BACTERIA AND ACTINOMYCETES ANTAGONISTIC TO FUNGAL ROOT PATHOGENS IN AUSTRALIAN SOILS

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Abstract

Techniques are described which make possible the large-scale isolation and screening of potential antagonists to fungal root pathogens. Sixty selected soils from five States have been sampled. Of more than 3500 isolates tested, about 40% inhibited one or more of nine pathogens on agar, and about 4% were effective in soil; a lower percentage might be effective under commercial conditions. Such large-scale methods are essential to success.

The ability of pathogens to grow through a soil sample in the laboratory, and to produce damping-off in glasshouse tests, generally agreed with the performance of the fungi in the same soil in the field. Some of the organisms found to be effective in inhibiting a pathogen on agar media also did so in soil, but those ineffective on agar were also ineffective in soil.

The effect of antagonists on pathogens is a continuum, ranging from stimulation, through no inhibition, to inhibition of one or several pathogens.

The antagonists isolated were mostly *Bacillus subtilis*, *B. megaterium*, and *Streptomyces* spp., with occasional *B. cereus*, *B. pumilus*, *B. polymyxa*, *B. badius*, *Pseudomonas putida*, and *P. fluorescens*.

Numbers of surviving *Pseudomonas* spp. declined rapidly in soil treated above 40°C, as did *Streptomyces* spp. above 60–70°C, but *Bacillus* spp. survived 80°C for 10 min.

Antagonists added to soil steamed at 100°C for 30 min multiplied there, and could be re-isolated; damping-off of seedlings grown in the soil was prevented by some. They did not increase, and failed to increase the protection against damping-off, in soil treated at 60°C for 30 min with aerated steam; this presumably depended on the balance between the quantity of surviving resident flora and the amount of antagonist inoculum added.

Four isolates of *Bacillus* and one of *Streptomyces* effectively controlled damping-off caused by *Rhizoctonia solani* in soil treated at 100°C. No antagonist reduced the survival in soil, or the spread through it, of *R. solani*. Disease reduction in this case apparently resulted from decreased infection, operating in the rhizosphere. Other *Bacillus* isolates increased the severity of damping-off by this pathogen. Some *Bacillus* isolates were specific for a *Rhizoctonia* strain, others had

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a wide spectrum of effectiveness. Thus, the above continuum of activity was also evident for disease control.

One *Bacillus* isolate effectively controlled damping-off caused by *Pythium*. *Pseudomonas* isolates were ineffective against *Rhizoctonia* or *Pythium* damping-off, and only a few were inhibitory on agar.

Some *Bacillus* isolates stimulated growth of pepper, snapdragon, and tomato seedlings in tests in nutrient-deficient, but not in fertile, soil. Some isolates of *Bacillus*, *Streptomyces*, and *Pseudomonas* inhibited seedling germination or reduced seedling growth. Still others were without noticeable effect. Thus, there is a continuum from stimulation of the host, through no effect, to inhibition of seedling growth, produced by the soil microflora.

Actinomycetes were more inhibitory to *Phytophthora* than to other pathogens tested on agar. The percentage of effective antagonists among actinomycetes were higher than among *Bacillus*. *Pythium ultimum* and *Sclerotium rolfsii* were least inhibited, of the fungi tested, by *Bacillus* and *Streptomyces* on agar.

There are three possible approaches to biological control of soil-borne plant pathogens: (1) Treat soil at 60°C for 30 min to eliminate plant pathogens and leave an effective antagonistic microflora to suppress later chance contamination. (2) Add suitable organic amendments to the soil to stimulate development of an antagonistic microflora. (3) Inoculate soil which has been steamed at 100°C for 30 min with selected antagonists. Microbiological evidence supporting (1) and evidence for the feasibility of (3) are supplied in this paper. Both (1) and (2) assume the presence of suitable effective antagonists in the soil. If these are not present, they may be added on the amendment in (2) and perhaps also in (1).

I. Introduction

Bedding-plant nurseries almost universally grow their seedlings in steamed or chemically treated soil to reduce losses from soil-borne pathogens such as *Pythium* spp., *Rhizoctonia solani* Kühn, and *Phytophthora* spp. High dosages of fumigants or steaming at 100°C for 30 min or longer tend to produce a "biological vacuum", and when a pathogen is accidentally introduced it may luxuriate, producing severe losses.

There are three general methods for preventing this without resorting to fungicidal drenches:

(1) Aerated steam treatment at 60–71°C for 30 min is now commonly used to kill root pathogens and leave a residual flora of saprophytes antagonistic to the pathogen (Baker 1962; Bollen 1969; Wuest, Baker, and Conway 1970).

(2) Addition to the soil of an amendment which stimulates antagonists, e.g. chitin (Mitchell and Alexander 1961); soybean straw (Weinhold and Bowman 1968).

(3) Addition of selected antagonists to the treated soil (Ferguson 1958) and their establishment in the rhizosphere of the seedling roots may check the development of a subsequently introduced pathogen and may continue the protection when seedlings are transplanted to untreated soil.

This paper, an extension of a previous abstract (Broadbent and Baker 1969), reports studies which may lead to the latter type of control.

II. Experimental Methods and Results

Fungal isolates used and their origin are set out in Table 1.

Table 1

FUNGAL ISOLATES USED IN PRESENT STUDY

Isolate No.	Name	Origin	Worker	Date of Isolation
3	*Armillaria mellea* (Vahl.) Sacc.	Valencia orange [*Citrus sinensis* (L.) Osb.] tree, Curlwaa, N.S.W.	P. Broadbent	November 1966
12	*Fusarium oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) Snyd. & Hans.	Tomato (*Lycopersicon esculentum* Mill.), Mildura, Vic.	P. Broadbent	May 1969
4	*Phytophthora cinnamomi* Rands	*Taxus baccata* L. f. *aurea* Carriere, Mt. Irvine, N.S.W.	A. L. Bertus	March 1967
14	*Phytophthora cinnamomi*	Apple fruit infected from contaminated water in nursery reservoir, Dural, N.S.W.	A. L. Bertus	January 1969
20	*Phytophthora cinnamomi*	Jarrah (*Eucalyptus marginata* Smith) dieback, Western Australia	P. Broadbent	July 1969
2	*Phytophthora citrophthora* (Sm. & Sm.) Leon.	Washington Navel orange fruit with brown rot, Richmond, N.S.W.	P. Broadbent	May 1967
6	*Phytophthora nicotianae* (B. de Haan) var. *parasitica* (Dast.) Waterh.	*Daphne odora* Thurb., Milperra, N.S.W.	A. L. Bertus	May 1967
8	*Pythium debaryanum* Hesse	Grape (*Vitis vinifera* L.)	J. McGechan	April 1964
7	*Pythium ultimum* Trow	Snapdragon (*Antirrhinum majus* L.), Dundas, N.S.W.	P. Broadbent	May 1969
15	*Rhizoctonia solani*	Damped-off red beet (*Beta vulgaris* L.) seedling, Blakehurst, N.S.W.	A. L. Bertus	February 1969
18	*Rhizoctonia solani*	Lucerne (*Medicago sativa* L.)	A. M. Smith	April 1969
75	*Rhizoctonia solani*	Diseased wheat coleoptile (*Triticum aestivum* L.), Ceduna, S.A.	R. L. Dodman	—
1	*Sclerotium rolfsii* Sacc.	*Colocasia antiquorum* Schott, Hong Kong	A. L. Bertus	January 1968

(*a*) *Selection of Soils for Isolation of Antagonists*

Areas selected for assay were of three types:

(1) A pathogen was known to be present without producing important disease loss to a susceptible crop.

(2) A pathogen has been introduced but failed to establish in the soil.

(3) Soils of special interest for various other reasons [e.g. pine forest soils whose microorganisms are known (Toussoun, Menzinger, and Smith 1969) to be antagonistic to *Fusarium*].

A total of 60 soils from New South Wales, Victoria, Queensland, Western Australia, and South Australia were examined. They included soils in which wheat, jarrah, vegetables, pine (*Pinus radiata* D. Don), ornamentals, avocado (*Persea americana* Mill.), citrus, saccaline (*Polygonum sachalinensis*, F. Schmidt), lucerne, and virgin bush were growing. Soil was collected from the upper 15 cm of the profile, placed in plastic bags, moistened if necessary, kept at 20–25°C, and assayed as soon as possible after collection.

(*b*) *Preliminary Appraisal of Antagonistic Potential*

To determine whether the microorganisms in a soil had potential for affecting the growth and survival of the root pathogens under study, the following procedures were adopted:

(1) A layer of treated (aerated steam at 60°C for 30 min) moist soil was placed in a glass vial (diam. 2·5 cm) over a culture of the fungus on 2 ml of dilute lucerne extract agar.* The aerated-steam treatment at 60°C for 30 min killed pathogenic fungi with minimal loss of antagonists (Baker 1962; Bollen 1969) and formation of soil phytotoxins (Dawson *et al.* 1965). A soil layer 3 cm deep was placed above *Phytophthora* spp., while the soil layer above other fungi with good competitive saprophytic ability was 9 cm deep. After 1- and 2-month intervals fresh lucerne stems which had been sterilized with propylene oxide were placed on the soil surface for 1–2 days and isolations made from them to determine whether the pathogen had grown through the soil to the surface. There was good correlation between this method of assessing antagonistic potential of a soil and its inhibitory effect on the pathogen in the field. *Rhizoctonia solani*, which grew through all soils thus tested, apparently is affected by a different mechanism of biological control, as explained later. Internal lysis of mycelium of this fungus, as found by Olsen and Baker (1968) in California, was not observed.

(2) In a modification of the above, a thin layer of dilute lucerne extract agar was slanted across half a Petri dish (10 cm diam.) and inoculated with the fungus under test. When the mycelium had covered the agar, soil to be tested (steamed at 60°C for 30 min) was placed in the dish to a depth of 1·5 cm and the fungus baited from the soil surface as above. After 1 and 2 weeks the soil was knocked from the inverted dish, and the exposed fungal mycelium examined microscopically for internal lysis. (Lysis is used in this paper for the process of dissolution, plasmolysis, and collapse of the protoplast. It does not refer to cell-wall degradation, which was rarely seen in this study.)

(3) Tests with a susceptible host growing in small punnets of soil were conducted to measure the ability of *Phytophthora cinnamomi* to cause root rot in selected soils: (i) untreated, or treated with (ii) aerated steam at 60°C for 30 min, or (iii) flowing steam at 100°C for 30 min. Small punnets were filled with treated soils, seeded with jacaranda (*Jacaranda acutifolia* Humb. & Bonpl.) or jarrah, and placed in a growth cabinet running on a 24°C day/18·5°C night 12-hr cycle. Cornmeal–sand inoculum (1 g per punnet) of *P. cinnamomi* was mixed with soil in half the punnets in each treatment.

One soil tested in the latter manner was obtained from an avocado grove where *P. cinnamomi* was present but had not caused detectable root rot. In the tube test [(*b*)(1)] *P. cinnamomi* had failed to grow through this soil. Jacaranda seedlings, used as test plants for *P. cinnamomi*, grew well in raw soil to which *P. cinnamomi* had been

* Water extract of 200 g succulent lucerne stems; 50 g agar; distilled water, 5 litres; sterilized at 15 lb/in^2 for 20 min.

added and in soil steamed at 60°C for 30 min and similarly inoculated, but not in soil treated at 100°C and similarly inoculated (see Fig. 2). Plants grew well in soil after all three treatments when *P. cinnamomi* was not added. The antagonists, which were effective in this soil when untreated, survived treatment at 60°C, but not 100°C, for 30 min.

(*c*) *Isolation of Antagonistic Microorganisms*

Isolations were made from either the surface of lysed mycelium or directly from soil or rhizosphere soil. In some of the soils subjected to procedure (*b*)(2) above, fungal mycelium was internally lysed or collapsed, and bacterial and actinomycete colonies had developed along the affected mycelium. This mycelium was picked from the plate with sterile forceps, shaken in sterile distilled water, and serial dilutions were made on to potato dextrose agar (PDA). Resulting bacterial colonies were streaked to establish homogeneity of the isolates.

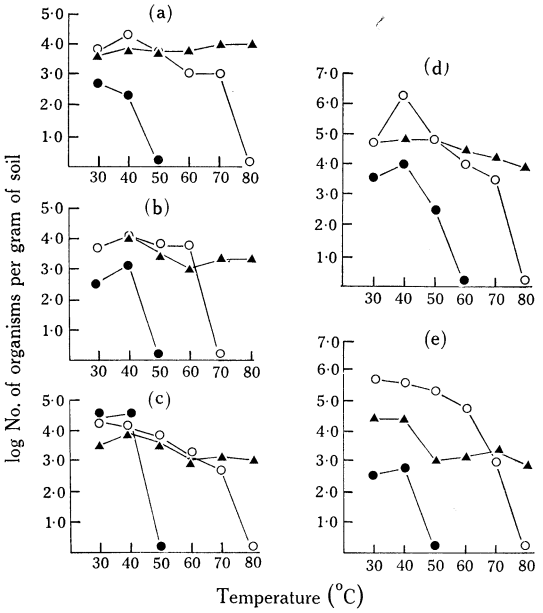


Fig. 1.—Comparative survival of *Bacillus* (▲), *Streptomyces* (○), and *Pseudomonas* (●) spp. following treatment of suspensions of five soils at six different temperatures for 10 min. Soils used were (*a*) vegetable, (*b*) garden, (*c*) pine duff, (*d*) vegetable, (*e*) pine duff.

Isolations of *Bacillus*, *Streptomyces*, and *Pseudomonas* spp. were also made from soil, and from the rhizosphere. Samples of 1 g of soil or 1 g lightly washed roots were shaken for 15 min in 10 ml sterile water. The suspension was drawn into Hawksley microhaematocrit centrifuge tubes which were sealed and placed in water at 30, 40, 50, 60, 70, or 80°C for 10 min, cooled, and plated in serial dilution on dried plates of yeast mannitol agar containing congo red, PDA, or soil extract agar (for *Bacillus* and *Streptomyces* spp.) and on modified King's medium B (Sands and Rovira 1970) for the isolation of fluorescent pseudomonads.

The survival of *Bacillus*, *Pseudomonas*, and *Streptomyces* spp. in a 1 in 10 soil suspension of five representative soils treated at different temperatures is shown in Figure 1. Above 40°C there were generally greater numbers than at 30°C, due possibly

to stimulation of germination or breaking of dormancy of spores. Numbers of *Pseudomonas* and *Streptomyces* decreased with increasing temperature. *Pseudomonas* usually did not survive treatment at 50°C for 10 min, and most *Streptomyces* spp. were killed at 80°C for 10 min.

Isolations from the rhizosphere of plants generally contained many bacteria forming spreading colonies and small numbers of *Bacillus* and *Streptomyces* (Fig. 3). *Pseudomonas* spp. were more prevalent in the rhizosphere than in the surrounding soil (Table 5). Soils high in organic matter (e.g. soils from avocado groves, pine forests, and the Western Australian forest krasnozem soil) contained many and varied micro-organisms; actinomycetes were very prevalent. Sandy loams planted to citrus had few actinomycetes, while marginal South Australian wheat soils, which were low in organic matter, yielded few *Bacillus* and *Streptomyces* (Table 2).

TABLE 2

NUMBER OF *BACILLUS* AND *STREPTOMYCES* ISOLATED FROM SIX REPRESENTATIVE SOILS HEATED AT DIFFERENT TEMPERATURES FOR 10 MIN

Results expressed as the number of cultures of the total number isolated which were antagonistic on agar to any of nine root pathogenic fungi

Soil and Soil Microorganisms	Treatment Temperature (°C)					
	30	40	50	60	70	80
Avocado soil 1						
Bacillus	8/40	9/30	11/20	16/26	6/32	11/40
Streptomyces	2/3	11/11	13/15	5/5	0/0	0/2
Avocado soil 2						
Bacillus	0/14	1/15	3/18	6/19	0/10	1/9
Streptomyces	4/6	3/5	15/25	0/1	0/9	0/0
Wheat soil 1						
Bacillus	—	7/19	2/3	2/10	3/8	3/11
Streptomyces	—	0/1	0/1	0/0	0/0	1/1
Wheat soil 2						
Bacillus	—	1/6	0/3	5/7	3/7	2/8
Streptomyces	—	0/0	0/0	0/0	0/0	0/0
Pine forest soil 1						
Bacillus	—	1/3	1/1	1/9	4/9	0/4
Streptomyces	—	0/0	7/8	29/35	1/1	0/1
Pine forest soil 2						
Bacillus	—	2/10	1/2	5/7	2/6	2/6
Streptomyces	—	2/2	4/4	26/27	1/1	1/1
Total *Bacillus*	8/54	21/83	18/47	35/78	18/72	19/78
Percentage *Bacillus*	14·8	25·3	38·3	44·9	25·0	24·4
Total *Streptomyces*	6/9	16/19	39/53	60/68	2/2	2/5
Percentage *Streptomyces*	66·6	84·2	73·6	88·2	100·0	40·0

Table 2 gives the percentage of antagonists, effective on agar against any of nine fungi, among 412 *Bacillus* and 165 *Streptomyces* isolated at different temperatures from six representative soils. The increase in total numbers of *Bacillus* at 60°C may reflect the breaking of dormancy of spores.

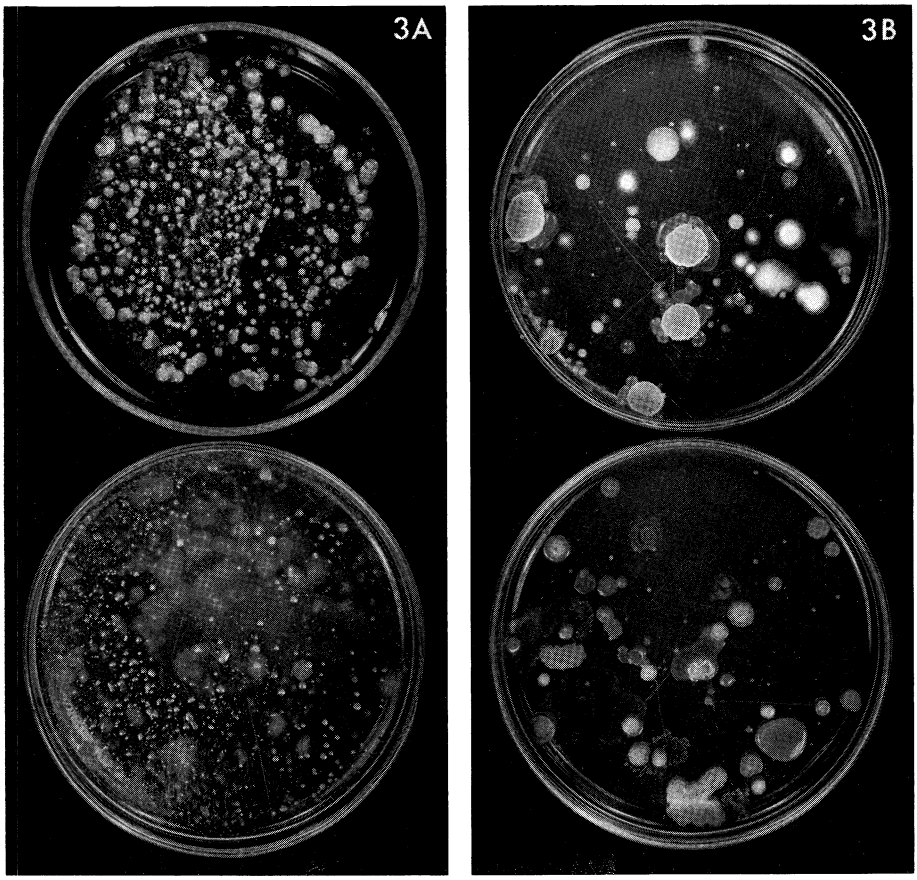
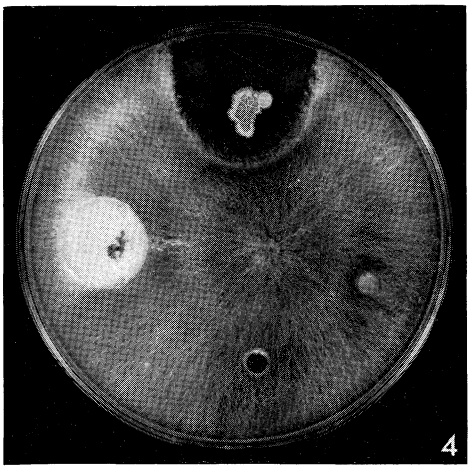
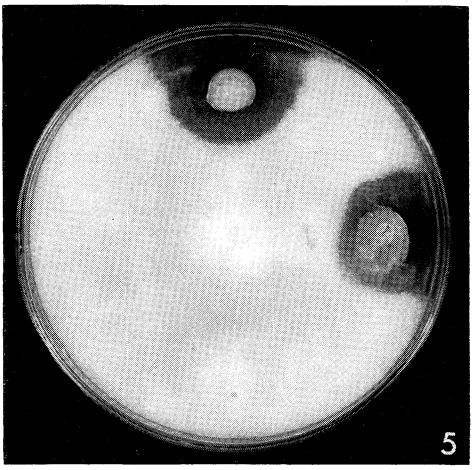


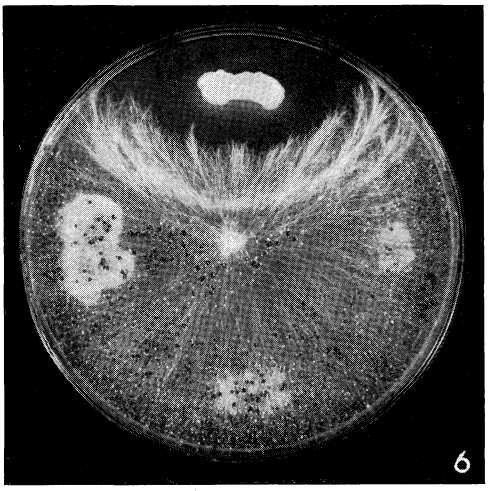
Fig. 2.—Soil from a Queensland avocado grove, in which *Phytophthora cinnamomi* was suppressed: untreated (left); steamed at 60°C for 30 min (centre); steamed at 100°C for 30 min (right). Each lot was then inoculated with a cornmeal–sand culture of *P. cinnamomi* and seeded to jacaranda. Photograph taken after 4 months at 23·5°C.

Fig. 3.—Isolations on yeast mannitol agar containing congo red (above) and on PDA (below) from rhizosphere (*A*) and the soil (*B*) around *Banksia grandis* plants in a lateritic soil from Western Australia. *Bacillus* and *Streptomyces* were abundant in soil, but spreading bacteria and *Pseudomonas* prevailed in the rhizosphere.

The percentage of *Bacillus* isolates antagonistic to plant pathogens increased with the temperature of soil treatment up to 60°C for 10 min, and then declined. The reason for this is undetermined. No such relationship was shown for *Streptomyces*. The data reaffirm the desirability of treating soil at no more than 60°C in order to retain antagonists while eliminating pathogens.







Figs. 4–6.—Zones of inhibition produced by *Bacillus* isolates against three root pathogens on PDA plates. *4*, *Bacillus* isolate AA43 effective against *Rhizoctonia solani* 15; three others were ineffective. *5*, *Bacillus megaterium* AA7 and *B. pumilis* AA15 causing inhibition of growth and lysis of hyphal tips of *Pythium ultimum*; two others were ineffective. *6*, *Bacillus* isolate $P_1 17$ effective against *Sclerotium rolfsii*, producing at the margin of the inhibited area frond-like growth similar to that produced in soil; three other *Bacillus* isolates were ineffective.

(*d*) *Presumptive Test for Antagonism to Certain Pathogens*

The various bacteria and actinomycetes isolated from hyphal surfaces, plant rhizospheres, and soil were tested for antagonism to the fungi *Phytophthora citrophthora*, *P. cinnamomi* and *P. nicotianae* var. *parasitica*, *Pythium ultimum* and *P. debaryanum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Sclerotium rolfsii*, and *Rhizoctonia solani* on PDA, Czapek (Dox) agar, and soil extract agar. Four bacterial or actinomycete isolates were placed on each plate simultaneously with a centrally placed PDA block taken from the actively growing margin of a young fungal colony. Bacteria growing on PDA

TABLE 3

ANTAGONISM ON AGAR TO EIGHT ROOT PATHOGENIC FUNGI BY *BACILLUS* AND *STREPTOMYCES* FROM 22 SELECTED SOILS

Results expressed as the number of cultures of the total number isolated which were antagonistic on agar to respective fungal isolate. Mean values for the number of soil samples examined are given

Soil and Soil Micro-organism*	Fungal Isolate No.†									% of Total Isolates Antagonistic
	1	2	4	14	6	7	8	12	15	
Avocado soils (6)										
Bacillus	4/19	52/272	32/263	11/61	24/266	11/273	16/44	6/30	4/23	12·8
Streptomyces	1/16	30/70	47/69	35/42	42/70	9/77	17/25	23/48	12/48	46·5
Pine forest soils (4)										
Bacillus	1/60	5/66	6/66	3/18	6/53	1/74	2/18	6/73	14/70	8·8
Streptomyces	5/107	56/102	52/104	19/25	84/101	20/105	16/25	35/106	28/108	40·2
Wheat soils (2)										
Bacillus	5/78	9/85	17/84	—	12/72	12/85	—	5/87	10/87	12·1
Streptomyces	0/3	0/3	1/3	—	1/2	0/3	—	0/2	0/3	10·5
Jarrah soils (10)										
Bacillus	—	59/289	45/289	16/78	46/289	2/289	—	—	—	13·6
Streptomyces	—	132/238	96/238	17/47	102/238	33/238	—	—	—	38·0
Percentage *Bacillus* antagonists	6·3	9·5	13·3	17·7	10·7	5·6	29·0	8·9	15·7	—
Percentage *Streptomyces* antagonists	6·5	49·1	56·8	80·6	73·4	15·7	66·0	38·5	25·2	—

* Numbers in parentheses indicate number of soil samples examined. † See Table 1 for name and origin of isolate.

showed greater inhibitory effect against the plant-pathogenic fungi than when growing on Czapek (Dox) or soil extract agar, but this did not determine the presence or absence of inhibition. The amount of bacterial growth could not be correlated with the extent of inhibition to root pathogens on agar.

TABLE 4

NUMBER OF *BACILLUS* AND *STREPTOMYCES* FROM WESTERN AUSTRALIAN FOREST SOILS ANTAGONISTIC ON AGAR TO PATHOGENIC PHYCOMYCETES

Results expressed as the number of cultures of the total number isolated which were antagonistic on agar to the respective fungal isolate

Soil*	Soil Micro-organism	Fungal Isolate No.†				% of Total Isolates Antagonistic
		1	4	6	7	
1*a*	*Bacillus*	8/42	6/42	5/42	1/42	11·9
	Streptomyces	27/37	23/37	22/37	11/37	56·1
1*b*	*Bacillus*	2/38	5/38	3/38	0/38	6·6
	Streptomyces	9/15	6/15	5/15	1/15	35·0
1*c*	*Bacillus*	3/24	3/24	4/24	0/24	10·4
	Streptomyces	28/42	11/42	14/42	3/42	33·3
2*a*	*Bacillus*	8/21	8/21	8/21	1/21	29·8
	Streptomyces	6/9	3/9	4/9	0/9	36·1
2*b*	*Bacillus*	3/28	1/28	2/28	0/28	5·4
	Streptomyces	11/32	7/32	8/32	4/32	23·4
3	*Bacillus*	0/24	0/24	0/24	0/24	0
	Streptomyces	11/34	9/34	8/34	2/34	22·1
4*a*	*Bacillus*	10/46	4/46	2/46	0/46	8·7
	Streptomyces	12/27	15/27	17/27	6/27	46·3
4*b*	*Bacillus*	3/25	1/25	3/25	0/25	7·0
	Streptomyces	17/23	13/23	14/23	3/23	51·1
5*a*	*Bacillus*	14/16	13/16	13/16	0/16	62·5
	Streptomyces	2/6	1/6	1/6	1/6	20·8
5*b*	*Bacillus*	8/25	4/25	6/25	0/25	18·0
	Streptomyces	9/13	8/13	9/13	2/13	53·8
Percentages all soils	*Bacillus*	20·4	15·6	15·9	0·7	—
	Streptomyces	55·5	40·3	42·9	13·8	—

* Soil origins as follows: 1, margin of jarrah dieback area in lateritic soil: *a*, from jarrah roots and soil; *b*, from *Banksia grandis* Willd. roots and soil; *c*, from *Eucalyptus calophylla* R. Br. roots and soil. 2, jarrah dieback area in lateritic soil: *a*, from jarrah roots and soil; *b*, from *B. grandis* roots and soil. 3, krasnozem soil, around *E. calophylla* and *E. patens* Benth. 4, margin of jarrah dieback in sand plain: *a*, from *B. grandis* roots and soil; *b*, from *Xanthorrhoea* sp. roots and soil. 5, jarrah dieback area in sand plain: *a*, from *E. calophylla* roots and soil; *b*, from *B. grandis* roots and soil.

† See Table 1 for name and origin of isolate.

Some isolates of *Bacillus* and *Streptomyces* had no effect on mycelial growth of the root pathogens. Others inhibited mycelial growth of one fungus only, some isolates inhibited mycelial growth of all fungi under test including several different isolates of some (Figs. 4–6). Others were stimulatory to the pathogen.

TABLE 5

NUMBER OF *PSEUDOMONAS* ISOLATED FROM SEVEN SOILS AND THEIR ANTAGONISM ON AGAR TO EIGHT ROOT PATHOGENS

Results expressed as the number of cultures of the total number isolated which were antagonistic on agar to the respective fungal isolate

Soil	Fungal Isolate No.*								% of Total Isolates Antagonistic
	1	2	4	20	6	7	12	15	
Pine forest duff	9/9	—	9/9	8/9	8/9	8/8	6/8	9/9	93·4
Corn									
Soil	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	2·5
Rhizosphere	0/11	0/11	0/11	0/11	0/11	0/11	0/11	1/11	1·1
Daphne									
Soil	0/7	1/7	1/7	1/7	0/7	0/7	0/7	0/7	5·4
Rhizosphere	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0
Citrus soil 1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0
Citrus soil 2	0/16	0/16	2/16	1/16	0/16	0/16	0/16	2/16	3·1
Saccaline									
Soil	0/7	0/7	2/7	0/7	0/7	0/7	0/7	3/7	8·9
Rhizosphere	0/2	1/2	0/2	2/2	0/2	0/2	0/2	2/2	31·3
Conifer nursery									
Soil	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0
Rhizosphere	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0
Percentage *Pseudomonas* antagonists	7·8	1·9	12·1	10·3	6·9	6·9	5·2	15·5	—

* See Table 1 for name and origin of isolate.

Antagonists isolated from 22 different soils are reported in Table 3; 723 of the *Bacillus* and 426 of the *Streptomyces* isolates were tested for inhibition in agar culture against nine pathogens. Details of organisms isolated from Western Australian jarrah soils are given in Table 4.

Actinomycetes were more inhibitory to *Phytophthora* spp. than to the other fungi tested (Table 3). The strongest antibiotic-producing actinomycetes were obtained from jarrah soils from Western Australia. *Pythium ultimum* and *Sclerotium rolfsii* were least inhibited by *Bacillus* and *Streptomyces* spp. on agar. The percentage of antagonists among actinomycetes was higher than for *Bacillus* (Tables 3 and 4).

Using the methods of Hsu and Lockwood (1969), approximately 80% of *Streptomyces* spp. tested inhibited fungal growth by antibiotic production while the remainder deprived the fungus of nutrients. Agar disks from the original inhibition zones when transferred to fresh agar plates seeded with the test fungi caused new inhibition zones, suggesting that most of our *Streptomyces* isolates were producing diffusible inhibitory substances.

All soils tested contained some bacteria and actinomycetes inhibitory to the root pathogens on agar, but soils with many antagonists effective on agar were not necessarily inhibitory to the pathogens. For example, soils 2*a*, 5*a*, and 5*b* (Table 4) had many antagonists but were from jarrah dieback areas, while soil 4 which had few antagonists effective on agar, was from an area where root rot had not developed.

Of the *Pseudomonas* isolates inhibitory to the fungi on agar, most had wide spectrum inhibition to fungi and also to *Bacillus* spp. Pine soil yielded the highest numbers of *Pseudomonas* antagonists effective on agar. The percentage of *Pseudomonas* isolates antagonistic to eight pathogenic root fungi are given in Table 5.

Twenty-six isolates of *Pseudomonas* pathogenic to plants were also included in tests for antagonism to the fungal root pathogens. These included four isolates of *Pseudomonas phaseolicola* (Burk.) Dowson and 22 isolates of *P. pisi* Sackett. Of these only one isolate of *P. pisi* inhibited growth of *Phytophthora* spp., *Fusarium oxysporum*, and *Rhizoctonia solani* on PDA.

Some lysis of mycelial protoplasts, particularly of *Phytophthora* and *Pythium*, by the action of *Bacillus*, *Streptomyces*, and *Pseudomonas* spp. has been observed on agar. Occasionally where plates had been inoculated simultaneously with the antagonist and the fungus a zone of inhibition of the fungus was formed (Fig. 5). The hyphae at the edge of the zone of inhibition ruptured immediately behind the hyphal tip. This was followed by coiling of the hypha and lysis. By growing the actinomycete or bacterium in Czapek (Dox) solution for 3 days then centrifuging (13,000 *g* for 10 min) and filtering the supernatant through a Millipore filter, it has sometimes been possible to obtain a cell-free solution which will inhibit mycelial growth when added directly to Czapek agar or placed in dialysing membrane on agar.

Certain actinomycetes clustered on the surface of mycelium of *Phytophthora* spp. resulted in death of the mycelium. Some *Bacillus* isolates stimulated sclerotium formation in *Sclerotium rolfsii* and initiated rhizomorph formation in *Armillaria mellea*.

One isolate of *Bacillus* from a South Australia wheat soil exhibited a "wetting action" on mycelium of *R. solani*. The mycelium was no longer water-repellant. Since *B. subtilis* Cohn emend. Prazm. produces antibiotics known to be surfactants

(Frobisher 1968), this effect may stimulate leakage from the mycelium providing nutrients for further bacterial colonization.

(*e*) *Identification of Antagonistic Microorganisms*

Most antagonistic *Bacillus* spp. were identified as *Bacillus subtilis* and *B. megaterium* de Bary. Some isolates of *B. cereus* Frankl. & Frankl., *B. pumilus* Gottheil, *B. polymyxa* (Prasm.) Migula, and *B. badius* Batch. emend Sag. & Apple. were also antagonistic to certain fungal root pathogens on agar.

Most isolates of *Pseudomonas* were Gram-negative, oxidase positive, fluorescent, and did not produce a necrotic reaction when injected into the mesophyll of tobacco leaves (Klement, Farkas, and Lovrekovich 1964). Depending on the soil, 10–100% of organisms isolated on Sand's modification of the King's B medium were *P. putida* (Trev.) Migula and *P. fluorescens* Migula.

The actinomycetes were only identified to genus.

(*f*) *Tests of Antagonists in Soil*

Potential antagonists were grown for 24 hr in shake cultures of yeast mannitol broth or nutrient broth, then concentrated by centrifugation, washed, and spun down again before being thoroughly mixed in U.C.-type mix (50 parts peat:50 parts sand+ fertilizers) (Baker 1957) which had been treated with flowing steam at 100°C for 30 min. One week later the soil was placed in punnets or flats uniformly sown to a susceptible crop, and randomized in a growth cabinet on a 12-hr cycle of 24°C day/18·5°C night. Wonder Bell pepper (*Capsicum frutescens* L. var. *grossum* Bailey) was used as the test plant for *Rhizoctonia solani* inoculations, and snapdragon (*Antirrhinum majus*) for *Pythium ultimum*. Treatments included uninoculated U.C. mix and the mix plus potential antagonists, with and without the fungal pathogen. The seeds were surface-sterilized with 5% sodium hypochlorite (0·5% available chlorine) for 2 min prior to planting to reduce surface contaminants. The punnets were inoculated in one corner (Ferguson 1958) with 0·2 g of a 5-week-old culture of the pathogen on cornmeal–sand medium, or a small piece of lucerne stem on which the fungus was established. The efficacy of the antagonist in controlling damping-off was measured by the percentage survival of seedlings, or distance of spread of the pathogens as evidenced by damping-off, or both.

One isolate of *Bacillus subtilis* (A13) stimulated growth of nitrogen-deficient peppers and tomatoes in the absence of *R. solani*, and also reduced damping-off caused by the pathogen (Fig. 7). Growth stimulation could not, however, be demonstrated in plants adequately supplied with nutrients. *Bacillus megaterium* (CC42) stimulated snapdragon growth (Fig. 8). Some *Streptomyces* isolates inhibited seedling germination and retarded plant growth, while certain *Pseudomonas* isolates had a phytotoxic effect on *Antirrhinum* seedlings. Reduction of germination of seed and inhibition of root growth by streptomycin were shown by Ferguson (1958) and Wright (1951).

Three *Bacillus* isolates (DD32, AA43, TX1) and one *Streptomyces* isolate ($Z_2$28) have effectively controlled damping-off by a virulent isolate (75) of *Rhizoctonia solani* in repeated tests (Figs. 9 and 10).

The ability of an antagonist to control *Rhizoctonia* damping-off varies with the strain of *Rhizoctonia*, some *Bacillus* isolates preventing pathogenicity of all isolates used and others effective only against the extremely pathogenic isolate 75 (Fig. 11). Such specificity may prove an obstacle to use of such isolates of antagonists in biological control. These results may reflect differences in growth rate or antibiotic specificity of the fungus. Some isolates (e.g. *Bacillus* G2A) increased the severity of damping-off (Fig. 7).

The effective antagonists did not influence the survival of *Rhizoctonia* in the soil, and protection against this fungus appears to occur in the rhizosphere, possibly by hindering infection rather than by destruction or inhibition of the fungus in the soil. It is to be recalled that this fungus spread through Australian soils in which seedling infection was later found to be drastically reduced. This is in accord with observations of Garrett (1970) in England. Sometimes a single infected seedling occurred in the midst of a group of healthy ones in soil which had been inoculated with the antagonist. This did not occur in punnets without the antagonist where damping-off occurred on a front, affecting all seedlings. It is possible that the single infected seedling did not have the antagonist colonizing its roots. Such a seedling became infected, and this could be the means whereby *Rhizoctonia* moves into the aerial phase of attack when the fungus advances up the stem. Under humid conditions, when capsicums reached 2–3 in. in height and the punnets became overcrowded, stem infection (wire-stem) occurred in plants previously protected from damping-off, and the fungus spread aerially from plant to plant.

Bacillus isolate WW27 has controlled *Pythium* damping-off (Fig. 12). All *Pseudomonas* isolates tested have failed to control damping-off either by *Rhizoctonia* or *Pythium*.

Build-up of *Bacillus subtilis* A13 was rapid for the first few days after the inoculation of U.C.-type mix treated at 100°C for 30 min, then numbers remained static (Fig. 13). Antagonistic *Bacillus* spp. were re-isolated from the rhizosphere of plants grown in soil treated at 100°C for 30 min and inoculated with the antagonist.

Attempts to re-isolate *Bacillus* and *Streptomyces* which had been introduced into U.C.-type mix treated with aerated steam at 60°C for 30 min have been unsuccessful, and no control of *Rhizoctonia solani* has been achieved.

Because of the large number of variable factors obviously involved in the operation of biological control, it would be expected that few isolates would be effective. In this regard it is comparable to the discovery and development of antibiotics for medical use. Of more than 3500 isolates of bacteria which were tested as potential antagonists, about 40% have shown activity on agar. In soil tests this number was further reduced. Five of 29 isolates antagonistic to *R. solani*, and 1 of 32 antagonistic to *P. ultimum* on agar were effective in a U.C.-type mix. About 4% of the original

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Fig. 9.—Reduction of damping-off and wire-stem of pepper seedlings produced by *Rhizoctonia solani* 75 by antagonistic *Bacillus* isolate DD32 (right). *B. subtilis* A13 (centre) was ineffective against this isolate of *R. solani* (cf. Fig. 7). Control, with no antagonist, at left.

Fig. 10.—Reduction of damping-off of pepper seedlings from *Rhizoctonia solani* 75 by antagonists. Control (left) without antagonists; *Streptomyces* isolate $Z_2$28 (centre); *Bacillus* isolate AA43 (right).

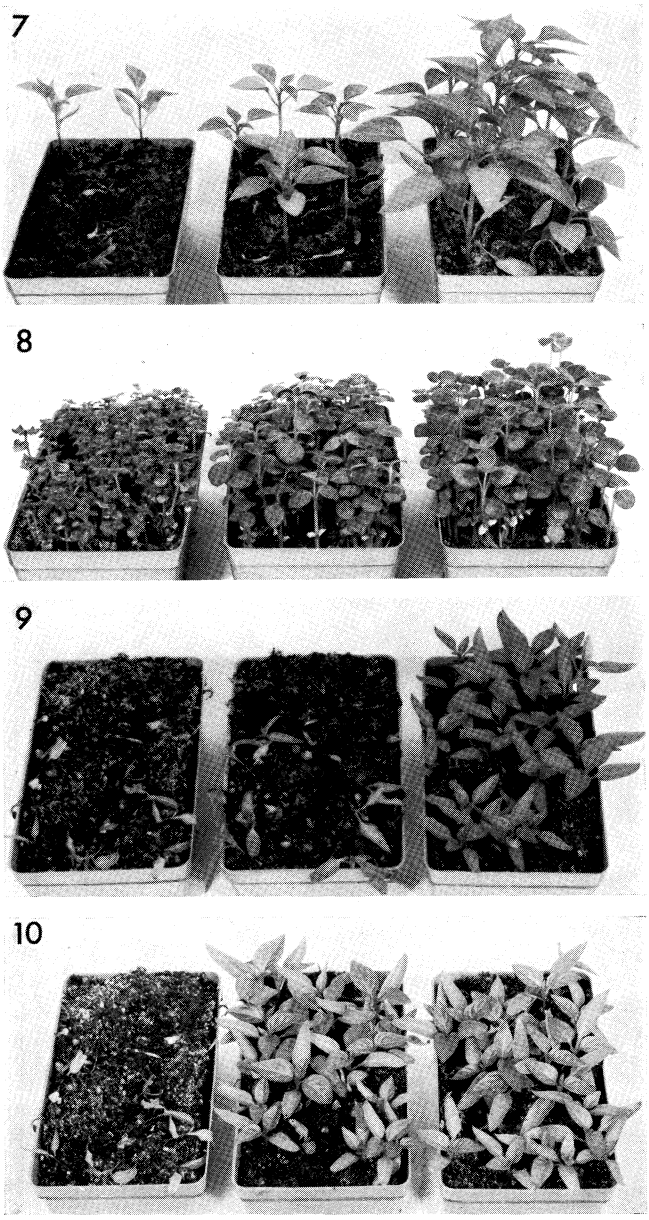

Fig. 7.—Reduction of damping-off and wire-stem of pepper seedlings caused by *Rhizoctonia solani* 15 by *Bacillus subtilis* A13 (right) added to the soil mix. Control (centre) inoculated with *R. solani* 15 at front left, but no antagonist added; note reduced size and stand. Addition of *Bacillus* isolate G2A (left) increased severity of infection by *R. solani* 15.

Fig. 8.—Retardation of growth and malformation of snapdragon seedlings by *Bacillus* isolate $T_4$15 (left) and stimulation of growth by *B. megaterium* CC42 (right) added to U.C.-type mix. Soil treated at 100°C for 30 min. Centre: control, no antagonist.

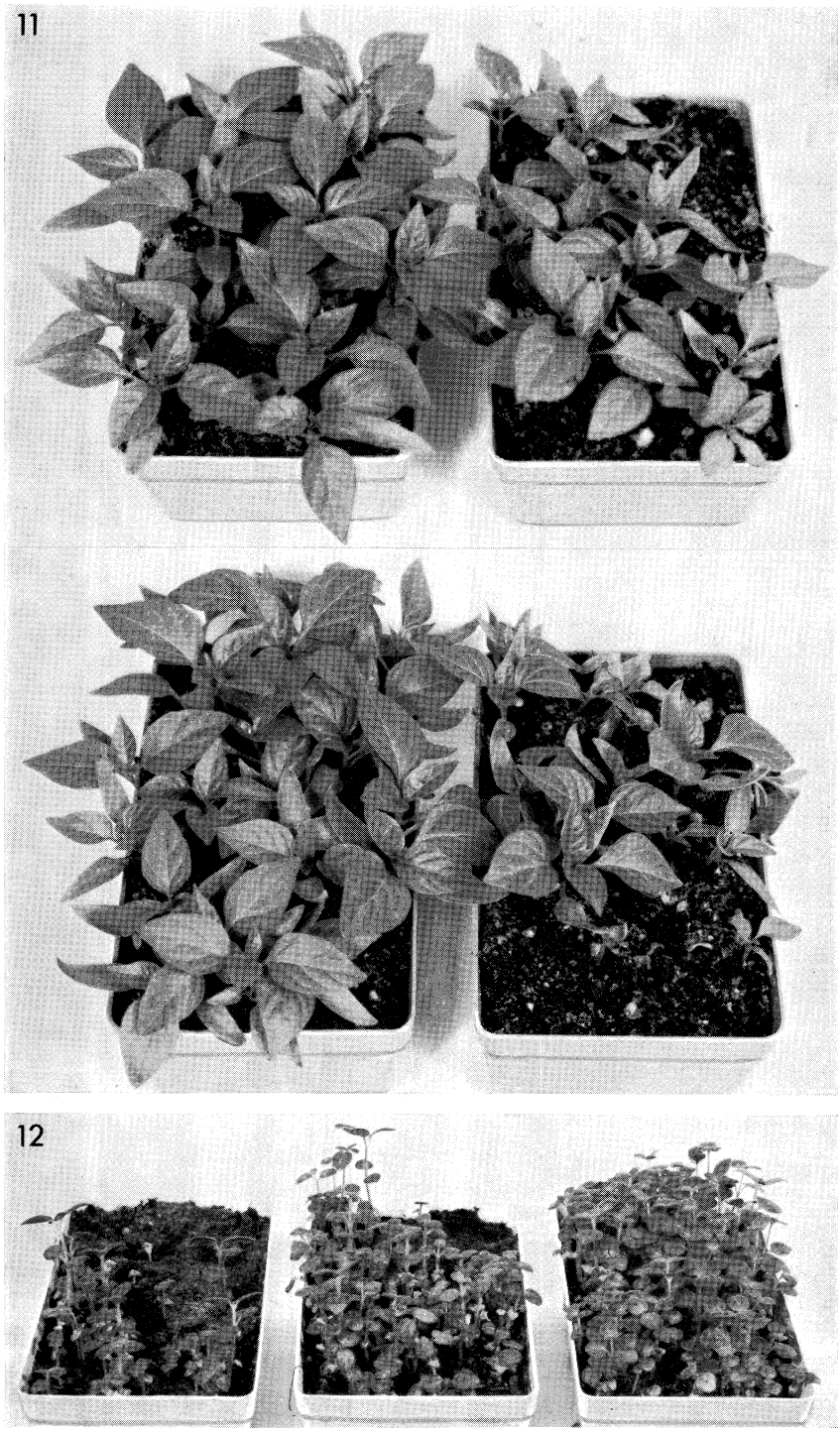

Fig. 11.—Differential effectiveness of two antagonists against three isolates of *Rhizoctonia solani* in preventing damping-off of pepper seedlings. *Bacillus* isolate TX1 (top row) and DD32 (bottom row). *R. solani* 75 (left, top and bottom), *R. solani* 18 (top right), and *R. solani* 15 (bottom, right). Note that both antagonists were effective against the virulent *R. solani* 75.

isolates, even from carefully selected soils, have proved useful in biological control under controlled conditions, and the value is likely to be even lower for commercial application. It is therefore necessary to work with large numbers of isolates in order to achieve success.

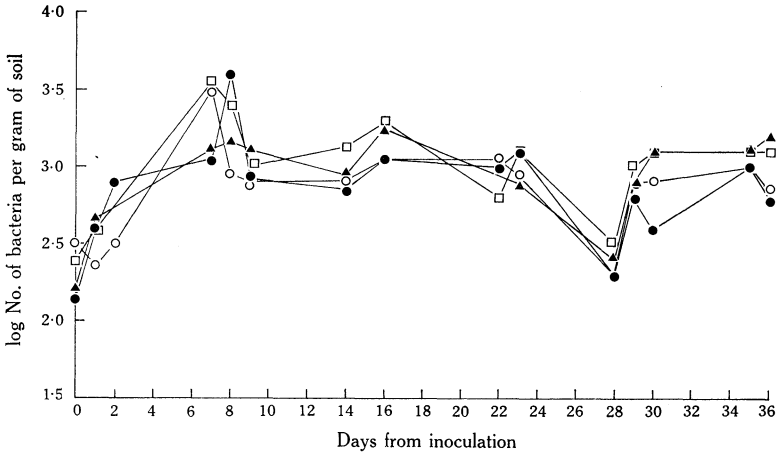

Fig. 13.—Multiplication of *Bacillus subtilis* A13 in sterile U.C.-type mix.

III. Discussion

Attention has been centred in these studies on species of *Bacillus* and *Streptomyces*, and to a lesser extent *Pseudomonas*, as potential antagonists, rather than on the frequently more conspicuous fungi. This choice was made because these bacteria and actinomycetes are ubiquitous and abundant in soil; tend to be well distributed through the surface-soil mass, rather than persisting in pockets, as many fungi do; have a more rapid reproduction rate than fungi; are efficient producers of antibiotics; and have features (enumerated below) particularly adapted to this role.

Pairing of antagonists with the fungal pathogen on agar media provides an initial screening for antibiotic-producing organisms, but does not mean that antibiosis will necessarily occur in that soil. Inactivation of antibiotics frequently occurs in soil by sorption on clay colloids and humus, by microbial degradation, and by instability due to pH (Brian 1957). Also very small amounts of antibiotics are produced in soil. Sometimes, too, the inhibitory zones produced by *Streptomyces* spp. result from depriving the fungus of nutrients and not from an antibiotic effect (Hsu and Lockwood 1969).

The number of microorganisms in a given soil shown by the plate method to be antagonistic does not accurately reflect the antagonistic potential of that soil. Most soils, particularly those rich in organic matter, have many antagonistic microorganisms.

Fig. 12.—Reduction of damping-off of snapdragon seedlings from *Pythium ultimum* by antagonists. Control (left) without antagonists; *Bacillus* isolate WA2b67 (centre); *Bacillus* isolate WW27 (right). Punnets inoculated with *P. ultimum* in top right-hand corner.

The antagonistic potential of *Bacillus* and *Streptomyces* spp. in a given soil is best appraised by assessing the growth of a pathogen through that soil treated at 60°C for 30 min, or by comparing damping-off or root rot of living plants produced by the fungus in that soil untreated, with the same soil treated at 60 and 100°C for 30 min. The seedling method is much more time- and space-consuming than the tube method, but is more reliable, particularly for *Phytophthora* spp. where mycelial growth is limited and infection is largely by zoospores (Campbell and Copeland 1954). The greater thermal resistance shown by *Bacillus* and *Streptomyces* spp. than by plant pathogens and most other saprophytes (Baker 1962; Bollen 1969; Wuest, Baker, and Conway 1970) makes it possible to use heat treatment to eliminate most of the other microflora. For this reason, a simplified soil flora still exhibiting antagonistic qualities may be obtained by treatment of the soil at 60°C for 30 min. In addition, treatments at 60°C for 30 min also cause the spores of *Bacillus* to break dormancy (Powell and Hunter 1955).

The difficulty of controlling *Pythium* damping-off in Australia is consistent with the small number of microorganisms antagonistic to it that have been found in Australian soils. Fewer actinomycetes were antagonistic to *P. ultimum* than to the other plant pathogenic fungi tested.

The isolation of *Bacillus* and *Streptomyces* spp. from internally lysed mycelium could support the interpretation of the role of these genera in mycolysis in nature. However, where external colonization of the fungus is accompanied by collapse of the mycelium, it is often difficult to determine if the organism is the cause of death or whether it is subsisting on exudates from mycelium dead or dying from other causes (Garrett 1965).

The stimulation of *Capsicum* and *Antirrhinum* growth by *Bacillus* spp. may have resulted from the production of a growth hormone, e.g. indoleacetic acid or gibberellic acid (Rovira 1965), stimulation of plant metabolism, mineralization of the soil, or destruction of soil toxins (Rovira and Bowen 1966), as well as from control of *Rhizoctonia solani*.

Success in establishing microorganisms in soil treated by steam or chemicals depends on the number of microorganisms remaining after treatment, and on the quantity of inoculum of the organism introduced and its competitive saprophytic ability. *Bacillus* spp. have characteristics particularly suited for studies on biological control: omnipresence in soils; high thermal tolerance; rapid growth in broth culture; and ready formation of resistant spores. They are said to be uniformly distributed through soil rather than concentrated in the plant rhizosphere (Peshakov 1965), and have been classed as poor competitors compared with *Pseudomonas* (Rovira 1965). That such generalization may not be applicable in specific cases is shown by the ease with which *Bacillus* isolates were obtained in these studies from the rhizosphere of plants in both cultivated and natural soils.

Streptomyces spp., by virtue of their wide distribution, thermal tolerance, active filamentous growth in soil (Lloyd 1969), and antibiotic production (Waksman 1967) may participate actively in establishing the microbiological equilibrium in soil, and may be a factor in affecting the incidence of certain soil-borne plant pathogens.

Fluorescent pseudomonads are metabolically very active, have a high growth rate (Vagnerova 1965), and predominate in the rhizosphere, but they have an

inhibitory effect on other rhizosphere bacteria (Rouatt and Katznelson 1961) as well as on *Bacillus* spp. In our initial tests they have not controlled damping-off due to *Pythium* or *Rhizoctonia*.

The high degree of specificity of action of some *Bacillus* strains for a given strain of the pathogen (e.g. for *R. solani*) may prove an obstacle in their practical use in biological control of damping-off fungi in steamed or chemically treated soils for bedding plants in nurseries. However, antagonists effective against a wide range of pathogens also occur, and may be used to inoculate soils, perhaps in combination with more specific antagonists.

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