium. Through methods of trial and error, a number of media in which inhibitors that would hypothetically suppress bacteria and actinomycetes but permit fungi to flourish have been tested.

The earlier methods used a naturally acid medium or, a medium artificially acidified by the addition of sulfuric, citric or lactic acids (Waksman, 1922b; Brierly et al., 1928; Smith and Humfeld, 1930; Ludwig and Henry, 1943). The latter method became more or less standard procedure, but nevertheless exhibited several important disadvantages (Martin, 1950).

With these disadvantages in mind, Tyner (1944) attempted to improve on the media used in the plating method for fungi. This investigator asserted that boric acid included in the fungal media effectively suppressed

bacteria and permitted satisfactory counts of fungi in composted soil, and maintained that counts were very much higher than when sulfuric acid was used as the inhibiting agent.

The results of Smith and Dawson (1944) were at variance with those of Tyner (1944). Boric acid in the fungal medium, at the concentrations recommended by Tyner, did not produce the desired results. Smith and Dawson (1944) questioned the findings reported by Eastwood (1944) that anisic acid, benzoic acid, and, to a less extent, chrysoidine Y had selective bacteriostatic action. Benzoic acid and chrysoidine Y were tested in glucose-nitrate-soil-extract agar and in Waksman's medium at the concentrations recommended by Eastwood. Both proved to be unsatisfactory, allowing bacteria to develop and fungi to spread.

Smith and Dawson (1944) suggested the use of rose bengal in the media for plate counts of soil fungi. They compared acid media popular at the time with media of the same composition except that, instead of acid, rose bengal was added after adjustment to pH 6.8. It was found that this dye at 1 part in 15,000 eliminated all actinomycetes and most bacteria and reduced the spreading of the fungal colonies to a minimum. Attempts were made to determine whether the concentration of rose bengal could be reduced in glucose-nitrate-soil-extract agar and, whether

the soil-extract itself made any appreciable difference in fungal counts. They found that decreasing the concentration of rose bengal from 1 part in 15,000 to 1 part in 25,000 resulted in more bacterial colonies on plates, and less inhibition of spreading fungal colonies. They also found that soil-extract in the medium gave higher fungal counts and reduced the toxicity of the dye for the fungi. In conclusion Smith and Dawson stated that the glucose-nitrate-soil-extract agar containing rose bengal at 1 part in 15,000 was the best medium for fungi. They noted that the comparatively few bacteria that survived in the presence of rose bengal produced large, soft, raised, glistening colonies that were readily differentiated from fungal colonies.

In a subsequent study, Dawson and Dawson (1946) carried out an investigation on the use of rose bengal with pure cultures of fungi. This was done in order to establish whether inhibition was due to the dye or to antagonistic effects by other organisms. They concluded that the only evidence of fungistatic activity of the dye was in reducing the size of fungal colonies.

Martin (1950) attempting to improve on the fungal media in use made a comparative study of acid, rose bengal, and streptomycin, singly and in various combinations as inhibitors of bacteria. In all three media tested by Martin, rose bengal (1 part in 15,000) plus streptomycin

(30 µg./ml. medium) or rose bengal (1 part in 30,000) plus streptomycin (30 µg./ml. medium) practically eliminated bacteria and reduced the spreading of fungal colonies to a minimum. Martin observed that bacterial colonies on glucose-nitrate-soil-extract agar were larger than on peptone-dextrose agar. These observations were in direct conflict with those of Smith and Dawson (1944).

Martin (1950) reported that streptomycin was superior to other common antibiotics for use as a bacterial inhibitor in fungal media. He stressed that the streptomycin should be added to the medium just prior to pouring the plates, and not before sterilization.

Actidione was reported by Whiffen et al. (1946), to be a chloroform and water soluble antibiotic produced by Streptomyces griseus. The antibiotic was isolated in pure crystalline form by Leach et al. (1947) who a year later (1948) determined its imperical formula.

Representative species of bacteria were tested by Whiffen (1948), using the broth tube method containing actidione in concentrations up to 1.0 mg./ml., and by Phillips and Hanel (1950) employing the surface plate count method on agar containing actidione at 0.1 mg./ml. medium. They concluded that actidione was non-inhibitory at the concentrations used.

The high degree of activity of actidione (cycloheximide) against many yeasts, molds, and saprophytic fungi combined with its low activity against bacteria led to its use in several selective media. Georg et al. (1951), Gray (1951), Green and Gray (1951), Kuzdas and Morse (1953), Jeffers (1954), Beech and Carr (1955), all found actidione to be effective.

Potassium sorbate (sorbic acid), a chemical agent used primarily in food products (Emard and Vaughn, 1952; Sheneman and Costilow, 1955; Costilow et al. 1955; Borg et al. 1955; Etchells et al. 1955; Costilow et al. 1957; Ferguson and Powrie, 1957), and nystatin (Lampen et al.1957), an inhibitor of endogenous respiration and anaerobic utilization of glucose by yeasts and other fungi according to reported results exhibited marked fungistatic activity.

EXPERIMENTAL

EXPERIMENTAL

1. The occurrence of bacterial colonies on glucose-nitrate-soil-extract fungal media

This preliminary study was carried out to extend the observations made by Smith and Dawson (1944) and by Martin (1950) that large, soft, raised, glistening bacterial colonies developed on glucose-nitrate-soil-extract fungal media. Smith and Dawson's medium follows:

Glucose	10.0 g.
$NaNO_3$	1.0 g.
K_2HPO_4	1.0 g.
Agar	15.0 g.
Soil extract	1000.0 ml.
pH 6.8-7.0	
Rose bengal	1:15,000

One ml. of 1/15 rose bengal solution was added to each litre of medium to yield a final dilution of 1 part in 15,000. The pH of the medium was adjusted with 1.0 N NaOH before the addition of the dye.

Martin used the same formula, but prior to plating added streptomycin at 30 µg./ml. medium.

The soil extract was prepared by autoclaving 500 g. soil in 1200 ml. tap water at 121°C. for one hour, filtering through paper and making up to one litre.

Various rhizosphere soil samples were used for platings from a) lawn soil, b) field soil and c) garden soil. These samples were obtained in the vicinity of the University of Manitoba. Duplicate samples of each soil were collected by the method outlined by Timonin (1940), and the procedure followed immediately. With each sample 10.0 g. soil was diluted serially in sterile water, care being taken to follow standard procedures at each step. One series of four plates was poured with Smith and Dawson's medium, and another with the medium used by Martin. Incubation was at 25°C. for seven days. The results are shown in Table 1.

Eighty-two large, raised, mucoid and glistening colonies were selected for detailed study. Each was isolated on an agar medium of the following composition:

Mannitol-calcium-glycerophosphate agar

Agar	15.0 g.
d-Mannitol	20.0 g.
KNO_3	5.0 g.
NaCl	3.8 g.
$MgCl_2.6H_2O$	1.0 g.
$MgSO_4.7H_2O$	0.6 g.

Mannitol-calcium-glycerophosphate agar

(continued)

Calcium glycerophosphate	0.8 g.
KCl	0.1 g.
Distilled water	1000.0 ml.

An identified culture of Agrobacterium radiobacter, designated as No. 426, was used as a control. The tests used were those of Hofer (1941) and certain others to check with the species as defined in Bergey (1957). The results are presented in Table 2.

About 65% of the isolates belonged to Agrobacterium radiobacter or were very closely related to it. Since a larger proportion of "radiobacter-like" colonies developed on Martin's medium in comparison to total numbers surface bacterial colonies, further studies were confined to this medium.

2. Effect of actidione on fungal growth

Since large numbers of fungi developing on the media inhibited bacterial growth during the preliminary platings, actidione (cycloheximide) was tested both for its fungistatic activity and for its effect on "radiobacter-like" colonies. Three concentrations of actidione (250, 500 and 1000 µg./ml. medium) were tested.

Actidione solutions were sterilized by passage through a sintered glass filter and added to the sterile, tempered medium prior to plating.

Duplicate samples of fresh garden soil were plated and count data recorded as follows:

a) Surface fungi

b) Subsurface fungi

c) "Radiobacter-like" surface colonies

d) Other surface bacterial colonies

e) Subsurface bacterial colonies

The results are presented in Table 3, and the effect of actidione on fungi illustrated in fig. 1.

In comparison to the control series actidione appeared to inhibit many fungi and to reduce spreading to a minimum. Higher concentrations showed the most marked effect and exhibited little inhibition of "radiobacter-like" colonies.

3. Biological activity of actidione tested at varying pH levels

In the paper published on "Cycloheximide (actidione) and its Agricultural Uses" (Ford, Klomparens and Hamner, 1956) a section was devoted to its stability at varying pH levels. The biological activity of actidione solutions decreased as pH increased. This phenomenon was tested with Martin's medium.

The medium was prepared in the usual manner. Four aliquots of one batch were adjusted to pH 4.7, 5.3, 7.1 and 8.2, respectively. The medium at each pH level was later divided into four parts. The first part was the check containing no actidione, to the second was added 250 µg., to the third 500 µg. and to the fourth 1000 µg./ml. medium.

A fresh sample of garden soil was plated as before. Incubation was at 25°C. for seven days. Count data were recorded as follows:

a) Surface fungi
b) Subsurface fungi
c) "Radiobacter-like" surface colonies
d) Other surface bacterial colonies
e) Subsurface bacterial colonies

The results are presented in Table 4, and the effect of actidione on fungi illustrated in figs. 2, 3, 4 and 5.

These results support previous findings that increase in pH decreases the biological activity of actidione. Interestingly, "radiobacter-like" colonies developed on this medium at relatively low pH. It was also observed that the lower the pH, the fewer other bacteria. Ninety-seven "radiobacter-like" colonies were isolated from media at pH 5.3 and studied in detail along with Agrobacterium radiobacter--426. The results are shown in Table 5.

4. A comparative study of rose bengal, streptomycin and actidione

Since Martin's medium at pH 5.3 containing actidione appeared promising as a selective medium for Agrobacterium radiobacter a study of the effect of rose bengal, streptomycin and actidione each singly and in various concentrations and combinations on Agrobacterium radiobacter--426 and Agrobacterium tumefaciens--AT_1 was carried out. The latter species was included on the assumption that the above inhibitors might show some selection within the genus.

Prior to this investigation an attempt was made without success to prepare a clear glucose-nitrate-soil-extract. Sterilization at 121°C. for 10 - 15 minutes repeatedly produced turbidity and in some cases a flocculant precipitate. This was found later to be associated with the phosphate radicle in K_2HPO_4. Even the use of a chelating agent, ethylene diamine tetraacetate, at concentrations up to 1000 mg./litre proved to be useless.

Since this study was to be made by measuring turbidity, a clear medium was essential. It was observed in previous tests that both cultures grew well on mannitol-calcium-glycerophosphate agar. A medium containing these constituents but no agar proved to withstand sterilization. Each culture was inoculated into this medium and incubated

at 25°C. Good growth appeared within 18 hrs. Accordingly, the mannitol-calcium-glycerophosphate medium was used in the following study.

To 100.0 ml. aliquots of this medium were added rose bengal, streptomycin and actidione in various concentrations and each singly and in various combinations. As before, the rose bengal was added prior to sterilization while the streptomycin and actidione were added aseptically after the medium had cooled to room temperature. Each flask in one series was inoculated with 1.0 ml. of a 48 hr. culture of Agrobacterium radiobacter--426 and each flask in a second series with Agrobacterium tumefaciens--AT_1. Incubation was at 25°C. and readings were made daily for seven days.

Growth response was determined by means of the transmittance scale on a Beckman Colorimeter. A set of 15 standardized test-tubes was used. The colorimeter was adjusted to 100% transmittance using an uninoculated control before each set of readings. Where readings were made with media containing rose bengal an uninoculated control containing the same concentration of rose bengal was used to adjust the colorimeter.

Prior to each reading, the medium was shaken vigorously and immediately 5.0 ml. dispensed aseptically to a standardized test-tube. Each test in a given medium with each culture was made in triplicate and the mean of

the three readings recorded. The data are presented in Table 6, and certain more outstanding results in graphic form in figs. 6, 7, 8, 9, 10, 11 and 12.

5. Fungistatic activity of potassium sorbate and nystatin

During the latter part of this investigation two fungistatic agents other than actidione were considered briefly. Various concentrations of potassium sorbate (0.01 - 0.75%) and of nystatin (570 - 6270 units/ml. medium) were used in Martin's medium. Potassium sorbate was added to the medium before sterilization, whereas the heat labile nystatin was added after sterilization and tempering.

One air-dried field soil sample was plated in duplicate. Potassium sorbate at 0.5% (w/v) and nystatin at 2400 units/ml. medium practically inhibited the fungi. The results are shown in figs. 13 and 14.

TABLES AND FIGURES

TABLE 1

NUMBERS OF LARGE, RAISED, MUCOID, GLISTENING BACTERIAL COLONIES ON:

(a) Smith and Dawson's medium

Soil type	Sample 1				Sample 2				No./g. of soil
	Plates				Plates				
	1	2	3	4	1	2	3	4	
a	2	3	1	1	1	1	2	3	1750
b	2	1	1	2	1	3	1	2	1625
c	3	3	1	4	2	5	1	2	2625

(b) Martin's medium

Soil type	Sample 1				Sample 2				No./g. of soil
	Plates				Plates				
	1	2	3	4	1	2	3	4	
a	19	17	27	7	16	16	13	24	1740
b	13	9	13	11	18	21	8	14	1340
c	14	39	23	24	27	34	31	24	2700

TABLE 2

CERTAIN DATA ON THE MORPHOLOGY AND PHYSIOLOGY OF ISOLATES[i] AND ON *Agrobacterium radiobacter*--426

Characteristics	Isolates		*Agrobacterium radiobacter* 426
	No. of cultures		
	Tested	+	+
Growth, mannitol-ca-glycerophosphate agar slants	82	63	+
Gram negative short rods	63	61	+
Motility	63	62	+
Gelatin liquifaction, 6 weeks	63	7	-
Browning of Ca-glycerophosphate agar	63	52	+
Turbidity in nutrient broth	63	61	+
Browning of litmus milk, 3 weeks	63	53	+
Nitrate utilization	63	57	+
Surface growth on agar slants	63	61	+

i See Table 1 for source of isolates

TABLE 2 (continued)

Characteristics	Isolated		Agrobacterium radiobacter 426
	No. of cultures		
	Tested	+	+
Large mucous colonies on nitrate glycerol agar slants	63	59	+
Growth, McConkey lactose taurocholate medium	63	63	+
Growth, Koser Na citrate medium	63	47	+
Reddish-brown pellicle, Ferric ammonium citrate medium	63	57	+
Starch hydrolized	63	0	-
Acid or Acid and gas, Lactose broth	63	3	-
Acid or Acid and gas, Sucrose broth	63	1	-
Acid or Acid and gas, Glucose broth	63	3	-
H_2S Formation, Lead acetate agar	63	54	+
Turbidity with heavy ring or pellicle, Veal infusion broth	63	56	+

TABLE 3

NUMBERS COLONIES IN MARTIN'S MEDIUM WITH DIFFERENT CONCENTRATIONS OF ACTIDIONE

(Fresh garden soil at dilution 1:1000)

(a) Surface Fungi

Days incubation	Actidione (µg/ml. medium)	Sample 1				Sample 2			
		Plates				Plates			
		1	2	3	4	1	2	3	4
4	0	61	27	48	31	39	34	46	74
	250	13	8	7	9	11	4	8	5
	500	4	7	2	4	3	1	5	6
	1000	-	-	-	-	-	-	-	-
7	0	⋇	94	⋇	⋇	87	101	⋇	⋇
	250	104	121	83	94	69	103	91	97
	500	69	74	62	81	77	84	67	74
	1000	39	53	41	27	44	37	28	34
10	0	⋇	⋇	⋇	⋇	⋇	⋇	⋇	⋇
	250	⋇	⋇	114	⋇	99	⋇	101	⋇
	500	77	75	66	84	79	87	71	80
	1000	41	53	42	29	44	38	30	34

⋇ Too numerous to count

TABLE 3 (continued)

(b) Subsurface Fungi

Days incubation	Actidione (µg/ml. medium)	Sample 1				Sample 2			
		Plates				Plates			
		1	2	3	4	1	2	3	4
4	0	116	91	212	149	162	183	141	187
	250	25	37	24	39	46	28	19	44
	500	31	28	40	24	23	25	37	31
	1000	21	18	29	23	17	22	13	19
7	0	⋇	106	⋇	⋇	149	172	⋇	⋇
	250	⋇	117	⋇	⋇	129	147	⋇	⋇
	500	65	74	41	60	27	49	54	62
	1000	51	64	39	31	61	37	23	30
10	0	⋇	⋇	⋇	⋇	⋇	⋇	⋇	⋇
	250	⋇	⋇	⋇	⋇	⋇	⋇	⋇	⋇
	500	64	81	54	58	40	47	71	66
	1000	50	65	41	34	54	39	29	33

⋇ Counts obscured by surface overgrowth

TABLE 3 (continued)

(c) "Radiobacter-like" Surface Colonies

Days incubation	Actidione (µg/ml. medium)	Sample 1				Sample 2			
		Plates				Plates			
		1	2	3	4	1	2	3	4
4	0	2	1	4	-	2	-	2	-
	250	-	-	1	-	-	-	1	-
	500	1	-	-	2	1	1	-	-
	1000	-	1	2	1	-	1	-	-
7	0	⊠	2	⊠	⊠	3	3	⊠	⊠
	250	1	1	3	2	2	1	1	1
	500	2	2	1	3	1	3	1	2
	1000	1	2	4	2	1	3	2	1
10	0	⊠	⊠	⊠	⊠	⊠	⊠	⊠	⊠
	250	⊠	⊠	3	⊠	2	⊠	1	⊠
	500	2	2	1	3	1	3	1	2
	1000	1	2	4	2	1	3	2	1

⊠ Counts obscured by fungal overgrowth

TABLE 3 (continued)

(d) Other Surface Bacterial Colonies

Days incubation	Actidione (µg/ml. medium)	Sample 1 Plates				Sample 2 Plates			
		1	2	3	4	1	2	3	4
4	0	1	1	-	-	2	-	1	-
	250	-	2	2	1	-	-	1	3
	500	1	1	-	2	-	-	2	1
	1000	1	-	1	-	-	2	-	-
7	0	※	2	※	※	2	1	※	※
	250	1	3	2	2	1	1	2	1
	500	2	1	1	2	1	-	2	2
	1000	2	-	3	1	-	2	1	1
10	0	※	※	※	※	※	※	※	※
	250	※	※	※	※	※	※	※	※
	500	2	2	1	2	1	1	2	2
	1000	2	1	3	1	-	2	1	1

※ Counts obscured by fungal overgrowth

TABLE 3 (continued)

(e) Subsurface Bacterial Colonies

Days incubation	Actidione (µg/ml. medium)	Sample 1 Plates				Sample 2 Plates			
		1	2	3	4	1	2	3	4
4	0	2	5	3	7	4	9	2	3
	250	4	-	2	5	7	3	4	1
	500	2	5	1	6	1	2	1	5
	1000	-	2	4	-	3	4	-	-
7	0	※	4	※	※	3	9	※	※
	250	※	2	※	※	5	8	※	※
	500	3	5	-	5	-	2	-	2
	1000	3	6	9	2	7	6	3	1
10	0	※	※	※	※	※	※	※	※
	250	※	※	※	※	※	※	※	※
	500	2	4	-	5	-	1	-	2
	1000	4	7	9	9	7	6	6	1

※ Counts obscured by fungal overgrowth

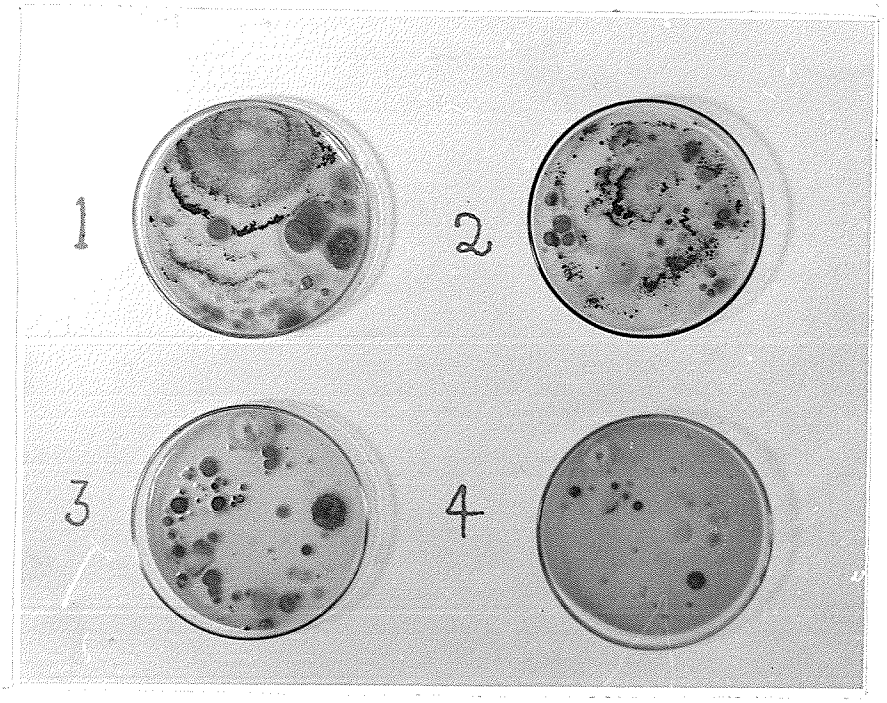

Fig. 1.

Fungi from garden soil (1:100)
in Martin's medium

Plate 1: without actidione

Plate 2: with actidione at 250 µg./ml.

Plate 3: with actidione at 500 µg./ml.

Plate 4: with actidione at 1000 µg./ml.

TABLE 4

NUMBERS COLONIES IN MARTIN'S MEDIUM WITH DIFFERENT CONCENTRATIONS OF ACTIDIONE AND AT DIFFERENT pH LEVELS

(Fresh garden soil at dilution 1:100)

(a) Surface Fungi

Actidione (µg/ml. medium)	pH of medium	Plates			
		1	2	3	4
0	4.7	※	※	※	※
	5.3	※	※	※	※
	7.1	※	※	※	※
	8.2	※	※	※	※
250	4.7	1	-	2	2
	5.3	8	7	4	2
	7.1	※	※	※	※
	8.2	※	※	※	※
500	4.7	-	-	-	-
	5.3	3	1	2	2
	7.1	※	※	161	※
	8.2	※	※	※	※
1000	4.7	-	-	-	-
	5.3	-	-	1	-
	7.1	※	※	※	※
	8.2	※	※	※	※

※ Too numerous to count

TABLE 4 (continued)

(b) Subsurface Fungi

Actidione (µg/ml. medium)	pH of medium	Plates			
		1	2	3	4
0	4.7	※	※	※	※
	5.3	※	※	※	※
	7.1	※	※	※	※
	8.2	※	※	※	※
250	4.7	9	4	12	7
	5.3	102	83	91	87
	7.1	※	※	※	※
	8.2	※	※	※	※
500	4.7	3	1	2	1
	5.3	83	77	91	78
	7.1	※	※	※	※
	8.2	※	※	※	※
1000	4.7	-	-	-	-
	5.3	9	4	7	10
	7.1	※	※	※	※
	8.2	※	※	※	※

※ Counts obscured by surface overgrowth

TABLE 4 (continued)

(c) "Radiobacter-like" Surface colonies

Actidione (µg/ml. medium)	pH of medium	Plates			
		1	2	3	4
0	4.7	※	※	※	※
	5.3	※	※	※	※
	7.1	※	※	※	※
	8.2	※	※	※	※
250	4.7	3	-	2	1
	5.3	19	17	23	14
	7.1	※	※	9	※
	8.2	※	※	※	※
500	4.7	1	4	2	1
	5.3	21	27	6	18
	7.1	※	4	1	※
	8.2	※	※	-	※
100	4.7	[illegible]	2[illegible]	[illegible]	[illegible]
	5.3	9	14	6	12
	7.1	1	※	※	※
	8.2	※	※	※	※

※ Counts obscured by fungal overgrowth

TABLE 4 (continued)

(d) Other Surface Bacterial Colonies

Actidione (µg/ml. medium)	pH of medium	Plates			
		1	2	3	4
0	4.7	※	※	※	※
	5.3	※	※	※	※
	7.1	※	※	※	※
	8.2	※	※	※	※
250	4.7	-	-	-	-
	5.3	1	-	-	-
	7.1	※	※	14	※
	8.2	※	※	※	※
500	4.7	-	-	-	-
	5.3	-	2	-	-
	7.1	※	7	9	※
	8.2	※	※	-	※
1000	4.7	-	-	-	-
	5.3	-	-	-	-
	7.1	4	※	※	7
	8.2	※	※	※	※

※ Counts obscured by fungal overgrowth

TABLE 4 (continued)

(e) Subsurface Bacterial Colonies

Actidione (µg/ml. medium)	pH of medium	Plates			
		1	2	3	4
0	4.7	※	※	※	※
	5.3	※	※	※	※
	7.1	※	※	※	※
	8.2	※	※	※	※
250	4.7	2	1	-	-
	5.3	3	5	9	7
	7.1	※	※	16	※
	8.2	※	※	※	※
500	4.7	1	4	2	-
	5.3	3	1	6	4
	7.1	※	※	17	※
	8.2	※	※	※	※
1000	4.7	-	-	1	-
	5.3	7	19	13	12
	7.1	※	※	※	※
	8.2	※	※	※	※

※ Counts obscured by fungal overgrowth

TABLE 5

CERTAIN DATA ON THE MORPHOLOGY AND PHYSIOLOGY OF ISOLATES[i]

FROM MARTIN'S MEDIA AT pH 5.3 AND ON

Agrobacterium radiobacter--426

Characteristics	Isolates		*Agrobacterium radiobacter* 426
	No. of cultures		
	Tested	+	+
Growth, mannitol-ca-glycerophosphate agar slants	97	84	+
Motility	84	79	+
Gram negative short rods	84	84	+
Gelatin liquifaction, 6 weeks	84	4	-
Browning of ca-glycerophosphate agar	84	72	+
Turbidity in nutrient broth	84	80	+
Browning of litmus milk, 3 weeks	84	61	+
Nitrate utilization	84	68	+
Surface growth on agar slants	84	77	+

i See Table 4 for source of isolates

TABLE 5 (continued)

Characteristics	Isolates		Agrobacterium radiobacter 426
	No. of cultures		
	Tested	+	+
Large mucous colonies on nitrate glycerol agar plates	84	74	+
Growth, Koser Na citrate medium	84	63	+
Growth, McConkey lactose taurocholate medium	84	84	+
Reddish-brown pellicle, Ferric ammonium citrate medium	84	74	+
Starch hydrolized	84	0	-
Acid or Acid and gas, Lactose broth	84	7	-
Acid or Acid and gas, Glucose broth	84	4	-
Acid or Acid and gas, Sucrose broth	84	6	-
H_2S formation, Lead acetate agar	84	73	+
Turbidity with heavy ring or pellicle, Veal infusion broth	84	71	+

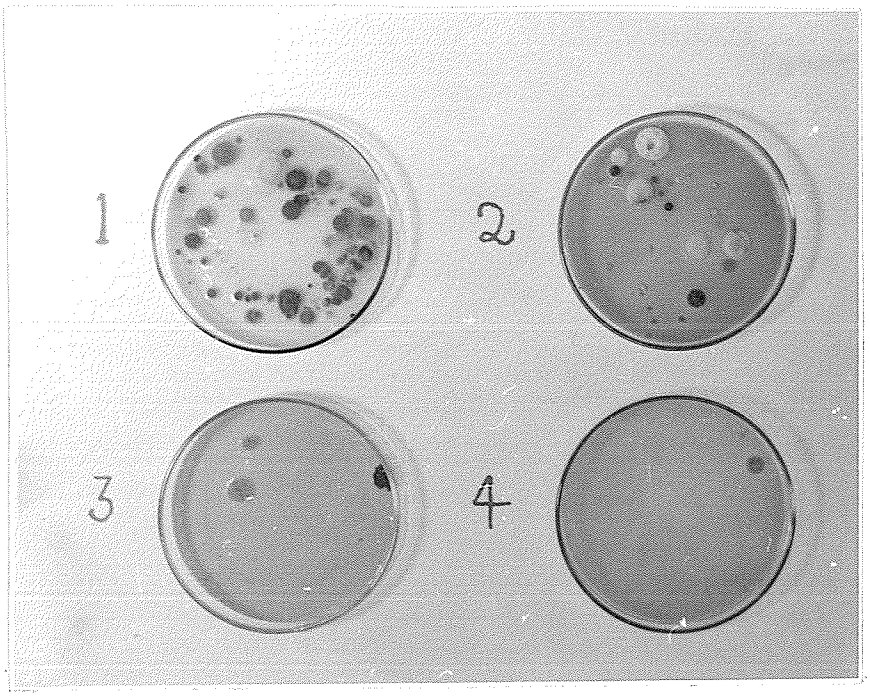

Fig. 2.

Fungi from garden soil
in Martin's medium at pH 4.7

Plate 1: at 1:10,000 without actidione

Plate 2: at 1:100 with actidione at 250 µg./ml.

Plate 3; at 1:100 with actidione at 500 µg./ml.

Plate 4: at 1:100 with actidione at 1000 µg./ml.

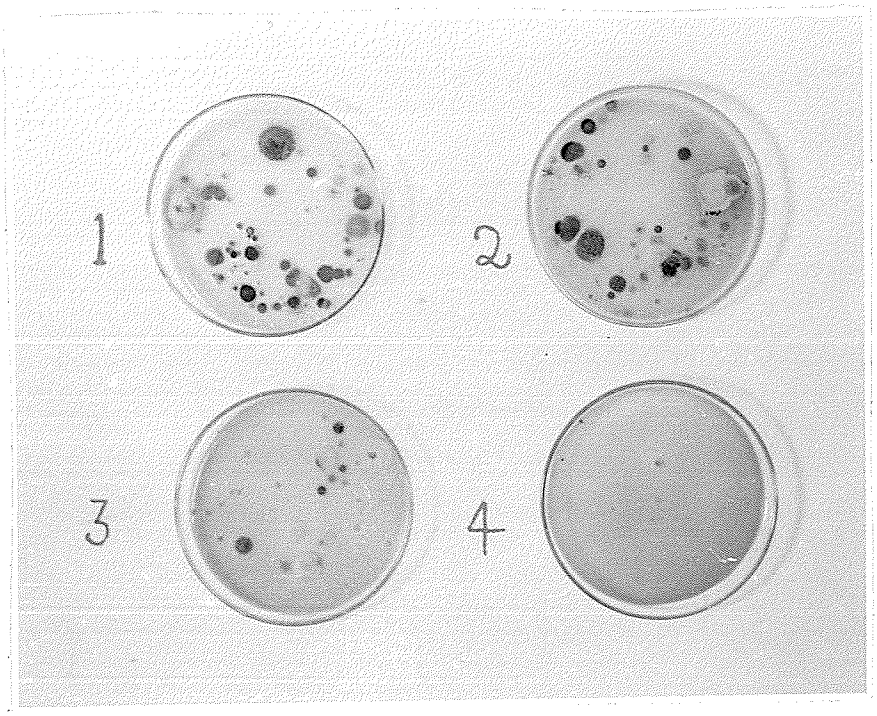

Fig. 3.

Fungi from garden soil
in Martin's medium at pH 5.3

Plate 1: at 1:10,000 without actidione

Plate 2: at 1:100 with actidione at 250 µg./ml.

Plate 3: at 1:100 with actidione at 500 µg./ml.

Plate 4: at 1:100 with actidione at 1000 µg./ml.

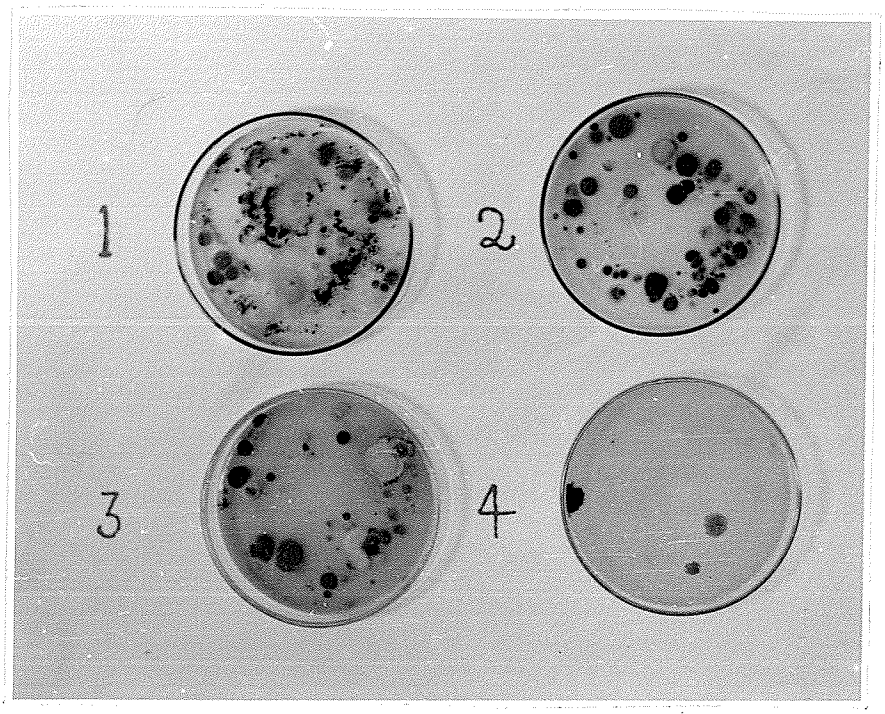

Fig. 4.

Fungi from garden soil (1:10,000)
in Martin's medium at pH 7.1

Plate 1: without actidione

Plate 2: with actidione at 250 µg./ml.

Plate 3: with actidione at 500 µg./ml.

Plate 4: with actidione at 1000 µg./ml.

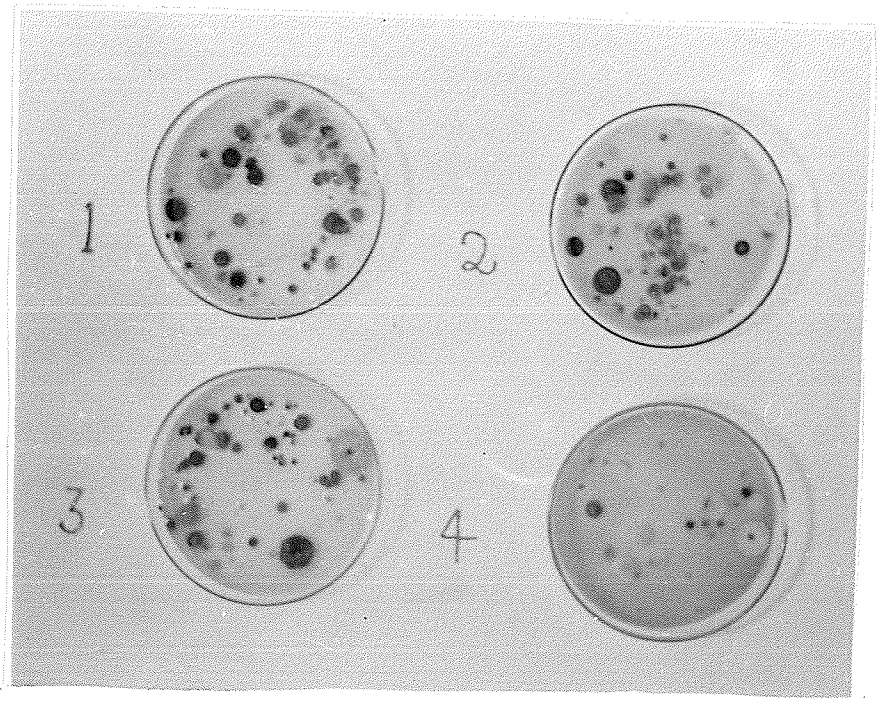

Fig. 5.

Fungi from garden soil (1:10,000)
in Martin's medium at pH 8.1

Plate 1: without actidione

Plate 2: with actidione at 250 µg./ml.

Plate 3: with actidione at 500 µg./ml.

Plate 4: with actidione at 1000 µg./ml.

TABLE 6

GROWTH RESPONSE OF *Agrobacterium radiobacter* (426) AND OF *Agrobacterium tumefaciens* (AT_1) IN MANNITOL-CALCIUM-GLYCEROPHOSPHATE MEDIUM CONTAINING ROSE BENGAL, STREPTOMYCIN AND ACTIDIONE EACH SINGLY, AND IN VARIOUS COMBINATIONS

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
Control	1	85.7	80.3
	2	74.3	63.0
	3	58.3	47.0
	4	48.0	34.3
	5	40.3	27.7
	6	35.3	21.3
	7	26.7	17.7
+ Streptomycin (30µg/ml.)	1	86.3	82.3
	2	77.3	62.0
	3	66.3	48.3
	4	59.7	36.7
	5	49.3	25.7
	6	43.7	21.3
	7	39.3	18.3
+ Streptomycin (60µg/ml.)	1	89.3	81.7
	2	78.3	58.3
	3	67.7	48.3
	4	58.3	38.7
	5	46.7	32.7
	6	42.3	27.3
	7	39.0	24.7

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Streptomycin (120µg/ml.)	1	97.0	85.0
	2	94.7	63.7
	3	92.3	59.3
	4	82.3	48.3
	5	63.7	41.3
	6	54.0	39.3
	7	46.3	39.0
+ Rose bengal (1:15,000)	1	96.0	91.7
	2	94.7	86.0
	3	94.7	68.0
	4	94.7	40.3
	5	94.7	25.0
	6	94.7	16.0
	7	94.7	8.0
+ Rose bengal (1:15,000) + Streptomycin (30µg/ml.)	1	95.0	94.7
	2	94.7	88.0
	3	94.3	75.3
	4	94.3	53.0
	5	94.3	30.3
	6	94.3	18.7
	7	94.3	10.3
+ Rose bengal (1:15,000) + Streptomycin (60µg/ml.)	1	97.3	95.3
	2	94.3	92.0
	3	94.3	92.0
	4	94.3	92.0
	5	94.3	90.0
	6	94.3	90.0
	7	94.3	90.0

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Rose bengal (1:15,000) + Streptomycin (120µg/ml.)	1	97.7	95.7
	2	95.7	92.0
	3	95.7	92.0
	4	94.7	92.0
	5	94.7	90.3
	6	94.7	90.3
	7	93.3	90.3
+ Actidione (200µg/ml.)	1	88.7	83.3
	2	78.7	67.7
	3	70.3	54.3
	4	63.7	39.7
	5	54.7	28.3
	6	52.7	23.7
	7	51.0	19.3
+ Actidione (400µg/ml.)	1	86.7	80.3
	2	79.3	77.3
	3	71.7	70.0
	4	65.7	61.3
	5	56.3	50.7
	6	51.7	47.0
	7	49.7	45.3
+ Actidione (800µg/ml.)	1	90.0	87.3
	2	83.3	75.7
	3	79.0	68.3
	4	73.7	59.3
	5	66.7	51.7
	6	61.3	49.0
	7	58.7	47.7

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Streptomycin (30µg/ml.) + Actidione (200µg/ml.)	1	88.7	83.7
	2	77.7	61.3
	3	68.0	52.7
	4	58.7	44.3
	5	50.7	36.7
	6	43.3	32.0
	7	40.7	28.7
+ Streptomycin (30µg/ml.) + Actidione (400µg/ml.)	1	90.7	82.3
	2	81.7	63.7
	3	77.0	53.3
	4	68.0	45.3
	5	59.3	37.7
	6	57.3	33.0
	7	56.0	31.3
+ Streptomycin (30µg/ml.) + Actidione (800µg/ml.)	1	90.0	86.3
	2	84.7	70.7
	3	78.0	61.7
	4	70.3	54.0
	5	68.7	46.3
	6	65.7	39.7
	7	64.0	34.0
+ Streptomycin (60µg/ml.) + Actidione (200µg/ml.)	1	87.0	83.7
	2	76.0	59.7
	3	68.7	47.0
	4	58.0	42.0
	5	47.7	39.7
	6	43.3	35.0
	7	40.3	32.7

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Streptomycin (60µg/ml.) + Actidione (400µg/ml.)	1	89.3	85.7
	2	81.7	75.0
	3	73.3	63.3
	4	65.7	54.0
	5	59.3	41.7
	6	52.0	39.7
	7	47.7	35.7
+ Streptomycin (60µg/ml.) + Actidione (800µg/ml.)	1	90.3	86.7
	2	83.7	77.7
	3	77.7	68.7
	4	70.0	60.7
	5	63.7	52.3
	6	60.7	46.7
	7	59.0	41.3
+ Streptomycin (120µg/ml.) + Actidione (200µg/ml.)	1	93.7	84.7
	2	90.7	61.7
	3	81.3	56.3
	4	70.3	49.0
	5	57.0	42.3
	6	52.7	41.0
	7	50.3	39.7
+ Streptomycin (120µg/ml.) + Actidione (400µg/ml.)	1	95.7	83.0
	2	93.3	77.7
	3	84.0	73.0
	4	72.7	65.0
	5	63.3	54.7
	6	58.7	50.3
	7	54.3	46.7

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Streptomycin (120µg/ml.) + Actidione (800µg/ml.)	1	95.7	87.7
	2	95.0	85.7
	3	89.7	78.3
	4	82.0	73.3
	5	66.7	68.3
	6	63.0	66.0
	7	61.7	65.3
+ Rose bengal (1:15,000) + Actidione (200µg/ml.)	1	98.7	95.0
	2	95.3	83.0
	3	95.3	69.0
	4	95.3	47.0
	5	95.3	31.0
	6	95.3	19.0
	7	95.3	10.3
+ Rose bengal (1:15,000) + Actidione (400µg/ml.)	1	100.0	95.0
	2	95.0	86.0
	3	95.0	77.0
	4	93.3	64.3
	5	93.3	54.0
	6	93.3	39.0
	7	93.3	23.7
+ Rose bengal (1:15,000) + Actidione (800µg/ml.)	1	100.0	100.0
	2	94.0	91.0
	3	94.0	82.3
	4	94.0	76.3
	5	94.0	66.0
	6	94.0	53.0
	7	94.0	36.3

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Rose bengal (1:15,000) + Streptomycin (30µg/ml.) + Actidione (200µg/ml.)	1	100.0	95.3
	2	97.7	88.0
	3	96.3	77.0
	4	93.7	64.3
	5	93.7	50.0
	6	93.7	32.0
	7	93.7	17.0
+ Rose bengal (1:15,000) + Streptomycin (30µg/ml.) + Actidione (400µg/ml.)	1	100.0	100.0
	2	97.0	91.7
	3	95.0	86.7
	4	95.0	83.0
	5	95.0	72.0
	6	95.0	67.0
	7	95.0	49.0
+ Rose bengal (1:15,000) + Streptomycin (30µg/ml.) + Actidione (800µg/ml.)	1	100.0	100.0
	2	96.3	91.3
	3	95.0	84.0
	4	95.0	77.0
	5	95.0	66.0
	6	95.0	56.0
	7	95.0	41.0
+ Rose bengal (1:15,000) + Streptomycin (60µg/ml.) + Actidione (200µg/ml.)	1	100.0	98.0
	2	96.0	93.3
	3	96.0	92.0
	4	95.0	90.3
	5	95.0	90.3
	6	95.0	90.3
	7	95.0	89.3

TABLE 6 (continued)

Medium	Time of incubation in days	% Transmittance	
		A. tumefaciens	A. radiobacter
+ Rose bengal (1:15,000) + Streptomycin (60µg/ml.) + Actidione (400µg/ml.)	1	100.0	96.3
	2	95.3	90.3
	3	95.3	89.0
	4	95.3	87.3
	5	95.3	86.0
	6	95.3	85.0
	7	95.3	85.0
+ Rose bengal (1:15,000) + Streptomycin (60µg/ml.) + Actidione (800µg/ml.)	1	100.0	100.0
	2	98.0	94.0
	3	96.0	93.0
	4	96.0	92.3
	5	96.0	90.0
	6	96.0	90.0
	7	96.0	90.0
+ Rose bengal (1:15,000) + Streptomycin (120µg/ml.) + Actidione (200µg/ml.)	1	100.0	100.0
	2	94.3	94.3
	3	94.3	92.0
	4	93.0	92.0
	5	93.0	92.0
	6	93.0	92.0
	7	93.0	92.0
+ Rose bengal (1:15,000) + Streptomycin (120µg/ml.) + Actidione (400µg/ml.)	1	100.0	100.0
	2	95.3	95.0
	3	95.0	93.0
	4	93.3	92.0
	5	93.3	92.0
	6	93.3	92.0
	7	93.3	92.0
+ Rose bengal (1:15,000) + Streptomycin (120µg/ml.) + Actidione (800µg/ml.)	1	100.0	100.0
	2	95.0	94.0
	3	95.0	94.0
	4	95.0	94.0
	5	95.0	94.0
	6	95.0	94.0
	7	95.0	94.0

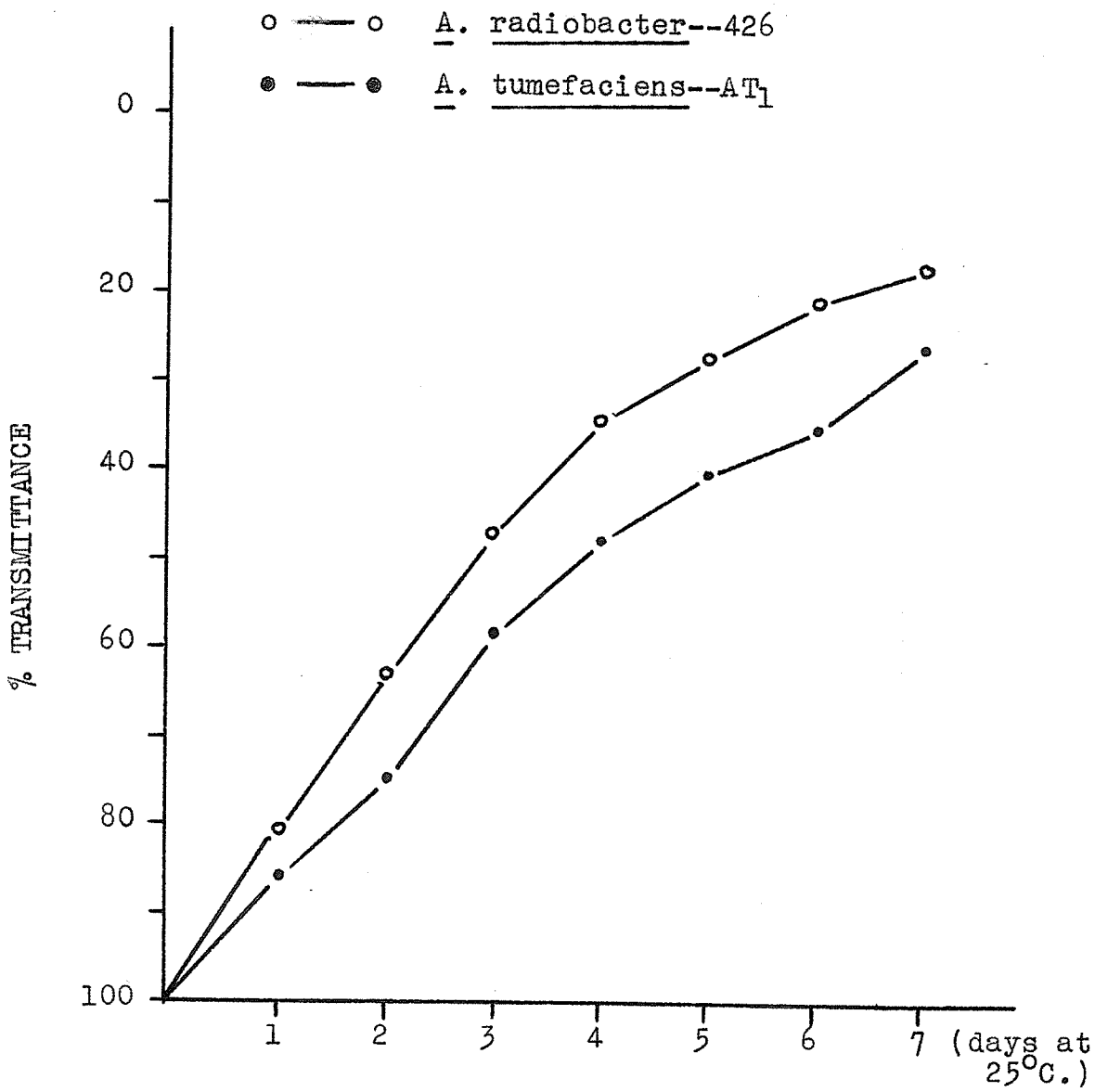

Fig. 6. Growth of Agrobacterium radiobacter--426 and of Agrobacterium tumefaciens--AT_1 in mannitol-calcium-glycerophosphate medium measured with a Beckman colorimeter

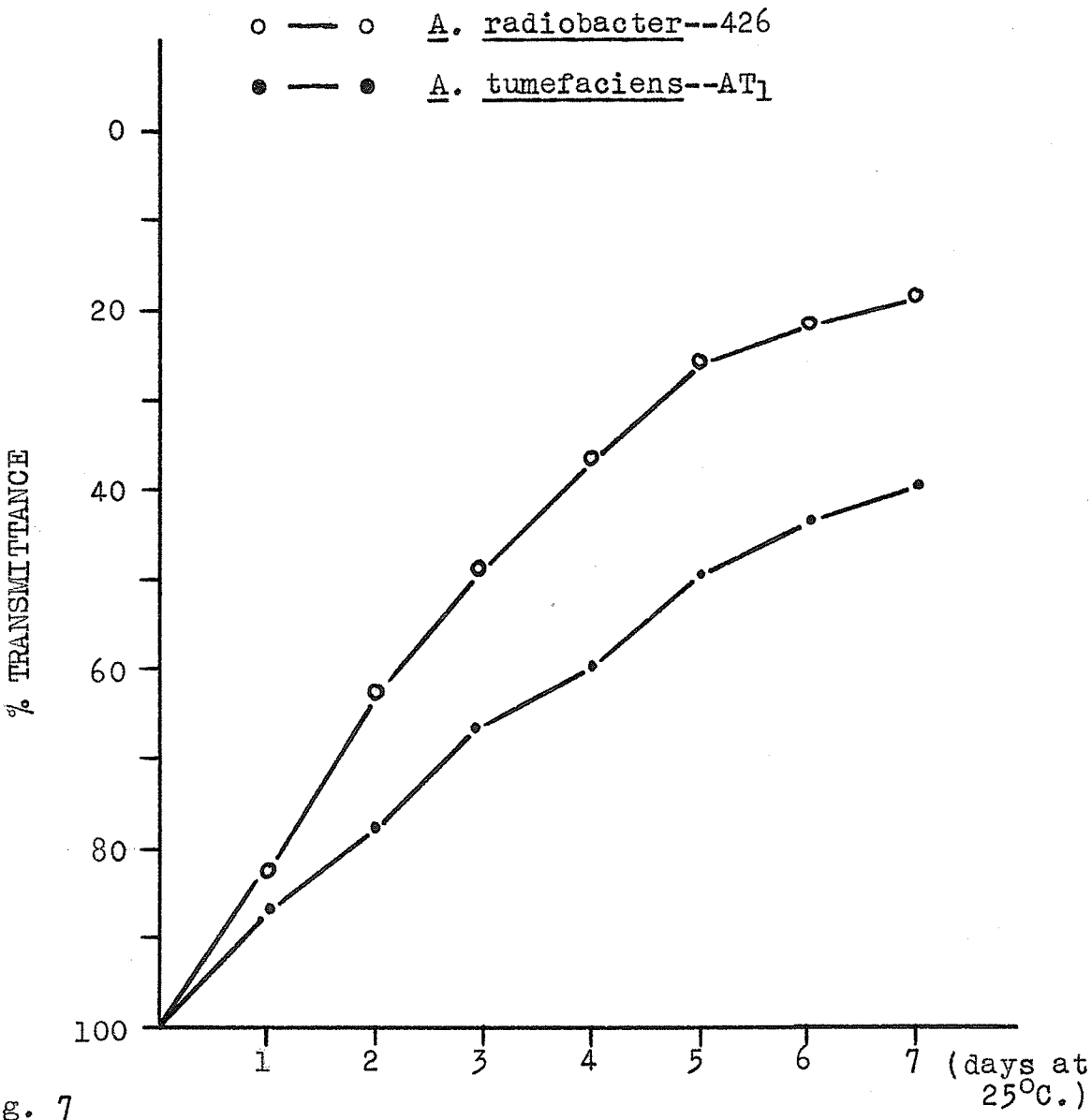

Fig. 7

Growth of Agrobacterium radiobacter--426 and of Agrobacterium tumefaciens--AT1 in mannitol-calcium-glycerophosphate medium containing streptomycin (30 µg./ml.) measured with a Beckman colorimeter.

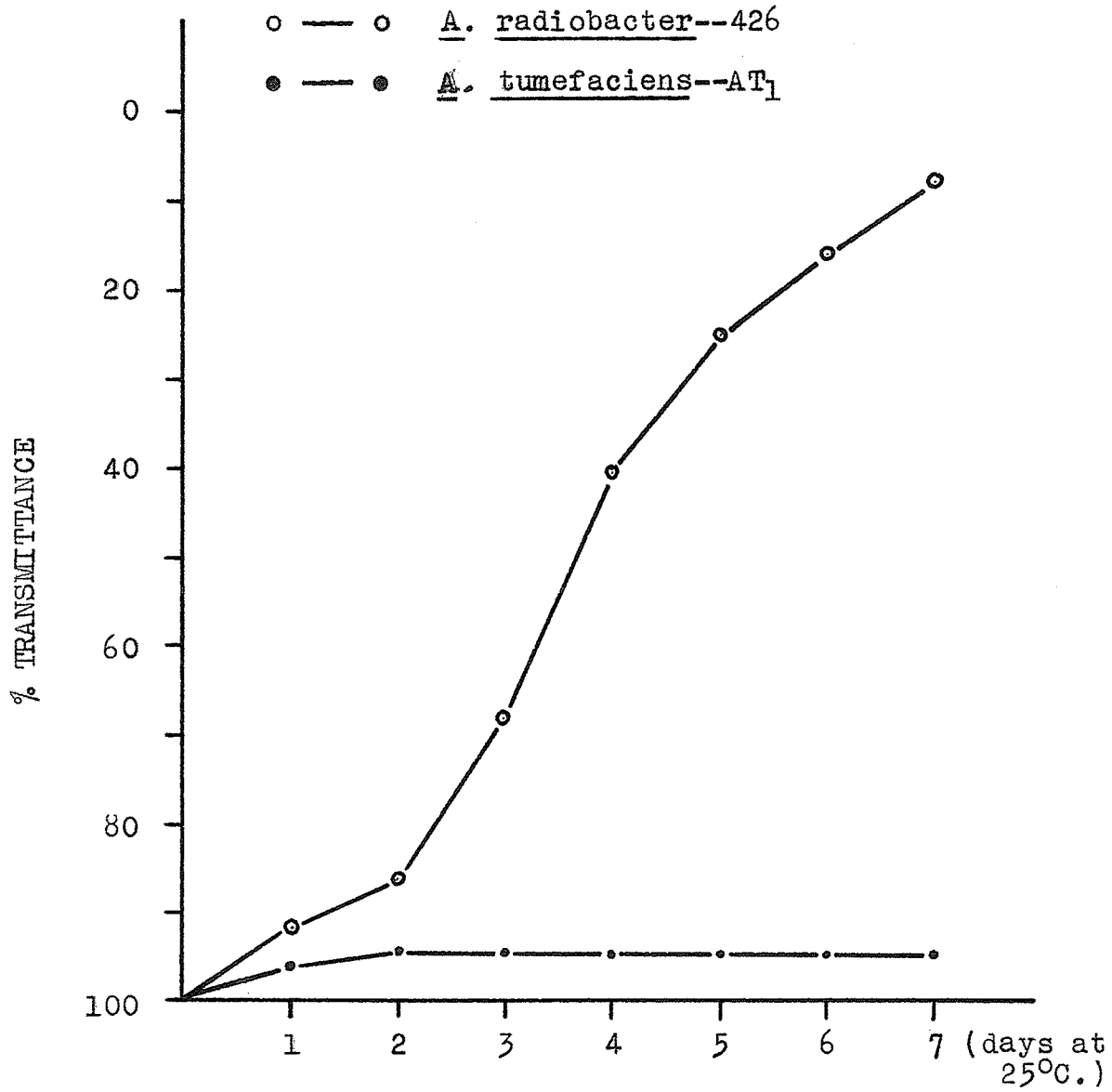

Fig. 8. Growth of Agrobacterium radiobacter--426 and of Agrobacterium tumefaciens--AT_1 in mannitol-calcium-glycerophosphate medium containing rose bengal (1 part in 15,000) measured with a Beckman colorimeter.

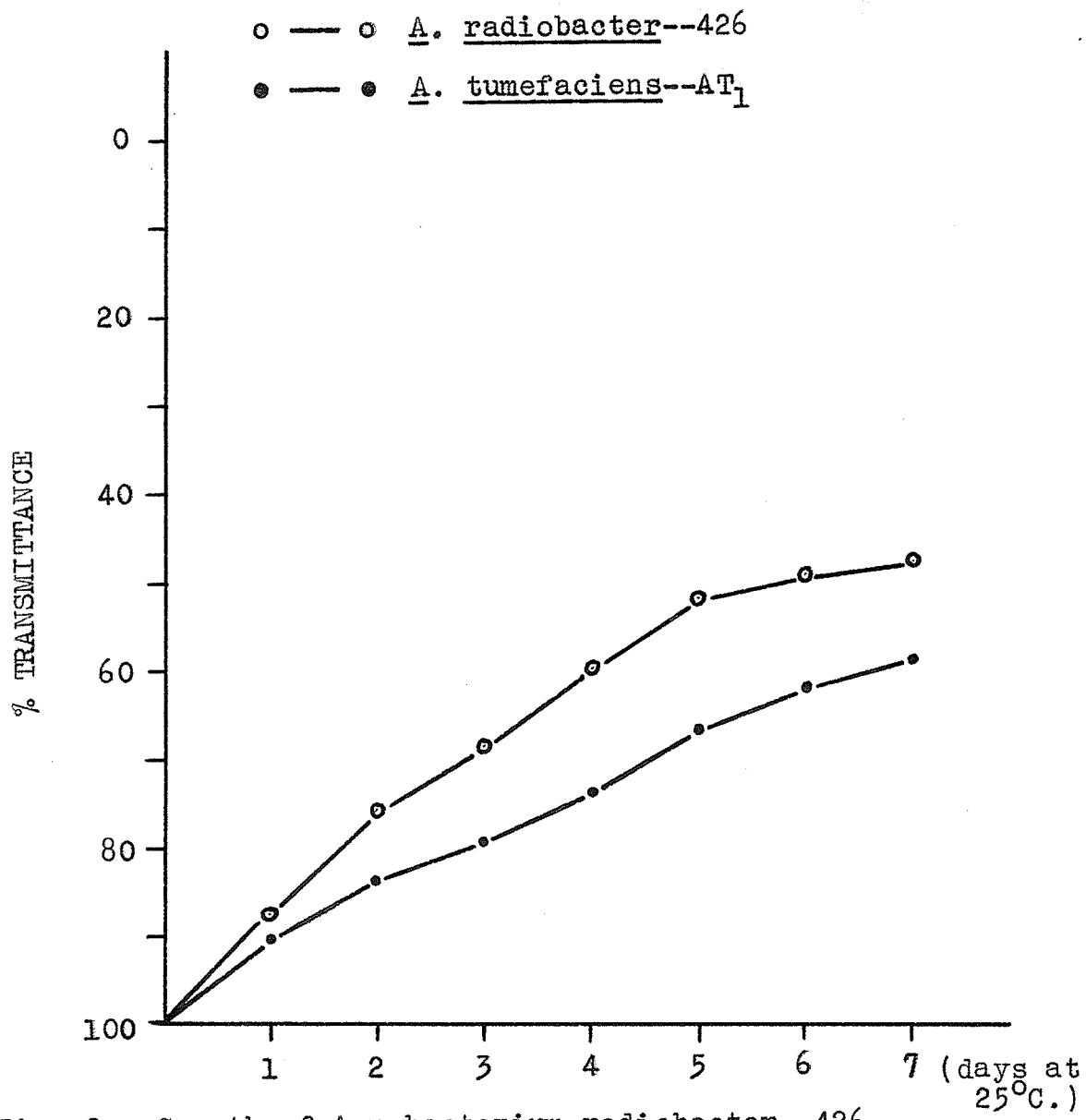

Fig. 9. Growth of Agrobacterium radiobacter--426 and of Agrobacterium tumefaciens--AT_1 in mannitol-calcium-glycerophosphate medium containing actidione (800 µg./ml.) measured with a Beckman colorimeter.

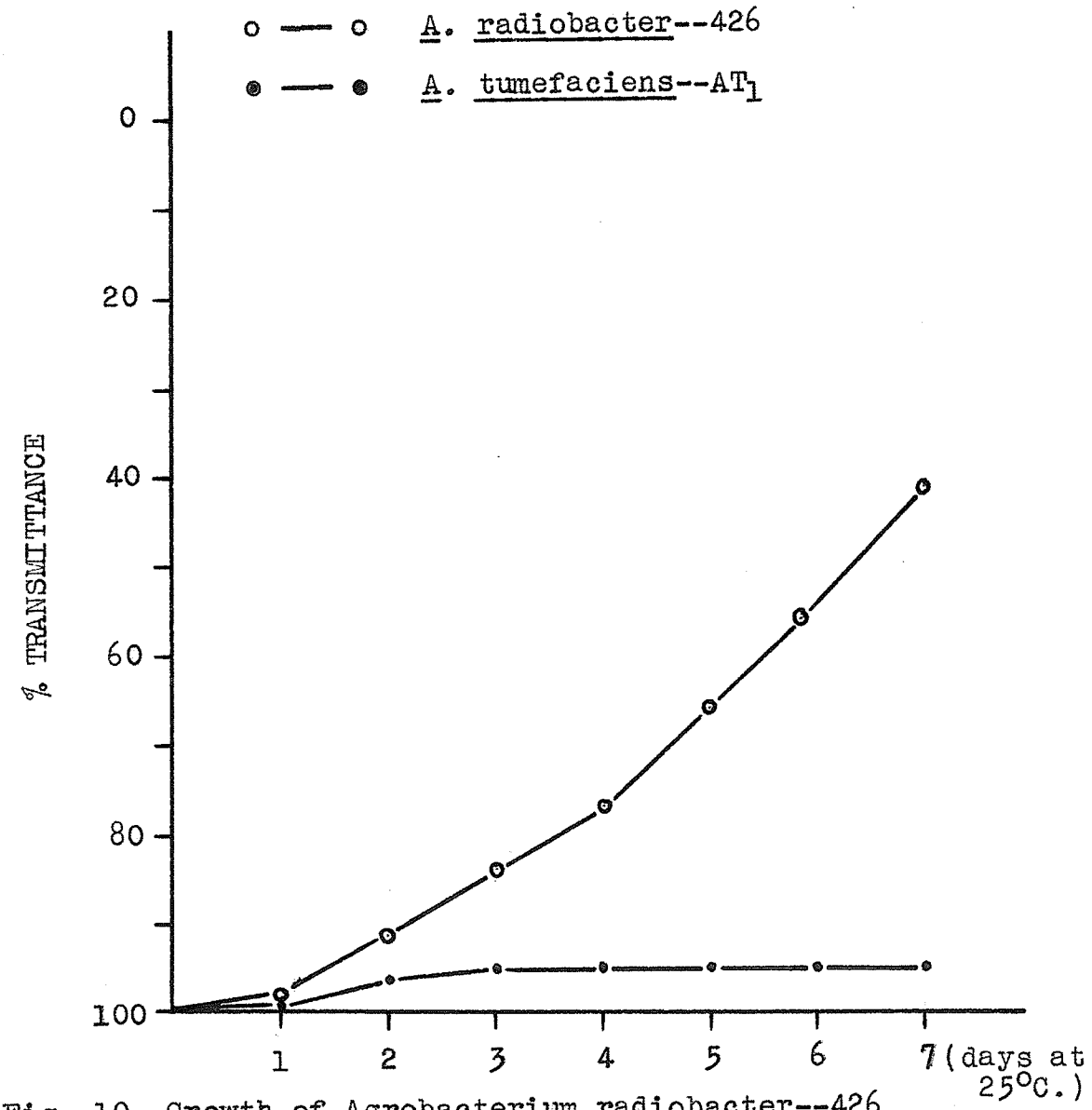

Fig. 10. Growth of Agrobacterium radiobacter--426 and of Agrobacterium tumefaciens--AT_1 in mannitol-calcium-glycerophosphate medium containing rose bengal (1 part in 15,000), streptomycin (30 µg./ml.), and actidione (800 µg./ml.) measured with a Beckman colorimeter.

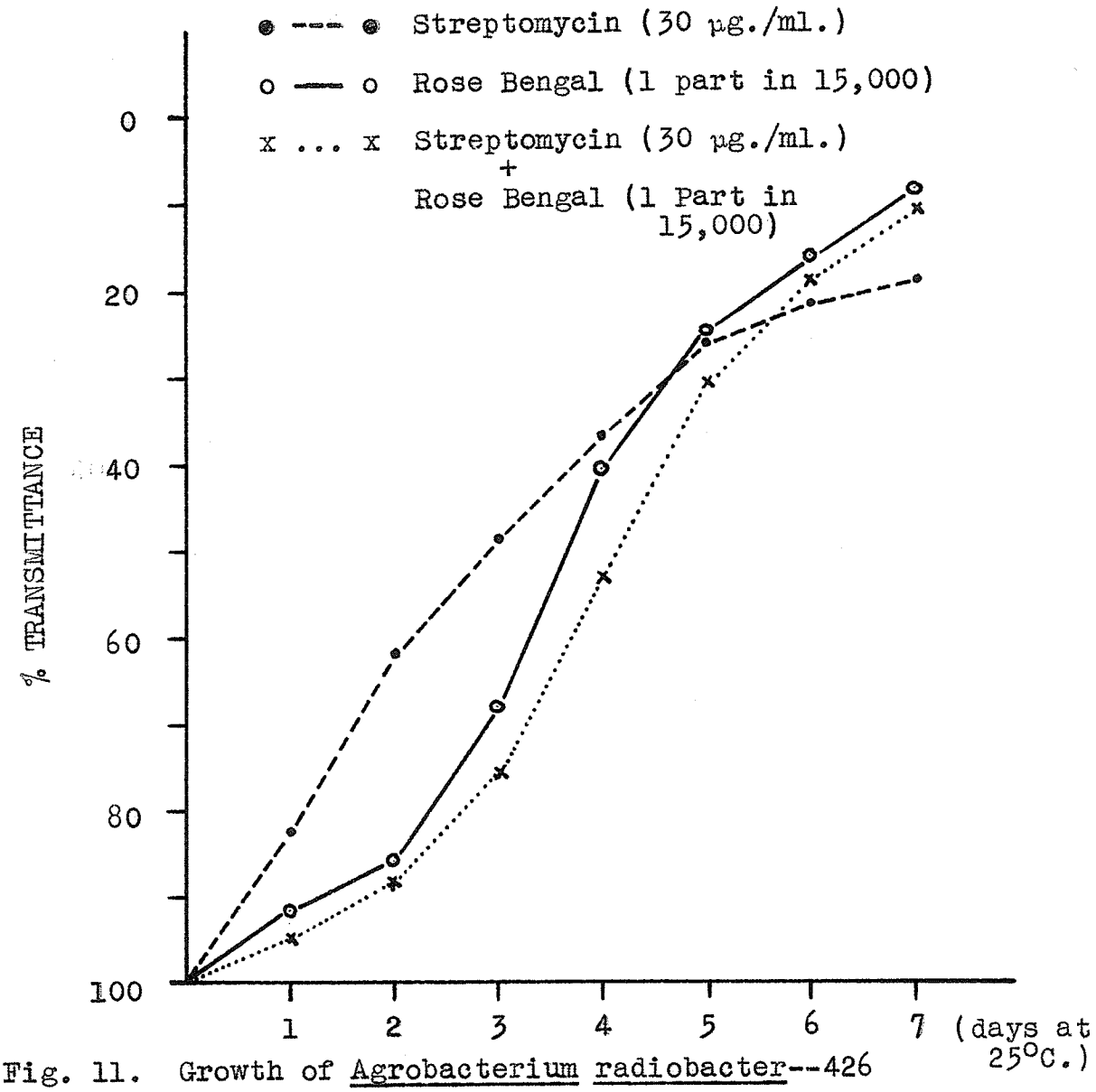

Fig. 11. Growth of Agrobacterium radiobacter--426 in mannitol-calcium-glycerophosphate medium containing streptomycin (30 µg./ml.) and rose bengal (1 part in 15,000), each singly and in combination, measured with a Beckman colorimeter.

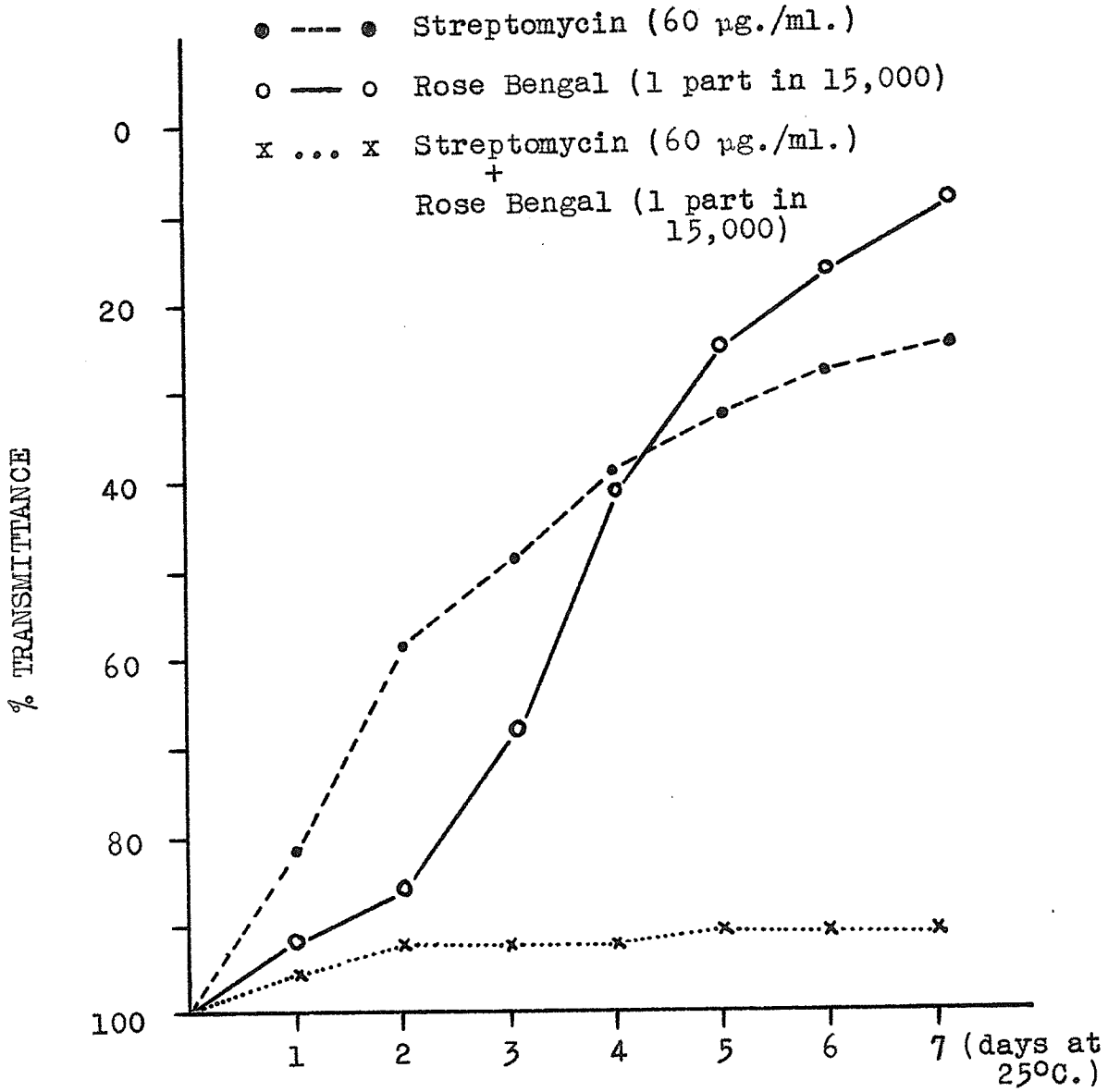

Fig. 12. Growth of Agrobacterium radiobacter--426 in mannitol-calcium-glycerophosphate medium containing streptomycin (60 µg./ml.) and rose bengal (1 part in 15,000), each singly and in combination, measured with a Beckman colorimeter.

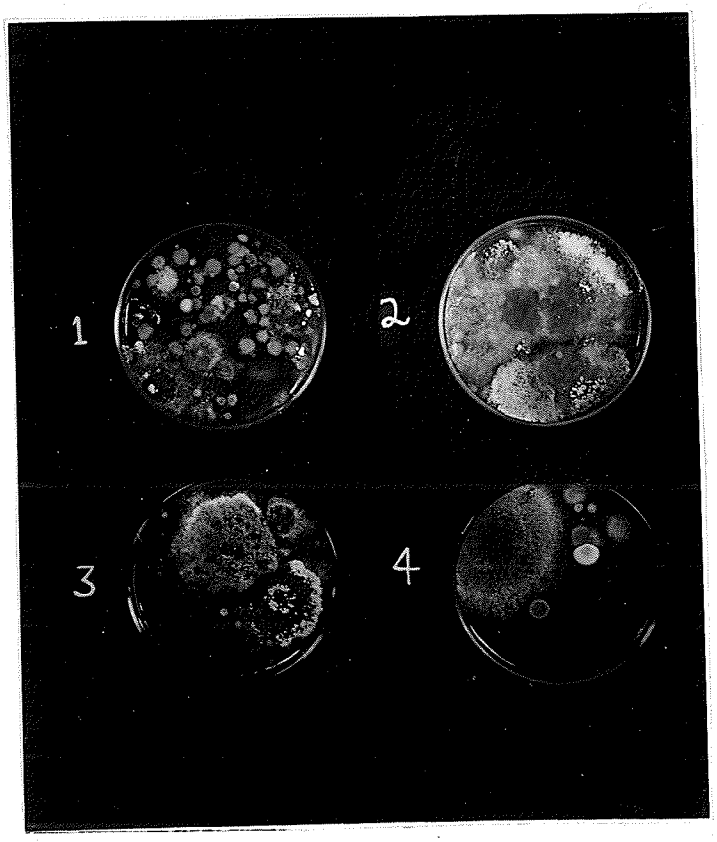

Fig. 13.

Fungi from field soil (1:10)
in Martin's medium

Plate 1: with potassium sorbate at 0.05% (w/v)

Plate 2: with potassium sorbate at 0.1% (w/v)

Plate 3: with potassium sorbate at 0.25% (w/v)

Plate 4: with potassium sorbate at 0.5% (w/v)

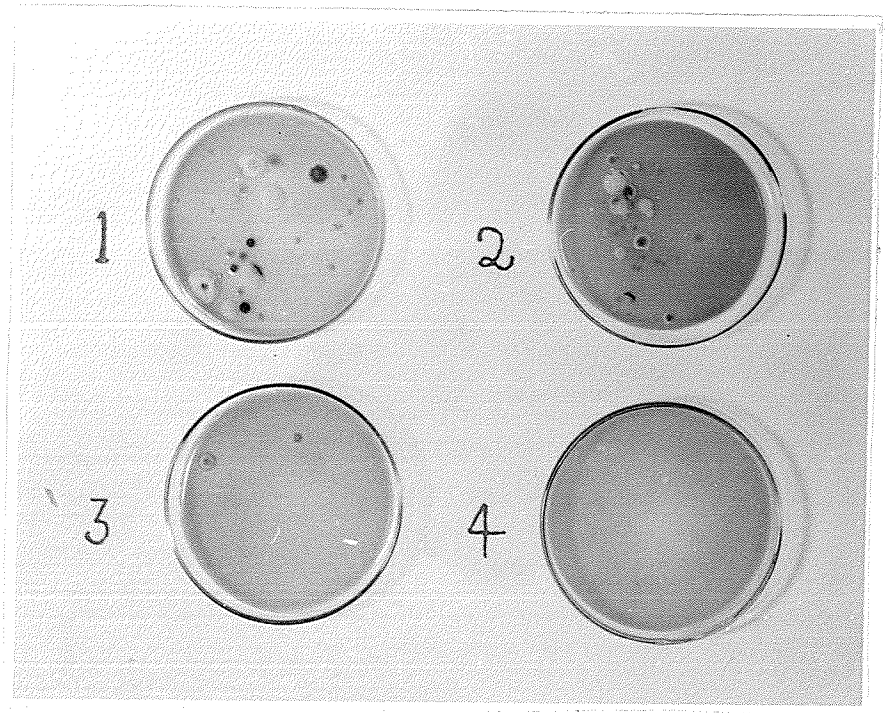

Fig. 14.

Fungi from field soil (1:100)
in Martin's medium

Plate 1: with nystatin at 600 units/ml.

Plate 2: with nystatin at 1200 units/ml.

Plate 3: with nystatin at 2400 units/ml.

Plate 4: with nystatin at 3600 units/ml.

DISCUSSION

DISCUSSION

Even though the development of a selective medium for Agrobacterium radiobacter may be deemed an accomplishment, it leaves unanswered the fundamental question as to what is different in the biological nature of this species that makes it respond selectively on this medium.

The fact that this species in many respects resembles Agrobacterium tumefaciens, a pathogen, and species of Rhizobium of agricultural significance points to the need for such a selective medium. Conceivably it could be used as a quick method for testing suspicious cultures in these practical groups of bacteria.

Probably it is worthy of note that identification of the cultures developing on this selective medium was limited to certain morphological and physiological tests and did not include phage testing. Conn et al. (1945) have reported that identification and differentiation from species of Rhizobium can be accomplished readily by a phage specific for Agrobacterium radiobacter.

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