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STUDIES IN THE RESISTANCE OF CERTAIN VARIETIES OF BANANA TO PANAMA DISEASE

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PART II. THE RHIZOSPHERE

Introduction. There have recently been many demonstrations of the presence in soils, of micro-organisms which are capable of antagonising plant pathogens in culture. For example, Meredith [5]) has shown that there are present in banana soils many actinomycetes which are antagonistic to *Fusarium oxysporum-cubense* in culture. He found however, no correlation between the resistance of soil type to the development of the disease and the abundance of antagonists. In a few cases it has been demonstrated that the introduction of an antagonist to a soil, sterile except for the presence of a plant pathogen, has depressed the incidence of the disease in plants subsequently grown in that soil. Weindling [7]) and Brian [1]) have reviewed much of the work on microbial antagonism to plant pathogens. The greatest claims for the significance of microbial antagonists to soil borne pathogens have been made by Russian workers, notably Novogrudskij [6]) who states that the 'bacterisation' of seeds of linseed, with cultures of organisms which have been shown to lyse *Fusarium lini* in culture, greatly reduces the incidence of disease in the developing seedlings.

The establishment of a root disease in a crop is frequently dependent on soil conditions. This subject has been reviewed by Garrett [3]). Frequently the addition of organic matter to soils heavily infected with some root disease organism, has resulted in a depression of the incidence of the disease, supposedly because of the stimulation of soil saprophytes which are antagonistic to the pathogens. The growing plant is itself responsible for making very

considerable additions of organic matter to the soil, in the form of decaying root cap cells, root hairs and the cortex of older roots, together with possible root excretions from living cells. The population of micro-organisms on the root surfaces is therefore expected to resemble that of an organically manured soil. L o c h h e a d and his associates have shown this to be true and have made an intensive study of the effects of growing plants in modifying the soil microflora. They have shown these effects to be both qualitative and quantitative [4]).

Not only is the soil microflora changed by the presence of plant root systems, but different plants may influence the soil population in different ways. The effect of this change on soil borne pathogens has been shown by D e y [2]) who has shown that the intercropping of peas with Sorghum, reduced the incidence of *Fusarium udum* in the peas. K a t z n e l s o n *et al.* [4]) review their own work on the differences between the rhizosphere flora of resistant and susceptible varieties of flax to the disease caused by *Fusarium lini*. In these cases the resistant varieties support a smaller microbial population in their rhizosphere than the susceptible varieties.

The experiments to be described, were designed to detect differences between the rhizosphere flora of banana varieties and to discover to what extent the differences which were found were related to the nutrient content of the underground parts of the banana varieties tested. For these experiments, banana varieties immune and susceptible to Panama disease were chosen to test the hypothesis that there is a correlation between disease resistance and the nature of the rhizosphere population. The organisms isolated from the rhizospheres were also tested for the production of antibiotics to *Fusarium oxysporum-cubense* in culture.

Experimental

(1) *The rhizosphere flora of banana varieties.* Two suckers were taken from each of the following banana varieties in the field.

Gros Michel	Silk Fig	(Both susceptible to Panama disease).
Congo	Guindy	(Both immune to Panama disease).

(See Part I, Table I for further details of these varieties).

The suckers were cleaned of attached soil and the roots were removed. The suckers were then planted in individual 10″ pots con-

taining a loamy soil. No bananas were planted in two control pots.

After one month, active growth of new roots had occurred and from each pot a 10 g sample of roots plus attached soil was added to one litre of sterile Ringer's solution at one tenth of normal strength. The flasks were shaken in a mechanical shaker for ten minutes. From each flask, 1 ml of suspension was taken into 99 ml of sterile Ringer/10 to give a dilution of 1 part of the washings of 1 g of root sample to 10,000 parts of diluent. After shaking, a further dilution was made by taking 1 ml of the above dilution to 99 ml of sterile Ringer/10 to give a final dilution of 1 part in 1,000,000. From this final dilution, samples of 2.0 ml, 0.6 ml and 0.2 ml were pipetted into tubes containing 20 ml of molten soil extract agar at 45°C and after rotation of the tubes to mix the inoculum with the agar the mixture was poured into sterile Petri dishes. In all, ten dilution series were prepared, two for each banana variety and two for the control soil. Three levels of plating density were obtained by using the three sizes of sample from the 1/1,000,000 dilution and each of these ultimate samples was replicated three times.

The soil extract agar medium used in this experiment was prepared by a modification of Löhnis method as follows. 1 kilogram of soil was autoclaved with 1 litre of distilled water for ½ hour at 1½ atmospheres pressure. The resultant turbid suspension was passed through a Sharple's vertical centrifuge until a clear extract was obtained. The original method of Löhnis which involved filtration and refiltration through filter paper to clear the liquid was slow, sometimes taking as long as two to three days to obtain 1 litre of extract. The centrifuge method reduces this period to about twenty minutes. The filtrate was made up to 1 litre with distilled water and 0.5 g of K_2HPO_4 was added with 20 g of agar. After steaming to dissolve the agar, the pH was adjusted to 6.8 and the medium was tubed in 20 ml aliquots and autoclaved for ½ hour at 1½ atmospheres.

The roots from which the rhizosphere samples were taken were weighed, after drying at 100°C to constant weight. The colonies which developed on the agar plates were counted, see Table I. The plates innoculated with 2.0 ml were discarded because the populations were too high for accurate counts to be made.

Discussion of results. The expression of bacterial counts from rhizosphere samples presents great difficulty. Lochhead and his co-workers have expressed their results in terms

TABLE I

Determination of banana rhizosphere population									
Variety of banana		Volume of 1/1.000.000 dilution per plate					Total difference of observations from mean soil count for each variety $(2) - \bar{X} + (3) - \bar{Y}$	Weight of Root = w	Estimated total bacteria per dry root $\frac{(2) - \bar{x} + (3) - \bar{y}}{4.8 \times 0.00{,}0001 \times w}$
		(1) 2.0 ml	(2) 0.6 ml	$(2) - \bar{x}$	(3) 0.2 ml	$(3) - \bar{y}$			
Gros Michel replication add sampling	A 1	—	33	(17)	15	(8)	220	0. 327 g	140,163,000
	A 2	—	51	(35)	11	(4)			
	A 3	—	55	(39)	18	(11)			
	B 1	—	42	(26)	22	(15)			
	B 2	—	36	(20)	21	(14)			
	B 3	—	37	(20)	18	(11)			
Congo	1	127	33	(17)	11	(4)	105	0. 180 g	121,458,000
	A 2	130	25	(9)	10	(3)			
	A 3	123	27	(11)	13	(6)			
	B 1	61	20	(4)	8	(1)			
	B 2	86	46	(30)	6	(−1)			
	B 3	75	32	(16)	12	(5)			
Silk Fig	1	89	21	(5)	5	(−2)	39	0.102 g	79,538,000
	A 2	71	16	(0)	9	(2)			
	A 3	75	26	(10)	7	(0)			
	B 1	49	28	(12)	4	(−3)			
	B 2	71	26	(10)	7	(0)			
	B 3	75	26	(10)	7	(0)			
Guindy	1	83	21	(5)	9	(2)	108	0.315 g	71,250,000
	A 2	81	17	(1)	4	(−3)			
	A 3	81	13	(−3)	9	(2)			
	B 1	122	52	(36)	6	(−1)			
	B 2	111	34	(18)	21	(14)			
	B 3	131	45	(29)	15	(8)			
Soil	1	86	8	$x = 16$	3	$y = 7$			
	A 2	56	12		8				
	A 3	68	14		7				
	B 1	62	14		11				
	B 2	61	29		6				
	B 3	61	18		5				

of the number of bacteria and other organisms per gram of soil washed off the root system of a plant which has been pulled from the soil and shaken. This method suffers from the disadvantage that the amount of soil which remains attached to a root system which has been shaken, is determined by the stickiness of the soil, varying with soil type and moisture content. Ideally, the expression of rhizosphere effect should take into account the amount of root present and perhaps also the area of root surface, which in most cases is extremely difficult to measure. In the final column of Table I the number of organisms developing per plate has been summed for each variety and for the control soils. The count obtained from the control soil has been subtracted from the rhizosphere counts, and the difference has been corrected to give the total estimated difference between the count from 10 g of control soil and that from 10 g of root plus attached soil. This has then been divided by the dry weight of roots. The order of the results is not of course changed by the subtraction of the control soil count, although the differences would be somewhat reduced if this was not done. In this form, the figures in the final column of Table I represent the increase in numbers of micro-organisms brought about by the presence of 1 g dry weight of roots in a soil sample. There is a very clear difference between the numbers of organisms supported by the root systems of the genetically related pair, Congo and Gros Michel and the Silk Fig and Guindy pair. In each pair it is noteworthy that the resistant variety supports a smaller rhizosphere flora than the susceptible, but in the absence of replication it is impossible to judge the significance of the differences found. The counts obtained however, support the view that resistant varieties bear a smaller rhizosphere flora than susceptible, which L o c h h e a d and his associates had suggested from experiments with flax and tobacco.

(2) *The interaction between the rhizosphere flora of banana varieties and their growth on media prepared from these varieties.* Agar media were prepared from the rhizomes of Congo, Gros Michel, Silk Fig and Guindy bananas as described in Part I of this paper. Soil extract medium was prepared as previously described and a synthetic medium, glucose-Marmite-peptone agar was made.

TABLE II

Plate Counts on Medium/Root sample interaction experiment									
Samples of soil and root microfloras	ml of 1 : 1,000,000 Dilution/ Plate	MEDIA							
		Gros Michel	Congo	Silk	Guindy	Soil extract	Glucose-Marmite peptone-agar	Dilution Totals	Totals
Gros Michel .	2.0	37	55	18	12	54	82	258	
	0.6	11	23	7	3	22	43	109	407
	0.2	5	13	2	2	5	13	40	
Congo	2.0	233	222	44	20	164	268	951	
	0.6	32	35	15	9	53	109	253	1301
	0.2	8	19	5	2	24	39	97	
Silk	2.0	80	118	57	32	80	185	552	
	0.6	10	24	5	2	17	50	108	742
	0.2	9	13	8	0	7	45	82	
Soil A	2.0	40	87	10	3	93	108	341	
	0.6	8	12	7	2	18	77	124	506
	0.2	1	4	3	1	15	17	41	
Soil B	2.0	34	26	15	19	30	65	189	
	0.6	4	5	1	0	17	35	62	259
	0.2	1	3	1	0	2	1	8	
Total . . .		513	659	198	107	601	1140	3215	3215

Glucose-Marmite-Peptone agar:

To 1 litre of distilled water was added 10 g of glucose, 5 g of peptone and 3 g of Marmite; after boiling the mixture, 20 g of agar was added and stirred until dissolved. The pH was then adjusted to 6.8 and the medium was tubed in 20 ml aliquots and autoclaved for ½ hour at 1½ atmospheres pressure.

Roots of Gros Michel, Congo and Silk Fig bananas were sampled in the field. Two soil samples, A and B were taken from midway between a row of Congo and a row of Gros Michel bananas. The soil samples were well out of the influence of the banana roots, but were under a mixed grass vegetation; no bare areas were available for sampling.

In the laboratory, 10 g subsamples of each of the root samples were taken — these were composed of root plus the soil particles which remained attached to the roots after shaking. 10 g subsamples of soil were taken from samples A and B.

Dilutions were prepared in the same way as in Experiment I. 2.0 ml, 0.6 ml, and 0.2 ml aliquots were taken from the 1/1,000,000 dilutions of each soil and root sub-sample, onto each type of agar medium. After seven days incubation at 25°C the colonies which had developed on the plates were counted and the counts obtained are presented in Table II.

Discussion of results. The results presented in Table II have been analysed in several ways. An analysis of variance of the logarithms of the plate counts in Table II is given in Table III.

TABLE III

Analysis of variance of Medium/Root Sample interaction experiment
(Analysis of the logs of values in Table II)

Main effects	Sum of squares	D.F.	Variance	Variance ratio	Probability
(Variety of root sampled	5.70	4	1.425	35.09	less than .001)
Type of medium . . .	12.22	5	2.444	60.19	*less than .001*
Dilution	16.08	2	8.041	198.05	less than .001
1st order interaction					
Variety/Medium. . . .	1.053	20	0.0526	1.295	—
Variety/Dilution . . .	0.628	8	0.0785	1.933	.05 — 0.1 *)
Medium/Dilution . . .	0.755	10	0.0755	1.859	.05 — 0.1 *)
2nd order interaction					
Variety/Medium/Dilution = Error	1.626	40	0.0406		
Total	38.062	89			

*) To be discussed in a later publication.

There is significant difference between the media used, in their power of supporting the microbial populations introduced from the soil samples. The statistically significant difference between the total numbers of the rhizosphere samples should be ignored as no correction was made in this experiment to express rhizosphere counts in terms of the amount of root present in each sample. However it is possible to compare the rhizosphere floras of the banana varieties with respect to their relative development on the various media. In Table IV the results presented in Table II have been transformed,

TABLE IV

The development of soil and banana root populations of micro-organisms on media derived from banana extracts, as percentages of the development on soil extract agar

Root and soil microflora samples	Root and soil media				
	Gros Michel	Congo	Silk	Guindy	Soil
Gros Michel . . .	65.4	112.3	33.3	20.9	100.0
Congo	113.2	114.5	26.5	12.4	100.0
Silk	95.2	149.0	67.3	32.7	100.0
Soil A	37.3	81.7	15.9	4.7	100.0
Soil B	79.6	69.3	34.7	38.7	100.0

by summing over dilutions and expressing the number of colonies developing on a banana extract agar as a percentage of the number of colonies developing from the same innoculum on soil extract agar. The results are also expressed in a histogram, Fig. 1. The differences between media show clearly, but the differences in reaction of rhizospheres to the various media are particularly interesting. The soil extract agar is a better nutrient medium for the soil organisms than any rhizome extract, but the rhizosphere organisms are more completely represented on Congo. Silk Fig and Guindy media are very much poorer in their power of supporting a mixed microbial population than Congo and Gros Michel, and this is to be expected from a consideration of the magnitude of the microbial populations which these varieties support in the field (Expt. 1).

It is clear from this experiment, that banana varieties differ in the nutrient content of their underground parts and it seems likely that it is this difference which determines the differences between the microbial populations which the varieties support on their roots in the field.

(3) *The presence of organisms antagonistic to Fusarium oxysporum-cubense in the rhizosphere of bananas.* After counting the colonies obtained in experiment (2) the plates were flooded with glucose-Marmite-peptone agar molten at 45°C which had been heavily seeded with spores of *Fusarium oxysporum-cubense.* Occasional actinomycetes showed antagonism to the development of the fungus,

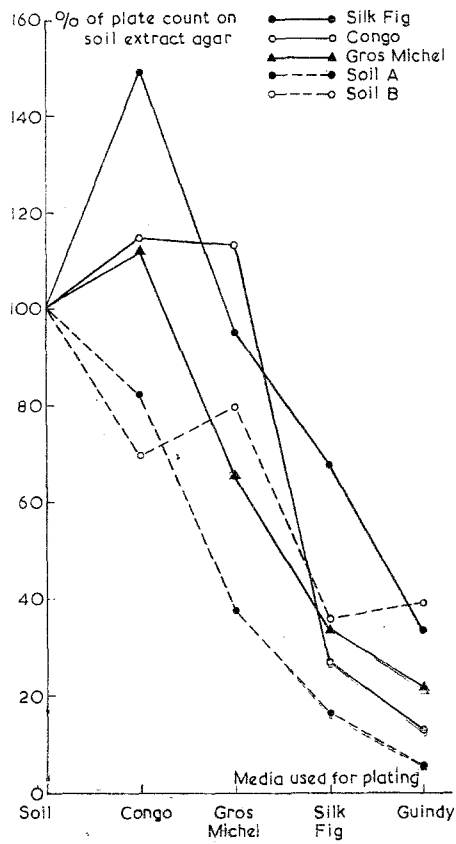

Fig. 1

demonstrated by rings of inhibition of the fungus in the agar above the colonies, but there was no correlation between the variety of banana and the abundance of these organisms.

A further test was made for the presence of antagonists to the fungus. Dilutions of the root washings of Congo and Gros Michel bananas were plated onto soil extract agar. After seven days, from

each of half the plates, 20 bacterial colonies and from the other half 20 actinomycete colonies were isolated and inoculated into tubes of sterile 1% Difco yeast extract broth. After ten days the broth was tested for antibiotic activity to *Fusarium oxysporum-cubense*. Each tube of extract was boiled for two minutes to effect partial sterilisation and 0.4 ml was added from a microdropper into a hole cut with a 1 cm cork borer in agar heavily seeded with spores of the fungus in Petri dishes. The results are given in Table V. A high pro-

TABLE V

Bacterial antagonists isolated from soil plates of the rhizospheres of Congo and Gros Michel bananas

Banana variety	Plate Count	Antagonists in 20 isolated	Percent Antagonists
A, B, = replicate root systems sampled			
a, b, c, d, = replicate plates made from each sample			
Gros Michel			
root sample . . . A (a)	186	1	5.0
(b)	49	0	0.0
B (a)	39	0	0.0
Congo			
root sample . . . A (a)	35	11	55.0
(b)	138	6	30.0
(c)	98	4	20.0
[(d)	303	0	0.0]
B (a)	43	2	10.0
[(b)	224	0	0.0]
(c)	215	3	15.0
C (a)	54	5	25.0

portion of bacteria isolated from the roots of Congo showed activity but only one antagonist was isolated from Gros Michel. At high plating densities the antagonist seemed to be suppressed and there was a sharp fall off in the percentage of antagonists obtained from any one root sample when the plating density was higher than 200 colonies per plate. (This plating density effect will be considered in detail in a later paper).

The 32 isolations of the antagonist were compared and from preliminary examination of growth rate, morphology, colony form and antibiotic production no difference was found between the isolations.

Further investigations of this organism are being made at the Colonial Microbiological Research Institute, Port of Spain, Trinidad.

The bacterium failed to produce antibiotics from subsurface colonies although its surface colonies were extremely active against *Fusarium oxysporum-cubense*.

Dr. A. C. Thaysen informs me that his investigations of this organism show that it requires fully aerobic conditions for antibiotic formation, which would account for the above observation.

Discussion of results. Further investigations are needed to determine whether the abundance of fungistatic organisms on the roots of the Panama disease immune Congo banana and its scarcity on Gros Michel roots was an ephemeral manifestation of the general difference in microflora between the two varieties or whether it is a permanent character of the varieties.

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Summary

The rhizosphere flora of banana varieties differs quantitatively from variety to variety. The comparison of rhizospheres is difficult, because the form of expression of the results may take many forms, none of them completely satisfactory. In the method adopted here, the two genetic pairs of banana, Gros Michel — Congo and Silk Fig — Guindy differ widely in the rhizosphere flora which they support, the former pair supporting the greater

number of micro-organisms. Within each pair, the higher number is found on the variety susceptible to Panama disease. The nutrient requirements of rhizosphere floras are related to the sap of the banana variety from which they have been derived.

A bacterium strongly antagonistic to *Fusarium oxysporum-cubense* was isolated in high numbers from the rhizosphere of the Panama disease immune Congo banana, although this was virtually absent from the susceptible Gros Michel.

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