OBSERVATIONS ON THE INHIBITORY ACTIVITY OF A BACTERIUM

Activity of the antifungal substance produced by the bacterium *Bacillus subtilis*

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IN a previous paper (Lily, *et. al.* 1952) it was stated that a bacterium belonging to the *Bacillus subtilis* group was antagonistic to *Helminthosporium halodes*, *Botryodiplodia theobromae*, *Rhizoctonia bataticola*, *Rhizoctonia solani*, *Gliocladium roseum* and *Fusarium* sp. Several other workers have also shown that *B. subtilis* produces active lethal products which can inhibit the growth of fungi which cause diseases in plants. Nandi and Sen (1953) reported that a strain B (x) of *B. subtilis* produced a substance which inhibited the growth of *Aspergillus niger*, *Penicillium notatum*, *Alternaria oryzae*, *A. solani*, *Fusarium* sp., *Botrytis cinerea*, *Curvularia* sp., *Verticillium* sp., *Phoma* sp., and *Trichophyton* sp. at concentrations varying from 0·2/*w* gm/ml to 12/*w* gm/ml. This active substance differed in its antibiotic activities from other antifungal products of *B. subtilis* like bacillomycin, mycosubtilin and fungistatin. Bobad, *et. al.* (1952) have stated that an antifungal polypeptide is produced by *B. subtilis* which is active against *Penicillium roqueforti* and *Aspergillus niger* at concentrations of 0·05—0·2 mgm/ml. *B. subtilis* added to steamed soil with *Helminthosporium sativum* was found by Anwar (1949) to result in complete protection of barley seedlings from root rot. Vasudeva and Chakravarthy (1954) have observed the antibiotic action of *B. subtilis* on *Rhizoctonia bataticola*, *R. solani*, *Alternaria solani* and *Fusarium udum*. Michner and Snell (1949) isolated two antifungal substances from *B. subtilis*; one termed the "Rhizoctonia factor" and another the "Aspergillus factor". A crystalline antifungal agent mycosubtilin was isolated by Walton and Woodruff (1949) from cultures of *B. subtilis*. Guntelberg and Ottesen (1952) prepared crystals containing Plakalbumin-forming enzyme from *B. subtilis*. Nine extra-cellular heat stable pohypeptides found to be antifungal were isolated from *B. subtilis* by sharon, *et. al.* (1954). Rangaswamy (1954) reported that an antibiotic produced by *B. subtilis* acted systemically in tomato plants when fed through roots and checked infection of *Alternaria solani*.

Antibiotic preparations from *B. subtilis* inhibited virus infections like Petunia mosaic, Chilli mosaic and Vinca mosaic. The inhibition was found to be due to

direct action of the antibiotic on the virus *in vitro* rather than a change brought about in the host tissue (Rangaswamy 1954). Toximycin, an antibiotic isolated from *B. subtilis* exerted a marked neutralizing action on tobacco mosaic virus *in vitro* (Stassel *et.al.* 1953).

Data on some trials made with *B. subtilis* and its metabolic products on certain fungi associated with coconut diseases are presented here.

EXPERIMENTAL

Preparation of the Bacterial Filtrate

The bacterium was grown in potato dextrose solution at the laboratory temperature in shallow layers in 500 ml. Erlenmeyer flasks. After incubating for four days the bacterial cells were filtered off and the filtrate autoclaved at 10 lb. pressure for 10 minutes. This culture filtrate was used to test the presence of the antibiotic factor.

Effect of Culture Filtrate on the Growth of Fungi in Pure Culture

Influence of the period of incubation of the bacterium on the production of the antibiotic factor.—In order to determine the influence of the period of incubation on the production of the antibiotic factor, a series of flasks with potato dextrose solution were inoculated with the bacterium simultaneously. The bacterial filtrate was collected at intervals of 24 hours for a period of 120 hours and was used to prepare potato dextrose agar for growth inhibition trials. The concentration of the culture filtrates used was 20% in each case.

Helminthosporium halodes, Botryodiplodia theobromae, Rhizoctonia bataticola and *R. solani* were grown on these media in Petri-plates. Radial growth of the fungi was recorded at intervals of 24 hours. As control, all the test fungi were grown in potato dextrose agar without the bacterial filtrate. Table I presents the observations recorded.

TABLE I

Showing the presence of antibiotic factor in the culture filtrate of the bacterium as indicated by the growth of certain fungi—Radial spread in cm.

Period of incubation of bacterium in hours	Incubation period in hours																			
	Helminthosporium halodes					Rhizoctonia solani					Rhizoctonia bataticola					Botryodiplodia theobromae				
	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
24	0·61	1·5	2·1	2·7	3·2	..	1·01	1·2	2·0	2·8	1·2	2·7	4·5	..	..	0·7	1·06	1·3	1·4	1·5
48	..	..	..	..	..	..	0·6	0·92	1·8	2 5	1·0	1·6	2·37	3·3	4·5	0·6	0·7	0·7	0 95	1·1
72	..	..	..	..	..	..	0·55	1·28	1·9	2·8	0·85	1·7	2·2	2·9	3·2	0·75	0·75	0·85	0·86	0·9
96	..	..	..	..	..	..	0·5	1·0	1·7	2·4	0·6	1·6	2·1	2·8	2·9	..	..	..	..	..
120	..	..	..	..	..	..	0·5	1·1	1·9	2·5	0·9	2·1	3·3	4·0	4·5	..	0·37	0·46	0·47	0·65
Control	2·3	4·5	..	..	..	0·5	1·5	3·03	4·5	..	2·5	4·5	..	..	..	2·3	4·5	..	..	..

Four days old bacterial filtrate found to exert maximum antibiotic effect was used for all further work. Potato dextrose solution was prepared containing different concentrations of the bacterial filtrate, *viz.*, 2, 4, 6, 8, 10, 15, 20 & 25 per cent. Aliquots of 50 ml. of these solutions were taken in 250 ml. flasks and were inoculated with *B. theobromae*, *R. bataticola*, *R. solani and H. halodes*. After two weeks incubation the fungal mats were harvested, dried at 60° C. for 48 hours and their dry weights determined. Results are given in Table II.

TABLE II

Dry weight (in mg.) of fungal mats in potato dextrose solution with different concentrations of the bacterial filtrate

Test fungi	Concentration of bacterial filtrate %								
	2	4	6	8	10	15	20	25	Control
R. bataticola ..	356·2	354·1	343·6	340·3	324·2	241·2	..	..	368·2
R. solani ..	532·0	523·5	511·4	501·3	485·3	355·9	162·2	..	566·4
B. theobromae ..	292·2	287·4	263·4	166·4	111·2	81·8	..	..	312·4
H. halodes ..	310·9	288·7	215·0	205·3	109·8	95·0	..	..	333·8

Inactivation of the Culture Filtrate by Soils

Soil samples collected from Kayangulam and Quilon Beach Q 1 (sandy), Quilon Kuripuzha Q 2 (red loam), and Adoor and Varkala (lateritic) were used to test the inactivation of the antibiotic factor in the bacterial filtrate. The method adopted for this determination was that of Hessayon (1953). 25 g. of soil were placed in 250 ml. Erlenmeyer flasks. The required quantity of distilled water was added to it to keep the moisture content of the soil at its saturation. One set of these soils was sterilized at 20 lb. pressure for 30 minutes. Duplicate flasks were maintained both for sterile and unsterile series. 20 ml. of the bacterial filtrate were added to all the flasks, shaken well and the flasks were incubated for two days. Controls for these were run by adding 20 ml. sterile distilled water instead of the bacterial filtrate. On the third day the contents of the flasks were filtered, 2 per cent agar was added to the filtrate and it was then sterilized at 10 lb. pressure for 10 minutes. For comparison of the activity of the bacterial filtrate before and after incubation in the soil, 20 ml. of the bacterial filtrate were diluted with distilled water equivalent to the quantity added to the soil to maintain its saturation capacity. The diluted bacterial filtrate was sterilized with 2 per cent agar as in the other cases. *R. bataticola* was grown in these agar media poured in Petri plates and the radial spread was measured. Results are presented in Table III.

TABLE III

Inactivation of the "antibiotic factor" of the bacterial filtrate by soil as indicated by the radial growth (in cm.) of *R. bataticola* in soil extract agar

Soil	Treatment	Sterilized			Unsterilized		
		24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
Adoor ..	a	0·7	1·9	2·8	0	0	0
	b	1·2	3·0	3·5	1	2·5	3·5
Kayangulam ..	a	0·5	1·0	1·4	0	0	0
	b	1·1	2·5	3·5	1·1	2·6	3·5
Quilon Beach ..	a	0·26	1·4	2·8	0	0	0
	b	1·1	2·4	3·5	1·1	2·7	3·5
Kuripuzha ..	a	0·6	1·5	2·4	0	0	0
	b	1·1	2·6	3·5	1·2	2·7	3·5
Varkala ..	a	0·7	1·5	2·5	0·6	1·3	2·0
	b	1·1	2·4	3·5	1·1	2·5	3·5
2 per cent agar ..		1·2	3·0	3·5	..	..	..
Diluted bacterial filtrate ..		0	0	0	..	..	..

a—Bacterial filtrate, b—Sterile water.

The water extracts of the different soils had no toxic effect on the test organism as shown by treatment in Table III. It was found that the "antibiotic factor" was partially inactivated by all the soils when the soils were sterilized and by Varkala soil alone when used without sterilization. The antibiotic factor remained active in unsterile Kayangulam, Quilon and Adoor soils.

Effect of Increased Concentration of the Filtrate on its Inactivation by Soil

Instead of adding 20 ml. of the filtrate 40 ml. of the same were added to each soil and the inactivation of the "antibiotic factor" was determined as in the previous experiment. Inactivation of the "antibiotic factor" was observed in Varkala soil (natural) alone; it was lesser than that recorded in the previous trials. Results are presented in Table IV.

TABLE IV

Inactivation of the "antibiotic factor" in soil when 40 ml. of the bacterial filtrate was added to Varkala soil

	Adoor	Kayangulam	Quilon Beach	Quilon Kuripuzha	Varkala
Radial growth of *Rhizoctonia bataticola* in 96 hours ..	Nil.	Nil.	Nil.	Nil.	0·5 cms.

Effect of Mixture of Soils on the Activity of the Filtrate

Kayangulam, Adoor, Quilon (Q 1 and Q 2) soils were mixed separately with Varkala soil in proportions of 5, 10, 25, 50, 75 and 90 per cent. The inactivation of the bacterial filtrate in these soil mixtures was determined as in the earlier experiment. It was found that in a mixture of these two groups of soils (that which inactivated the antifungal factor, namely Varkala and those which retained its potency, namely Kayangulam, Adoor and Quilon) the antifungal factor remained active as long as the percentage of the soil which retains its potency was high. Results are presented in Table V.

TABLE V

Inactivation of the "antibiotic factor" in mixtures of different soils as indicated by the radial growth of *R. bataticola* in cm. after 96 hours

Soil	Kayangulam	Adoor	Quilon Beach	Quilon-Kuripuzha
Varkala 5 per cent	Nil.	Nil.	Nil.	Nil.
" 10 "	Nil.	Nil.	0·9	Nil.
" 25 "	Nil.	Nil.	1·45	Nil.
" 50 "	1·98	Nil.	2·36	0·8
" 75 "	2	1·5	3·5	1·7
" 90 "	2·1	2·58	3·5	2·8

Results of the above two experiments reveal the variation in the effect of different soils on the inactivation of the antifungal factor of the bacterial filtrate. Varkala soil was capable of inactivating the antifungal factor, though partially, under unsterile conditions when used either alone or even in the presence of other soils which normally retained the activity of the antifungal factor. Explanation for this varying effect of the different soils on the activity of the antifungal factor was sought by the analysis of the soil samples, both mechanical and microbiological.

Mechanical Analysis of Soils

The dispersion of the soil as well as the determination of the percentage of silt, clay and fine sand were carried out by Robinson's method, the pH. of the soils were determined by Kuhn's calorimetric method as given by Wright (1939) and the moisture holding capacity as described by Keen and Raczkowskii (1921). The observations are recorded in Table VI.

TABLE VI

Presenting the results of the mechanical analysis of soils

Soils	Percentage of						
	Silt	Clay	Fine sand	Coarse sand	Moisture content	Moisture holding capacity	pH.
Varkala ..	2·2	49·1	17·5	32·0	16·5	48·8	5·1
Adoor ..	3·2	43·8	19·6	36·5	14·0	44·0	6·0
Quilon-Kuripuzha ..	1·9	13·5	29·3	54·2	8·5	34·4	5·2
Quilon-Beach ..	1·3	0·7	15·5	80·0	6·0	33·6	5·2
Kayangulam ..	1·5	3·0	20·0	74·0	8·0	31·2	6·4

The data presented above do not indicate any concrete relationship between the different soil factors and the activity of the antifungal factor. The lateritic soil samples, the one which retains the activity of the "antifungal factor", *i. e.*, Adoor soil, and that which partially inactivates it, namely Varkala soil, are more or less similar except for the difference in their pH. However, the activity of the antibiotic cannot be correlated with the hydrogen—ion concentration alone since it remained active in soils with a pH. range of 5·2—6·4.

Effect of the Bacterial Filtrate and Bacterial Cells on the Soil Microflora

The effect of the bacterium and its filtrate on the microflora of the above mentioned five soils was studied. 100 g. of the soils were placed in 250 ml. Erlenmeyer flasks and the moisture content was adjusted to 50% of their saturation capacity. In one series 20 ml. of the bacterial filtrate and in another 5 million bacterial cells suspended in 20 ml. steriel distilled water were added. Controls were maintained where instead of the bacterial cells and filtrate 20 ml. of steriel distilled water were added. Duplicate flasks were run for each treatment. The soil microflora was determined by the dilution plate method of Waksman and Fred (1922). Estimations of the fungal, bacterial and actinomycetes flora were carried out prior to the treatments and 15 and 25 days incubation after the treatments and their numbers were expressed on the basis of 10 g. moisture free soil.

TABLE VII

Showing the effect of *B. subtilis* cells and its filtrate on the soil microflora—Fungi in thousands and bacteria and actinomycetes in millions per g. soil

Treatment	Soil	Aspergilli	Penicillia	Fusaria	Rhizopus	Other fungi	B. Subtilis	Actinomycetes
Prior to treatment	K	0·75	2·5	1·0	0·25	0·25	6·25	1·0
	QB	0·5	2·0	0	0	0·75	4·75	3·0
	QL	0	6·5	0·5	0	1·25	2·75	2·0
	V	0·5	0·5	0·25	0	2·0	0·5	1·5
	A	0·5	1·25	0·25	0·25	0·5	1·0	1·0

TABLE VII—(*cont.*)

Treatment	Soil	Aspergilli	Penicillia	Fuseria	Rhizopus	Other fungi	B. Subtilis	Actinomyetes
After fifteen days incubation with water	K	0·5	4·75	1·25	0·25	1·0	20·5	1·5
	QB	1·5	24·25	0	0·25	0·25	6·0	9·0
	QL	0·5	65·75	0	0	0	9·75	11·0
	V	1·5	18·5	0	0	1·0	5·25	7·5
	A	0·5	0·75	0·5	0	0	18·0	1·5
After twenty-five days incubation with water	K	0·25	4·0	0	0	0·75	4·25	1·75
	QB	1·0	25·5	0	3·25	0	2·25	2·5
	QL	0·75	61·25	0	0	0·25	0·75	14·0
	V	3·25	43·25	0	0	0	0·75	5·75
	A	0	6·75	0	0	0·5	1·5	5·5
After fifteen days incubation with bacterial cells	K	0·25	8·25	1·0	0·25	1·0	27·0	1·0
	QB	0	1·25	0	0	1·25	12·75	1·0
	QL	0·75	9·0	0	0	0·25	13·5	1·75
	V	0·75	0·25	0	0	0·25	6·5	1·25
	A	0·5	1·5	0	0	2·25	10·25	1·0
After twenty-five days incubation with bacterial cell	K	0·75	6·75	0·25	0·5	2·25	20·5	1·75
	QB	0·25	2·0	0	2·0	0	0·5	7·25
	QL	0	30·25	1·75	0	0	3·0	6·75
	B	0·25	2·0	0·25	0·75	1·0	2·25	10·5
	A	0	8·0	0	0	0	9·25	5·5
After fifteen days incubation with bacterial filtrate	K	0·75	6·5	0	1·0	3·25	35·0	2·25
	QB	0	60·0	0	0	0	2·25	0·75
	QL	0	60·0	0	0	0	3·0	4·0
	B	0	60·0	0	0	0	2·0	0·5
	A	0	64·5	0	0	0	9·75	2·25
After twenty-five days incubation with bacterial filtrate	K	0	11·25	1·25	1·0	0	38·75	0
	QB	11·25	90·25	0	0	0	4·25	2·25
	QL	0	92·5	0	0	0	7·0	5·25
	B	0	92·75	0	0	2·5	1·75	0·75
	A	0	17·5	0	0	0	7·25	1·25

Results

1. The total number of fungi present in Quilon–2 sample was the highest (8·25 thousands), in the other soils it varied from 2·75 to 4·85 thousands. The number of *Bacillus subtilis* colonies varied from 0·5 in Varkala to 6·25 (millions) in Kayangulam soils. There was no perceptible variation in the numbers of actinomycetes in the different soils.

2. The numbers of Aspergilli and other fungi were not significantly affected

by the addition of the bacterium and its filtrate to the soils.

3. Penicillia increased even when soils with their moisture adjusted with sterile water were incubated. An increase in the numbers of Penicillia was also observed when the soils were treated with bacterial filtrate.

4. *Fusarium* and *Rhizopus* were absent in most of the soils after the treatments.

5. *Bacillus subtilis* increased when the soil was incubated for 15 days with the moisture adjusted to 50%. With increase in the incubation period their number increased. Kayangulam and Adoor soils recorded a greater increase of *B. subtilis* than Varkala and Quilon soils. With the addition of bacterial cells and its filtrate there was a further increase in the number of *B. subtilis* which remained high in Kayangulam and Adoor soils even after a long incubation period.

6. There was a uniform increase in the actinomycete flora when the soils were incubated with and without the bacterial cells and its filtrate.

Production of the Antibiotic Substance in Soil

To 100 g. each of Kayangulam soil in 250 ml. Erlenmeyer flasks were added (1) 2 per cent glucose,(2) 2 per cent farmyard manure, and (3) 2 per cent glucose plus 2 per cent farmyard manure. The contents of the flasks were mixed well and 20 ml. sterile water were added to them. Half the number of these flasks representing the three treatments were autoclaved for 30 minutes at 15 lb. pressure to form a sterile series. Both the sterile and unsterile series of flasks were inoculated with equal quantities of the washed suspension of the bacterium in sterile water. Flasks without the bacterial inoculum served as controls. The method adopted for the estimation of production of the antibiotic factor in the soil was the "soil sandwich" technique of Hessayon (1953) *R. bataticola* was the test organism used.

30 g. of soils from each of the above flasks were placed in Petri dishes and autoclaved at 10 lb. pressure for 10 minutes, after which about 10 ml. nutrient agar were added to each plate to anchor the soil layer to the base of the dish. After cooling another 20 ml. agar medium were added to form a smooth-surfaced layer of nutrient agar in which the soil to be assayed was embedded. The plates were then left to stand for 48 hours to allow diffusion into the agar of any antibiotic product present in the soil. In the centre of each assay plate cork borer discs of the inoculum of *R. bataticola* were placed. The radial spread of the fungus was measured every 24 hours. Results are presented in Table VIII and in Plate I.

TABLE VIII

Production of the "antibiotic factor" in soil with and without amendments by the bacterium as indicated by the growth of *R. bataticola*

(Radial spread in cm. after 48 hours)

Treatments	Soil					Soil + bacterium			
	Inoculation period	Control	a	b	c	Control	a	b	c
Sterile	10 days	3·5	4·5	4·5	4·5	3·1	0·7	3·3	1·6
	15 days	3·5	4·5	4·5	4·5	3·1	0·8	3·5	2·0
Unsterile	10 days	3·5	2·6	4·5	4·5	4·5	1·0	4·5	0
	15 days	3·5	3·5	4·5	4·5	3·8	0·5	4·5	0·3

(*a*) 2 per cent glucose.
(*b*) 2 per cent farmyard manure.
(*c*) 2 per cent glucose plus 2 per cent farmyard manure.

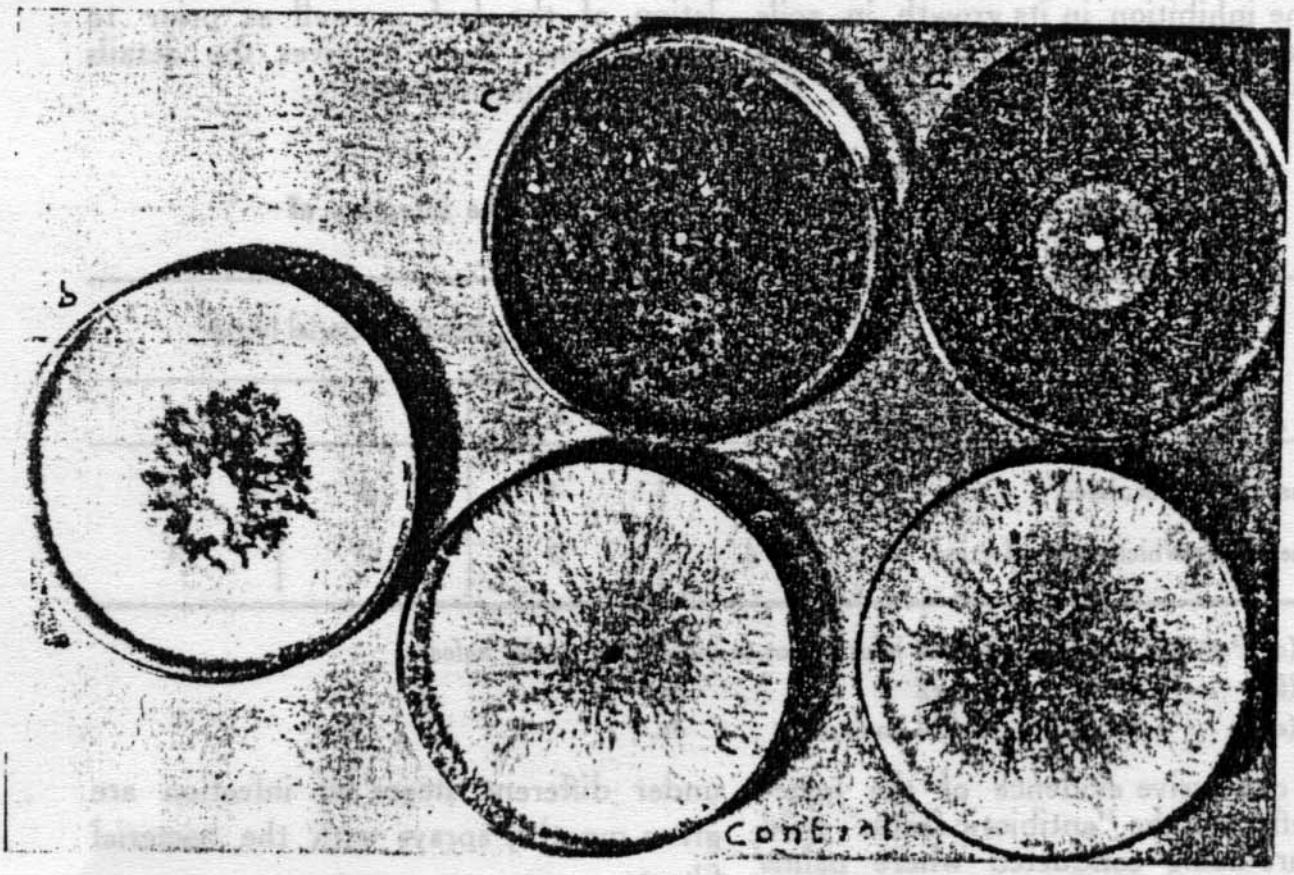

Plate 1. Showing the growth *R. bataticola* in the "Soil-sand-wich" plates representing the treatments a, b, c and the controls. (Soil and soil + bacterium) in the unsterile series after 10 days incubation.

Results

1. Addition of farmyard manure alone and in combination with glucose favoured the growth of the test organism in sterile and unsterile soils as compared to the control. On the other hand addition of glucose alone favoured its growth only in sterile soil and inhibited its growth in unsterile soil after a short incubation period, *i. e.*, 10 days. On longer incubation the adverse effect disappeared.

2. Inoculation of the soil, sterile and unsterile, amended and unamended, generally inhibited the growth of *R. bataticola*; the inhibitory effect being more perceptible when glucose alone and glucose plus farmyard manure were used as amendments.

3. The significant variation in the growth of *R. bataticola* in soils inoculated and uninoculated with *B. subtilis* indicate that the inhibition in its growth in soils inoculated with the bacterium might be due to the presence and activity of the latter which is boosted by the soil amendments (glucose and glucose plus farmyard manure).

Effect of the "Antibiotic Factor" on the Infection of Helminthosporium Halodes on Coconut Leaves

Three to five year old healthy coconut seedlings were selected for these trials. The method of inoculation of the seedlings was as reported earlier (Lilly *et. al*; 1952). Four days old bacterial filtrate was applied on the tender shoots of the coconut seedlings 72 and 24 hours prior and 24 hours after inoculation of the pathogen. Cotton pads soaked in the filtrate 50 ml. per seedlings were introduced between the leaflets. The seedlings were kept under observation for one week. The "antibiotic factor" inhibited the infection of *H. halodes* on coconut leaves when applied at the time of inoculation of the leaf as well as prior to inoculation. Table IX gives the details of the experimental results.

TABLE IX

Showing the effect of the bacterial filtrate on the infection of *H. halodes* on coconut leaves

Treatment	*H. halodes*	*H. halodes* bacterial filtrate		
		a	b	c
No. of seedlings inoculated ..	4	4	4	4
No. of seedlings which took infection ..	4	0	0	4

(*a*) Bacterial filtrate applied 24 hours prior to inoculation of *H. halodes*.
(*b*) Do. 72 do.
(*c*) Do. 24 hours after do.

For conclusive evidence of the beneficial effect of the "antibiotic factor" field trials are being conducted where palms affected by the leaf disease (*H. halodes*) under different stages of infection are given monthly sprays with the bacterial filtrate.

Discussion

The experimental results reported in this paper indicate that the antifungal factor produced by the bacterium *B. subtilis* inhibits the growth and activity of some of the fungi associated with coconut diseases in India. Whether this antifungal substance produced by *B. subtilis* is one among many of the antibiotics so far isolated from this bacterium by other research workers or whether it is a new one is a subject for further investigation.

The antibiotic factor in the bacterial filtrate is heat resistant and remains active in certain soils. Under sterile conditions all the soils tried inactivated it partially, whereas, under unsterile conditions only lateritic soils inactivated it and the "antibiotic factor" retained its potency in the loamy and sandy soils (Table III). Generally the inactivation of the antibiotic substances in soil is atributed to, (1) the activity of soil microorganisms, *i.e.*, the biological disintegration of the antibiotic, (2) the adsorption of the antibiotics by the clay or the organic matter present in the soil and (3) the effect of the hydrogen-ion concentration of the soil on the antibiotic. In the present instance, the clay contents of Varkala soil and Adoor soil are more or less equal, 49 and 43 per cent respectively (Table VI); nevertheless, the antifungal factor remained active in the Adoor sample but was inactivated, though partially, in Varkala soil. The acidity of the Varkala soil also may not be the factor responsible for the inactivation of the antibiotic since it retained its potency in Quilon soils which are as acidic as Varkala soil (Table VI). Detailed investigations which are in progress on the effect of hidrogen-ion concentration on the activity of the antibiotic in soil might throw further light on this aspect of the problem. That the biological disintegration of the antibiotic is not probable is proved by the data in Table III where we find that it remained active in unsterile soil while being partially inactivated in sterile soil. The inactivation of the antibiotic cannot be explained on the basis of a biological disentigration, nonetheless it appears to be closely related to the microbiological status of the soil. The data presented in Table VII indicate the changes involved in the microbiological equillibrium of the soil due to the addition of *B. subtilis* cells and its culture filtrate. Considering the two soils having a high clay content, namely Varkala and Adoor, we find that the former which inactivated the antibiotic did not respond so much in increasing the microbial flora as the latter which retained the potency of the antibiotic, to the addition of the bacterial filtrate. The increased *B. subtilis* colonies in Varkala soil was of a very short duration whereas it remained steady in Adoor soil (Table VII). The other soils where the antibiotic remained active also showed substantial increase of *B. subtilis* on incubation with the bacterial cells and its filtrate. Further evidence of the effect of Varkala soil in inactivating the antibiotic can be had from the data in Table V wherein the proportion of Varkala soil in the soil mixture is directly correlated with the inactivation of the antibiotic. It appears reasonable to presume that the increased *B. subtilis* colonies in the soil acts as a stimulant in retaining the potency of the antibiotic at a level lethal to the test organism. We have to hypothesize that in the soils which retain the activity of the antibiotic there might be a factor which favours the growth and survival of *B. subtilis* which is thus indirectly responsible for the activity of the antibiotic.

The evidence that *B. subtilis* can utilise the soil substrate as a medium for the production of the antibiotic substance (Table VIII) is important, for it indicates that in this case antibiotic production is a natural physiological mechanism and not an aberrant phenomenon appearing only under laboratory conditions of culture. Production of the antibiotic was favoured when the soil was supplemented with 2 per cent glucose with and without farmyard manure. A similar observation as to the effect of glucose on the antibiotic production of *Penicillium patulum* was reported by Grossbard (1952). However, she found that the antibiotic was produced only when the soil was autoclaved whereas in our experience natural soil was a better medium than autoclaved soil. This variation perhaps is due to the presence of naturally occurring *B. subtilis* in the soil as well as the favourable factor in the soil which increases it numerically.

In our experiments we found that the antifungal factor has got high inhibitory effect on *H. halodes*, which is associated with the leaf rot of coconut. In addition to the "Rhizoctonia factor" and the "Aspergillus factor" (Michner and Snell, 1949) there may be yet another factor present in the filtrate of *B. subtilis* which could be designated as the "Helminthosporium factor".

Summary

1. The bacterium identified as *B. subtilis* produces an antifungal substance both in pure culture and in soil.

2. The antifungal factor is thermostable. The culture filtrate inhibits the growth of *Rhizoctonia bataticola*, *R. solani*, *Botryodiplodia theobromae* and *Helminthosporium halodes* in pure culture at as low a concentration as 2 per cent. At 20 per cent concentration it completely supresses the growth of all fungi tried except *R. solani*.

3. The potency of the "antifungal factor" was adversely affected by five coconut soils, it was partially inactivated by all the five soils under sterile conditions and one soil under unsterile conditions. The inactivation of the antifungal factor was directly correlated to the concentration of the bacterial filtrate added to the soil.

4. In a mixture of the two types of soils, those which inactivated the antifungal factor and those which retained its potency, the antifungal factor remained active as long as the percentage of the soil retaining its potency was high.

5. Mechanical analysis of these soils indicated that the clay content has no bearing on the inactivation of the antibiotic. Similarly hydrogen-ion concentration of the soils which varies from 5·1 to 6·4 also seems to have little importance in inactivating the antifungal factor.

6. The study of the effect of the bacterium and its culture filterate on the microflora of the different soils revealed certain interesting relationship. Considerable increase in the *B. subtilis* colonies in soils which retained the potency of the "antifungal factor" was noticed on incubation at 50 per cent soil moisture content with and without the bacterial cells and their culture filtrate.

7. The "antifungal factor" was produced in the soil when supplemented with glucose and glucose plus farmyard manure in sterile and unsterile soils with an initial inoculum of *B. subtilis*. Production of the "antifungal factor" was more favoured by natural soil.

8. In a preliminary trial the "antifungal factor" inhibited the infection of *H. halodes* on coconut leaves when applied at the time of inoculation as well

as prior to inoculation (of the leaves with the pathogen). Further field trials are in progress to test the possibility of the antibiotic as a fungicide in controlling fungal infection.

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*Not seen in original.