MICROBIAL ANTAGONISMS AGAINST
A SALTANT OF
HELMINTHOSPORIUM SATIVUM

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INTRODUCTION

The disease complex known either as root-rot or foot-rot of cereals is world-wide in its distribution (7). It occurs throughout the three prairie provinces of Canada, and is frequently a limiting factor in the production of cereal crops (8). In Manitoba common root-rot of cereals and grasses is widespread. During the period 1930 to 1941 inclusive no wheat field in the province was found absolutely free from the disease. The average percentage of diseased plants for the three years, 1939, 1940 and 1941, was estimated at 38.3%. For the same period the average reduction in yield was estimated to be 12.1% (22). In our hungry world this is of no little consequence.

The problem of root-rot of wheat and other small grains is considerably complicated by the fact that several fungi may be involved as causal organisms, alone or in association, and each organism may be affected by differences in soil and climate. The methods which have been advanced for the control of this disease have, therefore, fallen into two general categories: (a) the production of resistant varieties (15), and (b) the investigation of the effect of the various soil environmental factors on the development of the pathogens (16). It is the purpose of this study to treat with one of these environmental factors; namely, the associative and antagonistic relationships of micro-organisms, with respect to a white fertile saltant of Helminthosporium sativum P. K. & B. This is one of the organisms of the root-rot complex.

This saltant, produced by Greaney and Machacek (13), 1933, by means of ultra-violet radiation, differs from the parent strain in that the mycelium is hyaline and the conidia almost colorless. It does not differ appreciably in pathogenicity. It was used in this investigation in preference to the parent strain since its striking lack of colour facilitates its recognition among other fungi in plates.

HISTORICAL

Investigation of microbial antagonism as a factor in the soil environment of root-rot pathogens was given its initial impetus by the work of Sanford and Broadfoot (28), 1931, and of Henry (16), 1931. The former workers demonstrated that certain species of fungi, actinomycetes and bacteria are effective in suppressing the pathogenicity of Ophiobolus graminis when filtrates or cultures of these organisms are combined with the pathogen and applied directly to the seed. The latter worker, Henry, studied the natural microflora of the soil in relation to the foot-rot problem of wheat. He found that the natural microflora had a marked inhibitive action on the growth of H. sativum in soil, with indication of a similar effect on Fusarium graminearum. All species of bacteria, fungi and actinomycetes isolated by Henry from the soil showed a suppressive action, most evident with fungi. A combination of the three groups proved the most effective. Henry also demonstrated that H. sativum sporulates readily in certain sterilized soils, while this is not the case if the soils are unsterilized. The fact that soils in which sporulation occurs may be rendered inimical to spore production by adding small amounts of unsterilized soil suggested that sporulation is inhibited by saprophytic soil microorganisms.

The work of Sanford and Broadfoot was confirmed by experiments conducted by Moritz, referred to by Garrard and Lochhead (11). In comparative pot culture tests, infection of wheat by O. graminis was found to be much less in unsterilized soil than in sterilized soil. This protective action varied with soil type, being greatest in soils showing the lowest incidence of the disease. Moritz believed that the antagonistic effect was of a quantitative rather than qualitative nature and was related to the microbiological activity of the soil.

Bisby, James and Timonin (2), 1933, while investigating fungi isolated from Manitoba soils by the plate method, observed that *Trichoderma lignorum* in pot culture tests prevented infection of wheat by *H. sativum* and *Fusarium culmorum*. Greaney and Machacek (14), 1935, reported that the pathogenicity of *H. sativum* on wheat seedlings was suppressed by the antagonistic action of *Trichothecium roseum*. In their opinion this effect was due to a toxic substance produced by the latter.

Novogrudski (24), 1937, found that certain bacterial strains isolated from soil and from diseased plants were lysogenic to *F. graminearum* and *Fusarium lini*. These provided reasonably good protection of wheat and flax seedlings in pot inoculation tests.

Henry and Campbell (17), 1938, reported the inactivation of certain seed-borne pathogens when infected seed was sown in natural soil. *Polyspora lini* and *Colletotrichum lini* were so affected both when naturally and artificially infected seed was used. This appeared to be due largely to the antibiotic action of the microorganisms of the soil since in sterilized soil similar seed produced significantly higher percentages of infection.

Sanford and Cormack (29), 1940, tested random isolations of *Penicillium* cultures and of other soil-inhabiting fungi for their effects on the virulence of *H. sativum* on wheat seedlings grown in steam-sterilized soil. They observed that some forms exerted a marked degree of suppression, some had no effect and others increased the virulence of the pathogen. *Trichoderma* reduced virulence by 50 to 58 per cent, *Absidia glauca* by 39 per cent and *Aspergillus nidulans* by 30 per cent. Marked variations in activity were observed among the different species of *Penicillium*. The reduction in virulence of the pathogen effected by these species ranged from 11 to 57 per cent.

Beresova and Naoumova (1), 1941, investigated the application of bacterial antagonists to seed for disease control. They designated their technique by the term "bacterization". By this method improved germination in flax and reduced infection by *F. lini* were obtained. Species of *Pseudomonas* and *Achromobacter* were found to be particularly effective in reducing seedling infection of wheat.

Ledingham (20), 1942, conducted green house and field inoculation studies in which *H. sativum* and *F. culmorum* were used both singly and in combination. Where the fungi were mixed, injury, as reflected in emergence and sometimes in yield, was less. Germination of *H. sativum* conidia was reduced in the presence of conidia of *F. culmorum*.

Simmonds (30), 1947, has recently submitted interesting evidence in support of the idea that seeds commonly harbour a bacterial surface flora which is antibiotic to *H. sativum*. This flora is believed to be separate and distinct from that of chance contamination.

Another phase in the investigation of microbiological antagonism has been an attempt in some cases to isolate the specific active principle from the organism involved and its application directly as a protective mechanism against pathogenic attack. Leeman (21), 1931, tested the action of various secretions and extracts of micro-organisms upon *H. sativum*. He concluded that micro-organisms, pathogenic or nonpathogenic, can supply substances which may be useful in the prevention of plant diseases.

Christensen and Davies (5), 1940, found that a strain of *Bacillus mesentericus* produced on artificial media an active substance that suppressed the growth of *H. sativum*. This substance increased sporulation of the fungus, inhibited or retarded spore germination, caused abnormal hyphal development and induced mutations in certain strains of the fungus. It was inactivated or destroyed, however, by certain fungi and bacteria.

Only recently, Goodman and Henry (12), 1947, reported that *Xanthomonas transluscens cerealis* exposed to a 1:1,000 dilution of subtilin was largely inactivated as measured by its ability to cause infection in barley seedlings. Comparable infection trends were obtained in sterilized and unsterilized soil. Subtilin also acted to reduce infection when applied to seed artificially infected with *X. transluscens cerealis* before treatment.

The above review has been confined to literature directly relevant to the subject under study. The general field of microbial associations and antagonisms has been dealt with by Waksman (33). Antibiosis in connection with cereal root diseases has been reviewed by Sanford (27), and by Garrard and Lochhead (11).

GENERAL CONSIDERATIONS

1. Criteria of Antagonism

The term microbial antagonism is a general expression of a phenomenon in which one organism is injured or even destroyed by the other (33). In plate cultures antagonism is manifested as a dwarfing or inhibiting effect on one species when two species are growing in association. The term usually implies that one species produces a substance that is toxic or "antibiotic" to the other.

According to Waksman (33), the three important types of antagonism are (a) the repressive, inhibitive or bacteriostatic (b) the bactericidal and (c) the bacteriolytic. Wolf and Wolf (35) cite a classification by Porter in which antagonistic interactions are further divided into four groups showing differences in degree of inhibitory action as follows:

1. One species overgrows and inhibits the other.
2. Each member of the pair exerts a slight mutual inhibition.
3. One of the pair grows close to but around the other.
4. Mutual inhibition is exhibited at a considerable distance and the two remain separate.

Examples of antagonism may be seen in Fig. 1.

It is conceivable, however, that other phenomena besides the production of antibiotic substances may be involved in antagonism. It is a common observation that when several microbes are growing in the same culture medium, some will be repressed in the course of time whereas others will survive and take their place. This may be due to the fact that these microbes compete for the use of the same nutrients or that conditions such as reaction, oxygen supply and temperature are more favourable to some organisms than to others, and an increasing share of the available nutrients will be utilized by the favoured organisms as they gain numerical preponderance over the others.

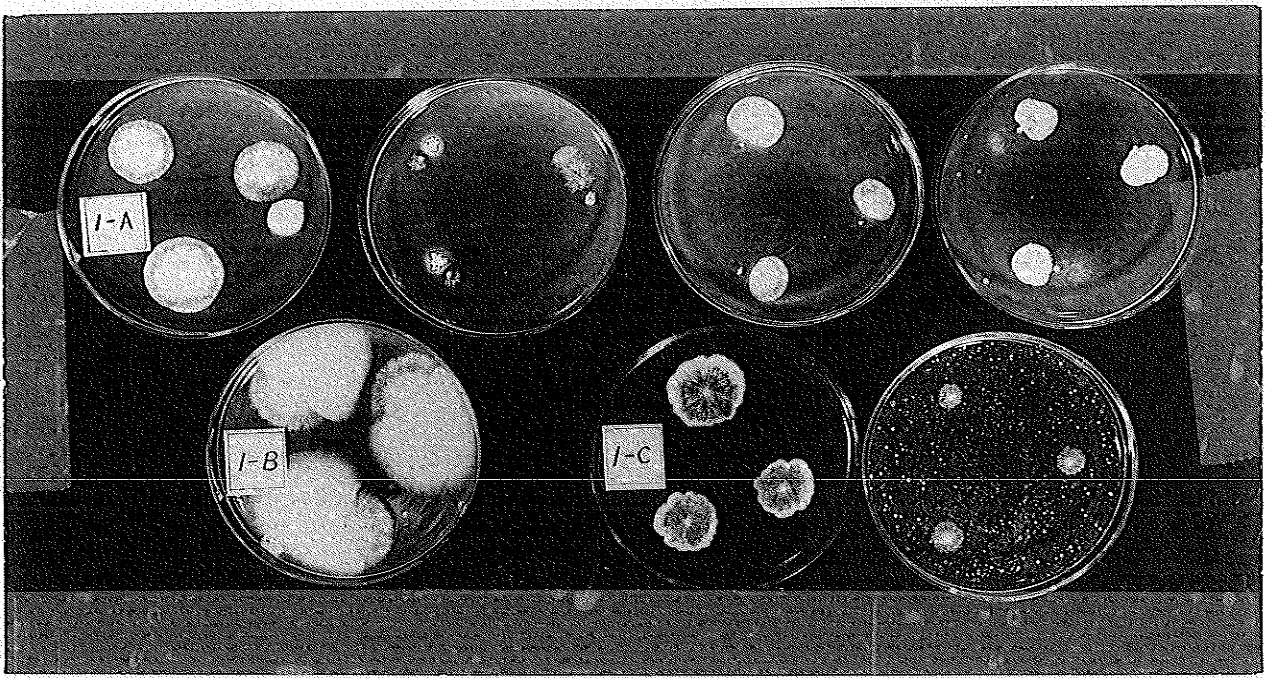

Figure 1. Antagonism in plate cultures.

1-A From left to right.
Control. H. sativum on Czapek's agar. Note the contaminant which apparently does not exhibit antagonism.

H. sativum and an Actinomyces. Note inhibition of saltant resulting in only slight growth.

H. sativum and an Actinomyces. Note Actinomyces (small speck) causing saltant hyphae to turn away.

H. sativum and Penicillium citrio-nigrum. Note fungal antagonist reducing saltant to slight feathery growth.

1-B H. sativum and Fusarium oxysporum. Note overgrowth effect.

1-C From left to right.
Control. H. sativum on potato dextrose agar.

H. sativum and a species of bacterium. Note the reduction in size of H. sativum.

Waksman and Starkey (34) present an interesting example of the influence of soil treatment upon the distribution of micro-organisms in the soil.

Treatment for a number of successive years	Reaction of soil pH	Bacteria	Fungi
No fertilizer	4.6	300,000	59,700
Lime, no fertilizer	6.4	5,200,000	22,450

This would indicate that pH conditions are created which are more favourable for bacteria. The decrease in the number of fungi would appear to be due primarily to competition for nutrients although it is possible that other factors may be involved. If this is true, antagonism in a broad sense must be considered to reflect competitive as well as antibiotic interrelations among micro-organisms.

Almost all micro-organisms inhabiting a natural environment such as soil or water are subject to numerous associative as well as antagonistic relationships. Some microbes living in association frequently develop characteristics which they do not possess when living in pure culture. When such associations result in changes that could not be brought about by the individual organisms alone, the process is termed "synergism" (18). Although many such reactions are recorded (33,35), no mention appears to be made of antagonism as a co-operative phenomenon that might not be produced by any of the associated organisms acting alone. Since microbes in the soil inevitably occur in mixed populations, the investigation of antagonism as a group rather than as an individual phenomenon may offer a larger insight into the matter.

Microbes grow and bring about many metabolic reactions in natural substrates, such as soils and water basins, in a manner quite different from those in artificial media (33). Broadfoot (3) working with cultures of bacteria and fungi used in previous experiments by Sanford and Broadfoot studied the antagonistic and compatible growth relationships of these organims towards Ophiobolus graminis on various culture media. He found that many of the organisms which exercised a marked degree of control on O. graminis on wheat in soil were not antagonistic in culture. This study demonstrated that the growth reactions of various organisms and O. graminis associated on artificial culture media is not a reliable indication that the same organisms will or will not suppress the pathogen in soil or open pot culture.

Brömmelhues (4), on the other hand, also working with O. graminis advanced a somewhat different view. She found that the pathogen was inhibited strongly in culture media by H. sativum and Penicillium sp. In pot soil tests when these were inoculated together with the pathogen the combined action gave less damage than that caused by O. graminis alone.

Such findings are perplexing indeed. Yet, it seems reasonable to assume that the final conclusion about antagonism must be based on studies with a susceptible host plant in soil harbouring the pathogen and the antagonist. A sterile soil, free from other organisms that might affect the relationship probably provides the best base for such an experimental study. It must be borne in mind, however, that pure cultures of microbes, free from the associative and competitive effects of other microbes found in natural substrates, may react in a manner quite different from those influenced by the growth of other organisms.

2. Antagonism to H. sativum by the "Natural" Flora of Cereal Seeds

Morgenthaler (23), 1918, suggested that it would be worthwhile investigating the biological importance of Bacterium herbicola and the epiphytic bacteria of grain. "One can assume," he wrote, "that the grain kernel and perhaps other plant parts are protected from damaging organisms under normal conditions by a layer of B. herbicola." (trans.)

Simmonds (30), 1947, as mentioned earlier, reported that certain bacteria commonly found on the surface of wheat seeds and other parts of the wheat plant are antibiotic to H. sativum. The most abundant bacterium in most cases of such antibiosis was found by Simmonds to be a short, motile rod.

James, Wilson and Stark (19), 1946, in a detailed study on the microflora of wheat found that two species predominated on plates prepared from washings from samples. One of these was identified as Bacterium herbicola aureum, the other as belonging to the genus Pseudomonas. Both were considered epiphytic on wheat. Subsequently, Stark (31) found that these two types constituted approximately 80% of the bacterial population on barley, flax and oat seeds. Since these two types may be considered truly representative of the epiphytic flora of Canadian cereal seeds, investigation as to whether or not they are antagonistic to H. sativum may in a sense provide an index of the antagonism to this pathogen by the "natural" flora of cereal seeds generally.

EXPERIMENTAL

1. Testing a Number of Isolates for Antagonism

(a) Single species vs. pathogen

Seven hundred isolates comprising 221 fungi, 193 bacteria and 286 Actinomyces were obtained from various sources as shown in Table 1. The purity of each culture was verified by plating and by microscopic study.

In order to determine whether isolates of fungi and Actinomyces were antagonistic to the saltant of *H. sativum*, a modification of the procedure originally introduced by Garré, as cited by Waksman (33), was adopted. Three pairs of spots 1 cm. apart were marked on the outside of the bottoms of petri plates. Czapek's medium was poured into the plates and allowed to harden. A needle transfer of a ten-day old culture of a fungus or an Actinomyces was made to each of the left spots, and of the saltant to each of right spots of a single plate. This procedure was applied to each of the various isolates of fungi and Actinomyces. Evidence of antagonism was based on examination after incubation at 25° C. for ten days.

Bacterial isolates were tested for antagonism to the saltant by the implantation method, as referred to by Waksman (33). Nutrient agar sticks were melted--one for each isolate examined--and tempered to 45° C. in a water bath. A tube was inoculated with a single loopful of a 24-hour culture and mixed by rolling between the hands. It was poured into a plate and allowed to harden. Three spots were marked on the outside of the bottom of each plate, and needle transfers of the saltant made to the surface of the agar above each. Plates were incubated at 25° C. and examined daily for evidence of antagonism.

As shown in Figure 1, certain fungi and Actinomyces manifested their antagonism to the saltant by slowing its growth and inducing its hyphae to turn away from the region in which the antagonist was growing. Other fungi suppressed the saltant by overgrowing it. Bacterial isolates demonstrated antagonism to the saltant by radically reducing its growth from that which it was normally able to attain. The various degrees of antagonism shown by the different isolates are represented in Table 1 by the symbols, +, ++ and + + +. The greatest degree of antagonism is designated as + + +, the least as +. Failure to produce evidence of antagonism is designated as -.

Table 1. Number of isolates antagonistic to a white fertile saltant of *H. sativum*

Source	Number Tested	Failed to show antag.	Number antagonistic +	+ +	+ + +
		Fungi			
Soil	198	176	12	6	4
Air	5	4	1	0	0
Flour	15	9	4	2	0
A.T.C. /	3	0	0	2	0
	221	190	17	10	4
		Bacteria			
Soil	153	148	0	2	3
Flour	20	8	1	2	9
Grain	10	2	0	0	8
A.T.C.	10	2	0	0	8
	193	160	1	4	28
		Actinomyces			
Soil	250	216	16	11	7
Wheat seedlings	34	25	4	3	2
Butter	2	0	0	0	2
	286	241	20	14	11
TOTALS	700	591	38	28	43

/ American Type Culture Collection

+, + +, + + + Increasing degree of antagonism

(b) Combinations of species vs. pathogen

An attempt was made to determine whether fungi which were not individually antagonistic to the saltant might exhibit such antagonism when grown together in combinations of two or three.

Fifty-two combinations were tested. In these trials needle transfers from two, or in some cases three, different isolates were made to each of the three left spots on the petri plate, and as before, a needle transfer of the saltant made to each of the three right spots. The multiple fungal inoculations to the same site resulted in a mixed, matted growth which, when antagonistic, manifested its antagonism by overgrowing the saltant. The incidence of antagonism among the various combinations of fungi tested is shown in Table 2.

Table 2. Number of combinations of fungi antagonistic to a white fertile saltant of H. sativum

Source	Isolates in comb.	Number Tested	Failed to show antag.	Number antagonistic +	+ +	+ + +
Soil	2	36	30	4	2	0
Soil	3	16	14	1	0	1
		52	44	5	2	1

+, + +, + + + Increasing degree of antagonism

(c) Antagonism in soil seeded with a susceptible host

As shown in Table 1, 31 fungi, 33 bacteria and 45 Actinomyces, a total of 109 organisms, were found to exert various degrees of antagonism toward the saltant in plate culture. An attempt was next made to determine whether these organisms could exert a comparable antagonism when grown in soil culture, and reduce the virulence of the saltant on barley seedlings.

The method followed was largely that described by Sanford and Cormack (29). O.A.C. 21 barley seed, grown in 1947, was soaked for three hours in water and then disinfected for seven minutes in 1:1,000 mercuric chloride. After being rinsed thoroughly in sterile water, approximately 100 seeds were transferred aseptically to each of four 6 oz. medicine bottles containing respectively: 60 cc. standardized suspensions of the pathogen alone, the antagonist alone, a mixture of the two and 60 cc. of sterile water as the control. They were soaked for an hour, at which time the bottles were inverted to remove the seed from direct contact with the water. This created moist chambers, in effect, in which the seeds were incubated overnight at 25° C. to enhance spore germination (26).

Following incubation, 20 seeds from each treatment were planted in duplicate in wide-mouthed one-litre Erlenmayer flasks. In order to give additional assurance of the presence of the organisms, 30 cc. of the suspension in which the seed had been treated were added to each flask. Each flask contained 300 gm. of a 3:1 soil-sand mixture of uniform moisture content, adjusted to make for optimum growth conditions. The flasks, containing the appropriate amount of the soil, had previously been autoclaved twice on successive days for a period of four hours at 15 lb. pressure. The 109 isolates were tested in an identical manner in 15 groups over a period of five months.

The seeds were grown at a room temperature ranging from 58° F. to 72° F. for ten days. At that time the seedlings were examined for infection and rated by the method of Greaney, Machacek and Johnston (15). In addition, the total green weight of the seedlings which had emerged in each flask was recorded. The correlation between green weight and disease proved low, however, inasmuch as the optimum conditions enabled the seedlings to withstand even severe lesioning[1]. For this reason the disease rating alone was used for determining the efficacy of the various antagonists in reducing the virulence of the pathogen. Since the control flasks and those treatments of antagonist alone inevitably resulted in disease free seedlings, they were omitted from consideration in the

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[1] Personal communication from Dr. J. E. Machacek

analysis of the data. Similarly excluded were those treatments of antagonist together with pathogen whose variance exceeded control limits established at the three-Sigma level (9). The results, shown in Table 3, reveal significant differences which extend even to the 1% level.

Table 3. Effect of isolates antagonistic to *H. sativum* on infection of barley seedlings

	DF	MS	F	5%	1%
Replicates	1				
Treatments	113	443.24	5.31	1.39	1.59
Error	113	83.52			

(d) Comparison of antagonism observed in the petri plate with that manifested as a reduction of infection on barley seedlings

The antagonism rating of each isolate in plate culture, together with the mean disease rating of each isolate in association with the saltant and of the saltant alone, is presented in Table 4.

A large number of the fungal isolates listed in this table were identified through the kindness of Drs. J. E. Machacek and W. L. Gordon, of the Dominion Laboratory of Plant Pathology, Winnipeg.

Table 4. Comparison of antagonism observed in the petri plate with that manifested as a reduction of infection on barley seedlings

Source	Isolate	Antag. in plates	Mean disease rating		Nec. diff.	
			Isolate & Saltant	Saltant Alone	5%	1%
			Fungi			
Soil	Aspergillus candidus	+	23.5	41.3	13.2	17.5
Soil	A. flavipes	+	2.0			
Soil	A. flavus 1	+ + +	1.0			
Soil	A. flavus 2	+ + +	10.5			
Flour	A. glaucus	+	20.0			
A.T.C.†	A. nidulans	+ +	1.0			
Soil	Cladosporium herbarum	+ +	6.5			
Soil	Fusarium oxysporum 1	+ + +	1.0			
Soil	Fusarium oxysporum 2	+ + +	1.0			
Soil	Mucor variens	+	6.0			
Flour	Penicillium canescens	+	7.5			
Flour	P. casei	+ +	3.5			
Flour	P. citreo-nigrum	+ +	50.0			
Soil	P. commune	+	15.0			

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† American Type Culture Collection

+, + +, + + + Increasing degree of antagonism

Table 4. (Cont'd)

Source	Isolate	Antag. in Plates	Mean disease rating		Nec. diff.	
			Isolate & Saltant	Saltant Alone	5%	1%
			<u>Fungi</u> (Cont'd)			
Soil	Penicillium corylophilum 1	+	2.5	41.3	13.2	17.5
Soil	P. corylophilum 2	+	5.5			
Soil	P. hagemi	+	18.0			
Soil	P. krzemieniewskii	+	8.5			
Soil	P. lilacinum	+	5.5			
Air	P. nigricans	+	15.5			
Flour	P. pfefferianum	+	7.5			
Flour	P. purpurogenum	+	5.5			
Soil	P. roqueforti 1	+ +	7.5			
Soil	P. roqueforti 2	+ +	5.0			
Soil	P. roseo-maculatum	+ +[x]				
Soil	P. steckii	+	13.5			
Soil	P. terrestre	+ +[x]				

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[x] Variance exceeded 3-Sigma control limit

Table 4. (Cont'd)

Source	Isolate	Antag. in plates	Mean disease rating Isolate & Saltant	Mean disease rating Saltant Alone	Nec. diff. 5%	Nec. diff. 1%
		Fungi (Cont'd)				
Soil	Scopulariopsis brevicaulis	+ +	1.0	41.3	13.2	17.5
A.T.C.	Trichoderma lignorum	+ +	3.0			
		Bacteria				
A.T.C.	Bacillus megatherium	+ + +	15.5			
A.T.C.	B. mesentericus	+ + +	3.0			
A.T.C.	B. mycoides	+ + +	36.0			
A.T.C.	Proteus vulgaris	+ + +	8.0			
A.T.C.	Pseudomonas aeruginosa	+ + +	7.5			
A.T.C.	Ps. fluorescens	+ + +	3.0			
A.T.C.	Staphylococcus albus	+ + +	8.0			
A.T.C.	S. aureus	+ + +	27.0			
Grain	Bacterium herbicola 1	+ + +	25.0			
	2	+ + +	10.0			
	3	+ + +	24.0			

Table 4. (Cont'd)

Source	Isolate	Antag. in plates	Mean disease rating		Nec. diff.	
			Isolate & Saltant	Saltant Alone	5%	1%
	Bacteria (Cont'd)					
Grain	Bacterium herbicola 4	+ + +	17.5	41.3	13.2	17.5
	7	+ + +	15.0			
Grain	Flavobacterium turcosum	+ + +	10.0			
Grain	Unidentified sp.	+ + +	29.0			
Grain	Pseudomonas sp.	+ + +	8.0			
Flour	Unidentified sp. 1	+ + +	26.5			
	2	+ + +	40.5			
	3	+ + +	17.0			
	4	+ + +	35.0			
	5	+ + +	40.0			
	6	+ + +	35.5			
	7	+ + +	31.0			
	8	+	36.5			
	9	+ +	40.5			
	10	+ + +	19.5			
	11	+ + +	12.0			
	12	+ +	10.5			

Table 4. (Cont'd)

Source	Isolate		Antag. in plates	Mean disease rating Isolate & Saltant	Mean disease rating Saltant Alone	Nec. diff. 5%	Nec. diff. 1%
			Bacteria (Cont'd)				
Soil	Unidentified sp.	1	+ +	39.0	41.3	13.2	17.5
		2	+ +	35.0			
		3	+ + +	2.5			
		4	+ + +	18.0			
		5	+ + +	38.0			
			Actinomyces				
Soil	Actinomyces sp.	1	+[x]				
		2	+ +	47.0			
		3	+ +	23.0			
		4	+	32.0			
		5	+ +[x]				
		6	+ +	38.0			
		7	+[x]				
		8	+[x]				
		9	+	29.5			
		10	+ + +	4.5			
		11	+ +	7.5			

Table 4. (Cont'd)

Source	Isolate		Antag. in plates	Mean disease rating		Nec. diff.	
				Isolate & Saltant	Saltant Alone	5%	1%
Actinomyces (Cont'd)							
Soil	Actinomyces sp.	12	+ + +	8.5	41.3	13.2	17.3
		13	+ + +	3.0			
			x				
		14	+				
		15	+ + +	15.5			
		16	+ +	2.5			
		17	+ + +	10.5			
			x				
		18	+				
		19	+ +	16.5			
		20	+	32.0			
		21	+ +	19.5			
		22	+ +	31.0			
		23	+	42.0			
		24	+	31.5			
		25	+	24.0			
		26	+ + +	26.5			
			x				
		27	+				
		28	+	15.5			
		29	+	41.5			
		30	+	20.5			

Table 4. (Cont'd)

Source	Isolate		Antag. in plates	Mean disease rating Isolate & Saltant	 Saltant Alone	Nec. diff. 5%	 1%
Actinomyces (Cont'd)							
Soil	Actinomyces sp.	31	+ +	11.0	41.3	13.2	17.3
		32	+ +	10.5			
		33	+ + +	1.0			
		34	+	15.5			
Wheat seedlings	Actinomyces sp.	1	+ + +	30.0			
		2	+ + +[x]				
		3	+ +	34.5			
		4	+ +	6.5			
		5	+ +	16.5			
		6	+	19.5			
		7	+	16.0			
		8	+	25.0			
		9	+	11.5			
Butter	Actinomyces sp.	1	+ + +	59.5			
		2	+ + +	23.0			

2. Testing the "Natural" Flora of Cereal Seeds for Antagonism to H. sativum

Ten cultures isolated by Stark (31) from cereal seeds were tested for antagonism to the saltant in the manner described for bacterial isolates. Seven of these were *Bacterium herbicola*; one was identified as *Flavobacterium turcosum* (Zimmermann) Bergey *et al*; one was an unidentified, yellow-pigmented motile rod; and one, a fluorescent organism belonging to the genus *Pseudomonas*. The results are shown in Table 5. They were also tested in soil culture and are included in the results shown in Table 4, as isolates from grain.

Table 5. Plate culture evidence of antagonism to a white fertile saltant of *H. sativum* by organisms isolated from cereal seeds

Organism		Degree of Antagonism
Bacterium herbicola	1	+ + +
	2	+ + +
	3	+ + +
	4	+ + +
	5	-
	6	-
	7	+ + +
Flavobacterium turcosum		+ + +
Unidentified sp.		+ + +
Pseudomonas sp.		+ + +

+, + +, + + + Increasing degree of antagonism
- Failure to show antagonism

Simmonds (30) outlined a test to demonstrate the antibiosis of seed surface bacteria to H. sativum. Incubated seed is placed at points on the margin of a colony of H. sativum growing in the centre of a potato dextrose agar plate. Inhibition of fungal growth is said to be noticeable in a day or so.

In this study the above investigator's method was tried on seeds of three varieties of wheat--Thatcher, Regent and Redman. Kernels of each variety incubated for 24 hours in a moist chamber at 25° C. were placed at points on the margin of a colony of H. sativum in separate plates. Each variety was tested on cultures of the fungus five, seven and ten days old. A typical yellow bacterial growth appeared on the agar around the seeds in about 24 hours. The antibiotic effect exerted by such growth on H. sativum appeared, however, to be only slight, even after incubation for ten days.

In a subsequent experiment three plates were seeded with 1.o cc., 0.1 cc. and 0.01 cc. respectively of a nutrient broth suspension of a bacterial antagonist isolated from wheat. Needle transfers in triplicate of the saltant were then made to each plate as before. This procedure was replicated three times for each of the three bacterial isolates tested in this manner. The plates were incubated at 25° C. and examined daily for ten days.

The results were the same for the three isolates tested. As shown in Fig. 2, the size of the saltant colony increased as the number of bacterial colonies decreased.

In order to determine whether the reduction of the number of bacterial colonies permitted a greater saltant growth only because of a concomittant reduction in the amount of antibiotic substance produced, the following experiment was carried out.

Transfers of *Bacterium herbicola* and of *Pseudomonas* sp., as representatives of the epiphytic flora of cereal seeds considered by Simmonds to be antibiotic to *H. sativum*, were made to separate 6 oz. medicine bottles containing 100 cc. of sterile nutrient broth. Similar transfers were made of twenty kernels of Redman and Regent wheat, grown in 1947, to provide a comparison with the mixed flora of seed. These cultures were incubated for ten days at 25° C. to permit the accumulation of antibiotic substances. Each of the four cultures was filtered through a Berkefeld filter and the filtrate, ostensibly containing the antibiotic substance, was tested for sterility by incubating overnight at 25° C. Potato dextrose agar plates were then poured in duplicate containing 1.0 cc., 0.1 cc. and 0.01 cc. of the filtrate. One was inoculated with needle transfers of *H. sativum*, the other with *Fusarium oxysporum* found previously to be antagonistic to the saltant. The entire experiment was duplicated using dilution of the actual organisms rather than of the filtrates.

After incubation for five days at 25° C. the plates were examined and contaminated ones discarded. As shown in Fig. 2, the filtrate at any dilution in no way affected the growth either of the saltant or of the Fusarium. The plates seeded with bacteria at varying dilutions gave results as before. As the number of bacteria present decreased, the size of the saltant and of the Fusarium increased.

Figure 2. Competitive rather than antibiotic relationships between cereal seed epiphytes and H. sativum

F-H From left to right.
Plate 1. Control. H. sativum on potato dextrose agar. Plates 2, 3 and 4. H. sativum on P.D.A. to which was added 0.01 cc., 0.1 cc. and 1.0 cc. of a filtrate derived from a ten-day old culture of Pseudomonas sp. obtained from wheat.

B-H From left to right.
Plate 1. Control. H. sativum on P.D.A. Plates 2, 3 and 4. H. sativum on P.D.A. in which was seeded 0.01 cc., 0.1 cc. and 1.0 cc. of a suspension of the same species.

Figure 2. (Cont'd)

F-F From left to right.
Plate 1. Control. Fusarium oxysporum on potato dextrose agar. Plates 2, 3 and 4. F. oxysporum on P.D.A. to which was added 0.01 cc., 0.1 cc. and 1.0 cc. of a filtrate derived from a ten-day old culture of Pseudomonas sp.

B-F From left to right.
Plate 1. Control. F. oxysporum on P.D.A. Plates 2, 3 and 4. F. oxysporum on P.D.A. in which was seeded 0.01 cc., 0.1 cc. and 1.0 cc. of a suspension of the same species.

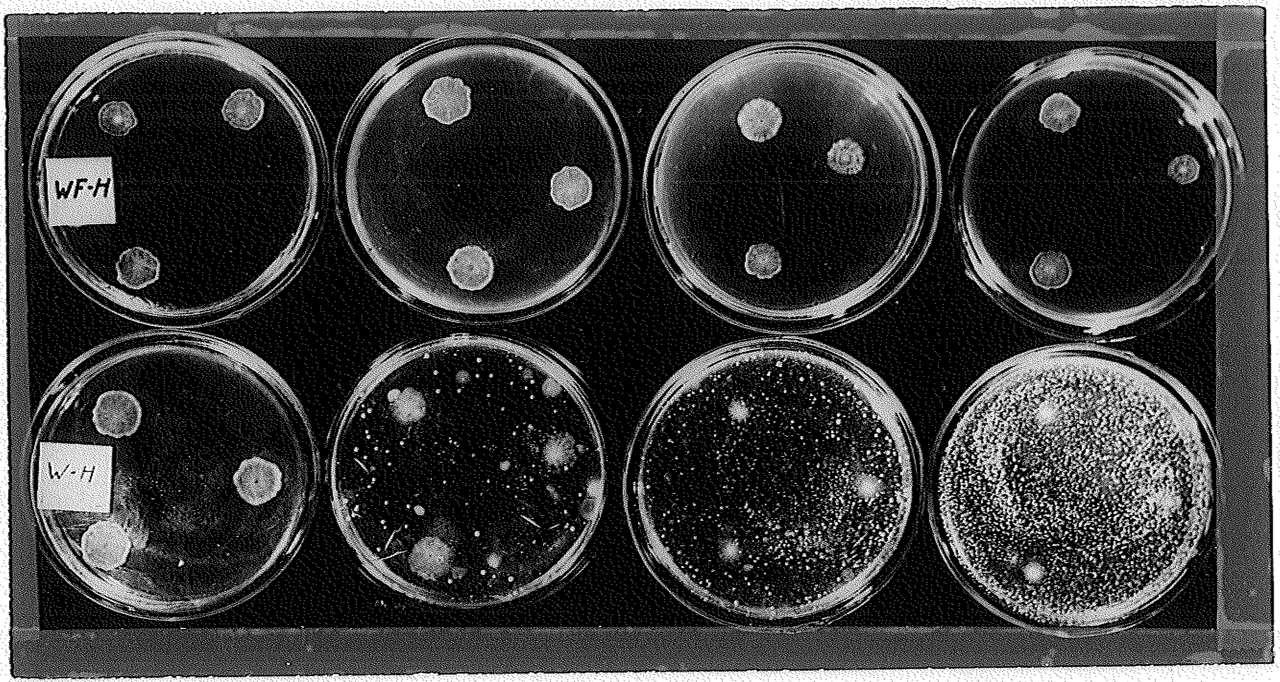

Figure 2. (Cont'd)

WF-H From left to right.
Plate 1. Control. H. sativum on potato dextrose agar. Plates 2, 3 and 4. H. sativum on P.D.A. to which was added 0.01 cc., 0.1 cc. and 1.0 cc. of a filtrate derived from a ten-day old culture of flora on Regent wheat seed.

W-H From left to right.
Plate 1. Control. H. sativum on P.D.A. Plates 2, 3 and 4. H. sativum on P.D.A. in which was seeded 0.01 cc., 0.1 cc. and 1.0 cc. of a suspension of the same flora.

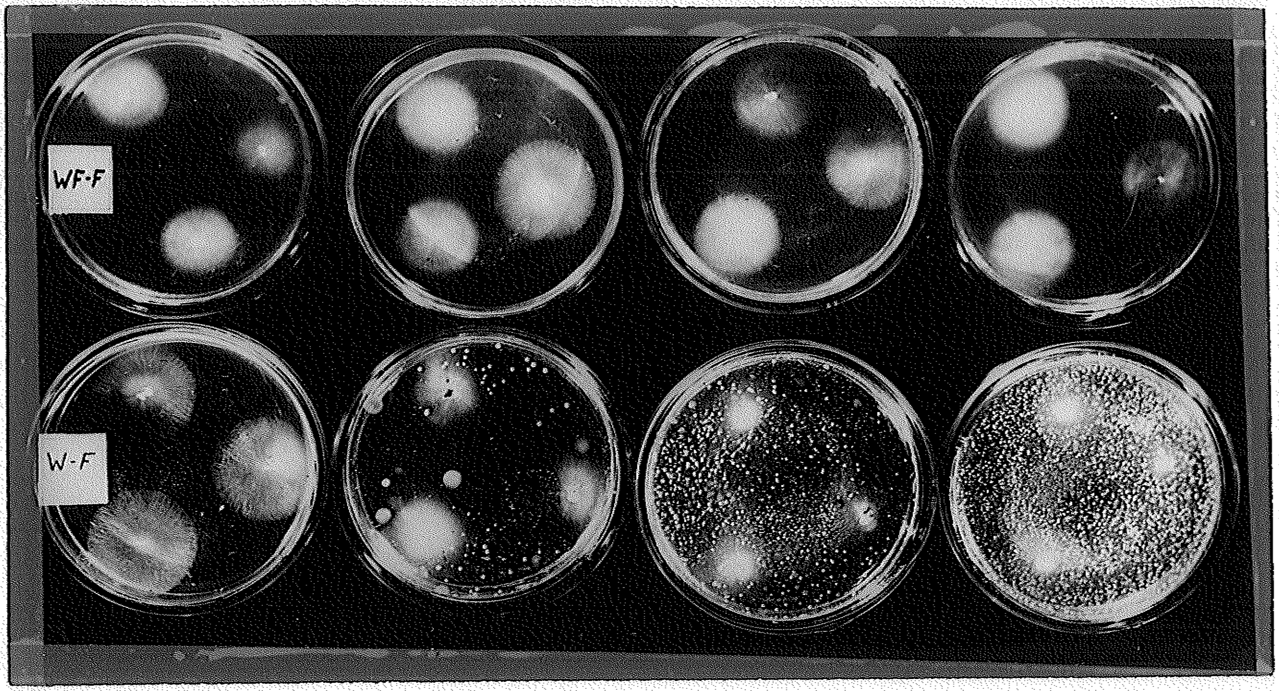

Figure 2. (Concl.)

WF-F From left to right.
Plate 1. Control. Fusarium oxysporum on potato dextrose agar. Plates 2, 3 and 4. F. oxysporum on P.D.A. to which was added 0.01 cc., 0.1 cc. and 1.0 cc. of a filtrate derived from a ten-day old culture of flora on Regent wheat seed.

W-F From left to right.
Plate 1. Control. F. oxysporum on P.D.A. Plates 2, 3 and 4. F. oxysporum on P.D.A. in which was seeded 0.01 cc., 0.1 cc. and 1.0 cc. of a suspension of the same flora.

DISCUSSION

From the foregoing it is apparent that a wide variety of micro-organisms are antagonistic to the saltant. In addition, there is good evidence in support of the idea that antagonism may arise by a process of synergism among organisms which are individually not antagonistic.

Among the isolates tested individually against the saltant, 31 fungi, 33 bacteria and 45 Actinomyces were found to be antagonistic in plate culture. Of these groups 28 fungi, 21 bacteria and 25 Actinomyces were found to be antagonistic in soil seeded with a susceptible host. While the bacteria and Actinomyces appeared to exert the greatest antagonism on petri plates, and the fungi the least, the reverse was true in soil culture. Here the fungi exerted the greatest antagonism presumably because of their relatively greater mass.

The relation between the degree of antagonism shown on plates and that manifested as a reduction of infection on barley seedlings was, for most antagonists, a broadly approximate one. A high degree of antagonism in the plate culture was not always associated with a similar level of antagonism in soil culture. However, approximately 70% of those found to be antagonistic in plate culture were found effective in a roughly corresponding manner in reducing infection in soil.

In general, the above results would appear to vindicate the use of plate culture tests for antagonism at least as a presumptive measure of this property.

The preliminary data presented appear to support the idea that the mechanism of antagonism by epiphytes on wheat seeds is competitive rather than antibiotic. Since there was evidence of inhibition of the pathogen by the bacteria in both petri plate and soil cultures, this distinction may appear more academic than practical. It indicates, however, that little of practical applicability in soil microbiology may be expected from attempts to isolate a specific antibiotic substance from wheat seed epiphytes. Rather it would appear advisable to search for means by which to foster soil conditions that would promote growth of antagonistic organisms. Even though such an antibiotic substance were shown to occur its effect would be contingent upon the presence and numbers of organisms capable of producing it.

Although the results of this study indicate that cereal seed epiphytes are capable of suppressing *H. sativum*, no consideration has been given to the possible effects that the interrelations of these epiphytes with other soil inhabitants might induce. Successful competition in a sterile medium, albeit soil, where there is only the pathogen to contend with can scarcely be accepted as providing an adequate criterion for suggesting that cereal seed epiphytes might appreciably inhibit infection of cereals by this fungus.

The contention that antagonism reflects the numerical preponderance of the epiphytic flora over the pathogen rather than the production of any antibiotic substance is in accord with the observation of Moritz, referred to previously, that an antagonistic effect observed by him was quantitative rather than qualitative.

The wide range of types shown to be antagonistic to the saltant--from suppurative staphylococci, to fungi and Actinomyces occurring naturally as soil saprophytes--precludes generalization as to which type of organism may be most effective in suppressing the pathogen, at least under test conditions in petri plates and in sterile soil. It confirms rather the contention of Leemann (21), referred to previously, that micro-organisms, both pathogenic and nonpathogenic, can supply substances which may be useful in the prevention of plant disease.

The fact remains, however, that under field conditions, micro-organisms inhabiting the soil live in a state of equilibrium and organisms inoculated into the soil are often rendered innocuous or disappear entirely as this equilibrium becomes established. In practice, this is confirmed by the fact that the application to the soil of organic materials which favour the development of antagonists has given much more favourable results than the use of pure cultures (33). For these reasons, results derived even from soil cultures, so long as these are free from micro-organisms other than the test ones, at best, must be considered as presumptive. They may be superior to petri plate results; but they are not field results. The statement made by Robison (25) in connection with antagonism to root nodule bacteria might well be quoted here. He writes:

> ".......More evidence than that presented by growing legumes in sterilized soil is needed to demonstrate that the phenomenon of antagonism as manifested in petri dishes will occur in such a highly complex system as soil under field conditions; a system in which the active material can easily become ineffective, either through physical or biochemical inactivation. Until these additional data are obtained, the role of antagonists as a factor influencing the activities of the root nodule bacteria in the soil will remain a mooted question."

Hitherto there has appeared to be tacit agreement among microbiologists that the complexity of the soil prohibits examination of it as a whole, and the interrelations of specific organisms must be examined as separate entities. But it is precisely this separation into separate entities that destroys the possibility of achieving any overall conception of micro-organism interrelations. It would seem that the direction to be taken is not the fragmentation of the soil population into arbitrary units matched one against the other, but rather the systematic exploration of methods by which to examine the soil microbial population <u>in situ</u>. As a point of departure microscopic methods embodying the Cholodny contact slide technique (6) might be used. This has already been utilized with striking success by Eaton and King (10) in connection with the inactivation of root-rot of cotton by the addition of organic manures to foster the growth of antagonistic organisms.

This approach must not be interpreted as implying that an attempt to achieve a total picture of microbial interrelations means the abandonment of that body of knowledge derived from the examination of the interrelations of specific groups of organisms as separate entities. It does infer that data on different types or groups should be considered along with findings derived from examination of the soil population as a whole.

Nor is such correlation without precedent. Timonin (32), in studying the microbial population of the rhizosphere in relation to resistance of plants to soil-borne diseases, found that results from the contact slide method agreed with those of the plating method.

Finally, it is the principle and not the method that is important at this stage. The contact slide method may not be the best method for accomplishing the result. More imperative is the conscious direction of effort on the part of microbiologists toward investigation of the soil population as a whole, rather than as fragments, the results of which in retrospect are assembled into a mosaic supposedly representative of the total picture.

SUMMARY

1. Seven hundred isolates comprising 221 fungi, 193 bacteria and 286 Actinomyces from various sources were tested for antagonism to a white fertile saltant of *H. sativum* P. K. & B.

2. Among these isolates 31 fungi, 33 bacteria and 45 Actinomyces were found to be antagonistic to the saltant in petri plate studies.

3. Of this smaller group, 28 fungi, 21 bacteria and 25 Actinomyces were found to reduce infection of barley seedlings in soil cultures harbouring only the pathogen and the test organism.

4. Fifty-two combinations of fungi were tested against the saltant. Eight combinations proved antagonistic in petri plate cultures. This is believed to be an expression of synergism.

5. Three concentrations of each of two species of bacteria believed to be epiphytic on cereal seeds and of the mixed flora on wheat were tested for antagonism to the saltant by the plate culture method. Antagonism was found to be in direct proportion to numbers of bacterial colonies on the plates.

6. Filtrates from ten-day old cultures of each of the above species and of the mixed flora added to the medium in three concentrations were found to exhibit no evidence of antagonism to the saltant.

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