I. THE FUNGAL FLORA ASSOCIATED WITH BLACK SPOT OF PINEAPPLES.

II. SOME ASPECTS OF THE MICROFLORA OF CITRUS SOILS.

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I

INTRODUCTION

The earliest reference to investigations of "black spot" in pineapples is made by Tryon (1898), who refers to the work of Dr A. A. Brown (1896) of the Stock Branch, Victoria (Aus.), who sectioned diseased areas of pineapples and found fungal spores and hyphae in the tissues. The symptoms of this so-called "fruitlet core rot," are described by Tryon as: "well defined dark brown markings immediately beneath the surface, and passing inwards to a depth of $\frac{1}{4}$" - $\frac{1}{2}$" - the malady commencing in separate fruitlets, the central core of the fruit remaining quite healthy." (This description agrees with the symptoms referred to as "black spot" in this country). Subsequent culture of the spores (found by Brown) on slices of healthy fruit resulted in growth of Mucor racemosus. Brown regarded an invasion by this fungus as a primary cause of the disease.

Tryon's investigations into the disease in Queensland in 1898, led him to conclude, however, that an injury to the pineapple fruit was initially necessary, before the fungus could produce diseased areas in the tissues. The fungus he isolated from the Queen pineapple variety, was Monilia sp. allied to M. candida, and not Mucor as reported by Brown. In support of his theory he quotes a reference by Peck (1892), to the effect that the hyphae of Monilia fructigena are

incapable of penetrating the unruptured epidermis of various fruits. In Cayennes he isolated Penicillium sp. from the withered flower parts as well as from the diseased areas. He does not consider this significant, as both fungi are saprophytic, and therefore secondary factors in the disease.

Tryon considered that injury to the fruit was caused by insects harboured in the floral cavity, and cites caterpillars of a small moth which consume the stamens and pistil, mealy bugs, mites, and thrips as being responsible. Mechanical fissuring found at the base of the receptacle, below the level of insertion of the stamens, did not serve as an entry for fungi, as the superficial layer of cells thus exposed was suberized. Invasion by the fungus followed the thin parenchymatous tissue forming the placental column and placenta of the ovary, and generally commenced immediately beneath the base of the style.

Despite these theories, however, Tryon distinguishes between the disease symptoms as shown in Queen and Cayenne fruits on the grounds that, in the Cayenne the disease spreads laterally from a point beneath the insertion of the stamens, while in the Queen, infection passes downwards from the base of the style to the ovary. He considers these differences to be "in accordance with the marked structural differences in matters of detail that exist between fruit and fruitlets of the two varities." He does not specify what these "marked structural differences" are.

He accounts for the prevalence of core-rot in the winter crop, by advancing the theory that certain chemical changes concerned in the ripening of the fruit may have an antagonistic effect on the changes constituting the disease, particularly in view of the fact that diseased fruitlets often show retarded development,- being small and pale green in colour. In the summer these chemical changes involved in ripening would be intensified, and would provide conditions unfavourable to the development of the fungus, thus checking the disease. In winter, when the ripening processes are protracted, the fungus is able to invade the tissues without interference.

While Tryon's theories on black spot have been largely borne out by other workers, his isolation of Monilia sp. as the causative fungus has not been duplicated, the majority of investigators attributing the disease to Penicillium sp. Simmonds (1933) considered Penicillium alone, as being the cause of "fruitlet core-rot" in pineapples in Queensland; the entry of the fungus being made possible by injuries in the floral cavities caused by the mealy bug, Pseudococcus brevipes, and a Tarsonemid mite. Parham (1934), working in Fiji, found Ceratostomella paradoxa and Helminthosporium sp., as well as Penicillium sp., associated with the disease which he refers to as "black fruit spot." An early worker in Hawaii, Larsen, (1910) described an "exogenous brown discolouration," in Cayenne pineapples, which he attributed to

Fusarium sp., as did Matz, (1920) in Porto Rico. Later workers, Sideris and Waldron, (1930) described the same condition in Cayennes without any accompanying hardness or crispness of the diseased tissues, and considered it to be caused by a "saprophytic Penicillium sp." When the brown discolouration was accompanied by hardness, they attributed it to the presence of an unidentified bacterium. Johannson, (1934) in Guatemala, came to the same conclusions as the above workers, (ie. Larsen, Sideris and Waldron) as did Serrano, (1928) in the Phillipines, and Barker, (1926) in Haiti. (see Thompson (1937) on Pineapple fruit rots in Malaya) Johnson (1935) in Hawaii, found both Penicillium sp. and Fusarium sp. responsible for "exogenous brown rot," and suggested that the rots attributed to Penicillium sp. in Queensland and Jamaica, and the "black spot" in South Africa, were the same as Larsen's exogenous brown rot.

Thompson, (1937) working in Malaya described a "brown fruitlet rot" distinguishing two types - one caused by bacteria and the other by fungi. There were no external symptoms, but the internal symptoms he described as a "wedge of brown, almost black decay extending to the core, and in such cases there may be a red-brown margin at the core or at the side of one or both fruitlets." He observed this brown tissue to be "harder and slightly drier" than healthy tissue. He also found that the loculi of the ovary were sometimes lined with a "skin or fluff" of white mycelium. Sections made of the

diseased areas showed hyphae of fungus commonly present at the outer margin of the rot, and bacteria at the inner margin. He assumed that fungal invasion probably followed that of bacteria. He isolated the fungus Penicillium sp. and the bacterium Erwinia ananas from the diseased areas, and in inoculation experiments he found that both produced brown rot in fruits inoculated when mature or in the flowering stage. However, he thought that the rot produced in the fruitlets inoculated when in flower, might be natural. No rot was produced in fruit inoculated when half grown, nor in immature fruits inoculated after flowering. He concluded that this was due to the fact that the wounded tissue became hard-walled by the time that the fruits ripened, and thus excluded the inoculum. This does not seem to be a very satisfactory explanation, as hardening of tissue would be unlikely to occur so rapidly that fungal hyphae in the inoculum, had no chance to grow into the surrounding tissues. His results were not conclusive as only three out of twelve pineapples inoculated with Penicillium sp. produced brown rot and yielded Penicillium sp. on reisolation from the treated fruitlets. The other nine pineapples were immature and showed no rot. Of thirty-six fruits inoculated with Penicillium sp. and Erwinia ananas only eight showed brown rot. On reisolation from the treated fruitlets the original inoculants were found in all cases, save two, where Thielaviopsis sp. and a yeast were present.

Thompson, (1937) suggested that Penicillium operating

in the absence of bacteria might produce an effect (viz. absence of hardness) on the tissues, different from that produced by bacteria operating alone, or accompanied by Penicillium. He concludes that as the rot in the majority of cases affects only a few fruitlets, and rarely extends to adjacent ones, the affected tissues must have been altered in some way so as to lower their resistance to invasion - the parasitic nature of the fungus and bacterium involved being doubtful.

Thompson is the only investigator who bears out the work of Serrano, (1928) who conducted a series of experiments in the Phillipines, proving that the bacteria Erwinia ananas and Phytomonas ananas were responsible for a "wet" type of brown rot, termed by him "fruitlet-rot." The only external symptoms Serrano observed were uneven ripening of the affected fruitlets. Internally, a brown discolouration appears to originate in the placentae of the ovary, where the three fissures running from the base of the three stamens end their course. These brown spots extend as "more or less granular radiations into the inner surface of individual fruitlets." The affected tissues are at first juicy and soft, but as the disease progresses, they become dry and hard. The disease develops only during the process of ripening, and does not seem to spread after ripening or during storage. In green immature fruits traces of the disease are only rarely found. The disease does not seem to affect the connective tissues, though the fibro-vascular bundles in the core may become brown.

Serrano isolated Penicillium sp. from "dry"brown rot areas, and did not regard it as being the cause of the "wet" condition. In field tests, where the fruit was sprayed during the flowering period, with water suspensions of both the bacterium and Penicillium sp. separately and together , the results obtained were as follows: 75% of the fruits sprayed with the bacterium became infected; 35% sprayed with Penicillium sp. alone, showed infection and 71% of the fruit sprayed with combined suspensions became infected. The control fruitlets showed 36% infection which was attributed to natural infection under field conditions. Using an inoculation technique, (mycelial hyphae in agar) in field and laboratory experiments, Serrano found that fruit inoculated with Penicillium sp. was "questionably positive," ie. discolouration being caused, but "the lesions produced were far from being typical of fruitlet brown-rot as caused by the bacterium Erwinia ananas." In his field inoculations he obtained similar results, though in a few cases fruits treated with Penicillium sp. did develop "characteristic lesions," these however, he considered as chance infections.

Serrano distinguished another type of fruitlet rot on the basis, chiefly, of darker colour. This he termed "black rot," and found crispness and hardness of the affected parts to be outstanding characteristics of infection in the early stages. He isolated the bacterium Phytomonas ananas from this condition. However, fruitlets inoculated with the

bacterium did not produce "hardness." This he explains by the hypothesis that hardness is probably only produced when infection takes place in green fruits, as at this stage the "upsetting of the metabolic processes in the plant system by the invading pathogen has a better chance of being decisive."

While acknowledging the fact that insects in the floral receptacle may cause injury which will facilitate the entry of the bacteria, Serrano regards mechanical cracks in the floral cavity as offering a mode of entry for the pathogen. He considers that the bacterial parasite remains inactive until the fruits begin to ripen with the warm weather, when an increase of sugar favours the pathogen, and rapid succulent growth of fruits increases their susceptibility.

In South Africa, the first report on black spot in pineapples grown in the Eastern Cape, appeared in 1924, by Pole-Evans. (It was first noted in the Phillipines at this time as well). He described a "wedge-shaped black spot" not previously studied, and due "possibly", to a mixture of Penicillium sp. and Fusarium sp., and a "soft, light brown, translucent, spotting" due to Fusarium sp.

Davies, (1928) investigating storage of pineapples mentions black spot as the only important field disease. Miss Bottomley, of the Division of Botany, in a private communication to Davies, suggested that black spot might be caused by several species or strains of Penicillium, one of which is more virulent than the rest. Infection is said to

occur during, or just after flowering. The only other reference to black spot occurs in a pamphlet on pineapple culture in the Eastern Cape, by Clark (1931) where it is stated that "the cause of the disease is thought to be due to Penicillium".

Unfortunately, there are no records of any actual experimental data in support of the generally accepted view that black spot in S. Africa is caused by Penicillium. The check list of S. African plant diseases, compiled by Doidge, Bottomley, van der Plank, and Pauer in 1951, states:- "Black spot caused by Penicillium funiculosum Thom. Fusarium rot, lesions more extensive and lighter brown than those of black spot caused by Fusarium moniliforme Sheld." An enquiry to the Division of Plant Pathology for any available data, states that the work consisted of isolating and proving the pathogenicity of the above fungi, but no published records exist.

For this reason, and in view of the fact that there is little agreement among workers in other countries as to the actual cause of the disease, or even whether the diseases variously referred to as brown spot, brown and black rot , eye rot, and fruitlet core rot, are one and the same, or the same as the S. African black spot disease, it is obviously necessary to reinvestigate the problem , as it occurs in this country.

This has been done along the lines of ascertaining exactly what organisms are associated with the disease, and proving their pathogenicity under various conditions. The pathogenic

fungi were then examined in culture under varying environmental conditions, in order to provide information as a basis for further work on the internal relationship between fungus and fruit. Finally, the organisms associated with other parts of the pineapple plant, (ie. floral cavities and leaves) and in the soil, were investigated in order to establish the relationship between them and the pathogenic fungi outside the pineapple fruit.

II

THE PINEAPPLE FRUIT AND SYMPTOMS OF THE DISEASE

A. Morphology of the flower and the fruitlet.

The pineapple, Ananas comosus (L) Merr. (Bromelia comosa L., A. sativus Schutt. f.) is a member of the monocotyledonous family Bromeliaceae, a native of tropical America, and widely cultivated in warm and tropical countries. In South Africa two varieties are cultivated, the Egyptian Queen, and the larger Smooth Cayenne. The name "Anan-as" is modified from the aboriginal S. American name.

The bisexual epigynous flowers are sessile, borne on a central axis, and each flower is subtended by a large bract. The three sepals are shorter than the three petals, and are arranged alternately with them - they are free at the tips. The corolla is shaded from white to purple, and each petal has a scale or ligule at its base. The petals are free, but connivent. The six stamens are free, arranged in two series of three, the outer whorl being attached to the base of the sepals, and the inner whorl to the base of the petals. The anthers are introrse and basifixed. The style is filiform and tripartite, with three stigmas. The tricarpellary ovary is inferior, with three locules each containing many abortive, anatropous ovules. In each carpel wall there is an internal nectary (see Figures 1, 2 and 3).

The fruit is a syncarpium, formed by the coalescence of

the thickened rachis of the inflorescence, together with the inferior ovaries of the flower, adhering withered floral parts, and the enlarged calices and bracts, into one large spherical to ovoid fleshy body, with a crown of sterile foliaceous bracts at the apex.

The inflorescence first appears as a small bud in the centre of the pineapple plant, the flowers aligned in three series of spiral rows, and takes about 3 - 4 weeks to reach the flowering stage. Flowering occurs over a period of one to two weeks, in acropetal sequence, the lowest flowers opening before the ones nearer the crown. After flowering the fruit takes from 3 - 4 months to ripen completely, the floral parts gradually wither and the calices and floral bracts become more fleshy and grow over the floral cavities. The base of the floral cavity and the ducts of the nectaries, (intercarpellary fissures) merge with each other, both showing the same process of lignification, suberization and formation of a periderm-like layer. (see Figure 4). Due to this rigidity of the lining of the floral cavity, it becomes incapable of stretching, and the enlargement of the underlying tissues results in irregular stresses which cause small cracks to appear in the cavity. (Okimoto, 1948, p 228)

It is during the flowering period that entry of insects to the floral cavity in search of nectar, is most likely to occur. Prior to flowering, the buds are tightly closed, and entry would be difficult, apart from the absence of nectar as

an incentive; and after flowering, the floral cavity is effectively closed over by the large fleshy bract and calyx. (Following the system adopted by Okimoto, (1948) the terms calyx and corolla have been used to describe the floral parts, instead of the terms outer and inner perianth, usually used in describing monocotyledonous flowers).

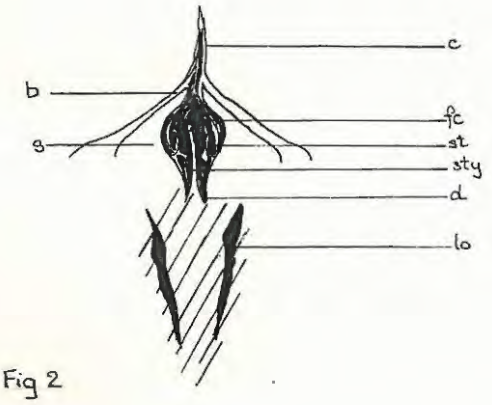


Fig 2

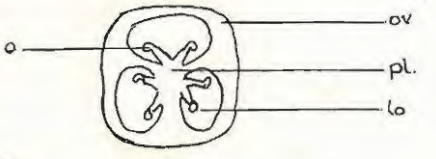


Fig.3

Fig.2. Longitudinal Section through Ripe Fruitlet. c,withered corolla; d,depression at base of style; f.c,floral cavity containing withered remains of style and stamens (st,sty); b,s,lo,same as Fig.1. Diagonal shading indicates area affected by black spot in early stages of the disease.

Fig.3. Transverse Section through Ovary. o,ovules; ov,ovary wall; lo,locule; pl,axile placenta.

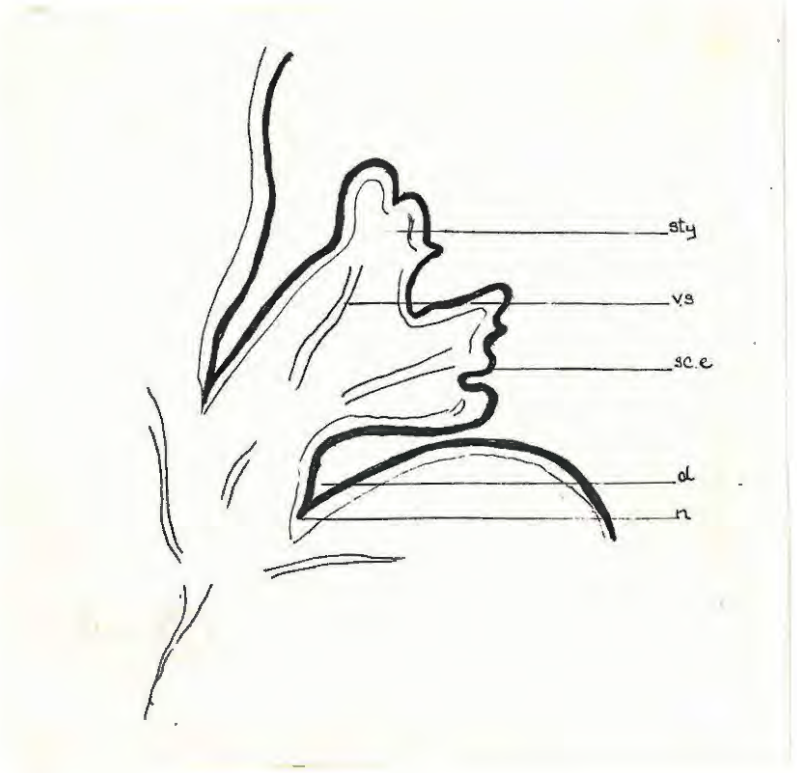


Fig.4.Diagram of a Longitudinal Section through Base of Floral Cavity.
sty,remains of style; vs,vascular strand; sc.e, sclerified epidermis; d,depression at base of style; n,sclerified nectary duct.

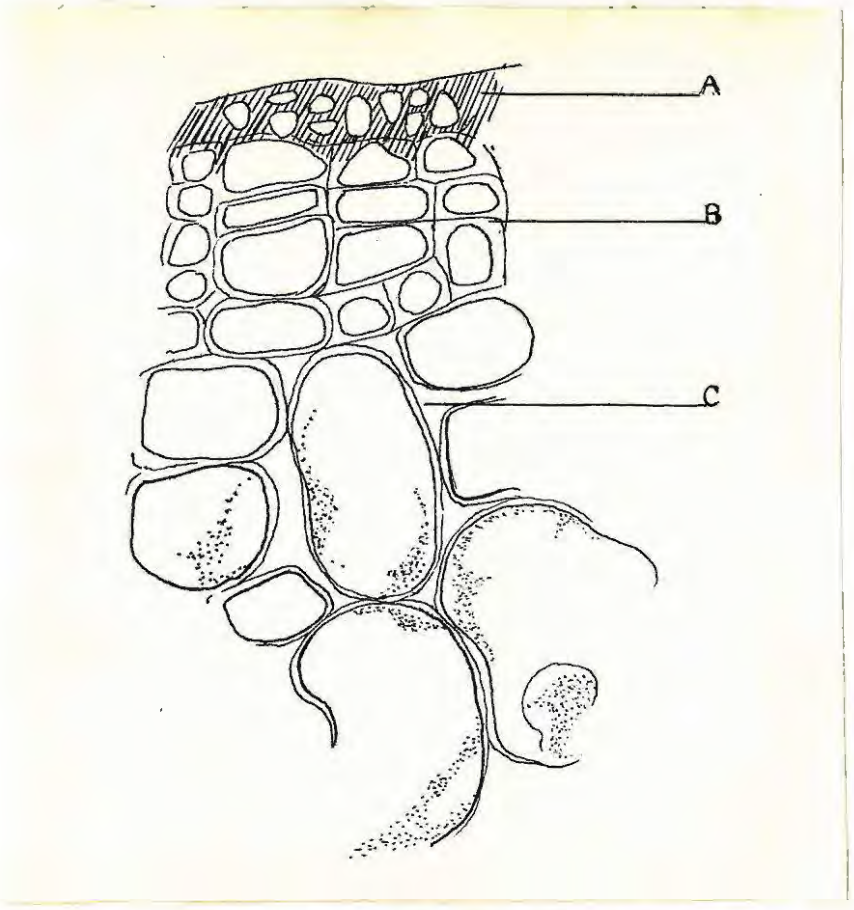


Fig.5. Drawing of Cells in region 1, fig.4.
A. Sclerified epidermal cells.
B. Periderm-like layer of sub-epidermal cells.
C. Thin-walled parenchyma with intercellular air spaces and cell contents.

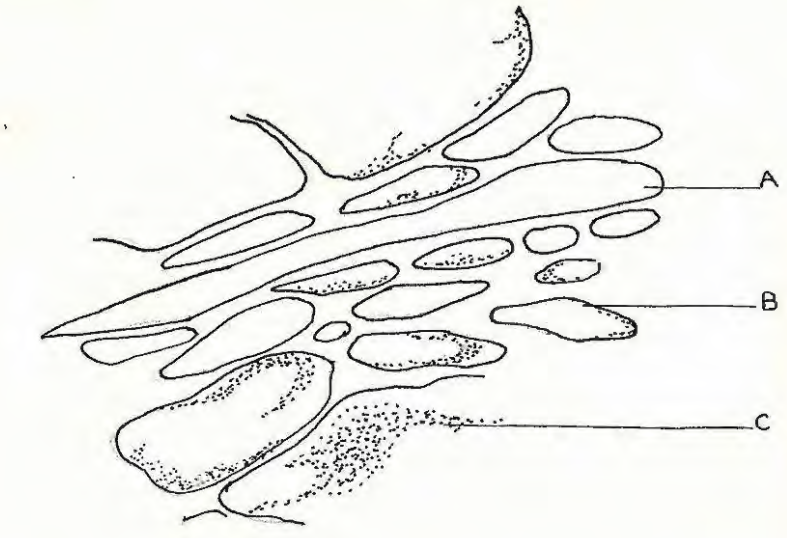


Fig.6. Drawing of Cells in region 2, fig.4.
A. Large intercellular space.
B. Parenchyma cells.
C. Cell contents.

B. Symptoms of black spot disease

In general, pineapple fruits affected with black spot show no symptoms of the disease externally, though underdevelopment of fruitlets and uneven ripening, (as shown by greenness of fruitlet as compared with yellow colour of surrounding fruitlets) is considered by some farmers as evidence of underlying disease. In these investigations this has not proved a reliable guide to diseased areas.

On cutting the fruit, black spot areas are easily distinguished. They occur immediately beneath the floral cavity and, except in extreme cases, are limited to individual fruitlets. A small patch of flesh (generally ± 1" x ½") becomes dark brown to black in colour (occasionally reddish-brown) and , depending on the amount of breakdown of cells, appears less firm and more juicy than the surrounding healthy tissue - this condition is referred to as "wet spot." (see Plate 1)

Another type of black spot occurs as hard and often brittle patches of brown tissue located beneath the floral cavity, but in this case the flesh is fissured and the sides of the fissures are callosed. This is termed "dry spot." (see Plate 2). It never appears to spread from one fruitlet to the other - the disease seemingly being held in check by the suberization of the tissues which prevents further invasion by the fungal hyphae.

Types of Black Spot.

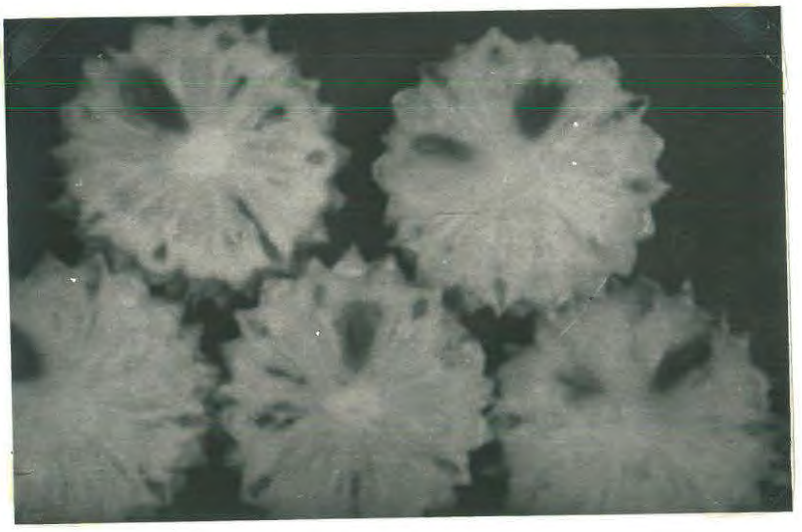


Plate 1 - Wet Spot

Plate 2. Dry Spot

C. Histology of Infected areas

Examination of a longitudinal microscopic section of the fruitlet reveals a strongly suberized layer of cells lining the three fissures at the base of the style, and covering the style as well. The underlying parenchyma consists of very thin-walled cells forming a more or less continuous tissue from beneath the style to the ovary, (the locule lining does not become thickened) - according to Tyron, (1898) the "path of least resistance" favoured by invading fungal hyphae. (see Figures 4, 5 and 6) After flowering, cell enlargement takes place, and the walls of the fully distended cells appear thinner. Loss of turgidity occurs after ripening, and the walls appear wavy. In over ripe fruit, the walls show evidence of softening and some disintegration. (Okimota, 1948)

Sections of diseased tissue are difficult to interpret accurately, as the mass of fungal hyphae and spores in the tissues precludes direct observation of the cell walls. As far as can be ascertained, the cell walls seem to separate from each other, as the fungal hyphae invade the tissues, the intercellular spaces thus formed, being filled with a yellowish substance, possibly resinous. Eventually, the cell walls break down under mechanical pressure of the hyphae and enzyme action, the dead cell contents turn brown and break up into refractive brownish globules. Finally, only the mass of hyphae remain in place of the tissues. Black spot lesions

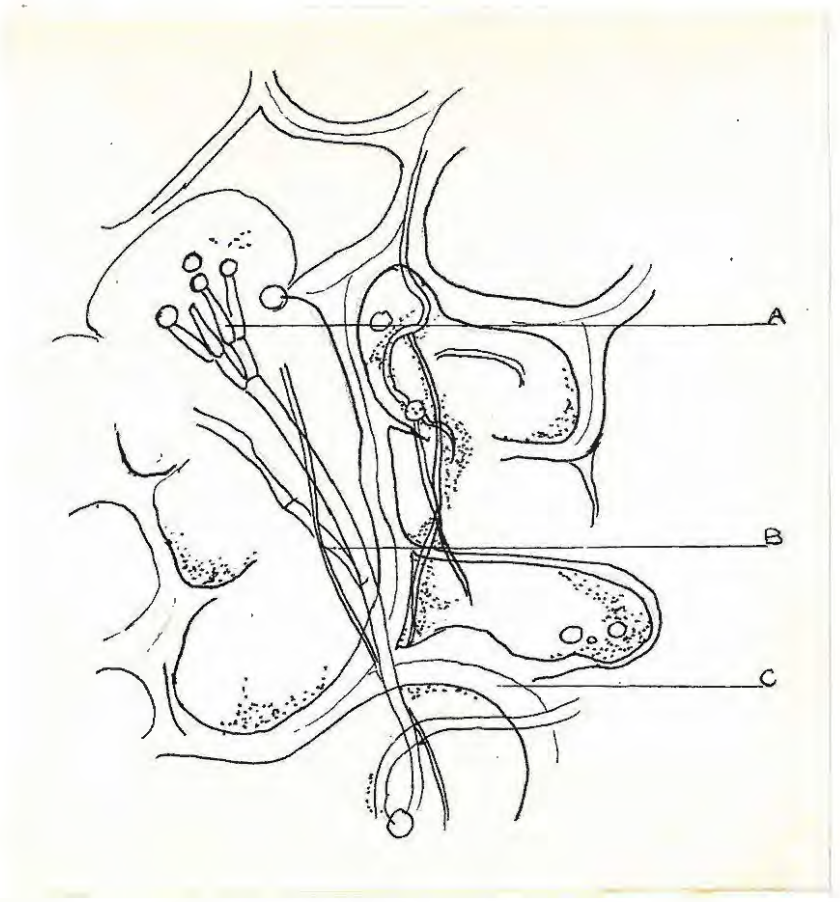


Fig.7. Drawing of Infected Tissue from Black Spot Area.
A. Penicillus of fungus.
B. Hyphae of fungus.
C. Thickened cell walls.

Fig.8. Drawing of Infected Tissues in Advanced Stages of Black Spot Disease.
The cell walls have completely broken down, and the cells are filled with a mass of hyphae, conidia and bacteria, and remains of the cell contents.

at this stage generally show blue-green spore masses lining the ovarial loculi and fissures of the infected area. (see Figures 7 and 8)

III

STUDY OF THE FUNGI ASSOCIATED WITH BLACK SPOT

A. Organisms isolated from black spot areas and their pathogenicity.

a) Organisms isolated.

In order to determine the fungal flora present in the black spot areas, isolations were made from diseased fruits, at various stages of development. Ripe and semi-ripe fruits were examined for any external symptoms, and then sliced with a knife. Notes were made on the type of black spot present, and the floral cavities were examined at the same time, for insects. The infected area was then cut out with a scalpel, (sterilized by dipping in alcohol and flaming) and transferred to a sterile Petri dish. Small pieces of tissue were then plated on to pineapple agar. The plates were incubated at 25°C and examined 24 hours later, when the resulting fungi were sub-cultured and pure cultures obtained. Stock cultures of the more prevalent fungi were kept on malt agar slopes, under normal laboratory conditions. Fruit examined in the field, was treated as above, the black spot areas being cut out and transferred to sterile specimen tubes, for transport to the laboratory.

The fruits were harvested over a period of five months, from January '54 to June '54. Of the 276 Cayenne fruits examined, 89 were infected with black spot, showing an average

Table I

Fungi isolated from diseased areas in Queen and Cayenne fruits.

Type of spot.	Fungus isolated									No. of spots.
	P	F	P-F	Th	Th-P	B-P	B-F	B-Th	B-Y	
Wet	49	28	18	2	1	5	1	1	1	106
Dry	19	7	4	-	-	1	-	-	-	31
Reddish	1	2	1	3	1	1	1	-	3	13
Pale gray	-	2	-	2	-	-	1	1	3	9
Total nos. fungi	69	39	23	7	2	7	3	2	7	159

P - P. funiculosum

F - F. moniliforme

Th- Thielaviopsis paradoxa

B - Bacterium Phytomonas (?)

Y - Yeast

of two black spot areas to each pineapple. Of the 178 Queen pineapples examined, 48 were infected, again with an average of two black spots per fruit.

The actual number of cultures made from black spot areas was 159, 100 cultures from Cayennes and 59 from Queens. The number of "wet" spots in both varieties altogether was 96, the number of "dry"spots 41, the remaining 22 infected areas were not typical black spots, but either reddish-brown or a pale, watery gray.

The fungus isolated most commonly from both wet and dry areas (see Table I) was Penicillium funiculosum Thom. Fusarium moniliforme Sheld. was isolated fairly frequently, both alone and in combination with P. funiculosum. Thielaviopsis paradoxa (de Seynes) Höhnel, was isolated occasionally, alone, and together with bacteria and P. funiculosum generally from reddish brown spots. A white bacterium, probably Phytomonas and an unidentified pink yeast were isolated together, and occasionally one or other with P. funiculosum or F. moniliforme. The bacteria and yeast were generally isolated from the pale gray type of spot - seldom from the typical black spot.

b) Pathogenicity of the fungi isolated from black spot.

In order to determine the pathogenicity of the two fungi isolated most frequently from black spot areas in pineapples, experiments were carried out in I. the field, and II. the laboratory.

I Field experiment.

The objects of this experiment were to determine:-

1. The pathogenicity of the fungi isolated from black spot areas.
2. Whether the age of the pineapple affected pathogenicity.
3. Whether the two varieties reacted differently to infection by the various fungal species.

With these aims in view, the several fungi available were introduced into the floral cavities, with and without accompanying damage to the underlying tissues. Both young and mature fruits of both Queen and Cayenne varieties were used.

The fungi used for inoculation were:-

1. Penicillium funiculosum Thom. isolated from black spots in fruit grown in the Division of Albany.
2. Penicillium expansum Link. emend. Thom. isolated from black spot areas, by the Division of Plant Pathology, Pretoria.
3. P. funiculosum. Thom., strain A: strain without profuse aerial hyphae; isolated from necrotic fruitlets infected with yellow spot virus as well as from black spot areas.

4. P. funiculosum Thom., strain B: strain with profuse development of aerial hyphae; isolated from necrotic, yellow spot virus areas, as well as from black spot.
5. Fusarium moniliforme Sheld. var. subglutinans Wr. and Rg. isolated from black spot areas.
6. F. moniliforme Sheld., var. subglutinans Wr. and Rg: isolated from black spot by the Div. of Plant Pathology, Pretoria.
7. Mixed fungal species: isolated by the Div. of Plant Pathology, Pretoria, from black spot. Identified as P. funiculosum Thom. and F. moniliforme Sheld.

These fungi were grown in Petri dishes on pineapple agar, under uniform conditions, at 25°C. They were ready for use as inocula after about 7 - 10 days growth.

Method of inoculation.

Fifty-six, uniform plants, (28 of each variety) were selected from double rows of pineapple plants growing at Bathurst Experimental Station. One series consisted of seven fruits, either in flower or in bud, the second series of seven fruits comprised pineapples which had just finished flowering, the flowers being withered and the fruit still green. Each series was duplicated for both Queens and Cayennes, and each fruit was treated with only one fungus, as follows.

Five fruitlets, (or "eyes") were selected at various positions on the fruit, and the surface of each sterilized by

swabbing with 95% alcohol. When this had dried off, an incision was made with a scalpel, (sterilized by dipping in alcohol and flaming over a spirit lamp) in such a way as to cut through the top of the fruitlet, and to expose the floral cavity, without completely removing the fleshy bract and calyx (see Figure 9).

A small portion of agar (about $\frac{1}{2}$ cm. square) including the fungal mycelium was then introduced into the floral cavity of one of the fruitlets, by means of a sterile spatula. The flap of the fleshy bract was pressed down over this and covered by a piece of sterile cotton wool. One other fruitlet was treated as above, but instead of the fungus, sterile pineapple agar was used as a control.

The remaining fruitlets were treated in the same way, plus the addition of a) slight damage, and b) extensive damage to the tissues of the fruit. This was effected in a) by puncturing the skin at the base of the floral cavity by means of a sterile needle, and then introducing the inoculum; and in b) by pushing the sterile needle to a considerable depth into the fruit and making a deeper puncture. A control fruitlet was also treated as under b), and sterile pineapple agar, introduced in place of the fungus. All the fruitlets were sealed by pressing back the cut bract and calyx, and packing with sterile cotton wool. Labels were then attached to the cotton wool over each treated fruitlet, indicating the type of inoculation and $\frac{3}{4}$" cotton tape was used to tie round the fruit,

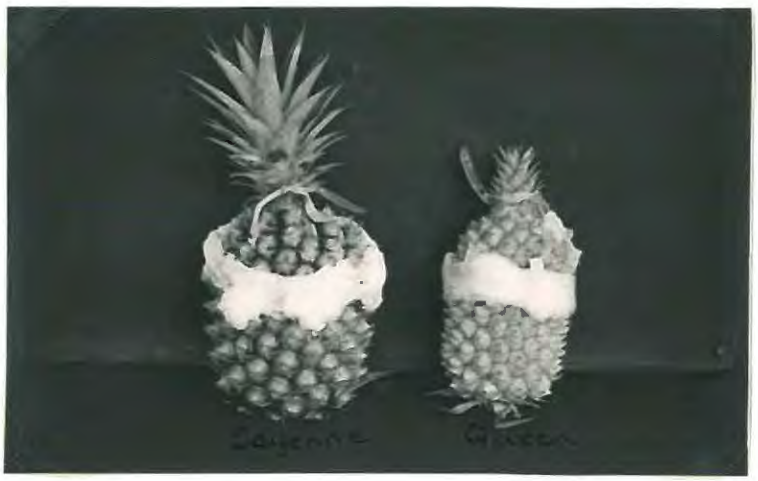


PLATE 3 - FRUITS AFTER INOCULATION

Method of securing treated fruitlets.

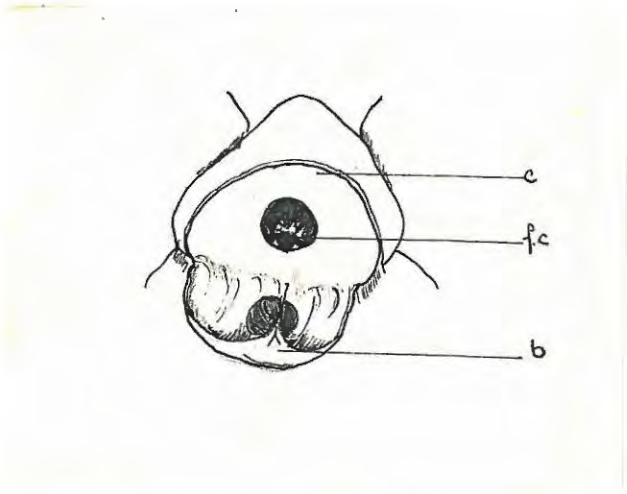


Fig.9. Diagram illustrating Method of exposing Floral Cavity.
f.c.,floral cavity; b,bract pulled back; c,cut surface of fruitlet.

keeping cotton wool and labels in place. (see Plate 3)

Harvesting of inoculated fruits.

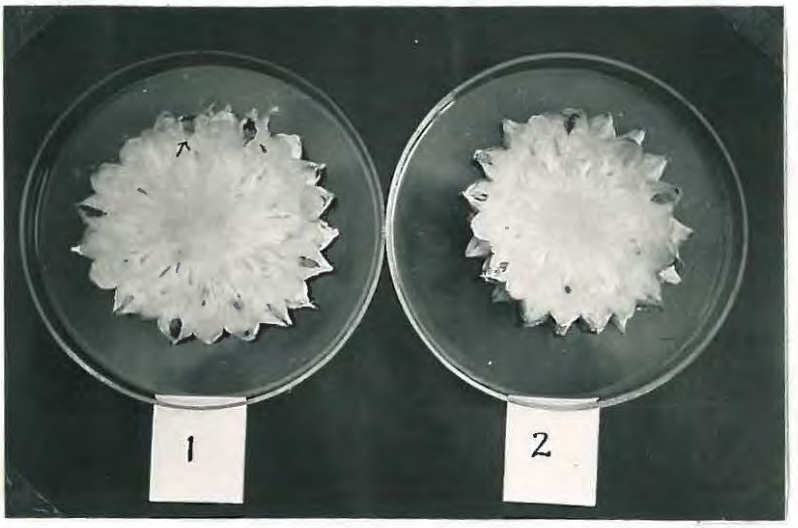
The mature pineapples of both Queen and Cayenne varieties ripened within 9 days, and were harvested at the beginning of February. One series of Cayennes and one of Queens, inoculated at the end of the flowering period, ripened 56 days later. The remaining fruits inoculated prior to, and during flowering, were ready for harvesting 95 days from the date of treatment.

The fruits were picked when ripe, and taken to the laboratory, where they were examined. Longitudinal cuts were made through the inoculated fruitlets (ie. transverse cut of the fruit) with a sterile knife, after sterilizing the surface in the usual way with alcohol. Notes were made on the condition of the underlying tissues, which were examined for changes in texture and colour. Small portions of affected tissue, (from fruitlets inoculated with extensive damage to floral cavity, and their controls) were removed under sterile conditions, plated on to pineapple agar and incubated at 25°C, until sufficient growth had taken place to permit identification of the fungi so isolated.

Results of inoculation (see Table II Plates 4 - 15).

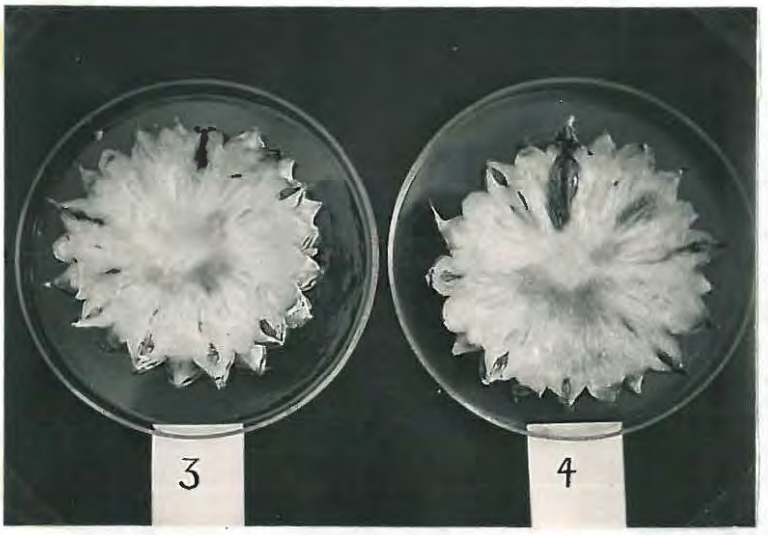
In every case, the flesh below the floral cavities of fruitlets inoculated without damage to the underlying tissues, and their controls, was unaffected, showing no signs of discolouration, or breakdown of cells resulting in fissures.

Results of Inoculation with Penicillium funiculosum.



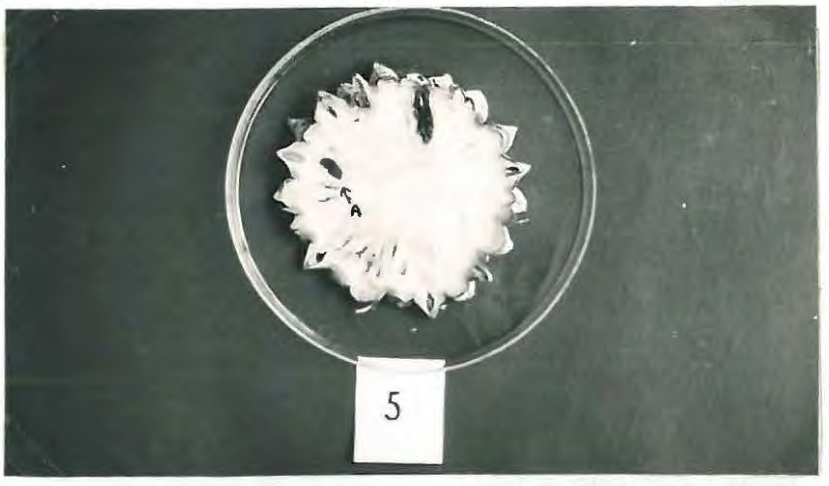
1, Control fruitlet inoculated with sterile agar, without damaging cavity.

2. Fruitlet inoculated with fungus, without damaging floral cavity.



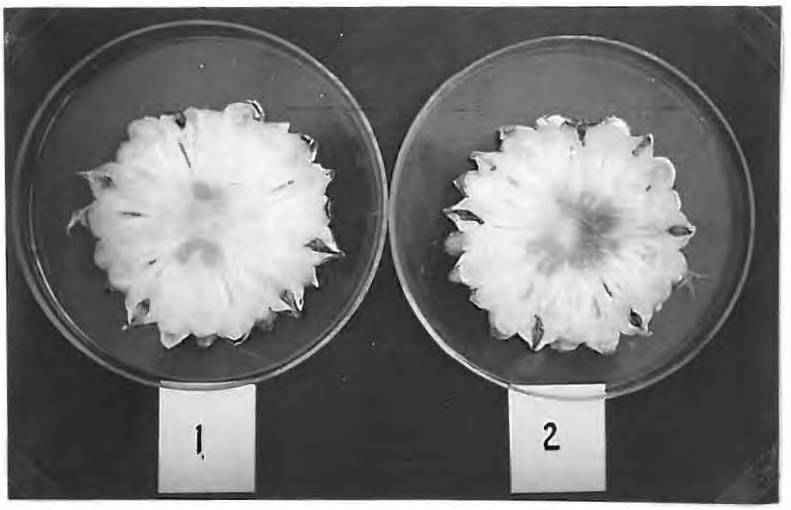
3. Fruitlet inoculated with fungus, with slight damage to cavity.

4. Fruitlet inoculated with fungus, and extensive damage to cavity.

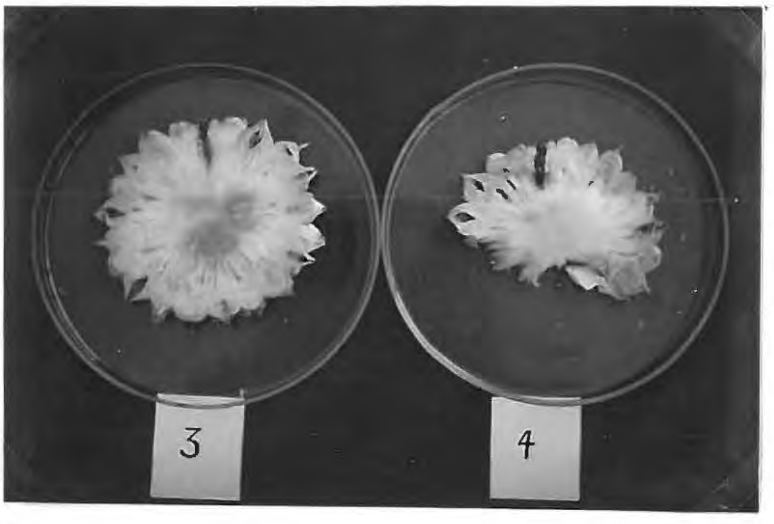


5. Control fruitlet inoculated with sterile agar, and extensive damage.
(A. Naturally produced black spot.)

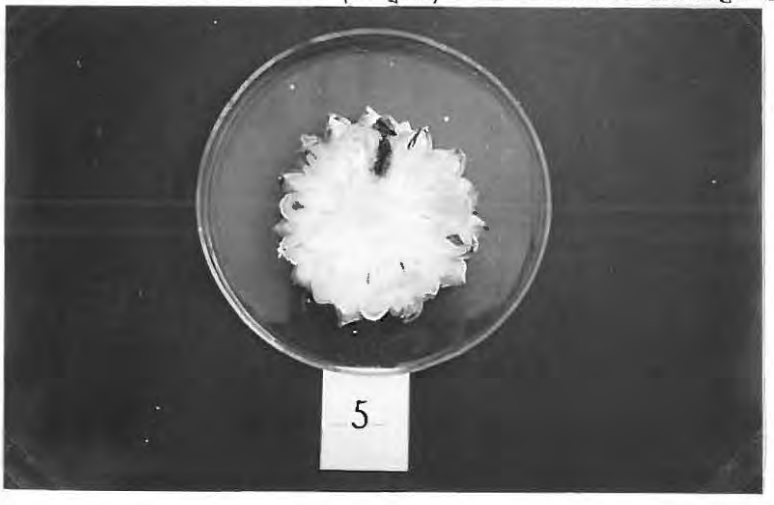
Results of Inoculation with Penicillium expansum



1. Control fruitlet inoculated with sterile agar, without damaging cavity.
   (Discolouration around core, produced naturally)
2. Fruitlet inoculated with fungus, without damaging cavity.

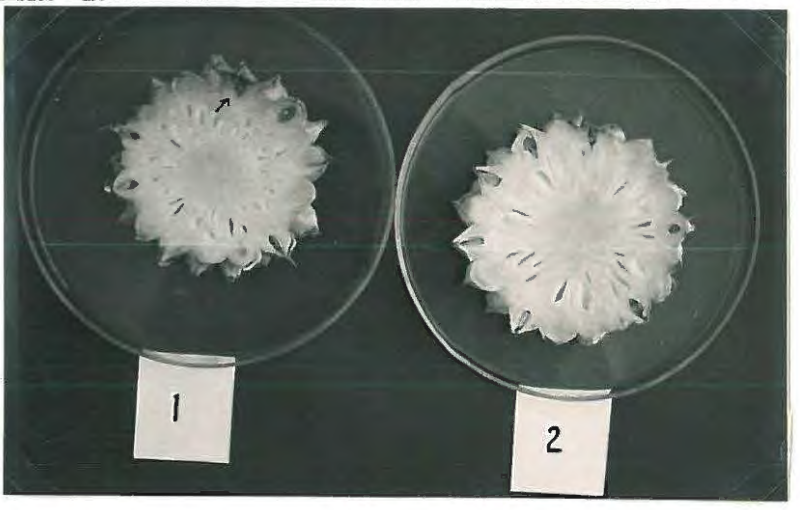


3. Fruitlet inoculated with fungus, and slight damage to cavity
4. Fruitlet inoculated with fungus, and extensive damage to cavity



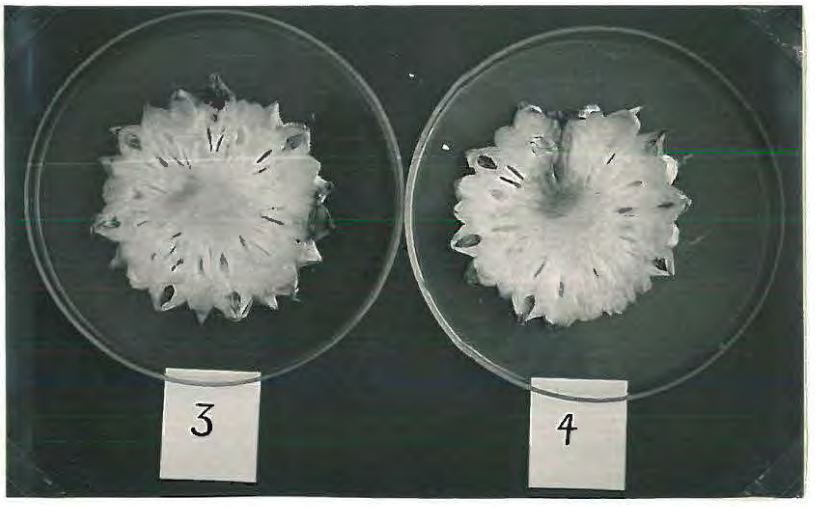
5. Control fruitlet inoculated with sterile agar, and extensive damage.

Results of Inoculation with Fusarium moniliforme



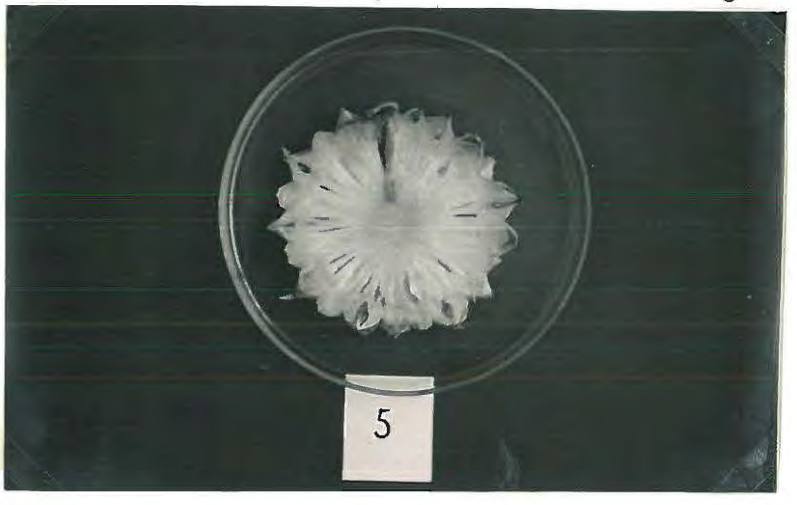
1. Control fruitlet inoculated with sterile agar, without damaging cavity.

2. Fruitlet inoculated with fungus, without damaging floral cavity.



3. Fruitlet inoculated with fungus, and slight damage to cavity.

4. Fruitlet inoculated with fungus, and extensive damage to cavity.



5. Control fruitlet inoculated with sterile agar, and extensive damage.

Field Experiment.

TABLE II.

Results of inoculation in treated fruitlets of pineapple.

1. Young Queens.

Fungal Inoculum	Method used in inoculation (a) no damage		(b) slight damage		(c) extensive damage		Controls -sterile agar (a) no damage		(c) extensive damage	
	RI	RII	RI	RII	RI	RII	RI	RII	RI	RII
P.funiculosum	-	-	+D	+WD	+D	+WD	-	-	+D	+D
P. -do - strain A	-	-	+W	+WD	+D	+W	-	-	+WD	+W
P. - do -strain B	-	-	+	+	+W	+D	-	-	+D	+D
P. expansum	-	-	+W	+	+D	+WD	-	-	+D	+WD
F. moniliforme	-	-	+	+W	+WD	+WD	-	-	+WD	+WD
- do - from Pretoria	-	-	-	+	+W	+D	-	ø	+D	+WD
Mixed species	-	-	+W	ø	+D	ø	-	-	ø	ø
2. Mature Queens.										
P.funiculosum	-	-	+	+D	+W	+D	ø	-	+W	+D
P. -do- strain A	-	-	+	+	+D	+D	-	-	+D	+D
P. -do- strain B	-	-	+	+	+D	+D	-	-	+W	+D
P. expansum	-	-	+	+	+W	+D	-	-	+W	+D
F. moniliforme	-	-	+	+	+O	+D	-	-	+O	+O
F. -do- from Pretoria	-	-	+	+D	+WD	+D	-	-	+WD	+D
Mixed species	-	-	+	+D	+O	+D	-	-	+O	+D
3. Young Cayennes.										
P.funiculosum	-	-	+D	+D	+D	+D	-	-	+D	+D
P. -do - strain A	-	-	+D	+D	+D	+O	-	-	+D	O
P. -do - strain B	-	-	+D	+W	+D	+D	-	-	+D	+D
P. expansum	-	-	-	-	+D	+D	-	-	+D	+D
F. moniliforme	-	-	+D	+W	+D	+D	-	-	+D	+D
F. -do- from Pretoria	-	-	+O	+D	+D	+WD	-	-	+WD	+W
Mixed Species	-	-	+D	+D	+D	+D	-	-	+D	+D
4. Mature Cayennes.										
P.funiculosum	-	-	+	+	+D	+D	-	-	+WD	O
P. -do- strain A	-	-	+D	+D	+D	+D	-	-	+D	+D
P. -do- strain B	-	-	+D	+D	+D	+D	-	-	+D	O
P. expansum	ø	-	+	+	+D	+D	-	-	+D	+D
F. moniliforme	-	-	+D	-	+D	+D	-	-	O	+D
F. -do- from Pretoria	ø	-	-	+	+W	+D	-	-	+D	+D
Mixed species	-	-	+	+O	+W	+O	-	-	+W	+W

\+ indicates production of black spot.
\- " no production of black spot.
D " dry spot.
W " wet spot.
WD " intermediate type of spot.
O " other types viz: red-brown and pale grey.
ø " fruitlets destroyed by rats and birds in the field.

Black spot under column (b) often too small to determine type.

Those fruitlets which were inoculated with slight, and with extensive damage to the base of the floral cavity, and the controls, (with extensive damage) all showed discolouration of the underlying tissues, varying from light brown to dark brown and in a few cases, reddish brown. In the majority of the fruitlets inoculated prior to and during flowering, there was breakdown of the cell walls, resulting in the formation of fissures with brown, corky walls - the same condition as is referred to as "dry spot." This type of spot was more prevalent in the Cayenne fruitlets. The remainder of the treated fruitlets showed discolouration of the tissues, but without any callosing - this condition is referred to as "wet spot."

An intermediate condition, combining the characteristics of both wet and dry spot, was produced in some fruitlets mainly those of the young Queens inoculated during flowering. A small number of inoculated fruitlets gave rise to a watery, gray-brown condition of the tissues below the floral cavity, (see Table III). In these cases small black beetles - (Nitidulidae) were often noticed crawling over the treated fruitlets, and reisolation generally yielded a white bacterium, probably Phytomonas, and an unidentified pink yeast.

In the case of dry spot it would seem that the development of callosed tissue in the fruit, acts as a barrier to the developing fungus, thus preventing the spread of the disease.

In the case of the wet spot condition, there is no such resistance, and the fungus spreads unchecked within the fruit. This is borne out by the fact that larger areas are involved in wet spot, than in dry spot, which in treated fruitlets, is limited mainly to the line of puncture.

Results of reisolations from treated fruitlets.

An average of 82% of the reisolations from black spot areas, produced as a result of inoculating fruitlets with a specific fungus and accompanying extensive damage, yielded the same fungus as that used in the inoculum. (see Table IV) Of these fruitlets, 27% yielded other fungi as well as those used in the inocula.

Penicillium funiculosum Thom. was the fungus isolated most frequently in association with the original inoculant, and Fusarium moniliforme Sheld. less frequently. Thielaviopsis paradoxa (de Seynes) Höhnel, and the white bacterium Phytomonas? were occasionally isolated.

Reisolations, made from black spot areas produced in control fruitlets, (inoculated with sterile agar and accompanying damage) gave P. funiculosum in 46% of the areas investigated. A mixture of P. funiculosum and F. moniliforme was found in 30% of the control fruitlets, and F. moniliforme alone, in 10%. Thielaviopsis paradoxa, Phytomonas?, and an unidentified yeast, were isolated occasionally, the former mainly in conjunction with P. funiculosum and F. moniliforme. (see Table VI)

TABLE III (see Table VII)

Type of "spot" produced by inoculations in pineapple fruits

Type of spot	QUEEN FRUITS						CAYENNE FRUITS						Total nos. of spots.
	Young		%	Mature		%	Young		%	Mature		%	
Black spot	RI	RII	Spot	RI	RII	Spot	RI	RII	Spot	RI	RII	Spot	
Dry	8	4	48.0	3	13	57.1	13	10	82.1	9	10	67.8	70
Wet	2	2	16.0	5	-	17.9	-	1	3.6	3	1	14.3	14
Intermediate	3	6	36.0	2	-	7.1	1	1	7.1	1	-	3.6	14
Other types													
Red-Brown	-	-		4	1	17.9	-	-		-	1	3.6	6
Gray	-	-		-	-		-	2	7.1	1	2	10.7	5
Total no.of spots in each series.	13*	12*		14	14		14	14		14	14		109

Black spot types examined were produced in fruitlets inoculated with fungus and accompanying extensive injury to floral cavity; and their controls.

* 3 fruitlets eaten out by rats or crows in the field.

TABLE IV.

Re-isolations from fruitlets inoculated with a particular fungus.

Fungus inoculated	QUEEN FRUITS				CAYENNE FRUITS				No. of times original inoculant re-isolated.
	Young		Mature		Young		Mature		
	RI	RII	RI	RII	RI	RII	RI	RII	
P.funiculosum	+	+	+	+	+	+	+	+	8/8
P. -do - strain A	+	+ +	+	+	-	+	+	+	7/8
P. -do- strain B	+	+	+	+	+	-	+	+	7/8
P. expansum	+ +	+	+	+ +	+	+	+ +	+ +	8/8
F. moniliforme	+ +	+ +	+	-	+	+ +	-	+	6/8
F.-do- from Pretoria	+ +	+ +	-	+ +	+ +	+ +	+	+	7/8
Mixed species	-	Ø	F	+	F	+	F +	F	2/7
No. of re-isolations in each series.	7	6	7	7	7	7	7	7	45/55

All re-isolations were made from black spot areas produced by inoculation with fungus and accompanying extensive injury to the base of the floral cavity.

R indicates replicate series. + indicates re-isolation of original fungus only.
+ + indicates re-isolation of original fungus and contaminant fungus as well.
- indicates no re-isolation of original fungus.
Ø indicates no re-isolation of any fungus, as the fruitlets had been eaten away by either crows or rats, while in the field.

Mixed species: In the case of re-isolations from fruitlets inoculated with a mixture of the two fungi P.funiculosum and F.moniliforme:- F indicates that only Fusarium moniliforme was isolated.
+ indicates that both were re-isolated.

TABLE V (See Table IV)

Fungi isolated in addition to inoculant P.funiculosum (Strain "A")

Fungi isolated	QUEEN FRUITS				CAYENNE FRUITS.			
	Young		Mature		Young		Mature	
	RI	RII	RI	RII	RI	RII	RI	RII
Fusarium moniliforme					+			
Bacteria		+ +						
Fungi isolated in addition to inoculant P.funiculosum (Strain "B")								
F.moniliforme						+		
Fungi isolated in addition to inoculant P.expansum								
P.funiculosum							+ +	
F.moniliforme	+ +							+ +
Thielaviopsis paradoxa				+ +				
Fungi isolated in addition to inoculant F.moniliforme								
P.funiculosum	+ +	+ +				+ +	+	+ +
Bacteria				+				
Fungi isolated in addition to inoculant F.moniliforme (ex Pretoria)								
P.funiculosum	+ +	+ +		+ +	+ +	+ +		
Thielaviopsis paradoxa				+ +				
Bacteria			+					
Fungi isolated in addition to inoculant mixed species.								
Bacteria	+						+ +	

+ indicates fungus isolated in place of original inoculant.
+ indicates fungus isolated in addition to original inoculant.

TABLE VI

Fungi isolated as contaminants from control fruitlets (with damage).

Fungus isolated.	QUEEN FRUITS				CAYENNE FRUITS				Total nos. of each fungus isolated.
	Young		Mature		Young		Mature		
	RI	RII	RI	RII	RI	RII	RI	RII	
P. funiculosum	3	2	3	1	4	2	3	3	21
F. moniliforme	-	-	1	-	-	2	1	1	5
Penicillium & *Fusarium*	2	2	2	2	3	2	1	1	15
Penicillium & *Thielaviopsis*	-	-	-	1	-	-	1	-	2
Fusarium & *Thielaviopsis*	1	-	-	2	-	-	-	-	3
Fusarium & Bacteria	-	-	1	1	-	-	-	-	2
Bacteria & yeast	-	2	-	-	-	1	1	2	6
No. of isolations in each series	6*	6*	7	7	7	7	7	7	54

* 2 fruitlets eaten out by rats in field.

Discussion and Conclusions.

From the foregoing, it is apparent that the fungi most frequently associated with the condition of black spot are Penicillium funiculosum Thom. and Fusarium moniliforme Sheld. Either fungus, by itself, or in conjunction with the other, is capable of producing breakdown of the tissues, and discolouration, irrespective of the age or variety of the fruit (see Table VII).

As all the control fruitlets inoculated, (with accompanying damage to the base of the floral cavity,) gave rise to black spot, from which P. funiculosum and F. moniliforme were isolated, it must be concluded that these fungi are present in the floral cavities of fruit, as spores, and once the epidermis is broken in any way, they germinate and are able to invade the underlying tissues, with ensuing development of black spot. Further experiments to determine whether pathogenic fungi are actually present in the floral cavity, and to identify them have been undertaken, and are reported in section B.

It will be seen from Table IV that fruitlets inoculated with F. moniliforme and P. expansum, but particularly the former, have more contaminant fungi on reisolation, than fruitlets where the original inoculant was P. funiculosum. This may indicate an inhibitory action exercised by Penicillium funiculosum on other fungi, and is possibly an explanation of the frequency with which it appears in association with

TABLE VII (See Table III)

Type of black spot produced by fungus isolated from treated fruitlets

Fungus isolated from black spot	Dry Spot	Wet Spot	Inter-mediate	Other types	Total no. of times fungus isolated.
P. funiculosum and *P. expansum*	34	7	5	1	47
F. moniliforme	9	2	3	-	14
Penicillium and *Fusarium*	18	4	6	-	28
Thielaviopsis with *Penicillium*/*Fusarium*	7	-	-	-	7
Bacteria with *Penicillium*/*Fusarium*	-	-	-	3	3
Bacteria and yeast	2	1	-	7	10
Total number of spots	70	14	14	11	109*

Isolations were made from fruitlets showing black spot produced by inoculations with fungus, and accompanying extensive injury to the base of the floral cavity, and their controls.

* 3 fruitlets eaten out by rats or crows in the field.

black spot areas. However, F. moniliforme seems to grow well in conjunction with it, (see Table V) provided the Fusarium gets a better start, as is the case when both hyphae and spores are used in the inoculum - the fungus thereby gaining the advantage over contaminant fungi (viz. Penicillium) which are probably present as spores only. Evidence of a stimulatory reaction exercised by F. moniliforme, has been established by Heald and Poole, (1908) who secured an abundance of perithecia of Melanospora pampeana, by growing it with a mixture of fungi including F. moniliforme.

From an examination of Table II, it also appears that the inoculated fruitlets of any one fruit, show a marked tendency to produce the same types of spot - either wet or dry. This supports the view, that conditions within the fruit, are the deciding factors in the production of wet or dry spot, and not the presence of a particular fungus. (see Table VII)

II Laboratory Experiments.

Method.

1. Fifteen mature Cayenne pineapples were kept in the laboratory, and inoculated with accompanying injury to the base of the floral cavity, using the fungi Penicillium funiculosum Thom. and Fusarium moniliforme Sheld. (The methods employed were the same as in the field experiment). After inoculation the fruits were kept under bell jars for ten to

fourteen days, thus increasing the humidity of the atmosphere in which they ripened. Control fruitlets were treated in the same way, but sterile pineapple agar was used as the inoculum.

2. Nineteen young pineapples, of both Queen and Cayenne varieties, were inoculated, as above, before the flower buds were fully developed, using the same fungi P. funiculosum and F. moniliforme. The fruits were allowed to ripen in the laboratory without being placed under bell jars. They were examined after 2 - 3 weeks, by which time the flowers had opened, and were beginning to wither.

Results of inoculation. (see Table VIII)

1. Conditions of increased humidity had no effect on the amount of damage caused by the fungi inoculated, but the majority of black spot produced was of the wet spot type. (see Chap. IV section B.) Controls also showed wet black spot.

2. In the 19 very young fruits, the damage produced in every case was in the form of callosed streaks, strictly limited to the line of puncture. Control fruitlets showed no discolouration.

These results indicate that in young fruits, fungal invasion does not proceed as rapidly as in mature fruits, either because the young fruit has greater powers of resisting and checking the advance, by suberization of the cell walls, or else

TABLE VIII

Laboratory Experiment 1.

Type of black spot produced by fungus isolated from treated fruitlets.

Fungus Isolated	Wet Spot	Dry Spot	Inter-mediate type	Red-brown/ pale gray types	Total nos of fungi
P.funiculosum	13	-	2	-	15
P. expansum	3	-	-	-	3
F. moniliforme	2	-	1	-	3
P. funiculosum + F. moniliforme	3	-	-	1	4
Thielaviopsis	1	-	-	1+P*	2
Phoma sp.	1	-	-	-	1
Total no of spots	23	0	3	2	28

Isolations made from fruitlets inoculated with fungus and their controls.

Two fruitlets contaminated - no cultures made.

* Penicillium sp. isolated as well as Thielaviopsis .

providing conditions unfavourable to the growth of the fungus.

Results of reisolation from treated fruitlets.

1. Reisolation of fungi from the damaged areas of the mature fruits, yielded the original inoculant in all cases; and in two cases, the original fungus, as well as another contaminant fungus. (see Table IX) These results show a higher number of contaminant free inoculations than in the field experiment; a probable explanation being the more sterile conditions possible when working in the laboratory rather than the field. (see below)

The control fruitlets, however, were contaminated in almost every case (see Table X). The fungus isolated most frequently was, as in the field experiment, Penicillium funiculosum Thom.

This discrepancy between the lack of contaminant fungi in those fruitlets inoculated with Penicillium funiculosum and Fusarium moniliforme, and the presence of contaminant fungi in the control fruitlets, despite increased conditions of sterility in the laboratory, may be accounted for in the following way:-

As fungal growth is favoured by increased humidity, (see Chapt. IV) rapid growth of the fungal inoculant might take place at the expense of other fungi only present in spore form in the floral cavities. (see section B.) In the control fruitlets, these fungal spores would be able to germinate without competition from fungal hyphae introduced in the inoculum.

TABLE IX

Laboratory Experiment 1.

Re-isolations from fruitlets inoculated with a particular fungus.

Fungus inoculated	MATURE CAYENNES RI	RII	RIII	No.of times inoculant re-isolated	Fungus isolated in addition to inoculant RI	RII	RIII
P.funiculosum	+	+	+	3/3	-	-	-
P. " Strain "A"	+	+	0	2/2	-	-	-
P. " Strain "B"	+ +	+	+	3/3	Thielaviopsis	-	-
P. expansum	+	+	+	3/3	-	-	-
F.moniliforme	+	+ +	+	3/3	-	Penicillium sp.	-
Total no. of re-isolations	5	5	4	14/14	1	1	0

0 - fruitlet contaminated on examination.
\+ - indicates re-isolation of original fungus.
\+ + - indicates re-isolation of original fungus together with a contaminant ~~and~~ fungus.

TABLE X

Laboratory Experiment 1.

Fungi isolated as contaminants from control fruitlets

Fungi Isolated	MATURE CAYENNES			No. of times fungus isolated.
	RI	RII	RIII	
P.funiculosum	3	2	2	7
F. moniliforme	-	1	1	2
Penicillium + Fusarium	1	2	-	3
Thielaviopsis	1	-	-	1
Phoma sp.	-	-	1	1
Total no. re-isolations	5	5	4*	14

* One re-isolation missing under RIII yielded no fungus.

TABLE XI

Laboratory Experiment 2.

Re-isolation from treated fruitlets producing same fungus as inoculium.

Fungus inoculated	YOUNG QUEENS & CAYENNES					No. of times original fungus re-isolated.
	RI	RII	RIII	RIV	RV	
P.funiculosum	+	+	+	+	+	5/5
P. expansum	+	+	+	+	+	5/5
F.moniliforme	+	+	+	+	+	5/5
F. moniliforme + P.funiculosum	+	+	+	+		4/4
Total no re-isolations	4	4	4	4	3	19/19

All these black spots were the "dry" spot type.
Control fruitlets were all negative - no contaminants isolated.

2. Reisolations from the black spot areas in the young pines, produced the same fungus as used in the inoculum in every case. (see Table XI) Control fruitlets gave no contaminants, though bacteria were present in a few cases.

Summary of Conclusions.

1. The fungi investigated are pathogenic when introduced into the tissues of the pineapple fruit. As they cannot invade the flesh unless the epidermis has been broken beforehand, they are thus a secondary cause of the disease.

2. In the case of Penicillium funiculosum Thom. there is a probable inhibitory action on other fungi, and in the case of Fusarium moniliforme Sheld. there is a probable stimulatory action.

3. There is no correlation between any specific fungus and the production of either wet or dry spot. All the fungi investigated caused both types of spot.

4. Increase of humidity during ripening, favours development of wet spot.

5. The age and variety of fruit at which inoculation is effected seems to affect the type of spot produced; dry spot is more prevalent in young fruit, and in Cayennes.

6. The age and variety of the pineapple fruit does not affect the pathogenicity of the fungi.

B. Organisms isolated from the floral cavities.

In view of the fact that fruitlets inoculated with sterile agar, and accompanying injury to the base of the floral cavity, consistently produced black spot areas, from which various fungi were isolated, (see Tables II and VI) experiments were undertaken to determine, a) what fungi constituted the flora of these cavities, in so far as cultural methods can give an indication of the natural flora. Further investigations were made into, b) methods of avoiding contamination of inocula by fungi in the cavities, by I) sterilization of the floral cavities, and II) extra-floral cavity punctures.

a) Isolation of organisms from the floral cavities.

Method.

Pineapple fruits of both varieties, at all stages of development, were examined in the following way:
The surface of the fruit was sterlized by swabbing with alcohol. The fruitlets selected, (at random) were then cut so as to expose the floral cavity, using a scalpel sterilized by dipping in alcohol and flaming. Scrapings were then taken from inside the cavities using a sterile rod, and these, together with the bases of the withered stamens, which were removed from the cavity with sterile forceps, were plated on pineapple agar. The plates were incubated at 25°C and examined after 24 hours, when isolates from the resultant fungi were subcultured for identification.

The total number of floral cavities examined was 56, made up of 44 mature fruits of Queen and Cayenne varities, and 12 young fruits. (At the time this experiment was undertaken, few young fruits were obtainable).

Results. (summarised in Tables XII and XIII)

The fungus found most frequently in the cavities was Penicillium funiculosum, which was isolated 20 times. Less frequently, Fusarium moniliforme and Thielaviopsis paradoxa were each isolated 12 times. Alternaria sp. and Phoma sp. were found in eleven cavities and ten cavities respectively. Fungi occurring occasionally were Botrytis sp. probably B. cinerea, Trichoderma viride, and an unidentified yeast. Bacteria and Actinomycetes were found in a few cavities. The flora of the cavities of young fruits, does not differ from that of mature ones.

The fungi P. funiculosum and F. moniliforme involved in the production of black spot, are thus shown to be present in floral cavities of healthy fruits.

Table XII, Fungi isolated from floral cavities.

No. of fungi isolated per cavity.	No. of Cavities Fungus isolated from.	Fungi isolated
1	9	*Phoma* sp.
	6	*Penicillium funiculosum*.
	5	*Thielaviopsis paradoxa*.
	3	*Alternaria*.
2	4	*P. funiculosum* + *F. moniliforme*.
	3	*P. funiculosum* + *T. paradoxa*.
	4	*F. moniliforme* + *T. paradoxa*.
	3	*F. moniliforme* + *Alternaria* sp.
	2	*P. funiculosum* + *Alternaria* sp.
	1	*Phoma* sp. + *Alternaria* sp.
	1	Yeast + bacteria.
	8	Actinomycetes + bacteria.
3	2	*P. funiculosum* + *Botrytis* + *Trichoderma*.
	3	*P. funiculosum* + *Alternaria* + *Cephalosporium*.
	1	*Botrytis* + *Alternaria* + *Trichoderma*
4	1	*F. moniliforme* + *Botrytis* + *Alternaria* + *Trichoderma*.

Table XIII.

Number of times a particular floral cavity fungus was isolated.

Fungal Species.	No. of times isolated.
Penicillium funiculosum.	20
Fusarium moniliforme.	12
Thielaviopsis paradoxa	12
Alternaria sp.	11
Phoma sp.	10
Botrytis sp.	4
Trichoderma sp.	4
Cephalosporium sp.	3
Yeast.	1
Actinomycetes.	8
Bacteria.	9

(Total number of cavities - 56)

b) Methods of avoiding contamination by fungi in the floral cavities.

I) Sterilization of the floral cavities.

Method.

Ten mature Cayennes were picked while still green, (Queen pineapples were not available at the time of the experiment) and on each fruit, two fruitlets were selected on opposite sides of the fruit. One fruitlet acted as a control, while the other was inoculated with Penicillium funiculosum or Fusarium moniliforme or a mixture of both fungi. The surface of the fruitlet was sterilized by swabbing with alcohol, then, using a sterile scalpel, the fleshy bract and calyx were cut across without removing them entirely, to allow access to the floral cavity. Alcohol, (95%) was then pipetted into the cavity, taking care not to damage the flesh in any way. The excess alcohol was absorbed by plugging the cavity for a few minutes with sterile cotton wool, which was then removed, and the cavity allowed to dry. The base of the floral cavity was then punctured by means of a sterile needle, and the fungal inoculum introduced. In the control fruitlets the same procedure was followed, except that the inoculum consisted of sterile pineapple agar. The bract and calyx, (if the latter had not fallen away) were then pressed back into position over the inoculum, the fruitlet was covered with sterile cotton wool, with label attached, and the whole held in place by tape.

TABLE XIV

Re-isolation from fruitlets inoculated through sterilized floral cavities.

Inoculant	MATURE CAYENNES				No.of times inoculant re-isolated
	RI	RII	RIII	RIV	
Penicillium funiculosum	+	+	+	+	4/4
Fusarium moniliforme	+	+	+	+	4/4
Penicillium & Fusarium funiculosum moniliforme	+	+	+	+	4/4
Control					No.of controls contaminated
Sterile pineapple agar	-	-	P*	-	1/4
" " "	-	-	P*B*	-	1 /4

*P = Penicillium sp.

*B = bacteria.

The fruits were allowed to ripen in the laboratory for a week or ten days, and then examined for production of black spot. The fruits were sliced with a sterile knife, and cultures, made in the usual way, from small portions of the flesh of the treated fruitlets.

Results of inoculation.

Inoculation with the fungi resulted in the production of black spot areas in all cases save one. The control fruitlets did not show black spot areas, the area round the line of puncture only appearing slightly gray.

Results of reisolation (see Table XIV),

The same fungus as that used in the original inoculant was reisolated in all cases. Reisolation from control fruitlets yielded Penicillium sp. in two cases, and a combination of bacteria and Penicillium sp. in one case.

Discussion and Conclusion.

From these results it is apparent that sterilizing the floral cavity, prior to injury, does reduce the amount of contamination by fungi present in it. Unfortunately sufficient pineapple fruits were not available to repeat the experiment, thus providing conclusive evidence. The reisolation of fungi from two of the control fruitlets can be explained by either, a) insufficient sterilization of the cavity, b) the presence of fungal spores resistant to the alcohol,or c) contamination during inoculation. The fact that no black spot was produced despite the isolation of a Penicillium sp., lends support to

the view that P. funiculosum is specifically pathogenic and responsible for black spot.

II) Extra-floral cavity punctures.

Method.

Five mature Cayennes were inoculated by means of puncturing the fruitlet in such a way as to avoid passing through the floral cavity.

In each pineapple, six fruitlets were selected (as the number of fruits available was limited) in a vertical line on one side of the fruit, and after swabbing with alcohol, the inoculum (P. funiculosum or F. moniliforme) was introduced through a needle puncture made at one side of the fruitlet, without passing through the floral cavity. The punctures were then sealed with a little melted paraffin wax, as cotton wool failed to prevent juice oozing out of the punctures thus carrying away the inoculum to a considerable extent. Two fruits were used for control fruitlets and treated in the same way, except that sterile agar was used as inoculum.

The fruit was allowed to ripen in the laboratory and then cut longitudinally, (with a sterile knife) to expose the damaged fruitlets. Cultures were made in the usual way from small portions of tissue from the treated fruitlets.

Results of inoculation.

Development of typical black spot areas occurred in fruitlets inoculated with the fungi. The control fruitlets, where

sterile pineapple agar was used, showed only a slight gray colour, and translucent appearance of the flesh at the site of the puncture. This latter effect may be due either to injury of cells and exudation of cell sap, or the effect of bacterial infection, or some fungus other than P. funiculosum and F. moniliforme.

Results of reisolation.(see Table XV)

Contamination of fruitlets inoculated with the fungi was low, ($^{3}/_{18}$), the original inoculant being isolated alone in almost all cases. The control fruitlets almost all showed contamination, reisolations yielding mainly bacteria and Penicillium sp.

Discussion and Conclusion

As considerable difficulty was experienced in getting the inocula into the punctures - this may be responsible for the contamination. The low incidence of contaminants in the fruitlets inoculated with fungus, may be due to the rapid establishment of the fungal inoculant at the expense of fungal contaminants present as spores.

This method of needle puncture outside the floral cavity does not appear to be any more satisfactory than that through the sterilized floral cavity. Where the floral cavity has been sterilized beforehand, it is more convenient to introduce the inoculum through it, than to force the inoculum into a small puncture, through which juice keeps flowing.

TABLE XV

Re-isolations from fruitlets inoculated without passing through cavities.

Inoculant	MATURE CAYENNES RI	RII	RIII	RIV	RV	RVI	No. of times inoculant re-isolated.
Penicillium funiculosum	+	+	+Th	+	+Th	+Th	6/6
Fusarium moniliforme	+	+	+	+	+	+	6/6
Penicillium & Fusarium funiculosum moniliforme.	+	+	+	+	+	+	6/6
Control							No. of controls contaminated
Sterile pineapple agar	B	B	B+F	P	P	P	6/6
" " "	F	P	P	P	B	P	6/6
" " "	B	P	B	B	P	P+F	6/6

+ indicates original inoculant only re-isolated.
+Th indicates original inoculant re-isolated together with Thielaviopsis.
B = bacteria.
P = Penicillium sp.
F = Fusarium sp.

C. Organisms associated with parts of the pineapple plant other than the floral cavities.

In order to shed further light on the occurrence and prevalence of the fungi associated with black spot, preliminary qualitative investigations of the fungal flora of a) the soil, b) the surface of the pineapple plant, and c) the flesh of undiseased fruits, were undertaken, in addition to those already described for floral cavities.

a) Organisms isolated from the soil.

Method.

Soil samples were taken at a depth of 6" from soil immediately below the pineapple plant. The soil was of two types 'light' or sandy soil, and 'heavy' or clay soil. Samples were taken from lands under cultivation from 2 - 3 years, and from old lands which had been under cultivation for 6 years.

About 20 gms. of soil were collected in a sterile specimen tube from beneath 3 plants in each soil category (2 - 3 year old lands - both light and heavy soils and 6 year old lands - both light and heavy soils). These samples from each type were lumped together and three soil plates prepared from each aggregate sample and treated as a unit. A fungus was given a positive record if it occurred on one or more plates, thus allowing for competitive growth and antagonism between fungi, and the chance absence of a fungus

from one particular plate. The absence of a fungus does not mean that it does not occur at that level in the soil, since the distribution of fungi is not uniform. (Warcup, 1951). Soil plates were made using pineapple agar (pH 4.6), Brown's agar (pH 5.8) and clear maize agar (pH 6.5) respectively, in each group of 3 plates. The plates were incubated at 25°C and examined at intervals over a period of 3 - 4 weeks.

The pH of the soils sampled was as follows:-

Light soils:	2 - 3 year old lands	pH 6.5
	6 year	pH 5.9
Heavy soils:	2 - 3 year	pH 6.0
	6 year	pH 5.75

Results. No differences in the fungal floras of the various soils were apparent. However, before any accurate conclusions can be drawn, more experiments both quantitative and qualitative, using samples from varying depths and at various seasons, must be made.

The following fungi were isolated from the soil plates:-

Alternaria sp. probably A. humicola Chaud.	Occurred very frequently; ie. more than twice in over two-thirds of the plates.
Trichoderma viride Pers. (ex Fr.	
Botrytis sp. probably B. terrestris Jensen.	
Phoma sp. probably P. humicola Oud.	
Penicillium expansum Link.	

Aspergillus sp. probably A. terreus Thom.	Occurred frequently; ie. more than once in two-thirds of the plates.
Verticilliastrum sp.	
Penicillium funiculosum Thom.	
Penicillium sp.	Occurred infrequently; ie. once in one-third of the plates.
Scopulariopsis sp.	
Fusarium moniliforme Sheld.	
Oospora sp. probably O. variabilis Lindau.	

Bacteria and Actinomycetes were frequently present.

These results show that the fungi associated with black spot, are present in the soil beneath the plants, but there is no marked prevalence in their numbers over that of other fungi. In a paper by Contois, (1953) on the microflora of the rhizosphere of the pineapple plant in Hawaii, the fungi isolated include the same genera as those isolated from the above soils. In the same report, he points out that the extreme variation obtained in the enumeration of fungi and Actinomycetes, precluded their further consideration in quantitative experiments.

b) Organisms from the surface of the pineapple.

Method.

Scrapings were made, (with a sterile scalpel) from the surface of the pineapple fruitlets, and from the leaves immediately surrounding the fruit, and plated on to pineapple - agar plates. The plates were then incubated at 25°C. 10 plates were made from scrapings from the leaves, and 10 plates from the surface of the fruitlets. The plates were

examined over a period of 4 weeks. Known fungi were recorded without further isolation, doubtful or unknown colonies were isolated for further investigation. A fungus was given a positive record if it occurred more than once.

Results. The fungi isolated were as follows:-

Alternaria sp probably *A. tenuis* Nees.	Occurred more than twice in over two-thirds (ie. 13) of the plates.
* *Monilia* sp. probably *M. humicola* Oud.	
Botrytis sp. probably *B. cinerea* Pers.	
Trichoderma viride Pers. (ex Fr.)	
Phoma sp. probably *P. humicola* Gilmann and Abott.	
Fusarium moniliforme Sheld.	Occurred more than once in two thirds of the plates.
Penicillium funiculosum Thom.	
Aspergillus sp.	
* *Cladosporium* sp.	Occurred once in one third (ie. 6) of the plates.
* *Thielaviopsis paradoxa* (de Seynes) Höhnel.	
Yeast.	Occurred once in any plate.
Cephalosporium sp. probably *C. curtipes* Saccardo.	
Pullularia sp.	
Stemphyliomma sp.	

Bacteria and Actinomycetes were isolated occasionally.

(* see end of p. 41.)

There was no particular prevalence of any one genus in any of the plates.

The fungi associated with black spot are therefore frequently present on the surface of the pineapple leaves and fruits - as would be expected.

C) Organisms isolated from flesh of undiseased fruits.

A trial experiment was undertaken using 26 young Queens, at pre-flowering, during flowering and after flowering stages. The fruits were cut under sterile conditions, and small portions of the flesh below healthy fruitlets were plated on to pineapple agar plates, which were then incubated at 25°C.

Growth of fungi on the plates indicated that very stringent precautions were necessary to avoid contamination from the fungi in the floral cavities, and establish completely sterile conditions, without which reliable data could not be assembled. This line of enquiry was left for study at a future date.

Conclusions.

While the foregoing experiments do show that the fungi associated with black spot are present in the soil and atmosphere, (though not, apparently, occuring with greater frequency than other genera) further work is necessary to establish whether or not there is any seasonal variation in the numbers and occurrence of spores of fungi concerned, and

whether or not the same fungi are found in natural land, or are a particular flora built up in response to the environment created by the pineapple plant.

* Fungi were submitted to the Division of Botany, Pretoria, for confirmation of identifications.

IV

THE STUDY OF BLACK SPOT FUNGI IN RELATION TO ENVIRONMENTAL CONDITIONS.

Both the susceptibility of the host, and the aggressive - ness of the pathogen are influenced by environmental factors. In order to discover how far the behaviour of the fungus in the host can be predicted from that in culture, it is necessary to establish what features of the fungus are constant, what influences may cause variation, and to what extent these variations may occur.

In dealing with the complex of factors which influence the reactions of both host and pathogen in nature, it is difficult to isolate the effects of any one particular condition; hence the results obtained in culture under constant laboratory conditions, cannot provide an entirely accurate parallel for behaviour of the fungus under natural conditions. While factors such as temperature and moisture are external conditions affecting the fungus, they cannot be separated from the internal conditions which are set up in response to them, and also influence the reaction of the fungus; viz. - the rate of accumulation and the concentration of staling products produced by the fungus, the changes produced in the character of the substrate following the initial reaction of the medium, and factors internal to the fungus itself, the age of the mycelium and physiological differences within the hyphae.

In this section the methods of culture and cultural characteristics exhibited by the fungus are described, followed by the reaction of the fungus to changes in temperature, moisture and H-ion concentration of the medium.

A. Cultural characteristics of the fungi.

a) Culture methods.

The three media used for culturing the fungi were pine-apple agar, malt agar, and Brown's synthetic agar, (see Appendix II). The fungi grew well on all these, showing steady growth with no development of abnormal spore or hyphal forms.

The fungi were cultured in 9 cm. Petri dishes, and a uniform depth of agar was maintained by using the same amounts of agar (generally 15 ccs.) when pouring plates. The plates were incubated at 25°C. Inocula used for making fresh cultures were small slabs of agar about 2 mm. square consisting of mycelium and spores. Stock cultures were kept on malt agar slopes in plugged test tubes, under ordinary laboratory conditions.

b) Description of cultural characteristics of fungi.

Penicillium funiculosum Thom. (see Figures 10 and 11, Plates 16 and 17)

The colonies are dark blue-green in colour, the reverse is dull yellow, later becoming red. The surface of the colony appears floccose, with ropes of hyphae bearing lateral conidiophores. The conidiophores sometimes arise directly from the substratum, giving the surface of the colony a powdery appearance. This condition seems to occur when the medium is old or when only a small quantity is available.

PLATE 17. PENICILLIUM FUNICULOSUM

X 1000 approx.

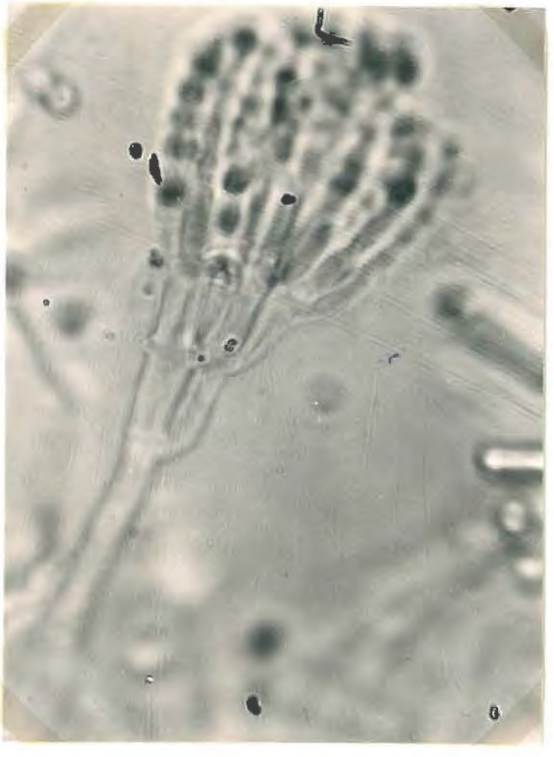


PLATE 18. PENICILLIUM EXPANSUM

X 1000 approx

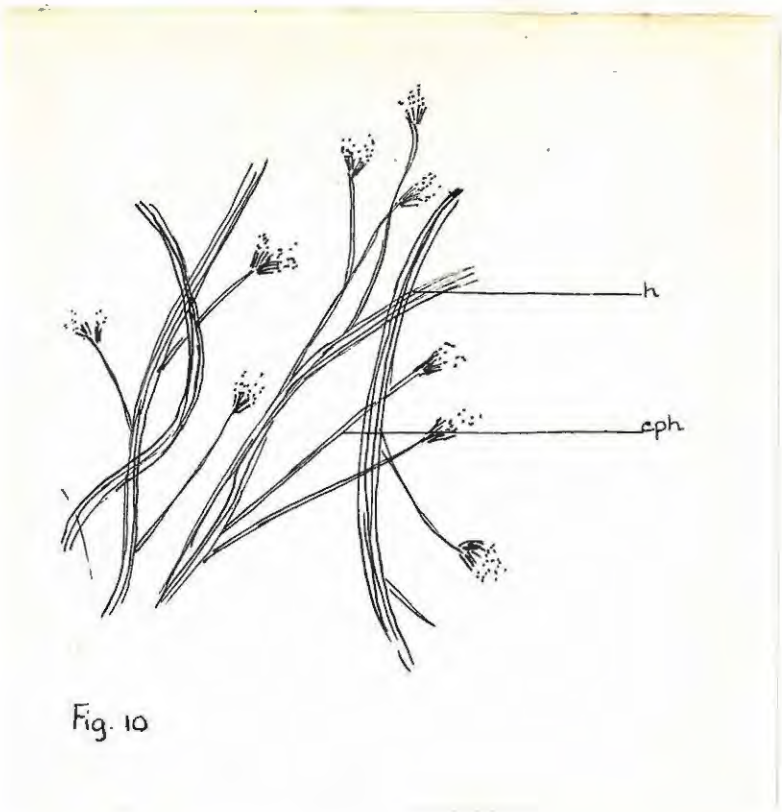


Fig.10. Semi-diagramatic Surface View of Colony of Penicillium.funiculosum.
h,hyphae in ropes; cph,conidiophores arising from them.

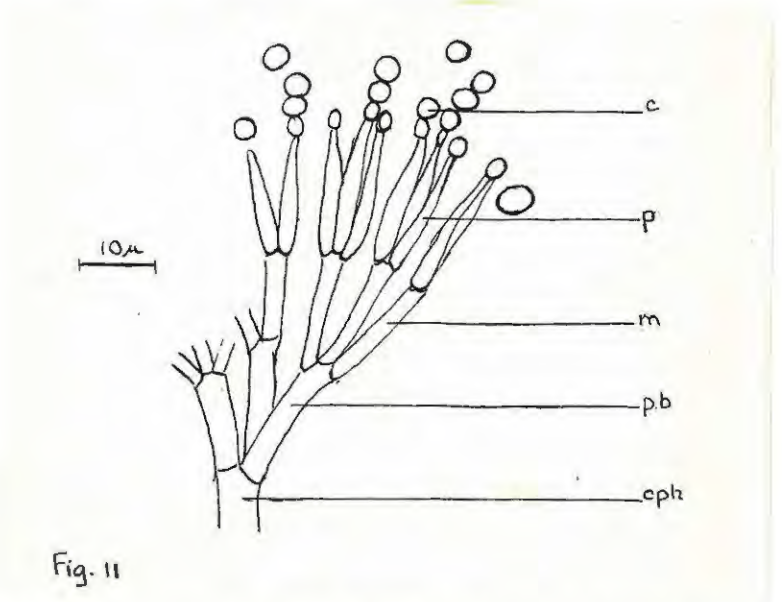


Fig.11. Penicillus of Penicillium funiculosum.
c,conidia; ph,phialides; m,metulae; pb,primary branches; cph,conidiophores.

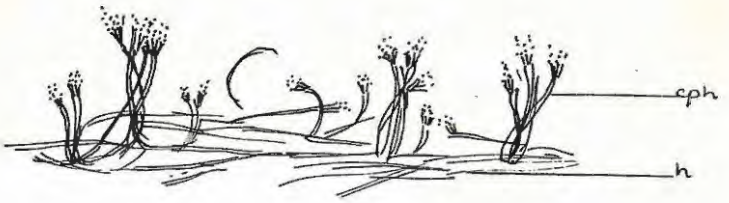


Fig. 12

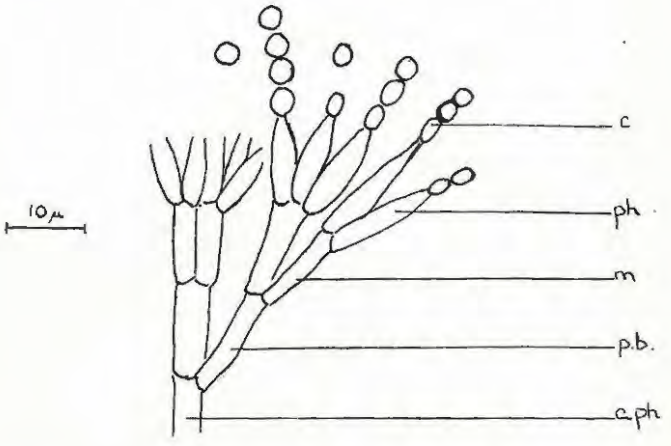


Fig. 13

Fig.12. Semi-diagramatic View of Colony of Penicillium expansum.
h, prostrate hyphae; cph, erect conidiophores in fascicles.

Fig.13. Penicillus of Penicillium expansum.
(Labels as for Fig.11).

The conidial fructification ($\pm$ 60 μ x 20 μ) is in three stages, consisting of one or two alternate, appressed branches bearing verticillate branchlets (metulae) and verticils of parallel phialides measuring $\pm$ 10 μ x 2.3 μ. The conidia are cylindrical at first, later elliptical, measuring $\pm$ 2 μ x 3 μ green in colour, and produced in chains which break up in fluid mounts.

The identification of the species was confirmed by the Commonwealth Mycological Institute, and two cultures nos. 57342 and 57343 were deposited in the herbarium.

Penicillium expansum Link emend. Thom. (see Figures 12 and 13, Plate 18)

The colonies are dull gray-green, the reverse becoming dull yellow later. The surface is powdery to tufted in appearance. The outer growing edge of the colony is generally white. The conidiophores ($\pm$ 90 μ) arise as lateral branches of aerial hyphae, and are often fascicled.

The conidial fructification ($\pm$ 40 μ x 25 μ) is in three stages, consisting of one or two primary branches bearing verticils of secondary branchlets (metulae) with dense whorls of phialides measuring $\pm$ 8 μ. The conidia are elliptical to globose measuring $\pm$ 4 μ x 2 μ. greenish in colour and produced in chains.

The identification of the species was confirmed by the Commonwealth Mycological Institute, and one culture No. 57344, was deposited in the herbarium.

Fusarium moniliforme Sheld. var. subglutinans Wr. and Rg.

Aerial mycelium is white to pink in colour, and the surface of the colony appears powdery to lanose. The microconidia are one-celled spindle shaped and oval, sometimes crescent shaped. They measure 4 μ - 15 μ x 2.3 μ, and are produced in chains at first, afterwards becoming scattered. (Plate 14) Macroconidia are crescent shaped or straight, tapering at both ends, sometimes constricted at the tip, sometimes hooked mainly 2 - 5 septate. They are sometimes gathered into pionnotes, and are salmon coloured at first later drying to pale brown. Sporodochia and sclerotia were not observed in culture, neither were chlamydospores. For spore measurements see Table XVI. Macroconidia 1 - septate: $\pm$ 13 μ - 28 μ; 2 - 3 septate: $\pm$ 20 μ - 45 μ; 4 - 5 septate: 40 μ - 70 μ.

Colonies grown on acid media (pH 4.5) coloured the medium pink at first, later blue-gray. On addition of a drop of phosphoric acid it again turned pink.

The species identification was confirmed by the Commonwealth Mycological Institute and two cultures Nos. 57345 and 57346 were deposited in the herbarium.

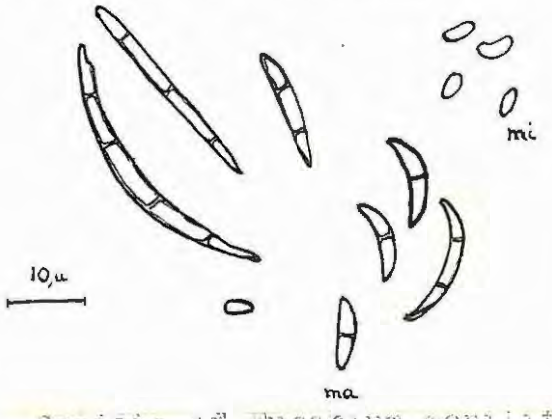


Fig.14. Conidia of Fusarium moniliforme var. subglutinans.
ma, macroconidia; mi, microconidia.

Table XVI

Measurements of conidia of Fusarium moniliforme

	Microconidia 0 - septate 4 μ - 15 μ.			
Range:	4 μ - 6 μ	7 μ - 11 μ	12 μ - 15 μ	
Numbers:	34	12	4	50
	Macroconidia 1 - septate 13 μ - 28 μ.			
Range:	13 μ - 17 μ	18 μ - 22 μ	23 μ - 28 μ	
Numbers:	35	4	11	50
	2 - 3 septate 20 μ - 45 μ			
Range:	20 μ - 28 μ	29 μ - 37 μ	38 μ - 45 μ	
Numbers:	30	10	10	50
	4 - 5 septate 40 μ - 70 μ			
Range:	40 μ - 50 μ	50 μ - 60 μ	61 μ - 70 μ	
Numbers:	17	10	23	50

c) Effect of Temperature.

Method.

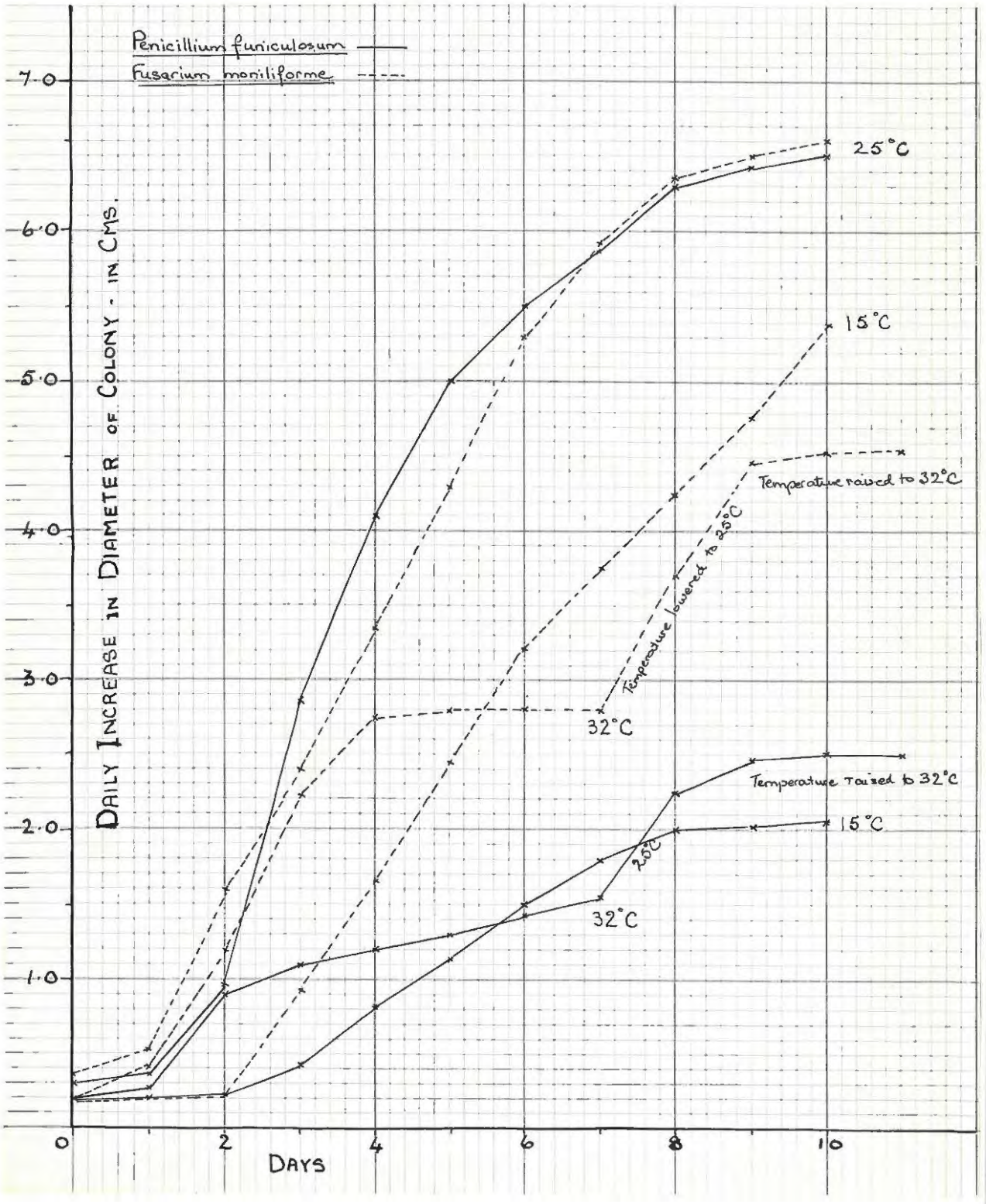
The fungi Penicillium funiculosum and Fusarium moniliforme were cultured on pineapple agar plates at temperatures of 15°C, 25°C, and 32°C. Two replicate plates were incubated at each temperature, and growth measurements of the fungi averaged to obtain the rate of growth. Growth measurements were made along two lines drawn at right angles to each other, on the bottom half of the Petri dish, their point of intersection being directly above the centre of the inoculum. This method of measuring the increase in diameter of the colony, does not take account of the fact that growth is vertical as well as horizontal, however, as the fungi were all grown on the same medium this method was adequate.

Results. (see Figures 15 - 16, and Tables XVII - XXII)

Growth of both the fungi takes place at all the temperatures investigated. In general the rate of growth was slowest at 32°C.

At 15°C, growth did not get under way in either fungus until after 48 hrs., the average rate of growth for Penicillium funiculosum during the following five days was 0.32 cms. per 24 hrs., and for Fusarium moniliforme 0.75 cms. per 24 hrs. Growth then gradually slowed up in both fungi, - the average diameter obtained by colonies of P. funiculosum being about 2.0 cms., and in F. moniliforme 5.0 cms.

FIG. 15. EFFECT OF TEMPERATURE ON GROWTH OF THE FUNGI



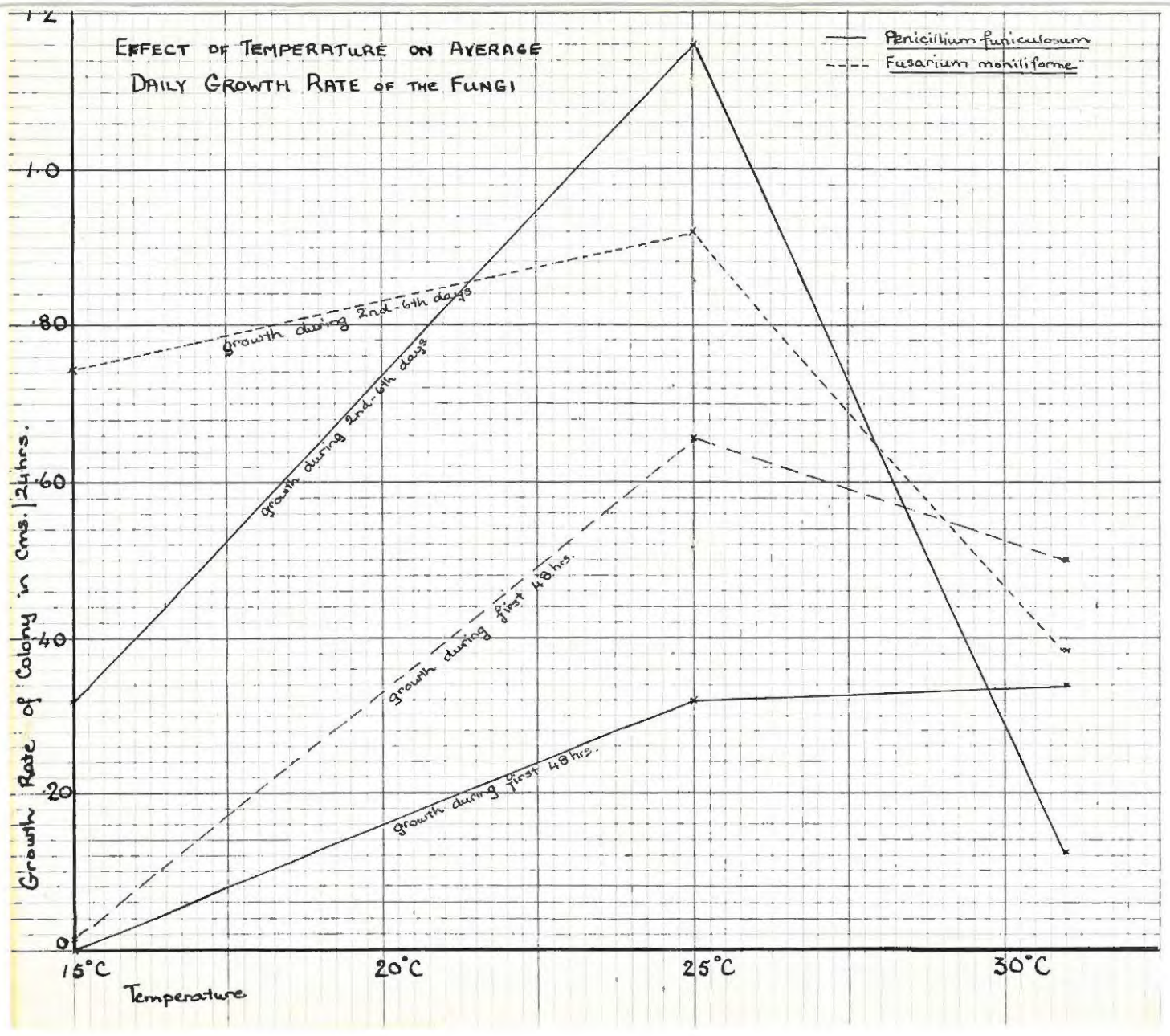

EFFECT OF TEMPERATURE ON AVERAGE
DAILY GROWTH RATE OF THE FUNGI
Penicillium funiculosum
Fusarium moniliforme
1·2
1·0
·80
·60
·40
·20
0
Growth Rate of Colony in Cms. / 24 hrs.
growth during 2nd-6th days
growth during 2nd-6th days
growth during first 48 hrs.
growth during first 48 hrs.
15°C
20°C
25°C
30°C
Temperature

FIGURE 16

At 25°C, growth took place within the first 24 hours, the ensuing growth rate being much faster than at 15°C. In the case of P. funiculosum this was 1.1 cms. per 24 hrs. and in F. moniliforme 0.9 cms. per 24 hrs. After the fifth day growth declined in both fungi, but the average diameter of colonies in both cases was about 6 cms.

At 32°C, the initial growth rate was higher than at 15°C, though not as high as at 25°C, growth taking place within the first 24 hours. P. funiculosum showed an average rate of 0.13 cms. per 24 hrs. during the next five days, while F. moniliforme averaged a daily increase of 0.39 cms. for the following two days, after which no further growth occurred. The diameter of colony reached was respectively 1.5 cms. (Penicillium) and 2.8 cms. (Fusarium) - less than the size at 15°C. Lowering the temperature to 25°C, resulted in growth being resumed in both fungi, an average increase of 0.7 cms. in 24 hours taking place. When the temperature was again raised to 32°C, growth ceased in both fungi.

This marked decline in the growth of F. moniliforme at the higher temperature is termed "staling," and is considered to be due to the accumulation of definite products formed by the fungus under certain conditions. (Brown, 1923, 1925, and Pratt, 1924). As growth is resumed in staled cultures when the temperature is lowered, this bears out the fact that staling is not due to exhaustion of the nutriment by drying of the medium. (Pratt and Boyle, 1924). The staling products must

therefore be regarded as growth inhibiting and not markedly toxic, or growth would not be resumed.

Examination of the results, suggests that the differential effect of the various temperatures is best shown from the second to the sixth days, as during the first two days there is an initial lag before the growth rate gets established, and after the sixth day growth declines, probably as a result of factors such as the accumulation of staling products, changes in the reaction of the medium, and as in the case of Penicillium, the production of numerous daughter colonies on the plate.

The optimum temperature is considered to be that temperature which permits the greatest metabolic activity to take place. This can be variously regarded as the temperature at which growth takes place most rapidly, or the temperature at which growth is longest sustained, at a high uniform rate. On the latter basis the optimum temperature will be lower for those forms, such as Fusarium, with a strong tendency to stale at high temperatures, despite the initial rapidity of growth. In the case of both the fungi under investigation, 25°C seems to be the optimum temperature. From the point of view of the fungus in nature, it is difficult to decide which interpretation is correct as higher temperatures under natural conditions are seldom sustained long enough to do more than stimulate the fungus.

Further effects of temperature are those connected with the other activities of the fungus, such as spore formation

and germination. The maintenance of a constant level of temperature may not necessarily favour all of these, and the optimum temperature for mycelial growth may not be ideal for reproduction.

In considering the two fungi investigated, the various temperatures seem to have little effect on the general morphology of each. At both 15°C and 25°C, colonies of Penicillium funiculosum were a dark blue-green, with very little production of aerial mycelium (and consequent short conidiophores). At 32°C, the colour of the colonies was lighter, and there was a pronounced development of aerial mycelium, showing typical 'ropes' of hyphae. However, formation of conidia was not nearly as profuse as at the lower temperatures, which apparently favour reproduction. In the case of Fusarium moniliforme very little aerial mycelium was developed at 15°C, and very few spores were produced. At 25°C, white aerial mycelium developed, and spores were freely produced. The reverse of the colony was colourless at first, later becoming typically pink to gray. Profuse marginal growth of pink aerial mycelium occurred at 32°C accompanied by spore production (cf. Brown, 1925). The centre of the colony showed less growth of aerial mycelium, the reverse being a yellow-pink colour, later becoming orange.

The effect of high temperatures seems to produce a similar reaction in the two fungi, P. funiculosum being stimulated to produce aerial mycelium, but few spores, and F. moniliforme to

produce aerial mycelium and profuse spores but mainly 1 - 2 septate. (cf. Brown, 1925, and Horne, 1926). At the lower temperatures, mycelial growth does not occur as readily, though spores are still produced. The increase in vertical growth (ie. aerial mycelium) over horizontal growth of the colony, at high temperatures, indicates a physiological unbalance rather than production of inhibiting substances. This view is supported by the fact that growth is resumed in staled cultures (grown at 32°C) when temperature is lowered to 25°C.

d) Effect of humidity.

Method.

The fungi P. funiculosum and F. moniliforme, were grown on pineapple agar plates at 25°C, with the addition of a shallow layer of sterile distilled water, in the centre of the lid, (the dishes were inverted). A ring of vaseline kept the water from spreading up the sides of the dishes. Two replicates were made of each fungus and the growth measurements averaged.

Results.

The results (see Figures 17, 18, and Tables XXIII and XXIV) closely approximate those obtained for the fungi when grown at ordinary atmosphere and 25°C; the growth rate over the first few days being almost the same for both fungi whether grown over water or not, - 0.94 cms. per 24 hours in the case of P. funiculosum and 1.0 cms. per 24 hours in the case of F. moniliforme. However, the maximum growth increase is sustained at a uniform daily rate for a longer period (6 days) when the fungi are grown over water, than at ordinary atmosphere (only 3 days). In so far as morphological changes were effected, P. funiculosum, when grown over water showed pronounced development of aerial mycelium and 'ropes' of hyphae. The conidiophores were long, but spore production was not as profuse as at ordinary humidity (cf. Hawker, 1950, p 176); neither was the reverse of the colony coloured yellow.
In F. moniliforme, there was little production of either aerial mycelium or spores, and no development of colour.

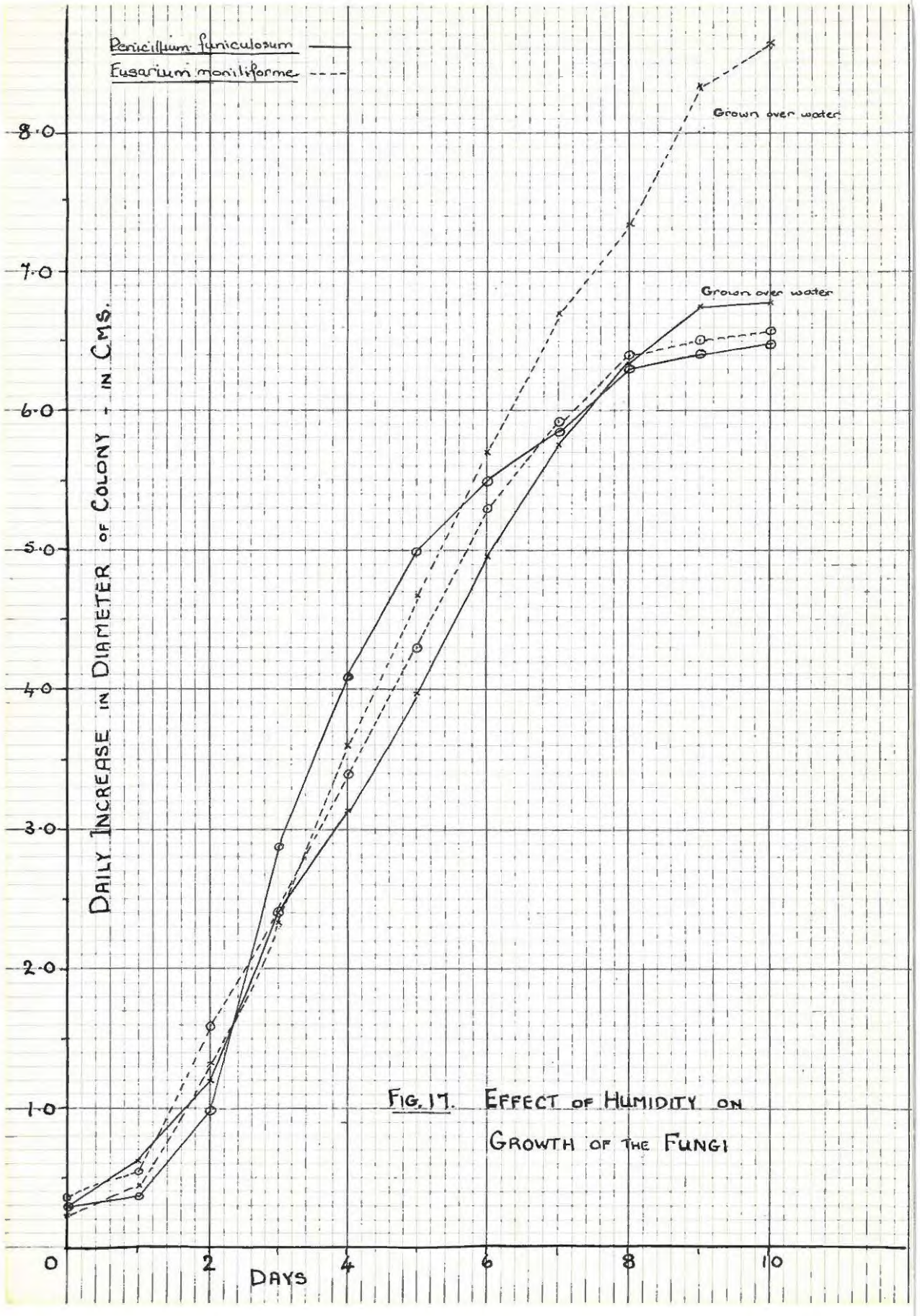


FIG. 17. EFFECT OF HUMIDITY ON GROWTH OF THE FUNGI

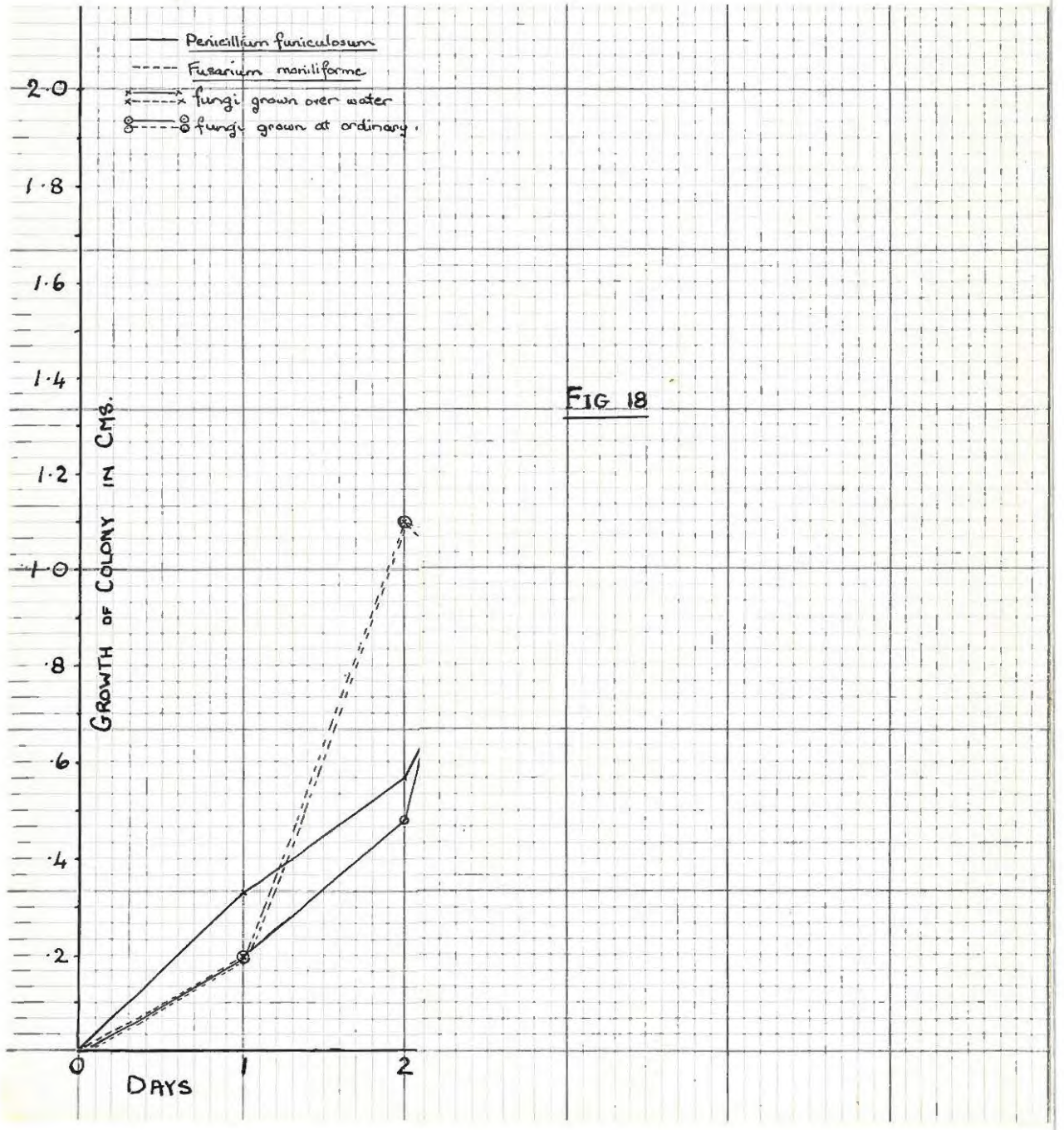

Penicillium funiculosum
Fusarium moniliforme
fungi grown over water
fungi grown at ordinary
2·0
1·8
1·6
1·4
1·2
1·0
·8
·6
·4
·2
GROWTH OF COLONY IN CMS.
0
1
2
DAYS

FIG 18

The effect of increased humidity is thus to prevent, or postpone staling.

e) Effect of pH.

Method.

The fungi P. funiculosum and F. moniliforme were cultured on pineapple agar plates at pH 4.0, 5.5, 6.5, and 7.5. The pH was adjusted by addition of 0.1 N sodium hydroxide or 5% phosphoric acid. Two replicate plates were cultured for each pH, incubated at 25°C, and the results averaged.

Results. The results (see Figures 19 - 23 and Tables XXV - XXXII) indicated that growth of both fungi took place at all the pH values investigated. The limits of H-ion concentration of the medium which would allow growth, were not determined. The differences in the average rate of growth per day, during the 3rd to 6th days, are less than 0.3cm., for both fungi at any pH level. However, it must be remembered that the effect of the pH of the medium on growth can only be judged during the first few days, as after this the initial reaction of the medium changes as long as fungal growth continues. This is particularly marked in the case of Fusarium species, where both alkaline and acid media become more alkaline (Boyle 1924). These changes are often accompanied by corresponding colour changes in the medium as observed by Horne and Mitter (1927) and W. Brown (1925, 26) working on this genus.

In this case, F. moniliforme var. subglutinans, the medium

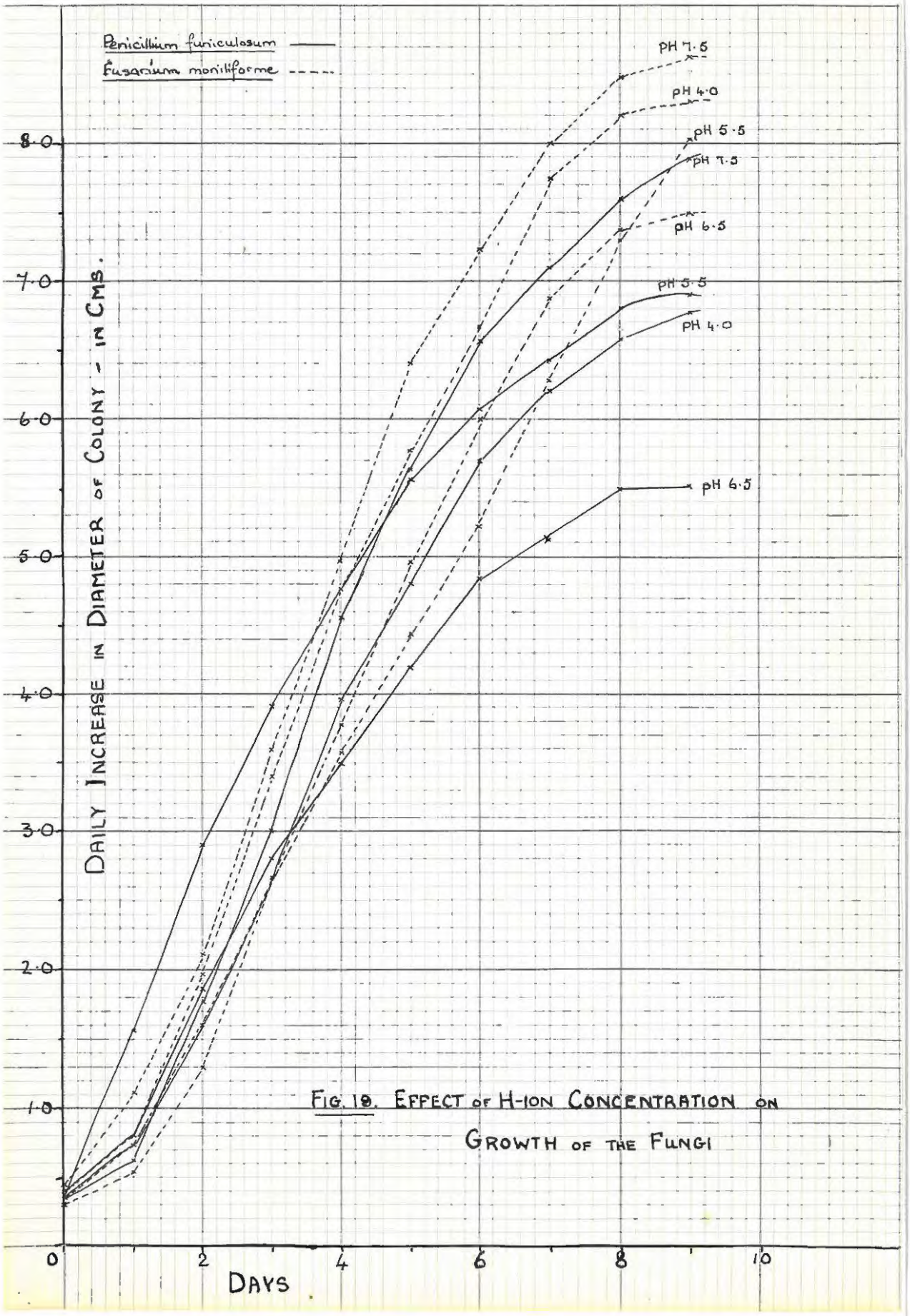


FIG. 19. EFFECT OF H-ION CONCENTRATION ON GROWTH OF THE FUNGI

EFFECT OF H-ION CONCENTRATION ON DAILY GROWTH RATE OF THE FUNGI

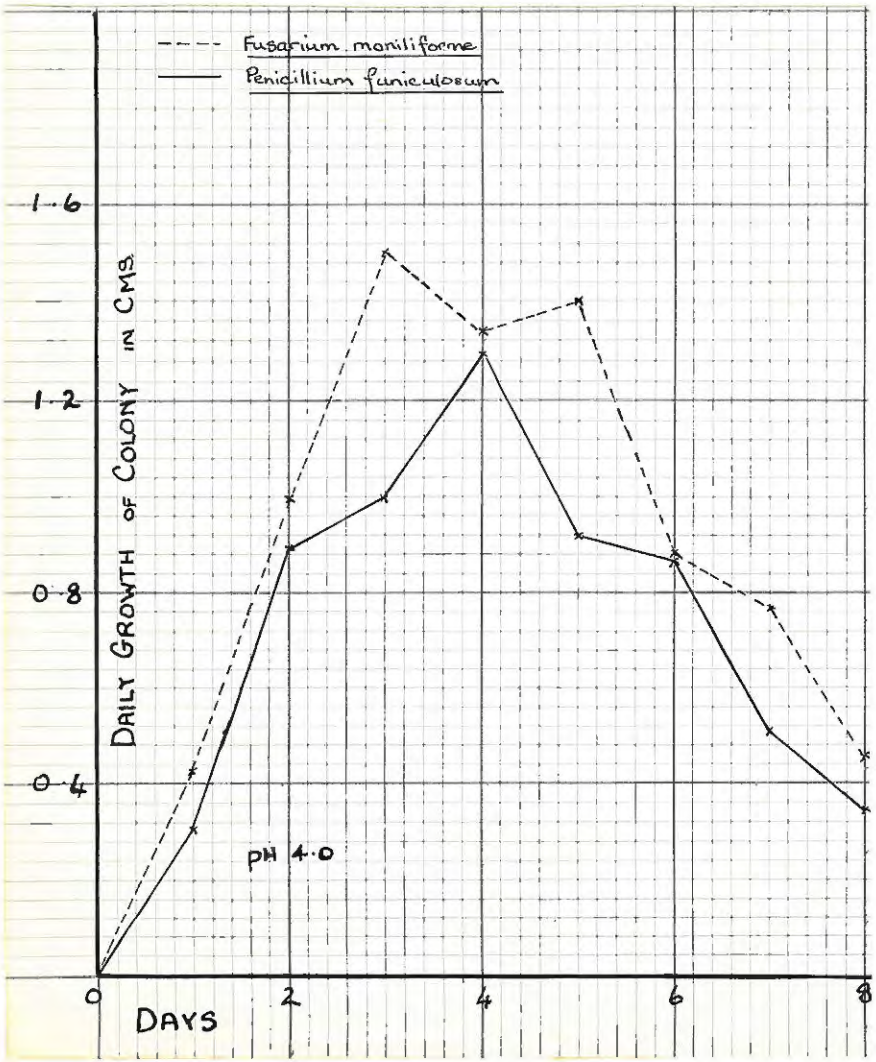


FIGURE 20

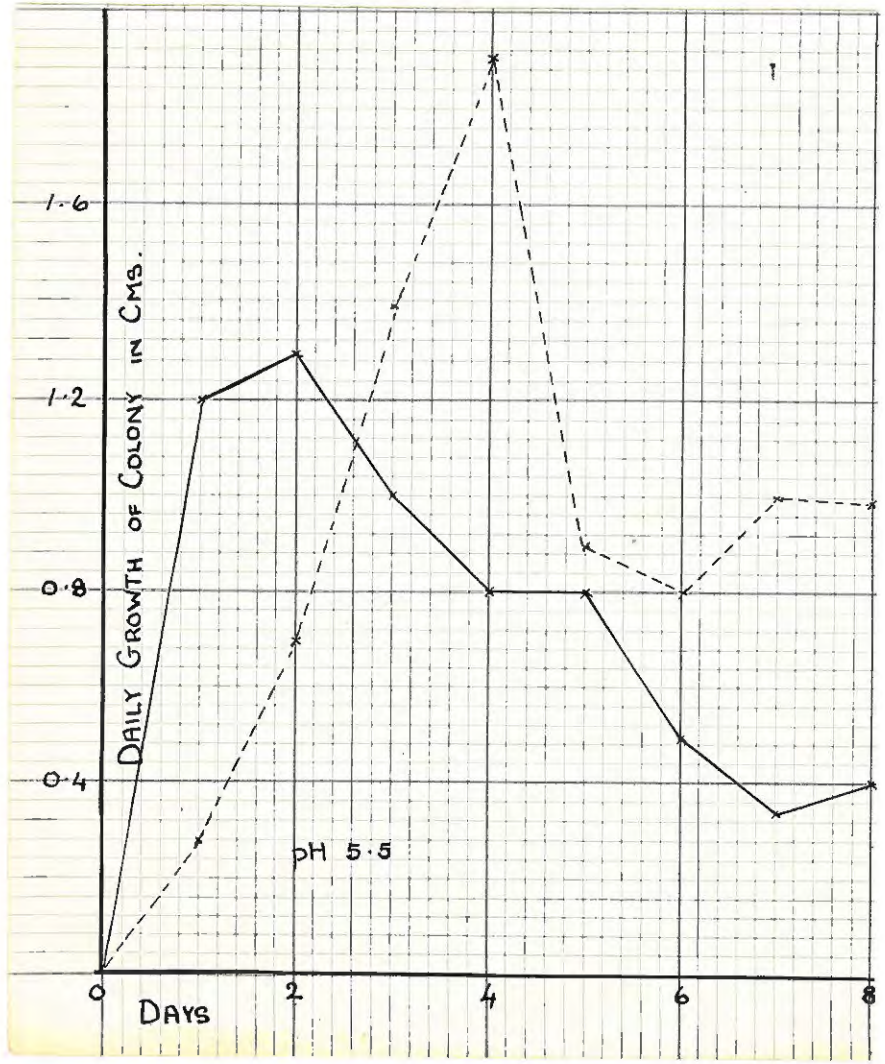


FIGURE 21

EFFECT OF H-ION CONCENTRATION ON DAILY GROWTH RATE OF THE FUNGI

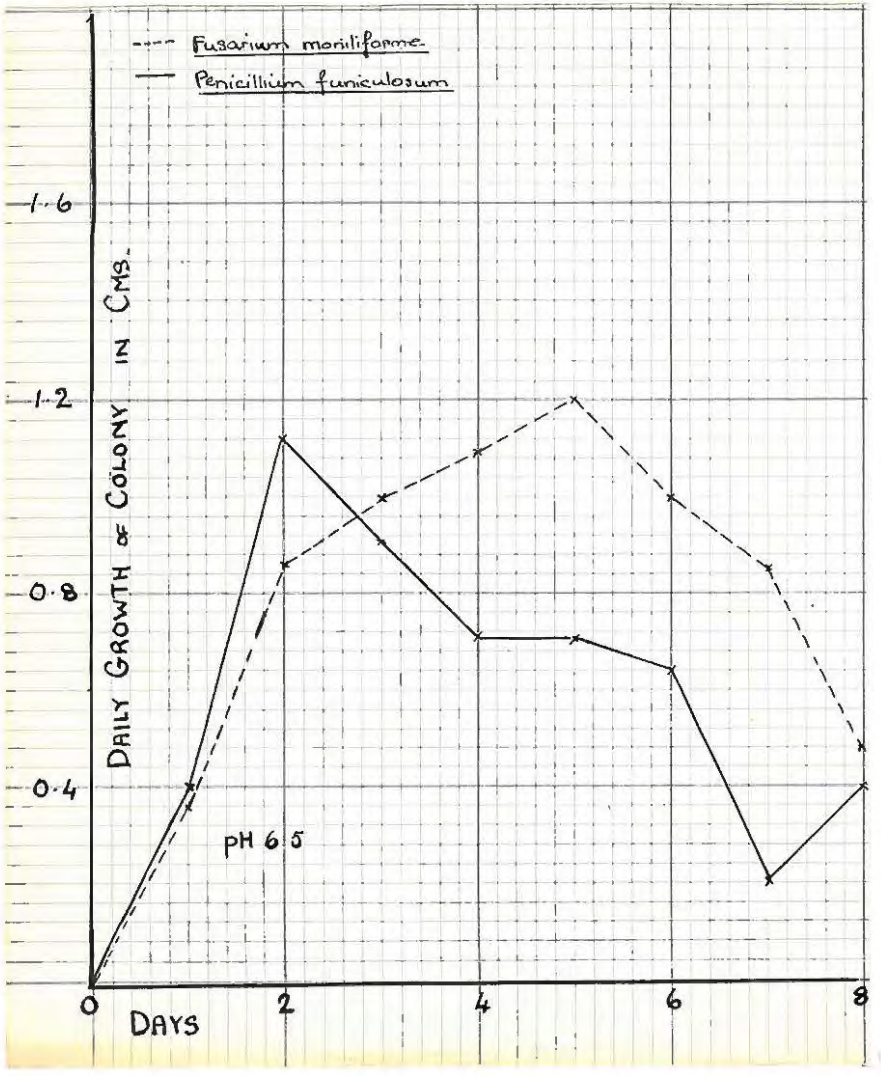


FIGURE 22

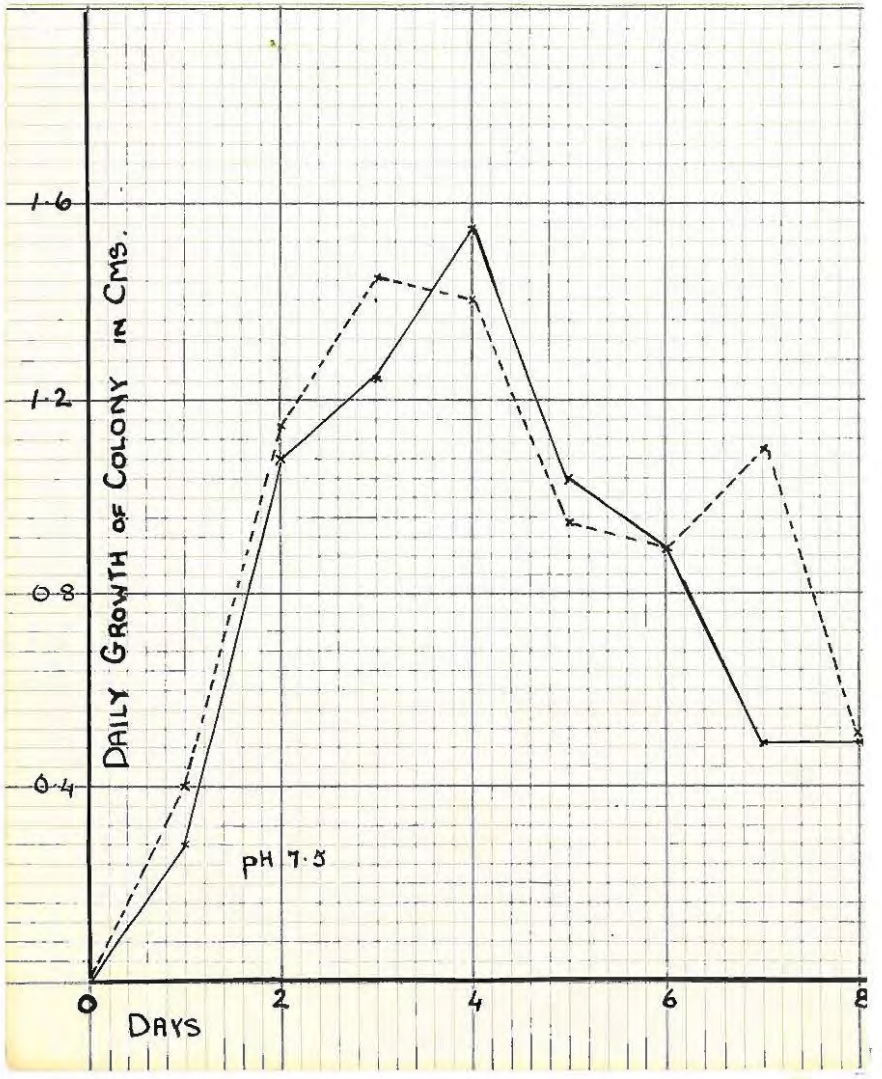


FIGURE 23

was gradually coloured pale pink to red, with the deepest colour developed at pH 6.5 and 7.5 (Similar development of colour, has been recorded by Horne and Mitter, 1927). At pH 4.0, the pale pink colour gradually changed to gray-blue after about 5 weeks. On addition of a drop of 5% phosphoric acid, the medium again turned red, showing that accumulated metabolic products of the fungus cause a change in the reaction of the medium. It has also been noticed that frequently when P. funiculosum and F. moniliforme are grown together, a bright red colour is produced in the medium, both when the fungi are grown on agar plates, and on slices of pineapple fruit, indicating the production of acid products by P. funiculosum. This production of colour when Penicillium and Fusarium are grown together, has also been observed by Brown (1925). Production of acid in P. glaucum. has been proved by Nikitinsky (1904).

No morphological changes were apparent either in - P. funiculosum or F. moniliforme under the various H-ion concentrations considered, aerial mycelium and spores being produced in all cases.

Spores of both fungi were found to germinate at all the pH levels investigated. Preliminary experiments with spores sown in juice of mature and young fruits indicated slightly quicker germination (within 6 - 12 hrs.) in the juice of young fruit. (no counts for percentage germination were made). The pH of juice from young fruit was 3.1, from mature fruit 4.8.

According to Waksman, (1931, p. 246) increase of acidity favourably influences the germination of fungal spores.

While H-ion concentration is an environmental factor of consequence in modifying the metabolic activities of fungi, change in pH level alone is not the limiting factor; temperature is also a critical factor, (Waksman, p. 791) as well as the accumulation of inhibiting metabolic substances produced by the fungus. (Boyle, 1924) In the case of the two fungi investigated, changes in the pH level between 4.0 and 7.5 do not affect growth in any way.

Conclusion.

The above experiments have been conducted using only pure cultures of one fungus at a time, and with one variable factor in each case. Under natural conditions this is seldom the case, as not only do all the environmental factors vary at the same time, but two or more organisms are frequently present together - as in black spot. (Chap. III) Thus, when any one factor affecting the growth rate is altered so as to lessen the rate, the resistance of the fungus to other factors will also be decreased. When a combination of fungi are present, the effects of changing environment will be more complex, as is the case where decay proceeds more rapidly as a result of the selective effect of temperature favouring rapid growth of one fungus at the expense of another, (Savastano 1929) in fruits inoculated with a combination of fungi. Further, the

associative interactions of fungi, whether antagonistic or stimulatory, (see Chap. III) will affect their response to external factors.

It is obvious from the foregoing that the results of the complex of interactions between fungi in the fruit and environmental conditions, cannot be predicted from the culture experiments undertaken in the laboratory. The effect of the fungal associations found in black spot will ultimately depend on the balance of all the factors concerned, on which further study is necessary.

B. Discussion of Pathogenicity.

Having considered the reactions of the fungi in culture, to changes in the environmental conditions, comparison with the reactions of the fungi in the natural state must be drawn. The pathogenicity of the fungi will be affected by such natural conditions as a) season, involving temperature and moisture changes and b) age of fruit involving changes in pH, and dependant on seasonal effects, and c) soil. It is therefore not possible to deal with these effects separately.

Most workers have noted a greater prevalence of black spot in the winter crop than in the summer crop. Tryon,(1898) accounts for this by advancing the theory that "certain (chemical) changes during ripening are antagonistic to the fungus (Monilia sp.)" and during winter these changes are delayed thus giving the fungus a better start. According to Bodenstein, (1936) the chief chemical change taking place during ripening is a decrease in the amount of acidity, (due mainly to citric acid.) The greatest proportion of sugars present is sucrose, a considerable amount of inversion taking place during increased temperature, depending on the concentration of acid in the fruit. Determination of pH in Cayenne fruits showed increased acidity in young fruits, pH 3.0 - 3.4; mature fruits had a pH level of 4.6 - 5.0.

Although the above two views might indicate that the higher acidity of the unripe fruit is a factor favourable to the

growth of the fungi involved, experiments in culture do not bear this out. The fact that black spot lesions are produced in both very young and mature fruits, (as inoculation experiments, and growing fungi on slices of tissue from mature and immature fruits have proved - see Chapter III) shows that the acidity of the fruit does not have any marked effect on the fungus.

The production of dry spot in young fruit inoculated with the fungi under investigation, (see Table II) may be due to the drier condition of the fruit inhibiting growth of the fungus, despite the fact that higher acidity usually favours growth. Shaw, (1935) working on fire blight of twigs caused by Bacterium amylovorum, found that the formation of a cork barrier in the cortex of pear and apple twigs, develops rapidly when the moisture is low, but is delayed when the air is humid. This probably explains, to some extent, the lack of callosing in fruits inoculated under humid conditions, (see Table VIII) apart from the fact that the fungi in culture grew better under humid conditions. The pathogenicity of the fungi Penicillium funiculosum and Fusarium moniliforme would seem therefore, to depend on the speed of fungal invasion, relative to the rapidity with which the host forms a cork barrier.

The increase of black spot in the winter crop may also be due to the effect of temperature - as higher temperatures inhibit growth, so the lower winter temperatures would be

more suited to fungal growth, provided they did not drop below the minimum temperature necessary.

It is interesting to note that the summer crop of 1953 - '54 in the Albany district, was marked by the large numbers of fruit infected with black spot. Fruit examined during this period showed 30% infection (see Figures 24 and 25). Normally the summer crop is free of black spot. Examination of Figures 24 and 25, shows that pineapples ripened during those months in which the rainfall was high, showed an increased incidence of black spot. The exception to this occurred during February when the highest percentage infection in both Queens and Cayennes was noted, although rainfall during the preceding month was low - 0.41" This can be explained by the heavy rains during September, October 9.59", and November 4.8", (December 1.4") which would no doubt contribute to the moisture available during the ripening period in January. Unfortunately the crop was not ready for harvesting till late January, so that no fruits were available for comparison in the period immediately following and preceding the rains.

The rainfall may have an effect on the water content of the host tissues. When transpiration is checked by rain, and the vessels of the plant are filled with water, flooding of the intercellular spaces may occur, causing not only favourable conditions for pathogens already in the tissues, (Shaw, 1934) but mechanical cracking and rupture of the epidermis, - thus providing the necessary means of entry for invading fungi,

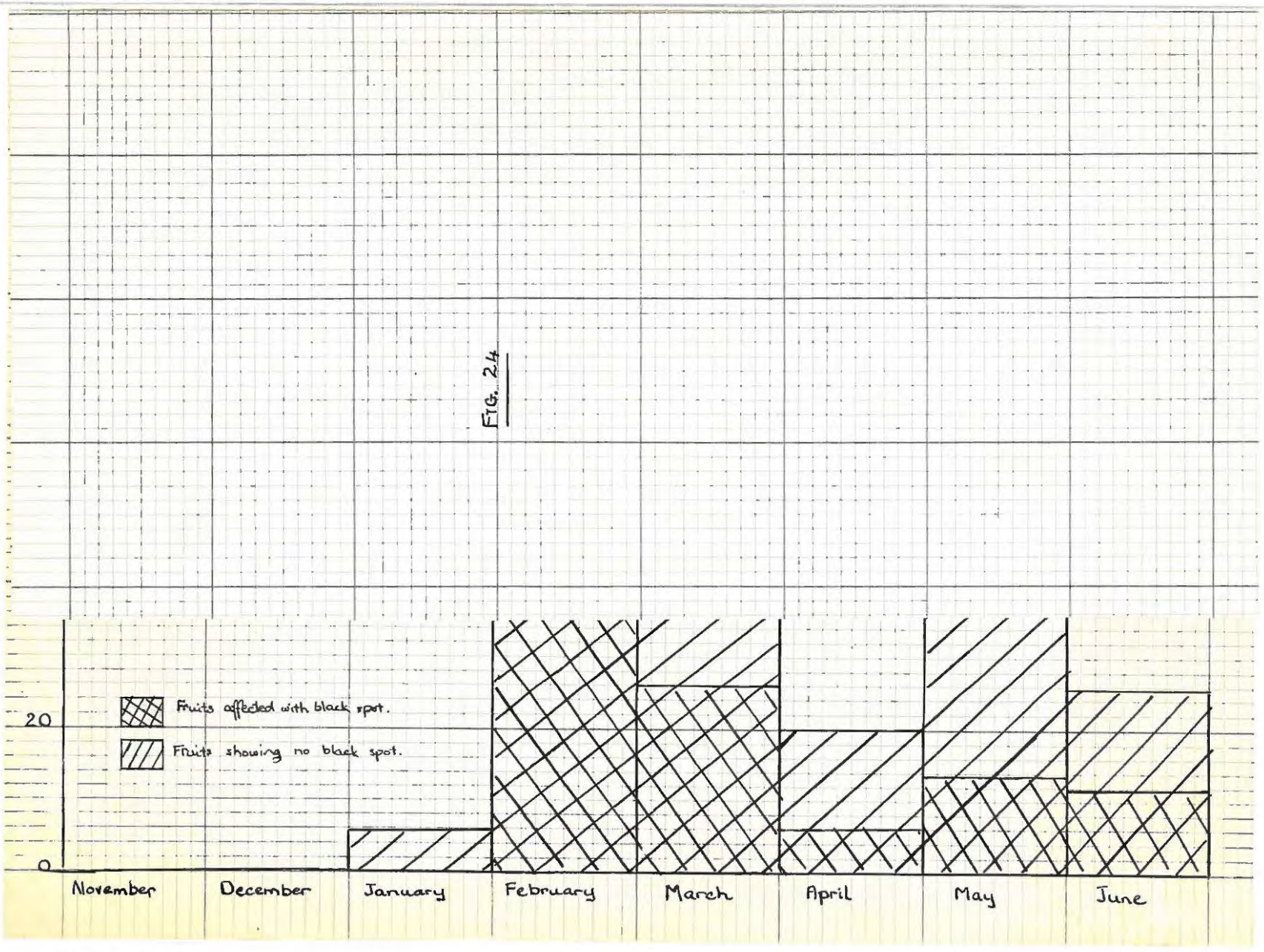


FIG. 24
Fruits affected with black spot.
Fruits showing no black spot.
20
0
November
December
January
February
March
April
May
June

FIGURE 24

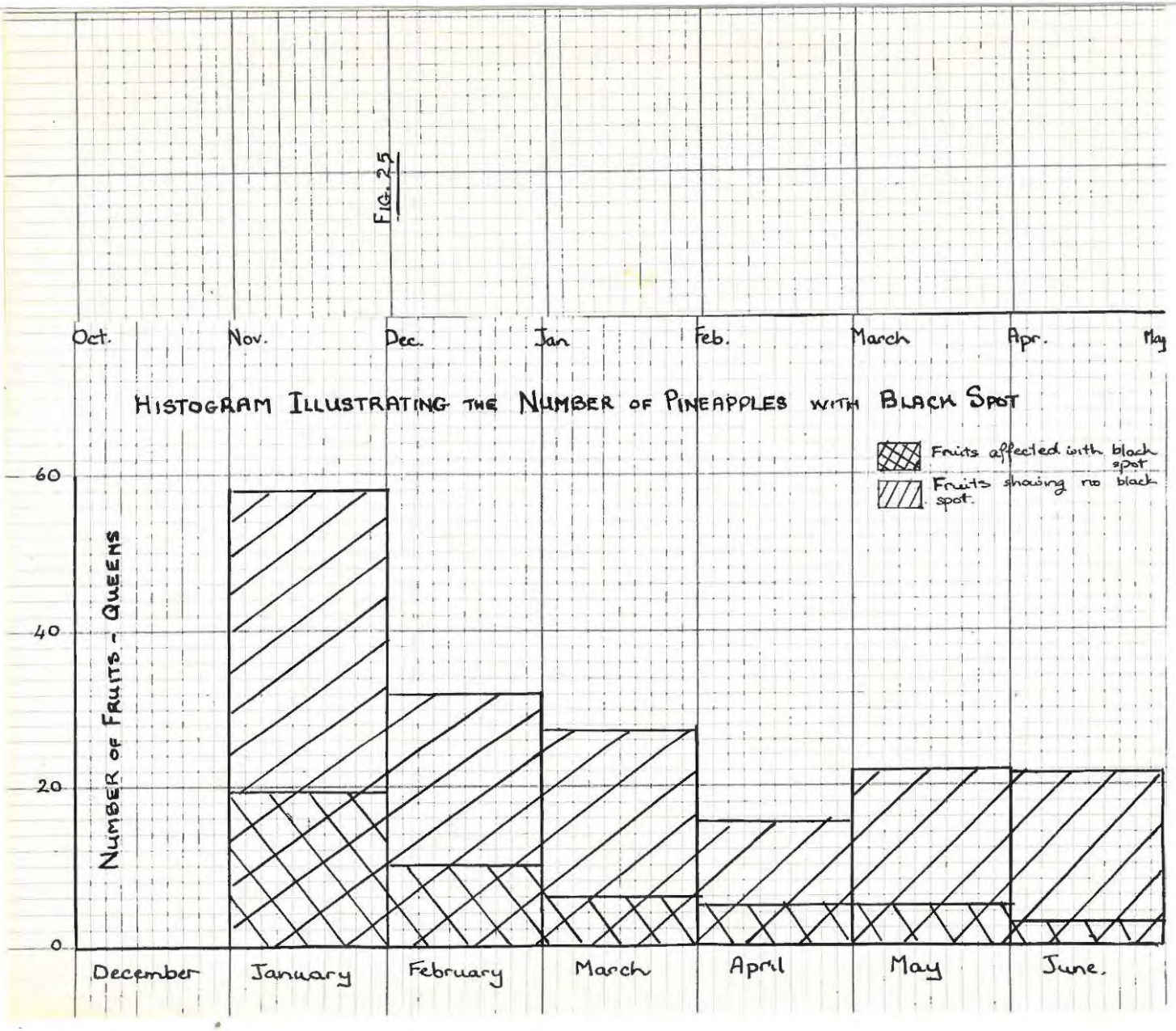


FIG. 2.5
Oct.
Nov.
Dec.
Jan
Feb.
March
Apr.
May
HISTOGRAM ILLUSTRATING THE NUMBER OF PINEAPPLES WITH BLACK SPOT
Fruits affected with black spot.
Fruits showing no black spot.
60
40
20
0
NUMBER OF FRUITS - QUEENS
December
January
February
March
April
May
June.

FIGURE 2.5

whose spores would probably germinate all the more readily under moister conditions (Gäumann, 1948, p 26) This factor is of importance in the prepenetration stages of invasion.

Increase in the numbers of fungi in the soil also occurs when the moisture content is raised, (Waksman, 1931) and would eventually result in an increase in the numbers of spores of pathogenic and other fungi. Penicillium expansum however, has been shown by Le Clerg and Smith, (1928) to occur abundantly in soils with a low moisture content. Clayton, (1923) states that in very wet soils an inhibition of infection takes place, (as in Fusarium bulbigenum which causes tomato wilt), which appears to be associated with the host metabolism, and is correlated with an absence of nitrates,- this does not inhibit the pathogen in the plant, but increases the plants resistance to disease. In the soil, deficiency of nitrogen is, however, unfavourable to most fungi (Waksman, 1931). According to Waksman, (1931, p. 753) and Gäumann, (1948, p. 29.) too high a water content of the soil is definitely injurious to pathogenic micro-organisms, as it may not only impede their respiration, but will also favour saprophytic competition and accelerate the development of the host.

The condition of the soil and its effect on the fungal population must therefore be of considerable importance to the pineapple plant, in so far as the presence of the pathogenic fungi associated with black spot are concerned. (Further work on this aspect is required).

It is difficult to draw a hard and fast line between saprophytism and parasitism. In the case of both Penicillium funiculosum and Fusarium moniliforme they are in the first instance saprophytic, entering through a wound. Their further invasion of healthy tissue, while partly mechanical due to pressure of the invading hyphae, must be in part, parasitic, - enzymes usually being produced in quantity and concentration sufficient to enable fungi to attack the living tissues, provided there is no cork barrier to be overcome. In the case of Fusarium oxysporum and P. expansum (which is a weak parasite) the production of toxins which affect the cell contents has been ascertained by Barnum (1924) and Sideris (1929). Infection under natural conditions must therefore be preceded by the saprophytic development of the fungi in the soil, the fungi attacking the plant only after they have reached a certain stage of development (Waksman 1931, p. 791).

The pathogenic potentialities of the fungi investigated, are thus obviously modified by the varying conditions of climate, soil, and plant. The factors influencing the communal life of both pineapple and fungi together, will obviously not have the same effect as on plant and fungus separately.

V

SUMMARY.

a) The objects of this investigation were as follows:-

1. To ascertain what fungi are associated with the condition known as "black spot" in pineapples.

2. To prove the pathogenicity or otherwise of these, and whether this is affected by a) the age of the fruit, b) the variety, and c) method of introducing the infection.

3. To study the ecology of the associated fungi and ascertain whether or not they are present in a) the soil b) floral cavities and surface of fruit, and c) the leaves of the pineapple plant.

4. To investigate the reactions of the associated fungi under varying conditions in culture of a) temperature b) moisture, and c) H-ion concentration.

b) Results obtained.

1. The fungi most frequently associated with black spot are Penicillium funiculosum Thom. and Fusarium moniliforme Sheld. var. subglutinans Wr. and Rg.

2. Inoculation experiments carried out on fruits in the field and in the laboratory, show that the above fungi are definitely pathogenic, irrespective of the age or variety of the fruit. Black spot lesions are only produced if there is injury to the fruit resulting in breaking the epidermis - infection can occur at any point on the fruit, provided there

is accompanying damage to the epidermis.

3. The above fungi are present in both the soil and surface of the pineapple plant. They are particularly prevalent in the floral cavities of the fruit.

4. a) High temperatures (above 30°C) definitely inhibit growth of both fungi, more particularly in the case of Fusarium moniliforme Sheld.

b) Humidity definitely favours rapid growth and postpones staling in both fungi.

c) Growth rates for both fungi at pH levels from pH 4.0 - 7.5 show no marked differences.

5. In addition to these results it is obvious that black spot lesions are of two types "wet" and "dry." The latter type is more prevalent under conditions of low humidity and in very young fruits, the former is more prevalent under conditions of high humidity and in ripe and semi ripe fruits. The two types of spot are found in fruits of both Cayenne and Queen varities.

6. There appears to be a slight correlation between the numbers of fruit infected with black spot and the amount of rainfall during the ripening period - more infection occurring after rainy months.

7. Insects have been noted in the floral cavities of the fruit, and on the surface of the fruits.

c) Conclusions.

Fungal infection of the pineapple fruit can only occur if the epidermis of the fruit is broken in some way. This injury almost certainly occurs through the floral cavity, as examination of the black spot lesions and presence of the pathogenic fungi Penicillium funiculosum and Fusarium moniliforme in the cavity, confirm. It is possible that injury occurs by insects, (which have been noted in the floral cavities and would be attracted there by the nectar) or by mechanical rupture of the tissues of the fruit. The extent of the damage caused by the invading fungi depends on the resistance of the fruit to attack - (in particular the speed with which it can produce a corky barrier,) and the reaction of the fungi, to the conditions prevailing at the time.

PART II

I INTRODUCTION

A routine preliminary investigation using micro-biological methods, was undertaken to estimate the effects of various fertilizers on citrus soils.

Martin (1950) working in America, found that poor growth in old citrus soils was due in part to the development of a "harmful fungal population." Fumigation in these old soils stimulated seedling growth, but in non-citrus soils had little effect on the growth. References to work on soil fungi in S. Africa are more limited. Cohen (1950), investigated the occurrence of soil fungi after burning and grazing had taken place. He found that burning and light grazing produced a richer flora as regards number of species, and a better balance between the three main fungal groups. Burning alone resulted in plots appearing sterile but actually having a dormant micro-flora. Control plots showed a poorer flora, but a more actively spore producing one.

II QUANTITATIVE EXPERIMENTS

a) Soil samples were taken from three plots at Belmont Valley, which had been under citrus since 1939. The plots had been treated with kraal manure, and ammonium sulphate and potash over a period of six years. (Five pounds of ammonium sulphate and superphosphate, and 1 lb. potash fertilizer were applied in winter to one plot, and 150 lbs. of kraal manure to the other plot; the

third plot was left as a control under natural conditions.) Each plot consisted of eight trees in two rows of four each, surrounded by a single row of guard trees.

Quantitative estimations of the numbers of fungi and bacteria in the soil, were made by plating a known dilution of the soil on to a selected medium and counting the number of colonies that developed. The method followed was that used by Brierley (1927). At the same time isolations were made of the fungi growing on the dilution plates, and these were grown in pure culture and identified.

It was hoped to establish some significant relationship between the soil treatment and its effect on the soil microflora, in this way.

b) Method.

Soil samples were collected by digging a hole approximately one foot square and about nine inches deep. Samples were taken by inserting a sterile specimen tube at a depth of six inches below the surface. About 20 gms. of soil were collected in each tube. These samples were taken from beneath each of the eight trees in the plot, and from three places between the trees. The samples for each plot were lumped together, (keeping those from under and between the trees separate) and from these aggregates soil was taken for determining pH, and for the quantitative estimation of the fungi present.

The soil was thoroughly mixed and any large lumps broken

up. Twenty five grammes of soil in 250 ccs. of sterile distilled water, was used to make a primary suspension of 1/10 - this was shaken by hand, slowly, for 15 - 20 minutes. Further dilutions of 1/100, 1/1000, 1/20,000 and 1/40,000 were made and each shaken in the same way for 15 mins. Dilution plates were made by adding 1 cc of each dilution to 9 ccs. of cooled medium, and pouring into a 9 cm. Petri dish. Two replicate plates were made of each dilution, incubated at 25°C and examined at intervals over 5 days. The medium used was Brown's agar (pH 6.0) - see Appendix II. All glassware was sterilized and the usual precautions taken to avoid contamination.

Counts of the fungi and bacteria were made after 60 hours and after 120 hours. The bottom of the Petri dish was divided into quadrates (by ruling two lines intersecting at right angles) and colonies were marked with a dot of indian ink as counted. Where the colonies were very small, and very numerous and uniformly distributed over the plate (as in the case of bacteria) counting was done in one sector only and the result multiplied by four.

In the first experiment the dilutions already mentioned were used. The soil was extremely dry. A second experiment was carried out in the same way but using dilutions of 1/100 (primary suspension made from 10 gms. soil) 1/1000, 1/5000 and 1/10,000. - these dilutions were used, as at dilutions of 1/20,000 and 1/40,000 (Exp. 1) no fungal colonies were produced.

A further experiment was carried out in duplicate (Exps. 3 and 4) using dilutions of 1/10 (primary suspension 25 gms. soil) 1/50, 1/100, 1/500 and 1/1000 - as no fungal colonies developed at the 1/10,000 dilution used in Exp. 2. Soil used for these two experiments was water logged following on heavy rains. Bacterial colonies were developed in such profusion on the plates that counting was impracticable.

H-ion concentration of soil.

The pH level of the soil was determined using a Beckman pH metre. Twenty grammes of soil were made up to 100 ml. with redistilled water, and shaken for 30 minutes. The decanted solution was used for measuring the pH. Results were as follows:

	Control plot.	Kraal manure plot.	N.P.K. plot.
Soil from under trees	pH 6.9	7.6	5.2
Soil from between trees	pH 6.7	7.25	5.6

As the nitrogen in plot N.P.K. was supplied in the inorganic form of ammonium sulphate this accounts for the comparitively low pH obtained for the soil sample.

c) Results. see Tables XVI A and B.

These results are based mainly on experiments 1 and 4, as in experiment 2 a large number of the plates liquified, due to failure of the thermostatic control in the incubator.
In experiment 3, bacterial colonies were so small, and so numerous that the entire surface of the plate was covered and

and counting was impracticable.

In all three plots, in experiment 1, fungal numbers (per gramme of soil) were higher under the trees, and bacterial numbers were higher between the trees. This might be due to increased acidity in the rhizosphere region, (Lochead, Katznelson and Timonin, 1948) but the pH readings do not bear this out, though not based strictly on soil from the immediate root zone only. (cf. Webley, Eastwood and Gimingham 1952) In experiment 4, fungal and bacterial numbers per gramme of soil are approximately the same, both under and between the trees, in all the plots. Soil used for this experiment was very moist.

The most obvious effect of the increased moisture content in the waterlogged soil used in experiments 3 and 4, was the numbers of bacterial colonies developed on the dilution plates. While various workers have pointed out that fungal numbers are reduced in waterlogged soils and bacterial numbers increased, (Waksman, 1931) the number of colonies on the plates does not necessarily indicate the same numbers in the soil.

In all the experiments (both dry and waterlogged soils) fungal and bacterial numbers (per grm. of soil) are highest in the plot treated with kraal manure, as would be expected. (Waksman, 1931 and Jensen, 1931) Bacterial numbers are lowest in the N.P.K. plot in all experiments, while fungal numbers are lowest in the N.P.K plot in experiment 1, in experiment 4, they are lowest in the control plot

T A B L E 16A

NUMBERS OF FUNGI PER GRAM OF MOIST SOIL

Exp.1 Dilution	Soil Sample	Plot A (N.P.K.) Plate I	Plot A (N.P.K.) Plate II	Plot A (N.P.K.) Average	Plot B (Kraalmanure) Plate I	Plot B (Kraalmanure) Plate II	Plot B (Kraalmanure) Average	Plot C (Control) Plate I	Plot C (Control) Plate II	Plot C (Control) Average
1/100	(Under Trees	800	700	750	1,000	1,300	1,150	2,000	2,800	2,400
	(Between Trees	400	600	500	1,700	1,600	1,650	1,450	2,000	1,700
1/1000	Under	6,000	6,000	6,000	5,000	4,000	4,500	8,000	13,000	105,000
	Between	4,000	4,000	4,000	11,000	15,000	13,000	6,000	5,000	5,500
1/20,000	Under	30,000	20,000	25,000	20,000	40,000	30,000	20,000	20,000	20,000
	Between	30,000	0	15,000	20,000	40,000	30,000	40,000	0	20,000
1/40,000	Under	80,000	0	40,000	0	160,000	80,000	40,000	120,000	750,000
	Between	0	0		80,000	40,000	0	0	40,000	20,000
Exp. 3 1/50	Under	0	765	385	810	1,000	950	Bact.	Bact.	-
	Between	400	250	325	Bact.	Bact.	-	Bact.	Bact.	-
1/100	Under	Bact.	Bact.	-	Bact.	Bact.	-	Bact.	Bact.	-
	Between	Bact.	Bact.	-	Bact.	Bact.	-	Bact.	Bact.	-
1/500	Under	Bact.	Bact.		Bact.	Bact.	-	Bact.	Bact.	-
	Between	Bact.	1,000	1,000	Bact.	Bact.	-	500	Bact.	-
1/1000	Under	Bact.	Bact.	-	Bact.	Bact.	-	Bact.	Bact.	-
	Between	2,000	Bact.	2,000	Bact.	Bact.	-	Bact.	Bact.	-
Exp. 4 1/50	Under	1,350	400	875	2,000	1,450	1,725	1,100	1,000	1,050
	Between	850	1,100	977	1,050	1,800	1,425	1,350	1,600	1,475
1/100	Under	2,100	2,300	2,200	1,700	1,700	1,700	1,100	1,400	1,250
	Between	1,200	1,600	1,400	1,600	2,500	2,050	2,300	1,800	2,050
1/500	Under	6,500	9,500	8,000	5,500	6,000	5,750	6,000	3,500	4,750
	Between	7,500	6,500	7,000	5,500	4,000	4,750	5,000	1,500	3,250
1/1000	Under	4,000	8,000	6,000	2,000	9,000	5,500	5,000	5,000	5,000
	Between	10,000	5,000	7,000	7,000	11,000	9,000	7,000	3,000	5,000

Bact. - plates covered with numerous bacterial colonies only.

Exp. 2. - Results omitted as agar liquified due to failure ofthermostat, in incubator.

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TABLE 16B NUMBERS OF BACTERIA PER GRAM OF MOIST SOIL

Exp. 1. Dilution	Soil Sample	Plot A (N.P.K.) Plate I	Plate II	Average	Plot B (Kraalmanure) Plate I	Plate II	Average	Plot C (Control) Plate I	Plate II	Average
1/100	(Under Trees	21,200	26,400	23,800	32,800	37,200	35,000	19,600	12,000	15,800
	(Between Trees	28,000	30,400	29,200	37,200	31,200	34,200	30,000	30,400	30,200
1/1000	Under	72,000	104,000	87,000	156,000	120,000	138,000	120,000	112,000	116,000
	Between	88,000	80,000	84,000	104,000	96,000	100,000	136,000	188,000	162,000
1/20,000	Under	560,000	900,000	680,000	460,000	260,000	360,000	520,000	980,000	750,000
	Between	180,000	80,000	130,000	320,000	800,000	560,000	100,000	200,000	150,000
1/40,000	Under	160,000	36,000	98,000	440,000	720,000	580,000	560,000	116,000	338,000
	Between	160,000	32,000	96,000	680,000	800,000	740,000	440,000	560,000	500,000
Exp. 3										
1/50	Under	Bacterial colonies too small and numerous for counting.								
	Between									
1/60	Under	Bacterial colonies too small and numerous for counting.								
	Between									
1/500	Under	70,000	70,000	70,000	85,000	80,000	82,500	68,000	71,000	69,500
	Between	35,000	195,000	11,500	52,000	65,000	58,500	50,000	43,500	46,500
1/1000	Under	47,000	48,000	47,500	60,000	84,000	72,000	80,000	80,000	80,000
	Between	24,000	11,000	17,500	80,000	80,000	80,000	32,000	46,000	39,000
Exp. 4										
1/50	Under	Bacterial colonies too small and numerous for counting.						9,200	12,800	11,000
	Between							4,800	-	4,800
1/100	Under	Bacterial colonies too small and numerous for counting.						5,000	6,400	5,700
	Between	11,200	10,800	11,000	-	12,000	12,000	5,600	4,400	5,000
1/500	Under	20,000	12,500	72,500	66,000	58,000	62,000	12,000	7,500	97,500
	Between	1,500	7,000	4,250	30,000	16,000	23,000	4,000	7,000	5,500
1/1000	Under	17,000	3,000	10,000	154,000	88,000	121,000	23,000	40,000	31,500
	Between	4,000	10,000	7,000	14,000	11,000	12,500	4,000	4,000	4,000

Exp. 2: RESULTS OMITTED AS AGAR LIQUIFIED DUE TO FAILURE OF THERMOSTAT IN INCUBATOR.

Owing to the varying soil conditions encountered at the times of sampling, the results are not sufficiently uniform to warrant drawing any definite conclusions.

III QUALITATIVE EXPERIMENTS

A. Soil plates.

a) The method used in the preparation of these plates was that of Warcup (1951). The media used were Brown's agar, malt agar and clear maize agar (see Appendix II). Approximately 0.01 gm. of soil was sprinkled in a sterile Petri dish, and about 15 ccs. of cooled medium was added. The dish was then rotated in order to spread the soil particles evenly throughout the agar. The plates were incubated at 25° C for several weeks and examined at intervals, fungi were isolated from the plates and grown in pure culture for identification. Two plates of each medium were used for soil from each category i.e. soil from under and between the trees of the three plots under investigation.

b) Results showed that the fungi grew on all three media, and there was no difference in the numbers and genera isolated from the various plots, or from under and between the trees. Genera isolated were as follows:

Penicillium expansum Link.	Occurring in 4/6 plates.
Penicillium sp.	
Mucor sp. probably *M. racemosus* Fres.	
Aspergillus sp. probably *A. terreus* Thom.	
Botrytis sp. probably *B. terrestris* Jensen.	
Trichoderma sp. probably *T. viride* Pers. ex Fr.	
Fusarium sp.	
Monilia sp. probably *M. geophilia* Oude.	
Cladosporium herbarum Link.	

Alternaria probably A. humicola Oud.	Occurring in one of the plates.
Chaetomium sp.	
Monotospora (?)	
Verticillium sp.	
Scopulariopsis sp.	

B. Water cultures.

Sparrow's (1940) method was used to isolate aquatic Phycomycetes present in the soil. A small quantity of soil (about 10 - 20 gms.) was placed in a flask containing about 200 ccs. sterile pond water. Halved boiled hemp seeds were suspended in this, by attaching them to spicules on a glass rod. The neck of the flask was plugged with sterile cotton wool. The flask was then incubated at 25°C for 10 days. The hemp seeds were removed after sufficient growth of fungal mycelium was observed on them, and the fungus was cultured on plates of oatmeal and clear maize agar (see Appendix II).

Fungi were isolated from all the soils. Difficulty was experienced in getting the fungi to produce sporangia on the agar plates, despite adopting the various procedures recommended by Legge, (1951) Goldie Smith (1949) and Hickman (1944).

As far as could be ascertained the fungi isolated were various species of Pythium.

IV

SUMMARY AND CONCLUSION.

A preliminary investigation of the microflora in citrus soils was made, in order to provide an indication of the effects of various soil treatments.

As soil conditions varied considerably at the times of sampling, the results of the four experiments (using the dilution plate method) were not comparable. From the numbers obtained, however, the indications are that fungal and bacterial numbers are highest in the plots treated with kraal manure; and fungal numbers in all the plots, are higher in soil under the trees, than between them. Isolations of fungi developed on soil plates, indicate that the flora of all three plots is the same.

As various workers (Waksman, 1931; Chesters, 1948; Warcup, 1951; Skinner, Jones and Mollison, 1952;) have pointed out, the number of colonies developed on the plates does not necessarily give an accurate picture of the microflora present in the soil. In these experiments, counts of fungi and bacteria have not proved a satisfactory method for estimating the effects of the soil fertilizers used.

APPENDIX I

Growth measurements of *Penicillium funiculosum* and *Fusarium moniliforme* under varying environmental conditions.

Tables XVII - XXXIV

The diameters a and b represent linear measurements made along two lines intersecting at right angles, the point of intersection being immediately above the centre of the inoculum. The columns 3 and 4 contain diameters a^1 and b^1 obtained from one plate and columns 5 and 6 the diameters a^2, b^2 obtained from the second plate. The 7th column contains the average of a^1 b^1, a^2, b^2.

TABLE XVII

Growth Measurements of Penicillium funiculosum at 15°C

Time in days	Time in hours	Diameter in cms. a^1	b^1	a^2	b^2	Average diameter	Growth rate in cms/24 hrs.
0	0	.20	.20	.30	.20	.22	.000
1	24	.20	.20	.30	.20	.22	.00
2	48	.20	.20	.30	.25	.23	.01
3	72	.40	.40	.50	.50	.45	.22
4	96	.80	.70	.90	.90	.82	.37
5	120	1.20	1.20	1.20	1.10	1.17	.35
6	144	1.50	1.50	1.60	1.40	1.50	.33
7	168	1.80	1.80	1.90	1.70	1.80	.30
8	192	2.00	2.00	2.00	1.90	1.97	.17
9	216	2.00	2.05	2.10	2.00	2.03	.06
10	240	2.00	2.10	2.10	2.10	2.07	.05

TABLE XVIII

Growth Measurements of Fusarium moniliforme at 15°C

Time in days	Time in hours	Diameter in cms. a^1	b^1	a^2	b^2	Average diameter.	Growth rate in cms/24 hrs.
0	0	.20	.20	.20	.20	.20	.00
1	24	.20	.20	.20	.20	.20	.00
2	48	.30	.25	.20	.20	.23	.03
3	72	1.00	1.00	.90	.90	.95	.72
4	96	1.60	1.70	1.70	1.60	1.65	.7
5	120	2.40	2.50	2.50	2.50	2.47	.82
6	144	3.20	3.25	3.20	3.20	3.21	.74
7	168	3.80	3.80	3.70	3.70	3.75	.54
8	192	4.30	4.30	4.20	4.20	4.25	.50
9	216	4.80	4.90	4.70	4.70	4.77	.52
10	240	5.45	5.50	5.40	5.40	5.44	.37

TABLE XIX

Growth Measurements of Penicillium funiculosum at 25°C

Time in days	Time in hours	Diameter in cms. a^1	b^1	a^2	b^2	Average diameter	Growth rate in cms/24 hrs.
0	0	.25	.30	.30	.35	.30	.00
1	24	.40	.50	.50	.55	.49	.19
2	48	.90	1.00	1.00	1.00	.97	.48
3	72	2.80	2.80	2.90	2.90	2.85	1.88
4	96	4.00	4.10	4.20	4.10	4.10	1.25
5	120	5.00	5.00	5.00	5.00	5.00	.90
6	144	5.50	5.50	5.50	5.50	5.50	.50
7	168	5.80	5.80	5.90	5.90	5.85	.35
8	192	6.20	6.20	6.40	6.40	6.30	.55
9	216	6.40	6.40	6.50	6.55	6.46	.16
10	240	6.00	6.40	6.60	6.60	6.50	.04

TABLE XX

Growth Measurements of Fusarium moniliforme at 25°C.

Time in days	Time in hours	Diameter in cms. a^1	b^1	a^2	b^2	Average diameter	Growth rate in cms/24 hrs.
0	0	.40	.30	.30	.30	.32	.00
1	24	.60	.50	.50	.50	.52	.20
2	48	1.60	1.60	1.60	1.60	1.60	1.12
3	72	2.40	2.35	2.40	2.45	2.40	.80
4	96	3.40	3.30	3.40	3.40	3.37	.97
5	120	4.30	4.20	4.35	4.40	4.31	.94
6	144	5.20	5.20	5.40	5.40	5.30	.99
7	168	5.80	5.80	6.00	6.00	5.90	.60
8	192	6.30	6.30	6.40	6.50	6.37	.47
9	216	6.40	6.40	6.50	6.60	6.47	.10
10	240	6.50	6.50	6.70	6.70	6.60	.13

TABLE XXI

Growth Measurements of Penicillium funiculosum at 32°C

Time in days	Time in hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.20	.20	.20	.20	.20	.00
1	24	.20	.20	.30	.30	.25	.05
2	48	.90	.90	.90	.90	.90	.65
3	72	1.00	1.10	1.10	1.10	1.07	.17
4	96	1.20	1.30	1.20	1.20	1.22	.15
5	120	1.35	1.40	1.30	1.30	1.33	.11
6	144	1.50	1.50	1.40	1.40	1.45	.12
7	168	1.60	1.60	1.50	1.50	1.55	.10
8	192	2.20	2.10	2.40	2.40	2.27	.72*
9	216	2.40	2.40	2.60	2.50	2.47	.20
10	240	2.40	2.40	2.60	2.60	2.5	.03**
11	264	2.4	2.4	2.6	2.6	2.5	.0

TABLE XXII

Growth measurements of Fusarium moniliforme at 32°C

Time in days	Time in hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.20	.20	.20	.20	.20	.00
1	24	.40	.40	.40	.40	.40	.20
2	48	1.20	1.20	1.20	1.20	1.20	.80
3	72	2.30	2.30	2.20	2.20	2.25	1.05
4	96	2.80	2.80	2.70	2.70	2.75	.50
5	120	2.80	2.80	2.80	2.80	2.80	.00
6	144	2.80	2.80	2.80	2.80	2.80	.00
7	168	2.80	2.80	2.80	2.80	2.80	.00
8	192	3.80	3.80	3.70	3.60	3.72	.90 *
9	216	4.60	4.50	4.50	4.40	4.50	.80
10	240	4.60	4.60	4.50	4.50	4.55	.05**
11	264	4.70	4.70	4.50	4.50	4.60	.10

* Temperature lowered to 25°C

** Temperature raised to 32°C

TABLE XXIII

Growth measurements of Penicillium funiculosum grown over water.

Time in days	Time in Hours	Diameter in cms. $^{1}_{a}$	$^{1}_{b}$	$^{2}_{a}$	$^{2}_{b}$	Average Diameter	Growth rate in cms/24 hrs.
0	0	.30	.30	.30	.30	.30	.00
1	24	.60	.60	.70	.65	.63	.33
2	48	1.20	1.20	1.20	1.20	1.20	.57
3	72	2.40	2.40	2.40	2.40	2.40	1.20
4	96	3.20	3.20	3.10	3.10	3.15	.75
5	120	4.00	4.00	3.90	3.90	3.95	.80
6	144	5.00	5.00	4.90	4.90	4.95	1.00
7	168	5.80	5.80	5.70	5.70	5.75	.80
8	192	6.40	6.40	6.20	6.30	6.32	.57
9	216	6.80	6.70	6.65	6.70	6.71	.39
10	240	6.80	6.80	6.70	6.70	6.75	.04

TABLE XXIV

Growth Measurements of Fusarium moniliforme grown over water.

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.30	.30	.20	.20	.25	.00
1	24	.50	.50	.40	.40	.45	.20
2	48	1.40	1.40	1.30	1.30	1.35	1.10
3	72	2.40	2.40	2.30	2.30	2.35	1.00
4	96	3.70	3.60	3.60	3.60	3.62	1.27
5	120	4.70	4.70	4.60	4.60	4.65	1.03
6	144	5.70	5.60	5.80	5.70	5.70	1.05
7	168	6.60	6.60	6.80	6.80	6.70	1.00
8	192	7.30	7.30	7.40	7.40	7.35	.65
9	216	8.30	8.20	8.40	8.40	8.32	.97
10	240	8.60	8.50	8.80	8.80	8.67	.35

TABLE XXV

Growth Measurements of Penicillium funiculosum grown at pH4.0

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.40	.40	.40	.40	.40	.00
1	24	.70	.70	.70	.70	.70	.30
2	48	1.60	1.60	1.60	1.60	1.60	.90
3	72	2.70	2.70	2.60	2.70	2.67	1.07
4	96	4.70	4.80	4.90	5.00	3.97	1.30
5	120	4.70	4.80	4.90	5.00	4.80	.93
6	144	5.80	5.90	5.60	5.70	5.70	.90
7	168	6.20	6.30	6.10	6.20	6.20	.50
8	192	6.50	6.50	6.60	6.60	6.55	.35
9	216	6.75	6.80	6.80	6.80	6.78	.33
10	240	6.90	6.90	6.90	6.80	6.87	.09

TABLE XXVI

Growth Measurements of Fusarium moniliforme grown at pH4.0

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.50	.50	.40	.50	.47	.00
1	24	1.00	1.20	1.10	1.20	1.12	.42
2	48	2.10	2.20	2.20	2.20	2.17	1.05
3	72	3.60	3.60	3.60	3.60	3.6	1.49
4	96	5.00	5.00	4.90	4.90	4.95	1.35
5	120	6.40	6.40	6.30	6.30	6.35	1.40
6	144	7.25	7.30	7.20	7.20	7.22	.87
7	168	8.00	8.00	8.00	8.00	8.0	.78
8	192	8.50	8.50	8.40	8.45	8.46	.46
9	216	8.70	8.70	8.60	8.60	8.65	.19
10	240	8.7	8.7	8.7	8.7	8.7	.05

TABLE XXVII

Growth Measurements of Penicillium funiculosum grown at pH5.5

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.30	.40	.40	.40	.37	.00
1	24	1.50	1.60	1.60	1.60	1.57	1.20
2	48	2.90	2.90	2.90	2.90	2.90	1.33
3	72	3.90	3.90	3.90	3.90	3.90	1.00
4	96	4.50	4.60	4.90	4.90	4.72	.82
5	120	5.50	5.50	5.60	5.60	5.55	.83
6	144	6.00	6.00	6.10	6.10	6.05	.50
7	168	6.30	6.30	6.50	6.50	6.40	.35
8	192	6.70	6.70	6.90	6.90	6.80	.40
9	216	6.70	6.70	7.10	7.10	6.90	.10
10	240	6.80	6.90	7.10	7.10	6.97	.07

TABLE XXVIII

Growth measurements of Fusarium moniliforme grown at pH5.5

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.30	.30	.30	.30	.30	.00
1	24	.60	.50	.60	.60	.57	.27
2	48	1.30	1.30	1.30	1.30	1.30	.73
3	72	2.60	2.60	2.70	2.70	2.65	1.35
4	96	3.50	3.60	3.60	3.60	3.57	1.92
5	120	4.40	4.40	4.50	4.50	4.45	.88
6	144	5.30	5.30	5.20	5.20	5.25	.80
7	168	6.30	6.30	6.20	6.30	6.27	1.02
8	192	7.30	7.30	7.30	7.30	7.30	1.03
9	216	8.10	8.10	8.00	8.00	8.05	.75
10	240	8.30	8.30	8.10	8.10	8.20	.15

TABLE XXIX

Growth measurements of Penicillium funiculosum grown at pH 6.5

Time in Days	Time in Hours	Diameter in cms. 1 a	1 b	2 a	2 b	Average Diameter	Growth rate in cms/24hrs.
0	0	.40	.40	.30	.40	.37	.00
1	24	.80	.80	.70	.80	.77	.40
2	48	1.90	1.90	1.80	1.90	1.87	1.10
3	72	2.80	2.80	2.80	2.80	2.80	.93
4	96	3.40	3.40	3.60	3.60	3.50	.70
5	120	4.10	4.10	4.30	4.30	4.20	.70
6	144	4.80	4.80	4.90	4.90	4.805	.65
7	168	5.00	5.00	5.10	5.10	5.05	.20
8	192	5.40	5.50	5.40	5.35	5.45	.40
9	216	5.50	5.50	5.40	5.40	5.45	.00
10	240	5.60	5.50	5.40	5.40	5.47	.02

TABLE XXX

Growth Measurements of Fusarium moniliforme grown at pH 6.5

Time in Days	Time in Hours	Diameter in cms. a	b	a	b	Average Diameter	Growth rate in cms/24 hrs.
0	0	.40	.40	.35	.30	.36	.00
1	24	.80	.80	.70	.70	.75	.39
2	48	1.60	1.70	1.60	1.50	1.60	.85
3	72	2.60	2.70	2.70	2.60	2.65	1.05
4	96	3.30	3.80	3.80	3.70	3.77	1.12
5	120	5.00	5.00	5.60	4.90	4.97	1.20
6	144	6.00	6.10	6.00	6.00	6.02	1.05
7	168	6.80	6.90	6.80	6.80	6.87	.85
8	192	7.40	7.40	7.30	7.30	7.35	.48
9	216	7.50	7.45	7.50	7.40	7.46	.11
10	240	7.50	7.50	7.50	7.40	7.47	.01

TABLE XXXI

Grow th Measurements of Penicillium funiculosum grown at pH 7.5

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24hrs.
0	0	.40	.40	.30	.35	.36	.00
1	24	.70	.70	.60	.60	.65	.29
2	48	1.80	1.80	1.70	1.70	.175	1.10
3	72	3.00	3.00	3.00	3.00	3.00	1.25
4	96	4.60	4.50	4.60	4.60	4.57	1.57
5	120	5.60	5.50	5.70	5.70	5.62	1.05
6	144	6.60	6.60	6.50	6.50	6.55	.93
7	168	7.10	7.10	7.00	7.10	7.07	.52
8	192	7.60	7.60	7.60	7.60	7.60	.53
9	216	7.90	7.90	7.80	7.90	7.88	.28
10	240	7.90	7.95	7.90	7.90	7.91	.03

TABLE XXXII

Growth Measurements of Fusarium moniliforme grown at pH 7.5

Time in Days	Time in Hours	Diameter in cms. a^1	b^1	a^2	b^2	Average Diameter	Growth rate in cms/24 hrs.
0	0	.40	.40	.40	.40	.40	.00
1	24	.80	.80	.80	.80	.80	.40
2	48	1.96	1.90	2.00	2.00	1.96	1.16
3	72	3.40	3.40	3.40	3.40	3.40	1.44
4	96	4.80	4.80	4.80	4.80	4.80	1.4
5	120	5.70	5.70	5.80	5.80	5.75	.95
6	144	6.60	6.60	6.70	6.70	6.65	.90
7	168	7.80	7.70	7.80	7.70	7.75	1.10
8	192	8.30	8.25	8.30	8.20	8.26	.51
9	216	8.30	8.30	8.30	8.30	8.3	.04
10	240	8.30	8.30	8.30	8.40	8.31	.01

TABLE XXXIII

Average daily growth rate of fungi at various temperatures and increased humidity.

Fungus	15°C	25°C	32°C	Over water (25°C)	Growth Period.
Penicillium funiculosum	.0	.33 cms.	.35 cms.	.45 cms.	during first two days.
Fusarium moniliforme	.01	.66 cms.	.5 cms.	.65 cms.	
P. funiculosum	.32 cms.	1.16 cms.	.13 cms.	.94 cms.	during 2nd - 6th days.
F. moniliforme	.75 cms.	.93 cms.	.39 cms.	1.09 cms	

TABLE XXXIV

Average daily growth rate of fungi at various H-ion concentrations.

Fungus	pH 4.0	pH 5.5	pH 6.5	pH 7.5	Period of growth
Penicillium funiculosum	.6 cms	1.26 cms	.75 cms	.69 cms	during first two days.
Fusarium moniliforme	.73 cms	.5 cms	.62 cms	.58 cms	
P. funiculosum	1.05 cms	.79 cms	.74 cms	1.20 cms	during 2nd-6th days
F. moniliforme	1.28 cms	1.25 cms	1.10 cms	1.17 cms	

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APPENDIX II

a) Media used for culturing fungi.

Pineapple agar.

200 ccs. pineapple juice was expressed from fresh peeled pineapple fruits, and autoclaved at 20 lbs. pressure and 126°C for 25 minutes. This was added to 1 litre of distilled water and 30 gms. agar. A large percentage of agar is required to ensure that the medium will gel, as the juice is highly acid. The agar was autoclaved at 10 lbs. pressure and 115°C for 15 minutes.

Malt agar.

20 gms. malt extract and 20 gms. agar in 1 litre of distilled water, autoclaved at 20 lbs. pressure for one hour.

Clear maize agar.

50 gms. of freshly ground mealies in 1 litre water, cooked at 60°C for one hour, then filtered until clear, 15 gms. of agar were added and the mixture heated. Autoclaved at 15 lbs. pressure for one hour.

Oat agar.

50 gms. of oats were made into a paste with 300 ccs. of water, and steamed for one to two hours until the mixture had reached the 600 cc. mark. 15 gms. of agar were dissolved in 400 ccs. water, added to the oat mixture and steamed for half an hour. The agar was autoclaved at 15 lbs. pressure for

one hour.

Brown's synthetic agar.

Asparagin 1 gm., glucose 1 gm., potassium dihydrogen phosphate 0.625 gm., magnesium sulphate 0.375 gm., agar 10 gms., distilled water 500 ccs. Autoclaved at 10 lbs. pressure for 15 mins.

b) Staining.

Small portions of fungal mycelium were mounted directly in lactophenol and acid fuchsin, or cotton blue. The slide was warmed gently. Cover slips were sealed by ringing with Kronig's cement.

c) Microtomed sections.

Diseased pineapple tissue was embedded in paraffin wax for microtoming. The sections were mounted in Canada Balsam.

Double stains used were:-

1. Picric aniline blue and saffranin.
2. Eosin Y and fast green in clove oil.

The former method was found to give better results than the latter. Both cotton blue and acid fuchsin were used as well, being quicker than the double staining methods.

VI

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