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INFECTION BY *CORYNEBACTERIUM FASCIANS*: ANTIBIOTIC AND STIMULATORY FACTORS.

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I. INTRODUCTION.

Historical Account and Scope of the Thesis.

The phenomenon of resistance and susceptibility among plants and that of pathogenicity and non-pathogenicity in micro-organisms has been recognised for a very long time, but the factors which contribute to these conditions have only recently been investigated in any detail. In connection with plant infections we must, it is obvious, consider both the reaction of the plant to the invading organism and the pathogen itself. The inevitable presence of other micro-organisms in addition to the pathogen, introduces a complication and from this arises the very important subject of antagonism.

In this thesis, both are considered. The spasmodic occurrence of plants infected with Corynebacterium fascians has been noted as a feature of the disease. This is in contrast with that shown by the majority of other bacterial and particularly fungal diseases, where under favourable conditions the disease rapidly spreads from plant to plant, and once established in the soil, will infect any susceptible plant grown in that soil. Fasciated plants however invariably occur as isolated specimens in a stand of normal healthy plants. It may be concluded from these considerations that either microbiol antagonism or plant resistance are the controlling factors in infection with

this organism.

Antagonism, either the result of competition for food or due to the production of a toxic substance by one of the organisms which inhibits the other, forms the basis of one section of this thesis.

The subject is very wide and has received very considerable study in the direction of the discovery of organisms which produce toxic substances or antibiotics, which are active against human pathogens. A vast amount of literature is available on this subject, but this has been surveyed by Waksman (1937) and Florey et al (1949).

The activity of these antibiotics against the plant pathogenic bacteria and fungi has received less attention in the past, but interest in this field is now increasing. In one branch of the subject, the effect of addition of known antibiotics to the plant tissue has been studied. Ark and Hunt (1941) , and Ark (1947) reported the partial control of angular leaf spot caused by Pseudomonas lachrymans, and canker caused by Corynebacterium michiganese by soaking the cucumber and tomato seeds in streptomycin. The same antibiotic was used by Brown and Heep (1946) to kill off X. pruni which causes the serious disease of stone fruit. In this case the bud sticks were soaked in the antibiotic solution. Again, streptomycin has been suggested by Brown (1948) and Hampton (1948) as a means for the control of

Crown Gall. The galls were either immersed in the solution of the antibiotic or this was injected into the galls.

When considering antagonism as a factor in the control of a disease, it is obvious that certain conditions must be fulfilled if it is to be effective. First the antibiotic must be produced where it is required to antagonise the pathogen. This may be on the surface of the plant or in the soil. Secondly, the antibiotic must remain active under these conditions.

The production of antibiotics in soils with which we are here concerned, has been the subject of considerable work. Mitchell et al (1941) for example showed that the addition of 3% organic material to soil controlled the development of the cotton root rot fungus, P. omnivorum, under field conditions. Similarly, King et al (1934) by the addition of organic manure to soil were able to control the same disease in Arizona and Thankam (1949) using stable manure, groundnut cake or urea, found them to be effective against Fusarium vasinfectum, the causal organism of cotton wilt. In the latter case, the effect was to reduce the survival period of the organism. Finally, the control of potato scab by green manuring with rye grass reported by Millard (1921) and also confirmed by Sanford (1926) may be cited. It must be emphasised that in all this work no direct evidence that

antagonism was responsible for the control was obtained. It was assumed that such a process took place, the added organic material encouraging the growth of bacteria and fungi which were antagonistic to the pathogen.

However, more direct evidence has been obtained by using mixed culture inoculations under controlled conditions. Thus Millard and Taylor (1927) found that a mixture of Actinomyces scabies and a saprophyte A. praecox, in soil in pots, reduced the occurrence of potato scab to a negligible amount as compared with the action of the parasite alone. Porter (1924) demonstrated the antagonistic effect of mixing certain bacteria with Helminthosporium on the infection of wheat seedlings and similarly with Fusarium on flax seedlings. Bamberg (1930) obtained almost complete control of smut in maize by the use of a mixed inoculum of Ustilago zeae and a certain bacterium.

Weindling and Fawcett (1936) were able to control damping off due to Rhizoctonia in citrus seedlings grown in pots by adding Trichoderma lignorum to the soil. Anwar (1949) used a soil isolate of B. subtilis and two fungi, T. lignorum and a species of Penicillium to reduce the incidence of the root rot disease of barley caused by Helminthosporium sativum.

Little attention has however been given to the bacterial plant pathogens although it has been assumed that

antagonism would play an impprtant part in their control. In the case of C. fascians which belongs to the Gram positive group of organisms which are known to be most susceptible to antibiotics, this factor is obviously very important, and this is supported by the difficulty of isolation of the pathogen. This has been attributed to antagonism on the isolation plates which owing to the slow growth rate of the pathogen, frequently prevents its appearence at all.

The presence of antibiotic substances in soil was demonstrated by Winter (1940) and Waksman and Woodruff (1942) who by the use of suitable extracting agents obtained certain substances of the actinomycin type which had bacteriostatic and bacteriocidal properties. There is considerable evidence for the production of antibiotics in soils enriched by the addition of certain carbon sources. In this connection the work of Grossbard (1952) may be mentioned. In this work Penicillium patulum, Aspergillus clavatus and A. terreus produced antibiotic substances in autoclaved soil supplemented with certain carbon sources. There was also evidence that some activity occurred in soil only partially sterilised, but the activity was of a lower order. Previously evidence of antibiotic production in supplemented soils was obtained by Lewis (1929) using bacteria and by Nickell and Burkholder (1947) with actinomycetes. Similar results were

described by Grossbard (1947, 1948a) with Penicillium patulum and also in later papers (1948b, 1949, 1950) the same author describes further evidence using unidentified species of Penicillimm , Aspergillus terreus, and A. clavatus. Gottlieb and Siminoff (1950) also showed that A. clavatus was active in this way. Brian et al (1951) obtained evidence of the formation of griseofulvin in the soil by P. griseofulvum. Nandi (1948) was able to detect the presence of antibiotic substances in the aqueous phase of unsterile soils which had been supplemented with food substances.

In considering the possible action ~~action~~ antibiotics may exert on the soil population it is necessary to consider whether they are able to function in the soil. It is known that the basic antibiotics such as streptomycin are strongly adsorbed on the soil colloids. This was clearly demonstrated by Nandi (1948), Hutchinson et al (1949), Sinha (1950), and Gottlieb (1950). In addition, Waksman (1950) stated that even if antibiotics were formed in the soil, they would not persist owing to the destruction brought about by the growth of other micro-organisms. However Nandi (1948), Sinha (1950), Nimkar (1950), and Monib (1953), showed that certain purified antibiotics when added to non-sterile soil do persist for varying periods, during which time they exert an influence on the soil microflora. Evidence of the persistance of antibiotics in soil was also found by Jefferys (1950)

and Gottlieb and Siminoff (1950).

In this thesis the effect of four species of bacteria which were shown to be antagonistic to C. fascians in plate culture have been studied under laboratory conditions in an effort to determine how far antagonism may be a controlling factor in infection with the pathogen. The effect of these antagonists in pure culture experiments in sand has been followed by work in sterile and non-sterile soil to see how far pure culture work can be correlated with more natural conditions. Jacobs and Mohanty (1951) recorded some effect of B. mesentericus on the percentage of infection of sweet pea seedlings in sand culture experiments, but the results were inconclusive.

As a branch of this work, it was considered desirable to investigate the survival of C. fascians and the antagonists in sterile and non-sterile soils, as this would have a considerable bearing on the interpretation of any results from infection experiments done in these media. In this work, which was only a preliminary survey of survival of the pathogen, the prospect of using a selective medium was also investigated. Considerable work on this was done by Mohanty (1947), but, on the whole, the results obtained suggested that there is little prospect of obtaining a medium specifically selective for the pathogen, but some reduction in the amount of growth of the majority of

the soil microflora with only a partial inhibition of the pathogen was obtained with potassium dichromate as the selective agent.

A further section of this thesis deals with the possible effect that the plant, and in particular the seed, might have on the infectivity of the pathogen. Tilford (1936) in work with pure culture infections found that the average infection of sweet pea seedlings to be only 64%, when the seeds were inoculated at sowing time. Lacey (1936) also recorded that not all isolates of the pathogen exhibited 100% infection, a fact that was further confirmed by Jacobs and Mohanty (1951) who examined a large number of strains and recorded widely varying percentages of infection.

The presence of antibacterial and antifungal substances in plants has been recorded for a wide range of genera and species. Link et al (1929, 1929a), and Link and Walker (1933) showed that the resistance of pigmented onion scales was due to the presence of catechol in the scales, which prevented attack by Colletotrichum circinans, the causal organism of onion smudge. White onions which do not contain this substance are susceptible.

Sherman and Hodge (1936) examined a number of plant juices for bactericidal properties and Osborn (1943) in an examination of 2300 plant species found 134 of these to give inhibitory extracts. Particularly potent extracts were

given by plants of the family Ranunculaceae, a fact confirmed by Seegal and Holden (1945). Further examinations of a wide range of plants have been made by Little and Grubaugh (1946), Sanders et al (1945), Carlson et al (1946, 1948), and Carlson and Douglas (1948).

In all this work, the emphasis has been on the discovery of antibiotic substances active against human pathogens and in none has any attempt been made to demonstrate the effect of these substances in the plant on its susceptibility to plant pathogenic fungi or bacteria. In the case of the tomato, Irving et al (1946) were able to isolate the antibiotic tomatin, and show it to be active against a wide range of bacteria and fungi.

Lacey (1936) showed that the age of sweet pea seedlings was important in infection with C. fascians. Seeds inoculated immediately upon germination gave high percentages of infection, those inoculated after 10 days were less easily infected and after 17 days the plants were apparently immune. It would appear then that the early stage of germination is the important time for infection and it is possible therefore that the diffusion of substances from the seed may be an important factor.

The influence of the seed on the pathogen and on the other soil microflora, which may include antagonistic

organisms, has been rather fully investigated here. The presence of antibiotic substances in seeds was recorded by Osborn (1943) who found that extracts of the seeds of cabbage, cauliflower, broccoli, kohlrabi and clematis were active against B. coli and Staph. aureus. These results were confirmed by Lucus and Lewis (1944). Ivánovics and Horváth (1947) were able to extract the active principle from radish seeds and obtained the antibiotic raphanin, which was shown to be active against B. coli and S. aureus and also to inhibit the germination of seeds.

A further factor which has been investigated is the possible effect of the diffusion from seeds of substances which act as microbiol food. This it was thought might influence infection by the pathogen, by stimulating its growth and also affect the development of other micro-organisms present in the soil which may act as antagonists. We have already seen that the production of antibiotics in soil is largely dependent on supplying a supplement in the form of organic matter and the possibility that the necessary materials may diffuse from seeds was considered worth investigation.

The diffusion of substances from roots is a well known phenomenon as is the influence these substances exert on the soil population, producing a completely different

micro-flora in their zone of influence or rhizosphere. Wallace and Lochhead (1951) showed that the surface of the seeds of various plants had their own individual micro-flora which is in itself suggestive that the seed may play a considerable part in controlling the micro-flora which is able to develop in the soil around it.

Finally, in connection with the work on seeds, a short investigation was undertaken to determine a suitable agent which could be used for the surface sterilisation of seeds.

II. SOURCE, PATHOGENICITY, AND GROWTH CHARACTERISTICS OF STRAINS OF C. FASCIANS, AND SOURCE AND GROWTH CHARACTERISTICS OF OTHER ORGANISMS USED.

During the course of the work to be described, seven pathogenic and non-pathogenic strains of C. fascians isolated from various host plants, were used. Details of the origin, pathogenicity, and growth characteristics of these are given in Table I. In addition, five other organisms largely bacteria antagonistic to the pathogen, were used and details of these are given in Table Ia.

The strains of C. fascians were selected on grounds of pathogenicity and maintenance or loss of this factor when kept on artificial media. For example, strains 505 and 12S were both 100% pathogenic to sweet pea when isolated, but culture on artificial media resulted in the complete loss of pathogenicity by strain 12S whereas strain 505 remained 100% pathogenic.

As far as possible, a brief survey using all the strains was made in each section, and from this a smaller number showing different reactions selected for further study. Strain 505 was used throughout the work as typical of the organism, and where only one strain could be dealt with, this was selected.

All stock cultures were kept on nutrient agar medium

Table I.

The origin, pathogenicity, and growth characteristics of strains of *C. fascians* used.

Strain no. and type*	Host	Signs of disease	Isolated by	Date of isolation	Pathogenicity on sweet pea		Growth† on soil extract	
					On isolation	At commencement of present work	diluted ×5	diluted ×5 plus 1% mannitol
2WR	White mutant of a normal strain		Mohanty	-	66.7%	50.0%	+	+
S/100S	Straw-berry	Cauliflower	Lacey	April 1951	100%	100%	(+)	(+)
503S	Dahlia	Leafy gall	Lacey	1948	-	68.8%	(+)	++
505S	Sweet pea	Fasciation	Lacey	February 1949	100%	100%	+	+++
516S	Chrysanth.	Leafy gall	Lacey	April 1951	66.7%	66.7%	+	++
12S	Sweet pea	Fasciation	Lacey	1935	100%	nil	+	+++
515S pink	Chrysanth. maximum	Cauliflower	Lacey	1950 April	nil	nil	+	++

* The symbols S and R refer to smooth and rough phases respectively.

† Amount of growth shown by symbols ranging from (+), very feeble to +++, very good.

Table Ia.

Origin and growth characteristics of other organisms used.

Organism	Source	Isolated by	Date of isolation	Growth* on Soil extract Diluted × 5	Soil extract Diluted × 5 plus 1% mannitol
F.A.1. (*Pseudomonas* sp.)	Dahlia, leafy gall.	Lacey	March 1949	-	-
F.A.6. (*Pseudomonas* sp.)	Dahlia, leafy gall	Lacey	March 1949	-	-
F.A.7. (*Bacillus* sp.)	Contaminated sweet pea seed	Dadd	January 1950	-	-
B. subtilis	Stock culture	-	-	+	+
B. mesentericus 6353	Stock culture	-	-	-	(+)

* For an explanation of symbols see footnote to Table I.

and cultures for the preparation of inocula grown on a similar medium. In the case of C. fascians, these were incubated for 48 hours at 25°C, and for all other organisms for 24 hours at 30°C.

The soil extract used was prepared by autoclaving 500 grams of soil with 500 ml. of tap water for 20 minutes at 15 lb. pressure, and filtering off the supernatant. This was re-filtered, if necessary, until clear, and diluted with tap water according to requirements.

The effect of time of inoculation on pathogenicity.

Two methods of inoculation of seeds have been used by other workers when determining the pathogenicity of C. fascians. They differed only in the time of application of the inoculum. In some cases this was applied at the time of sowing the seed, while in others the seeds had been previously germinated. It was considered desirable therefore, before adopting any one method for this work to see if, during the early stages of germination and growth, the time of inoculation influenced the percentage of infection obtained.

Three sets, each of 20 sand culture tubes, were set up as described in Section IVa, using soil extract mannitol as the medium (soil extract prepared as described above, with the addition of 1% mannitol). Sweet pea seeds were surface

sterilised in 1% chloramine T for 15 minutes and given four washings, each of 2 to 3 minutes, in sterile distilled water. One seed was placed in each tube. One set was inoculated immediately with a suspension of strain 505 of C. fascians in sterile tap water, 0.2 ml. of a fairly dense suspension being dropped on to each seed. The second set was inoculated similarly on appearance of the radicle, and the third set on appearance of the plumule. The number of seeds which germinated and the number infected was recorded after 3, 4, and 5 weeks growth, and are shown in Table II.

The results clearly indicate that over the period of growth chosen, no effect of time of inoculation on the percentage of infection could be demonstrated. Although seeds inoculated on appearance of the plumule received the inoculum 7 to 9 days after those inoculated at the time of sowing, after 3 weeks growth the percentage of infection was not significantly different. All three sets continued to show this similarity until the experiment was discontinued after 5 weeks growth of the seedlings.

This observation is in accordance with that found by Lacey (1936), in which sweet pea seedlings inoculated 10 days after germination gave similar percentages of infection to those inoculated immediately after germination of the seed.

Table II.

The effect of time of inoculation on the pathogenicity of strain 505 of *C. fascians* on sweet pea seedlings in soil extract mannitol medium.

Time of inoculation	Period of growth								
	3 weeks			4 weeks			5 weeks		
	No. of plants	Plants fasciated		No. of plants	Plants fasciated		No. of plants	Plants fasciated	
		No.	percentage		No.	Percentage		No.	Percentage
Sowing time	19	12	63.2	19	14	73.7	19	16	84.2
On appearence of radicle	15	8	53.3	15	12	80.0	15	12	80.0
On appearence of plumule	18	12	66.7	20	16	80.0	20	16	80.0

In this case however, the severity of the attack was reduced in the later inoculations. In the experiment described above, no difference in the severity of the attack could be demonstrated between the three sets of seedlings.

IIIa. METHODS OF SURFACE STERILISATION OF SEEDS IN RELATION TO THE PRODUCTION OF ZONES OF INHIBITION.

Various substances including silver nitrate, sodium hypochlorite, bromine, and alcohol have been used by workers as surface sterilising agents for seeds, but by far the most common one employed is an aqueous solution of mercuric chloride. Being highly active against both fungi and bacteria, it would appear ideal for this type of work, and due to the mechanical barrier of the seed coat, damage to the embryo is eliminated. The removal of the mercuric chloride from the seed has normally been effected by washing in one or more changes of sterile distilled water.

Kemp (unpublished work) in examining the diffusion of substances from sweet pea seeds and gram seeds, obtained very large zones of inhibition against C. fascians from seeds surface sterilised with mercuric chloride. At first these were considered due to the diffusion of naturally occurring water soluble substances from the seeds, but further consideration led to the view that the removal of all the mercury may not have been effected by the one washing given. To avoid this the work was repeated using silver nitrate as the surface sterilising agent with four washings, including one

in 1% sodium chloride. Zones of inhibition were again obtained but their diameter was substantially reduced. It would appear therefore that the large zones obtained when mercuric chloride was used, were due, in part at least, to the diffusion from the seed of mercury retained by it during washing. No attempt was made in this work to confirm this, or to compare the zones obtained from seeds given no treatment with them.

1. Selection of a medium.

Before an investigation into the diffusion of substances from seeds could be undertaken, it was necessary to select a suitable medium in which the tests could be made. As the work as a whole was related to conditions in the soil, it was considered desirable that, as far as possible, a medium bearing some relation to these conditions should be chosen. Alternatively, if this proved impossible, a different type of medium but one giving similar results in relation to the diffusion of inhibitors from seeds should be used.

The growth of C. fascians is very poor on soil extract medium prepared as described in Section II. Various media, mostly soil extract with the addition of an energy source, with and without thiamin, were examined todetermine one on which the pathogen would make

satisfactory growth with as short an incubation period as possible, while the inhibition zones produced by seeds were distinct and of a reasonable size for measurement. Potato extract, on which the organism was known to make good growth, was included.

Seeds of sweet pea, var. Powerscourt, were surface sterilised in 0.1% silver nitrate for one minute and given four washings, three in sterile distilled water and one in 1% sodium chloride. Two plates of each medium inoculated with strain 505 of C. fascians were poured, and three seeds placed in each dish before the agar had solidified. Sufficient medium was used to just cover the seeds. The plates were incubated at 25°C and examined daily for the presence of growth. The diameter of the zones of inhibition were recorded as soon as sufficient growth had been made. The degree of growth was recorded after 10 days incubation for all media. The results are shown in Table III.

Regarding growth indicated by ++ and +++ as sufficient and an incubation period for visible growth of not more than 3 days as desirable, six media, four of the soil extract media and the two potato extract media must be considered suitable from this point of view. Within the soil extract group, the addition of thiamin reduced the size of the zones of inhibition to almost half that exhibited

Table III.

The growth of *C. fascians* (strain 505), and inhibition zones produced by silver nitrate surface sterilised sweet pea seeds, on various media.

Medium	Incubation period at 25°C for visible growth. (days)	Diam. of inhibition zone (millimetres)	Growth *
Soil extract dil. 10 with tap water	6	17.7	(+)
" " " 5 " " "	4	12.5	+
" " " 10 " " " plus 0.001% thiamin	4	15.4	+
0.5% glucose	5	12.0	(+)
Soil extract dil. 5 with tap water plus 1% mannitol	3	12.8	++
1% mannitol and 0.001% thiamin	3	7.4	++
1% sucrose	3	11.4	++
1% sucrose and 0.001% thiamin	3	6.4	++
Potato extract (20%) plus 1% sucrose	2	8.2	+++
" " " " 1% mannitol	2	8.3	+++

* Symbols indicate degree of growth as follows: +++ good (equivalent to that obtained on nutrient agar); ++ moderate; + poor; (+) feeble.

in a similar medium where this substance was absent without increasing the degree of growth made. This consideration alone makes them unsuitable for the work. A similar result was shown by the potato extract media, where without the addition of thiamin, the zones were small.

Of the two remaining media, soil extract with the addition of mannitol or sucrose, both were satisfactory in the size of inhibition zone produced, but owing to the less stable nature of sucrose, mannitol , which could be autoclaved with little chance of appreciable breakdown, was considered superior. This medium, soil extract diluted times five with tap water plus 1% mannitol has therefore been used throughout the work.

2. Adsorption of mercuric chloride.

(a). Inhibition zones from treated and untreated seeds.

Batches of ten sweet pea, gram, and maize seeds were surface sterilised by soaking in a solution of mercuric chloride. Methods employed followed those used by previous workers with regard to the strength of the mercuric chloride solution and the number of washings given. Thus, seeds were sterilised in 0.1 or 0.2% mercuric chloride and given washings in sterile distilled water ranging from one to ten in number. The seeds were then plated out in soil extract and soil extract mannitol media inoculated with

strain 505 of C. fascians, as described in the previous experiment. As the experiment was first tried on sweet pea seeds only and little difference between the two media in the size of zones produced was evident, seeds of gram and maize were only tested in soil extract mannitol medium. Controls of seeds not treated but given three washings in sterile distilled water only, were included.

Zones of inhibition round the seeds were measured after 3 and 6 days incubation at 25°C for soil extract mannitol and soil extract media respectively, and are shown in Table IV, together with details of the methods of surface sterilisation employed.

In every case, zones from treated seeds were several times the size of those from untreated ones. A photograph appears in fig. 1. Two explanations for this would appear possible. Firstly, the mercuric chloride may be adsorbed on to the seed coat during sterilisation, and diffuse out slowly into the medium. Mercury is known to be readily adsorbed under many conditions, and a simple adsorption without any chemical reaction could be used to explain the results obtained. Alternatively, the mercuric chloride may exert an action on the seed coat, releasing inhibitory substances in the process. Mercury readily reacts with a wide variety of organic compounds, and it is possible that during this process, highly inhibitory substances may be released

Fig. 1. Inhibition zones against C. fascians around sweet pea seeds treated with mercuric chloride.



Table IV.

Zones of inhibition produced by untreated seeds, and seeds after surface sterilisation with mercuric chloride, in soil extract media, against strain 505 of *C. fascians*.

Seed	Method of sterilisation†	Zones of inhibition (mm.)			
		Treated		Untreated	
		Soil extract	Soil extract mannitol	Soil extract	Soil extract mannitol
Sweet pea, Powerscourt	A	40.6	40.6	9.1	8.6
"	B	42.8	41.4		
"	C	44.5	40.5		
Maize	C		53.8		nil
"	D		43.6		
Gram	A		52.1		5.2

† Symbols refer to methods as follows: A, 0.2% mercuric chloride for 15 min., 1 washing for 45 min.; B, 0.2% mercuric chloride for 15 min., 3 two min. washings; C, 0.2% mercuric chloride for 15 min., 10 two min. washings; D, 0.1% mercuric chloride for 5 min., 3 two min. washings.

from the seed.

The following experiments were designed to determine as far as possible which of these factors, or indeed whether both, were operative.

(b). Removal of the inhibitor by soaking.

If adsorption of the mercuric chloride takes place during the surface sterilisation process, and is the cause of the large inhibition zones, it is obvious that the 10 washings given were insufficient to remove it. Soaking in sterile water was therefore tried.

It was necessary to be able to remove any adsorbed mercury in order that a determination of the amount of the substance taken up by the seeds and that recovered could be made and the results compared. These allowing for the quantity lost in the washings, should be comparable if adsorption is to account for the large zones obtained in the previous experiment.

Two sets, each of 50 sweet pea seeds were surface sterilised in 0.2% mercuric chloride for 15 minutes and given ten washings in sterile distilled water. Each set was transfered to a boiling tube, and 20 ml. of distilled water added. The tubes were kept in the refrigerator and the plugs covered with metal foil to reduce evaporation to a minimum. After 24 hours the seeds were removed to fresh

tubes and a further quantity of distilled water added. This was repeated after 48 and 72 hours and 5 and 8 days soaking. The liquid from the soaking of one set of seeds was assayed, after removal of the seeds, in soil extract mannitol agar medium against strain 505 of C. fascians. Five seeds from the second set were tested similarly at the time of each transfer.

The results, shown in Table V., indicate that a considerable period of soaking is required to remove all the inhibitor. Only after 8 days soaking was an absence of inhibitor indicated. Approximately two thirds was however removed during the first 24 hours and the majority of that remaining during the next 24, but a relatively small amount is firmly bound to the seed, and diffuses out very slowly. For the purpose of determining the amount recoverable from the seed, 5 days soaking when 99% or more has been removed, may be considered satisfactory.

(c). Estimation of mercuric chloride adsorbed.

Two methods were tried. In the first, seeds were treated in mercuric chloride solution and washed in the usual way, and then soaked in several changes of distilled water to remove the sterilising agent. This was then assayed by the cup plate method in soil extract mannitol

Table V.

The diffusion of adsorbed mercuric chloride from treated seeds into water after various periods of soaking.

Soaking time	Inhibition zone (millimetres)	
	Seeds†	Soakate
nil	40.5	-
24 hours	23.4	28.4
48 hours	9.1	14.8
72 hours	5.9	11.1
5 days	4.6	9.0
8 days	4.0*	8.9

† Average zone from one seed (average of 5 replicates)

* Approx. diameter of seed.

agar medium seeded with C. fascians. The mercuric chloride solution used for the sterilisation and the washings were similarly assayed. From a standard assay curve for mercuric chloride it was possible to determine the amount taken up by the seeds and the amount recoverable from them, and to compare the two. Several difficulties were however evident. First, the amount taken up by the seeds from a 0.1% solution was very small and to overcome this, a very large number had to be treated in a small quantity of a more dilute mercuric chloride solution, to obtain a significant adsorption. Secondly, the assay of the mercury solutions was difficult in the low concentration range, and could possibly be influenced by the presence of naturally occurring antibiotics. It was obvious therefore that the method was very unsatisfactory and in spite of several attempts using a large umber of replicates, no satisfactory correlation was obtained.

The second method involved the estimation of mercury by the dithizone colorimetric method. This was found to give more satisfactory results and will be described in more detail.

5 successive batches of 20 seeds were surface sterilised in the same 10 ml. of 0.001% mercuric chloride solution and given 10 washings in 10 ml. quantities of distilled water. The washings and sterilising solution were retained for future estimation. The seeds were soaked in 50 ml. of

distilled water and this was changed every 24 hours for 5 days. The soakates were collected together and then the bulk was reduced to not more than 10 ml. by distillation under reduced pressure. The resulting liquid was made up to 10 ml. with distilled water before estimation. The washings were also collected together, concentrated in a similar way, and made up to 10 ml.

The estimation of mercury was carried out as follows. 5 ml. of the mercury solution was shaken with two successive 5 ml. portions of dithizone (a 0.001% solution of freshly purified dithizone in chloroform) and the chloroform layer separated off. Each extraction was timed to take 30 seconds. The two portions were mixed and read in a spectrophotometer at a wavelength of 600 millimicrons, 90 seconds from the commencement of the first extraction. The precise timing of the readings was found essential as the colour of the mercury dithizone compound rapidly fades. It was also necessary to standardise the shaking of the liquids during extractionas the dithizone is rapidly oxidised to diphenylthiocarbodiazone with a corresponding change in the colour of the solution. The results are shown in Table VI, together with the quantities of mercury as read from the standard curve shown in fig. 2.

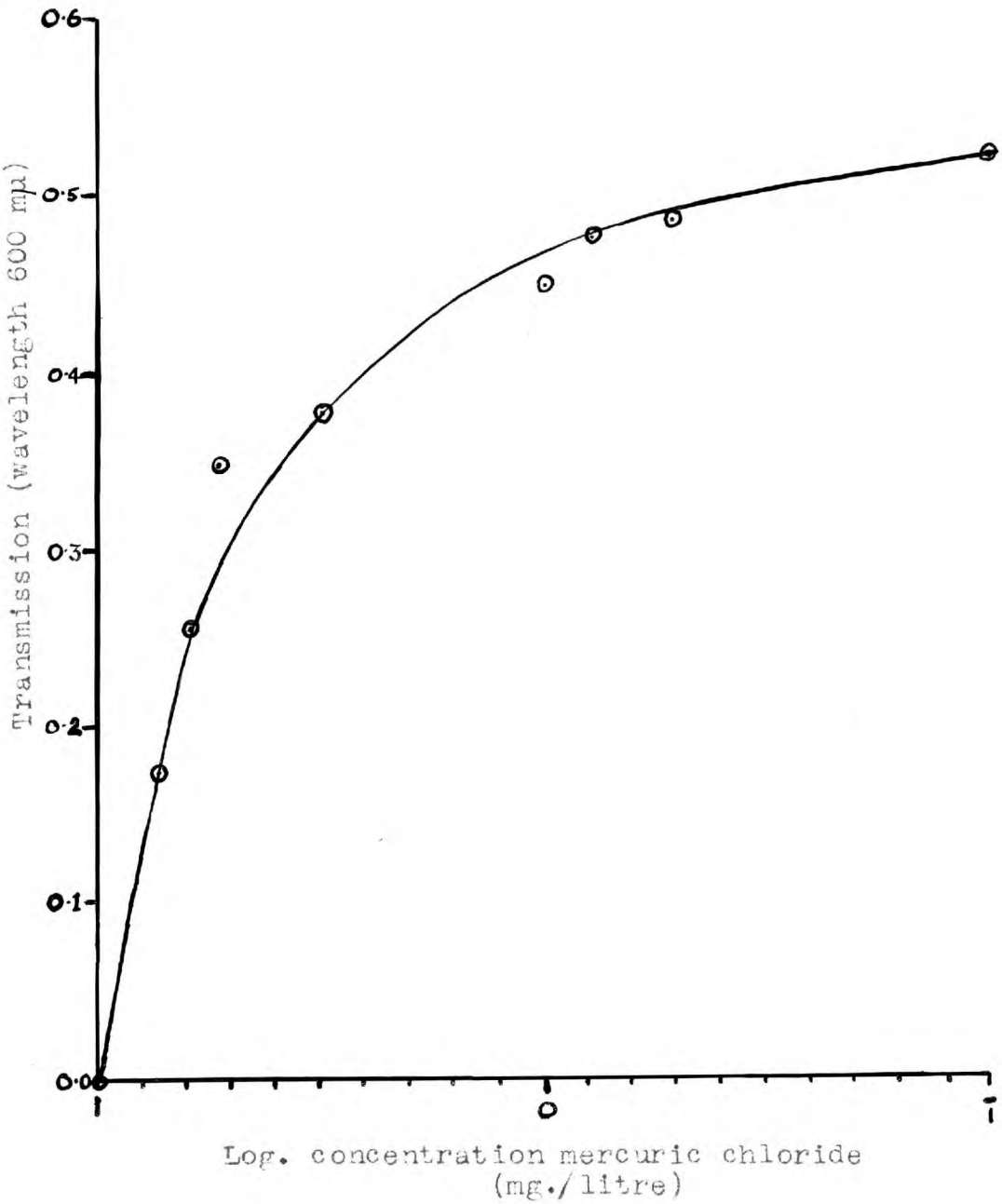
It will be seen that the quantity recovered from the seeds plus that lost in the washings was very nearly

Table VI.

The uptake of mercuric chloride by sweet pea seeds and the recovery of the substance by soaking.

	Spectrophotometer reading	mg. Hg/litre
Sterilisation solution (0.001% mercuric chloride)	0	10.0
Sterilisation solution after treating 200 seeds	0.325	4.37
Quantity adsorbed on seeds		5.63
Washings from 200 seeds	0.515	0.16
Soakate from seeds	0.285	5.38
Mercury not accounted for		0.09

Fig. 8. Determination of mercury with dithizone by the mixed colour method.



equivalent to that removed from the sterilising liquid. The actual quantity lost, 0.09 milligrams of mercury, can be easily accounted for by failure to remove all of this substance from the seed and also from adsorption on the glass of the flasks in which the washings and soakings were carried out.

It must be emphasised that even though the quantity taken up by the seeds is recoverable, this does not prove that no reaction took place between the mercuric chloride and substances in the seed coat. To determine this, a measure of the activity of the solutions before and after being in contact with the seed is necessary and for reasons already discussed, the assay of such solutions is difficult and although this was attempted no satisfactory results were obtained. In the light of previous results however, it seems likely that simple adsorption of the sterilising agent was responsible for the large zones obtained.

3. Adsorption of silver nitrate and other surface sterilising agents.

In the light of the previous work, it was decided to examine the adsorption of three other commonly used sterilising agents, namely silver nitrate, sodium hypochlorite and chloramine T, to find one which could be used in

future work where the retention of the sterilising agent would be undesirable. In the first experiment, the seeds of eleven common plants were treated with silver nitrate, and the zonation compared with that shown by untreated seeds. In addition to recording the diameter of the inhibition zones, the frequency of occurrence was also noted to see if the 100% frequency recorded for mercuric chloride treatment was duplicated with other surface sterilising agents.

The silver nitrate treatment consisted of soaking the seeds in a 0.1% solution for one minute followed by two three minute washings in sterile distilled water, one in 1% sodium chloride and a final one in water. The seeds were then plated out as previously described for mercuric chloride treatment. Controls of washed seeds were included as before.

The results, recorded after 3 days incubation at 25°C, are shown in Table VII. It will be seen that in every case the average diameter of the inhibition zones was increased by silver nitrate treatment, presumably due to the diffusion of adsorbed silver. Some seeds of all plants tested, whether normally exhibiting inhibition zones or not, did so after treatment. It must be noted however that the frequency of occurrence of zones was not

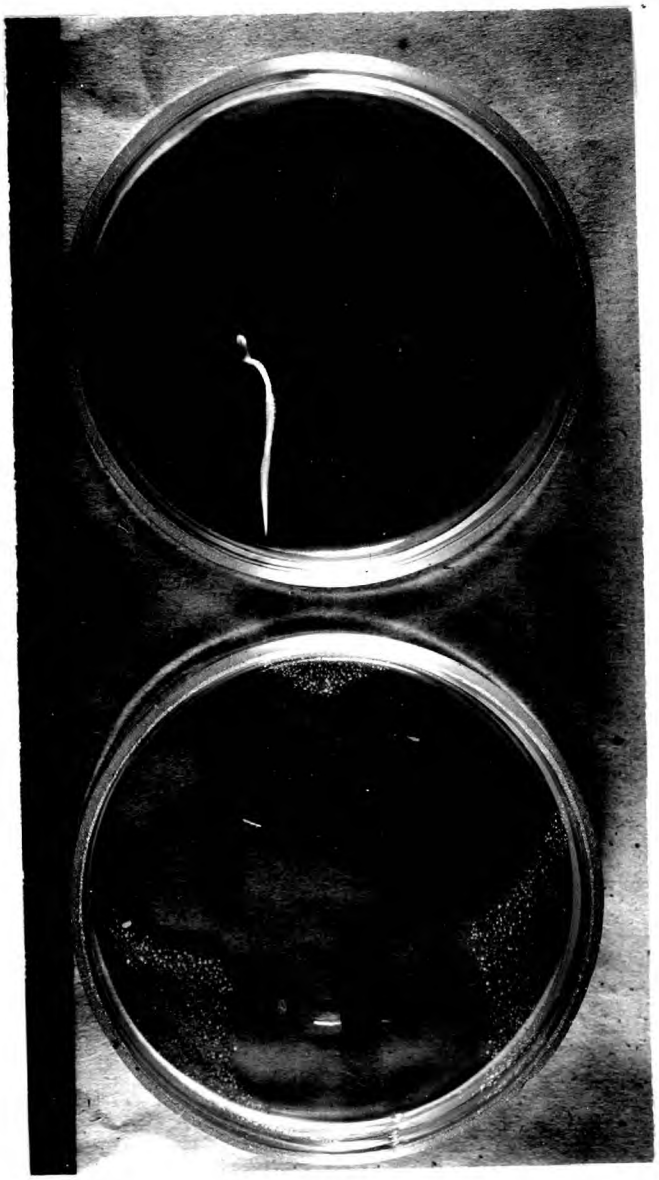
Table VII.

The effect of surface sterilisation with silver nitrate on the zones of inhibition produced by various seeds against strain 505 of *C. fascians* in soil extract mannitol medium.

Seed	Inhibition zones* (mm.)		Percentage of treated seeds showing inhib. zones.
	Treated seeds	Untreated seeds	
Barley	17.0	nil	100
Carrot	16.7	nil	100
Flax	30.2	nil	100
French bean, Lightning	21.9	19.7	100
Lettuce, Cheshunt Early Giant	5.4	nil	91.6
Mustard, white	5.3	nil	66.7
Pea, Eight Weeks	13.5	nil	100
Radish, Delicacy	8.0	nil	33.3
Sweet pea, Powerscourt	15.4	8.6	100
Wallflower, Orange King	5.2	nil	50.0
Wheat	11.1	nil	100

* Average of 15 replicates, recorded after 3 days incubation at 25°C.

Fig. 3. **A comparison of inhibition zones from seeds treated with mercuric chloride and silver nitrate.**



Top: Small zones of inhibition against C. fascians around sweet pea seeds treated with silver nitrate.

Bottom: Very large inhibition zones from mercuric chloride treated seeds.

always 100% as in the previous case. This was at first viewed with reserve, but subsequent replicate experiments confirmed the result. The reason for this is unknown.

The other two substances, sodium hypochlorite as a 15% solution and chloramine T as a 0.1 and 1% solution for 15 minutes followed by 4 washings in distilled water, were examined similarly. The sweet pea was used as test seed and the zonation recorded as before and compared with controls of untreated seeds.

Seeds treated with sodium hypochlorite all showed large inhibition zones averaging 15.3 mm. in diameter against 8.9 mm. for the controls, where zonation was restricted to only a few seeds. This substance was apparently retained by the seeds and so was considered[1] unsatisfactory for future work.

Chloramine T treated seeds exhibited no large zones and the frequency of small zones was identical with that shown by untreated seeds. Sterilisation was complete with both concentrations of the substance. Further trials on french bean and maize confirmed this result.

It appeared therefore that this substance was satisfactory for seed treatment and was not retained. It has been used as a 1% solution for all future work. It must be emphasised however that the substance is not fungicidal, at least at this concentration, and so would not be effective in cases of heavy fungal contamination.

IIIb. THE EFFECT OF SEEDS AND DIFFUSATES FROM SEEDS, ON THE GROWTH AND PATHOGENICITY OF C. FASCIANS, AND ON THE GROWTH OF ORGANISMS ANTAGONISTIC TO THE PATHOGEN.

1. Natural antibiotics in plant seeds.

The presence of antibacterial and antifungal substances in the extracts of plant tissues has been recorded for a wide range of genera, but the role these substances play in resistance to disease has so far not been clearly demonstrated. Antibiotic substances have been demonstrated in seeds (Osborn, 1943 ; Ivanovics and Horvath, 1947) and the possibility of the seed playing some part at least in the control of infection was considered worth investigation.

Some indication that the seed has a positive role was obtained from the fact that many strains of C. fascians fail to show 100% infection of sweet pea seedlings when grown under pure culture conditions, and inoculated at the time of sowing. Similarly, the french bean is very difficult to infect in this way, but axillary bud inoculations are more frequently positive.

A range of seeds of different genera have therefore been investigated for the presence of diffusible antibiotic substances, and in the case of the sweet pea seed the reaction of different strains of C. fascians has been

studied in relation to their virulence.

(a). Inhibition zones from various seeds.

To avoid any possible retention of the sterilising agent and to reduce the soaking time to a minimum, the seeds to be examined, mostly seeds of common food crops, were not surface sterilised but only washed in three changes of sterile distilled water. One exception to this was tomato seed, where the nature of the coat led to very heavy contamination. These were surface sterilised with chloramine T and given four washings in sterile distilled water.

Six strains of C. fascians, namely 2WR, S/100, 503, 505, 516, and 515 pink were used, and ten seeds of each plant tested against each strain. The medium, soil extract mannitol agar, was inoculated with the strain of C. fascians from a suspension of the organism in tap water, and the plates poured. While the agar was still liquid the washed seeds were placed in the dishes. Sufficient medium was used to just cover the seeds. The number of seeds exhibiting zones of inhibition was recorded for each plant and strain, and these divided into groups depending on the diameter of the inhibition zone. In the case of seeds that were not spherical, such as the french bean, the diameter of the inhibition zone was measured in two directions at right angles, and the mean taken.

The results are shown in Table X. Photographs of inhibition zones from the seeds of four plants are shown in figs. 5 to 8 inclusive.

Of the 20 different genera and species, just over half showed the presence of antibiotic substances active against C. fascians in varying degrees, Considerable variation in response by the strains used was also evident. Strain S/100 appeared to be somewhat resistant to these antibiotic substances, a reaction shown also by strain 505 but to a lessextent. The remaining strains were considerably more susceptible. The strongest inhibition was given by seeds of the french bean, which is interesting in view of the difficulty of infecting this plant through the seed. Further experiments on this aspect will be discussed later. Gram, clover, sweet pea and garden pea showed the presence of moderately active antibiotic substances, while only weak activity was shown by broccoli, flax, mustard, radish, tomato and wheat.

Of those giving completely negative results, i.e. carrot, lettuce, lupin, maize, neem, Nicandra physaloides, parsley, Solanum panduraeforme, and tobacco, from only two, namely lettuce and tobacco, has C. fascians been recorded. It appears probable therefore that the resistance of these plants is controlled by other factors. Indeed, all

Figs. 5 to 8 incl. Zones of inhibition from various seeds.

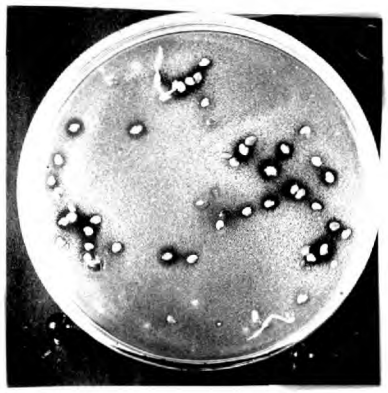


Fig. 5. Clover



Fig. 6. Flax



Fig. 7. French bean

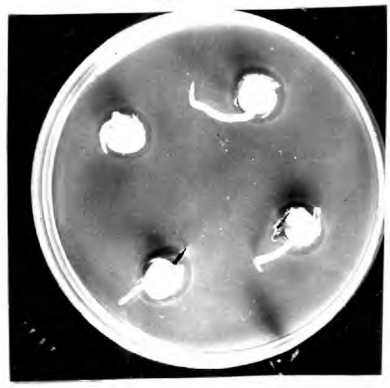


Fig. 8. Pea

Table X.

The variation in sensitivity of strains of C. fasciana on soil extract mannitol medium to antibiotics contained in the coats of various seeds.

Seed	Frequency of occurrence of inhibition zones of various size groups† against strain number																									
	2WR					S/100					503					505					515 pink					516				
	3	2	1	½	0	3	2	1	½	0	3	2	1	½	0	3	2	1	½	0	3	2	1	½	0	3	2	1	½	0
Broccoli, Early Purple	0	1	1	0	8	0	0	0	0	10	0	0	1	0	9	0	0	0	0	10	6	0	0	0	4	0	2	1	2	5
Clover, mixed	6	2	1	0	1	0	0	0	2	8	0	8	0	0	2	0	1	2	0	7	0	2	4	0	4	3	2	2	0	3
Flax	0	0	0	4	6	0	0	0	2	8	8	0	0	1	1	0	0	0	0	10	1	0	2	1	6	0	0	0	0	10
French bean, Lightning	10	0	0	0	0	0	0	1	3	6	0	0	8	2	0	10	0	0	0	0	10	0	0	0	0	0	4	6	0	0
Gram	1	4	5	0	0	0	1	3	0	6	0	0	0	10	0	0	10	0	0	0	0	0	5	2	3	0	0	3	2	5
Mustard, white	0	0	0	0	10	1	0	0	0	9	0	0	0	0	10	0	0	0	0	10	1	0	0	1	8	0	0	0	3	7
Pea, Eight Weeks	0	1	4	2	3	0	1	1	0	8	0	1	2	4	3	0	0	0	0	10	0	0	1	2	7	1	5	0	3	1
Radish, French Breakfast	0	0	1	1	8	0	0	0	1	9	0	0	0	2	8	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10
Sweet pea, Powerscourt	0	2	3	0	5	0	0	0	0	10	0	0	5	0	5	0	1	1	0	8	0	1	7	1	1	0	0	2	3	5
Tomato, Sunrise*	0	0	0	0	10	0	0	0	0	10	0	0	0	3	7	0	0	10	0	0	0	0	0	0	10	0	0	0	2	8
Wheat	0	0	0	1	9	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10	0	1	5	2	2

The following seeds were also tested and gave negative results: Carrot, Short Horn; lettuce, Cheshunt Early Giant; lupin, Russel Hybrids; maize, Wisconsin 255; neem; Nicandra physaloides; parsley; Solanum panduraeforme; and tobacco.

† Zonation divided into the following size groups: 0, no zone; ½, zone up to 2 millimetres greater than diameter of seed; 1, zone 2.5 to 4 mm. greater; 2, zone 4.5 to 8 mm. greater; 3, zone more than 8 mm. greater.

✱ Seeds were surface sterilised in 1% chloramine T and given four washings in sterile distilled water to reduce the heavy contamination of the seed coat.

efforts to infect the tomato, which shows the presence of little antibiotic substance in the seed coat, have failed. In this case, it is known that the plant contains an antibiotic, tomatin, and it may be that in other plants antibiotics are present which exert an influence on infection by C. fascians.

(b). Location of the antibiotic substances.

Seeds of the french bean were soaked in sterile distilled water for one hour and the seed coats removed, as far as possible avoiding damage to the kernel. The two parts of the seed were then tested in a similar manner to that described for whole seeds. In every case where there was antibiotic activity, it was shown by the seed coat and never by the kernel. This was further confirmed on sweet pea seeds.

(c). Sensitivity of strains to the antibiotic in sweet pea seeds.

(i). As shown by inhibition zones.

To obtain a more detailed picture of the variation seen from the first experiment, seeds of sweet pea, var. Powerscourt, were examined in larger numbers against seven strains of C. fascians, namely 2WR, S/100, 503, 505, 516, 12S, and 515 pink, for the presence of diffusible antibiotic substances. 100 seeds were used against each strain. The method was similar to that described in (a) above. Zones of inhibition were recorded after 3 and 7 days incubation at 25°C, and are shown in Table XI.

The considerable variation in the sensitivity of the strains seen previously was confirmed. For example, after 3 days incubation strain S/100 exhibited only 2% zonation against 55% by strain 2WR. The strains used can be conveniently divided into two groups. (i). The sensitive group, where there was zonation shown by over 30% of the seeds, and including strains 2WR, 503,516, and 515 pink. (ii) The insensitive group,in which the highest zonation recorded was 11%. This included strains S/100, 505, and 12S.

The zones also exhibited great variation in size,

Table XI.

The effect of strain variation on the sensitivity of *C. fascians* to antibiotics in the seed coat of the sweet pea in soil extract mannitol medium.

Strain no.	Frequency of occurrence of inhibition zones of various size groups* after incubation at 25°C for									
	3 days					7 days				
	3	2	1	½	0	3	2	1	½	0
2WR	2	9	30	14	45	0	2	5	14	79
S/100	0	0	1	1	98	0	0	0	1	99
503	2	4	8	17	69	0	1	2	16	81
505	0	1	3	7	89	0	0	3	5	92
516	3	6	8	15	68	0	2	4	18	76
12S	0	0	0	1	99	0	0	0	1	99
515 pink	4	12	28	11	45	0	4	5	24	67

* Zones of inhibition divided into following groups:

3, zone diameter over 15 millimetres.
2, zone diameter 10.5 to 15 mm.
1, zone diameter 7 to 10 mm.
½, zone diameter up to 6.5 mm.
0, no zone.

but the production of large zones was restricted to strains in the sensitive group. This variation in size of zones, representing a variation in the amount of diffusible antibiotic present, is not surprising when it is remembered that the variety of seed being used does not represent anything approaching a genetically pure line, being selected for vigorosity and colour and size of flower only.

With continued incubation for up to 7 days there was a rapid loss of zonation due to the development of colonies within the original zone. The antibiotic is therefore only bacteriostatic.

(ii). The relationship between zonation and pathogenicity.

To access the value of diffusible inhibitory substances in seeds towards the control of infection with C. fascians, the results from the previous experiment were compared with the percentage of infection of the sweet pea by each of the strains used.

From Table XII where the results are shown, it can be seen that there is a marked inverse correlation between percentage of infection after 5 weeks growth of the seedlings and the percentage zonation in the case of strains 2WR, 503, and 505. Apparently, the remaining strains do not confirm this, but if the correlation is made between percentage

Table XII.

The pathogenicity of strains of C. fascians in relation to their sensitivity to antibiotics contained in the coat of sweet pea seeds.

Strain no.	% of seeds exhibiting zones of inhibition*		% of infection of seedlings various periods after inoculation[1]			% of infection shown by strain when isolated
	including group $\frac{1}{2}$	excluding group $\frac{1}{2}$	3 weeks	4 weeks	5 weeks	
2WR	55.0	41.0	21.1	50.0	50.0	66.7
S/100	2.0	1.0	5.6	5.3	5.3	100.0
503	31.0	19.0	46.6	66.7	68.8	not recorded
505 [2]	11.0	4.0	11.1 (76.5)	44.4 (73.7)	66.7 (94.7)	100.0
516	32.0	17.0	nil	6.7	6.3	Approx. 66.7
12S	1.0	nil	nil	nil	nil	100.0
515 pink	55.0	44.0	nil	nil	nil	nil

* After 3 days incubation at 25°C. Data extracted from Table XI.

1. Data extracted from Table XXI.

2. Results in brackets obtained from a repeat experiment.

of zonation and the percentage of infection shown by the strain at the time of isolation, a more satisfactory result is obtained. Thus strain 128, although non-pathogenic when used in this work, when freshly isolated exhibited 100% pathogenicity, and this was reflected in the 1% zonation recorded for this strain. Strain 515 pink must be regarded as differing from the rest as it was never pathogenic. In this case, non-pathogenicity may be considered due to the absence of a factor, or factors, not connected in any way with susceptibility or resistance to seed coat antibiotics. Indeed no records have been found of pathogenic pink strains of the organism.

From the above considerations it can be seen that, if a satisfactory correlation is to be obtained for all strains (excluding 515 pink for reasons already discussed), the percentage of infection of the strain at the time of isolation must be used. It would appear possible that there exist 'stable' and 'unstable' strains of the organism, strains retaining their original pathogenicity after culture on artificial media for a considerable period being regarded as 'stable' and those showing a declining pathogenicity as 'unstable'. If this is so, we must assume that 'unstable' strains lose their pathogenicity as the result of the loss of a factor (or factors) which is distinct from that

controlling the susceptibility to seed coat antibiotics, a factor which, under these circumstances it is assumed remains relatively stable in all strains. In this way such strains after culture on artificial media would not be expected to exhibit a correlation as is shown by the 'stable' group.

The mode of action of these two factors can now be postulated. Pathogenic and non-pathogenic strains are distinguished by the presence or absence of a 'pathogenic factor'. When this factor is present the strain may be considered potentially 100% pathogenic. However a second factor controlling the reaction to seed coat antibiotics is also present. Thus strains may be resistant or susceptible to these antibiotics, and it is this factor which control the level of the infection.

The position of strain 515 pink can now be re-considered and is seen to fit into the scheme outlined above. Here the 'pathogenicity factor' was absent from the strain when isolated, but the factor controlling susceptibility to seed coat antibiotics remains operative.

For any particular strain the correlation between actual numbers is not complete. Indeed, no effort was made to obtain such a numerical relationship, but rather to illustrate that a possible correlation does exist.

However, a few possible reasons for the apparent numerical discrepancies will illustrate the difficulties involved in such a determination.

Firstly, the number of replicates used in determining the percentage of infection of each strain was relatively small (actually 20). Secondly, the percentage of infection was of course recorded only on seeds that germinated, while the percentage zonation was recorded from all seeds. No way of avoiding this was found for, if the recording of zonation was restricted to germinating seeds a further error would be introduced, as the germination in agar plates was found to be lower than that obtained in sand culture. Finally, as will be discussed later, C. fascians apparently exhibits a 'seasonal effect' with regard to pathogenicity, and as little is known about this its possible effect must be considered. Strain 505 for which two sets of results are shown in Table XII, illustrates this. The first set of results were observed to indicate a much lower percentage of infection than was normal for this strain, and the experiment was therefore repeated at a later date with the apparently normal result shown in the second set of figures.

Table XII also shows the percentage zonation excluding the $\frac{1}{2}$ group rating. It was at first considered doubtful if such small zones would exert an effect, and perhaps a better correlation may result from ignoring

them. The reverse however, was found to be the case.

(d). Consistency of zonation.

(i). In different batches of seed.

As shown previously, only in a few cases can the presence of diffusible inhibitory substances be demonstrated in all seeds of any one variety. It was considered of interest therefore to see if the percentage zonation recorded for one batch of seed remained constant in future batches. This was important as several batches of seed had to be used in this work, and any significant variation between batches might influence the results obtained in other experiments.

From each batch of seeds of sweet pea, var. Powerscourt, 100 were examined in soil extract mannitol medium, and the number and size of zones of inhibition recorded as in the previous experiments. The examination was made immediately the seeds were received, which in each case was within about three months of harvesting. Three such batches were obtained representing seed harvested in 1949 and the two succeeding years. The results are shown in Table XIII.

It will at once be seen that there is no great difference in the percentage zonation, actually, a variation of only 10% in the three batches. Considering both percentage zonation and the amount of diffusible

Table XIII.

The variability of different batches of sweet pea seeds in relation to the sensitivity of strain 505 of *C. fascians* to antibiotics contained in the seed coat.

Seed batch †	Frequency of occurrence of inhibition zones of various size groups * after incubation at 25°C for									
	3 days					7 days				
	3	2	1	½	0	3	2	1	½	0
1949	0	0	7	12	81	0	0	0	4	96
1950	0	0	1	20	79	0	0	0	3	97
1951	0	1	3	7	89	0	0	3	5	92

† Year of harvesting.

* Groups as shown in Table XI.

antibiotic present, the 1949 batch of seed was the most effective. In the 1950 batch the percentage remained constant, but the zones were practically all of the small size group. In the 1951 batch, the percentage was reduced while the size of the zones remained similar to the 1949 batch. The only noted difference in the three batches was that while the seeds of the 1949 batch were ripened under exceptionally dry conditions, those of the 1950, and to a less extent the 1951 batches were ripened under very wet conditions, a factor which may account for the lower activity of the latter two batches.

(ii). Effect of storage.

Seeds of the 1949 batch of sweet pea were stored for one year and the percentage zonation again determined. This was further repeated after 2 years storage. It was found that the percentage zonation was slowly reduced. After one year it had been reduced from 19% to 15%, and after two years to 12%. The loss of activity was also indicated by a reduction in the size of the zones obtained, all of which were in the $\frac{1}{2}$ group rating.

(e). Inhibitory effect of seeds on antagonistic organisms.

In view of the work undertaken in Section IVa of this thesis, a study of the reaction of two organisms which had been shown to be antagonistic to C. fascians

in plate culture, to the diffusate from the seeds of various plants, was made. The range of seeds examined and the method employed in the test was identical with that previously described in Sub-section 1a. The two antagonists used were B. subtilis, and F.A.1. The zones of inhibition were recorded after incubation at 25°C for 3 days and are shown in Table XIV.

Very few seeds produced inhibitory substances active against either or both of the organisms, a contrast with the results obtained for C. fascians. In fact only seeds of two plants, namely french bean and sweet pea, were active. The sweet pea showed the presence of an antibiotic active against B. subtilis in approximately 60% of the seeds, but inactive against F.A.1., while in the case of the french bean, it was 100 and 10% respectively. F.A.1. being highly resistant to the antibiotics must be considered to be a potentially highly efficient antagonist, while B. subtilis was susceptible, and must be considered a relatively inefficient one on these grounds. This is further considered in Section IVa in the light of results obtained from the use of various antagonists in infection experiments with C. fascians.

It should be noted that as F.A.1. failed to grow in soil extract mannitol medium, the recording of zones of inhibition relied upon the simultaneous stimulation

Table XIV.

The sensitivity of two organisms, B. subtilis and F.A.1., to antibiotics contained in the coats of various seeds.

Seed	Frequency of occurrence of inhibition zones of various size groups† against antagonist									
	B. subtilis					F.A.1.				
	3	2	1	½	0	3	2	1	½	0
French bean, Lightning	0	0	10	0	0	0	0	0	1	9
Sweet pea, Powerscourt	0	0	6	0	4	0	0	0	0	10

The following seeds were also examined and gave negative results:
Broccoli, Early Purple; clover, mixed; flax; gram; mustard, white; pea, Eight Weeks; radish, French Breakfast; tomato, Sunrise; wheat; carrot, Short Horn; lettuce, Cheshunt Early Giant; lupin, Russel Hybrids; maize, Wisconsin 255; neem; Nicandra physaloides; parsley; Solanum panduraeforme; tobacco.

† Size groups as shown for Table X.

of the organism by the seed, which as can be seen from reference to Table XVI, was shown by the majority of seeds tested.

(f). Diffusion of the antibiotic into water and its activity in aqueous solution.

In order that seeds containing an inhibitory substance active against the pathogen may be expected to exert an influence on infection either under natural conditions or in laboratory culture work, it would seem that two conditions must be fullfilled. Firstly the antibiotic must be water soluble in order that it can form a protective sphere round the seed. Secondly it must be either stable in solution for several days, or diffuse out slowly over the period of germination, or, preferably both. An examination of two seeds was made therefore to determine how far these conditions are met by seeds indicating relatively high antibiotic activity in plate culture.

Seeds of french bean were surface sterilised in 1% chloramine T for 15 minutes and given 4 washings in sterile distilled water. Half the number were plated out in soil extract mannitol agar medium as previously described, the other half were soaked in sterile distilled water for 24 hours at laboratory temperature, and then tested in a similar way. After 3 days incubation at 25°C zones of

inhibition were recorded against strain 505 of C. fascians from all the untreated seeds while no zones were exhibited by the treated ones, indicating complete diffusion of the active substance into the water.

To determine the activity of the antibiotic in solution, 20 seeds, after surface sterilisation as above, were soaked in 50 ml. of sterile distilled water at laboratory temperature for 24 hours, and the resulting extract assayed against strain 505 of C. fascians, in soil extract mannitol medium by the cup plate method.

Zones of inhibition averaging 15 mm. in diameter were recorded, indicating that the antibiotic was present in the aqueous extract. A further assay carried out after the extract had been stored for 3 days, indicated a reduced activity.

The experiment was repeated for sweet pea seeds, and a similar result obtained, with the exception that after 24 hours soaking not all the seeds were inactive. A few still exhibited small zones of inhibition, which indicates that diffusion of the antibiotic was not as fast as in the case of the french bean.

(g). Effect of removal of the antibiotic on infection.

If the conception previously put forward that the seed coat antibiotics of the sweet pea control the level

of infection by C. fascians, is correct, assuming the absence of other factors, the removal of these substances if complete, should result in 100% infection under pure culture conditions by all the 'stable' group of strains. Removal of the antibiotic was effected by soaking surface sterilised seeds of sweet pea in sterile distilled water for 36 hours before sowing.

Two sets of 25 sand culture tubes were set up, the method being identical with that described in Section IVa. Soil extract mannitol was used as medium. One set of tubes contained freshly surface sterilised seeds of sweet pea, and the other, seeds from which the antibiotic had been removed. Both sets were inoculated with strain 2WR of C. fascians. This strains was chosen because it exhibited a stable pathogenicity of approximately 55% so that a significant increase in the percentage of infection was possible.

The percentage of infection was recorded after 3, 4, 5, 6, and 7 weeks growth of the seedlings, and the results are shown in Table XV and fig. 9.

It was at once obvious that contrary to the anticipated increase in the percentage of infection due to the removal of the antibiotic from the soaked seeds, the reverse was the case. The lower percentage of infection in the soaked seeds was shown throughout the time ofthe

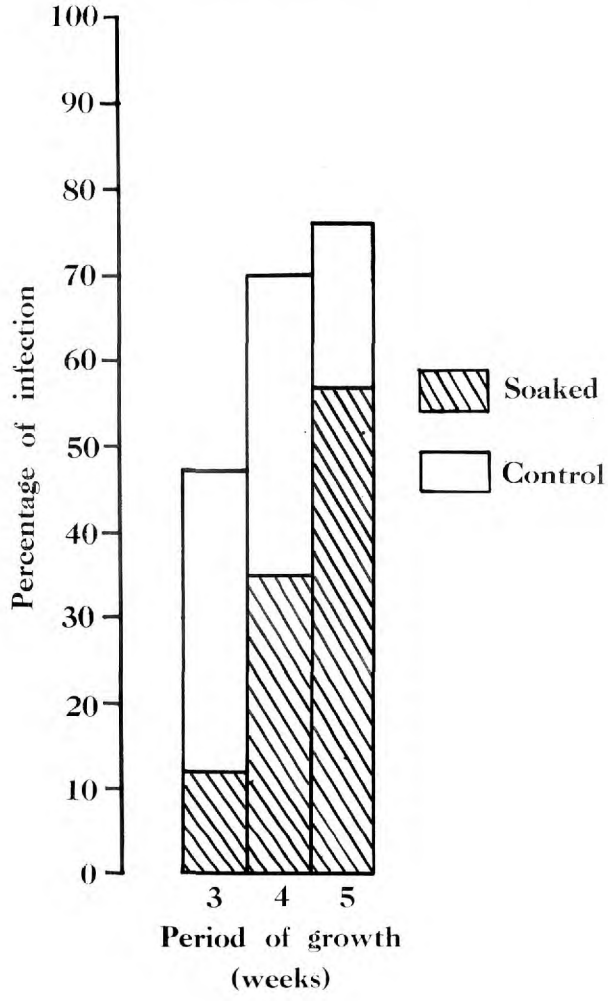
Table XV.

The effect of removal of the seed coat antibiotic by soaking, on the percentage of infection of sweet pea seedlings by strain 2WR of *C. fascians*, in sand plus soil extract mannitol medium.

Treatment	Period of growth								
	3 weeks			4 weeks			5 weeks*		
	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
Seeds soaked 24 hours	17	2	11.8	20	7	35.0	21	12	57.1
Control	19	9	47.4	20	14	70.0	21	16	76.2

* Results after 6 and 7 weeks did not significantly differ from those shown here.

Fig. 9 The effect of removal of the antibiotic on infection



experiment, but it was most pronounced after 3 weeks growth when the first record was taken, the difference being reduced after 4 weeks and still further reduced after 5 weeks growth. It would appear that a lag in attack took place, to be followed by normal infection. However, although the experiment was run for 7 weeks, the maximum time culture under these conditions could be continued, no significant increase in the percentage of infection took place.

No explanation of this would appear possible without reference to work described later, where it will be noted that a large number of seeds, including sweet pea, exhibit stimulation of C. fascians in plate culture on soil extract mannitol medium. As can be seen by reference to Table XVIII, strain 2WR was stimulated by all seeds of sweet pea, and it is considered that the low percentage of infection described above may be the result of the soaking out of, or partial soaking out of, the stimulating agent which is essential for attack to take place. If this is so, it is possible that the pathogen was unable to infect the plant until stimulated by later exudates.

Support for this conception was obtained from a further experiment in which seeds of sweet pea, after soaking for 24 hours, were examined in plate culture for

stimulation of the pathogen. The results showed the absence of stimulation as compared with a 100% positive result in the control of untreated seeds.

From this explanation however, it would be expected that the percentage of infection would have reached if not exceeded that of the control with longer growth of the seedlings. It may be that resistance of the plant as demonstrated by Lacey (1936), is concerned. This work showed that inoculation of sweet pea seedlings after 10 days growth resulted in a percentage of infection of only 75, as compared with 100 from those inoculated immediately after germination. Surface inoculations made after 17 days growth were negative. It was concluded that the plants developed a resistance to infection as growth proceeded, and that seedlings must be infected at a very early stage for severe fasciation to result.

It is reasonable to suppose therefore that failure to reach or exceed the percentage of infection of the control with treated seeds, was due to this effect. Apparently during the time infection was checked by the absence of the stimulatory factor, a resistance was built up by the plants which, in some, was sufficient to make them immune from attack.

The high percentage of infection exhibited by strain 2WR in the control series was exceptional and not

repeated during future work.

It must be emphasised that the results obtained were from one experiment only, which owing to limitation of time could not be repeated. Whether they represent the whole picture and are applicable to all strains of the pathogen requires further study. They must be regarded only as an indication of the number of factors which apparently influence infection by C. fascians even under pure culture conditions.

A similar experiment with seeds of the french bean was attempted, but great difficulty was experienced in obtaining satisfactory growth of this plant in sand culture and the experiment had to be abandoned.

2. The effect of alcohol on the antibiotics in seeds.

During preliminary work on the use of various surface sterilising agents, alcohol was examined as a possible one for seeds. It was at once noticed however, that seeds so treated exhibited exceptionally large and clear zones of inhibition as compared with those from untreated seeds. As it seemed very unlikely that retention of alcohol was the cause of this, a short investigation was made into the effect of alcohol treatment on the diffusion of inhibitory substances from sweet pea seeds.

(a). Treatment of seeds with alcohol.

(i). Experiments with the 1949 batch of seed.

20 seeds were treated in 50% alcohol (V/V) followed by three washings in sterile distilled water of 2 to 3 minutes each. The seeds were then tested in soil extract mannitol agar medium against strain 505 of C. fascians, as described in previous work on untreated seeds, for the diffusion of inhibitory substances. The diameter of the zones of inhibition were recorded after 3 days incubation at 25°C.

The results showed the diffusion of an antibiotic substance from all the seeds. The inhibition zones were clear and distinct with an average diameter of 12.2 millimetres, but individual zones varied from 8.0 to 20.0 mm. A control of untreated seeds exhibited only two zones of inhibition averaging 5.0 mm. in diameter. The experiment was repeated twice with substantially the same result.

It can be seen that treated seeds exhibited both a higher frequency of zonation and an increased zone diameter over the untreated ones. 50% alcohol was shown to be non-toxic in cup assay and so cannot be considered responsible for the zonation even if retained within the seed coat. It would appear that there was an action by the alcohol on the seed, which resulted in the release of a water soluble

antibiotic. Whether the inhibiting substance was the same as that which diffuses from untreated seeds, will be discussed after consideration of the results of further experiments.

(ii). Experiments with the 1950 batch of seed.

The previous experiment was repeated using the 1950 batch of seed immediately it became available. The results however showed no effect of alcohol treatment, the zones being small and showing a similar frequency to that of the control.

To confirm this the experiment was repeated using alcohol concentrations of 50, 70 and 95% for 2, 5, 10, 20, 40 and 60 minutes. No effect of alcohol treatment was however shown by any of the series.

It appears that this batch of seed was lacking in a sufficient concentration of the antibiotic to be effective. This is in accordance with the result obtained from untreated seeds, shown in Table XIII., where it was shown that the zones obtained from the 1950 batch were almost all in the $\frac{1}{2}$ size group.

(b). Alcohol extracts.

15 seeds were soaked for 24 hours in 3 c.c. of 50% (V/V) alcohol. The resulting extract was assayed in soil extract mannitol agar medium against strain 505 of

C. fascians for the presence of inhibitory substances. However, after 3 days incubation at 25°C no zones of inhibition were present.

It was considered that the negative result might be due to a concentration effect, so it seemed desirable to increase the number of seeds per ml. of alcohol. To do this it was necessary to remove the seed coats of the sweet pea seeds and use these alone. If this was not done the seeds imbibed the majority of the liquid and little was available for assay.

36 seeds were soaked for 5 hours in sterile distilled water, and the coats removed. These were soaked in 3 ml. of 50% alcohol for 24 hours in a refrigerator to reduce evaporation. The liquid was assayed as previously described. Zones of inhibition with an average diameter of 13.5 millimetres were obtained, indicating the presence of an active antibiotic in the seed coat.

(c). The effect of storage.

The 1949 batch of seed was stored for 12 months and the effect of alcohol treatment again studied. 21 seeds were examined for the diffusion of inhibitory substances as previously described.

The frequency of occurrence and size of the zones obtained showed an obvious decrease on those recorded

12 months previous. The percentage zonation was reduced from 100 to 47, and the average diameter of the zones from 12.2 to 10.6 millimetres.

This reduction in antibiotic activity can be correlated with a similar effect shown by untreated seeds after storage. This has been fully explained previously.

From the results obtained in these experiments, some indication of the effect of alcohol can be deduced. The alcohol has been shown to have an action on the seed coat resulting in the release of an antibiotic substance. This substance may be the same as, or different from that which diffuses from untreated seeds. However, from consideration of the similar relationship between loss of the inhibitor as the result of storage for both treated and untreated seeds, and the correlation of lower activity in the case of the 1950 batch of seed, there is a strong indication that they are if not the same substance, at least closely related.

The action of the alcohol may be physical or chemical, but it would seem unlikely that 50% alcohol would react with anything normally present in the seed coat, especially as the period of contact was only 2 to 3 minutes. The formation of an active ester, which is one reaction that must be considered, would appear very unlikely, at least

in sufficient concentration to be effective.

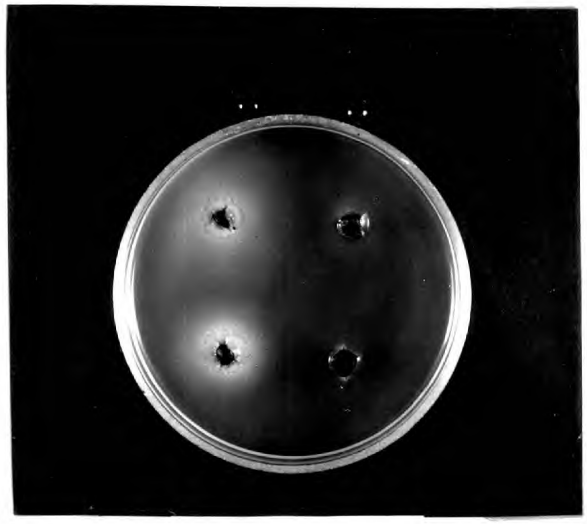
If it is a physical action the effect of the alcohol may be to increase the rate of diffusion of the antibiotic from the seed, so that an inhibitory concentration is reached in the medium before growth of the organism can take place. This must be considered likely, as 50% alcohol would have a greater penetrating power than water and so would rapidly 'wet' a larger area of the seed coat, thus allowing the diffusion of the antibiotic from a much greater area than in untreated seeds.

3. The stimulatory effect of seed diffusates.

During the course of the work on seed coat antibiotics it was noticed that many seeds exhibited a marked stimulation of both C. fascians and antagonistic organisms. The stimulation was apparently independent of the presence of an antibiotic, occurring with equal frequency from seeds exhibiting zones of inhibition and those failing to do so.

This type of stimulation was visible after 3 days, and pronounced after 7 days incubation at 25°C on soil extract mannitol medium. A second type was distinguished by the fact that it occurred spasmodically, and was many times heavier than that normally shown. In every case where this abnormal stimulation was evident, the seed was found to be attacked by a contaminating organism, which, it

Fig. 10. Stimulation of C. fascians in soil extract mannitol agar medium by decomposing seeds.



Left: Two decomposing sweet pea seeds exhibiting very heavy stimulation.
Right: Control of uncontaminated seeds

appeared, was an internal contaminent of the seed. This type of stimulation was well developed after 3 days but continued to develope for up to 14 days on soil extract mannitol medium, when the growth was many times that obtained from a culture of the organism on nutrient agar. This will be considered later, and only the normal type as shown by uncontaminated seeds will be considered here.

(a). Stimulation by various seeds.

The same range of seeds as examined for zones of inhibition, were tested for the diffusion of stimulatory substances against strains of C. fascians, F.A.1., and B. subtilis. The method employed was similar to that described previously in Sub-section 1a; i.e. 10 seeds of each plant were tested against each organism. The seeds were washed in three changes of sterile distilled water and plated out in soil extract mannitol medium inoculated with the test organism, using sufficient medium to just cover the seed. As before, seeds of tomato were surface sterilised in chloramine T and given 4 washings to reduce the heavy contamination of the coat. The plates were incubated at 25°C for 7 days.

No effort was made to record the diameter of the zones of stimulation owing to the difficulty of deciding where the zone terminated and the complication that some

seeds also exhibited an inner zone of inhibition. Stimulation was therefore simply recorded as positive or negative, and the frequency of occurrence as 100% or less than 100%. The results are shown in Table XVI.

Considering first the reaction of strains of C. fascians, it can be seen that all seeds used with the exception of neem, stimulated one or more strains. The most active response was shown by strain 515 pink, which was stimulated by seeds of all the plants tested, although in some cases, the frequency of occurrence of stimulation zones was not 100%. Strains 2WR and S/100 responded to diffusates from the majority of seeds, while strain 516 showed little response. Strains 503 and 505 were intermediate between the last two.

Seeds of a few plants, namely french bean, lettuce, lupin, and sweet pea, stimulated all strains, while the least active were radish, tobacco and carrot.

It is obvious that no direct correlation between susceptibility to infection by C. fascians and the response of the strain to stimulatory diffusates is possible. For example, all strains were stimulated by both carrot and lupin, but from neither of these plants has infection been recorded. However, it must be noted that highly

Table XVI.

The variation in response of strains of *C. fascians* and two antagonistic organisms, in soil extract mannitol medium, to stimulatory diffusates from various seeds.

Seed	Occurrence of zones of stimulation† against strain of *C. fascians* or antagonistic organism							
	Strain no.						Antagonist	
	2WR	S/100	503	505	516	515 pink	F.A.1.	*B. subtilis*
Broccoli, Early Purple	+	+	(+)	+	-	(+)	+	+
Carrot	+	-	-	-	-	+	+	+
Clover, mixed	+	+	(+)	(+)	-	+	+	+
Flax	-	(+)	+	+	-	(+)	+	+
French bean, Lightning	+	+	(+)	+	+	+	(+)	+
Gram	+	(+)	-	-	-	+	-	-
Lettuce, Cheshunt Early Giant	+	+	+	+	(+)	+	+	+
Lupin, Russel Hybrids	(+)	(+)	(+)	+	(+)	+	+	+
Mustard, white	+	-	-	(+)	-	(+)	+	+
Maize, Wisconsin 255	(+)	-	-	(+)	-	+	+	+
Nicandra physaloides	+	+	+	-	-	+	+	+
Parsley	(+)	(+)	+	-	-	+	+	+
Pea, Eight Weeks	(+)	(+)	-	-	-	+	+	+
Radish, French Breakfast	-	+	-	-	-	+	+	+
Solanum panduraeforme	+	+	-	-	-	+	+	+
Sweet pea, Powerscourt	+	+	+	(+)	(+)	+	(+)	+
Tobacco	-	-	-	+	-	+	(+)	-
Tomato, Sunrise*	+	+	(+)	(+)	-	+	+	+
Wheat	(+)	-	+	(+)	-	+	(+)	(+)

Note: Neem seed failed to stimulate any organism.

† Symbols indicate ~~degree~~ frequency of stimulation as follows: +, 100%; (+), less than 100%; -, no stimulation.

* Seeds surface sterilised in chloramine T to reduce heavy contamination.

susceptible plants such as the sweet pea, exhibited stimulation of all strains.

It would appear from these and previous results that the effect of the stimulatory diffusates from seeds is restricted to assisting an early infection, which as as discussed earlier results in an increase in the percentage of infection and the severity of the attack. Under certain circumstances this may be a controlling factor especially in the case of plants where an early resistance is built up thus preventing later infection.

During the course of this experiment, it appeared that in many cases seeds around which there was a stimulatory zone had failed to germinate. This was pronounced with such seeds as wheat and clover. These two were therefore examined in larger numbers and the percentage of seeds exhibiting stimulation of strain 505 of C. fascians recorded for germinated and ungerminated seeds. The method was identical with that previously described, with the exception that 300 seeds of each plant were tested. The results are shown in Table XVII. It should be noted that only seeds showing no contamination when the results were recorded after 7 days incubation at 25 C were included.

It can be seen that although stimulation was not

Table XVII.

The effect of germination of the seed, on the diffusion, in soil extract mannitol agar medium, of substances stimulatory to strain 505 of *C. fascians*

Seed	Percentage stimulation by seeds*	
	Germinated	Ungerminated
Clover	11.0	98.6
Wheat	25.6	91.0

* After 7 days incubation at 25°C.

restricted to ungerminated seeds, the majority of them did exhibit stimulation. Stimulation was also shown by a small percentage of the germinated seeds, but it was clear that while the majority of seeds germinated during the first 3 to 4 days, seeds that exhibited stimulation had only just commenced to germinate after 7 days.

As expected, it appears that germination of the seed reduced the amount of nutrients diffusing from the seed, which therefore exhibited little or no stimulation of the pathogen. If the seed failed to germinate, the diffusion of nutrients was high and stimulation resulted. In the case of seeds germinating at a late date, it is likely that as germination was delayed, nutrients diffused out before being stopped by the germinating process.

It must not be thought from the above experiment that for all plants any possible influence of stimulation on infectivity is restricted to seeds that are slow to germinate, but it is obvious that any attempt at correlation between pathogenicity of a strain of C. fascians and the reaction of the strain to diffusible nutrients from the seed would be difficult to demonstrate. For example in the case of sweet pea, while the majority of seeds exhibit stimulation of several strains, even here there is a more pronounced effect from ungerminated and slowly germinating seeds.

(b). Variation in strain response.

The reaction of various strains to diffusates from sweet pea seeds was considered worth further investigation, and a more accurate estimation of the percentage stimulation was therefore made for all strains.

100 seeds of sweet pea, var. Powerscourt, were tested against each strain of C. fascians, namely 2WR, S/100, 503, 505, 516, and 515 pink. The method was identical with that described in previous experiments of this type. The presence of stimulation was recorded after 3 and 7 days incubation at 25°C.

From Table XVIII where the results are shown, it can be seen that with the exception of two strains, namely 505 and 12S, all strains exhibited 100% or nearly 100% stimulation. The two odd strains exhibited only 16 and 19% respectively, and must therefore be considered unresponsive to the diffusible nutrients. The absence of any correlation between percentage of infection and response to diffusible nutrients is again seen. For example, strains 505, S/100, and 12S, were all 100% pathogenic when isolated, but the only one to show 100% stimulation was strain S/100.

It is clear that the part played by these stimulatory substances, if indeed they do exert an influence on infectivity, is obscure, and no general explanation can be

Table XVIII.

The effect of strain variation on the response of *C. fascians*, in soil extract mannitol agar medium, to stimulatory diffusates from sweet pea seeds.

Strain no.	Percentage stimulation after incubation at 25°C for	
	3 days	7 days
2WR	95	100
S/100	100	100
503	100	100
505	16	16
516	96	98
12S	19	20
515 pink	100	100

suggested which will account for all the results obtained.

(c). Activity of seed extracts.

30 seeds of sweet pea var. Powerscourt, were soaked in distilled water for a few hours until removal of the coats was possible, The kernels were then ground up with 5 ml. of soil extract mannitol medium, and the solid material centrifuged off. The resulting supernatant was high speed centrifuged until clear, and sterilised by passege through a grade 5 sintered glass filter. The extract was stored in a refrigerator until required.

The activity of this extract was examined by the cup plate assay method, 25 ml. of soil extract mannitol agar medium inoculated with the test organism, being used in each plate. Three cups each 8 mm. in diameter were cut in each plate, and two plates used for each organism. Each cup received 0.2 ml. of the extract, and the plates were then incubated at 25°C. All seven strains of C. fascians and all the antagonistic organisms used in this work were examined. The degree of stimulation was recorded after 3 and 7 days incubation and the results are shown in Table XIX.

All strains of C. fascians and the antagonistic organisms exhibited stimulation round the cups after 3 days incubation and this continued to develope for 7 days when it appeared, the maximum had been attained. With the

Table XIX.

The stimulatory effect of cold water extracts of sweet pea seeds on strains of C. fascians and antagonistic organisms in soil extract mannitol agar medium.

Organism		Stimulation† after incubation at 25°C for 3 days	7 days
C.fascians strain no.	2WR	+++	+++++
	S/100	+++	++++
	503	+++	+++++
	505	+++	+++++
	516	+	++
	128	+	++
	515 pink	+	++
F.A.1.		+++	++++
F.A.6.		+++	++++
F.A.7.		+++	++++
B. subtilis		+++	++++
B. mesentericus		+++	++++

† Symbols refer to degree of stimulation as follows:
+++++, very heavy;
++++, heavy;
+++, moderate;
++, light;
+, very light.

exception of 3 strains of C. fascians, namely 516, 12S and 515 pink, the degree of response was similar for all the organisms. The three exceptions exhibited only a feeble response after 3 days which showed little development after 7 days incubation.

If the feeble response shown by these strains is considered due to the inability of the organism to make full use of the nutrients contained in the extract, such strains may be considered to have less chance of infecting a seed than the highly responsive ones, for a similar reason. Although no complete correlation between pathogenicity and the response to stimulatory substances is possible for each strain, it should be noted that the three strains showing feeble response were all either non-pathogenic or nearly so, at the time of the experiment. Those exhibiting a good response were, with the exception of strain S/100, all highly pathogenic.

However, considering the results as a whole, although it would appear that by simple correlation stimulation cannot be regarded as a controlling factor under normal conditions, it is possible that it plays an important part in the building up of inocula, without which infection is doubtful.

4. Stimulation by decomposed seeds.

As mentioned previously, seeds which are contaminated by a bacterium or fungus frequently exhibit very heavy stimulation of C. fascians in plate culture. From one such seed, a species of Bacillus was isolated, and this organism, to be known as F.A.7., was used in all work on the decomposition of seeds. The organism was later shown also to be antagonistic to the pathogen both in plate culture and in sand culture experiments.

(a) Inoculation of seeds with F.A.7.

30 seeds of sweet pea, var. Powerscourt, after surface sterilisation by the chloramine T method, were inoculated with F.A.7. in three sets each of ten seeds as follows. In set one, the organism was smeared direct on to the seed coat. In set two, a small piece of the coat was removed and inoculation made at this point. Seeds of the last set were soaked for a short period, the coats removed, and the kernel inoculated. A control of uninoculated seeds was included.

The seeds were plated out in soil extract mannitol agar medium inoculated with strain 505 of C. fascians and the plates incubated at 25°C for 4 days, at the end of which they were examined for decomposition of the seeds and exhibition of stimulation zones.

The results showed that only where the seed coat had been removed was there any heavy stimulation. Seeds in the first set were not decomposed, but in the other two sets, attack by F.A.7. was evident, although this was most pronounced in the set where the coats had been completely removed. It would appear therefore that the coat was effective as a mechanical barrier preventing entry of the organism. This supports the view already expressed athat under natural conditions, seeds exhibiting decomposition were internally contaminated. Seeds in the second set, although decomposed, failed to exhibit a heavy stimulation although some response to C. fascians was evident. This was probably due to the slower rate of decomposition as compared with seeds from which the coats had been removed. The amount of stimulatory material available for diffusion would therefore be less. In addition, it is possible that the seed coat reduced the rate of diffusion of the stimulatory substance.

The above work was subsequently repeated using seeds of the french bean, and similar results obtained.

(b) Resistance of seeds to decomposition.

It was noted during the previous experiment that only a percentage of seeds in any one batch could be decomposed by inoculation with F.A.7. A further investigation of this was therefore undertaken.

36 sweet pea seeds were soaked for a few hours and the coats removed. The kernels were placed on glass beads in petri dishes containing a little water, and heavily smear inoculated with organism F.A.7. After 3 days at 25°C the number of seeds completely decomposed and those showing small areas of attack was recorded. Those not completely decomposed were further incubated and again examined after 7 days.

The results showed that after 3 days approximately half were showing almost complete decomposition while the remainder were either germinating normally or showed small areas of attack only. After 7 days one third of the original number were growing normally, while a small percentage had lesions, where it appeared, attack had been arrested at an early stage. The rest, approximately 60%, were completely decomposed.

It appears that nearly one third of the seeds in the sample tested exhibited a complete resistance to attack by F.A.7. In a smaller number of cases, this resistance was not complete and some attack took place. The nature of this resistance is unknown and owing to insufficient time, the investigation was not continued.

The experiment was repeated several times during the decomposition of seeds for the preparation of extracts, and a similar result was always recorded. In some cases,

an attempt was made to inoculate resistant seeds after 3 days incubation with fresh smears of F.A.7., but in almost every case this failed to produce attack.

(c) Extract of decomposed seeds.

(i) Stimulatory effect on C. fascians.

The method used for the preparation of the extract was as follows.

Sweet pea seeds were surface sterilised and inoculated with F.A.7. as previously described. Those seeds decomposed after 3 to 4 days incubation were removed and placed in a mortar with soil extract mannitol medium at the rate of 10 seeds to 5 ml. of medium. The whole was thoroughly macerated. The resulting fluid was high speed centrifuged until clear and then sterilised by filtration through a grade 5 sintered glass filter.

The extract was tested for ability to stimulate growth of strain 505 of C. fascians in soil extract mannitol agar medium, both in cups and when mixed with the medium at the rate of 0.5 ml. to 5 ml. of medium. A control of the same batch of soil extract mannitol agar and one of nutrient agar was included, the latter for comparative purposes.

After 4 days incubation at 25°C there was heavy stimulation round the cups and growth on the medium to which

the extract had been added was very heavy as compared with that on soil extract mannitol agar only. Growth continued where stimulated by the extract for at least 10 days when it was many times that obtained on soil extract mannitol agar. When compared with growth on nutrient agar, it was seen to be several times greater. See fig 11.

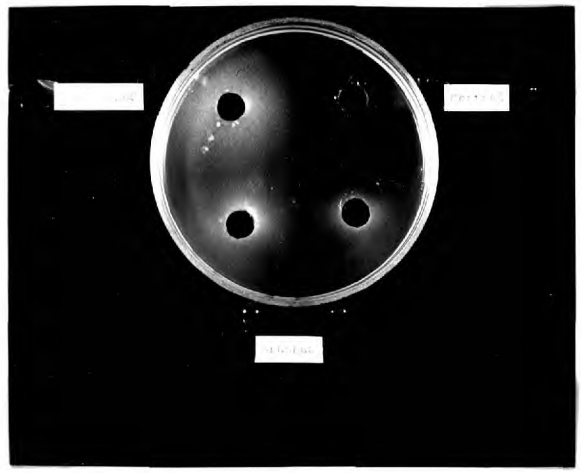
The type of growth was modified, being very mucoid. It was considered that the presence of a large amount of mucus might be giving a false impression of the number of cells actually present. A smear was therefore made from a stimulated area and examined under the microscope. It was at once clear that while a large amount of mucus was present, this could not account for the apparently very heavy growth obtained.

(ii) Activity on other strains of C. fascians and antagonistic organisms.

A further examination of the stimulatory effect of the extract was made on all strains of C. fascians and on the five antagonistic organisms used.

The extract was tested by the cup plate method (0.2 ml. of the extract per cup) in soil extract and soil extract mannitol agar media seeded with the appropriate organism. The plates were incubated at 25°C and examined after 3 and 7 days for stimulation. The degree of

Fig. 11. Stimulation of C. fasciens by extracts of crushed and decomposed seeds.



stimulation recorded was based on the stimulation shown by strain 505 after 7 days incubation. This was given five plus signs and the rest graded accordingly. The results are shown in Table XX.

It will be seen that some variation in response to the extract was evident among the strains of C. fascians as well as among the antagonistic organisms, but in every case, some stimulation was evident after 7 days incubation on either medium. In particular while the majority of strains of the pathogen exhibited an almost similar degree of stimulation, strains S/100 and 503 showed less response. It would appear that these two strains are unable to make use of the additional nutrients provided, possibly due to the absence of an essential growth factor in the required concentration in the basal medium. Strain S/100 makes poor growth in soil extract mannitol medium as can be seen by reference to Table 1, but this is not true of strain 503.

In all cases response in soil extract agar was poor, again probably due to nutritional limitations of the basal medium.

Among the antagonistic organisms, F.A.7. exhibited the least response, while F.A.1., and F.A.6., the two species of Pseudomonas, exhibited a high response.

It should be noted that response to decomposed seed extract does not follow similar lines to response to extract

Table XX.

The stimulatory effect of an extract of decomposed seeds on strains of C. fascians and organisms antagonistic to the pathogen in soil extract and soil extract mannitol media.

Organism	Degree of stimulation† on two media after incubation at 25°C.			
	Soil extract		Soil extract mannitol	
	Incubation period 3 days	7 days	3 days	7 days
C.fascians strain no. 2WR	(+)	(+)	+++	+++
S/100	(+)	+	++	+++
503	(+)	+	++	+++
505	+	+	+++	+++
516	-	(+)	+++	+++
12S	-	(+)	+++	+++
515 pink	-	(+)	+++	+++
F.A.1.	(+)	+	+++	+++
F.A.6.	+	++	+++	+++
F.A.7.	(+)	+	++	+++
B. subtilis	+	++	+++	+++
B. mesentericus	++	++	+++	+++

†Degree of stimulation denoted by symbols as follows:

+++, Very heavy
+++, heavy
+++, moderate
++, fair
+, poor
(+), very poor
-, no stimulation

of crushed seeds, for in the latter case, strains S/100 and 503 exhibited a high response. In view of this, it was decided to repeat the two experiments using one batch of medium in order that possible variations in composition of the basal medium should be avoided.

The results were similar to those already described. S/100 again showed little response to the decomposed seed extract, but a rather heavier response was exhibited by strain 503 on soil extract mannitol agar.

(d) Influence of extract on pathogenicity.

During work on the pathogenicity of various strains of C. fascians in sand culture, three different media were used to saturate the sand, namely tap water, soil extract and soil extract mannitol. It was noted that in the majority of cases the percentage of infection for any one strain was highest in the best medium, i.e. soil extract mannitol, and always the most severe type of infection was predominant where this medium had been used. It seemed reasonable therefore to assume that further improvement of the nutritive value of the medium might still further increase the pathogenicity of the organism. The following experiments in which decomposed seed extract was added to sand culture infections were therefore undertaken in view of the high degree of stimulation exhibited by the extract in plate culture.

In view of the amount of extract required for these experiments, a cheaper seed that could be rotted down in a similar manner was considered essential. The french bean seed was chosen as it is largely composed of protein and was easily and cheaply obtained. The seed coats were again removed and the kernels decomposed as previously described for sweet pea, except that 7 days was required before sufficient decomposition had taken place for preparation of the extract. This was examined to see if it exhibited similar reactions to that previously described. It was found to give identical reactions in all respects and was therefore used in all future work.

Sterilisation of the extract by filtration was extremely slow owing to its colloidal nature. The activity of autoclaved extract was therefore examined, and it was found that, provided the autoclaving time was reduced to a minimum, 10 minutes at 15 lb pressure was used, little if any reduction in activity was evident. This method was therefore adopted, the extract being autoclaved in sealed bottles.

(i) Experiments in soil extract mannitol medium.

The pathogenicity of all seven strains of C. fascians used in this work was determined in sand culture plus soil extract mannitol as medium, with and without the addition of decomposed seed extract. The tubes were set up as

described in the general method in Section IVa.

40 tubes were used for each strain, 20 of these having the extract added, the other 20 acting as a control. The extract was added at the time of inoculation as follows. A heavy suspension of the pathogen in soil extract mannitol was prepared from cultures grown on nutrient agar. To one half of this was added an equal volume of the decomposed seed extract, and to the other an equal volume of soil extract mannitol. The decomposed seed extract had previously been roughly standardised by determining the highest concentration which would stimulate but not inhibit the organism when tested by the cup plate method in soil extract mannitol agar medium. Dilution with the inoculum was so arranged that the resulting mixture was at the optimum concentration for stimulation. Inoculation was achieved by dropping 0.5 ml. of the suspension on to each seed.

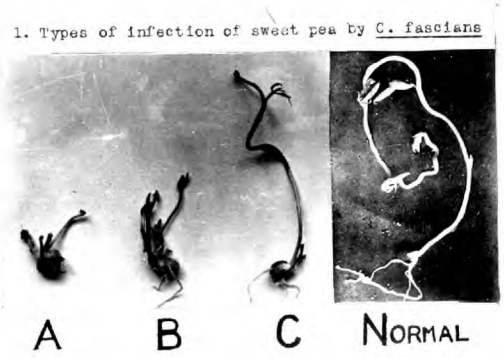
The tubes were kept in the light but away from direct sunlight and the moisture content of the sand maintained by the addition of soil extract mannitol as required. The number of germinated seeds and the number showing fasciation was recorded after 3, 4, 5 and 6 weeks, when the experiment had to be discontinued owing to the size of the seedlings which had grown out of the 3 in. by 1 in. tubes used. The severity of infection was recorded after 6 weeks growth and this was divided into three groups depending on the

degree of stunting and basal swelling of the shoots (see footnote to Table XXIa). The results are shown in Tables XXI and XXIa and graphically in fig. 13.

From the results three main points are indicated. Firstly, all the stable group of strains, i.e. those having a reasonably high consistent pathogenicity, namely 2WR, 503 and 505, were stimulated by the extract to produce a higher percentage of infection. Normally this increase over the control was 10 to 15%. This was shown after 3 weeks growth and the trend was maintained until the experiment was discontinued. Secondly, in addition the severity of infection was markedly increased. The number of seedlings having very stunted growth with basal swelling of the shoots was very high where the extract was present, but rarely occurred in the control. Lastly, the non-pathogenic strains 12S and 515 pink were not stimulated to attack the host, a similar negative reaction being exhibited by the feebly pathogenic strains S/100 and 516.

It would appear from the results that with the exception of the nonpathogenic and feebly pathogenic strains, the degree of growth made by the pathogen in the surrounding medium determines to some extent the percentage of infection obtained. However as the inoculum used was a heavy one, and the majority of strains made fair growth on the soil extract mannitol medium, it would be expected that more

Fig. 12. Types of infection of sweet pea by C. fascians



A, Severe; B, moderate; C, slight.

Table XXI.

The effect of addition of decomposed seed extract on the percentage of infection of sweet pea seedlings in sand plus soil extract mannitol medium, by various strains of C. fascians

Strain number	Treatment	Period of growth: 3 weeks			4 weeks			5 weeks			6 weeks		
		No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
2WR	Extract	15	5	33.3	16	10	62.5	18	11	61.1	18	11	61.1
	Control	19	4	21.1	20	10	50.0	20	10	50.0	20	10	50.0
S/100	Extract	18	1	5.6	18	1	5.6	19	1	5.3	19	1	5.3
	Control	18	1	5.6	19	1	5.3	19	1	5.3	19	1	5.3
503	Extract	18	13	72.2	18	14	77.8	18	17	94.4	18	17	94.4
	Control	15	7	46.6	15	10	66.7	16	11	68.8	16	13	81.2
505*	Extract	19 (18)	6 (16)	31.6 (88.9)	19 (18)	11 (17)	57.6 (94.4)	19 (19)	15 (19)	78.9 (100.0)	19 (19)	15 (19)	78.9 (100.0)
	Control	18 (17)	2 (13)	11.1 (76.5)	16 (19)	8 (14)	44.4 (73.7)	18 (19)	12 (18)	66.7 (94.7)	18 (19)	12 (18)	66.7 (94.7)
516	Extract	14	2	14.3	14	2	14.3	14	2	14.3	14	2	14.3
	Control	15	nil	nil	15	1	6.7	16	1	6.3	16	1	6.3
12S	Extract	18	nil	nil	18	nil	nil	18	nil	nil	18	nil	nil
	Control	17	nil	nil	17	nil	nil	18	nil	nil	18	nil	nil
515 pink	Extract	12	nil	nil	12	nil	nil	14	nil	nil	14	nil	nil
	Control	16	nil	nil	16	nil	nil	17	nil	nil	17	nil	nil

*Figures in brackets represent results from a replicate experiment.

Fig. 13 Stimulation of infection with decomposed seed extract

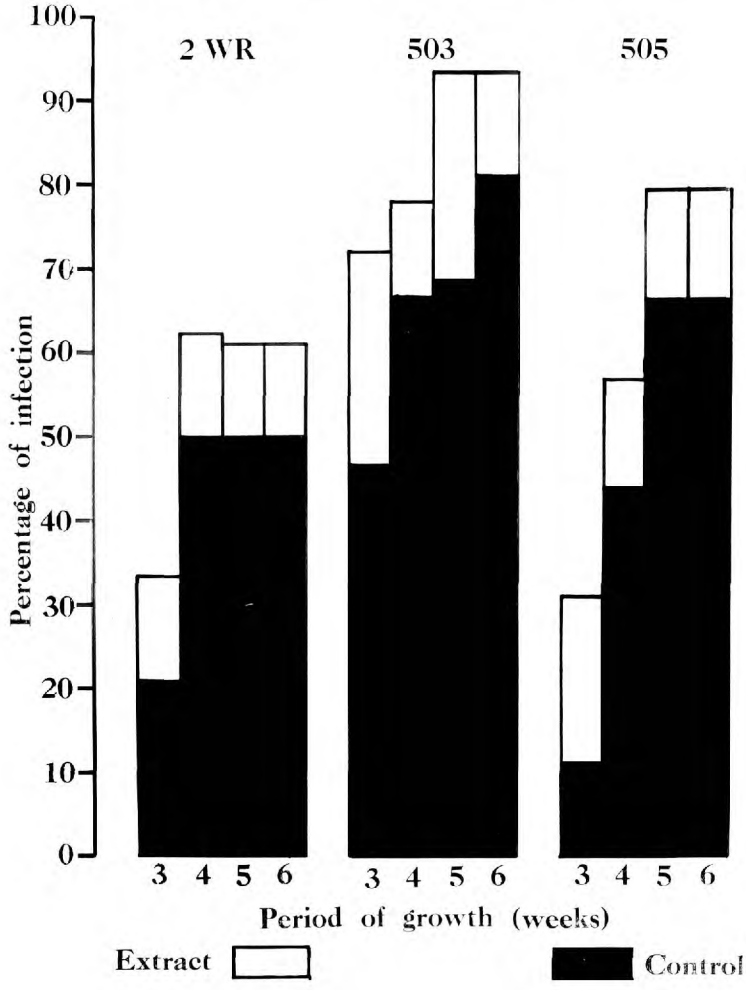


Table XXIa.

The effect of the addition of decomposed seed extract on the severity of infection of sweet pea seedlings in sand plus soil extract mannitol medium by various strains of C. fascians.

Strain no.	Treatment	Severity of infection* after 6 weeks growth		
		A	B	C
2WR	Extract	4	0	7
	Control	0	2	8
S/100	Extract	0	0	1
	Control	0	0	1
503	Extract	3	8	6
	Control	0	3	10
505†	Extract	1 (11)	4 (3)	10 (2)
	Control	0 (6)	4 (3)	8 (5)
516	Extract	0	0	2
	Control	0	0	1

* Severity graded into three groups, A, B, and C, and the number of plants in each group recorded.

A. Seedlings exhibited multiple shoots not more than one inch in length and all had swollen bases.

B. Multiple shoot development without basal swelling. Shoots up to 2 inches in length.

C. Development of one normal shoot followed by further basal shoots. No basal swelling.

A photograph appears in fig. 12.

† Figures in brackets represent results from a replicate experiment.

than sufficient organisms would be available to ensure infection taking place.

Considering these results in the light of those obtained for the diffusion of antibiotic substances from seeds, it appears possible that the effect of the stimulatory substance was to partly antagonise the effect of this inhibitor, thus enabling the organisms to attack seeds which would otherwise be protected. Seeds which contained only a low concentration of the inhibitor would then become susceptible to attack, and the percentage of infection therefore increased. This will be further considered later.

As a confirmation of these results, the experiment was repeated using strain 503 only with a larger number of replicate tubes. 100 tubes were set up as previously described, half being inoculated with the mixture of C. fascians and decomposed seed extract, the remainder acting as a control. The results were recorded as in the previous experiment withthe exception that they were discontinued after 5 weeks owing to the size of the seedlings. From the previous and other experiments it was found that, normally, the maximum percentage of infection is reached after 5 weeks growth. The results are shown in Tables XXII and XXIIa, and graphically in fig. 14.

It is at once seen that the percentage of infection was not significantly increased by the extract, although

Table XXII.

The effect of addition of decomposed seed extract on the percentage of infection of sweet pea seedlings in sand plus soil extract mannitol medium by strain 503 of C. fascians.

Treatment	Period of growth								
	3 weeks			4 weeks			5 weeks		
	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
Extract	35	25	71.4	39	31	79.5	42	39	92.9
Control	44	30	68.2	44	37	84.1	44	37	84.1

Fig. 14 Stimulation of infection of strain 503 with decomposed seed extract

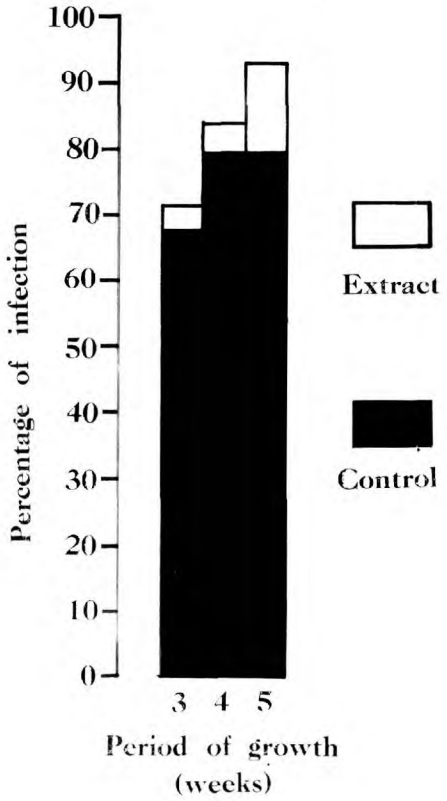


Table XXIIa.

The effect of the addition of decomposed seed extract on the severity of infection of sweet pea seedlings in sand plus soil extract mannitol medium by strain 503 of *C. fascians*.

Treatment	Severity of infection* after 5 weeks growth		
	A	B	C
Extract	25	6	8
Control	5	6	26

* See footnote to Table XXIa.

there was a trend in this direction. The severity of infection however, was again greatly increased by the presence of the extract, the majority of infected plants being in the most severely attacked group where the extract was present, against a majority in the least severe group where it was absent.

The experiment was further repeated five times with results which with the exception of one set, showed a definite increase in the percentage of infection. The severity of the infection was always increased.

The reason for this variation is not known, but it is probable that an uncontrolled factor which had a considerable influence on the infection, was responsible. Unavoidably the experiments were performed at various periods of the year, under varying light and temperature conditions, and in view of what has already been mentioned regarding a 'seasonal effect' which the pathogen appears to exhibit, it is likely that such an external factor or combination of factors was responsible for the variation seen. From Table XXI where two sets of results from replicate experiments are shown for strain 505, it is clear that even with the 'stable' strains considerable variation in the percentage of infection occurs from one experiment to another. It is realised that the number of replicates was small, but it

appears that this cannot account for the variation seen, as during any particular period all the results exhibited a similar trend whether this was an increase or a decrease on the normal.

(ii) Experiments in soil extract medium.

The effect of decomposed seed extract was also examined in a sand plus soil extract medium. It has previously been shown that the stimulatory powers of the extract in plate culture were much reduced in the absence of mannitol, and it was considered desirable to see if a similar reduction in activity was shown in sand culture infections.

40 tubes were set up for each of the two strains of the pathogen used, namely strains 503 and 505. The method was identical with that described in the previous experiment with the exception that soil extract was substituted for soil extract mannitol. In each set 20 tubes received the extract, the other 20 acting as a control. The percentage of infection was recorded after 3, 4 and 5 weeks growth of the seedlings and the severity of infection at the end of the experiment. These are shown in Tables XXIII and XXIIIa and graphically in fig 15.

Again both the percentage of infection and the severity of infection were increased by the presence of the extract, and it would appear that its activity was not

Table XXIII.

The effect of addition of decomposed seed extract on the percentage of infection of sweet pea seedlings in sand plus soil extract medium by strains 503 and 505 of *C. fascians*.

Strain	Treatment	Period of growth								
		3 weeks			4 weeks			5 weeks		
		No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
503	Extract	17	12	70.6	17	14	82.4	18	18	100
	Control	15	10	66.7	15	11	73.4	16	14	87.5
505	Extract	16	10	62.5	17	13	76.5	17	15	88.2
	Control	18	8	44.4	18	11	61.1	19	14	73.7

Fig. 15 Stimulation of infection with decomposed seed extract

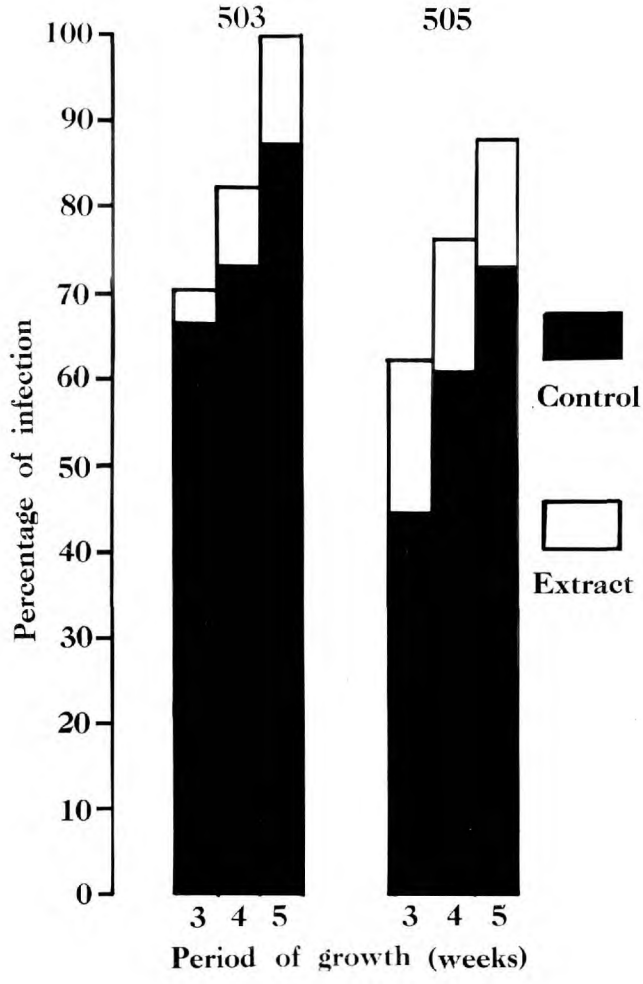


Table XXIIIa.

The effect of the addition of decomposed seed extract on the severity of infection of sweet pea seedlings in sand plus soil extract medium by strains 503 and 505 of *C. fascians*.

Strain no.	Treatment	Severity of infection† after 5 weeks growth		
		A	B	C
503	Extract	8	2	8
	Control	3	2	9
505	Extract	6	0	9
	Control	0	0	14

† See footnote to Table XXIa.

reduced in the absence of the mannitol. It is obvious from the plate culture work previously described that the amount of growth made in the absence of mannitol was greatly reduced, and this would appear to support the idea already put forward that the action of the extract was to antagonise the effect of the seed coat antibiotics rather than simply stimulate multiplication of the organism.

5. Stimulatory effect of known substances.

In the previous work on stimulation, the active principle in the extract was unknown. The protein nature of the seed suggested that amino acids formed by breakdown of the kernel may be present and these might have been responsible for the stimulation. A range of substances was therefore examined in the hope of finding one or a combination which would exhibit a similar degree of stimulation.

(a). Stimulation in plate culture.

39 substances including amino acids, purine and pyrimidine bases, and growth factors, were examined by the cup plate method for the ability to stimulate strain 505 of C. fascians and the antagonistic organisms in soil extract mannitol agar medium. The substances were dissolved in water, some only with difficulty, the concentration being that recommended by other workers for a variety of

organisms. Thus the amino acids and bases were made up at a concentration of 0.1%, the growth factors at 0.001% and the other substances at 0.1% with the exception of casein hydrolysate where 1.0% was used, as it was known that in other media this concentration was stimulatory. 0.2 ml. of the solution was added to each cup, and the plates incubated at 25°C. Those inoculated with the antagonistic organisms were examined after 2 days and those with C. fascians after 4 days, for zones of stimulation. The degree of stimulation was graded using the heavy stimulation of C. fascians induced by casein hydrolysate as a maximum. The results are shown in Table XXIV.

It will be seen that only a relatively few substances produced a stimulation of C. fascians, and of these only casein hydrolysate produced a degree of stimulation in any way comparable with that obtained from decomposed seed extract. Three amino acids, namely aspartic acid, glutamic acid and leucine, produced a small stimulation. Guanine, calcium pantothenate, glutaric acid and glutathione were other substances which had a positive effect. When a mixture of all the amino acids was used the stimulation was very small and only small/ stimulations were obtained with mixtures of amino acids plus growth factors and with all 39 substances together.

Considering the antagonistic bacteria, F.A.1. and

Table XXIV.

The stimulatory effect of various substances in soil extract mannitol agar medium on strain 505 of *C. fascians* and organisms antagonistic to the pathogen.

	Substance	*C. fascians* strain 505	Degree of stimulation † F.A.1.	F.A.6.	F.A.7.	*B.subtilis*
Amino acids (0.1%)	Alanine	-	-	-	-	-
	Arginine	-	+	+	-	+
	Aspartic acid	(+)	(+)	(+)	(+)	+
	Cystine	-	(+)	(+)	-	-
	Glutamic acid	+	+	+	(+)	++
	Glycine	-	-	-	-	-
	Histidine	-	-	++	-	-
	Isoleucine	-	-	-	-	-
	Leucine	+	(+)	+	-	-
	Methionine	-	-	-	-	-
	Norleucine	-	-	-	-	-
	Phenylalanine	-	(+)	(+)	-	-
	Proline	-	++	++	-	-
	Serine	-	-	-	-	-
	Threonine	-	-	-	-	-
	Tryptophane	-	-	-	-	-
	Tyrosine	-	(+)	(+)	-	-
	Valine	-	-	-	-	-
Bases (0.1%)	Adenine	-	-	-	-	-
	Guanine	+	+	++	+	++
	Uracil	-	-	-	-	-
Growth factors (0.001%)	Aneurin	-	-	-	(+)	-
	Ascorbic acid	-	-	+	(+)	(+)
	Asparagin	-	-	-	-	-
	Biotin	-	-	-	-	-
	Calcium pantothenate	(+)	-	-	-	-
	Folic acid	-	-	-	-	-
	Nicotinic acid	-	-	-	-	-
	Riboflavin	-	-	-	-	-
Casein hydrolysate 1.0%		+++	++	++	(+)	-
Creatine 0.1%		-	-	-	-	-
Glutaric acid 0.1%		+	(+)	+	(+)*	++
Glutathione 0.1%		+	+	+	(+)	-
Lysin 0.1%		-	-	+++	-	-
Nucleic acid 0.1%		-	-	-	++	++
Sodium succinate 0.1%		-	-	++	-	-
All amino acids		(+)	-	++	-	-
Amino acids plus growth factors		+	+	++	-	-
All substances		+	+	++	+	+

† Degree of stimulation graded as follows:
+++, heavy;
++, moderate;
+, light;
(+), very light;
-, no stimulation.

* Stimulation in two concentric bands separated by a zone of normal growth.

F.A.6., although previously thought to be very similar, exhibited some differences in their reaction to these substances. Both were moderately stimulated by casein hydrolysate and proline, but F.A.6. responded equally well to histidine, lysin and sodium succinate, to all of which F.A.1. was negative. F.A.1. also exhibited a much greater response to the mixtures of amino acids, amino acids plus growth factors and all 39 substances together. F.A.7. showed only a feeble response toa few substances with the exception of nucleic acid, which produced a moderate stimulation. B. subtilis also generally failed to respond but moderate stimulation was produced by glutamic acid, glutaric acid and nucleic acid. The mixtures produced little if any, effect.

It would appear from these results that the heavy stimulation produced by the extract of decomposed seeds cannot be attributed to the presence of one or more amino acids or to a common growth factor requirement. It is realised that not all possible substances have been examined, only those readily available were tested, and also that a concentration effect may have influenced the degree of stimulation obtained. In view of this last consideration, three of the substances, namely glutamic acid, glutaric acid, and casein hydrolysate, were re-examined at various concentrations from 0.1 to 1.0% to determine the optimum one

for stimulation of strain 505 of C. fascians. The method was identical with that described above.

The optimum concentrations were found to be as follows. Glutamic acid, 0.1%; glutaric acid, 0.05%; casein hydrolysate, 1.0%. Generally, above these concentrations the substances proved to be inhibitory. In all cases, the degree of stimulation remained similar to that already described, even in the case of casein hydrolysate this never reached the very heavy level obtained with the decomposed seed extract.

(b). Effect on pathogenicity.

The effect of three substances, glutamic acid, glutaric acid, and casein hydrolysate, on the pathogenicity of strain 503 of C. fascians was studied. Four sets each of 20 sand culture tubes were set up in accordance with the general method described in Section IVa. The substances were added to soil extract mannitol medium so as to give a final concentration which corresponded with the optimum one previously determined. This was used to saturate the sand in the tubes. Thus set one contained soil extract mannitol plus 0.1% glutamic acid, set two 0.05% glutaric acid, and set three 1.0% casein hydrolysate. The fourth set acted as a control.

Inoculation of the sweet pea seeds was made at the time of sowing and the moisture content of the sand maintained

by the addition of the appropriate medium as and when required. The number of seeds germinating and the number showing infection was recorded after 3, 4, and 5 weeks growth of the seedlings and are shown in Table XXV and diagrammatically in fig. 16. The severity of infection was recorded after 5 weeks growth and divided into the 3 groups previously described. This is shown in Table XXVa.

A marked increase in the percentage of infection in each set where a stimulatory substance was added is at once obvious. The increase was generally higher than that obtained using decomposed seed extract, averaging over 20%. The increase in percentage of infection was, as before, accompanied by an increase in the severity of the attack. This was well shown by glutamic and glutaric acids, but only feebly by casein hydrolysate. If this result was confirmed it would suggest that perhaps two factors are operating, one affecting the percentage of infection and the other the severity, but from this one experiment no definite conclusions can be drawn. The experiment was not repeated.

In previous work using decomposed seed extract, it was shown that the degree of stimulation exhibited in plate culture showed no relation to the percentage of infection obtained in sand culture experiments. This was again demonstrated, for although the degree of stimulation produced

Table XXV.

The effect of addition of various substances on the percentage of infection of sweet pea seedlings in sand plus soil extract mannitol medium by strain 503 of *C. fascians*.

Treatment	Period of growth								
	3 weeks			4 weeks			5 weeks		
	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage
Glutamic acid 0.1%	15	9	60.0	19	14	73.7	19	16	84.2
Glutaric acid 0.05%	13	8	61.5	13	10	76.9	14	13	92.9
Casein hydrolysate (vitamin free) 1.0%	12	7	58.3	13	11	84.6	13	12	92.3
Control	12	5	41.7	15	8	53.3	16	11	68.8

Fig. 16 The stimulatory effect of various substances on infection

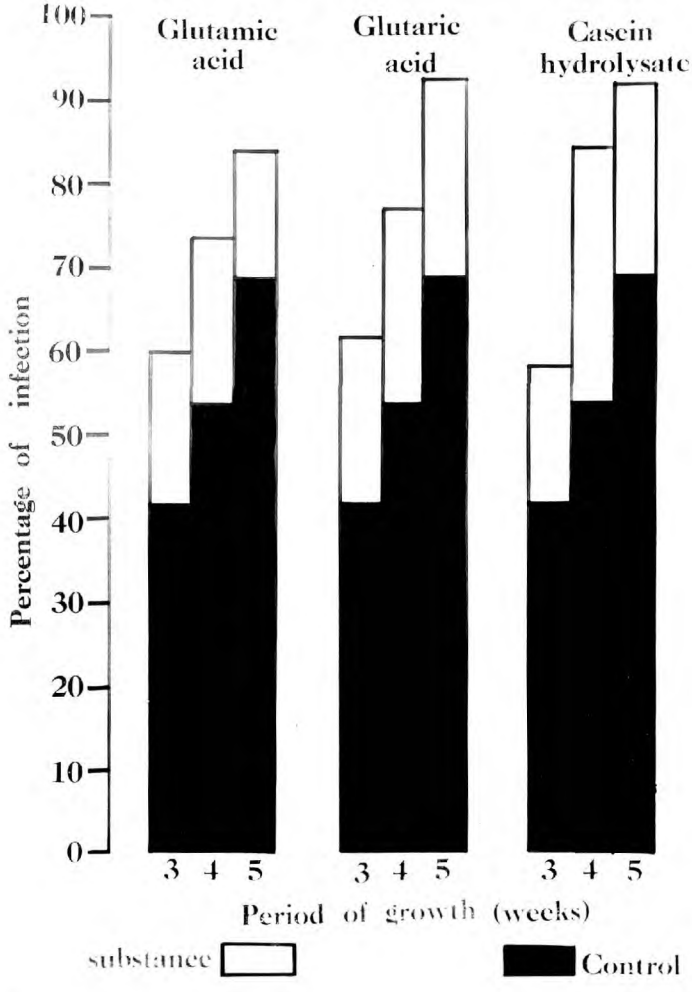


Table XXVa.

The effect of the addition of various substances on the severity of infection of sweet pea seedlings in sand plus soil extract mannitol medium by strain 503 of C. fascians.

Treatment	Severity of infection* after 5 weeks growth		
	A	B	C
Glutamic acid 0.1%	11	2	3
Glutaric acid 0.05%	8	2	3
Casein hydrolysate (vitamin free) 1.0%	2	4	6
Control	1	1	9

*See footnote to Table XXIIIa.

in plate culture by, for example, glutamic acid, was very small compared with that produced by decomposed seed extract, the increase in percentage of infection was higher when the former substance was used. This is further evidence that stimulation of growth of the organism does not provide a satisfactory explanation for the increase in infection obtained.

A check was made to see that the degree of stimulation shown in plate culture was similarly shown in the sand culture tubes. Counts taken indicated that there was a similar relationship, those for decomposed seed extract being 100 times those where glutamic acid was present.

IVa. THE EFFECT OF ANTAGONISTIC BACTERIA ON INFECTION BY C. FASCIANS.

The spasmodic occurrence of the fasciation disease, previously discussed, led naturally to an investigation of the relationship between the pathogen and the soil microflora. Many instances of antagonism between two organisms have already been cited and this approach was therefore considered worth attention. The difficulty of isolating the pathogen has for a long time been attributed to the effect of inhibitory organisms on the isolation plates, but no direct evidence that such organisms could exert an influence in pure, or mixed culture, with the pathogen, was available.

1. Method of cultivation of seedlings.

In all work designed to determine the percentage of infection of the pathogen, the following general method was adopted as a basis, any slight modifications being made where the experiment demanded them.

All seedlings were grown in 3 in. by 1 in. glass specimen tubes, at the bottom of which there was about a half inch of sand or soil according to the particular experiment. The tubes were plugged and sterilised in the hot air oven. The sand was saturated with the particular medium required avoiding the accumulation of surface water. Seeds of sweet pea, var. Powerscourt were surface sterilised

in 1% chloramine T for 15 minutes and given 4 washings of 2 to 3 minutes each in sterile distilled water. One was then placed in each tube. The seeds were inoculated with a suspension of the organism or organisms in tap water, 0.2 ml. of a dense suspension being dropped on to each seed.

The tubes were placed in racks in the light but away from direct sunlight, and the moisture content of the sand maintained by addition of medium as and when required. During the winter months additional artificial lighting was used in the form of two 500 watt lamps covering an area of approximately 10 square feet. A water screen was used to remove excessive heat. The artificial lighting was arranged such that the period of light for any one week averaged 12 hours per day. Under these conditions growth of the seedlings in December and January appeared normal. No additional heating was used above that of the laboratory which was found to fluctuate considerably, but did not appear to seriously affect growth of the seedlings.

When the seedlings reached the cotton wool plugs they were allowed to grow out of the tubes, the plug being replaced round the shoot to prevent contamination. This was normally necessary on uninfected seedlings during the fifth week of growth.

2. Antagonism on plates.

Several organisms were examined for antagonism against

all the strains of C. fascians. They included two known to be antagonistic to certain strains of the pathogen, and stock cultures of B. subtilis and B. mesentericus, as used by Mohanty (1951). F.A.7., a species of Bacillus used previously for the decomposition of seeds, was also included.

The nutrient agar medium was seeded with the appropriate strain of C. fascians and poured into petri dishes. The suspected antagonist was then streaked on to the surface of the agar in the form of a cross, and the plates incubated at 30°C.

Growth of the antagonist and inhibition of the pathogen if present, was recorded after 48 hours incubation. The results are shown in Table XXVI.

All strains of the pathogen were inhibited by F.A.1. and F.A.6., but F.A.7. and B. subtilis were not 100% effective. Two strains of C. fascians, namely S/100 and 516 were not inhibited by F.A.7., and B. subtilis also failed to inhibit strain 516. This result was considered with some degree of suspicion, but a replicate experiment gave a similar result. The particular strain of B. mesentericus used was not active against any strain of the pathogen, a contrast to that found by Mohanty (1951) where this organism was shown to be effective as an antagonist.

Table XXVI.

The antagonistic effect of various bacteria against strains of *C. fascians* on nutrient agar medium.

Strain of *C. fascians*	Antagonist				
	F.A.1.	F.A.6.	F.A.7.	*B. subtilis*	*B. mesentericus*
2WR	+	+	+	+	-
S/100	+	+	-	+	-
503	+	+	+	+	-
505	+	+	+	+	-
516	+	+	-	-	-
515 pink	+	+	+	+	-

In all cases, inhibition was complete and continued incubation of the plates for up to three weeks failed to show growth of any colonies of C. fascians within the inhibition zones.

3. Antagonism in sand culture.

180 tubes were set up according to the general method described at the commencement of this Section. These were divided into 9 sets of 20 tubes each. Three sets had tap water as medium, a second three soil extract, and the final three, soil extract mannitol. One set of each medium was inoculated with a mixture of strain 505 of C. fascians and the antagonist F.A.1. A second series was similarly inoculated using F.A.6., and the third had the pathogen only and acted as a control series. The suspensions for inoculation were prepared as follows. The organisms were grown on nutrient agar slopes and the growth washed off with sterile tap water. The resulting suspension was roughly standardised to give a dense suspension of the organism. Equal quantities of the suspensions of C. fascians and the antagonist were thoroughly mixed and this constituted the inoculum. The control suspension of C. fascians only was, after standardisation, diluted with an equal volume of tap water in order that the number of pathogenic organisms in all the tubes should be the same.

The percentage of infection was recorded for each set after 3, 4, and 5 weeks growth of the seedlings and are shown in Table XXVII and graphically in fig. 17. The severity of attack was recorded after 5 weeks growth and graded as described in the previous Section. This is shown in Table XXVIIa.

The results indicate three main points. Firstly in all the series, the presence of an antagonist substantially reduced the percentage of infection although nowhere was it completely controlled. Secondly as expected, the highest level of control was obtained where soil extract and soil extract mannitol were used as the medium. This is most probably the result of better growth of the antagonist, but it must be noted that even in tap water, a considerable degree of control was obtained. Presumably the antagonists were able to make some growth in the seed diffusates but less than in the improved soil extract media. In tap water medium a reduction of between 40 and 50% was recorded, but in soil extract mannitol this was increased to nearly 75%. Thirdly, the severity of attack was reduced by the presence of the antagonists, but this was clearly shown only in the series containing soil extract or soil extract mannitol as medium. In the tap water series seedlings in the control set never exhibited severe infection so that itis not surprising that in this medium, no response to the presence of antagonists

Table XXVII.

The effect of two antagonistic bacteria, F.A.1. and F.A.6., on the percentage of infection of sweet pea seedlings in sand plus various media by strain 505 of *C. fascians*.

Antagonist	Medium	Period of growth								
		3 weeks			4 weeks			5 weeks		
		No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
F.A.1.	Tap water	18	6	33.3	18	8	44.4	18	9	50.0
	Soil extract	15	nil	nil	15	1	6.7	15	1	6.7
	Soil extract mannitol	17	1	5.9	17	2	11.8	17	4	23.5
F.A.6.	Tap water	15	6	40.0	15	7	46.7	15	7	46.7
	Soil extract	13	1	8.3	13	2	15.4	16	4	25.0
	Soil extract mannitol	19	2	10.5	19	4	21.0	19	5	26.3
Control	Tap water	17	11	64.7	17	13	76.5	17	15	88.2
	Soil extract	17	11	64.7	18	12	66.7	18	12	66.7
	Soil extract mannitol	19	19	100.0	19	19	100.0	19	19	100.0

Fig. 17 The effect of two antagonists on infection by strain 505 in sand culture

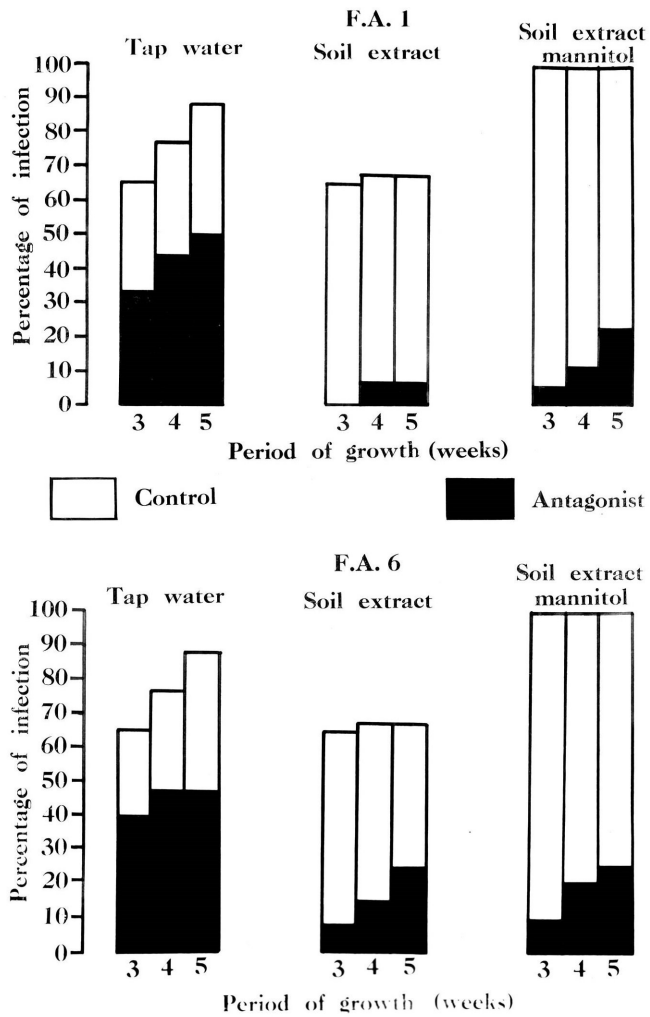


Table XXVIIa.

The effect of two antagonistic bacteria, F.A.1. and F.A.6. on the severity of infection of sweet pea seedlings in sand plus various media by strain 505 of C. fascians.

Antagonist	Medium	Severity of infection* after 5 weeks growth		
		A	B	C
F.A.1.	Tap water	nil	3	6
	Soil extract	nil	nil	1
	Soil extract mannitol	nil	1	3
F.A.6.	Tap water	1	4	2
	Soil extract	nil	nil	4
	Soil extract mannitol	nil	nil	5
Control	Tap water	nil	2	13
	Soil extract	4	3	5
	Soil extract mannitol	13	5	1

*Severity graded into three groups, A, B, and C, and the number of plants in each group recorded.

A. Seedlings exhibited multiple shoots not more than one inch in length and all had swollen bases.

B. Multiple shoot development without basal swelling. Shoots up to two inches in length.

C. Development of one normal shoot followed by further basal shoots. No basal swelling.

These groups are illustrated photographically in fig.12.

could be demonstrated.

This experiment was repeated using in addition, two other strains of the pathogen, namely 2WR and 503. The results are shown in Tables XXVIII, XXVIIIa, XXVIIIb, and XXVIIIc, and graphically in figs. 18 to 20 inclusive.

It will be seen that the results were substantially the same as those previously recorded with the exception that in four series, complete control of infection was obtained. In every such case, soil extract mannitol had been used as the medium, and in all cases where this medium was employed, the percentage of infection was very low, i.e. less than 7.0%. It would appear that both antagonists were about equally effective in the media used.

A subsequent replicate experiment gave substantially the same results as described above, complete control however being obtained in all the series using soil extract mannitol as medium.

The effect of other organisms on the pathogenicity of strain 505 of C. fascians was also examined. Two organisms, F.A.7., and B. subtilis, shown previously to be antagonistic to the pathogen in plate culture, and B. mesentericus which was not active under these conditions, were used. Soil extract mannitol only was employed as medium in order to reduce the amount of apparatus required for the experiment. The results are shown in Tables XXIX and XXIXa, and graphically

Table XXVIII.

The effect of two antagonistic bacteria, F.A.1. and F.A.6., on the percentage of infection of sweet pea seedlings in sand plus various media by strain 2WR of C. fascians.

Antagonist	Medium	Period of growth								
		3 weeks			4 weeks			5 weeks		
		No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage
F.A.1.	Tap water	14	2	14.3	17	3	17.6	17	3	17.6
	Soil extract	16	2	12.5	18	2	11.1	18	2	11.1
	Soil extract mannitol	16	nil	nil	16	nil	nil	16	nil	nil
F.A.6.	Tap water	17	4	23.5	19	4	21.1	19	4	21.1
	Soil extract	16	1	6.3	16	2	12.5	17	3	17.6
	Soil extract mannitol	16	nil	nil	16	1	6.3	16	1	6.3
Control	Tap water	12	3	25.0	12	6	50.0	14	6	42.9
	Soil extract	14	5	35.7	15	7	46.7	16	9	56.3
	Soil extract mannitol	11	6	54.5	11	7	63.6	11	7	63.6

Table XXVIIIa.

The effect of two antagonistic bacteria, F.A.1. and F.A.6., on the percentage of infection of sweet pea seedlings in sand plus various media by strain 503 of *C. fascians*.

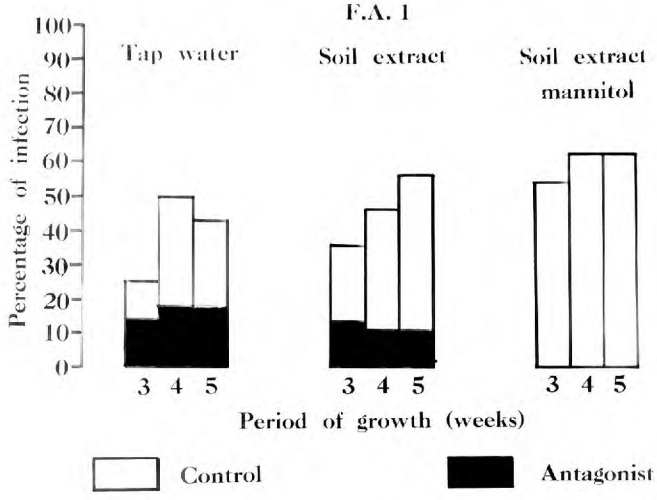
Antagonist	Medium	Period of growth								
		3 weeks			4 weeks			5 weeks		
		No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage
F.A.1.	Tap water	17	4	23.5	17	4	23.5	18	7	38.9
	Soil extract	17	2	11.8	18	3	16.7	18	6	33.3
	Soil extract mannitol	17	nil	nil	20	nil	nil	20	nil	nil
F.A.6.	Tap water	15	1	6.7	18	1	5.6	19	8	42.1
	Soil extract	15	1	6.7	15	2	13.3	15	4	23.7
	Soil extract mannitol	18	nil	nil	18	nil	nil	18	1	5.6
Control	Tap water	19	2	10.5	19	3	15.8	19	11	57.9
	Soil extract	18	15	83.3	18	15	83.3	18	15	83.3
	Soil extract mannitol	17	10	58.4	19	15	78.9	19	16	84.2

Table XXVIIIb.

The effect of two antagonistic bacteria, F.A.1. and F.A.6., on the percentage of infection of sweet pea seedlings in sand plus various media by strain 505 of C. fascians.

Antagonist	Medium	Period of growth								
		3 weeks			4 weeks			5 weeks		
		No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
F.A.1.	Tap water	19	4	21.0	19	7	36.8	19	11	57.9
	Soil extract	18	3	16.7	19	4	21.1	19	10	52.6
	Soil extract mannitol	18	nil	nil	18	nil	nil	18	nil	nil
F.A.6.	Tap water	18	1	5.6	19	5	26.3	19	6	31.6
	Soil extract	18	nil	nil	19	1	5.3	19	4	21.1
	Soil extract mannitol	16	nil	nil	19	nil	nil	19	nil	nil
Control	Tap water	19	10	52.6	19	17	89.5	20	19	95.0
	Soil extract	20	15	75.0	20	19	95.0	20	19	95.0
	Soil extract mannitol	19	12	63.2	20	18	90.0	20	19	95.0

Fig. 18 The effect of two antagonists on infection by strain 2 WR in sand culture



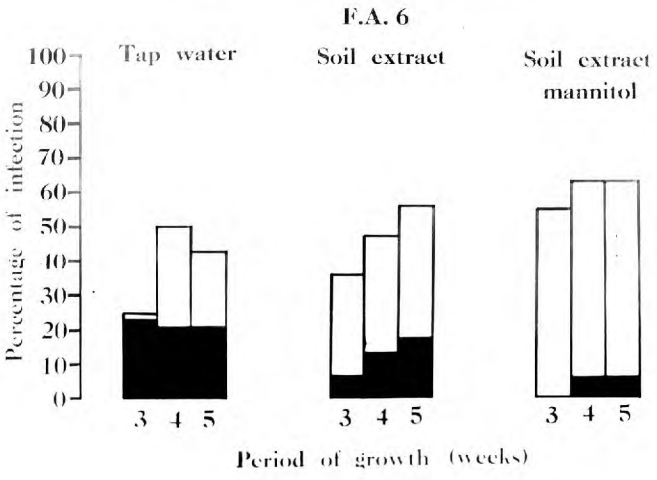


Fig. 19 The effect of two antagonists on infection by strain 503 in sand culture

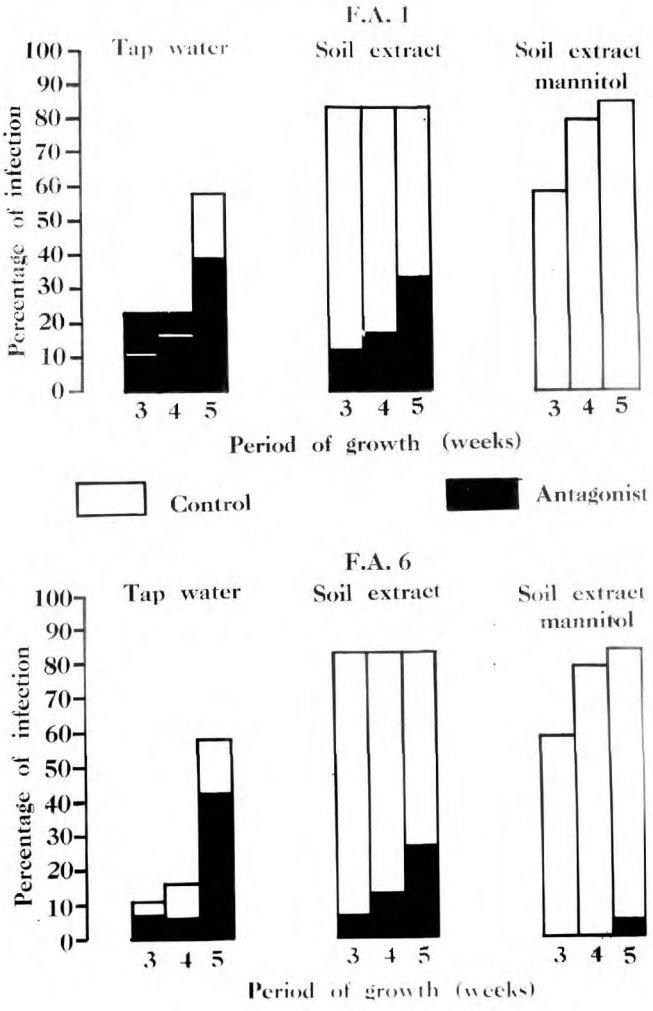
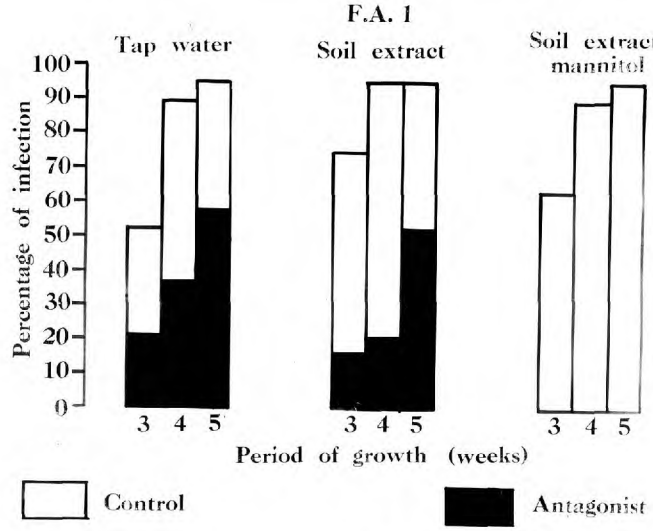


Fig. 20 The effect of two antagonists on infection by strain 505 in sand culture



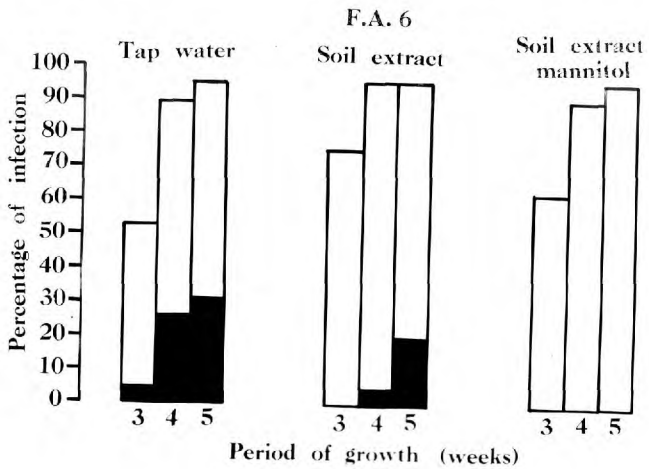


Table XXVIIIc.

The effect of two antagonistic bacteria, F.A.1. and F.A.6., on the severity of infection of sweet pea seedlings in sand plus various media by strains 2WR, 503 and 505 of *C. fascians*.

Antagonist	Medium	Severity of infection† after 5 weeks growth								
		2WR			503			505		
		A	B	C	A	B	C	A	B	C
F.A.1.	Tap water	nil	nil	3	2	1	4	1	1	9
	Soil extract	nil	1	1	nil	1	5	nil	nil	10
	Soil extract mannitol	nil	nil	nil	nil	nil	nil	nil	nil	nil
F.A.6.	Tap water	1	nil	3	nil	nil	8	nil	1	5
	Soil extract	nil	nil	3	nil	1	3	nil	nil	4
	Soil extract mannitol	nil	nil	1	nil	nil	1	nil	nil	nil
Control	Tap water	2	1	3	nil	2	9	2	4	13
	Soil extract	4	2	3	5	5	5	2	10	7
	Soil extract mannitol	4	2	1	5	nil	11	3	1	15

† See footnote to Table XXVIIa.

Table XXIX.

The effect of F.A.7., B. subtilis, and B. mesentericus on the percentage of infection of sweet pea seedlings in sand plus soil extract mannitol medium by strain 505 of C. fascians.

Antagonist	Period of growth								
	3 weeks			4 weeks			5 weeks		
	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage
F.A.7.	12	nil	nil	13	nil	nil	13	nil	nil
B. subtilis	16	1	6.3	19	5	26.3	19	5	26.3
B. mesentericus	17	12	70.6	20	20	100.0	20	20	100.0
Control	16	10	62.5	20	18	90.0	20	18	90.0

Fig. 21 The effect of various antagonists on infection by strain 505 in sand culture

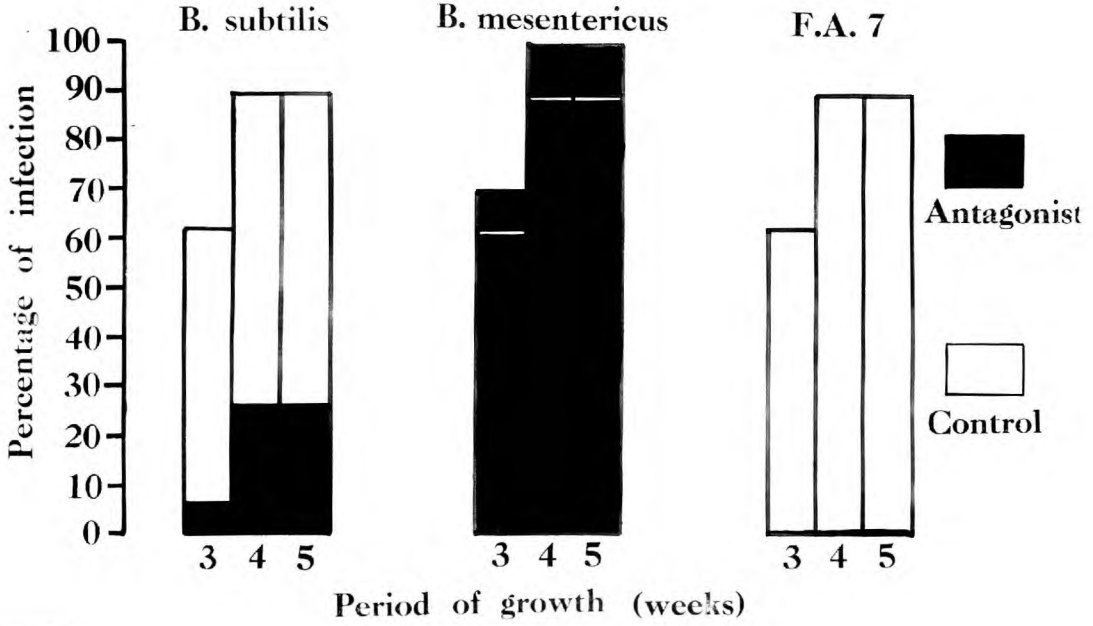


Table XXIXa.

The effect of F.A.7., B. subtilis, and B. mesentericus on the severity of infection of sweet pea seedlings in sand plus soil extract mannitol medium by strain 505 of C. fascians.

Antagonist	Severity of infection* after 5 weeks growth		
	A	B	C
F.A.7.	nil	nil	nil
B. subtilis	3	1	1
B. mesentericus	15	3	2
Control	14	4	nil

*See footnote to Table XXVIIa.

in fig. 21.

Complete control of infection was exhibited by F.A.7. and a high degree of control by B. subtilis. B. mesentericus was without effect on the pathogen, and in fact, this series exhibited a higher percentage of infection than that of the control one. This however cannot be considered of any significance as the increase was only 10%, representing infection of 2 extra seedlings in the experiment. There was no variation between the control series and this one in the severity of the attack. The experiment was not repeated.

This is a reversal of the effect recorded by Mohanty (1951) where some degree of control using B. mesentericus as the antagonist was obtained, while B. subtilis was ineffective. However as it is not known whether the strains employed in both sets of work were the same, no useful purpose is served in making direct comparisons. Strain variation could easily explain the variation observed.

4. Antagonism in sterile soil

The relationship between sand culture infections and those under natural conditions must be considered before the possible effect of antagonistic organisms in the control of the disease can be discussed. It is known that many antibiotics are strongly adsorbed on the colloidal clay particles in the soil, and it is necessary to consider

whether they remain active under these conditions. The work of Sinha (1950) already refered to, indicates that an adsorbed antibiotic has an effect on the soil population. Problems connected with the demonstration of antagonism in non-sterile soil include possible counter antagonism and breakdown of the antibiotic by other soil inhabitants, but under sterile conditions, several workers have demonstrated antagonistic activity. This was therefore investigated in connection with infection by C. fascians.

Soil, a loam collected from Slough Research Station, with the addition of 1% mannitol as an energy source, was brought to a moist crumb condition by the addition of tap water. 6 in. by 1 in. boiling tubes were employed for culture of the seedlings in place of the specimen tubes used in the sand culture work, as the former could be autoclaved with safety. About one inch of soil was placed in each one of the tubes which were plugged and then sterilised at 20 lb. pressure for one hour. They were then allowed to stand for 5 days before use.

Sufficient sterile tap water was added to each tube to bring the soil back to a moist crumb condition, the remaining operations being similar to those described in the general method. Four antagonistic organisms were employed, plus a control series of strain 505 of C. fascians only. Each series consisted of 20 tubes making a total of

100 for the whole experiment.

The growth of the seedlings in soil was found to be slower than in sand culture, so much so that the stage of development reached after 4 weeks corresponded with that attained by 3 week old seedlings in the sand culture work. The percentage of infection was therefore recorded after 4, 5, and 6 weeks growth, and the severity of attack after 6 weeks so as to make the results more comparable to those already obtained. The results are shown in Tables XXX and XXXa and graphically in fig. 22.

After 6 weeks growth, all series containing an antagonist exhibited a lower percentage of infection than the control series, and in the case of all except the F.A.7. series, some degree of control of infection was evident throughout the period when results were taken. The series containing F.A.7. however exhibited a percentage of infection identical with that of the control after 4 weeks and it was only after further growth that a divergence occurred. In the control series further infection took place, but this was apparently arrested where F.A.7. was present. The highest degree of control was given by F.A.6., the rest being similar but much less effective.

The experiment was repeated with substantially the same result but with one exception. F.A.7. was without effect, the percentage of infection for this and the control

Table XXX.

The effect of various antagonistic bacteria on the percentage of infection of sweet pea seedlings in sterile soil plus 1% mannitol medium by strain 505 of *C. fascians*.

Antagonist	Period of growth								
	4 weeks			5 weeks			6 weeks		
	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage	No. of plants	Plants fasciated number	percentage
F.A.1.	14	7	50.0	16	10	62.5	16	10	62.5
F.A.6.	16	6	37.5	18	6	33.3	18	6	33.3
F.A.7.	17	11	64.7	17	11	64.7	17	11	64.7
B. subtilis	15	2	13.3	17	10	58.8	17	11	64.7
Control	17	11	64.7	18	15	83.3	18	15	83.3

Fig. 22 The effect of antagonists on infection by strain 505 in sterile soil plus 1% mannitol

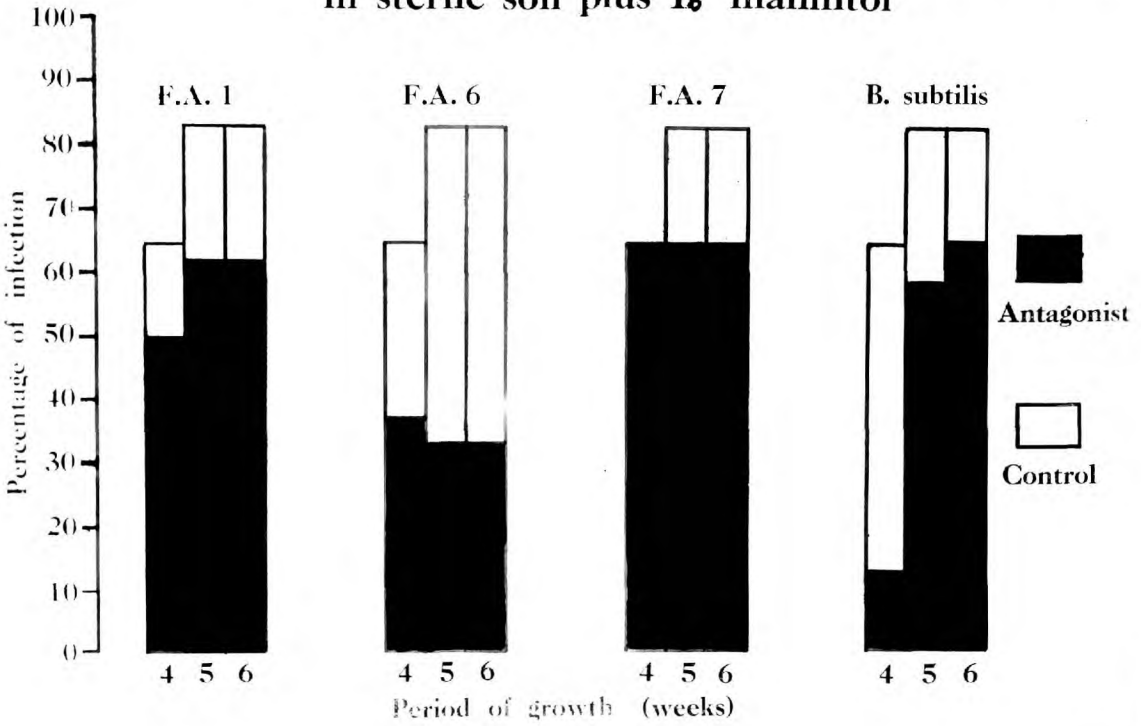


Table XXXa.

The effect of various antagonistic bacteria on the severity of infection of sweet pea seedlings in sterile soil plus 1% mannitol medium by strain 505 of *C. fascians*.

Antagonist	Severity of infection* after 6 weeks growth		
	A	B	C
F.A.1.	5	3	2
F.A.6.	3	0	3
F.A.7.	6	2	3
B. subtilis	1	1	9
Control	8	1	6

*See footnote to Table XXVIIa.

series being identical throughout. It would appear doubtful therefore if, in view of the inconclusive result previously obtained, F.A.7. can be ascribed any antagonistic action. It is possible that the organism is unable to produce its antibiotic under these conditions. In plate culture, this organism failed to grow on soil extract mannitol medium but was stimulated to grow by the diffusates from sweet pea seeds in a similar manner to the two antagonists F.A.1. and F.A.6.

In the previous sand culture work the severity of infection was always reduced by the presence of an antagonist. In these experiments in sterile soil however, with the exception of the B. subtilis series, no effect on the severity of attack could be demonstrated. The B. subtilis series however exhbited a marked reduction in the severity, so much that only about 20% of the infected seedlings could be classified in the two most severe classes as against 60% in the control.

Many workers have found that the addition of an energy source essential to the demonstration of antagonism in sterile soil. In some cases added carbohydrate may be necessary for growth of the antagonist, but in others it is possible that while the organism is able to make growth it is unable to produce its antibiotic, at least in any quantity, unless an additional energy source is provided.

This is important when antagonism under natural conditions is considered and the point will be further discussed later.

The previous experiment was therefore repeated omitting the addition of 1% mannitol to the soil. The method was otherwise identical in all respects to that of the previous experiment, and the results are shown in Tables XXXI and XXXIa, and graphically in fig. 23.

After 6 weeks growth of the seedlings the results closely followed the pattern of those in sterile soil with an energy source. Again F.A.7. was more or less ineffective, with F.A.1. also exhibiting a low degree of activity. F.A.6. was, as before, the most active, effecting a reduction of over 50% in the percentage of infection of the pathogen. B. subtilis was moderately effective exhibiting its most pronounced effect during the early stages, i.e. after 4 weeks growth its antagonistic activity was pronounced but this partially faded as growth of the seedlings continued. This pattern of antagonism was also seen in the previous experiment in the series containing this antagonist. It is possible that the antibiotic produced by B. subtilis was only bacteriostatic and active for a short period only. After growth of the antagonist ceased due to lack of sufficient nutritive substances, C. fascians was apparently able to attack the plants.

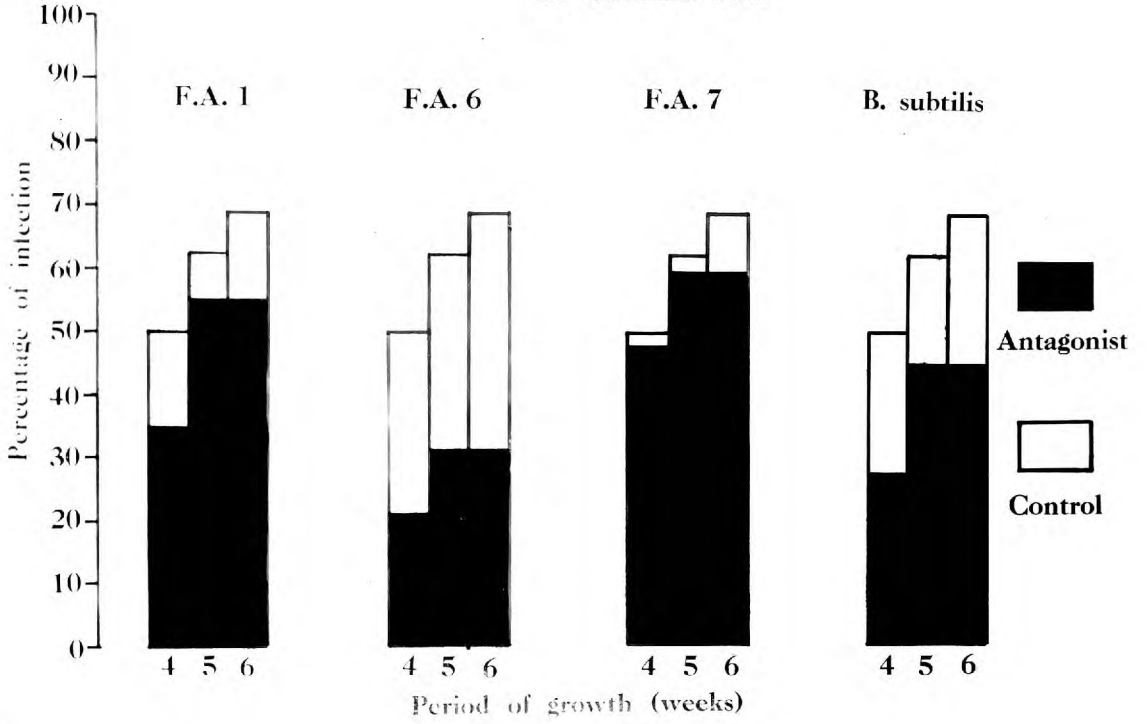
A contrast to the results obtained in the previous

Table XXXI.

The effect of various antagonistic bacteria on the percentage of infection of sweet pea seedlings in sterile soil by strain 505 of *C. fascians*.

Antagonist	Period of growth								
	4 weeks			5 weeks			6 weeks		
	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
F.A.1.	20	7	35.0	20	11	55.0	20	11	55.0
F.A.6.	14	3	21.4	16	5	31.3	16	5	31.3
F.A.7.	19	9	47.4	19	11	59.9	19	11	59.9
B. subtilis	18	5	27.8	18	8	44.4	18	8	44.4
Control	14	7	50.0	16	10	62.5	16	11	68.8

Fig. 23 The effect of antagonists on infection by strain 505 in sterile soil



experiment was afforded by the severity of attack recorded. In all cases including the control, the seedlings were easily classified into the least severe group, i.e. type C. As mentioned previously, from work by Lacey (1936) it seems that early attack is necessary for a severe form of infection to take place. It is likely therefore that the effect of the mannitol in the previous experiment was to encourage more rapid growth of the pathogen and consequently a greater chance of an early attack. Conversely, where the mannitol was absent infection took place during a late stage of germination of the seedlings resulting in a mild form of infection.

5. Antagonism in non-sterile soil.

As discussed previously, under natural soil conditions the picture is very complex, factors operating against both the pathogen and the antagonist, and due to this it is extremely difficult to demonstrate the operation of one particular factor. However the work would not have been complete without an attempt being made to demonstrate antagonism under something approaching natural conditions and the following experiments were therefore undertaken.

Two parallel experiments were set up, one in the laboratory, growth of the seedlings being carried out in 3 by 1 inch specimen tubes as described in the general

method, and the second in an unheated greenhouse.

In the former, 100 plugged tubes were dry sterilised and about a half inch of moist soil placed in each. Sweet pea seeds were surface sterilised, placed in the tubes and inoculated as previously described. It should be noted that the soil used, Slough top soil loam, had been kept in the laboratory in a dry condition for about 10 months and its viable count was low. This was used to reduce the chance of antagonism between the natural soil microflora and the pathogen, which could easily mask any effect exhibited by the added antagonists. The same four antagonists were used as in the previous experiment using sterile soil, 20 tubes being allotted to each with a control series of a similar number.

This work was duplicated using 3 inch pots in place of the plugged tubes. The pots were washed out but not sterilised. Tap water suspensions of the organisms were prepared as described in the general method, and the appropriate one used to moisten the soil for each particular series. Ten untreated sweet pea seeds were placed in each pot, the seeds being pushed about a half inch deep into the soil. The pots were covered with sheets of glass and brown paper to retain moisture, and placed on the greenhouse staging. Watering was carried out as required, and the paper removed as soon as the seedlings appeared above the soil.

The temperature fluctuations were great under these conditions. The work was done in the early spring when the greenhouse temperature frequently reached 80°F during the day dropping to 40°F. or lower at night.

In both experiments the results were recorded after 4, 5, and 6 weeks growth, although the seedlings in the greenhouse experiment were not in such an advanced stage as those grown in tubes in the laboratory. The results for the laboratory work are shown in Table XXXII.

In the greenhouse work only a few isolated seedlings exhibited fasciation after 6 weeks growth and these were indescriminately distributed throughout the series. The experiment was continued for 10 weeks but no further infection appeared. It must be remembered when considering the failure of the control series that comparison with the laboratory work is difficult, so many extra factors being involved. For example, no sterilisation of the seeds or pots was undertaken, and therefore contamination from these may have introduced antagonistic organisms. The seed itself was germinating under different conditions, in the laboratory work it rested on the surface of the soil, but in the pots it was buried. In addition the temperature fluctuations may have exerted an adverse effect.

In the laboratory experiment also trouble was experienced, a low percentage of infection in the control

Table XXXII.

The effect of various antagonistic bacteria on the percentage of infection of sweet pea seedlings in soil by strain 505 of C. fascians.

Antagonist	Period of growth								
	4 weeks			5 weeks			6 weeks		
	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
F.A.1.	10	3	30.0	10	4	40.0	10	4	40.0
F.A.6.	12	3	25.0	16	3	18.8	16	3	18.8
F.A.7.	13	4	30.8	13	4	30.8	13	4	30.8
B. subtilis	9	nil	nil	9	1	11.1	9	1	11.1
Control	13	1	7.7	13	1	7.7	13	3	23.1

series making the results inconclusive. However some slight indication of antagonism was shown by B. subtilis, the remaining antagonists appearing to be ineffective.

This experiment was repeated twice with larger numbers of replicate tubes in each series, but in each case the percentage of infection in the control series never exceeded 30%. In both, B. subtilis reduced the percentage of infection by more than 50%, while the remaining antagonists were again relatively ineffective.

It is obvious that these results give no proof of antagonistic activity. The low percentage of infection in the control series combined withthe relatively small number of replicate tubes (never more than 40 per series) makes the result nothing more than an indication of possible antagonistic activity. It was considered that selection of a soil with an even lower number of naturally occurring antagonists, combined with a much larger number of replicates, might yield some more definite results, but owing to restriction of time, this could not be attempted.

6. Axillary bud infections.

During the isolation of C. fascians from infected shoots, considerable trouble is often experienced. The cause of failure to isolate the pathogen is attributed to the presence of large numbers of antagonistic organisms on the

isolation plates, which owing to the slow growth of C. fascians often completely prevents its appearence. F.A.1. and F.A.6. were obtained from such plates. It was therefore decided to examine the four antagonists used in the previous work for possible antagonistic properties against two strains of C. fascians, namely 503 and 505, in axillary bud inoculations.

Sweet pea seedlings were grown under sterile sand culture conditions in 6 by 1 inch specimen tubes as previously described. At the end of about 4 weeks the majority had reached the level of the cotton wool plugs and had at least two well developed leaves. In most cases two axillary buds were inoculated on each plant, but in a few cases owing to slower germination of the seed this had to be reduced to one per plant. In every case 10 tubes were allotted/ to each combination of antagonist and pathogen, giving normally 20 inoculated buds.

The method of inoculation of the axillary buds was a modification of that used by Mohanty (1950). Suspensions of the organisms were prepared in tap water and the required mixtures of pathogen and antagonist made as described in the general method. Sterile absorbent cotton wool was soaked in these suspensions for a few seconds and small pads of this made to lodge in the axils of leaves. As far as possible the cotton woll was also wrapped round the main stem of the seedling to assist retention of the pad

in position and reduce loss of moisture.

The cotton wool was kept moist by the daily addition of sterile tap water, and the moisture content of the sand maintained by addition of tap water as required. The number of buds stimulated in each series was recorded 16 days after inoculation, and the results are shown in Table XXXIII. Buds not showing stimulation were examined again after 24 days but no further development had taken place.

It will be seen that all the antagonistic bacteria with the exception of B. subtilis exhibited considerable control of infection, F.A.1. being the most effective against both strains of the pathogen. It is likely that the failure of B. subtilis to antagonise was due to its inability to grow under these conditions where the only available nutrients were those diffusing from the plant.

It is important to note that the percentage of infection in the the control series for strain 503, was higher than that obtained under normal conditions in seed inoculations. Mohanty (1950) regularly recorded 100% infection under these conditions, but this was not obtained in either control series, although it did occur in the series containing strain 505 of the pathogen and B. subtilis.

7. Adsorption of antibiotics.

In view of the evidence by other workers of the adsorption of many antibiotics on soil particles, and the

Table XXXIII.

The effect of various antagonistic bacteria on the percentage of infection of axillary buds of sweet pea seedlings by strains 503 and 505 of C. fascians.

Strain number	Antagonist	No. of axillary buds inoculated	Buds stimulated after 16 days	
			number	percentage
503	F.A.1.	20	2	10.0
	F.A.6.	18	6	33.3
	F.A.7.	19	4	44.4
	B. subtilis	19	18	94.7
	Control	20	19	95.0
505	F.A.1.	16	nil	nil
	F.A.6.	18	6	33.3
	F.A.7.	18	9	50.0
	B. subtilis	20	20	100.0
	Control	18	15	83.3

suggestion of a positive antagonism under sterile soil conditions obtained in the preceeding experiments, it was decided to determine if the active substance produced by two of the antagonists used, namely F.A.1. and F.A.6., were adsorbed. In addition the active cold water extract of the french bean seed was also included as an indicator of the reaction of seed coat antibiotics to soil conditions.

Cultures of F.A.1. and F.A.6. were grown in a medium consisting of soil extract mannitol to which decomposed seed extract (for preparatory details see Section IIIb) was added in the ration of 10 parts soil extract to one of decomposed seed extract. This was placed in flat bottles, about 20 ml. being used in each, and heavily inoculated with the antagonist. After incubation for 36 hours at 30°C they were centrifuged to remove the bulk of the sells.

Kaolin and bentonite were used as adsorbing agents, 0.1 gm. of the material being added to 5 ml. samples of the culture filtrates. These were shaken up and allowed to stand for half an hour before being high speed centrifuged until the supernatants were visibly clear. They were then sterilised by passage through grade 5 sintered glass filters.

The bean seed antibiotic was prepared by soaking 30 franch bean seeds in 50 ml. of sterile distilled water for 24 hours at laboratory temperature. The liquid was

subsequently treated with kaolin and bentonite and sterilised as described above.

These six treated extracts with the addition of controls of untreated ones were tested by the cup plate method for inhibition of strain 505 of C. fascians in soil extract mannitol agar medium. The diameter of the zones of inhibition round th e cups were recorded after 4 days incubation at 25°C and are shown in Table XXXIV.

Adsorption of the active substances in the filtrates of F.A.1. and F.A.6. was complete with either kaolin or bentonite as the absorbent. In the case of the french bean seed antibiotic, this was not so, although its activity was reduced by both adsorbents. It would appear therefore that if antagonism is assumed to have been demonstrated in soil, the antibiotics responsible must have remained active against the pathogen in the adsorbed state.

Table XXXIV.

The adsorption of antibiotics contained in the culture filtrates of F.A.1. and F.A.6., and in the cold water extract of french bean seeds, by kaolin and bentonite.

Antibiotic	Inhibition zones* against strain 505 of C. fascians on soil extract mannitol agar medium after treatment with the adsorbant.		
	Kaolin	Bentonite	Control
Culture filtrate of F.A.1.	nil	nil	11.0
Culture filtrate of F.A.6.	nil	nil	10.5
Extract of bean seeds	9.0	11.5	15.0

* Diameter in millimetres. Diameter of cup 8.0 mm. Results recorded after 4 days incubation at 25°C.

IVb. THE PERSISTANCE OF C. FASCIANS IN SOIL.

1. Selective media for C. fascians.

In view of the results obtained in the previous Section, it was considered desirable to make a short investigation into the survival of C. fascians in both sterile and non-sterile soil. Such an examination is simple in sterile soil, but samples from non-sterile soil, as has previously been discussed, frequently fail to show the presence of the pathogen, although infected plants are growing in it. This has been attributed to the presence of antagonists on the isolation plates which prevent growth of the pathogen. Before therefore an attempt was made on this problem, it was decided to re-examine the possibility of using a selective agent in the medium used for the plate counts to reduce the number of contaminating organisms. The results of Mohanty (1947) were not promising in this direction, only potassium dichromate showing any effect, but the work was repeated and in addition the same selective agent was employed in liquid medium as a simple means of detecting the presence of the pathogen where only small numbers are present.

(a). Potassium dichromate as a selective agent.

Two series of test tubes were set up each containing 10 ml. of the selective medium. The concentrations of potassium dichromate were obtained by adding 1 ml. of the

appropriate concentration of the substance to 9 ml. of nutrient broth or soil extract mannitol, after autoclaving. The final concentrations of dichromate used ranged from 1:1000 to 1:100,000. Three replicate tubes were used for each concentration. Each tube was inoculated with two drops of a suspension of the organism in tap water, one series with strain 505 of C. fascians and the other with a suspension of soil organisms. The tubes were incubated in an almost horizontal position at 25°C and examined daily for signs of growth. The results were recorded after 5 days for the nutrient broth series and 8 days for soil extract mannitol ones. These are shown in Table XXXV. The growth has been estimated using that made in the medium without the selective agent as the control. This was given five plus signs and the rest graded accordingly.

It was obvious that at the higher concentrations where the soil organisms were completely inhibited, C. fascians was also either unable to grow at all or was strongly inhibited. In nutrient broth there was very little difference in the rate of growth of the two, no growth occurred at 1;1000 but both made very slow growth at 1;5000. The growth of the pathogen appeared better than that of the soil organisms at concentrations of 1:10,000 and 1:20,000 but it is doubtful if this would help much in detection of the pathogen.

In soil extract mannitol the picture was more promising. At concentrations of 1:5000 and 1:10,000 the

The growth of C. fascians and soil organisms in the presence of various concentrations of potassium dichromate in nutrient broth and soil extract mannitol media.

Conc. of pot. dichromate	Growth† after incubation at 25°C in			
	Nutrient broth		Soil extr. mannitol	
	C. fascians	Soil orgs.	C. fascians	Soil orgs.
1:1000	-	-	-	-
1:5000	(+)	(+)	+	-
1:10,000	++	++	++	+
1:20,000	++(+)	++	+++	++
1:30,000	+++	+++	+++	+++
1:50,000	+++	++++	++++	+++++
1:75,000	+++	+++++	+++++	+++++
1:100.000	+++++	+++++	+++++	+++++
Control	+++++	+++++	+++++	+++++

† Growth graded from (+), very feeble to +++++, heavy (degree of growth made on medium without addition of dichromate).

soil organisms were strongly inhibited while considerable growth was made by C. fascians. At higher dilutions there was again no difference in the two series.

It is possible therefore that potassium dichromate might be used as a selective agent for isolation of the pathogen but since at such concentrations it is obviously inhibitory to it, it would be of little use as a medium for estimating the number of cells in soil, as a large number would probably be unable to develope at all. For the same reason the detection of very small numbers would be unreliable.

The experiment was repeated in agar media in plates with the same concentrations of dichromate. The results taken afeter 5 and 8 days were similar to those obtained by Mohanty (1947) where it was shown that while some inhibition of the soil micro-flora was obtained at a concentration of 1:20,000, C. fascians was apparently able to grow normally. Counts made on this selective medium were however much lower than those on nutrient agar and so the method would not be of great value for the estimation of populations.

(b). Potassium tellurate with Potassium dichromate.

Crosse and Pitcher (1952) successfully used a medium containing potassium dichromate and potassium tellurate for the isolation of C. fascians from plant material. The composition of the medium was Difco nutrient agar plus 5% sucrose and 0.02% potassium tellurate. Before pouring the medium, sufficient potassium dichromate solution was

added to give a final concentration of 0.0033%.

The medium was examined first to see if it could be used for the detection of the pathogen in soil when there in small numbers only in relation the that of other organisms, and secondly to determine the difference between counts on the medium and those on nutrient agar.

20 plates of the medium seeded with C. fascians and soil organisms were poured, and then incubated for 10 days at 25°C. A number of colonies appeared and all showing reduction of the tellurate were subcultured on to nutrient agar for identification. The plates showed no fungi and no spreading organisms and from subsequent identification it appeared that the organisms present were confined to three types including C. fascians as one of them.

In the next experiment counts were made on the selective medium and on nutrient agar froma standardised suspension of the pathogen. 4 replicate sets of 5 plates were used for each, and the counts taken after 10 days for the selective medium and after 5 days for the nutrient agar, and compared.

The counts on the selective medium were found to be only 2% of those on nutrient agar although this figure may not be accurate as the number of colonies appearing on the plates of the selective medium was small due to the use of too high a dilution. It was obvious however that a very large proportion of the organisms had been unable to grow on the medium and so it would be unsuitable for the estimation of a population.

In addition, its use involves a considerable amount of work as mentioned previously, because other organisms in the soil are able to reduce the tellurate and it is necessary to streak all colonies showing this reduction on to a further nutritive medium for identification of the pathogen. The use of this medium for future work was therefore abandoned.

2. Disappearence from sterile soil.

Three strains of the pathogen, namely 2WR, 503 and 505 were examined to determine the viability of the organisms after incubation in soil for varying periods.

Six 150 ml. flasks each containing 25 grams of soil which had been brought to a moist crumb condition by the addition of tap water, were sterilised in the autoclave at 20 lb. pressure for 1 hour. They were then allowed to stand for 5 days before use, when the moisture content was again adjusted by the addition of sterile tap water.

Two flasks were used for each strain. The inoculum consisted of a suspension of the pathogen in tap water, the dilution being so arranged as to give initial counts of a reasonable order, i.e. around 100×10^6. Care was taken when inoculating the soil not to over moisten it and so prevent aeration. The flasks were plugged with cotton wool and these were covered with metal foil to reduce evaporation. They were incubated at 25 C.

Initial counts were made from the inoculated soils on to three media, nutrient agar, soil extract mannitol, and Thornton's medium. Samples were taken from both flasks of each strain and mixed. From this 5 grams was weighed out for preparation of the suspension. All dilutions were done in tap water, and 5 replicate plates used for each medium and strain. These were incubated at 25°C for 7 days and then counted.

Counts were subsequently taken after 3 and 10 days incubation of the soil in a similar manner. The results are shown in Table XXXVI.

Two points are worth consideration. First all strains rapidly died out under these conditions. After 10 days few viable cells could be detected in any of the flasks. Secondly, strain 505 which is the most active pathogen, was more resistant. One flask with this strain which was further examined after 25 days still had a few viable cells in it while one of 2WR showed none.

The reason for the rapid death of the cells is not known. However the process of sterilisation by autoclaving is known to cause many chemical and physical changes to take place, and it was assumed that such was the case here. It is generally agreed however that the toxic properties of autoclaved soil are largely lost by allowing it to stand, after treatment, before inoculation, and this was, as mentioned previously, carried out.

Table XXXVI.

Counts of viable cells from soil inoculated with three strains of *C. fascians* before and after incubation at 25°C.

Strain	Nutrient agar			Soil extract mannitol			Thornton's medium		
	Initial count $\times 10^3$	Count after days at 25°C. $\times 10^3$		Initial count $\times 10^3$	Count after days at 25°C $\times 10^3$		Initial count	Count after days at 25°C $\times 10^3$	
		3	10		3	10	$\times 10^3$	3	10
2WR	42000	<100	<1	54300	100	< 1	45000	<100	<1
503	44300	<100	<1	34900	<100	< 1	15700	<100	< 1
505	171000	21700	2	185300	39700	3	172600	15700	6

Some effort was made to detect the presence of toxic substances in the soil first by placing sterilised soil on or in an agar plate seeded with the pathogen and later by the preparation of aqueous extracts, but these were all unsuccessfull. Further work is obviously needed and possibly the use of other solvents may be more effective in the extraction of a toxic substance.

It is reasonable however to consider that the rapid death of the pathogen in sterile soil may have been the reason for the low pathogenicity obtained in the controls of the infection work in this medium. With this in mind, it was decided to carry the work a stage further and examine the influence of the seed on the persistance of the pathogen in soil.

3. Influence of the seed on persistance of the pathogen and F.A.1.

Two flasks of soil were sterilised by autoclaving as previously described and allowed to stand for 5 days. Half inch internal diameter glass tubing was cut into 6 inch lengths, the ends covered with Oxoid metal test tube caps, and dry sterilised. The soil was inoculated with either strain 505 of C. fascians or F.A.1., and then placed in the tubes under aseptic conditions. The soil was packed down

with the aid of a glass ram rod made to fit the tubes, and in the centre of the soil column, as it was being packed in, was placed one seed of sweet pea which had been surface sterilised in chloramine T.

Non-sterile soil inoculated with C. fascians was treated similarly in a third set of tubes, all of which were incubated in moist chambers at 25°C.

Initial counts were made on the soils as described in th e previous experiment. At the end of 10 days the tubes were taken out and with sterile glass rods the soil was pushed out as a core which remained intact. This was then cut so that the area round the seed (a quarter of an inch either side of the seed) was separated from the rest and this was considered the zone of influence of the seed. A further sample was taken of the general soil at least one and a half inches from the seed zone. Counts were then made on the two samples as previously described. The results are shown in Table XXXVII and diagrammatically in fig. 24.

In the case of the non-sterile soil tubes, the number of colonies of C.fascians was counted separate from that of the general soil flora, the differentiation of the pathogen being made entirely on colony appearence. It will be seen that the initial counts on this soil were very low. This was due to the fact that the soil had been stored in a dry condition in the laboratory for a considerable period

Table XXXVII.

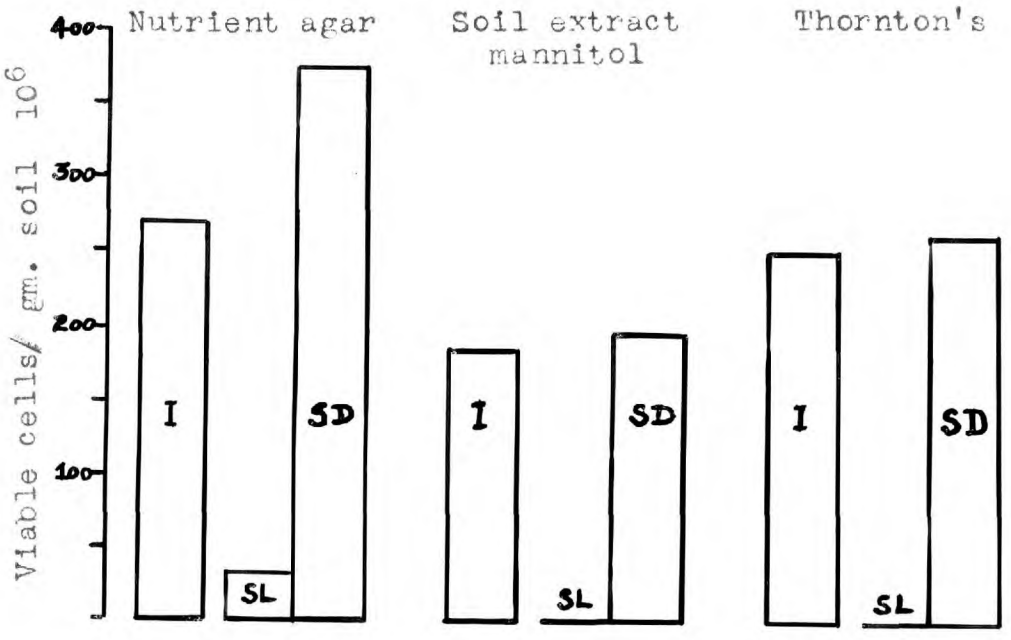
The influence of the sweet pea seed on the persistance of C. fascians and F.A.1. in sterile and non-sterile soil.

Inoculum and medium	Sample †	Nutrient agar		Soil extract mannitol		Thornton's medium	
		Initial count $\times 10^6$	Count after 10 days at 25°C. $\times 10^6$	Initial count $\times 10^6$	Count after 10 days at 25°C. $\times 10^6$	Initial count $\times 10^6$	Count after 10 days at 25°C. $\times 10^6$
C. fascians sterile soil	G	268	34	189	<1	246	<1
	S	268	373	189	194	246	250
F.A.1. sterile soil	G	19	<1	-	-	11	<1
	S	19	<1	-	-	11	<1
C.fascians Non-sterile soil *	G	153	<10	148	70	162	50
	S	153	300	148	170	162	190
Soil organisms	G	22	750	2	1510	3	900
	S	22	410	2	780	3	690

† G, general soil zone; S, zone of influence of the seed.

* Non-sterile soil inoculated with a suspension of C. fascians.

Fig. 24. The influence of the sweet pea seed on the persistance of C. fascians in soil.



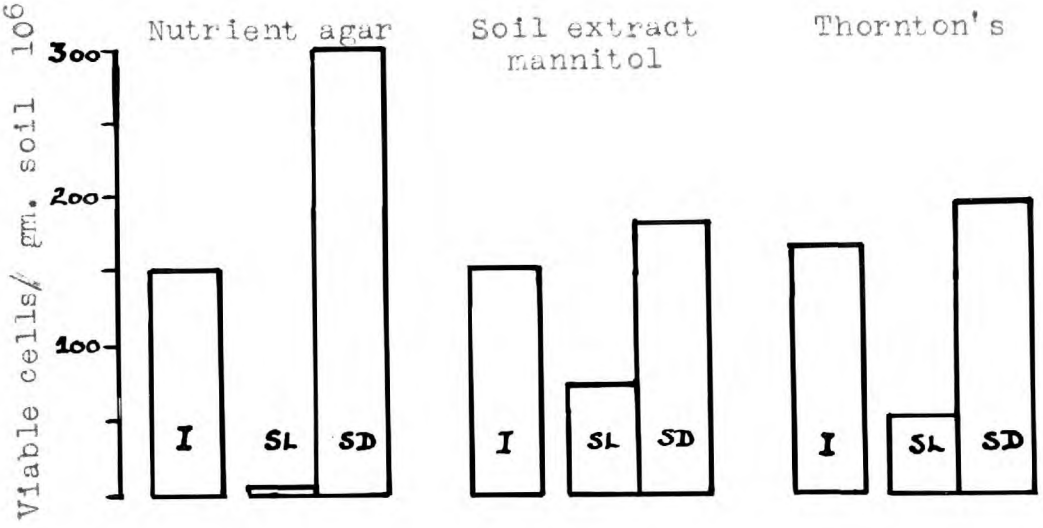
Legend:

I, initial count of viable cells before incubation.
SL, count of organisms in the general soil zone after incubation for 10 days.
SD, count of organisms in the zone of influence of the seed after incubation for 10 days.

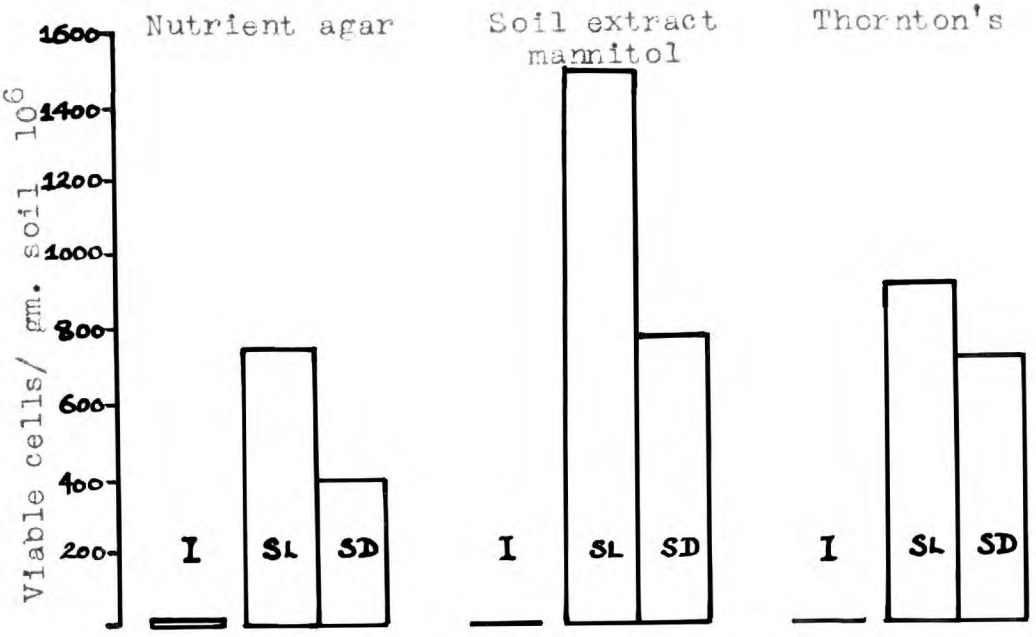
Fig. 24 (contd.).

Non-sterile soil

C. fascians



Other soil organisms.



and a large proportion of the microflora had died out. This was used, as it was considered less likely to interfere with the detection of the pathogen than a soil rich in possible antagonistic organisms.

From the results it can be seen that the effect of the seed in both sterile and non-sterile soil was to enable the pathogen not only to survive but to increase in numbers, while in the general soil zone there was a similar dying out to that shown in the previous experiment. The antagonist F.A.1. however, on the other hand was not influenced bythe seed and rapidly died out in both zones. It must also be noted that the number of other organisms developing in the zone of influence of the seed in non-sterile soil was less than that in the general soil, although the results obtained must be considered only as an indication and need further confirmation. If this is so however, it appears that the sweet pea seed has selective stimulatory powers towards C. fascians, and this may be in part at least the reason why this plant is so easily infected even in non- sterile soil.

From these two experiments it does appear that the seed plays an important part in the control of the microflora which is able to grow around it, and must therefore be considered in the same wayas the rhizosphere of the root, which is known to have its own microflora.

To determine the part this plays in the control of infection however requires a considerable amount of further work and this was not attempted.

V. THE COMBINED EFFECT OF STIMULATORY AND ANTAGONISTIC FACTORS ON INFECTION BY C. FASCIANS.

To complete the investigation it was decided to examine the effect of the addition of the extract of decomposed seeds on the antagonistic power of two organisms F.A.1. and F.A.6. in sand culture infection. Some effect on infection has been shown by both these factors and the experiment to be described was carried out to give some indication whether stimulation could be regarded as important in view of the apparently more powerful effect exerted by antagonistic bacteria.

160 sand culture tubes were prepared as described in Section IVa. The sand was saturated with soil extract mannitol and one surface sterilised seed placed in each tube. The sweet pea was used as test seed. The tubes were divided into sets of 20 each and inoculated as follows. Set one had strain 505 of C. fascians only. Set two had the pathogen plus F.A.1.; set three the pathogen with decomposed seed extract as described in Section IIIb, and the final set the pathogen plus both the other factors. The remaining four sets of tubes were inoculated similarly using F.A.6. as the antagonist.

The results were recorded after 3, 4, and 5 weeks as previously described and are shown in Table XXXVIII.

Table XXXVIII.

The combined effect of antagonism and stimulation on infection of sweet pea seedlings by strain 505 of C. fascians in sand plus soil extract mannitol medium.

Antagonist	Treatment	Period of growth								
		3 weeks			4 weeks			5 weeks		
		No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage	No. of plants	Plants fasciated number	Plants fasciated percentage
F.A.1.	Antagonist	16	1	6.3	16	1	6.3	18	2	11.1
	Extract	14	13	92.9	15	14	93.3	16	16	100.0
	Antagonist & extract	15	6	40.0	15	6	40.0	15	6	40.0
	Control	16	10	62.5	19	17	89.5	19	18	94.7
F.A.6.	Antagonist	14	0	nil	16	nil	nil	16	nil	nil
	Extract	15	13	86.6	17	16	94.1	17	16	94.1
	Antagonist & extract	12	2	16.7	14	3	21.4	15	3	20.0
	Control	14	10	71.4	17	14	82.4	18	16	88.9

The antagonistic effect of both F.A.1. and F.A.6. was apparently affected by the presence of the stimulatory extract, a higher percentage of infection being recorded in both series containing the two factors than in those with either antagonist alone. This was however still less than the control series and those containing the stimulatory extract only. The latter as previously shown, increased the percentage of infection over that of the control and similarly, the antagonists were highly effective, reducing the infection to nearly zero.

It would appear from these results that the presence of the stimulatory extract was favourable to C. fascians to a higher degree than it was to the antagonists although it has previously been shown that all the antagonists used were heavily stimulated by the extract.in plate culture. It does seem possible therefore that stimulation as a factor in infection of plants by this pathogen may play an important part under certain conditions, although it must be remembered that the experiment above used a very powerful stimulatory extract which may rarely be present under natural conditions.

The severity of attack was not apparently influenced by the extract where the antagonists were present as all were in the least severe group (type C) as were those

where only the antagonists were present. In contrast, where the extract only was used, the majority fell into the most severely infected group (type A).

VI. DISCUSSION.

Considering the data obtained, it appears that two sets of factors operate in infection by C. fascians, one inhibitory and the other stimulatory, and it is the combined effect of the two that controls the pathogenicity of the organism. The two sets of factors vary in their degree of activity, and in some cases only one may be operative.

The presence of an inhibitor in the coats of some seeds has been demonstrated and the possible effect they have on the control of infection has been investigated, but it must be remembered that under natural conditions the effectiveness of such inhibitors would depend to a very large extent on the conditions in the soil. Rapid leaching of the soil during wet periods might remove the inhibitor quickly, and the protection afforded the plant would be lost. Seeds containing only a small quantity of the inhibitor would particularly easily become susceptible to attack and the infectivity would rise. The adsorption of these antibiotic substances must however be considered, as it has already been shown that they are likely to be strongly adsorbed on the soil particles. That antibiotics remain active under these conditions is now fairly well established, and it does therefore raise some doubt as to whether the soil moisture content is as important as at first considered.

The first stage in this work however was to obtain a satisfactory explanation of the variation in pathogenicity shown from one strain to another when examined under pure culture conditions in the laboratory, and here a possible inverse correlation between percentage of infection and the presence of seed coat antibiotics has been demonstrated. It seemed doubtful at first if the small zones of inhibition shown in plate tests by many seeds could form effective barriers to the pathogen, especially as the antibiotics were infrequently bactericidal, and the zones were quickly overgrown. However the age of the plant when attack takes place has been shown previously by Lacey (1936) to be important and thus any delay in the infection of the seed reduces the chance of infection and decreases its severity.

An effort made at removing the antibiotic from seeds by soaking, although failing to induce increased infection, did throw some light on the presence of other factors which may under some circumstances be controlling ones. The failure to obtain an increased pathogenicity with seeds from which the antibiotic had been soaked out was ascribed to the simultaneous removal of stimulatory substances and this was supported by evidence obtained in agar plates. The importance of this stimulation was not clear as it was found that the majority of seeds exhibited a stimulation

of the pathogen in plate culture, but it does seem that its presence is, in some cases at least, essential for infection to take place. It is not absolutely controlling but as has been shown, both the percentage of infection and the severity of attack were reduced by its absence, presumably because the pathogen was unable to make sufficient growth duringthe early stages of germination. Strains may vary in this respect, to some this stimulation may be essential while to others it is only an additional factor which increases the pathogenicity. It was found that by providing a better medium in which the organism was able to grow round the seed, the percentage of infection was increased as was the severity of the attack. Thus the pathogenicity of any one strain was higher in sand saturated with soil extract mannitol than where tap water was used.

The more pronounced stimulatory effect shown by the use of an extract of decomposed seeds must be considered in relation to natural conditions. The nutritive value of the soil is very variable, especially the organic matter content, which would be high for example round the roots of plants where dead parts of the root system are usually abundant. The seed extract is essentally composed of the breakdown products of proteins, and it is reasonable to suppose that similar foci of broken down or partially broken down proteins would occur in the soil and could act similarly

as stimulatory areas for the growth of C. fascians. Under such conditions, heavy local inocula could be built up and this would tend to encourage very localised infections, which is a noted feature of the disease. That such a process may actually take place is only speculation, but it does illustrate a means by which heavy stimulation may be important under natural conditions and it does fit in with the observed nature of the disease.

Under what conditions the stimulation of the pathogen becomes controlling it is difficult to say, but it is probably correct to consider that there is a balance between the inhibitory effect of the seed coat antibiotics and the stimulatory powers of the seed, and that the final result as to whether infection takes place depends on the relative activity of the two factors, which is in turn controlled by the local conditions in the soil surrounding the seed.

The precise effect of the decomposed seed extract also remains in doubt. That it stimulates growth has been shown, but also in two experiments in two different media where the amount of growth made by the pathogen was of a completely different order, the stimulated increase in pathogenicity was similar. Thus the activity of the extract towards the increase of infection was the same in soil extract as in soil extract mannitol medium, although on the

latter medium the pathogen was stimulated to a very much higher degree than on the former one.

It seems therefore that the original explanation that the extract functions simply as a growth stimulant is not the whole story. A second explanation which has already been suggested is that the extract, and this also applies to the other stimulating substances, glutaric and glutamic acids and casein hydrolysate, enabled the pathogen to overcome at least partially, the effect of the seed coat antibiotics, and thus seeds that were normally protected became susceptible. It is known that the antibiotic activity of many substances depends on the medium in which the tests are carried out, and it is suggested that a similar factor is operating here.

Also on the inhibitory side is the possible effect antagonistic bacteria may exert on the pathogen. The literature on this aspect has been surveyed in the introduction to this thesis and it has been fairly conclusively shown that the presence of antagonistic organisms can influence the pathogenicity of other micro-organisms, although previous work was mainly with fungi. In the preceeding work, the effect of bacterial antagonists on infection with C. fascians has revealed that this is another factor which under natural conditions is likely to be important.

Some difficulty was experienced in obtaining a control of

the diseased by antagonistic bacteria under soil conditions, but although no conclusive results have been obtained in non-sterile soil, sufficient has been done to make it suggestive that given suitable conditions, the growth of antagonistic bacteria might control at least partially, the growth of the pathogen. The exact nature of these conditions was not investigated, but it was shown that these antagonists were able to effectively produce their antibiotics in sterile soil without the addition of an energy source, the required nutrients apparently diffusing from the seed. In the majority of work on the production of antibiotics in soil, the addition of an energy source has been found essential, but it does seem that the seed is able to supply this and so would enable the development of a sphere of of actively growing possibly antagonistic bacteria around it.

An attempt to obtain evidence of this by counts of viable cells in soil without any addition and in the zone of influence of seeds, failed to confirm this. The antagonist F.A.1. was used and it was found to rapidly die out under both conditions. The reason for this was not established, but it seems fairly certain that this was not so in the infection work done in this medium as repeated replicate experiments always showed a reduction in infection where this antagonist was used, although the effect was not pronounced.

The diffusate from seeds was found to be favourable to the development of the pathogen, and counts taken showed that while in the general soil the pathogen fairly rapidly died out, it was able to survive and increase in numbers round seeds of the sweet pea. The diffusates were apparently supplying the required growth substances for C. fascians and from the results in non-sterile soil, they appeared to be selectively favourable to this organism. This adds further emphasis to the idea already expressed that the location of C. fascians in the soil is in the form of foci around decomposing protein material where the required diffusible nutrients are present.

During the work as a whole one feature noted was the variation in the percentage of infection obtained in the controls of experiments undertaken at different periods of the year. In particular, ~~one feature~~, that of decreased pathogenicity during the period June to August, was particularly noticable. The highest percentages of infection were obtained in the period February to April. This has been described as a 'seasonal effect' and a similar effect has been noted by Lacey (private communication). The nature of this effect is unknown. It is mentioned to explain the variable infection percentages obtained in apparently identical experiments as owing to limitation in the number of infection tests that could be carried out at any one time, the work had to be continued throughout the year. It was

however found completely impossible to carry out any experiments of this type during the period June to August owing to the extremely variable and almost invariably low pathogenicity exhibited by otherwise 'stable' strains.

To sum up, several factors have been shown to exert an influence on infection by C. fascians, including that of inhibitors diffusing from seeds and antibiotics produced during the growth of antagonistic bacteria. In addition stimulatory substances which also diffuse from seeds and similar stimulants resulting from the breakdown of protein seeds, appear to be important. It has been suggested that although infection is normally controlled by the inhibitory factors, stimulation is, at least in some cases, essential.

VII. SUMMARY.

1. A study has been made of the parts played by the seed and antagonistic bacteria in infection by Corynebacterium fascians. Work connected with the seed included a study of both antibiotic and stimulatory properties exhibited by the seeds of various plants.

2. From a number of media tested, soil extract prepared by the autoclaving of 500 gm. of soil with 500 ml. of tap water for 15 minutes at 20 lb. pressure, and diluting the filtrate times 5 with tap water, plus the addition of 1% mannitol, was adopted for the work. This medium allowed good growth of the majority of strains of C. fascians in 3 days at 25°C, and zones of inhibition produced round seeds were clear cut and of a reasonable size for measurement.

3. Large inhibition zones exhibited round seed surface sterilised in 0.2% mercuric chloride and given up to 10 washings in distilled water, were shown to be due to adsorbed mercury. All seeds exhibited this adsorption, and soaking for several days was required to effect removal. Silver nitrate and calcium hypochlorite were shown to exhibit a similar effect.

4. Chloramine T at a concentration of 1% was effective as a surface sterilising agent providing fungi were not

present. This substance was removed by 4 washings in distilled water and used throughout the work.

5. Seeds of 20 plants from 18 genera were examined for the presence of diffusible inhibitory substances as shown by the presence of zones of inhibition against C. fascians in soil extract mannitol agar medium. About half showed the presence of antibiotic substances active against one or more strains of the pathogen.

6. These antibiotic substances were located in the coats of the seeds and never occurred in the kernel.

7. A detailed examination of the antibiotic substances in the coat of sweet pea seeds led to an inverse correlation between percentage of zonation and percentage of infection for any one strain. Where a strain had lost its pathogenicity either partially or wholly through culture on artificial media, the correlation was possible only between the percentage zonation and the percentage of infection at the time of isolation of the strain. This it was suggested, indicated that the loss of pathogenicity due to culture on artificial media was not due to an increase in the susceptibility of the organism to seed coat antibiotics, but to another independent factor.

8. Storage of the seed was shown to produce a reduction in

its antibiotic activity. Batches of seed harvested in successive years were also shown to exhibit considerable variation in their antibiotic activity.

9. Removal of the seed coat antibiotic from sweet pea seeds by soaking for 24 hours did not result in an increase in the percentage of infection as expected, but had the reverse effect. This was attributed to the simultaneous soaking out of stimulatory substances from the kernel. Soaked seeds failed to exhibit stimulation in plate culture.

10. Treatment of one batch of sweet pea seeds with 50% alcohol resulted in an increase in their antibiotic activity possibly due to the greater 'wetting' power of the alcohol which subsequently allowed diffusion of the antibiotic from a greater area. A second batch of seeds harvested in the succeeding year, failed to exhibit this effect. The only observed difference between the two batches was that while the first was ripened under dry conditions, the second was ripened under exceptionally wet conditions. The lack of activity of the second batch was correlated with a low antibiotic activity exhibited by untreated seeds of the same batch.

11. Many seeds exhibited stimulation of the pathogen in

plate culture and this stimulation was intensified if the seed was undergoing decomposition either by the action of bacteria or fungi. In Nature these organisms occurred as internal contaminants of the seed. A bacterium, F.A.7., a species of Bacillus was isolated from one such seed.

12. Extract of the kernels of crushed seeds exhibited a moderate stimulation of the pathogen and various antagonistic organisms.

13. Extracts of decomposed seeds exhibited a very heavy stimulation of all strains of the pathogen in soil extract mannitol medium, growth at 25°C continuing for 10 days when it was several times that obtained on nutrient agar.

14. The addition of this extract to sand culture infections resulted in an increase in the percentage of infection of between 10 and 15% and a marked increase in the severity of the attack. A similar increase was shown when either soil extract or soil extract mannitol was used as medium, although the degree of stimulation in the former medium was only a fraction of that in the latter. This suggested that stimulation, resulting in a rapid build up of the inoculum was not responsible for the increased pathogenicity, especially in view of the fact that the initial inoculum was very heavy. It was considered that the effect of the

decomposed seed extract might be to partially antagonise the seed coat antibiotic thus allowing infection of seeds containing only a low concentration of the antibiotic. Non-pathogenic strains and those which had almost lost their pathogenicity, were not influenced.

15. A range of substances including amino acids and growth factors was examined to see if stimulation equivalent to that shown by decomposed seed extract could be obtained. However, only light stimulation was exhibited by a few substances against C. fascians, including glutamic acid, leucine, guanine and glutaric acid. A heavier stimulation was shown by casein hydrolysate (vitamin free) but it was not of the same order as that obtained with decomposed seed extract.

16. The effect of addition of stimulatory substances to sand culture infections in soil extract mannitol medium was studied, using 0.1% glutamic acid, 0.05% glutaric acid and 1.0% casein hydrolysate as stimulatory substances. In each case the percentage of infection and the severity of attack was considerably increased, a rise of approximately 20% in the percentage ofinfection being exhibited.

17. A study was made of the effect of the addition of suspensions of organisms shown to be antagonistic to C. fascians in plate culture on the infection of sweet pea

seedlings in sand, sterile soil and non-sterile soil.

18. Under pure culture conditions in sand, four organisms F.A.1., F.A.6., F.A.7. and B. subtilis were shown to reduce the percentage of infection and the severity of the attack of three strains of C. fascians in tap water, soil extract and soil extract mannitol media. Antagonism was greatest in soil extract mannitol medium due presumably to the better growth of the antagonist. A reduction of 75% in the percentage of infection was demonstrated in this medium.

19. In sterile soil, with and without the addition of 1% mannitol as an energy source, one antagonist, F.A.6. exhibited marked inhibition of the pathogen, a reduction of 50 to 65% in the percentage of infection being shown. A less pronounced control was exhibited by the other antagonists. Reduction in the severity of attack was evident where an energy source was present, F.A.6., and B. subtilis being particularly effective.

20. Results in non-sterile were inconclusive owing to the difficulty of obtaining a satisfactory control series. However some degree of control of infection was indicated where two of the antagonists, F.A.6. and B. subtilis were present.

21. Axillary bud infection was reduced in the presence of

antagonistic organisms.

22. Antibiotics produced by two of the antagonists were shown to be strongly adsorbed by bentonite and kaolin from culture filtrates of the organisms.

23. The rapid death rate of C. fascians in sterile soil was demonstrated. Counts of viable cells of one strain, showed that after 3 days at 25°C the count was reduced by nearly 2o% and after 10 by about 70%. Other strains exhibited a greater susceptibility, showing few viable cells after 3 days. The cause of this rapid death rate was not investigated.

24. Counts of viable cells of C. fascians taken from the bulk of a soil mass and from the zone of influence of a seed, showed that in both sterile and non-sterile soils the rapid death rate of the pathogen was checked by diffusates from the seed, and actually a small increase in the number of cells took place. One of the antagonistic organisms examined similarly showed no response and rapidly died out.

25. The combined effect of stimulatory and antagonistic factors indicated that there was some reduction in the effect exhibited by antagonistic bacteria on the pathogenicity where the stimulatory extract of decomposed seeds was present.

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X. APPENDIX.

THE PRESENCE OF ANTIBACTERIAL AND ANTIFUNGAL SUBSTANCES IN THE EXTRACTS OF VARIOUS VARIETIES OF TOBACCO AND OTHER SOLANACEAE.

Many plants have been examined for the presence of antibiotic substances, and a short survey of the literature is included in the introduction to this Thesis. In all the work however the emphasis has been on the detection and isolation of substances active against human pathogens. Few of these investigators have included plant pathogens among the test organisms.

No record could be found in the literature of tobacco (Nicotiana tabacum) having been examined for possible antibiotic substances and the examination of other Solanaceae was confined to the tomato (Lycopersicum esculentum) from which the antibiotic tomatin has been isolated (Irving, Fontaine and Doolittle, 1946), Datura stramonium and two species of Physalis. From the latter three, the presence of active inhibitory substances against both B. coli and Staphylococcus aureus has been demonstrated (Carlson, Douglas and Robertson, 1948).

A further consideration was the fact that the susceptibility of various tobacco varieties to Wildfire disease was markedly different, some varieties appearing to be

relatively resistant while others are highly susceptible. It was of interest therefore to see if this resistance could be related in any way to the presence of inhibitors in the plant tissues.

9 varieties of tobacco and five other members of the Solanaceae were examined. Seeds of the tobacco varieties were supplied by the Tobacco Growers Association in S. Rhodesia and the plants were raised in a greenhouse. The remaining Solanaceous plants were supplied as dried specimens.

Preparation of extracts.

Extracts were prepared from leaves, shoot tips and fruits. The shoot tip comprised the top one and a half inches of the plant, any developing leaves being included. For the leaf extracts, only fully green leaves were used, any showing yellowing being discarded.

The expressed plant juice was obtained as follows. 5 grams of material was surface sterilised by immersion in 1.0% chloramine T for 15 minutes followed by washing in four changes of sterile distilled water. The tissue was transfered to a sterile pestle and mortar, removing as much as possible of the surplus water by shaking, and thoroughly macerated. The juice obtained was used directly in the tests.

The ether extracts of the fresh material were prepared

by addind 5 ml. of purified ether to 5 grams of the plant material, which had previously been macerated as described above. This was placed in a refrigerator and allowed to stand for 24 hours after which it was assayed. The dried material was treated similarly except that 15 ml. of ether was used to every 2 grams of plant material.

Acid alcohol extracts were prepared in a similar way, using absolute alcohol containing 1% hydrochloric acid as the extraction medium. Before assay, the extracts were neutralised by the addition of sodium hydroxide and the supernatant subsequently diluted to twice the original volume of acid alcohol used. Acid alcohol only treated in a similar way was shown to be non-toxic to all the test organisms.

Method of assay.

The plant juice extracts were examined by the Oxford Cup method of assay, and the ether and acid alcohol extracts by the paper disc method (Vincent and Vincent, 1944, and Epstein, Foley and Perrine and Lee, 1944).

The test organisms consisted of two strains of Bacterium coli, and Staphylococcus aureus, organisms normally adopted as standard for this type of work, and various plant pathogens, namely, Bacterium tumefaciens, Corynebacterium fascians, Pseudomonas tabaci, Xanthomonas campestre, and Bacterium carotovorum. Three fungi, two

strains of Alternaria longipes and Botrytis cinerea were also included. All the bacteria with the exception of B. carotovorum were cultured on an Oxoid medium (meat extract, peptone and sodium chloride) , and the fungi on potato dextrose agar. B. carotovorum made unsatisfactory growth on this and was therefore grown on Difco Yeast Beef agar.

60ml. of the agar medium seeded with the test organism was poured into each of the 6 inch diameter petri dishes and allowed to set. The plates were then allowed to stand at laboratory temperature for one hour before placing the sterile porcelain cylinders on the surface. These were filled with the aqueous plant extracts. The paper discs used for the ether and acid alcohol extracts were sterilised and each dipped separately in the extract and immediately placed on the agar surface. The fungal tests were carried out in 4 inch diameter plates, the inoculation being made at the centre, and the cylinders or discs placed in a circle approximately a half inch from this point.

The bacterial plates were incubated at 30°C and the fungal ones at 25°C. With the exception of C. fascians and X. campestre, the presence of zones of inhibition and stimulation was recorded after 24 hours incubation, the rest after 48 hours. The fungal plates were observed at intervals and the results recorded as complete inhibition if the fungus failed to grow up to the edge of the cups or discs

when the general level of spread had reached their outer edges. If there was a delay in the growth of the fungus up to the cups or discs, this was recorded as partial inhibition. The results are summarised in Tables I to V inclusive.

In the above work, only mature plants were used. The age of the tobacco plants was at least 5 months. It was decided therefore to examine four tobacco varieties in the seedling stage for the presence of antibiotic substances. Large numbers of seedlings were grown and the expressed juice assayed when the plants were 10 weeks old. Apex and leaf extracts were tested separately. These results are shown in Table VI.

Results.

Almost all the varieties of tobacco and other species of Solanaceae examined yielded extracts which were active against one or more of the test organisms. Considerable variation between the activity of the expressed juice of the various tobacco varieties towards some of the plant pathogens was evident, although all appeared to contain an active inhibitor of Ps. tabaci.

The majority of extracts were active against Staph. aureus, although the inhibition was only partial. Bacterium coli strain 86 was generally unaffected but one variety,

Table I.

The activity of the expressed plant juice of various varieties of tobacco against several bacteria and fungi in plate assay.

Variety	Tissue	Test organism								
		B.coli 86	B.tumefaciens	C.fascians 505	Ps.tabaci	X.campestre	Staph. aureus	Botrytis cinerea	Alternaria TRS 43	longipes MR 7054
Bonanza	Apex	S	S	S	+	-	SP	-	-	-
	Leaf	S	S	S	+PS	P	SP	-	-	-
Broad Leaf	Apex	S	PS	S	+PS	-	SP	-	-	-
	Leaf	S	PS	S	+PS	-	SP	-	-	-
C7	Apex	S	SP	PS	+	-	SP	-	-	-
	Leaf	S	SP	PS	+	-	SP	-	-	-
Gold Dollar	Apex	S	SP	+	+S	P	SP	-	-	-
	Leaf	S	SP	+S	+S	PS	S	-	-	-
Harisons Special	Apex	S	S	S	+PS	-	PS	-	-	-
	Leaf	S	S	S	PS	-	PS	-	-	P
Jamaica Wrapper	Apex	S	S	+S	+	-	SP	P	-	-
	Leaf	S	S	+S	+	-	SP	-	-	-
Oxford 26	Apex	PS	S	S	PS	P	PS	P	P	P
	Leaf	PS	S	S	+S	P	PS	P	P	P
Special 400	Apex	S	S	S	+PS	P	PS	-	-	-
	Leaf	S	S	S	+PS	P	S	-	-	-
White stem Orinoco	Apex	S	S	S	+PS	-	S	-	P	P
	Leaf	S	S	S	+PS	S	PS	-	P	P

Table II.

The activity of the ether extract of the leaves of various varieties of tobacco against several bacteria and fungi in plate assay.

Variety	Test organism									
	B.coli 86	B.coli 163	B.tumefaciens	C.fascians 505	Ps.tabaci	X.campestre	Staph. aureus	B.carotovorum	Botrytis cinerea	Alternaria longipes MR 7054
Bonanza	-	-	-	-	+P	+	P	-	-	-
Broad Leaf	-	-	P	-	P	-	P	-	-	-
C7	-	-	-	-	+	+	PS	-	-	-
Gold Dollar	P	-	P	-	+P	-	+	+	-	-
Harrisons Special	-	-	-	-	+P	P	+	-	-	-
Jamaica Wrapper	-	-	+	-	+	-	P	-	-	-
Oxford 26	+	-	+	-	+P	+	+	P	-	-
Special 400	-	-	-	-	+P	P	P	-	-	-
White stem Orinoco	-	-	-	-	+P	+	P	-	-	-

Table III.

The activity of ether extracts of various plants of the family Solanaceae against several bacteria and fungi in plate assay.

Plant	Tissue	Test organism									
		B.coli 86	B.coli 163	B.tumefaciens	C.fascians 505	Ps.tabaci	X.campestre	Staph. aureus	B.carotovorum	Botrytis cinerea	Alternaria longipes MR 7054
Datura ferox	Leaf	-	-	-	-	-	-	-	-	-	-
	Fruit	-	-	-	-	-	-	-	-	-	-
Datura stramonium	Leaf	-	-	-	P	P	+	-	-	-	-
	Fruit	P	S	-	P	-	-	P	-	-	-
Nicandra physaloides	Leaf	-	-	-	-	+	+	+	P	-	-
	Fruit	-	-	-	-	-	-	P	-	-	-
Physalis peruviana	Leaf	P	+	P	+	+P	+	-	-,	-	-
	Fruit	-	-	-	-	-	-	-	-	-	-
Solanum incanum	Leaf	-	-	-	-	-	-	+	-	-	-

Key to symbols used: +, zone of complete inhibition; P, zone of partial inhibition; S, stimulation; -, no inhibition or stimulation.
Order of zones is from cup outwards. Thus, +PS means a zone of complete inhibition followed by one of partial inhibition, followed by stimulation.

Table IV.

The activity of the acid alcohol extract of the leaves of various varieties of tobacco against several bacteria and fungi in plate assay.

Variety	Test organism									
	B.coli 86	B.coli 163	B.tumefaciens	C.fascians 505	Ps.tabaci	X.campestre	Staph. aureus	B.carotovorum	Botrytis cinerea	Alternaria longipes MR 7054
Bonanza	S	S	-	-	PS	+	+	-	-	-
Broad leaf	S	S	-	-	+S	-	P	-	-	-
C7	-	-	-	-	+S	P	P	-	-	-
Gold dollar	S	S	-	-	+S	-	+	-	-	-
Harrisons special	S	S	-	-	+S	-	-	P	-	-
Jamaica wrapper	S	S	-	-	PS	-	P	-	-	-
Oxford 26	S	S	-	-	PS	-	+	-	-	-
Special 400	S	S	-	-	+S	P	P	-	-	-
White stem Orinoco	S	S	P	-	+S	-	+	P	-	-

Table V.

The activity of acid alcohol extracts of various plants of the family Solanaceae against several bacteria and fungi in plate assay.

Plant	Tissue	Test organism									
		B.coli 86	B.coli 163	B.tumefaciens	C.fascians 505	Ps.tabaci	X.campestre	Staph. aureus	B.carotovorum	Botrytis cinerea	Alternaria longipes MR 7054
Datura ferox	Leaf	-	-	-	P	-	+	+	-	-	-
	Fruit	-	-	-	P	P	+	P	-	-	-
Datura stramonium	Leaf	-	P	-	+	+	+	+	-	-	-
	Fruit	-	-	-	+	P	P	P	-	-	-
Nicandra physaloides	Leaf	-	-	-	-	P	-	-	-	-	-
	Fruit	-	-	-	-	-	-	-	-	-	-
Physalis peruviana	Leaf	-	-	P	-	P	P	P	-	-	-
	Fruit	-	-	-	-	P	-	P	-	-	-
Solanum incanum	Leaf	-	-	-	-	-	-	-	-	-	-

Table VI.

The activity of the expressed juice of 10 week old seedlings of four varieties of tobacco against several bacteria and fungi in plate assay.

Variety	Tissue	Test organism								
		B. coli 86	B. tumefaciens	C. fascians 505	Ps. tabaci	X. campestre	Staph. aureus	Botrytis cinerea	Alternaria TRS 43	longipes MR 7054
Bonanza	Apex	-	-	P	P	-	+S	-	-	-
	Leaf	-	-	-	P	-	+S	-	-	-
Gold dollar	Apex	P	-	-	-	-	+S	-	-	-
	Leaf	-	-	-	-	-	+S	-	-	-
Oxford 26	Apex	-	-	-	-	-	S	-	-	-
	Leaf	-	-	S	-	-	S	-	-	-
White stem Orinoco	Apex	-	-	-	-	-	S	-	-	-
	Leaf	-	-	-	-	-	S	-	-	-

namely Oxford 26, was active against this organism. There was little activity against the fungi used, only a few scattered instances of partial inhibition being recorded.

Considering the activity of the tobacco varieties, Gold Dollar was the most effective, followed by C7 and Broad Leaf.

The results obtained from the ether and acid alcohol extracts of the tobacco varieties followed a similar pattern to those for the expressed juice. Again, some activity against Ps. tabaci was shown by all varieties, and the majority were also active against Staph. aureus. The ether extract of Oxford 26 was active against strain 86 of Bacterium coli, the rest again being negative.

There was nothing to suggest that the ether and acid alcohol extracts were more active than the expressed juice, although there was some variation between the results obtained with the three methods of extraction. All the ether and acid alcohol extracts were ineffective against the fungi.

The ether and acid alcohol extracts of the other Solanaceous plants all yielded active extracts with the exception of Solanum incanum which was notably ineffective. Datura stramonium was particularly active. It should be noted that in addition to the inhibition of Ps. tabaci and Staph. aureus, which was again shown by most extracts,

several were active against other plant pathogens, namely X. campestre and C. fascians. In all cases extracts prepared from the fruits of these plants were less active than those from the leaves.

Extracts prepared from 10 week old seedlings instead of mature plants were much less active, and it was notable that while there was still considerable inhibition of Staph. aureus, the effect on Ps. tabaci was greatly reduced.

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