Studies in the infection of oranges by *Penicillium digitatum* Sacc.

by

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Ph.D. thesis presented to the University

of London.

January, 1965.

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ABSTRACT

In a study of the infection of oranges by Penicillium digitatum Sacc. it was found that spores suspended in water, different rind extract media, or citric acid solution did not infect uninjured fruit. Wounds were necessary for infection but wounds between the vesicles of the flavedo were resistant to infection whereas other wounds were not. Infection of fruit through wounds into the rind tissues was affected by spore load, depth and position of wounds, number of pricks/inoculum site, method of inoculation and wound-healing period.

It was found from a microscopic study of the penetration of rind tissues by hyphae in wounds that hyphae in inter-vesicle wounds did not penetrate the flavedo tissues prior to the penetration of the albedo, that leads to the formation of lesions. Hyphae in wounds into the oil vesicles passed down the thin-walled cells of the oil vesicles and into the albedo before lesions were formed.

Inoculated wounds which developed red spots were generally resistant to infection.

Flavedo was more liable to infection when spores were suspended in rind extracts, essential oil, orange juice, citric acid and buffered pectin solutions.

Germination of spores was good in sugar-phosphate, glucose-Yeastrel, glucose-casamino acid mixtures, and in extracts of rind tissues. Emanations from rind infected with P.digitatum stimulated germination of spores in water. The stimulatory effect of wounding between and into the oil vesicles on germination of spores was similar.

Growth of mycelium was good in different rind extract media but was not increased by addition of a vitamin mixture to a synthetic medium.

Active PME was found in culture filtrates in vitro, but PG, Cellulase (Cx), and Macerating Enzyme were of low activity.

PME was active in 0.2M NaCl extract of infected rind tissues, Macerating Enzyme was not very active, PG almost inactive and Cx was not found.

The development and control of wastage in the transport and storage of fruit was also studied.

CONTENTS

INTRODUCTION

The infection of oranges by *Penicillium digitatum* Sacc. is the subject of this thesis. This fungus is the cause of Green Mould, a post-harvest disease of Citrus fruit.

Green Mould and the causal fungus, *P.digitatum* have been subjects of study in Citrus fruit growing areas throughout the world for sometime past because of the large losses of fruit in transport and storage caused by it. A major aim of these investigations has been the development of a non-toxic fungicide (or fungistat) capable of controlling wastage of Citrus fruit caused by *P.digitatum*.

From a review of the literature on work done on Green Mould and *P.digitatum* it appeared that the more fundamental aspects of the parasitism of Citrus fruit by *P.digitatum* had been neglected. Details of the infection process and subsequent parasitism of oranges by *P.digitatum* were thus made the main aims of this investigation.

Also included, as part of the thesis, is an account of work done in conjunction with Dr. R.A. Christ, of the South African Co-operative Citrus Exchange Ltd., on the development and control of wastage in shipments of oranges from South Africa during 1961 and 1962.

ANATOMY OF ORANGE RIND

The detailed anatomy of Citrus fruit is described by Bartholomew and Reed in volume 1 of "The Citrus Industry" (edited by Webber and Batchelor, 1943).

A general description of the anatomy of orange rind is given here, as infection of fruit through injuries into the different rind tissues is frequently referred to throughout the text.

For the purpose of this work, the rind is divided into (a) epidermis, (b) an outer orange coloured flavedo layer and (c) an inner white albedo layer (Plate 1).

(a) Epidermis

The epidermis forms the outer wall of the flavedo layer, is heavily cutinised and interspersed with stomata.

(b) Flavedo

The flavedo is a compact tissue 20 cell layers deep (approx.) and is divided into an inner and outer layer. The outer layer is composed of isodiametric collenchyma cells, which become progressively larger and more thin walled in the inner layer. Many chromatophores are contained in these cells.

The oil vesicles (or glands) are embedded in the inner layer and usually extend into the albedo. The walls of the oil vesicles are composed of large thin-walled parenchyma cells. The cells of the outer flavedo covering the oil vesicles are called oil vesicle cover cells.

The flavedo extends 1.5 mm. (approx.) below the epidermis.

There is no sharp distinction between the inner layer of the flavedo and the albedo.

(c) Albedo

The albedo layer is white, containing few chromatophores and is composed of thin-walled parenchyma cells, which in mature fruit are elongated and branched forming an intricate network of cells and large intercellular spaces. This gives a spongy texture to the mature peel.

The main vascular bundles are situated in the albedo but they are also found in the flavedo.

The thickness of the albedo layer varies considerably between different varieties and fruit of the same variety.

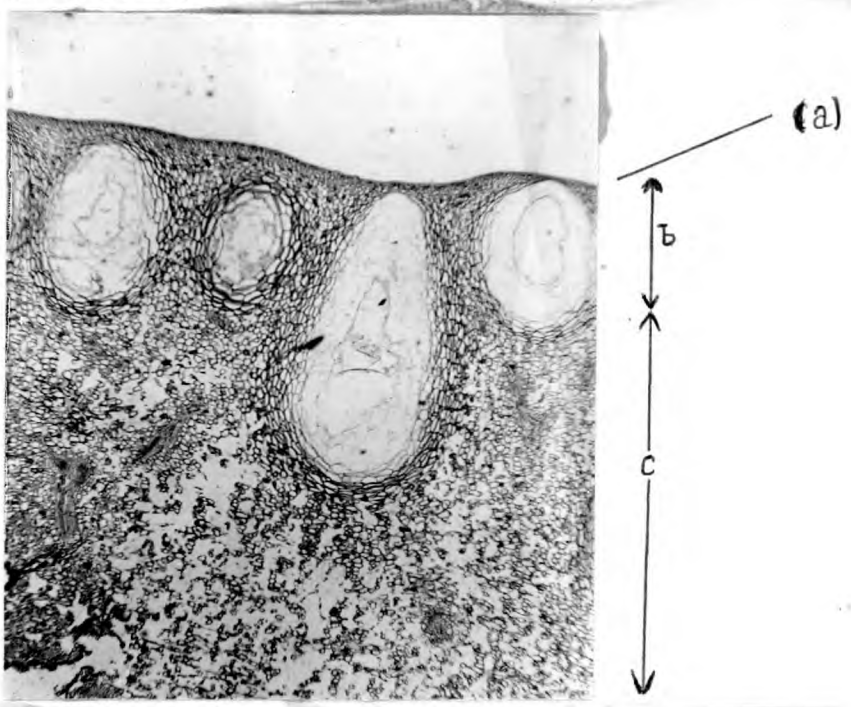

Plate 1. Longitudinal section (L.S.). Orange rind.

(a) Epidermis
(b) Flavedo
(c) Albedo

PART 1

SECTION 1 - REVIEW OF LITERATURE

Disease

Green Mould is a post harvest disease of Citrus fruit and is caused by Penicillium digitatum Sacc. The cultural characteristics of the fungus are described by Thom (1910) and also by Raper and Thom (1949).

The fungus is almost specific to Citrus fruit and is found in almost all Citrus fruit growing areas throughout the world.

Symptoms

The disease symptoms caused by the fungus were described by Fawcett (1936) who pointed out the characters which distinguish it from Blue Contact Mould caused by Penicillium italicum Wehmer.

Plate 2 shows the stages in development of Green Mould on orange fruit.

Both Green Mould and Blue Contact Mould appear as water-soaked soft areas which are often referred to as "water rot", "blister rot" or "sneak rot". A little later white mycelium develops at the centre of each water soaked area and it is not until spores are produced that the two diseases can be distinguished. Green Mould produces olive-green spore masses whereas Blue Contact Mould produces masses of blue spores.

Green Mould rapidly spreads through the whole fruit which becomes a soft shrunken mass covered with a layer of olive-green spores. If the humidity is low the fruit shrinks to a wrinkled dry mummy, but in high humidity other moulds and bacteria enter, and the fruit

Plate 2. Stages in development of Green Mould on orange fruit.

Top left: healthy fruit; middle: water rot; right: white mycelium rot.
Bottom left: Green Mould; middle: advanced Green Mould; right: decomposed Green Mould fruit.

flattens into a soft decomposing mass.

The main characteristic features of Green Mould are (1) the wide white margin ahead of the green area while the soft areas are enlarging; (2) the white area of mycelium usually pasty and wrinkled; (3) the green spore masses confined to the surface of the fruit and (4) the wrapper adhering readily to the damaged part of the fruit.

Infection

In a study of the infection of oranges by P.digitatum and P. italicum, Green (1932) found that wounds were necessary for infection when fruit were inoculated with spores in water. However, shallow minute wounds in the form of pricks were resistant to infection. She suggested that the outer rind tissues (flavedo) were resistant to infection because this tissue was hard and compact and contained no soluble pectin. She found that an inoculum of spores in citric, malic, oxalic and sulphuric acids or orange juice promoted infection through minute wounds, and also through uninjured rind. In uninjured rind the stomata were the channels of entry. Green suggested that the acids and orange juice hydrolyzed the insoluble precursor of pectin in the flavedo cell walls into soluble pectin. This caused softening of the cell walls of this tissue and led to loss of resistance to infection.

This work was followed by that of Bates (1933) who showed that the oil vesicles provide a suitable avenue for infection.

Bates (1936), in his inoculation of fruit, unlike Green (1932), distinguished between inoculations into, and between, the oil

vesicles. He found that the outer oil-bearing portion (flavedo) of the rind and the inner white (albedo) tissue were resistant to infection when they were inoculated with dry spores between the oil-vesicles. Infection was promoted by an inoculum of spores in water, but very shallow wounds still remained comparatively resistant to invasion. The amount of infection increased with deeper wounding of the flavedo and wounds extending into the albedo showed no resistance to infection when inoculated in this way. He also found that oranges became infected in the absence of wounds when fruit were left to stand on their stem ends in an inoculum of spores in water contained in a petri dish.

Tosco (1951) found that P.digitatum acted as a wound parasite and that healthy fruit, when placed in contact with infected fruit, became infected themselves. He also observed that spores placed in an oil gland did not develop mycelium.

Littauer and Nadel-Schiffmann (1952) found that a period of 2-3 days incubation was necessary for the formation of lesions on Valencia and Shamouti oranges, lemons and grapefruit, when these fruit were inoculated and incubated at temperatures in the range 18-27°C. When inoculated fruit of the same varieties were incubated at 32-33°C. only grapefruit was attacked and a period of 4 weeks was necessary before lesions were observed. The incubation period for the formation of lesions increased with decrease in temperature.

Dellerē (1953) stated that penetration does not take place mechanically because a thin covering of collodion provided

protection of Citrus fruit against contact infection.

Nadel-Schiffmann and Littauer (1956), studying the mode of infection of Citrus fruit by the fungus, found that infection-rate was higher with scratched than with pricked inoculations and also that infection-rate increased with depth of wound. Inoculations pricked into the oil glands gave consistently higher rates of infection , regardless of depth, than did those between the glands.

Miyakawa (1958) investigating the infection of Satsuma oranges by P.digitatum and P.italicum found that fruit were easily infected by both fungi when oil glands were inoculated by pricking. It was also found that P.digitatum only infected fruit through fresh wounds and that freshness of wound did not affect infection by P.italicum.

Penetration

Both Klotz (1930) and Green (1932) studied the penetration of fruit by the fungus but their work dealt mainly with the spread of the fungus through the host in well established lesions.

Klotz (1930) found that the hyphae penetrated the tissues of the rind intracellularly as well as intercellularly. At first the fungus penetrated all the cells of the albedo and flavedo tissues but avoided the space containing the oil. Hyphae also invaded the juice vesicles but the expanded part of the vesicle containing the juice was the last to be invaded.

In addition, Green (1932) found that the first effect of the fungus at the advancing margin of decay was to cause the albedo

cells to swell. Growth of hyphae through this tissue caused dissolution of cell walls. The cell walls of the flavedo were unchanged in the soft zone (margin of decay) but at the region where conidiophores emerged, they became swollen and were penetrated by hyphae.

Growth

Fergus (1952) reviewing reports in the literature on various aspects of the nutruitional requirements of the fungus found that the results of various workers were not in agreement, particularly in regard to the growth factor requirements and the suitability of different nitrogen compounds for the growth of the fungus.

The following is a summary of conclusions drawn by Fergus from previous cultural studies. "P.digitatum requires thiamin and will not grow unless it is added to the medium (Wooster and Cheldelin, 1945); P.digitatum requires the addition of extracts of natural materials or organic compounds to grow satisfactorily, although it will grow slightly in a synthetic medium without their addition (Thom,1910; Camp,1923; Marloth, 1931; Raistrick et al., 1931); or P.digitatum will grow satisfactorily in a synthetic medium without added growth factors or extracts of natural materials (Woltje, 1918; Chrzaszcz and Tiukow, 1930; Kursanoff and Alexeyeva, 1938; Pratt, 1944)".

Fergus (1952) studied the effect of carbon, nitrogen and various growth factors on growth. He found that the addition of a mixture of vitamins to a basal medium allowed growth to start earlier than

in the basal medium alone but the mycelium finally produced was practically the same in both sets of treatments. These results agreed with those of Pratt (1944) but directly contradicted those of Wooster and Cheldelin (1945), who found that thiamin or the thiazole moiety of thiamin was required.

Miyakawa (1958) found that growth of the fungus was more vigorous on media of smashed rind tissue than on juice agar. Growth was also stimulated on a synthetic agar medium containing fruit rind extracts. The growth stimulating substance or substances were soluble in water, 80% methanol or 80% acetone but not in ether. Desiccation or pasteurization of the rind had no effect on the stimulation.

Miyakawa (1959) continuing his studies on the stimulating effect of the contents of Satsuma orange fruit rind on growth of P.digitatum found that the growth stimulating substance (or substances) was readily adsorbed by active carbon; on agar media containing the rind extract treated with carbon the mycelial growth of P.digitatum was decreased even when the amount of carbon added was very small.

Spore germination

Comparatively little is known about the germination of spores of the fungus. Marloth (1929) found that spores did not germinate below pH 2.3 and that the optimum range of pH for germination in modified Duggar's solution was 3.5 – 8.0.

Tosco (1951) found that fruit inoculated with dry spores in the absence of wounds did not germinate to produce a mycelium when fruit were kept at 76-77% relative humidity.

The germination of spores was also investigated by Dellerć (1953) and he found that the spores did not germinate in double-distilled water, and also that modification of the osmotic pressure of different salt solutions failed to induce germination.

Studying the effect of orange rind extract on spore germination, Miyakawa (1958) found that this extract stimulated spore germination. The stimulative factor was readily extracted from the rind by water and absorbed by activated charcoal (active carbon).

The component of the rind extract absorbed by charcoal was released by 60-80% acetone and the concentrated material from acetone extract stimulated spore germination only when added with the filtrate (charcoal treated extract) or with a very small amount of phosphate. Thus, the phosphoric compounds were found to activate the germination stimulant.

Pectic enzymes

Green (1932) suggested that pectic enzymes may be secreted by the fungus while rotting oranges but she was unable to demonstrate the presence of such enzymes in extracts of rotted fruit.

However, Miyakawa (1962) studying the decomposition and utilization of pectin by P.digitatum and P.italicum showed that protopectinase was produced by both fungi but that activity of both species was much lower than those of the less pathogenic P.expansum and P.purpurogenum.

SECTION II - MATERIALS AND METHODS

A. Fungus

An isolate of *Penicillium digitatum* Sacc. was obtained from a rotting Valencia orange. A monospore culture was established and pathogenicity of this culture was confirmed. Stock cultures were grown on V8 vegetable juice agar and stored by freezing at - 20°C., and at 4°C. under liquid paraffin.

Subcultures of the fungus were also grown on V8 vegetable juice agar in McCartney tubes at 22-24°C. for the production of spores. An even carpet of mycelium and spores resulted within 4 days of inoculation. The period between sowing and harvesting varied from 7 to 15 days.

B. Fruit

Experimental fruit were of the Navel, Valencia and Shamouti varieties and each fruit measured 3" approx. in diameter at the equator i.e. midway between the stem and blossom ends of the fruit. Fruit of this size are described as count 150 in the trade i.e. there are 150 fruit in a standard packing case measuring 26" x 12" x 12".

The Navel and Valencia fruit were imported from South Africa and were the produce of the South African Co-operative Citrus Exchange Co., Ltd. The Shamouti fruit were imported from Israel and were the produce of the Citrus Marketing Board.

The fruit were stored in a cold room at 4°C. for a period not

exceeding 3 weeks. All fruit were washed in tap water, dried and carefully examined before use so that those with bad blemishes or any sign of fungal infection could be rejected.

C. Inoculation experiments

1. Spore suspensions

The carpet of mycelium and spores, produced by a subculture of the fungus in McCartney tubes, were rubbed with a sterile wire loop and transferred to McCartney tubes containing Tween 80 distilled water (1 part Tween 80: 10,000 parts water).

After shaking, the suspension was filtered through 3 folded cones of sterile muslin to remove most of the hyphae. The resulting spore suspension was washed 4 times with sterile distilled water by centrifugation at 2,500 r.p.m. for 30 seconds.

The spore suspension was made in the appropriate medium and the spore concentration was adjusted to contain not less than 1×10^6 spores per ml. This eliminated the possibility of variation in results caused by density of inoculum.

2. Preparation of fruit

All fruit were washed in tap water to remove any debris or residual fungicide on the fruit surface. After drying in a clean cloth, the fruit were surface sterilized by swabbing with 95% alcohol which was allowed to dry without flaming.

At the equator of each fruit a site free from injury and of area 0.4 sq. cm. (approx.) was marked out with 4 spots of indian ink from a camel hair brush (Pl.5, f.28).

3. Wounding

The number and relative position of wounds varied between experiments. Wounds, in the form of pricks, were made using No. 1 entomological pins sealed into glass tubes with "Araldite" so that definite lengths of pin projected (Pl. 3). The instruments thus formed were robust, easily cleaned and sterilized by flaming in alcohol.

4. Inoculation

With the aid of a dissecting microscope (X12) drops of spore suspension were placed on the inoculum sites from the hypodermic needle of an "Agla" syringe. The use of this microscope (X12) ensured complete coverage of wounds by the inoculum drop.

The volume of the inoculum drop varied between experiments; drops of volume 0.01 and 0.02 ml. with careful handling remained fixed in position at the inoculum site. Larger drops however, were held in position by glass rings, 1 cm. diameter, sealed to the fruit surface with Vaseline.

5. Design of inoculation

Fruit of the one variety and from the same consignment of fruit were used in each experiment. One inoculum site per fruit was selected as above. In general, 10 fruit were inoculated for a particular treatment in each experiment and fruit of the same treatment were incubated in the one box.

The choice of 10 fruit per treatment was considered to be a fair sample and it was chosen in preference to a smaller number of

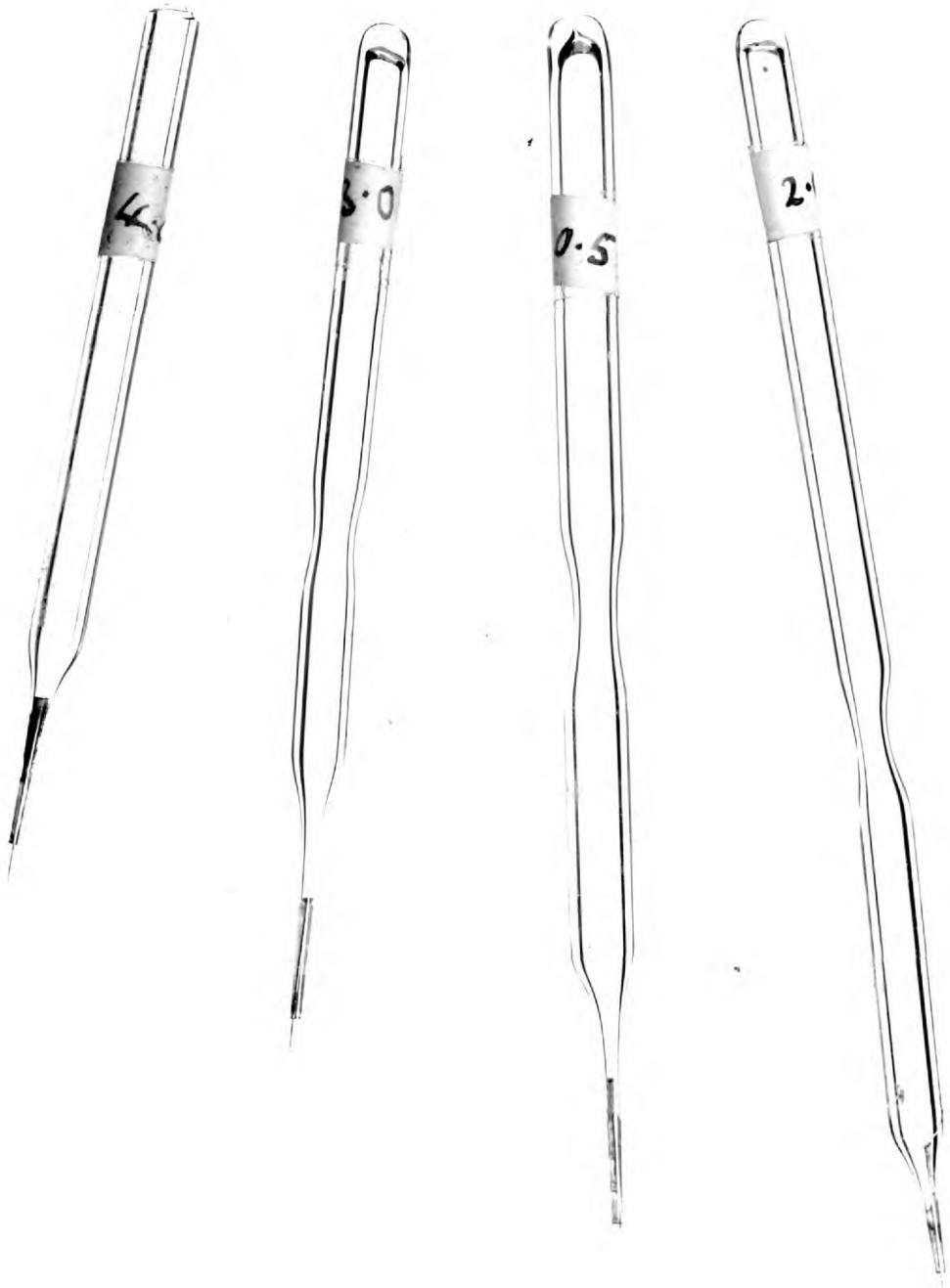

Plate 3. Wounding instruments.

fruit, each with a greater number of inoculum sites per fruit.

6. Incubation

After inoculation each fruit was placed on an aluminium ring (Pl. 4) and transferred to a plastic box which had a tightly fitted transparent lid (Pl. 5). A humid atmosphere was maintained by the addition of water sufficient to cover the bottom of the box. The size of the boxes, 13" x 9½" x 6", was such that 10 fruit could be placed side by side but not in contact in one box. Boxes with fruit were incubated at 22-24°C. in the light (fluorescent).

7. Assessment of results

After the inoculated fruit had been incubated in the humid chambers, the number of inoculum drops producing lesions were counted. The term "lesion" is used to describe the initial softening or "water rot" caused by the spores at the inoculum site.

D. Chemical materials

1. Pectic substances

Pectin (P) and sodium polypectate (NaPP) were obtained from Sunkist Growers Inc., California.

P is a high methoxyl pectin, in which a number of carboxyl groups of the anhydro -galacturonic acid units are esterified with methyl groups. NaPP is the sodium ammonium salt of polygalacturonic acid.

These substances were washed with 60% ethyl alcohol containing 0.1N HCl. The acid was removed by filtration through No. 541 Whatman filter paper and the residue washed in 95% ethyl alcohol.

Plate 4. Fruit mounted on aluminium ring.

Plate 5. Plastic box containing inoculated fruit. Black spots mark inoculum sites.

Ethyl alcohol was removed by evaporation in a 70°C. oven for 24 hours. The dry powders were left at room temperature for 48 hours to equilibrate with air and stored in screw cap bottles. Final moisture content of P and NaPP were 9.12% and 9.65% respectively.

Solutions of 1% (w/v) concentration were prepared by slow addition of powdered pectic substance to distilled water stirred by a magnet. Adjustments in the pH of these solutions were made by addition of 0.1N NaOH or 0.1N HCl. Solutions were stored for periods up to 7 days under a few drops of toluene at 4°C.

2. Cellulose

Soluble cellulose was carboxymethylcellulose (CMC) (Hercules Powder Co., U.S.A.). CMC composed of high chain length polymers with a degree of substitution of 0.4 was used in growth experiments and testing culture filtrates for cellulose activity. CMC, high chain length polymers with a degree of substitution of 0.7, was used in the preparation of a cellulose gum seal for Van Tieghem cells.

Insoluble cellulose used in growth experiments was Solka Floc wood cellulose stated to contain 99.5% cellulose and obtained from Brown and Co., Boston, U.S.A.

Solutions of CMC were prepared as described for pectic substances.

3. Sugars

Sugars were obtained from B.D.H., Poole and Light and Co., Ltd., Colnbrook.

4. Casamino acids

Bacto-vitamin free casamino acids were obtained from Difco Laboratories, Detroit, U.S.A.

5. Vitamins

Vitamins were supplied by B.D.H., Poole.

6. General chemical reagents

General chemical reagents were of analar grade and supplied by either B.D.H, or Hopkins and Williams, Chadwell Heath, Essex.

7. Tween 80

Tween 80 was supplied by Honeywill-Atlas Ltd., London.

8. Orange essential oil

Pure essential oil from oranges, prepared by the "cold-press method" was supplied by Dr.T Gutter, The National and University Institute of Agriculture, Rehovoth, Israel. The oil was kept in a tightly stoppered bottle wrapped in black paper at $4^{\circ}C$.

E. Microtechnique

1. Fixation

(a) Formalin - propiono - alcohol (FPA)

50% ethyl alcohol	90 ml.
Propionic acid	5 ml.
Formalin	5 ml.

The material was left under suction in the fixative for 30 min., fresh fixative was added and the material left to stand for 24 hr. in specimen tubes.

2. Dehydration

The material was dehydrated prior to embedding by taking it through the following series of mixtures of tertiary butyl alcohol, ethyl alcohol and water.

Mixture	Water	95% Ethyl alcohol (ml.)	Tertiary Butyl alcohol (ml.)	100% Ethyl alcohol (ml.)	% Total alcohol (approx.)	Time
1	50	40	10	-	50	2 hours
2	30	50	20	-	70	overnight
3	15	50	35	-	85	1 hour
4		45	55	-	95	1 hour
5			75	25	100	2 hours

A few dry pieces of erythrosin dye were added to mixture 5 to stain the material superficially so that it could be easily orientated during embedding.

From mixture 5 the material was taken through 3 changes of pure tertiary butyl alcohol; the tertiary butyl alcohol was changed after 1, 24 and 1 hour intervals. The dehydrated material was then transferred to a 1:1 mixture of liquid paraffin and tertiary butyl alcohol. The method of embedding followed was the same as that described by Johansen (1940) P. 134-135. Paraffin wax (M.P.56°C.) was used for embedding.

3. Staining

Serial microtome sections (L.S. and T.S.), 8 to 12 μ thick

were fixed to slides smeared with Haupt's adhesive (Haupt, 1923). Each slide was flooded with 3% formalin in distilled water and the wrinkles were removed from the paraffin ribbon by gentle heating over an alcohol flame. The excess water was drained off and the slides were left to dry at room temperature for 48 hours before removal of paraffin.

Paraffin was removed according to the following schedule.

Xylol 5 min.

Xylol-100% ethyl alcohol mixture (1:1), 5 min.

100% ethyl alcohol-ether mixture (1:1), 5 min.
(ether contained less than 1% Celloidin)
Slides removed and allowed to remain in air until whitish opaque.

95%, 70% and 35% ethyl alcohol 5 min. in each

Water . 5 min.

Sections were stained in thionin - orange G. The technique adopted, with a few modifications, was similar to that described by Gurr (1953) for infected plant tissues.

(a) Stains

(i) Carbol thionin

	Thionin (Ehrlich)	0.125g.
	Phenol crystals	1g.
10%	Alcohol	100 ml.

Heat to dissolve and filter. The solution deteriorates after a few weeks.

(ii) Orange G

0.2% Orange G in 100% alcohol.

(b) Technique

Stain in thionin 1 hour.

Rinse in 70% ethyl alcohol.

Rinse in 90% " " .

Rinse quickly in 2 changes of 100% ethyl alcohol.

Differentiate in orange G solution ½ - 1 min.

Rinse in 100% ethyl alcohol-xylol mixture (1:1).

Pass through 2 changes of xylol.

Mount in balsam.

(c) Results

Hyphae and lignified cell walls of host stained violet-purple. Unlignified cell walls of host stained orange.

F. Spore germination

1. Buffers

Solutions of buffers at pH 5.2 were prepared as follows:

Sorensen's phosphate mixture (Clark, 1923)

0.25 ml. M/15 $Na_2HPO_4 . 2H_2O$ + 9.75 ml. M/15 KH_2PO_4

Citric acid - sodium citrate (Dawson et al., 1959)

6.1 ml. 0.1M $C_6H_8O_7 . H_2O$ + 13.9ml. 0.1M $C_6H_5Na_3O_7 . 2H_2O$

Sodium acetate - acetic acid (Dawson, et al., 1959)

7.9 ml. 0.2M $C_2H_3NaO_2 . 3H_2O$ + 2.1 ml. CH_3COOH.

2. Media

(a) Carbohydrates

Solutions of carbohydrates were sterilized by autoclaving at 15 lb.p.s.i. for 10 minutes. Buffered solutions of all

carbohydrates except pectin were prepared by addition of sterile buffer (autoclaved as above) to the carbohydrate solution after sterilization. Buffered pectin solution was prepared by adjustment of pH to 5.2 with 0.1N NaOH prior to sterilization and addition of buffer.

(b) Nitrogen compounds

Nitrogen compound solutions (buffered and unbuffered) were adjusted to pH 5.2 with 0.1N NaOH or 0.1N HCl before sterilization by autoclaving at 15 lb.p.s.i. for 10 minutes. Buffered solutions were prepared by addition of sterile buffer (autoclaved as above) to the nitrogen-compound solutions after sterilization.

(c) Vitamins

Solutions of biotin and thiamin (aneurine hydrochloride) were prepared according to the methods described by Barton-Wright (1946). Solutions were sterilized by autoclaving at 15 lb.p.s.i. for 10 minutes.

(d) Rind extracts

Flavedo, albedo and complete rind extracts were removed from Valencia fruit as on P. 40 , 25g. of each tissue were finely macerated in a Moulinex coffee mill and transferred to 125 ml. distilled water in a 250 ml. conical flask. The flasks were plugged with cotton wool and left at 5°C. for 18 hours. The extracts were strained through 3 layers of muslin (without squeezing) into a 200 ml.

graduated cylinder, the tissues were washed with distilled water and the final volume of filtrate made up to 125 ml. Large suspended particles were removed by filtration through No. 541 Whatman filter paper under suction. The filtrates were made cell free by centrifugation at 9,000 r.p.m. for 10 minutes and the supernatant cell free extracts (pH5.0) were stored in McCartney tubes at - 20°C.

Flavedo, albedo and complete rind extracts contained 1.48, 1.54 and 1.73g. water soluble material in 100 ml. of each extract respectively. These extracts are referred to as $^{N}/_{1}$ concn. in some experiments.

Orange juice was extracted from peeled Valencia fruit using a hand juice extractor. The juice was centrifuged at 9,000 r.p.m. for 10 minutes and the supernatant stored in McCartney tubes at - 20°C.

(e) Spore suspensions

Aqueous spore suspensions were prepared as for infection experiments on P. 23 .

Spore suspensions in the different media were prepared by dilution of equal volumes of aqueous spore suspension and the particular medium under investigation.

3. Spore germination techniques

(a) Nutruitional requirements for spore germination

In studying the nutruitional requirements for spore germination a technique similar to that of Peterson (1941) was used. Glass slides, 3"x1", were sterilized by flaming in 95% alcohol and

each slide was smeared with 3 spots of Vaseline, spaced equidistantly apart.

A sterile grease free coverslip $\frac{3}{8}$" diameter was placed on each spot. The coverslips had been cleaned with "Chemico" (obtained from Chemico Works, Solihull, England), washed in tap and distilled water and stored in absolute alcohol. Coverslips were left to dry on clean blotting paper before being placed on the Vaseline spots with a forceps. Coverslips were fixed to the slides by warming the slides over an alcohol flame.

One slide was placed on a "Z" shaped glass rod in a sterile petri dish. A saturated atmosphere was maintained in the petri dish by covering the bottom with sterile water.

A standard loopful (.01 ml. approx.) of spore suspension was transferred to the coverslips on each slide giving 3 replicates per treatment. After replacing the petri dish cover the spores were incubated in the dark at 25°C. The period of incubation, unless stated otherwise, was 24 hours. Growth of spores was arrested by adding a drop of lactophenol in cotton blue to the drop containing the spores.

(b) Effect of emanations on spore germination

In a study of the effect of emanations from infected orange rind tissues on spore germination the following technique was used.

Van Tieghem cells, "pyrex" glass rings, $\frac{1}{2}$"x$\frac{9}{16}$" diam, were sealed to sterile glass slides with cellulose gum (prepared by mixing CMC 7HP with sterile water). One cell was sealed to each

slide and a plug of orange rind, 5-6mm. x 5mm. diam., was placed in each cell. Plugs of rind were removed from fruit, surface sterilized by flaming in 95% alcohol, with a sterile cork boring device. Each plug was inoculated with 0.2 ml. spore suspension in sterile distilled water, containing 2×10^6 spores per ml. (approx).

A standard loop of aqueous spore suspension was placed in the centre of a sterile coverslip, $\frac{3}{4}$" diam.; the coverslip with drop in position was inverted and sealed to the upper rim of the cell with cellulose gum as above. The spore density in the hanging drop varied between experiments and will be mentioned specifically in each experiment. Three to 4 replicates per treatment were used.

In experiments studying the stimulation of spore germination by emanations from rind infected by P.digitatum two controls were used.

(i) Cells + rind + 0.2ml. sterile water.

(ii) Cells + 0.2ml. sterile water.

In experiments studying the stimulation of spore germination by emanations from rind infected by other fungi one control as in (i) above was used.

Replicates of the same treatment were placed in transparent plastic sandwich boxes, 7"x4$\frac{1}{2}$"x1$\frac{5}{8}$ ", and incubated in the light (fluorescent) at 24°C. ± 1°. Growth of spores was arrested by adding a drop of formalin to each cell after 24 hours.

(c) Stimulation of spore germination on fruit surface

The stimulation of spore germination by substances diffusing from artificially injured and uninjured rind was studied with thin water agar disks placed on the surface of fruit, which had been previously washed in tap water, sterilized by swabbing in 95% alcohol and the alcohol allowed to evaporate.

The agar disks, 6mm. diam., were cut from flat bottom petri dishes, 9cm. diam., containing 8 ml. 2% distilled water agar. Disks were placed at sites, free from visible wounds, selected with a dissecting microscope (X12), at the equator of fruit. One disk was placed on each fruit site with a small sterile spatula. In a study of the stimulation of spore germination by substances diffusing from artificial wounds, the sites were wounded immediately before placing the agar disks in position on the wounds. One drop (0.01ml.) aqueous suspension containing 2,000 spores (approx.) was applied to each disk.

Fruit + inoculated disks, supported on aluminium rings as in plate 4 , were incubated in plastic boxes as described for infection experiments on P. 26 . The number of replicates per treatment varied and only replicates of the same treatment were placed in the one box.

Controls were of two types:

(i) A box control consisted of 3 inoculated water agar disks on sterile glass slides, supported on an aluminium ring and incubated with fruit in plastic boxes

as above.

(ii) A plate control consisted of 3 inoculated water agar disks on sterile glass slides, placed on a "Z" shaped glass rod in a sterile petri dish containing water.

Germination at 24°C. ± 1° in the light (fluorescent) was studied after varying periods of incubation. Growth of spores was arrested by addition of a drop of cotton blue in lactophenol to each disk.

(d) Assessment of Results

Percentage germination and average germ tube length were used as criteria to determine the effect of various agents on germination.

In determining percentage spore germination, as a standard procedure the number of germinated and ungerminated spores in 10 fields of view for each of 3-5 replicates per treatment were counted. Not less than a total of 250 spores in any one treatment were counted.

Average germ tube length in the presence of active stimulants was taken as the mean length of 30 germ tubes per treatment unless stated otherwise.

G. Growth experiments

1. Agar media

The following media were used and the pH was not adjusted unless stated otherwise.

(a) V8 vegetable juice agar

The contents of one 12½ oz. can of V8 vegetable juice (obtained from Campbell's Soups Ltd., King's Lynn, Norfolk) were diluted to 1.5 l. with distilled water and 37.5g. agar added.

(b) Malt extract agar

Twenty g. malt extract and 25g. agar in 1 l. distilled water.

(c) Flavedo extract agar

Two hundred g. fresh weight of flavedo tissues were removed from fruit with a "Skyline" peeler and boiled for 15 minutes in 1 l. distilled water. After cooling the liquid was strained through 3 layers of muslin, 25g. agar were added and the solution made up to 1 l. with distilled water.

(d) Albedo extract agar

The albedo tissues were obtained after removal of the outer flavedo layer of the rind with a "Skyline" peeler. The tissues were cut into thin slices and the medium was prepared as described above for flavedo extract agar.

(e) Complete rind extract agar

Rind, containing both flavedo and albedo tissues, was removed from fruit by hand and cut into thin slices. The medium was prepared as described above for flavedo and albedo extract agar media.

(f) Orange juice agar

Orange juice obtained by pressing peeled fruit with a hand

juice extractor, was centrifuged at 7,000 r.p.m. for 10 minutes. One hundred ml. of the cleared juice was then adjusted to the pH of the other rind extracts, pH 4.7, with 0.1N NaOH. Twenty five g. agar were added and the solution made up to 1 l. with distilled water.

(g) Sterilization

Agar media were steamed for 30 minutes in a steam bath before autoclaving at 15 lb. p.s.i. for 20 minutes.

(h) Inoculation and incubation

Eighteen ml. of medium were added to each petri dish and dishes were inoculated with a disk of mycelium 0.5cm. in diameter. Four replicates per treatment were used and all cultures were incubated in the dark at temperatures in the range 5-30°C.

(i) Assessment of results

At regular intervals the average diameter of each colony was measured in cm. along two fixed lines, at right angles through the centre, marked on the bottom of each petri dish. The average diameter recorded per treatment was therefore the mean of 8 readings (4 replicates per treatment, 2 readings per replicate).

2. Liquid media

(a) Fruit extract media

Flavedo, albedo, complete rind and orange juice liquid media were prepared as above except that no agar was added. The pH of each medium was 4.7 before sterilization.

(b) Fergus' medium

The basal medium of Fergus (1952) was composed as follows:

Sucrose	25.7g.
NH_4NO_3	4g.
KH_2PO_4	13.61g.
$MgSO_4.7H_2O$	1.23g.
$FeSO_4.7H_2O$	0.02g.
Minor elements	1ml. minor element solution (see below).
Distilled water	1 l.

The pH of this medium was 4.4.

(c) Minor element solution

A stock solution was made up in glass distilled water as follows:

Compound	Weight of compound in stock solution	Essential element	Element in stock solution p.p.m.
$CuSO_4.5H_2O$	78.6mg.	Cu	20
$ZnSO_4.7H_2O$	220mg.	Zn	50
$Na_2MoO_42H_2O$	25.2mg.	Mo	10
$MnSO_4.4H_2O$	2.03g.	Mn	500
$Na_2B_4O_7.10H_2O$	4.406g.	B	500
Water	1 l.		

(d) Vitamin solutions

The solutions of aneurine hydrochloride, pyridoxine hydrochloride, calcium panthothenate and biotin were prepared

according to the methods described by Barton-Wright (1946).

(e) Modified basal medium

This medium was used for the production of pectic and cellulolytic enzymes. Glucose, pectin, sodium polypoctate, carboxymethylcellulose and Solka Floc wood cellulose were used as carbon sources. Solutions were adjusted to pH 5.0 with 0.1N NaOH. The medium was composed as follows:

Carbon source	10g.
Casamino acids (Difco vitamin free)	8g.
KH_2PO_4	5g.
$MgSO_4.7H_2O$	0.5g.
$FeSO_4.7H_2O$	0.02g.
Ycastrel *	1g.
Minor elements	1ml. minor element solution (see above).
Distilled water	1 l.

*obtained from Brewer's Food Supply Company Ltd., Edinburgh.

(f) Sterilization

All liquid media were sterilized by autoclaving at 15 p.s.i. for 20 minutes.

(g) Culture vessels

"Pyrex" conical flasks fitted with cotton wool plugs and containing 50ml. of medium were used.

(h) Inoculation and incubation

Each culture vessel was inoculated with 1ml. of dense

spore suspension. Cultures were either grown in stationary culture in the dark at 25°C. or in shake culture on a mechanical shaker in the light (fluorescent) at 23°C. ± 1°.

(i) Assessment of growth

Growth was calculated from dry weight of mycelium. After harvesting by filtration through No. 541 Whatman filter paper and transferring the mycelium to a weighed aliminium foil cup, the mycelium was dried at 70°C. for 24 hours, cooled over $CaCl_2$ in a desiccator and weighed.

(j) Collection of Culture filtrates

Culture filtrates for pectic and cellulolytic enzyme work were collected after 5 days incubation in shake culture. After removal of mycelium the filtrates were centrifuged at 9,000 r.p.m. for 10 minutes to make cell free. The cell free solutions were then stored in McCartney tubes at -20°C. until required.

H. Estimation of Activity of pectic and cellulolytic enzymes

The activity of two enzymes, polygalacturonase (PG) and pectinmethylesterase (PME), acting on pectic substances were studied. PG hydrolyses 1:4 glycosidic linkages between anhydro-galacturonic acid units (GA) in a GA polymer and its activity was estimated by its ability to reduce the viscosity of NaPP and P solutions. PME removes the methyl ester groups from GA units of P polymers. Its activity was measured by titration of liberated carboxyl groups with alkali (Kertesz,1951, P.362).

Cellulase, Cx (Siu and Reese, 1953), hydrolyses 1:4 glucosidic linkages in cellulose molecules and its activity was estimated by its ability to reduce the viscosity of CMC solutions.

1. Estimation of viscosity reducing activity

The viscosities of reaction solutions were measured in Cannon-Fenske size 200 viscometers in a water bath at $25(\pm \frac{1}{4})^{\circ}C$. The flow time for each viscometer was found with 10 ml. distilled water.

Reaction mixtures consisting of 5 ml. 1% pectic substance, or 0.35% CMC, X ml. 0.1N NaOH or HCl to bring to the desired pH, (2-X) ml. distilled water and 2 ml. 0.05M sodium citrate buffer were pipetted into the viscometers. One ml. culture filtrate was added at zero time and mixed by bubbling air through the mixture. Flow times were measured at regular intervals from time zero. Control flow times were determined with culture filtrates, previously held at $100^{\circ}C$. for 5 minutes and cooled.

The relative viscosity of a particular solution was found by dividing its flow time by the flow time for water in the same viscometer. This is permissible because the densities of the mixtures were very close to that of water.

Activity was calculated from the curves drawn by plotting relative viscosity against time. Activity = $100/t$ where 't' is the time in minutes for (a) 10% reduction in viscosity of CMC and P solutions using infected and healthy rind extracts or (b) 25% and 50% reduction in viscosity of CMC and P, or NaPP solutions,

respectively using culture filtrates of the fungus.

2. Estimation of liberated carboxyl groups

Reactions were performed at a room temperature of 21° ± 1°C. in "pyrex" glassware.

Reaction mixtures consisted of 15ml. 1% pectin, X ml. 0.1N NaOH or HCl to bring to the desired pH and (3-X) ml. distilled water. To the mixture 4 ml. culture filtrate or rind tissue extract (P. 48) were added at zero time.

The pH of the reaction mixture was recorded at time zero on a Cambridge pH meter and maintained at this pH by the dropwise addition of 0.01N NaOH with constant stirring. The volumes of alkali used after known intervals were recorded and the reactions were allowed to continue for 30 minutes. Culture filtrates or rind tissue extracts previously held at 100°C. for 5 minutes and cooled, were used as controls.

The difference between the volumes of NaOH to neutralize carboxyl groups in the control and the reaction mixture is equivalent to the weight of methyl groups removed.

Equivalent weight of methoxyl group (CH_3O) = 31

1ml. 0.01N NaOH= 0.31mg. methoxyl group.

The methoxyl groups present in pectin were calculated from the fresh weight of pectin and the methoxyl content was estimated at 7.5%.

PME activity was expressed as percentage hydrolysis of methyl ester groups in the polymer.

$$\% \text{ hydrolysis} = \frac{\text{mg. methoxyl group liberated in time} \times 100}{\text{mg. methoxyl group originally present}}$$

3. Determination of Macerating Enzyme Activity

Culture filtrates and extracts of infected rind tissue were tested for macerating activity by the disc method of Brown (1915).

Potato discs, 8 mm. diameter and 0.5mm. thick, were cut from cylinders of tuber medulla tissue that had been previously injected with distilled water for 30 minutes under a pump. The discs were then thoroughly washed in distilled water.

Four ml. of culture filtrate, or infected rind extract solution were adjusted to pH 5.0 by addition of X ml. 0.1N HCl and (1.1-X) ml. distilled water. Three ml. of each test solution was transferred to shallow glass jars and ten potato discs were placed in each solution. Disks suspended in distilled water, adjusted to pH 5 as above, were used as controls.

The maceration time (minutes) was determined from the mean time for the 10 discs to lose their coherence. This is called the reaction time (R.T.) and macerating activity is recorded as $^{100}/_{R.T.}$

4. Preparation of healthy and infected rind extracts

Infected rind tissues were obtained by inoculating Shamouti fruit, wounded with a multiple pricking device with an aqueous suspension of spores from an atomiser. Fruit were incubated for 3 days in plastic boxes at 24°C. as described on P. 26 . Infected rind tissues, which had reached the "white mycelium" stage in

development were removed by hand and stored in screw cap bottles for 48 hours before preparation of salt extracts.

One hundred g. of infected rind tissues on removal from storage were macerated in 0.2M NaCl, in a Moulinex liquidizer. Extraction of the enzymes was completed by transferring the macerated tissues (pH 3.3) to a 1 l. beaker, the pH was adjusted with N NaOH to 7.0 and maintained at this value for 30 minutes, during which time the mixture was stirred magnetically.

The macerated tissues were transferred to a graduated cylinder and made up to 1 l. with 0.2M NaCl. Crude fibre was removed by straining and squeezing through 4 layers of muslin, followed by filtration through No.541 Whatman filter paper. The filtrate was made cell free by centrifugation at 9,500 r.p.m. for 10 minutes and the supernatants stored in McCartney tubes at -20°C.

Salt extracts of healthy rind tissues were prepared as described for infected tissues above.

SECTION III - EXPERIMENTAL RESULTS

A. THE FORMATION OF LESIONS IN FRUIT UNDER CONTROLLED CONDITIONS

The diagnostic features of lesions formed in the field have been described earlier (P.14). There follows a description of the symptoms that appear on Shamouti fruit inoculated in a standard manner and incubated under carefully controlled conditions.

Ten fruit were inoculated with 0.01 ml. aqueous spore suspension containing 20,000 spores (approx.) after one site per fruit had been pricked six times into, and between the oil vesicles to a depth of 4 mm. The fruit were incubated at 22-24°C. as described on P.26 .

Twenty four hours after inoculation a white "halo" of germinating spores appeared at each inoculum site, but it was not until 48 hours after inoculation that lesions could be distinguished. By this time lesions were approximately 6 mm. in diamater, the infected rind was smooth and water soaked, and growth of hyphae appeared at the points of pricking on most fruit (Plate 6).

Three days after inoculation, the inoculum sites were covered by a white growth of mycelium and tufts of hyphae had burst through the stomata (Plate 7). Lesions were now 1-3 cm. in diamater.

After 4 days the first sporulation was observed in the centre of the mycelium mat and appeared as a change in colour from bright white to dull, olive-green. At this stage the advancing edge of each lesion consisted of a water soaked band, 1-1.5 cm. wide surrounding the central mat of mycelium. In different fruit, lesions varied from 4 to 8 cm. in diameter, reflecting the variation in

resistance to spread of the fungus.

Five days after inoculation there was heavy sporulation in the central part of each lesion, which varied from 5 to 9 cm. in diameter. This area was surrounded by a white band of mycelium, 2-3 mm. wide, which in turn was enclosed by a water soaked advancing front that showed no superficial mycelium.

Most fruit were infected completely 6 days after inoculation and remained fairly firm in consistency. Little shrinkage of "covered" fruit was observed initially and this only became noticeable after a prolonged storage period.

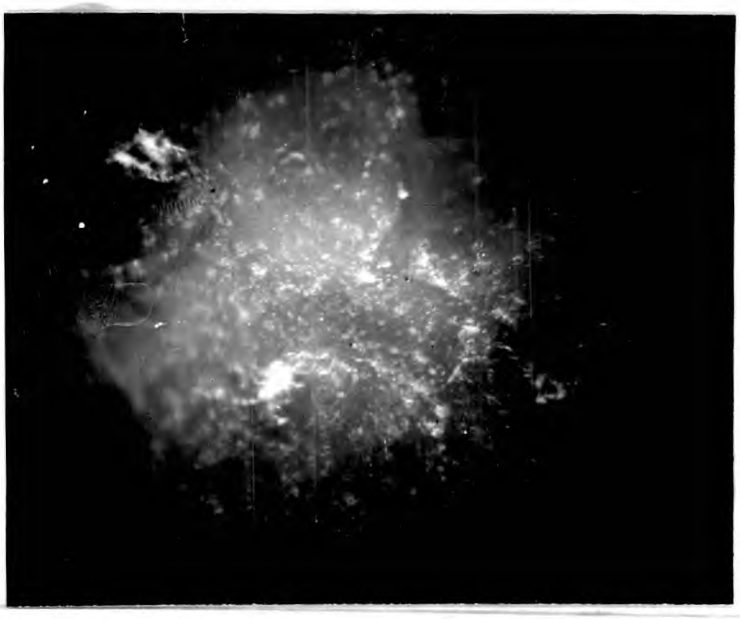

Plate 6. Growth of hyphae in wounds 2 days after inoculation. X8.

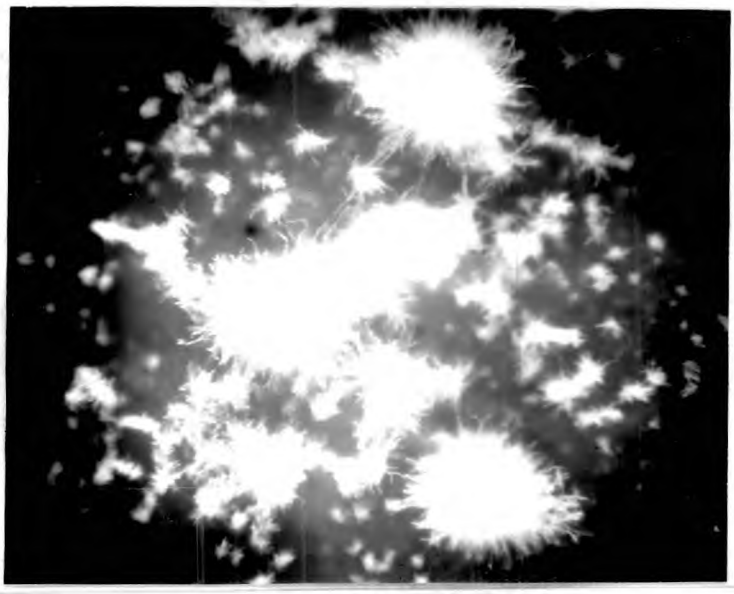

Plate 7. Tufts of mycelium emerging from wounds and stomata 3 days after inoculation. X8.

B. INFECTION OF FRUIT THROUGH UNINJURED RIND

1. Infection of fruit by an inoculum of spores in water

The aim of this investigation was to determine whether or not fruit could be infected through uninjured rind when inoculated with an aqueous spore suspension.

Fruit were inoculated in two ways and the fruit selected for inoculation by each method were carefully examined with the aid of a dissecting miscroscope (X 12) in order to avoid the occurrence of wounds at inoculum sites.

Using the first method of inoculation oranges of the Valencia, Navel and Shamouti varieties were used. Forty fruit of each variety, all having green stems, were used in separate experiments with each variety.

In an experiment, fruit were inoculated with 0.01 ml. aqueous spore suspension (1) at 4 sites/fruit 2 mm. from the stem end; (2) at 4 sites/fruit 2 mm. from the blossom end; (3) on stems of fruit, and (4) into cavities left after removal of stems. Ten fruit/ treatment were used and fruit were incubated as described on P. 26 for 50 days. The results of these experiments are summarized below.

No lesions developed on fruit inoculated at the stem and stylar ends and growth of mycelium was not observed at these sites either. However, this did not mean that spores had not germinated at these sites (see P.187).

Fruit inoculated over the stems frequently showed sparse growth

of hyphae but lesions were produced only in 2 Shamouti fruit. These lesions developed after an incubation of 30 days and a Brown Rot caused by Diplodia natalensis (P.245), was found at the sites of developing lesions. This suggested in mind of the other results that the primary infection was not caused by P.digitatum.

There were no lesions on fruit inoculated after the removal of the stems although in most cases there was sparse growth of hyphae in the cavities.

These results agree with those of Green (1932), but not with those of Bates (1936), who claimed to have infected through the rind of uninjured fruit at the stem end.

The method of inoculation used by Bates was not the same as that used above and an experiment using Bates' method is described below.

Shamouti fruit free from visible injuries were placed on their stem ends, blossom ends and on their sides in the bottom half of petri dishes containing 10 ml. of dense spore suspension in sterile water; 10 fruit for each position were used. The lesions developed within 20 days of inoculation are shown in Table 1 .

Table 1. Infection of "uninjured" Shamouti oranges placed on their stem ends, blossom ends and on their sides in 10 ml. of spore suspension in sterile water contained in the bottom half of petri dishes

Days in spore suspension	Number of lesions amongst 10 fruit in each series		
	Fruit placed on stem ends	Fruit placed on sides	Fruit placed on blossom ends
4	2	1	1
6	6	2	4
8	10	3	4
11	10	6	6
13	10	8	7
20	10	8	7

Lesions developed in all fruit standing on their stem ends and also in those placed in other positions but more slowly and to a somewhat lesser extent.

These results are in contrast to those obtained by the other method of inoculation and would seem to suggest that fruit are infected through uninjured rind when fairly large areas of fruit surface are suspended in an aqueous suspension of spores.

However, fruit from the same consignment stained with a solution of 0.1% Light Green in 70% ethyl alcohol, revealed small wounds on the rinds. Stain, absorbed by the wounds remained after the fruit had been washed in cold water, and was readily detected by means

of a dissecting microscope (X 12).

Wounds were larger and more numerous at the stem end than at the other positions inoculated. These probably resulted from damage caused in handling and clipping at the stem end during harvesting. It was also observed that when fruit were suspended with their stem ends in water cracks appeared in the outer rind after 5 days. These cracks probably resulted from differences in expansion of the flavedo and albedo, which was spongy and may have absorbed more water. The formation of cracks and the greater number of wounds were the cause of the more rapid development of lesions at the stem end.

The lesions formed at other positions were probably the result of infection through minute wounds. The development of these was aided by substances leached out from the rind which promoted germination of spores in water in contact with fruit.

When those spore suspensions contained in petri dishes, in which fruit had remained sound after a 20 day incubation period, were examined microscopically it was found that germ tube growth was profuse. This growth however was not sufficient to cause lesions in the absence of a suitable avenue for infection.

The technique of Bates used above seemed unsuitable for determining whether wounds were necessary for infection and in experiments to be described in the text later it was rejected in favour of a technique in which a much smaller inoculum site and defined quantities of inocula were used as described on P.24 .

2. Infection of fruit by an inoculum of spores in rind extracts and orange juice

In the last experiment it was seen that Shamouti fruit were not infected even when inoculum sites were covered by growth of hyphae.

In this experiment Shamouti and Navel fruit were inoculated with 0.01 ml. suspension containing 10,000 spores in (a) water, (b) water extract of flavedo, (c) water extract of albedo, (d) water extract of complete rind and (e) orange juice to see if growth of hyphae in these media promoted infection of fruit through uninjured rind. Extracts of rind tissues were prepared as described on P. 34.

In this and other experiments in this section there was one inoculum site/fruit and 10 fruit/treatment.

Table 2 shows the number of lesions 30 days after inoculation.

Table 2. Infection of "uninjured" Shamouti oranges by spores suspended in water, water extract of rind tissues and orange juice

Variety	Number of lesions out of 10 in each series				
	Suspension medium				
	Water	Flavedo extract	Albedo extract	Complete rind extract	Orange juice
Shamouti	0	0	1	0	1
Navel	0	1	0	0	1

Growth of spores in the rind extracts and orange juice was such as to allow the development of clumps of sporulating hyphae at the inoculum sites but resistance to infection was not overcome (Plate 8). It was clear, therefore, that in spite of this good growth the fungus did not penetrate the unbroken cuticular layer of the epidermis.

The results of this experiment did not agree with those of Green (1932) who infected Spanish oranges in the absence of wounds using an inoculum of spores in orange juice.

In view of this, Shamouti fruit were again inoculated with spores in orange juice and an attempt was made to break the rind resistance by increasing the volume of inocula to 0.05 ml. or 0.1 ml. In the controls, spores in 0.01 ml. water were used.

"Perspex" rings, 1 cm. diameter and 1 cm. deep, were sealed to the fruit with Vaseline to keep inocula in position.

Table 3 shows the number of lesions formed within 25 days of inoculation.

Table 3. : Infection of "uninjured" Shamouti oranges by spores suspended in orange juice

Days after inoculation	Spores in orange juice		Spores in water
	0.05 ml./site	0.1 ml./site	0.1 ml./site
4	0	0	0
6	0	1	0
8	1	1	0
11	1	2	0
25	1	2	0

Plate 8. Growth and sporulation of P.digitatum on uninjured Shamouti orange.

The results of the previous experiment were confirmed. Only a few lesions developed in fruit inoculated with spores in juice and these developed slowly. The action of acids in the juice on small superficial wounds was thought to result in infection (see P.123).

Another experiment repeated the above with Navel fruit and gave similar results. Only one lesion developed on a fruit inoculated with 0.1 ml. inoculum in juice within 15 days of inoculation.

Although Green (1932) infected Spanish oranges with an inoculum of spores in orange juice in the absence of wounds it is possible that minute wounds did occur on these fruit. The presence of such wounds would not have been detected unless the fruit surface was stained specially to reveal them and this was not done by Green. Alternatively, the infection of uninjured Spanish oranges by her could have been a characteristic of the fruit used. Whether this was a varietal characteristic is not known as the variety used by Green was not stated.

3. Infection of fruit by an inoculum of spores in citric acid

Green (1932) successfully inoculated Spanish oranges with spores in citric acid in the absence of wounds. A similar experiment with Shamouti oranges is described below.

Sites on fruit specially selected as being free from visible wounds were marked with indian ink and around each a "perspex" ring, 1 cm. diameter and 1 cm. deep, was sealed with Vaseline.

Inocula of spores in 0.3 ml. of 1%, 3% and 5% solutions of citric acid were used. Controls were inoculated with spores in water. The development of lesions after different periods of incubation is shown in Table 4 .

Table 4. Infection of "uninjured" Shamouti fruit by spores suspended in citric acid

Days after inoculation	Number of lesions out of 10 in each series			
	Citric acid			Water
	1%	3%	5%	
7	0	0	0	0
9	0	0	1	0
12	1	1	2	0
13	1	2	2	0
15	1	2	2	0

Few lesions developed and these did so very slowly. After incubation of fruit for 3 days slight depressions in the form of small pits developed on some fruit inoculated with spores in 3% and 5% acid solutions. With further incubation, growth and sometimes sporulation of the fungus was seen at these sites. Growth of mycelium on some fruit caused lesions to develop (Plate 9) while on others growth was confined to the rings at each site and only depressions were formed (Plate10). Thirteen days after inoculation growth of the latter type was found on 4, 6 and 7 fruit

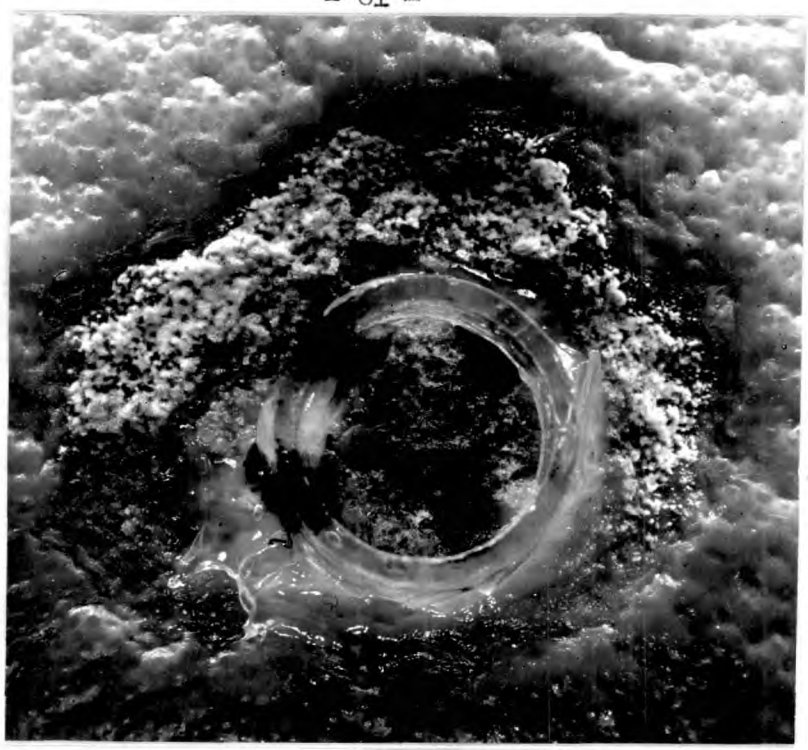

Plate 9. Infection of an uninjured Shamouti orange by P.digitatum spores suspended in 5% citric acid solution.

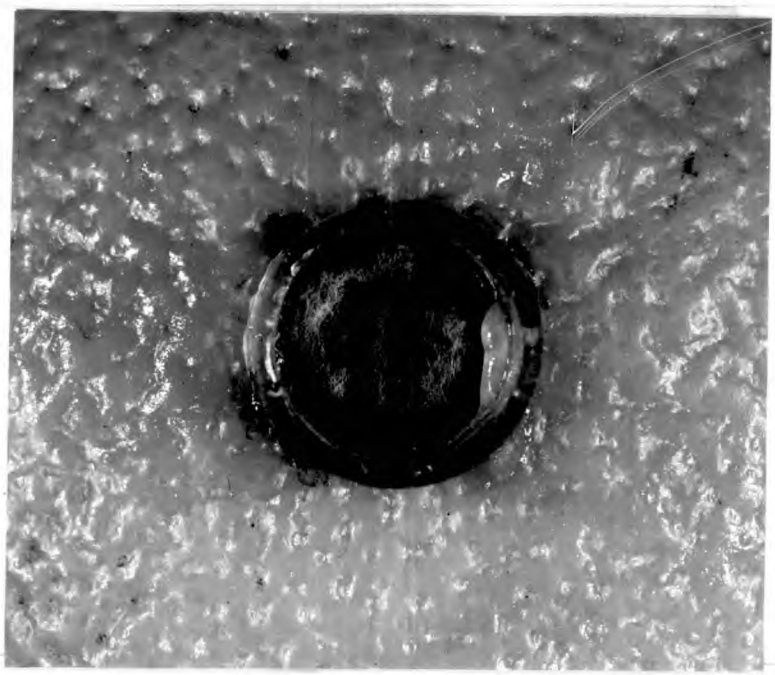

Plate 10. Growth of P.digitatum spores in 3% citric acid solution causing a depression at an inoculum site on uninjured Shamouti orange.

treated with spores in 1%, 3% and 5% citric acid solutions respectively. Penetration of the rind tissues by the fungus was not observed.

These results are in direct contrast to those of Green (1932) who found that all inoculations gave lesions after 8 days incubation. The period of incubation in the above experiment was double that used by Green.

Growth of spores at inoculum sites was not a direct result of stimulation of growth by the acid, because very few spores germinated when suspended in 1% acid and incubated on sterile glass slides for 24 hr. at 24-25°C. The spores did swell considerably however. Growth of the fungus on the fruit may have depended on an increase in the passage of stimulative substances from the rind after it had been damaged by the citric acid.

4. Infection of sound fruit by contact with infected fruit

Fawcett (1936) stated that infection may start by contact of sound fruit with fruit already infected by the fungus, but that this did not take place so readily as with Blue Mould caused by P. italicum.

To clarify this point, three experiments with Shamouti, Valencia and Navel fruit were done. Ten sound fruit of each variety, free from distinguishable surface blemishes, were chosen. Each fruit was attached to a fruit infected with Green Mould, by a tight elastic band so that the periphery of that lesion on the adjacent infected

fruit came into contact with the sound fruit. The fruit were then incubated for 6 days.

In each experiment a distinct softening of the rind tissues of the sound fruit adjacent to the infected fruit was detectable after removal of the elastic bands. These soft areas subsequently developed into green sporulating lesions.

In another experiment with Navel fruit, sound fruit, placed in contact with Green Mould infected fruit as before but not under pressure, did not become infected.

It appears therefore that under pressure sound fruit are infected by contact with infected fruit.

Under transport conditions the development of wastage in sound fruit after contact with Green Mould infected fruit was frequently observed. One of the factors contributing to this spread is the pressure under which the fruit are packed in cases.

C. INFECTION OF FRUIT THROUGH WOUNDS

The infection of fruit through wounds was investigated to discover the part played by injury in providing avenues for infection by the fungus.

Valencia fruit were wounded by pricking each fruit three times into or between the oil vesicles to varying depths. An inoculum of 0.01 ml. aqueous spore suspension containing 10,000 spores was applied to the wounds at each inoculum site. There was one site/fruit and 10 fruit/depth of prick. Fruit were incubated for 39 days and the results are shown in Table 5 .

Table 5. Promotion of infection by P.digitatum through wounds of varying depth into or between the oil vesicles of Valencia oranges, using spore suspensions in water.

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series												
	Wounds between oil vesicles							Wounds into oil vesicles					
	Depth of Prick (mm.)												
	0	0.25	0.5	1.0	2.0	3.0	4.0	0.25	0.5	1.0	2.0	3.0	4.0
2	0	0	0	0	2	0	3	1	1	3	8	9	9
3	0	0	0	1	2	1	5	2	3	4	10	10	10
4	0	0	0	1	2	2	7	2	5	6	10	10	10
6	0	0	0	1	3	2	8	2	5	6	10	10	10
7	0	0	0	1	3	3	8	3	5	6	10	10	10
39	0	0	0	1	3	3	8	3	5	6	10	10	10

The results show clearly the effect of wounds on infection. Fruit were infected more readily when spore suspensions were put into

the oil vesicles compared with between the oil vesicles. Generally, the rind between vesicles was resistant to infection and this resistance was very marked to inocula placed 2 mm. deep or less. Lesions developed more slowly in the "inter-vesicle" zone and the increase in number of lesions formed with increased depth of prick was not as striking as when vesicles were inoculated directly.

Bates (1933) also found that vesicles provided a suitable avenue for infection but the resistance of the "inter-vesicle" flavedo to infection by spores in water was not reported.

The further experiments on wounding between and into the vesicles will now be considered later under separate headings.

1. Effect of healing wounds on infection of fruit

A process called "wilting" of fruit and probably more correctly described as "healing" of wounds on fruit is practised widely throughout the Citrus industry. In this fruit harvested in the field are left for approximately 1 day before being treated in the packhouse. During this time injuries made on the fruit during harvesting have time to heal and thus it is found that fruit, left to "wilt" in this way, become less wasty than those receiving packhouse treatment immediately after harvesting.

The effect of healing wounds on the initiation and development of lesions from wounds into and between oil vesicles was studied as described below.

Shamouti fruit, pricked three times into the oil vesicles to a

depth of 2 mm. or between the vesicles to a depth of 4 mm. were left at room temperature for 1 and 7 days to allow the wounds to heal before being inoculated. Fruit were inoculated with 0.01 ml. aqueous spore suspension containing 10,000 spores and the number of lesions that developed within the 20 day period of incubation is shown in Table 6 .

Table 6. Effect of healing wounds, 2 mm. deep into or 4 mm. deep between oil vesicles of Shamouti oranges, on infection by P.digitatum spores in water

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series					
	Wounds between oil vesicles			Wounds into oil vesicles		
	Days between wounding and inoculation					
	0	1	7	0	1	7
2	4	2	0	3	0	0
4	9	4	0	8	3	0
5	9	4	0	9	4	0
6	9	4	0	9	4	1
8	9	4	2	9	4	2
10	9	6	6	9	4	4
20	9	6	6	9	4	4

Healing of wounds increased the resistance of fruit to infection from inoculation into or between the oil vesicles. This was slightly more striking in fruit pricked into the vesicles. Healing of wounds for longer than 1 day did not increase this resistance. Not only was the number of lesions less in the "wilted" fruit but

the lesions that did develop did so more slowly because with both types of wound the longer the period of healing the greater was the incubation period necessary for the first appearance of lesions. Fruit, pricked into the vesicles and inoculated immediately after wounding developed lesions after 2 days, whereas those left to heal for 7 days did so only 6 days after inoculation.

D. INFECTION OF FRUIT THROUGH WOUNDS BETWEEN THE OIL VESICLES

1. Resistance of Shamouti fruit to infection

Fruit of the Shamouti variety were used to give a comparison with the results obtained in the previous experiment with Valencia fruit.

The method of inoculation was the same as that used with Valencia oranges except that the number of pricks/site was increased to 6 and the number of fruit /treatment to 12. The effect of increase in depth of prick on infection was looked at more closely by having more intermediate values in prick depth than in the previous experiment.

The fruit were incubated for 49 days. The rate and development of lesions at the different depths inoculated is given in Table 7 .

Table 7. Effect of depth of wound between oil vesicles on infection of Shamouti oranges by an aqueous suspension of P.digitatum spores

Six wounds per fruit.

Days after inoculation	Number of lesions out of 12 in each series											
	Depth of prick (mm.)											
	0	0.25	0.5	0.75	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0
3	0	0	0	0	0	0	0	3	1	4	10	7
4	0	0	0	0	0	0	2	3	3	4	12	11
5	0	0	0	0	0	0	2	4	3	4	12	12
6	0	0	0	1	0	0	2	4	3	4	12	12
8	0	0	0	1	0	0	2	4	3	4	12	12
19	0	0	0	1	0	0	2	4	3	4	12	12
49	0	0	0	1	1	2	2	4	3	4	12	12

It is clear that the tissues of the inter-vesicle zone of the rind of Shamouti fruit has considerable resistance to infection. This resistance was very marked at a depth of 1.5 mm. and somewhat less at a depth of 3.5 mm. After incubation for 49 days there was a slight decrease in this resistance because more fruit became infected but after this prolonged period occasional infections from fungi other than P.digitatum developed at the stem ends of fruit. These were mostly caused by Alternaria citri and Diplodia natalensis. Infection by these fungi might have reduced the resistance of the fruit to infection by P.digitatum and so the results after 49 days are best overlooked.

Wounds deeper than 3.5 mm. showed no resistance to infection, and the development of lesions from these wounds was very rapid. Pricks 5 mm. deep were still within the outer rind and had not penetrated into the juice vesicles.

At infection sites which did not develop into lesions, red marks or stains were frequently observed around the pricks. The occurrence of these red spots is dealt with in another section (P.115).

The marked resistance observed with wounds no deeper than 1.5 mm. corresponded to the limit of the oil-bearing flavedo tissue in the outer rind, this tissue extending into the rind to a depth of 1.5 mm. approximately. Thus one could more accurately attribute this marked resistance to infection to the inter-vesicle tissues of the flavedo layer.

The underlying factors and mechanisms capable of breaking this

resistance were subjects of further investigation later on in the work.

2. Resistance of Navel fruit to infection

Resistance of the inter-vesicle tissues of Shamouti and Valencia fruit to infection has already been described. It was now of interest to investigate if Navel fruit also showed this resistance because fruit of this variety are frequently described as being more wasty i.e. more susceptible to disease than Valencia fruit.

The same procedure was used as in the corresponding experiment with Valencia fruit. The fruit were incubated for 36 days to establish whether an extended period of incubation after lesion formation appeared to be complete, had any effect in lowering resistance to infection. The number of lesions developed after different periods of incubation is shown in Table 8 .

Table 8. Effect of depth of wound between oil vesicles on infection of Navel oranges by an aqueous suspension of P.digitatum spores

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series					
	Depth of Prick (mm.)					
	0	0.5	1.0	2.0	3.0	4.0
2	0	0	0	2	3	5
3	0	0	0	2	6	8
4	0	0	0	3	7	9
5	0	0	0	5	7	10
36	0	0	0	5	7	10

Like the other varieties, Navel fruit show a resistance to infection in the inter-vesicle tissues of the flavedo to a depth of between 1 and 2 mm. in the rind. A large proportion of inoculations into the tissues of this zone were successful only when they were made to a depth of 3 or 4 mm.

A careful examination of fruit still sound 10 days after inoculation revealed that red spots were again present in the form of halos around the pricks. After 19 days it was found that 16 out of 20 fruit pricked to a depth of 0.5 and 1 mm. showed these spots on close examination. Fruit pricked to the same depths but not inoculated did not develop red spots.

At one inoculum site where profuse growth of sporulating hyphae was observed no lesion was formed (Plate 11). The occurrence of good growth of hyphae in inter-vesicle flavedo wounds would not, therefore, appear to be a prerequisite for the formation of lesions within the period of incubation.

It was, however, observed that the number of lesions formed after inoculation at depths of 2 and 3 mm. were more than twice as numerous as those formed at corresponding depths in Valencia fruit although the conditions of both experiments were identical. This may explain, at least in part, the fact that under commercial conditions Navel fruit are more susceptible to infection by P.digitatum than are Valencia fruit.

3. Variation in susceptibility of Navel and Valencia fruit to infection

Although in earler work on infection it was observed that

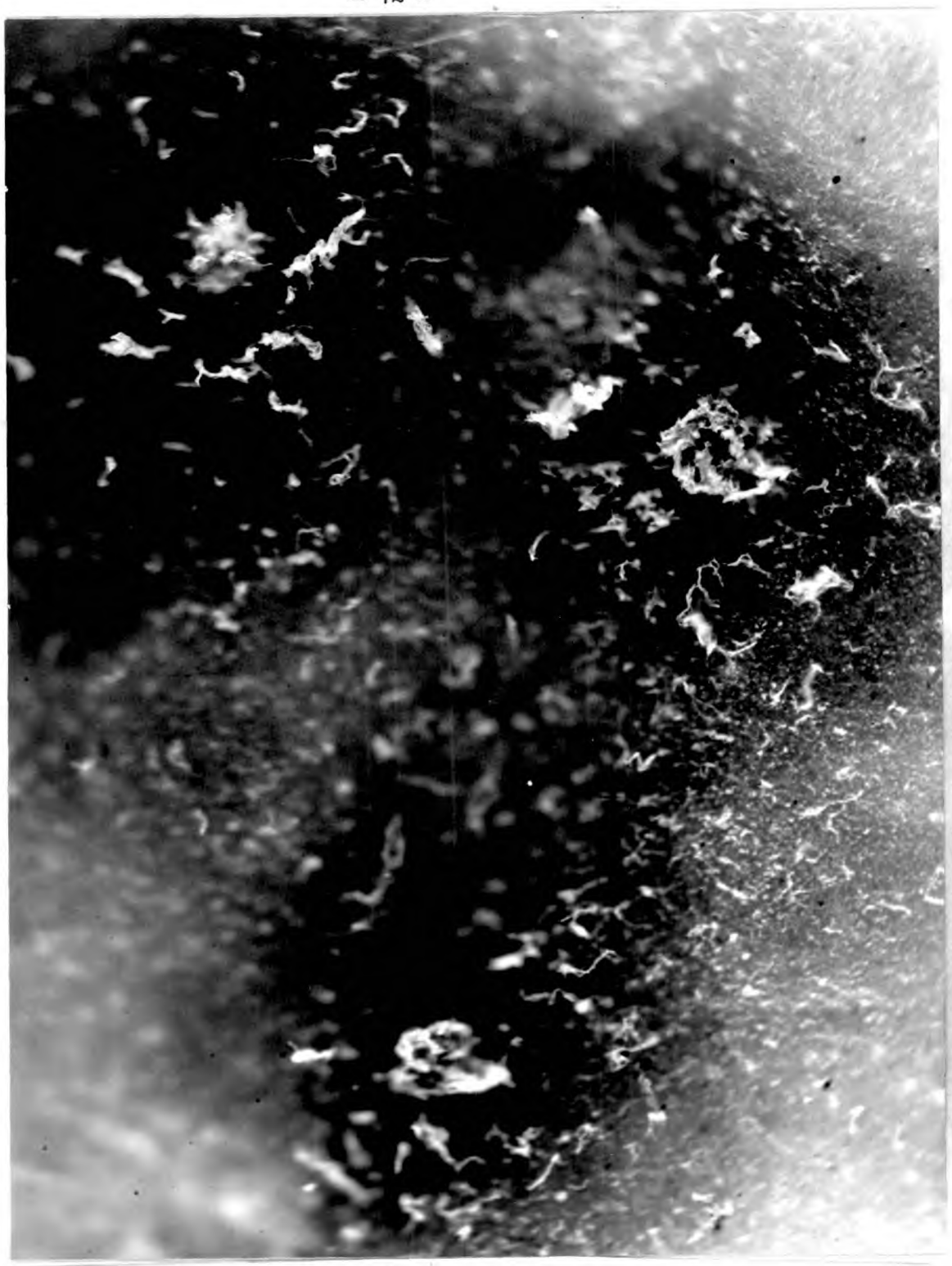

Plate 11. Growth and sporulation of P.digitatum in wounds 1 mm. deep between the oil vesicles of Navel orange. The dark areas around each wound are 'red spots'.

the 3 varieties Navel, Valencia and Shamouti, all showed a definite resistance to infection following inoculations into the inter-vesicle flavedo tissues, nonetheless a certain variation in this resistance was exhibited by fruit of any one variety. Examples of this are shown in Tables 9 and 10 which contain the results of a number of experiments done at different times with Navel and Valencia oranges taken from different consignments.

In each experiment 10 fruit of either Navel or Valencia variety, from different consignments A-L, were wounded by pricking three times to 1.0 mm. depth between oil vesicles and inoculated with 0.01 ml. aqueous spore suspension containing 10,000 spores. The number of Navel and Valencia fruit developing lesions is shown in Tables 9 and 10 respectively.

Table 9. Variation in resistance of Navel oranges, from different consignments A-H, to infection by an aqueous suspension of spores of P.digitatum when inoculated through pricks 1.0 mm. deep between oil vesicles

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	A	B	C	D	E	F	G	H
3	0	0	0	0	0	0	1	7
5	0	0	0	0	0	2	5	8
8	0	1	0	0	0	2	6	8
10	0	1	0	0	0	2	6	8
11	0	1	0	3	0	2	6	8

Table 10. Variation in resistance of Valencia oranges, from different consignments I-M, to infection by an aqueous suspension of spores of P. digitatum when inoculated through pricks 1.0 mm. deep between oil vesicles.

Three wounds por fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	I	J	K	L	M
3	0	0	0	1	0
5	1	1	0	3	0
8	1	1	0	5	0
11	1	1	0	5	0

It is clear that in the different experiments the resistance of the fruit varied considerably and that in most experiments, most of the fruit were resistant.

Keeping in mind that fruit of both varieties were chosen at random, the results of the experiments taken as a whole suggest that Navel fruit are less resistant to infection than Valencia fruit. This corresponds with field observations because Navel fruit in transit normally develop more wastage from infection by P.digitatum than Valencia fruit.

4. Resistance of inter-vesicle flavedo tissues at the stem and blossom ends of fruit to infection

In this experiment the resistance of the inter-vesicle flavedo tissues at the stem and blossom ends of orange fruit was studied. This was of particular interest because infections at the

stem end by P.digitatum are much more frequent in the field than at other parts of the fruit.

Two experiments, one with Shamouti and the other with Navel fruit were done.

In the first experiment, Shamouti fruit were pricked three times/inoculum site between the oil vesicles to a depth of 1 and 1.5 mm. at the stem and blossom ends of each fruit. Ten fruit were used for each depth of prick and 0.01 ml. aqueous spore suspension containing 10,000 spores was applied to each inoculum site. The fruit were placed on their sides before inoculation and the inoculum drops were kept in position by a smear of Vaseline. The fruit were incubated for 10 days and the number of lesions developed within this period are shown in Table 11.

Table 11. Resistance of the inter-vesicle flavedo tissues, at the stem and blossom ends of Shamouti oranges, to infection by a suspension of P.digitatum spores in water

Three wounds per inoculum site.

Days after inoculation	Number of lesions out of 10 in each series			
	Depth of prick (mm.)			
	1.0		1.5	
	Stem end	Blossom end	Stem end	Blossom end
3	1	0	1	0
10	1	0	1	0

A definite resistance to infection was shown at both ends of the fruit but lesions did develop at the stem ends in 2 fruit.

A similar experiment was done with Navel oranges.

No lesions had developed at either end of the fruit at both depths of inoculation after incubation for 11 days.

Therefore, the flavedo tissues at both the stem and blossom ends of the fruit are resistant to infection and the greater susceptibility of fruit to infection at the stem end in the field cannot be attributed to lower resistance of the inter-vesicle tissues of the flavedo at this part of the fruit.

The increased infection known to occur at the stem end in the field probably results from the fact that this end of the fruit is more easily injured because it is the part most exposed in the handling of the fruit at harvesting when clippers are used to remove the stems. Such injuries would rupture the oil vesicles and provide the type of wound through which the fungus readily enters the fruit.

5. Resistance of lemon and grapefruit to infection

In studying the infection through inter-vesicle flavedo tissues of different varieties of orange it was seen that most lots of fruit showed some resistance. It was now of interest to see if there were similar resistance in other species of Citrus in which the rinds of the fruit have the same basic structure as that of orange.

Lemons and grapefruit were used for this purpose. Ten fruit of each were pricked three times/inoculum site to a depth of 1 mm.

between the oil vesicles, then inoculated with 0.01 ml. aqueous spore suspension containing 10,000 spores and incubated for 11 days.

No lesions developed on any of the fruit. So it would seem that these fruit have a resistance to infection in the flavedo inter-vesicle tissues similar to that observed for oranges.

6. Effect of spore numbers in the inoculum drop on the production of lesions

The effect of spore numbers in the inoculum drop on infection was investigated with Shamouti fruit which were pricked between the oil vesicles 6 times/inoculum site to a depth of 4 mm. There were 16 fruit for each treatment.

Spore concentration was altered by dilution and the densities were checked by the use of a haemacytometer counting cell; 0.01 ml. aqueous spore suspension was applied to each inoculum site. The fruit were incubated for 24 days and the number of lesions developed within this period is shown in Table 12 .

Table 12. Effect of spore numbers on the infection of Shamouti fruit, pricked between the oil vesicles to a depth of 4 mm.

Six wounds per fruit.

Days after inoculation	Number of lesions out of 16 in each series					
	Number of spores/0.01 ml. inoculum					
	20,000	2,000	200	20	2	0
2	9	1	0	0	0	0
3	13	11	2	0	0	0
4	16	15	2	1	0	0
5	16	15	2	4	0	0
6	16	15	4	4	0	0
8	16	15	5	4	1	0
9	16	15	5	5	1	0
11	16	15	5	5	1	0
12	16	15	5	6	2	0
13	16	15	5	6	2	0
14	16	15	6	6	2	0
24	16	15	6	6	2	0

Increasing the spore load led to an increase in the number of lesions formed and the rate of lesion formation was more rapid in fruit inoculated with the most spores.

An inoculum of 2 spores resulted in the formation of lesions in 2 out of 16 fruit and these did not develop until after an incubation period of 8 to 12 days. In contrast, all lesions developing in those fruit inoculated with drops containing 2,000 and 20,000 spores did so within 4 days. With smaller inocula however lesions continued to appear and develop until the fourteenth day of incubation.

In summary, therefore, the effect of larger inocula at the inoculation sites was to increase the speed at which lesions developed and the total number of lesions formed.

Next an experiment was carried out using those fruit, wounded to a depth of 4 mm., that had remained sound in the above experiment. These were thought to have remained sound because the inocula were too small. It was thought that reinoculation of the inoculum sites on these fruit with a denser spore suspension would result in infection in most of the fruit.

Twenty eight fruit were inoculated with 0.01 ml. aqueous suspension, containing 10,000 spores, at each inoculation site.

After incubation for 22 days only one fruit became infected, a rather surprising result.

Examination of the inoculum sites with a dissecting microscope (X 12) revealed that the orifices of the pricks were traversed by hyphae and that these hyphae were visible without staining; growth was not extensive. Transverse sections of the rind, cut through the pricks and stained with lacto-phanol in cotton blue did not show penetration of the flavedo and albedo tissues by hyphae.

The resistance to infection observed in the wounded fruit above was similar to, but more striking than, that observed with fruit of the same variety, left to heal at room temperature (P. 65). The healing of wounds, which is thought to be responsible for the increased resistance of fruit to infection, would therefore appear to be more effective in resisting infection when fruit, wounded

between the oil vesicles, are kept at a high humidity in closed plastic boxes than at comparatevely lower humidity at room temperature.

7. Effect of variation in number of pricks at the inoculum site on the production of lesions

The effect of varying the number of wounds at the inoculum site was studied in this experiment. Shamouti fruit were pricked 1, 2, 4 or 6 times/site to a depth of 4 mm., this depth ensured high infection. All wounds were made close to one another to allow coverage by the inoculum drops; 0.01 ml. aqueous spore suspension containing 10,000 spores was applied to each inoculum site. Fruit were incubated for 11 days and the number of lesions developed within this period is shown in Table 13 .

Table 13. Effect of number of pricks/site on the development of lesions in Shamouti oranges, wounded between the oil vesicles to a depth of 4 mm. and inoculated with an aqueous suspension of P. digitatum spores

Days after inoculation	Number of lesions out of 10 in each series				
	Number of pricks/site				
	6	4	2	1	0
2	6	7	4	3	0
3	10	8	8	7	0
4	10	10	10	8	0
5	10	10	10	9	0
11	10	10	10	9	0

Incubation for 2 days was necessary and sufficient for the formation of lesions. The greater the number of pricks/site the faster did lesions develop but even pricking only onee/site to this depth caused lesions in 9 out of 10 fruit. Thus, spores in conjunction with one very small wound on the fruit can produce a lesion that can result in total wastage of the fruit.

8. Effect of inoculating fruit by different methods on the production of lesions

In this experiment Navel oranges were pricked to a depth of 3.5 mm. i.e. into the albedo. There were 6 pricks/site and 10 fruit/treatment.

The fruit were inoculated in the following ways:-

(a) By an inoculum of 0.01 ml. aqueous spore suspension containing 10,000 spores applied to each inoculation site : after wounding.

(b) By an aqueous spore suspension as in (a), which was applied to inoculation sites and left to dry at room temperature for approx. 2 hours before the fruit were wounded.

(c) By wounding fruit with an inoculation pin coated with dry spores.

(d) By wounding fruit with a pin coated with dry spores, after which 0.01 ml. sterile water was added to each inoculation site.

(e) By brushing dry spores on a camel hair brush over inoculation sites wounded shortly beforehand.

After inoculation the fruit were incubated for 30 days and

the number of lesions developed within this period is shown in Table 14 .

Table 14. Effect of inoculating Navel oranges in different ways on the production of lesions [1]

Six wounds, 3.5 mm. deep between the oil vesicles, per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Method of inoculation				
	(a)	(b)	(c)	(d)	(e)
2	10	0	4	9	3
3	10	0	6	10	4
4	10	1	7	10	4
5	10	1	7	10	5
7	10	1	7	10	5
10	10	1	7	10	6
13	10	1	7	10	6
23	10	2	7	10	6
30	10	2	7	10	6

These results clearly indicate the effect of water at the inoculum site in promoting infection. The formation of lesions in the presence of water was completed within 3 days, whereas with dry spores lesion development was slower and lesions did not develop at 3 or 4 of the inoculated sites. The inefficiency of method (b) is particularly striking and is not easily understood in light of the results by method (c).

9. Effect of emanations from fruit infected by P.digitatum on resistance of flavedo to infection

The use of ethylene in some Citrus packhouses to "degreen" fruit, and the fact that this gas is produced by growth of P.digitatum in culture and on Citrus fruit suggested an investigation of the effect of emanations from fruit infected by this fungus on the resistance of the flavedo tissues to infection.

Biale and Shepherd (1941) found that gaseous products from lemons infected by P. digitatum caused a marked increase in the rate of carbon dioxide evolution by green fruit and accelerated colour development in the same fruit.

The occurrence of wastage of oranges in clumps or "pockets" in packing cases also suggests that volatile products emanating from infected fruit weaken the resistance of adjacent fruit to infection.

Navel fruit were pricked to depths of 0.5, 1.0 and 1.5 mm. between the oil vesicles and inoculated with 0.01 ml. aqueous suspension containing 10,000 spores. There were 3 pricks/inoculation site and 5 fruit/depth of prick. Fruit were incubated in closed plastic boxes after the introduction of two fruit infected by P. digitatum to each box. Controls each of 5 fruit/depth of prick were incubated in the absence of infected fruit.

After incubation for 20 days none of the inoculated fruit became infected. This suggested that the increased respiratory rate of the inoculated fruit, resulting from the emanations from the infected

fruit, did not result in an increase in susceptibility to infection.

After 20 days had elapsed and no lesions had formed, each of the inoculated fruit was placed in contact with a fruit infected by P.digitatum as described on P. 62 . Within one week of doing this, lesions had developed on all the fruit attached in this way.

From this therefore, it would appear that emanations from fruit infected by P.digitatum have little effect on resistance to infection and that spread by contact appears to be the cause of the "pockets" of wasty fruit that are common during transport and storage.

Further studies on the effect of emanations on the germination of spores in water is described on P. 180.

E. INFECTION OF FRUIT THROUGH WOUNDS INTO THE OIL VESICLES

1. Effect of depth of wound on the production of lesions

In an earler experiment (P.64) it was seen that Valencia oranges were readily infected by inoculation through the oil vesicles. Navel oranges were also used in a similar experiment.

Ten fruit/depth of prick were pricked into adjacent oil vesicles 6 times/inoculum site to depths varying from 0 to 5 mm. To each inoculum site 0.01 ml. aqueous suspension containing 10,000 spores was added. The fruit were incubated for 21 days and the lesions formed within this period are shown in Table 15.

Table 15. Effect of depth of wound into oil vesicles on infection of Navel oranges by an aqueous suspension of P.digitatum spores

Six wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series											
	Depth of prick (mm.)											
	5.0	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.75	0.5	0.25	0
2	10	8	9	7	8	7	3	1	0	1	0	0
3	10	10	10	10	10	10	8	4	1	2	1	0
5	10	10	10	10	10	10	9	5	4	5	4	0
6	10	10	10	10	10	10	9	6	5	5	5	0
8	10	10	10	10	10	10	9	6	6	5	6	0
10	10	10	10	10	10	10	9	6	7	5	6	0
13	10	10	10	10	10	10	9	6	7	6	6	0
21	10	10	10	10	10	10	9	6	7	6	6	0

It was seen that with wounds of 2 mm. deep, and deeper, lesion formation was completed within 3 days. With wounds less than 2 mm. deep a considerably longer incubation was necessary to allow all lesions that were going to develop to do so; as long as 13 days for some treatments. Even with the most shallow wound, only 0.25 mm. deep, lesions did ultimately develop in as many as 6 out of the 10 fruit inoculated.

Quite minor damage to the rind at the site of an oil vesicle will therefore produce a site through which the fungus can enter into the fruit, and the fact that the oil vesicles do provide such an easy avenue for infection is of considerable practical interest as this is most probably the main way in which infection occurs after the fruit are harvested. A scratch by a thorn or by the finger nail of a packer, or by a splinter in a packing case will rupture many oil vesicles and so provide a number of avenues for entry of the fungus.

The effect of the essential oils that are released on wounding the oil vesicles on the spores of the fungus are described later (p. 196).

2. Effect of variation in the number of pricks into the albedo on the production of lesions

Navel oranges were used and the number of pricks/inoculum site varied from 1 to 6; the pricks were 4 mm. deep and 10 fruit/treatment were inoculated. The pricks were made into adjacent oil vesicles to allow coverage by an inoculum consisting of 0.01 ml.

aqeuous suspension containing 10,000 spores. Fruit were incubated for 20 days and the number of lesions developed within this period is shown in Table 16 .

Table 16. Effect of number of pricks/site on the development of lesions in Navel oranges, wounded into the oil vesicles to a depth of 4 mm. and inoculated with an aqueous suspension of P. digitatum spores

Days after inoculation	Number of lesions out of 10 in each series						
	Number of pricks/site						
	6	5	4	3	2	1	0
2	10	10	10	10	8	4	0
3	10	10	10	10	8	6	0
20	10	10	10	10	8	6	0

An incubation period of 2 days was necessary for the formation of lesions in the most favourable circumstances. The greater the number of pricks/site the more rapidly did the lesions develop. Only 1 prick/site in the presence of spores in water was needed to allow a high proportion of the inoculations to give lesions. At the inoculum sites of fruit remaining sound after 3 days, germinated spores were observed as a white halo around the pricks and this was associated with a red staining of the tissues around the pricks.

It would appear that substances passing into the inoculum drop

as a result of wounding provide nutrients sufficient to ensure germination of the spores and growth of the germ tubes but this still did not permit infection of the fruit.

3. Effect of variation in the number of pricks into the flavedo on the production of lesions

The procedure adopted was the same as in the previous experiment except that fruit were pricked into the flavedo to a depth of 0.5 mm. The fruit were incubated for 27 days and the number of lesions developed within this period is shown in Table 17.

Table 17. Effect of number of pricks/site on the development of lesions in Navel oranges, wounded into the oil vesicles to a depth of 0.5 mm. and inoculated with an aqueous suspension of P.digitatum spores.

Days after inoculation	Number of lesions out of 10 in each series					
	Number of pricks/site					
	6	4	3	2	1	0
2	0	0	1	0	0	0
3	1	0	3	0	0	0
11	1	1	3	0	0	0
23	1	2	3	0	0	0
27	1	2	3	0	0	0

The fruit appeared resistant to infection through the oil vesicles at this depth and there was no obvious relation between the number of pricks and the formation of lesions.

These results are unlike those found in earlier experiments with Valencia (P. 64) and Navel fruit (P.85) which became infected to a greater extent with this type of treatment. A repeat of the above experiment confirmed this result.

It seems that apart from differences in susceptibility between varieties there are also differences in susceptibility between fruit of the same variety to infection by the fungus.

Germinated spores around the pricks were observed at most inoculum sites and red staining at the pricks accompanied this growth of germ tubes.

4. Effect of spore numbers in the inoculum drop on the production of lesions

The effect of spore load in the inoculum drop was investigated with Navel oranges which were pricked into the oil vesicles 3 times/inoculum site to a depth of 4 mm; 10 fruit/spore concentration were used.

The spore concentration was altered by dilution and spore counts were made with a haemacytometer counting cell; 0.01 ml. of the different spore suspensions was applied to each inoculum site. The fruit were incubated for 22 days and the number of lesions developed within this period is shown in Table 18.

Table 18. Effect of spore numbers on the infection of Navel oranges, pricked into the oil vesicles to a depth of 4 mm.

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series								
	Number of spores/0.01 ml.inoculum								
	40,000	20,000,	2,500	625	150	40	20	10	1
2	1	0	0	0	0	0	0	0	0
3	9	7	1	2	0	0	0	0	0
4	10	10	8	7	3	4	0	0	0
6	10	10	10	9	4	5	1	2	0
7	10	10	10	9	4	6	1	2	0
10	10	10	10	9	4	6	2	3	1
11	10	10	10	9	5	6	2	3	1
22	10	10	10	9	5	6	2	3	1

The number of lesions and the rate at which they appeared increased with increase in the spore load. However, a concentration of less than 625 spores/drop had a marked limiting effect on lesion production. An inoculum of 1 spore/drop produced lesions in 1 out of 10 fruit and this required an incubation period of 10 days. Generally the more dilute the spore suspension the greater the period needed to complete the formation of lesions.

The occurrence of red spots around the pricks of the fruit remaining sound was again frequently observed, fruit showing this coloration around the pricks did not become infected within 22 days of inoculation. Whether this red "spotting" at the pricks indicates some kind of resistance to infection by the fruit is not known but this aspect will be considered in greater detail on P.115.

5. Effect of inoculating fruit by different methods on the production of lesions

The effect of various methods of inoculation on the production of lesions following inoculations between the oil vesicles has already been described. A similar experiment was done with Navel fruit pricked into the oil vesicles to a depth of 4 mm. There were 3 pricks/site and 10 fruit/treatment.

The fruit were inoculated in the following ways:-

(a) By an inoculum of 0.01ml. aqueous spore suspension containing 10,000 spores applied to each inoculation site after wounding.

(b) By an aqueous spore suspension as in (a), which was applied to inoculation sites and left to dry at room temperature for approx. 2 hours before the fruit were wounded.

(c) By wounding fruit with an inoculation pin coated with dry spores.

(d) By wounding fruit with a pin coated with dry spores, after which 0.01 ml. sterile water was added to each inoculation site.

(e) By brushing dry spores on a camel hair brush over inoculation sites wounded shortly beforehand.

After inoculation the fruit were incubated for 7 days and the number of lesions developed within this period is shown in Table 19.

Table 19. Effect of inoculating Navel oranges in different ways with an aqueous suspension of P. digitatum spores

Three wounds, 4 mm. deep into the oil vesicles, per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Method of inoculation				
	(a)	(b)	(c)	(d)	(e)
2	3	0	6	7	8
3	10	10	10	10	9
7	10	10	10	10	9

Within 3 days lesions had appeared at 49 of the 50 inoculation sites. No differences were produced by the different methods of inoculation except perhaps in the number of lesions appearing after 2 as compared with 3 days incubation and these differences are not likely to be significant.

These results stand in marked contrast to those obtained when the inoculations were made between the oil vesicles (P. 81), when method (b) proved to be an inefficent method of inoculation and method (e) only about half as efficient as method (a) and (d).

F. INFECTION OF FRUIT THROUGH INSECT WOUNDS

The suggestion of Bates (1936) that scale infected oranges were more liable to infection than sound oranges was investigated.

The occurrence of scale insects (Coccidae) on Citrus fruit is of great economic importance because no fruit infested with scale to any extent is allowed to be exported.

Scale insects feed on plant tissues by means of a proboscis thrust into the superficial cells. Like other insects they avoid the oil vesicles when inserting the proboscis. Once the proboscis has been inserted into the fruit it remains fixed in the same position throughout the life of the scale and persists after death of the scale (Plate 12).

Two experiments with fruit infected by scale insects were carried out to see if such fruit were more liable to infection.

In the first experiment Shamouti fruit, infected with Black Scale, Chrysomphalus aonidum were inoculated as follows:

(a) By an inoculum of 0.02 ml. aqueous spore suspension containing 20,000 spores applied to 1 scale insect/fruit.

(b) By an inoculum of 0.02 ml. aqueous spore suspension containing 20,000 spores applied to each fruit after the removal of 1 scale at the inoculum site.

(c) By an inoculum of 0.02 ml. spore suspension containing 20,000 spores in orange juice applied to 1 scale/fruit.

(d) By an inoculum of 0.02 ml. spore suspension, containing

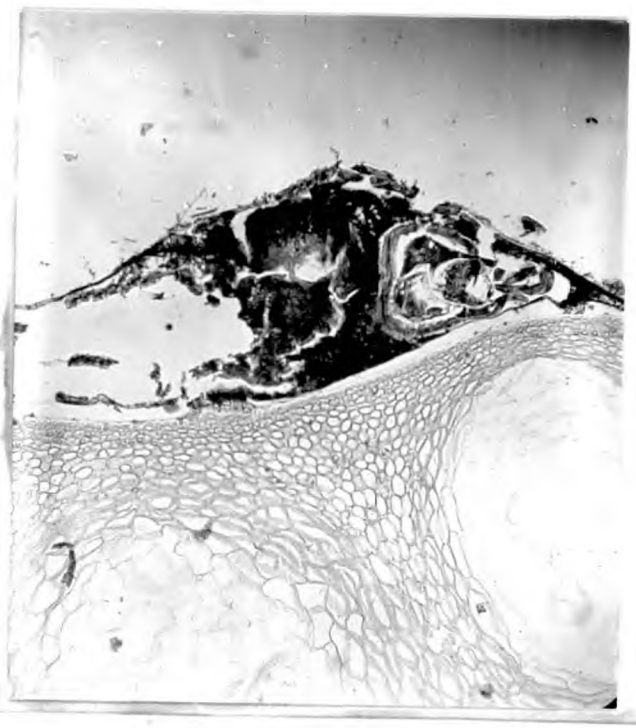

Plate 12. L.S. Black Scale (Chrysomphalus aonidum) on a Shamouti orange . X30.

20,000 spores, in orange juice applied to each fruit after the removal of 1 scale at the inoculum site.

Ten fruit/treatment were inoculated and incubated for 17 days. The number of lesions that developed within this period is shown in Table 20 .

Table 20. Effect of inoculating scale infested Shamouti oranges with P.digitatum spores suspended in water and orange juice.

Days after inoculation	Number of lesions out of 10 in each series			
	Method of inoculation			
	(a)	(b)	(c)	(d)
5	0	0	0	0
6	0	0	1	0
17	0	0	1	0

The inoculation of the scale insects, or the marks left after the removal of scale insects did not lead to the formation of lesions and although there was profuse growth of hyphae after fruit had been inoculated with spores in orange juice only one fruit + scale inoculated in this way became infected 6 days after inoculation.

In the second experiment each fruit was inoculated with 0.05 ml. spore suspension in (a) distilled water and (b) orange juice containing 50,000 spores. The inoculum was held in position by a

glass ring 1 cm. diameter, sealed to the surface of the fruit by Vaseline. Ten Shamouti fruit, with one scale/inoculum site were inoculated in each treatment. The fruit were incubated for 17 days and the lesions formed within this period are shown in Table 21.

Table 21. Effect of inoculating scale infested Shamouti oranges with P. digitatum spores suspended in water and orange juice

One scale insect/inoculum site.

Days after inoculation	Number of lesions out of 10 in each series	
	Spores suspended in water	Spores suspended in orange juice
5	0	1
10	0	2
17	0	2

Two lesions developed following inoculation of fruit with spores in orange juice.

The results of these experiments suggest strongly that the points of attachment of the scale insect Chrysomphalus aonidum are not suitable avenues for the entrance of the fungus into the rind from a suspension of spores in water.

There was a limited amount of infection from spores suspended in orange juice but this may have been due to the action of acid on other wounds at the inoculum site and not to wounds caused by the proboscis of the insect. It would of course be difficult to

distinguish between these.

The low amount of infection in fruit + 1 scale/inoculum site, inoculated with spores in orange juice is in general agreement with that of Bates (1936). Bates however found that fruit containing 3 scales/inoculation site were readily infected by spores suspended in orange juice.

An experiment to confirm this was not carried out as the area needed to cover 3 scale insects would have been much greater than that area, 0.4 sq. cm., used in other experiments. Neither the area covered nor the amount of inoculum used by Bates was stated in his work.

G. A MICROSCOPIC STUDY OF THE PENETRATION OF HYPHAE IN WOUNDS INTO THE RIND TISSUES

It was shown in Section D that the inter-vesicle tissues of the flavedo were resistant to infection. This resistance was lost when the oil vesicles were ruptured by wounds of comparable depth. In this section the growth of hyphae in wounds into, and between the oil vesicles was studied microscopically.

Navel fruit, pricked three times to depths of 0.25 to 4.0 mm. between the vesicles, and into the vesicles, were inoculated with an aqueous suspension of spores and incubated for periods of 24,48 and 72 hours.

After each period of incubation the inoculated tissues were fixed in formalin-propiono-alcohol. After fixation for 24 hours the material was dehydrated, embedded and stained as described in the Materials and Methods (P. 31).

1. Penetration by hyphae in wounds between the oil vesicles

Table 22 summarizes the sequence in penetration of the rind tissues by hyphae in wounds between the oil vesicles 48 and 72 hours after inoculation.

Table 22. Penetration of rind tissues by hyphae in wounds of different depth between the oil vesicles

Depth of Prick (mm.)	Time (hours) after inoculation	Spores	Flavedo penetrated	Albedo penetrated
0.25	48	Germinated	No	No
0.5	48	"	"	"
1.0	48	"	"	"
1.5	48	"	"	"
1.5	72	"	"	"
3.0	48	"	"	Yes
3.0	72	"	"	"
4.0	48	"	"	"
4.0	72	"	Yes	"

(a) Colonization of flavedo

(i) Wounds 0.25 to 1.5mm. deep

Spores germinated in pricks 0.25 to 1.5 mm. deep into the flavedo tissues. Transverse sections through pricks 1mm. deep 48 hours after inoculation are shown in Plate 13 and through pricks 1.5 mm. deep after 72 hours in Plate 14 .

Hyphae, 3 μ in thickness, were observed in all pricks within this range of depth. These hyphae penetrated the ruptured cells that resulted from wounding but they did not invade the compact cells of the flavedo.

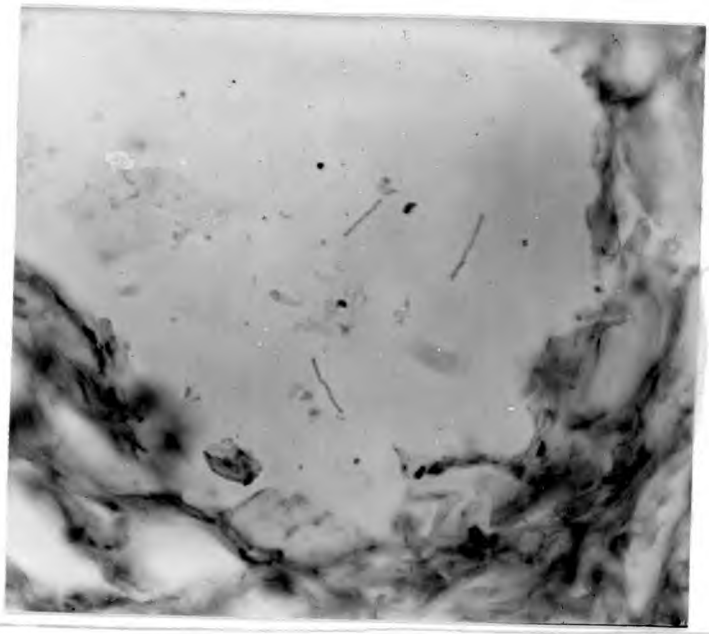

Plate 13. Transverse section (T.S.): hyphae in wounds 1 mm. deep between the oil vesicles 48 hours after inoculation. X240.

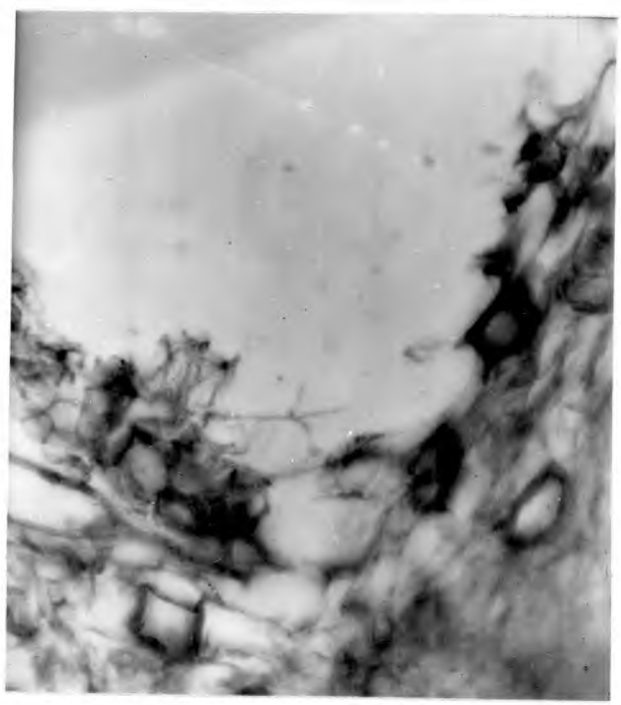

Plate 14. T.S. Hyphae in wounds 1.5 mm. deep between the oil vesicles 72 hours after inoculation. X240.

(b) Colonisation of albedo

(i) Wounds 3.0 mm. deep

A longitudinal section through a prick 3 mm. deep is shown in plate 15. Germination of spores resulting from wounding led to initial penetration of the albedo tissues after 48 hours. Penetration of the flavedo tissues before invasion of the albedo tissues was not observed (Plate 16).

Seventy two hours after inoculation the invading hyphae 3.5 to 6 μ thick had dissolved the albedo cell walls (Plate 17).

(ii) Wounds 4.0 mm. deep

After 48 hours dissolution of albedo cell walls by invading hyphae was observed (Plate 18). Hyphae did not penetrate the flavedo cells on initial entry into the pricks.

Seventy two hours after inoculation hyphae had penetrated the compact flavedo cells but not extensively (Plate 19). At the orifice of each wound the flavedo cells although not penetrated by hyphae lost their coherence (Plate 20).

Extensive penetration of the albedo tissues by hyphae resulted in dissolution of cell walls to a distance of 1 mm. from each prick. Hyphae were both intercellular and intracellular and branched repeatedly in the tissues (Plate 21). They varied in thickness from 3 to 7 μ.

2. Penetration by hyphae in wounds into the oil vesicles

Table 23 summarizes the sequence in penetration of the

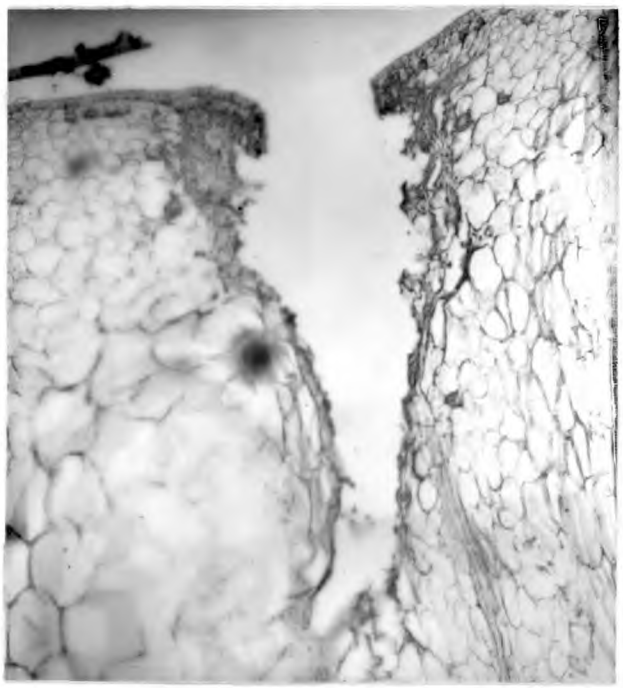

Plate 15. L.S. Wound 3 mm. deep between the oil vesicles. X60.

Plate 16. L.S. Hyphae in wound 3 mm. deep between the oil vesicles 48 hours after inoculation. X240.

Plate 17. L.S. Dissolution of albedo cell walls by hyphae 72 hours after inoculation of wounds 3 mm. deep between the oil vesicles. X240.

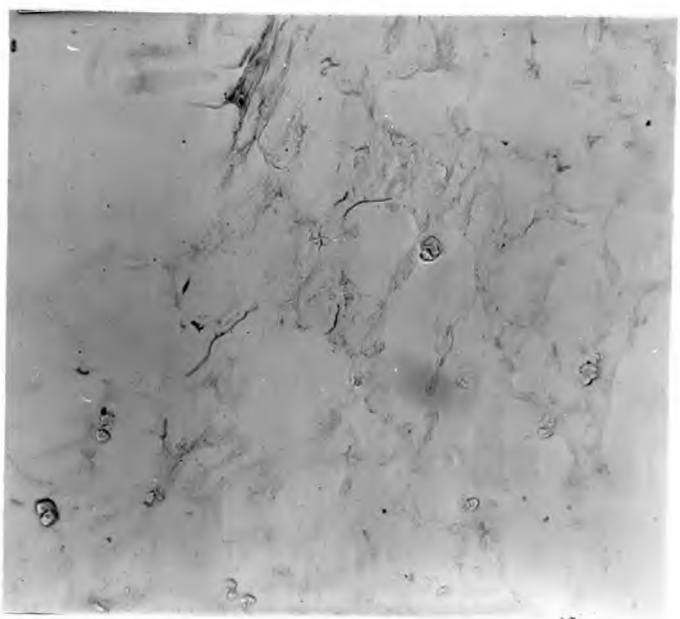

Plate 18. L.S. Invasion of albedo by hyphae 48 hours after inoculation of wounds 4 mm. deep between the oil vesicles. X120.

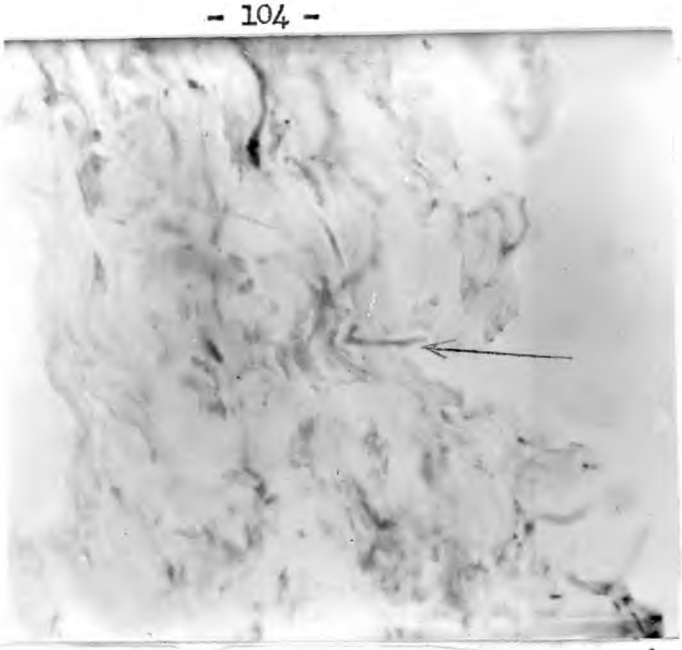

Plate 19. L.S. Initial penetration of flavedo by hyphae from wounds 4 mm. deep between the oil vesicles 72 hours after inoculation. X240.

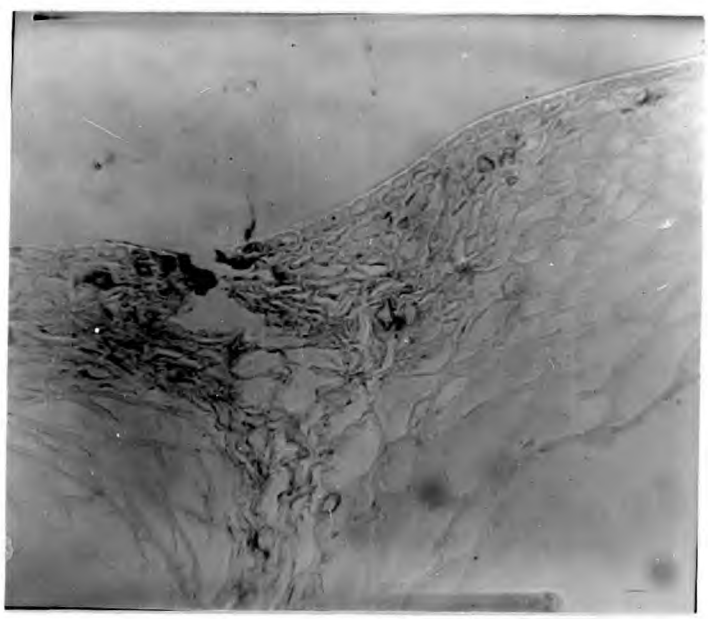

Plate 20. L.S. Loss in coherence of flavedo cells around wound. X100.

rind tissues by hyphae in wounds into the oil vesicles.

Table 23 .Penetration of rind tissues by hyphae in wounds of different depth into the oil vesicles

Depth of prick (mm.)	Time (hrs.) after inoculation	Oil vesicle cells penetrated	Inter-vesicle flavedo penetrated	Albedo penetrated
0.25	24	Yes	No	No
0.5	24	"	"	"
1.0	24	"	"	"
1.5	24	"	"	"
3.0	24	"	Yes	Yes
4.0	24	"	"	"
0.25	48	"	"	No
1.0	48	"	"	Yes
1.5	48	"	"	"
3.0	48	"	"	"
4.0	48	"	"	"
0.25	72	"	"	"
0.5	72	"	"	"

(a) Penetration of tissues 24 hours after inoculation

(i) Wounds 0.25 - 1.5 mm. deep

Hyphae were confined to the dead cover cells of the oil vesicles and initial penetration of the oil vesicle cells was observed (Plate 22). The cover cells of the oil vesicles were

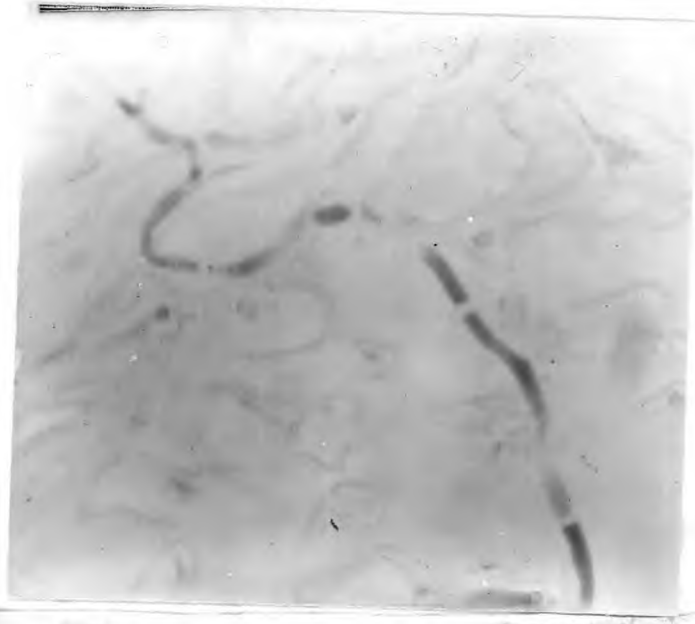

Plate 21. L.S. Inter-cellular and intra-cellular penetration of albedo cells by hyphae. X240.

Plate 22. L.S. Hyphae in oil vesicle cover cells 24 hours after inoculation of wounds 0.25 mm. deep into the oil vesicles. X240.

dead before being penetrated by the hyphae and the death of these cells resulted from the absorption of phytotoxic essential oil released after wounding the oil vesicles (Plate 23).

(ii) Wounds 3.0 -4.0 mm. deep

The hyphae penetrated down into the albedo tissues through the thin walled cells of the oil vesicles and caused complete dissolution of these cells (Plate 24). Penetration of the flavedo tissues was limited to those cells adjacent to the oil vesicles. Initial penetration of the albedo tissues was also observed.

(b) Penetration of tissues 48 hours after inoculation

(i) Wounds 0.25 mm. deep

Flavedo cells adjacent to the oil vesicles were dissolved by invading hyphae (Plate 25). Penetration of the albedo tissues was not observed.

(ii) Wounds 1.0 - 1.5 mm. deep

Initial penetration of the oil vesicle cells was followed by penetration of the inter-vesicle flavedo tissues (Plate 26). This was followed by penetration of the albedo tissues. The hyphae in the flavedo and albedo were 3 to 14 μ in thickness and were both intercellular and intracellular (Plate 27).

(iii) Wounds 3.0 - 4.0 mm. deep

Penetration of the albedo tissues, followed by an extensive invasion of the flavedo tissues by granular hyphae from the albedo tissues led to sporulation on the fruit surface (Plate 28).

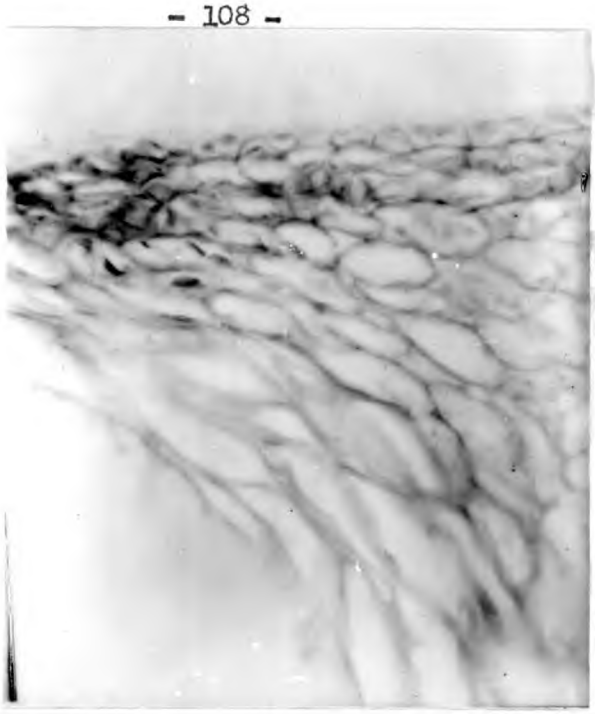

Plate 23. L.S. Oil vesicle cover cells killed by absorption of phytotoxic essential oil. X240.

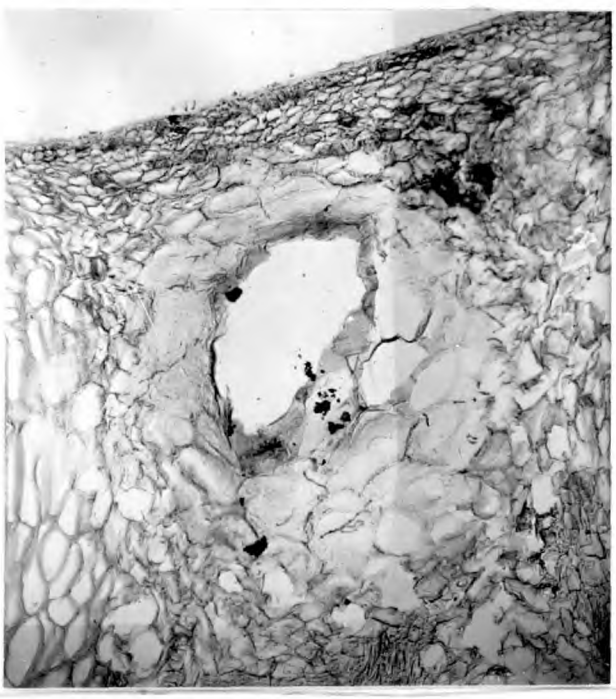

Plate 24. L.S. Dissolution of thin-walled oil vesicle cells by hyphae 24 hours after inoculation of wound 3 mm. deep into the oil vesicles. X60.

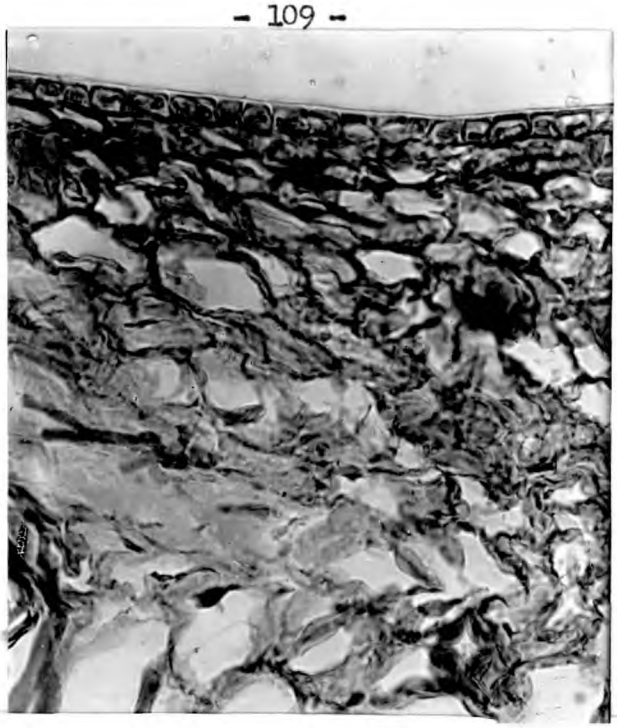

Plate 25. L.S. Dissolution of flavedo cells adjacent to an oil vesicle by hyphae from wounds 0.25 mm. deep into the oil vesicles 48 hours after inoculation. X240.

Plate 26. L.S. Inter-cellular and intra-cellular penetration of inter-vesicle flavedo cells by hyphae 48 hours after inoculation of wounds 1 mm. deep into the oil vesicles. X240.

Plate 27. L.S. Penetration of albedo cells by hyphae 48 hours after inoculation of wounds 1 mm. deep into the oil vesicles. X240.

Plate 28. L.S. Sporulating hyphae on the surface of fruit 48 hours after inoculation of wounds 3 mm. deep into the oil vesicles. X60.

The flavedo cells when first penetrated by hyphae, 12 μ thick, were not extensively disintegrated by the invading hyphae (Plate 29). Subsequently however, they were completely ramified and dissolved by hyphae where sporulation occurred (Plate 30).

At the edge of a lesion the epidermal cells were filled with hyphae (Plate 31). Nearer the centre of the lesion these hyphae clumped together (Plate 32) and then ruptured the cuticular layer to sporulate (Plate 28).

In the older parts of the lesion growth and sporulation of hyphae in the space containing the oil was observed (Plate 33).

(c) Penetration of tissues 72 hours after inoculation

(i) Wounds 0.25 - 0.5 mm. deep

Lesions were formed as described above.

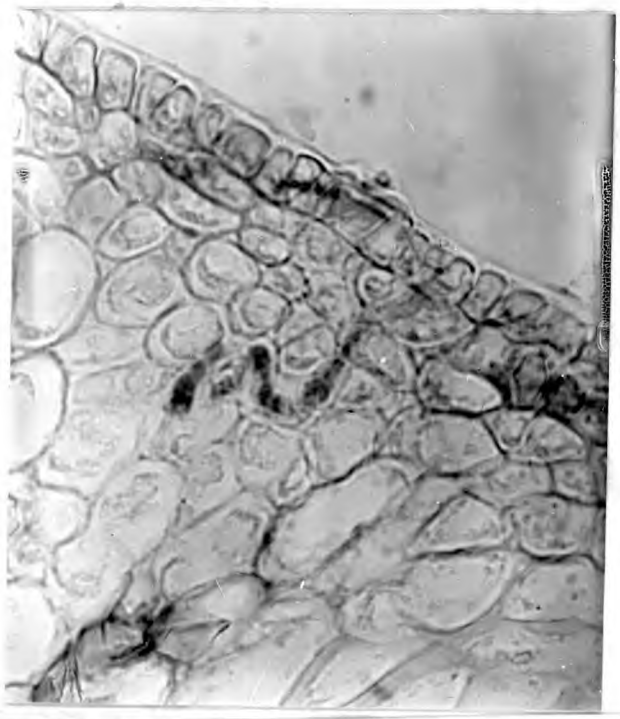

Plate 29. L.S. Flavedo cells invaded by hyphae. X240.

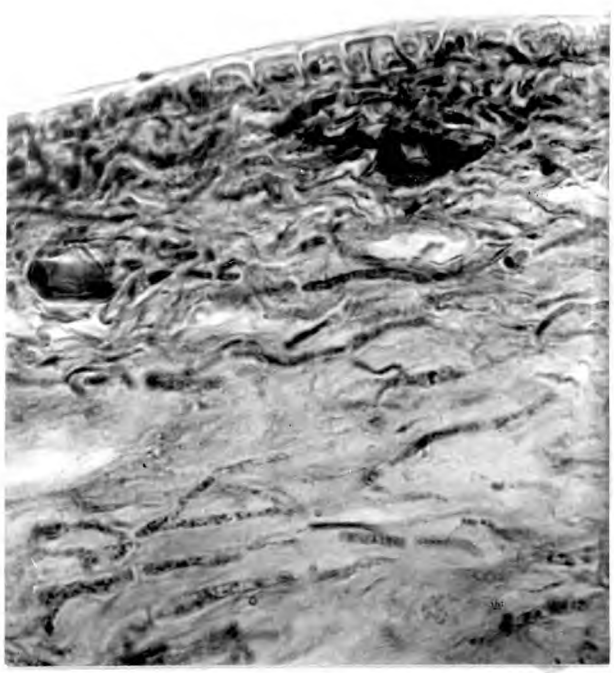

Plate 30. L.S. Dissolution of flavedo by hyphae. X240.

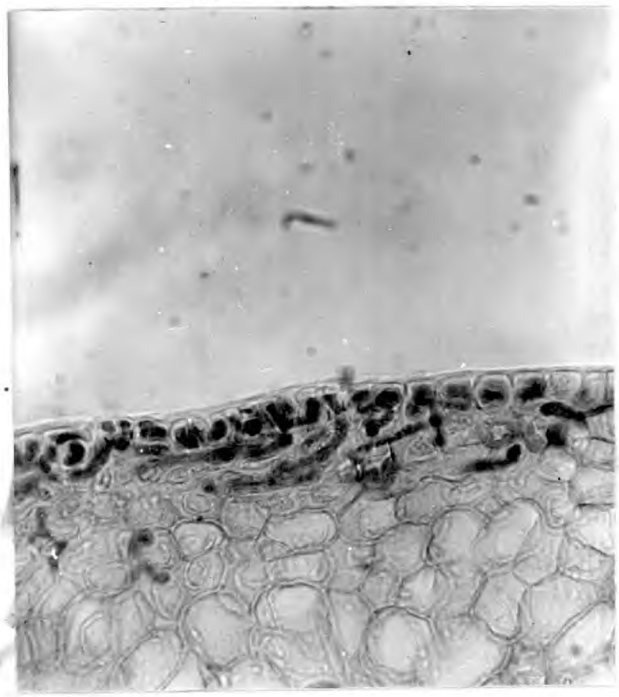

Plate 31. L.S. Epidermal cells at the edge of a lesion filled with hyphae. X240.

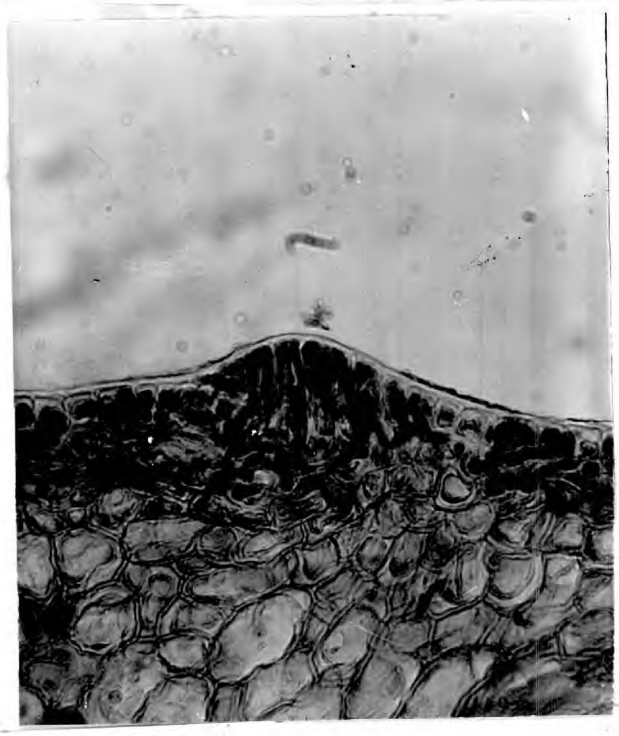

Plate 32. L.S. Aggregation of hyphae before bursting through the outer cuticular layer of the epidermis. X240.

Plate 33. L.S. Sporulating hyphae in an oil vesicle close to the centre of a lesion. X60.

H. DEVELOPMENT OF RED SPOTS

Shamouti, Valencia, and Navel oranges inoculated with an aqueous spore suspension into the flavedo tissues frequently developed red spots at the periphery of pricks at inoculum sites 3 to 6 days after incubation.

Generally, these spots denoted a resistance of the fruit to infection by the fungus. Only occasionally did profuse growth at the points of inoculation lead to infection in the presence of red spots. Plate 11, (P.72) shows one site with red spots, where profuse growth and sporulation of the fungus 20 days after incubation did not result in the formation of a lesion and in an experiment with Navel fruit only 4 out of 50 fruit inoculated showing red spots at the pricks developed lesions.

Having established that resistance to infection was frequently accompanied by red spots at the points of pricking, the formation of these spots was studied in some detail.

Navel fruit, pricked to a depth of 0.5 and 1.5 mm. between, and 0.5 and 1 mm. into the oil vesicles, were inoculated with 1.0 ml. aqueous spore suspension containing 10,000 spores and incubated for 7 days. After this period, sites showing red spots and those not showing red spots, were cut out from the rind and fixed in formalin-propiono-alcohol (P. 30). Serial microtome sections 12 μ thick were then cut and stained with carbol-thionin and orange G by the method already described on P. 31. Microscopic

examination of these sections showed the reaction of the flavedo cells and the mode of penetration of these cells by the fungus.

With tissues not showing red spots, hyphae were detected in the pricks at a depth of 0.5 mm. between the vesicles, but there was no distortion of cells other than those ruptured in wounding.

The same picture was obtained with pricks made at a depth of 1.5 mm. between the vesicles (Plate 34).

With tissues showing red spots growth of hyphae in the pricks was observed at depths of 0.5 and 1.5 mm. between the oil vesicles. The cells of the compact flavedo bordering the pricks had lost their coherence to each other and appeared to have been dead at the time of fixation (Plate 35). No fungal hyphae were detected in these dead cells although they did stain blue with carbol-thionin.

Good growth of hyphae was observed in pricks of 0.5 mm. depth into the vesicles. The sub-epidermal cells of the flavedo around the periphery of the pricks had lost their coherence and this resulted in the appearance of the red spots around the pricks on the surface of the fruit (Plate36).

With inoculations made 1.0 mm. deep into the vesicles numerous dead cells causing the red spots on the surface were present in the sub-epidermal cells of the flavedo tissues. These tissues were not extensively penetrated by hyphae and the growth of hyphae appeared restricted to the flavedo tissues (Plate 37).

These examinations showed that red spots were caused by the dead, sub-epidermal cells of the flavedo on the periphery of the pricks and that there was growth of hyphae at all the red spots.

Plate 34. L.S. Wound 1.5 mm. deep between the oil vesicles. X240.

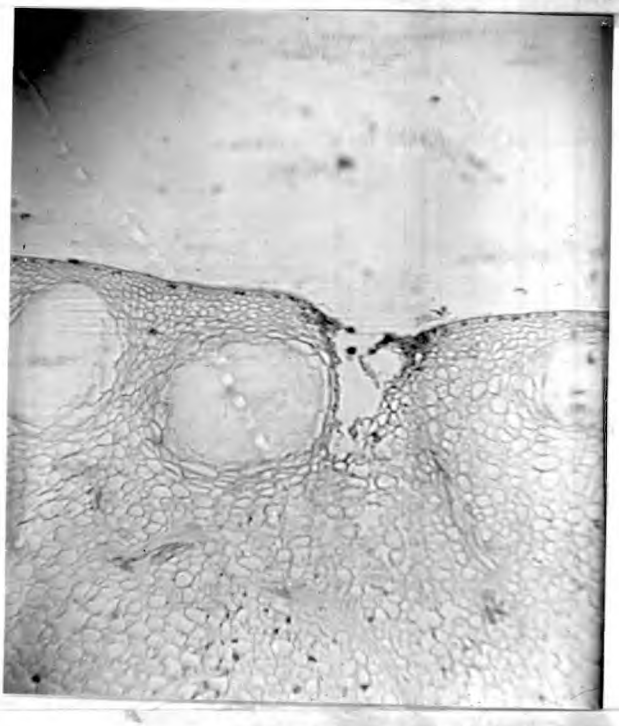

Plate 35. L.S. 'Red spot' at a wound 1.5 mm. deep between the oil vesicles. X12.

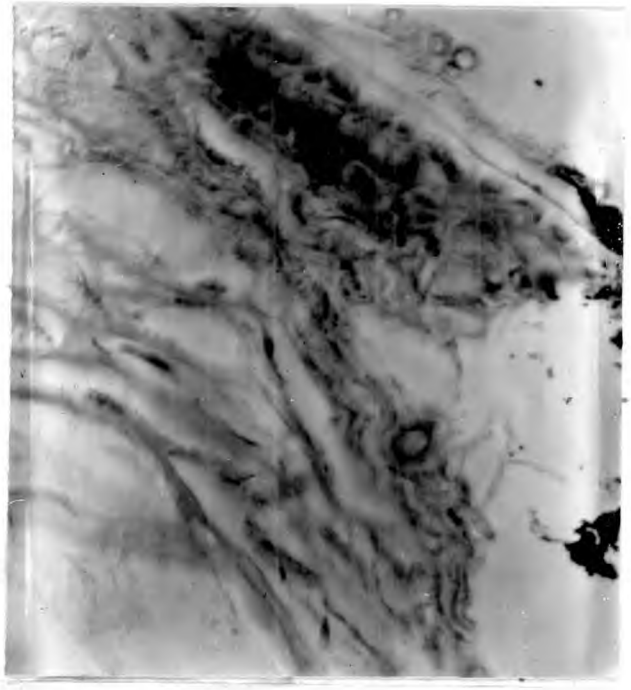

Plate 36. L.S. Loss in coherence of flavedo cells at a 'red spot'. X240.

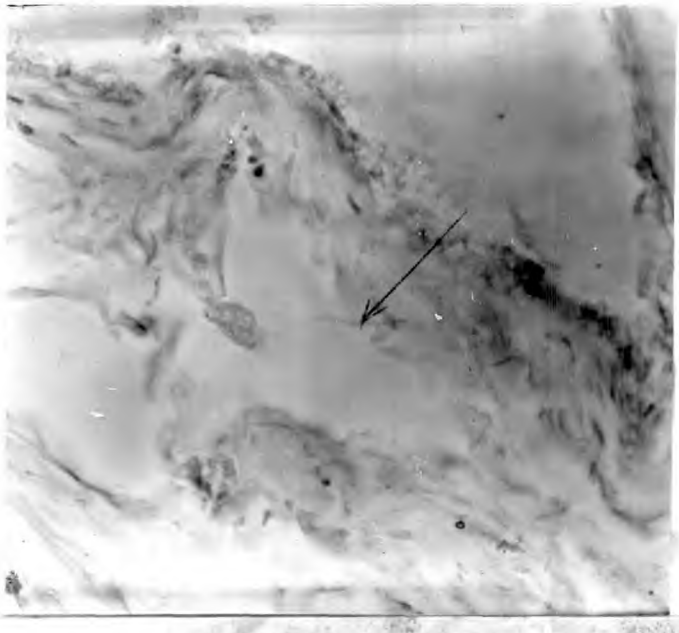

Plate 37. L.S. Initial penetration of flavedo by a thin hypha at the periphery of a wound 1 mm. deep into an oil vesicle. X240.

But in some pricks, growth of hyphae did not result in red spots developing after 7 days incubation.

The lack of coherence in the sub-epidermal cells appeared to result not from growth of hyphae through these cells but rather to substances produced by growth of hyphae in the pricks and the production of these substances by the fungus seemed limited to the immediate vicinity of the pricks.

1. The appearance of red spots in inoculated oranges, lemons and grapefruit

In studying the resistance of the inter-vesicle tissues of the flavedo to infection it was observed that for any one method of inoculation, the number of red spots developing varied considerably between varieties of orange.

In this experiment the appearance of red spots was investigated using Navel and Valencia oranges, grapefruit and lemons.

Ten fruit of each type were inoculated three times into the flavedo between the oil vesicles to a depth of 1 mm. Each fruit was inoculated with .01 ml aqueous spore suspension containing 10,000 spores. The appearance of the 'spots' with time is recorded in Table 24.

Table 24. The appearance of red spots in Navel and Valencia oranges, lemons and grapefruit inoculated with an aqueous suspension of spores of P.digitatum between the oil vesicles

Three pricks, 1 mm. deep/inoculum site.

Days after inoculation	Number of red spots out of 10 in each series							
	Oranges						Lemons	Grapefruit
	Navels					Valencias		
	A	B	C	D	E			
3	6	4	6	3	6	6	4	9
7	6	5	9	4	7	7	5	9
11	6	5	9	4	7	7	5	9

Navels A,B and C were part of different consignments from the same packhouse.

The development of red spots was not followed by the formation of lesions in any fruit. 'Spots' developed at the points of inoculation of both varieties of orange, lemons and grapefruit. There were marked differences in the number of 'spots' developing in each type of fruit. Even Navel oranges from the same packhouse varied in the number of 'spots' produced.

As it was possible that the 'spots' may have developed as a result of chemical treatment in the packhouse, untreated fruit were used in the following experiment.

Navel fruit were inoculated as above. Four out of a possible 10 fruit developed red spots within 6 days. So it would appear that 'spots' at the points of inoculation can develop in chemically

untreated fruit.

It can be concluded that the development of these spots is a character of various Citrus fruit, and that it is probably a resistant reaction to infection. The development of 'spots' is accompanied and possibly caused by growth of the fungus at the points of inoculation and is not thought to be caused by the application of chemicals in the control of wastage.

I. BREAKDOWN OF RESISTANCE OF FLAVEDO TO INFECTION

It has been shown that fruit inoculated between the oil vesicles with an aqueous spore suspension show a resistance to infection but that this resistance is largely lost when the wounds extend into the albedo. There is also little resistance when wounds are made into the oil vesicles. The problem now is this: why are the inter-vesicle tissues of the flavedo resistant, and why are the albedo tissues more susceptible to infection?

With this problem in view a further study of resistance of the flavedo was made paying particular attention to the role of different substances in the rind that might be released on wounding and which might therefore alter the resistance of the tissues to infection in the presence of spores.

1. Effect of rind extracts in the inoculum drop on resistance of flavedo to infection in Shamouti, Navel and Valencia fruit

In this experiment Shamouti fruit were used and spores were suspended in water infusion extracts of the flavedo, albedo and complete rind, orange juice (see P. 34), 1% citric acid and orange rind essential oil. In this and all subsequent experiments fruit were wounded by pricking three times between the oil vesicles to 1.0 mm. An inoculum of 0.02 ml. of the appropriate medium containing 20,000 spores (approx.) was applied to the wounds at each inoculum site. There were 10 fruit for each treatment and each fruit was inoculated at one point. The fruit were incubated

for 24 days and the number of lesions formed are shown in Table 25.

Table 25. Effect of P.digitatum spores suspended in different rind extracts and citric acid on resistance of flavedo in Shamouti fruit to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per . . . . per fruit.

Days after inoculation	Number of lesions out of 10 in each series						
	Water	Flavedo extract	Albedo extract	Complete rind extract	Orange juice	1% Citric acid	Essential oil
2	0	0	0	0	0	0	1
3	0	0	1	2	5	7	3
4	0	3	4	5	7	8	5
6	0	5	8	6	10	9	5
8	0	5	9	8	10	9	6
11	0	5	9	8	10	9	6
13	0	5	9	8	10	9	7
24	0	5	9	8	10	9	7

As expected spores suspended in water did not cause lesions to be produced but substantial numbers of fruit inoculated with spores in rind extracts, citric acid and essential oil produced lesions through the wounds 1.0 mm. deep.

Orange juice was the most efficient treatment in promoting infection. Citric acid was almost as good although it had been found to have no direct effect on the growth of the fungus (P. 62). Of the

rind extracts the flavedo was the least effective and the albedo extract the best. Each of the rind extracts, and the orange juice allowed the fungus to grow and sporulate well at the inoculum sites prior to infection.

Essential oil also promoted infection but not so well as the other treatments. The oil killed the cells bordering the wound and in so doing probably provided the fungus with a suitable substrate for growth and this promoted infection and colonization of the healthy rind tissues. The other substances probably promote infection in the same way but their action is more direct in the sense that they are also readily available energy sources so that growth of the fungus is stimulated immediately. This probably explains why they are somewhat more effective in promoting infection than is the essential oil. However, the fact that flavedo extract was not able to promote infection so efficiently as the other rind extracts could not be explained on this basis because the fungus grew very well on this medium (see P.153).

This leads to the idea that some factor is produced by the growth of the fungus in albedo and complete rind extracts which reduces the resistance of the rind to infection and that this factor is less readily produced in the flavedo extract.

It must also be assumed that the factor is produced after growth in orange juice, and in citric acid at the site of a wound. It would appear that good growth of the fungus at the inoculation

site does not necessarily mean that the factor is produced in sufficient quantities to ensure infection. Once sufficient of the factor has been produced to ensure infection, it can be assumed that it is then produced cumulatively after growth on the dead tissues of the expanding lesion so that a progressive lesion is formed.

In another experiment the ability of spores suspended in rind extracts to promote infection through wounds between the oil vesicles in Navel fruit was investigated.

The methods used were those already described for the experiment with Shamouti fruit. The number of lesions formed within 19 days after inoculation are shown in Table 26.

Table 26. Effect of P.digitatum spores suspended in different rind extracts on resistance of flavedo in Navel fruit to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Water	Flavedo extract	Albedo extract	Complete rind extract	Orange juice
3	0	1	4	3	3
5	0	3	6	5	10
7	0	3	8	6	10
19	0	3	8	6	10

All extracts promoted infection but to a varying degree. Again the juice was most efficient in doing this and of the rind

infusion extracts the flavedo was least efficient and the albedo best.

In general, these results agree with those obtained in the experiment with Shamouti fruit. The ability of the juice, albedo and complete rind extracts to promote infection substantially and the lower ability of the flavedo extract to do so were confirmed.

A further experiment dealt with the effect of spores suspended in rind extracts in promoting infection through wounds between the oil vesicles in Valencia fruit.

The fruit were incubated for 19 days after inoculation and the number of lesions formed within this period are shown in Table 27.

Table 27. Effect of P.digitatum spores suspended in different rind extracts on resistance of flavedo in Valencia fruit to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Water	Flavedo extract	Albedo extract	Complete rind extract	Orange juice
2	0	0	1	0	2
4	0	4	8	4	9
5	0	5	9	4	10
8	0	6	9	4	10
15	0	6	9	5	10
19	0	6	9	5	10

Infection was promoted most readily by using the orange juice. Of the rind extracts the albedo extract promoted infection best. The results are therefore in general agreement with those of the earlier experiments with Shamouti and Navel fruit and that the capacity of spores to break the resistance of the flavedo to infection is shared by all three varieties of fruit tested.

2. Effect of rind extracts in the inoculum drop on resistance of flavedo to infection in Shamouti fruit wounded 0.5 mm. deep between the oil vesicles

In this experiment the effect of spores suspended in rind extracts on the resistance of flavedo to infection was investigated when 3 wounds 0.5 mm. deep were made between the oil vesicles.

The method of inoculation and incubation was the same as in the last experiment. The fruit were of the Shamouti variety but were taken from a different shipment than were those used previously (P.123).

The lesions formed within 19 days after inoculation are shown in Table 28 .

Table 28. Effect of P.digitatum spores suspended in different rind extracts on resistance of flavedo in Shamouti fruit to infection

Three wounds, 0.5 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Water	Flavedo extract	Albedo extract	Complete rind extract	Orange juice
3	0	0	4	1	7
5	1	0	5	3	10
7	2	2	6	4	10
11	2	3	6	4	10
19	2	3	6	4	10

The ability of orange juice to cause infection was not reduced by the more shallow wounding whereas with the other extracts it was. The flavedo extract had very little effect in promoting infection and the albedo extract, while still quite active in the promotion of lesions, was less so than when deeper wounds were used. The complete rind extract was also considerably less effective.

The effect of orange juice in promoting infection through very shallow wounds was thought to result from the action of acids in the juice, mainly citric and malic acids, on the cells exposed by the wounds. The results of another experiment supported this view.

By titration the amount of acid in the juice was estimated at 1%, so each Shamouti fruit was wounded three times to a depth of

0.5 mm. and inoculated with 0.1 ml. of spore suspension in 1% citric acid. Within 5 days of incubation 10 lesions out of a possible 10 had developed.

In the juice the organic acids occur mostly in the free form, whereas in the peel they are present largely as salts (Kefford,1959). This together with the fact that the concentration of organic radicals in the peel is much lower than in the juice would explain in part at least, why peel extracts are less effective in promoting infection.

3. Effect of flavedo extract in the inoculum drop on resistance of flavedo to infection in Shamouti, Navel and Valencia fruit wounded to different depths

In the previous experiment it was seen that with pricks 0.5 mm. deep the effect of flavedo extract in promoting the formation of lesions was not very marked.

In this experiment it was decided to investigate the effect of depth of wound in the promotion of infection by an inoculum of spores in flavedo extract, to see if by increasing the depth of wound there would be a corresponding increase in the ability of the spores to reduce the resistance to infection.

Wounds 0.5, 1.0 and 1.5 mm. deep were made between the oil vesicles. After inoculation the fruit were incubated for 20 days and the lesions formed within this period are shown in Table 89.

Table 29. Effect of depth of wound between oil vesicles on infection of Shamouti oranges by P.digitatum spores suspended in flavedo extract

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Depth of prick (mm.)							
	0		0.5		1.0		1.5	
	Water	Flavedo extract	Water	Flavedo extract	Water	Flavedo extract	Water	Flavedo extract
3	0	0	0	0	0	0	0	2
5	0	0	1	2	0	2	1	6
7	0	0	2	2	1	3	1	6
8	0	0	2	2	1	3	1	7
9	0	0	2	2	1	4	1	8
13	0	0	2	3	1	5	1	8
20	0	0	2	3	1	5	1	8

With the extract the deeper the wound the shorter was the period of incubation necessary for the formation of lesions and the more effective was the promotion of infection. Depth of wounding had no effect on the number of lesions formed, or on the speed of formation when the spores were in water.

It thus appears that although the resistance of flavedo can be broken by suspending the spores in flavedo extract this is

effective only when wounds are sufficiently deep.

A similar experiment was done with Navel fruit. After inoculation the fruit were incubated for 20 days and the lesions formed within this period are shown in Table 30.

Table 30. Effect of depth of wound between oil vesicles on infection of Navel oranges by P. digitatum spores suspended in flavedo extract

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Depth of prick (mm.)							
	0		0.5		1.0		1.5	
	Water	Flavedo extract	Water	Flavedo extract	Water	Flavedo extract	Water	Flavedo extract
3	0	0	0	0	0	0	0	0
5	0	0	0	1	0	4	0	4
9	0	0	0	2	1	5	0	7
20	0	0	0	2	1	5	0	7

Again, suspending the spores in flavedo extract promoted infection, the efficiency of this depending on the depth of wound made. The deeper the wound the more efficient the extract was in promoting infection.

In another experiment Valencia fruit were used. The results are shown in Table 31.

Table 31. Effect of depth of wound between oil vesicles on infection of Valencia oranges by P.digitatum spores suspended in flavedo extract.

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Depth of prick (mm.)							
	0		0.5		1.0		1.5	
	Water	Flavedo extract	Water	Flavedo extract	Water	Flavedo extract	Water	Flavedo extract
2	0	0	0	0	0	0	0	1
4	0	0	0	0	0	4	0	5
8	0	0	0	1	0	6	0	6
15	0	0	0	1	0	6	0	7
19	0	0	0	1	0	6	0	7

Wounds 1.0 mm. deep were necessary for promotion of infection with an inoculum of spores in flavedo extract. Increasing the depth to 1.5 mm. only resulted in a slight increase in the number of lesions formed. In general, these results agree with those obtained with Navel and Shamouti fruit.

The effect of deeper pricks in promoting infection in the three varieties is attributed to the fact that the deeper the wound the thinner is the layer of cells which is able to resist penetration by the fungus.

4. Effect of orange essential oil in the inoculum drop on resistance of flavedo to infection in Shamouti fruit

In an earlier experiment with Shamouti fruit (P.123) orange essential oil substantially promoted infection through wounds 1.0 mm. deep between the oil vesicles. This effect was now studied in more detail.

Spores suspended in essential oil were applied to unwounded fruit and to those wounded 0.5, 1.0 and 1.5 mm. deep between the oil vesicles. The fruit were incubated for 19 days and the number of lesions that developed within this period is shown in Table 32.

Table 32. Effect of P.digitatum spores suspended in orange essential oil on resistance of flavedo in Shamouti oranges to infection

Three wounds per fruit.

Days after inoculation	Number of lesions out of 10 in each series			
	Depth of prick (mm.)			
	0	0.5	1.0	1.5
2	0	0	1	2
3	0	0	4	9
5	0	0	6	10
15	0	0	7	10
19	0	0	7	10

Infection was not promoted in the absence of wounds nor with wounds 0.5 mm. deep. With wounds 1.0 and 1.5 mm. deep the formation

of lesions was promoted and there were more lesions with the deeper wounding.

In fruit wounded to a depth of 1.0 and 1.5 mm. contact of the essential oil with the cells at the inoculum site resulted in a slight sinking of the tissue between the oil vesicles in the rind, so that the oil vesicles stood out prominently at the affected areas. This symptom developed within 3 days.

These spots are the result of rapid penetration of the rind cells by the oil, which is phytotoxic and kills the cells at the surface. The development of these 'oil spots' in the field is known as 'olleocellosis'.

Although it can be assumed that the death of the surface cells followed contact with the oil, nonetheless with this method of inoculation infection through the uninjured cuticle or through pricks 0.5 mm. was not promoted presumably because the deeper cells of the flavedo were not affected.

It would seem that the oil promotes infection because it kills the flavedo cells, and not because it has any direct effect in promoting growth of the fungus (see P.196).

5. Effect of carbohydrates and nitrogen compounds in the inoculum drop on resistance of flavedo to infection

It was seen in earlier experiments that spores suspended in albedo extract and orange juice were very effective in the promotion of infection when shallow wounds were made between the oil vesicles.

It was suggested that in the orange juice it was the action of acids, such as citric and malic acids, on the exposed flavedo cells of the pricks which was responsible for increasing the infection rate by spores. However, because the acid content of the albedo is low (Kefford, 1959), it was thought that some substance other than acid might play a part in overcoming resistance to infection by spores suspended in albedo extract.

In the following experiments the effect of suspending spores in solutions of carbohydrates found in orange rind on the breakdown of resistance of flavedo to infection was studied in an attempt to ascertain what components of the albedo extract were active in the promotion of infection. Solutions of some nitrogen containing compounds were also tested for their effect on spore germination.

(a) Effect of buffered solutions of carbohydrates in the inoculum drop on resistance of flavedo to infection in Shamouti and Navel fruit

In this experiment the methods used were similar to those described in earlier experiments (P. 122). Fruit were wounded between the oil vesicles to a depth of 1 mm. and inoculated with spores suspended in 1% glucose, fructose, sucrose and pectin solutions containing 0.01M phosphate buffer (Sorensen's) at pH5.2. This pH was selected as it was within the pH range of the peel (5.0 - 5.5). The fruit were incubated for 16 days and the number of lesions formed within this period are shown in Table 33.

Table 33. Effect of P.digitatum spores suspended in different carbohydrate solutions containing 0.01M phosphate buffer (pH 5.2) on resistance of flavedo in Shamouti oranges to infection

Three wounds, 1.0 mm. deep between the oil vesicles, per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Suspension medium				
	Buffer	1% Glucose	1% Fructose	1% Sucrose	1% Pectin
3	0	1	0	0	1
5	1	1	1	1	4
8	1	2	1	1	6
10	1	2	1	1	9
16	1	2	1	1	10

Pectin was the only compound effective in promoting lesion formation through wounds of this type.

Growth of germ-tubes and the formation of red spots were observed at those sites inoculated with spores in glucose, fructose and sucrose but the number of lesions formed as a result of these treatments did not appear to be significant.

This experiment was repeated with Navel fruit, with the results shown in Table 34.

Table 34. Effect of P.digitatum spores suspended in different carbohydrate solutions containing 0.01M phosphate buffer (pH 5.2) on resistance of flavedo in Navel oranges to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Suspension medium				
	Buffer	1% Glucose	1% Fructose	1% Sucrose	1% Pectin
3	0	0	0	0	3
5	0	0	0	0	7
7	0	1	0	0	9
9	0	1	0	0	10
20	0	1	0	0	10

Again, only spores suspended in pectin solution promoted infection.

(b) Effect of concentration of buffered pectin in the inoculum drop on resistance of flavedo to infection in Navel fruit

In this experiment fruit were pricked between the oil vesicles to a depth of 1.0 mm. and inoculated with spores suspended in 0.01%, 0.1%, 0.5% and 1% pectin solutions buffered with 0.01M phosphate buffer (Sorensen's) at pH 5.2. The fruit were incubated for 27 days and the number of lesions produced

within this period are shown in Table 35.

Table 35. Effect of P.digitatum spores suspended in buffered pectin solutions of different concentration on resistance of flavedo in Navel oranges to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series				
	Suspension medium				
	Buffer	0.01% Pectin	0.1% Pectin	0.5% Pectin	1% Pectin
2	0	0	1	0	0
4	1	1	5	7	4
6	2	1	7	9	9
7	2	1	8	9	9
9	2	1	8	10	10
27	2	1	8	10	10

Concentrations of pectin in range 0.1 to 1% were effective in promotion of infection.

(c) Effect of depth of prick on resistance of flavedo in Navel fruit to infection by spores suspended in buffered pectin

In this experiment fruit were wounded between the oil vesicles, to depths varying from 0 to 1.5 mm., and inoculated with spores suspended in 0.5% pectin solution containing 0.01M phosphate buffer (Sorensen's) at pH 5.2. Corresponding treatments with spores

in water acted as controls. The number of lesions formed within an incubation period of 20 days is shown in Table 36 .

Table 36. Effect of depth of wound on resistance of flavedo in Navel oranges to infection by P.digitatum spores suspended in buffered 0.5% pectin solution

Three wounds, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Water	Pectin	Water	Pectin	Water	Pectin	Water	Pectin
	Depth of prick (mm.)							
	0		0.5		1.0		1.5	
2	0	0	1	0	0	0	0	1
4	0	0	1	7	0	7	0	10
6	0	0	1	10	0	9	0	10
9	0	0	1	10	0	10	0	10
20	0	0	1	10	0	10	0	10

Spores did not promote infection in the absence of wounds but when suspended in pectin solution infection took place even with wounds as shallow as 0.5 mm.

It would thus appear that spores in pectin are only effective in promoting infection when some sort of superficial wound has been made in the fruit.

(d) Effect of polysaccharides in the inoculum drop on resistance of flavedo to infection in Navel fruit

In the preceding experiments it was seen that fruit, wounded 1.0 mm. between the oil vesicles, were infected by spores suspended in buffered pectin. It was now of interest to see if spores suspended in unbuffered pectin and other polysaccharide solutions infected fruit wounded in a similar way.

Spores were suspended in 0.5% solutions of the following buffered and unbuffered polysaccharides, pectin (P), sodium polypectate (NaPP), and carboxymethylcellulose (CMC). The buffered solutions contained 0.01M phosphate buffer (Sorensen's) at pH 5.2. The fruit were pricked between the oil vesicles to a depth of 1.0 mm. prior to inoculation. The fruit were incubated for 25 days and the lesions formed within this period are shown in Table 37 .

Table 37. Effect of P.digitatum spores suspended in different polysaccharide solutions on resistance of flavedo in Navel oranges to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Suspension medium							
	Water	Buffer	P	B-P	NaPP	B-NaPP	CMC	B-CMC
3	0	0	2	4	0	0	0	0
5	0	1	2	9	0	0	0	0
7	0	2	2	9	0	0	0	4
10	2	2	2	9	0	2	0	7
22	2	2	3	9	0	2	0	7

+B - = Buffered.

Both buffered pectin and buffered carboxymethylcellulose solutions promoted infection whereas unbuffered solutions of these polymers did not. This would seem to indicate that the phosphate buffer aids in the promotion of infection.

Another experiment with Navel fruit from a different consignment was carried out to confirm the observations above. The design, method of inoculation and incubation were the same as in the previous experiment. The lesions formed after 15 days incubation are shown in Table 38.

Table 38.Effect of P.digitatum spores in different polysaccharide solutions on resistance of flavedo in Navel oranges to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Suspension medium							
	Water	Buffer	P	B-P	NaPP	B-NaPP	CMC	B-CMC
15	1	1	8	10	4	3	1	1

Spores suspended in buffered and unbuffered pectin solutions promoted infection but unbuffered pectin did so to a lesser extent. The production of lesions was stimulated to a slight extent by sodium polypectate also.

Comparing the results of the two experiments it is seen that only spores suspended in buffered pectin were consistent in

promoting infection.

In an attempt to clarify some of the inconsistent results observed in the two previous experiments a similar experiment was done with Valencia fruit. The fruit were incubated for 14 days and the number of lesions formed within this period are shown in Table 39.

Table 39. Effect of P.digitatum spores in different polysaccharide solutions on resistance of flavedo in Valencia oranges to infection

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Suspension medium							
	Water	Buffer	P	B-P	NaPP	B-NaPP	CMC	B-CMC
3	1	3	9	10	5	1	1	0
4	1	3	10	10	6	2	1	0
5	1	3	10	10	7	4	2	2
14	1	3	10	10	7	4	2	2

Buffered and unbuffered pectin and to a lesser extent unbuffered sodium polypectate promoted infection.

The results of this experiment are consistent with those of the previous experiment with Navel fruit. The results of the three experiments as a whole show that spores in buffered or unbuffered pectin promote infection through wounds into the flavedo between the oil vesicles. Infection is also promoted but to a lesser

extent with spores suspended in unbuffered sodium polypectate but carboxymethylcellulose is ineffective.

(e) Effect of buffered pectin in the inoculum drop on resistance of flavedo to infection in Valencia fruit from different packhouses

In this experiment Valencia fruit from different packhouses (i) - (iv) were used. Fruit were wounded to a depth of 1.0 mm. between the oil vesicles and inoculated with spores suspended in 0.5% pectin solution buffered with 0.01M phosphate buffer (Sorensen's) at pH 5.2. The number of lesions formed within an incubation period of 10 days is shown in Table 40.

Table 40. Effect of P.digitatum spores in buffered pectin on resistance of flavedo to infection in Valencia oranges from different packhouses

Three wounds, 1.0 mm. deep, between the oil vesicles per fruit.

Days after inoculation	Number of lesions out of 10 in each series							
	Packhouse (i)		Packhouse (ii)		Packhouse (iii)		Packhouse (iv)	
	Suspension medium							
	Bfr*	0.5% B-P*	Bfr*	0.5% B-P*	Bfr*	0.5% B-P*	Bfr*	0.5% B-P*
3	0	6	0	8	1	8	0	9
5	1	9	5	10	1	10	0	10
7	1	10	5	10	1	10	0	10
10	1	10	5	10	1	10	0	10

Bfr* = Buffer
B-P* = Buffered Pectin

Resistance of flavedo to infection was overcome in all fruit inoculated with spores in buffered pectin solution. A variation in the ability of spores in buffer to promote infection with fruit from the different packhouses was also observed.

The ability of spores suspended in buffered pectin solutions to break the resistance of flavedo to infection was therefore found in the three varieties of orange Shamouti, Navel and Valencia. Three modes of action of the buffered pectin in promoting the development of lesions in the presence of shallow wounds seem possible. The pectin could act either in the production of germ tubes, in promotion of germ tube growth or by its effect on the metabolism of the host. The effect of carbohydrates (including pectin) on spore germination and hyphal growth is examined on P.169 and 199 respectively, while the pectic enzymes produced in vivo and in vitro by fungus are studied on P.207 and 200 .

(f) Effect of spores suspended in culture filtrates of the fungus grown on synthetic media, and 0.2M NaCl extract of infected rind tissues on flavedo resistance to infection

In this experiment spores were suspended in culture filtrates of the fungus grown on basal medium containing 1% pectin, NaPP, CMC, and Solka Floc wood cellulose and glucose, and in 0.2M NaCl extract of infected rind tissue. Drops of inoculum, 0.2ml., containing 2,000 spores were added to each inoculum site on Shamouti fruit. Drops were kept in position by a circular ring of vaseline. Each site was pricked 3 times between the oil vesicles to a depth of 1.0mm. and there were 10 fruit for each treatment. The number of lesions formed are shown in Table 41.

Table 41. Promotion of infection through wounds between the oil vesicles in the rind of Shamouti fruit using spore suspensions in water, culture filtrates of the fungus grown on synthetic media and 0.2M NaCl extract of infected orange rind

Three wounds, 1.0 mm. deep, per fruit.

Medium	Number of lesions amongst 10 oranges in each series				
	Days after inoculation				
	4	5	6	12	14
Basal medium + 1% pectin filtrate	0	0	0	0	0
A " " + " " "	0	1	1	1	1
" " + 1% NaPP "	0	0	0	1	2
A " " + " " "	0	0	0	0	0
" " + 1% CMC "	0	0	0	0	0
A " " + " " "	0	0	0	0	0
" " + 1% Wood cellulose filtrate	0	0	0	1	1
A " " + " " "	0	0	0	0	0
" " + 1% glucose "	2	3	5	5	5
A " " + " " "	1	1	1	1	1
0.2M NaCl extract of infected rind tissue	1	1	1	1	1
A " " " " "	0	0	0	0	0
Water	1	2	2	2	2

A = autoclaved for 10 minutes at 15 lb.p.s.i.

Spores suspended in culture filtrate of the fungus grown on 1% glucose promoted infection to some extent and this effect was eliminated by autoclaving.

Spores suspended in the other autoclaved and non-autoclaved culture filtrates of the fungus and infected rind extract did not promote infection. However the non autoclaved culture filtrates of the fungus grown on glucose, pectin and NaPP did cause a browning reaction around the pricks as shown in Plates 38, 39 and 40 respectively. "Browning" was most extensive with that culture filtrate of the fungus grown on glucose. Autoclaved culture filtrates of the fungus grown on glucose, pectin and NaPP did not result in "browning" at inoculum sites and culture filtrates of the fungus grown on CMC and wood cellulose did so only to a very slight extent.

"Browning" occurred in depressions at inoculum sites and was generally accompanied by growth of hyphae in pricks but in some fruit it was observed in the absence of visible growth. This reaction is distinct and usually more extensive than the "red spots" resulting from growth of spores in pricks as described on P.115.

Promotion of infection by spores suspended in the culture filtrate of the fungus grown on glucose was confirmed in a subsequent experiment with fruit from the same consignment.

The ability to promote infection with this filtrate is thought therefore to be caused by the presence of some thermolabile

metabolic product of the fungus as the same filtrate autoclaved did not promote infection.

"Browning" also only occurred when spores were suspended in non-autoclaved culture filtrates of the fungus. It was therefore concluded that this reaction was also caused by a thermolabile product of the fungus in the culture filtrates.

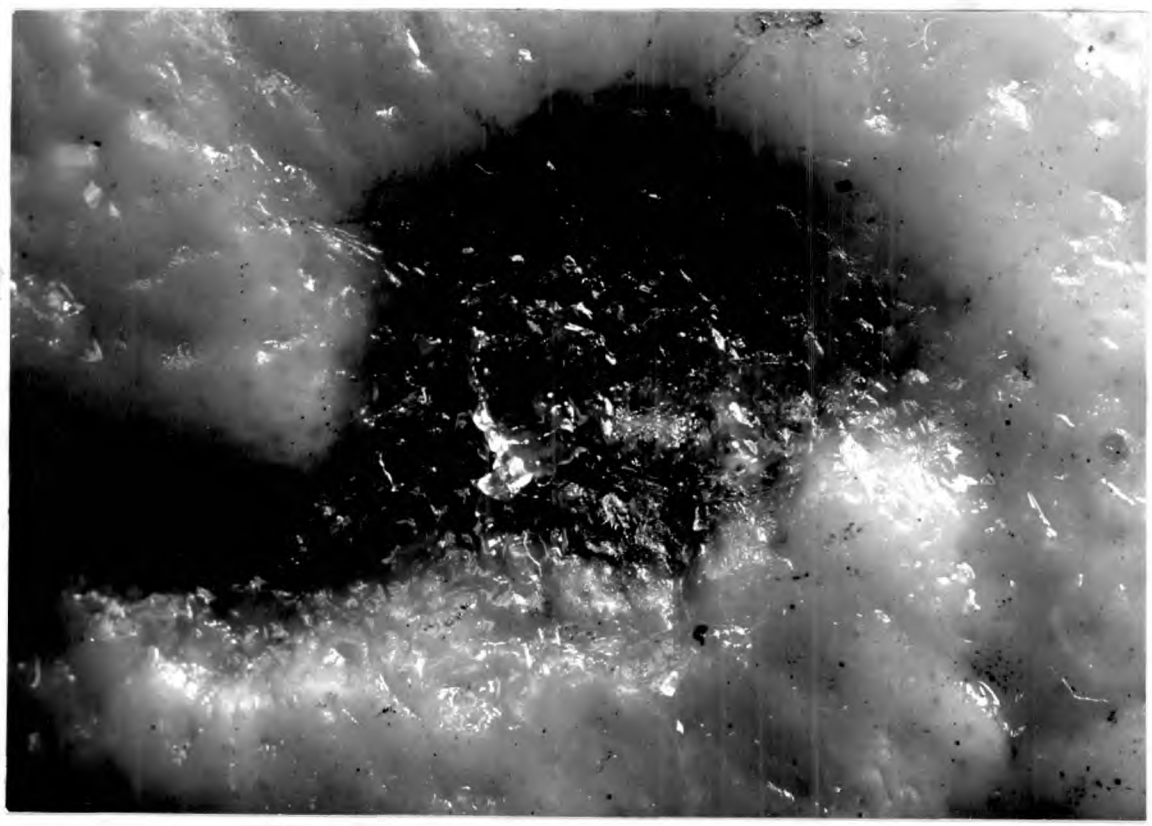

Plate 38. 'Browning' of rind tissues by spores suspended in a culture filtrate of P.digitatum grown on a synethetic medium containing glucose. X6.

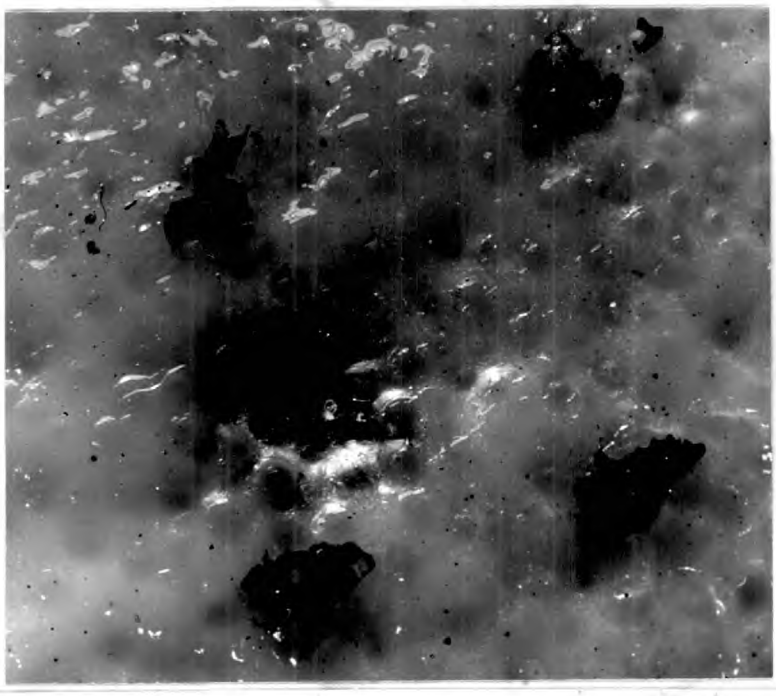

Plate 39. Rind tissues sunken and brown following inoculation with spores suspended in a culture filtrate of P.digitatum grown on a synthetic medium containing pectin.

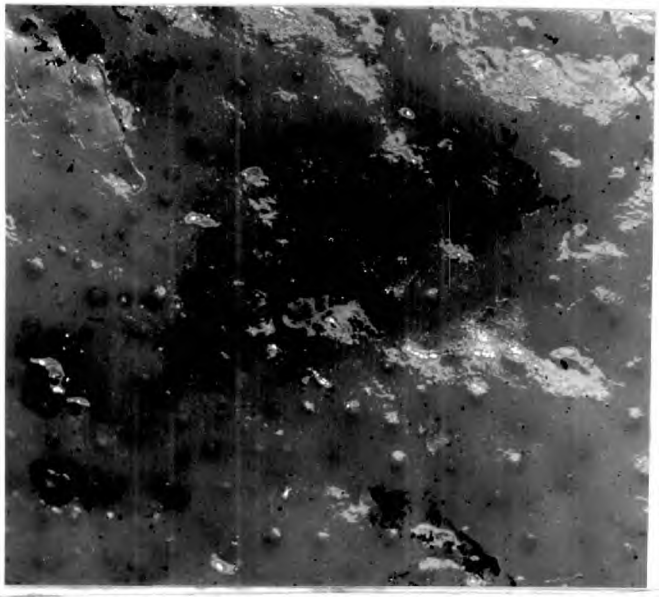

Plate 40. 'Browning' of rind tissues by spores suspended in a culture filtrate of P.digitatum grown on a synthetic medium containing NaPP. X6.

J. GROWTH OF MYCELIUM IN CULTURE

The aim of these experiments was to investigate the growth of the fungus in rind extract media and also to determine whether or not the fungus can grow in a synthetic medium with no vitamins added.

1. Effect of temperature on growth in agar media

The effect of temperature on growth in flavedo extract, albedo extract, complete rind extract, orange juice, V8 juice and 2% malt extract agar was studied at 5°, 9°, 18°, 24°, 25° and 30°C.

After 10 days incubation the optimum temperature for growth on the different media varied between 19.5°C. on orange juice to 23°C. on V8 juice agar (Fig.1). The optimum temperature for growth of the fungus in fruit was near 24°C. as observed by Fawcett and Barger (1927), and by Savastano and Fawcett (1929).

Growth and sporulation were recorded at all temperatures within the range 5-30°C. No growth was recorded at 30°C. within the 11 day period of incubation.

Growth was most rapid on V8 juice. Differences in the rate of growth on rind extract media were not very marked but a comparison of growth at the optimum temperature for each medium after 10 days showed that growth was best on albedo extract and least on orange juice.

At 24° and 25°C. colonies on rind extract media were 'ragged' in outline (Plate 41) while at 18°C. and lower temperatures they were more circular in outline (Plate 42). This 'ragged' appearance of colonies was caused by the accumulation of staling products

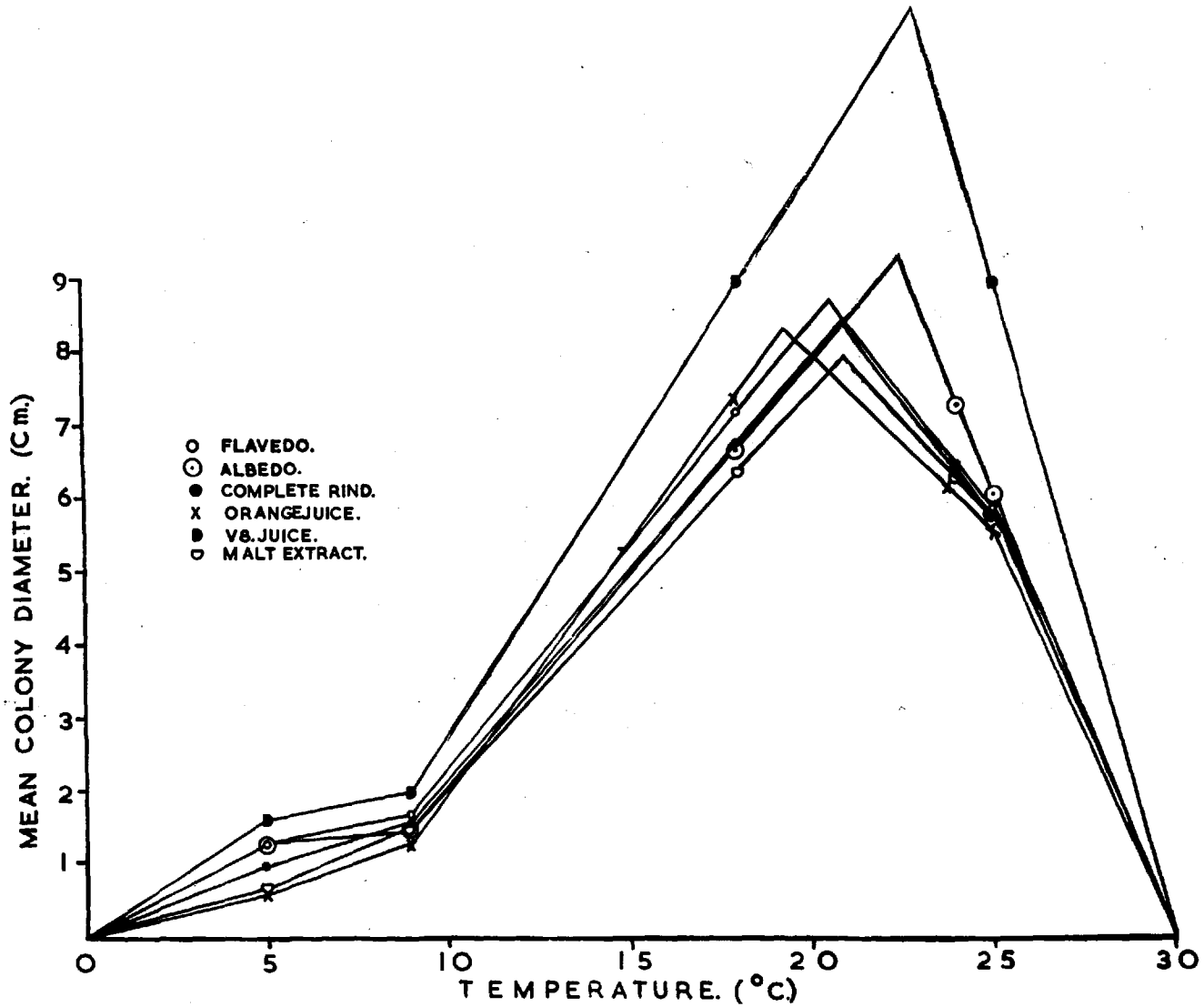

Fig. I. EFFECT OF TEMPERATURE ON GROWTHON AGAR MEDIA AFTER 10 DAYS INCUBATION.

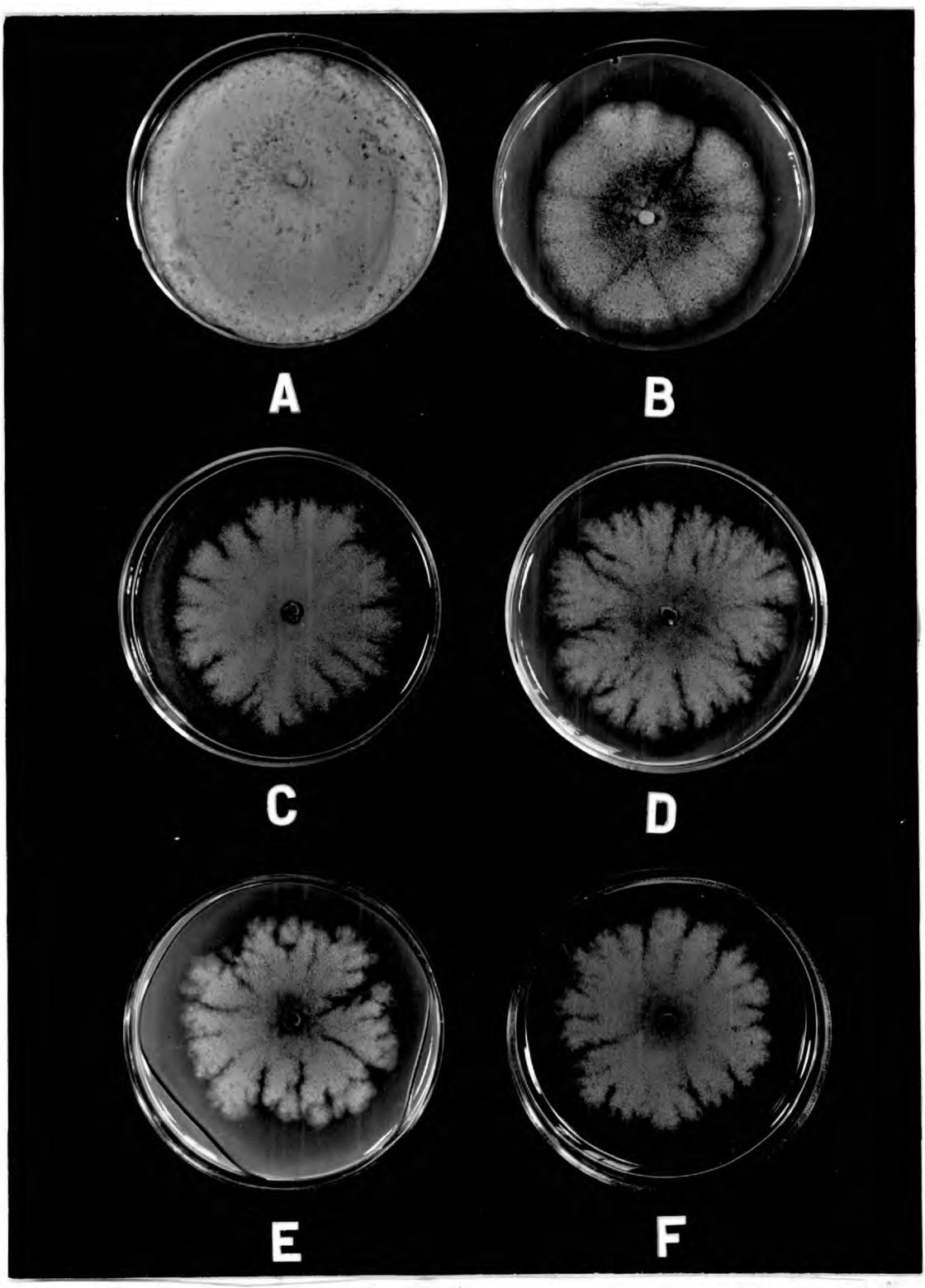

Plate 41. Growth of P.digitatum on agar media at 25°C.

A. V8 vegetable juice. B. 2% malt extract.
C. Flavedo extract. D. Albedo extract.
E. Orange juice. F. Complete rind extract.

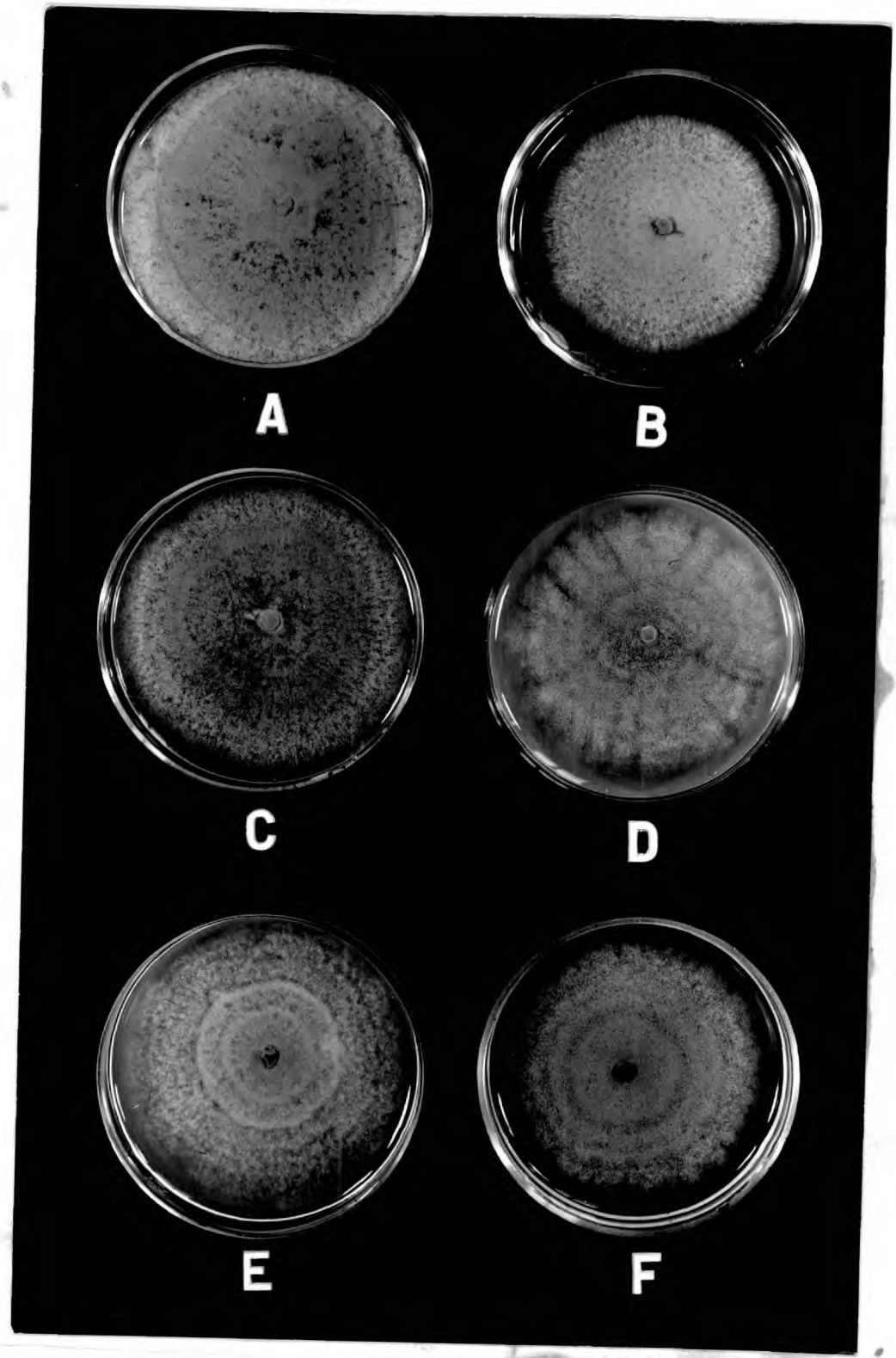

Plate 42. Growth of P.digitatum in agar media at 18°C.

A. V8 vegetable juice. B. 2% malt extract.
C. Flavedo extract. D. Albedo extract.
E. Orange juice. F. Complete rind extract.

in the rind extract media.

The effect of staling products on linear growth in complete rind extract agar at 25°C. is shown in Fig. 2 . Growth at 25°C. on this medium was initially more rapid than at 18°C., however, a reduction in rate of growth due to the accumulation of staling products at 25°C. within the first 4 days of incubation resulted in a more rapid rate of growth at 18°C. A steady rate of growth was maintained at 18°C. throughout the 11 day period of incubation (Fig. 2).

At 18-25°C. sporulation was heavy on V8 juice, fairly heavy on malt extract, flavedo and complete rind extract; good on albedo and orange juice media. At 5° and 9°C. sporulation was light on all media.

2. Growth in liquid rind extract media

The growth of the fungus in flavedo extract, albedo extract, complete rind extract and orange juice in liquid media was studied to determine whether any significant differences in growth were found on these media. Another aim of the experiment was to observe the changes in pH with growth. It was suggested in earlier experiments (P.128) that pH may be a possible factor affecting the susceptibility of the inter-vesicle flavedo tissues to infection.

Cultures were incubated in the dark at 25°C. in stationary culture. Three replicates per treatment were removed after 3,7,11 and 16 days. After harvesting the mycelium the pH of the filtrates

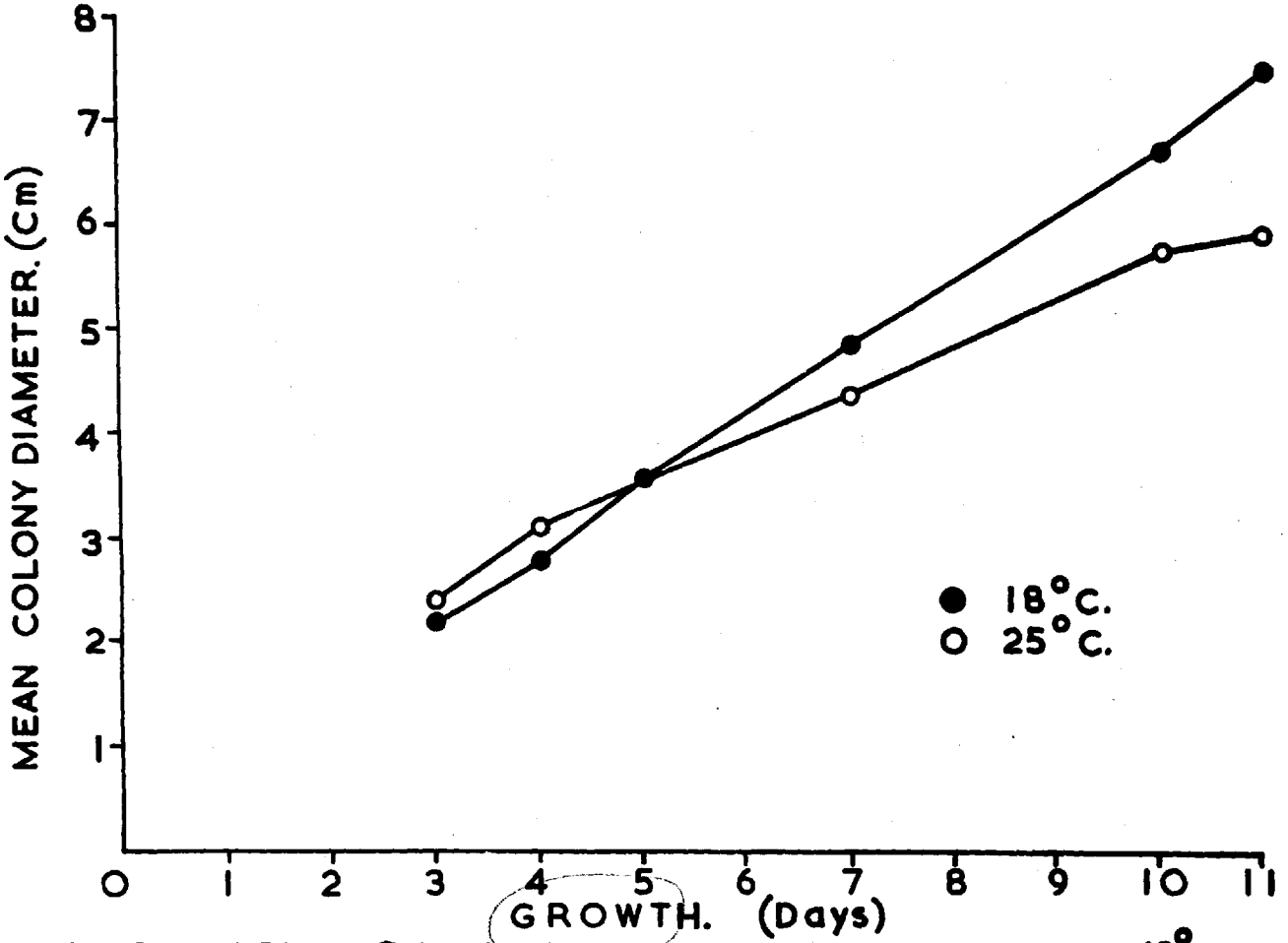

Fig. 2. GROWTH ON COMPLETE RIND EXTRACT AGAR AT 18° AND 25°C.

was determined using a Cambridge pH meter. The mean dry weight of mycelium for each treatment and period of incubation are shown in Fig. 3 , the corresponding pH changes in the media are shown in Fig.4.

Maximum growth was produced on all media after 10 days, growth was best on flavedo extract (Fig. 3). Plate 43shows growth and sporulation in the 4 media after 16 days.

Sporulation was fairly heavy in flavedo, albedo and complete rind extracts but light in orange juice.

Drift in pH in the different media resulting from growth in the rind extracts is shown in Fig. 4 . The lowest pH in each medium was recorded after 7 days, at that time the pH of the flavedo extract was 4.0 compared with 2.9 for albedo extract and 3.2 for orange juice. Therefore, the better growth in flavedo extract did not result in a lower pH of this growth medium.

These results suggest that the low infection of fruit through inter-vesicle flavedo wounds by a spore suspension in flavedo extract was not caused by less growth in this medium. The increased infection through inter-vesicle flavedo wounds by spores in albedo extract and orange juice may be partly due to the effect of the low pH of these extracts, resulting from growth of spores, in overcoming flavedo resistance.

3. Effect of vitamins and Yeastrel on growth

In a study of the effect of a mixture of vitamins and 0.1% Yeastrel in Fergus' synthetic medium the following treatments

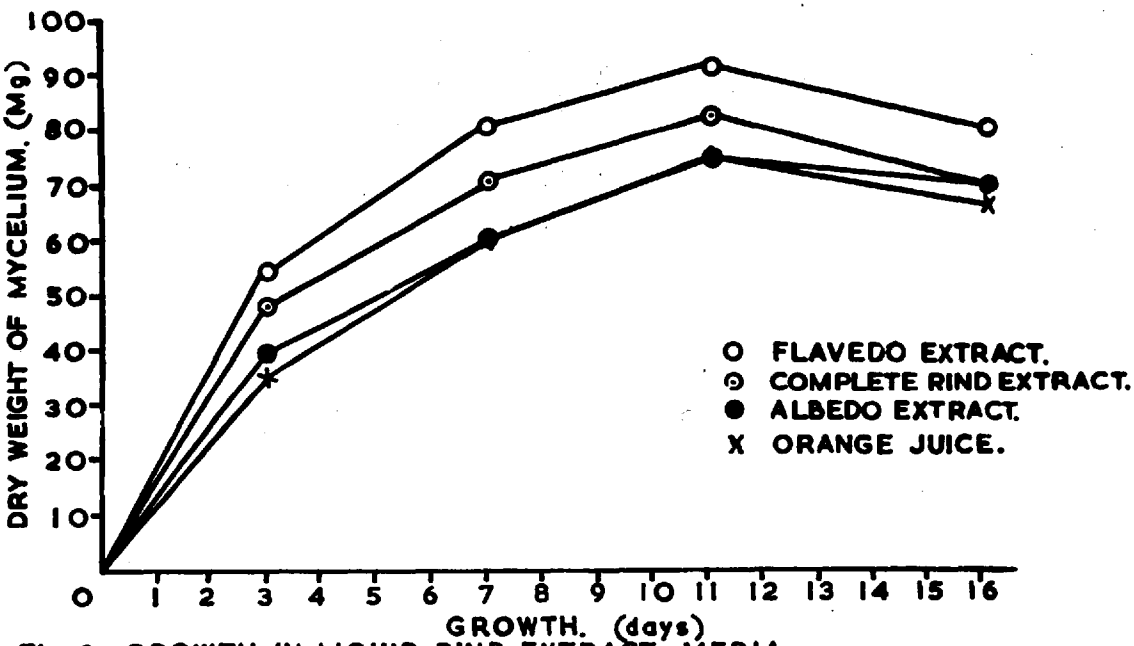

Fig. 3. GROWTH IN LIQUID RIND EXTRACT MEDIA.

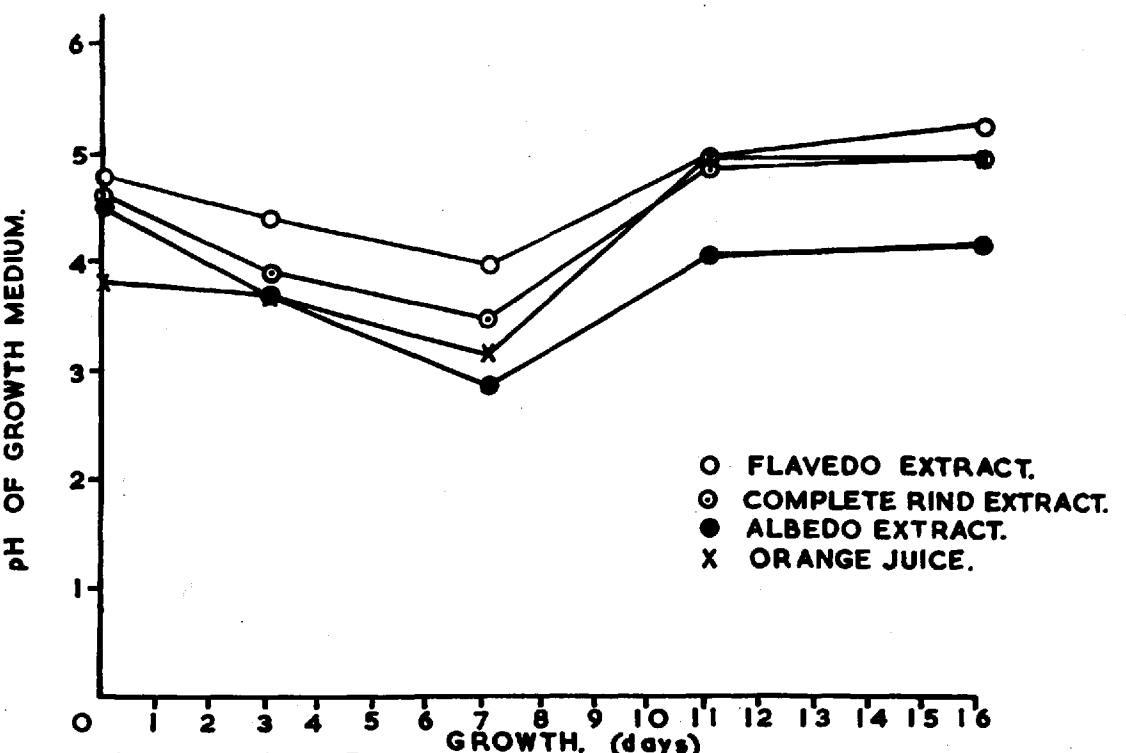

Fig. 4. CHANGE IN pH RESULTING FROM GROWTH IN LIQUID RIND EXTRACT MEDIA.

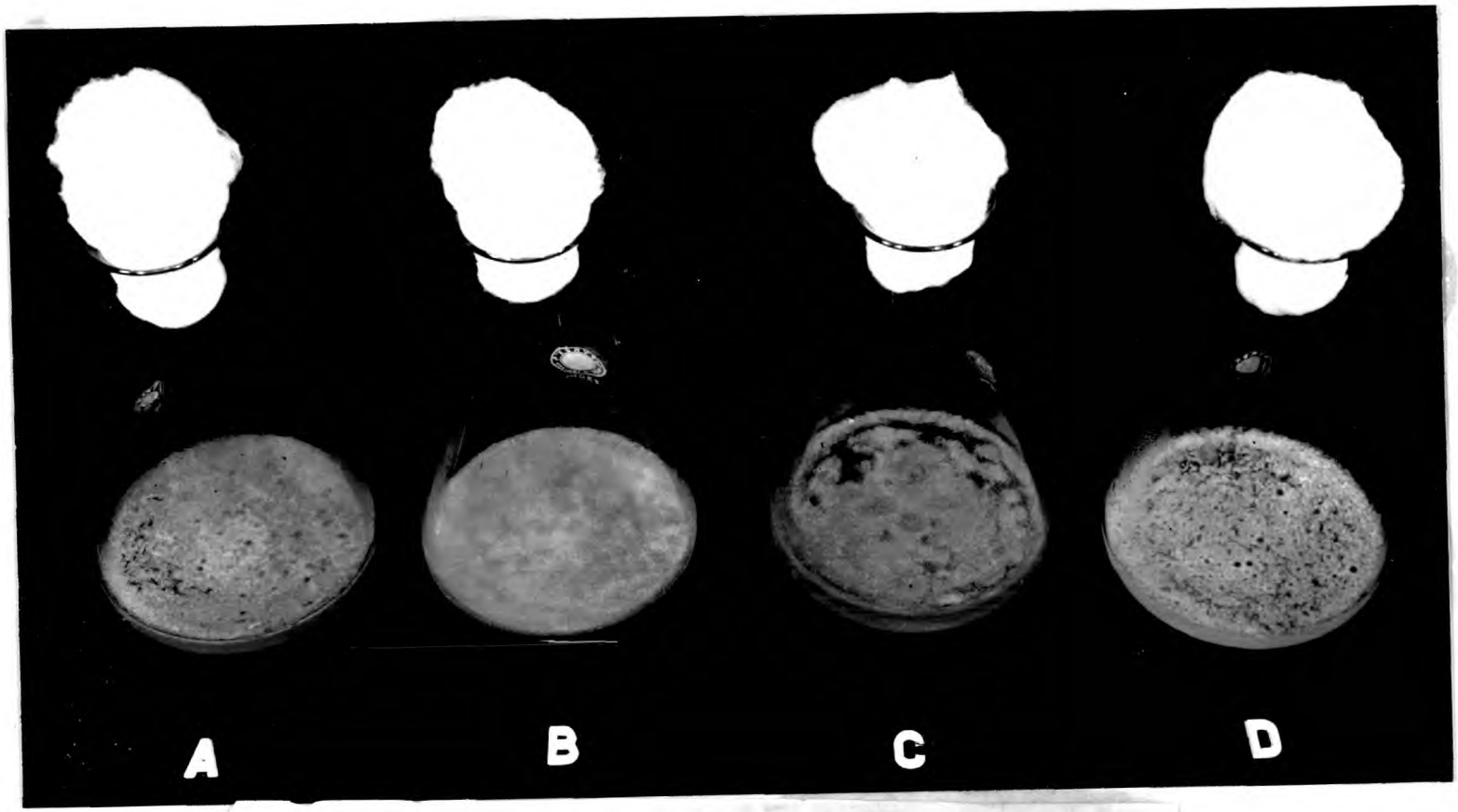

Plate 43. Growth of P.digitatum in liquid media at 25°C.

A. Complete rind extract.
B. Flavedo extract.
C. Albedo extract.
D. Orange juice.

were used:

(a) 50 ml. basal medium + 1 ml. water.

(b) 50 ml. basal medium containing 0.1% Yeastrel + 1 ml. water.

(c) 50 ml. basal medium + 1 ml. vitamin solution containing 20 µg. aneurine hydrochloride, 40 µg. pyridoxine hydrochloride, 30 µg. calcium panthothenate and 0.2 µg. biotin.

The techniques and media used are described in Materials and Mathods.

Cultures were incubated in the dark at 25°C. in stationary culture. Four replicates per treatment were removed after 3,7,11,16, 19 and 22 days and the mean dry weight of mycelium for each treatment and period of incubation are shown in Fig.5 .

Both rate of growth and the amount of mycelial growth was greatest in that medium containing 0.1% Yeastrel (Fig. 5). Addition of a mixture of vitamins to the basal medium allowed an earlier initiation of growth but the maximum mycelium produced was practically the same as that in the basal medium alone. Growth and sporulation in the 3 media after 12 days incubation is shown in Plate 44.

Sporulation was fairly heavy in basal medium + 0.1% Yeastrel and light in the other media.

These results agree with those of Pratt (1944) and Fergus (1952) but directly contradict those of Wooster and Cheldelin (1945), who found that thiamin or the thiazole moiety of thiamin was required for growth.

It is thought that either a vitamin (or vitamins) other than

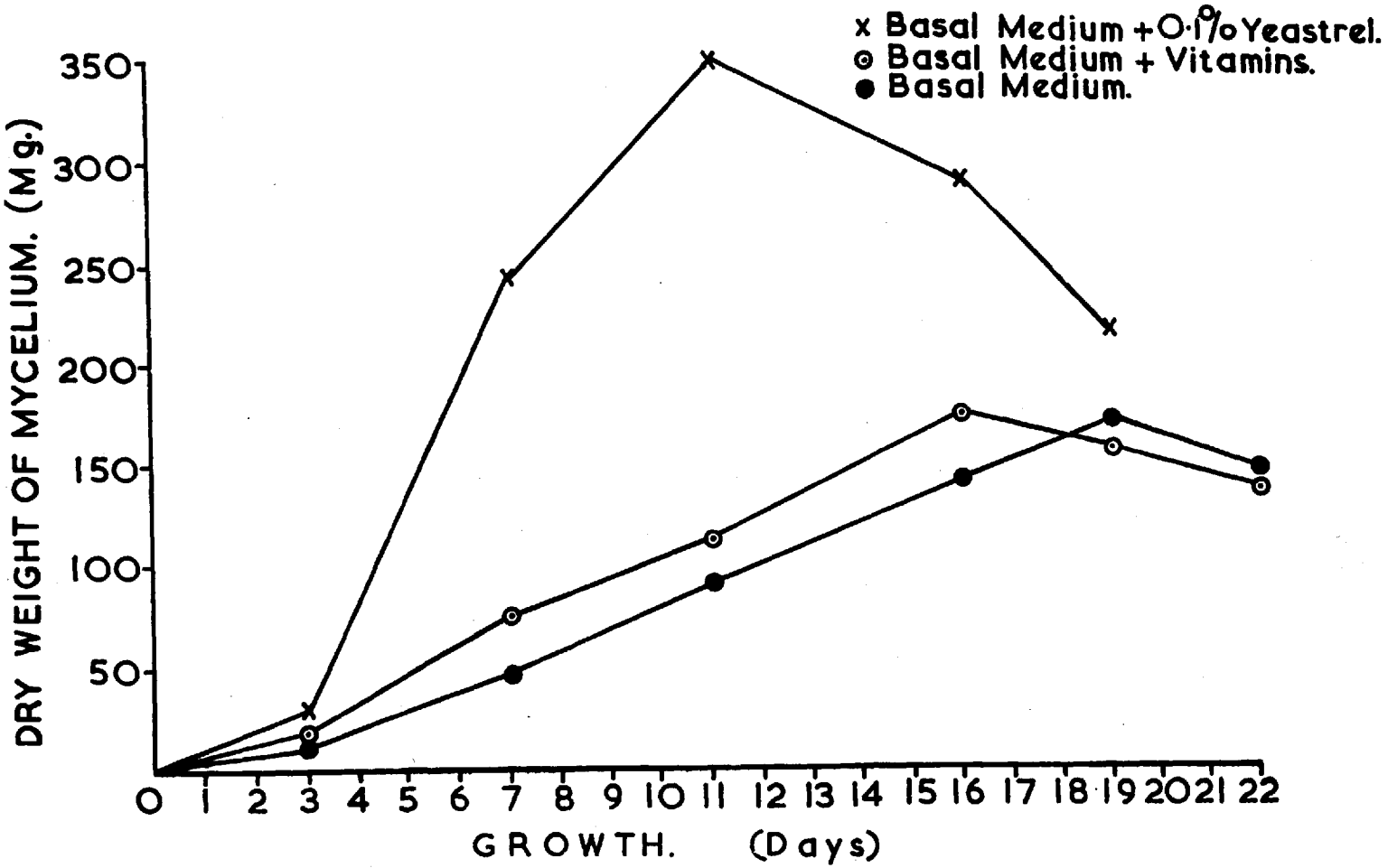

Fig. 5. EFFECT OF VITAMINS AND YEASTREL ON GROWTH AT 25°C.

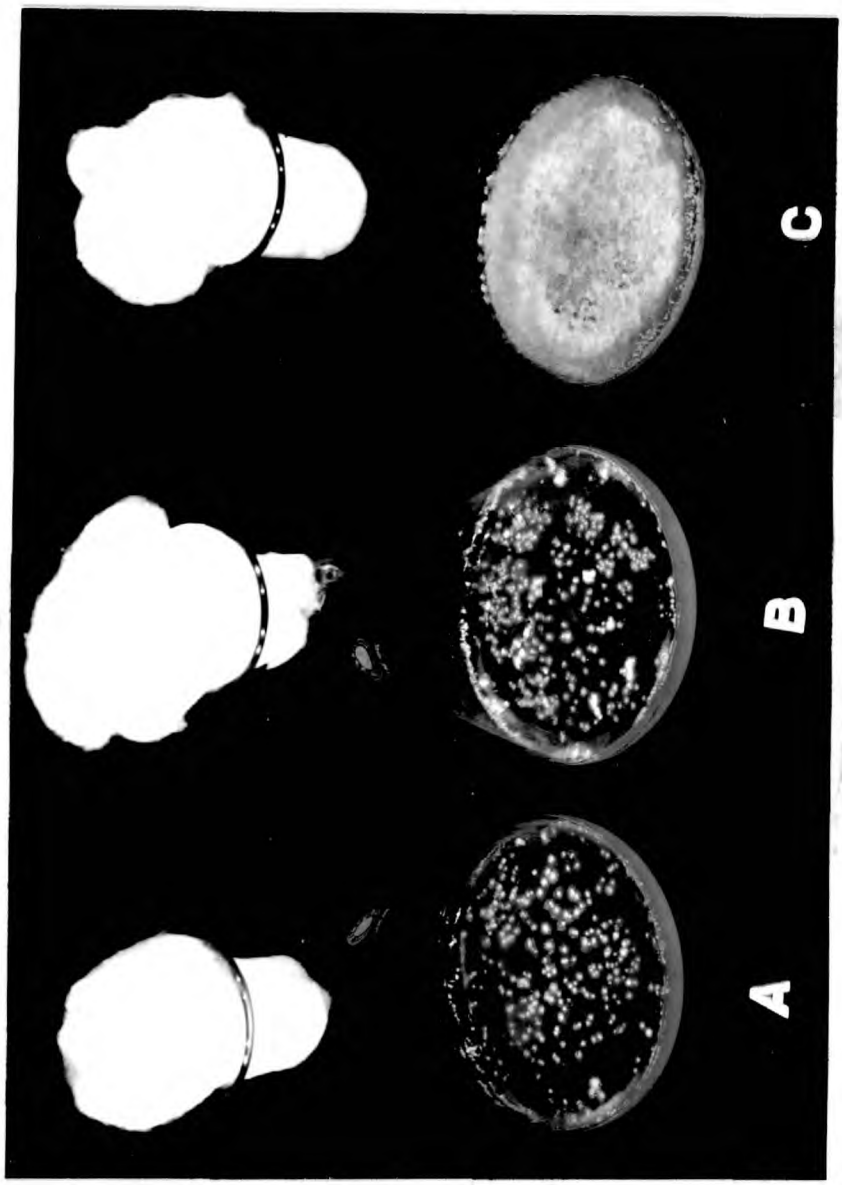

Plate 44. Growth of P.digitatum after 12 days at 25°C.

A. Basal synthetic medium.
B. Basal synthetic medium containing a mixture of vitamins;
C. Basal synthetic medium containing 0.1% Yeastrel.

the four used in the vitamin mixture above, or some other stimulating substance (or substances) such as purines, pyrimidines or amino acids is present in the yeast extract and resulted in the increased growth.

K. SPORE GERMINATION

The nutruitional requirements for spore germination were studied to determine the effect of some known rind components on germination. This was done with the aim of acquiring information on which water soluble substance or substances, diffusing from wounds, stimulate germination of spores on the fruit surface.

1. Germination of spores in synthetic media

(a) Effect of spore concentration in 1% glucose soln. on germination of spores

Spore suspensions of varying concentrations in 1% glucose were incubated for 24, 53 and 72 hours. Percentage germination in each treatment after the 3 periods of incubation is shown in Table 42.

Table 42. Effect of spore concentration in 1% glucose soln. on spore germination after 24, 53 and 72 hours incubation

Spores/ml.	% Germination		
	Incubation time (hr.)		
	24	53	72
1,090,000	0	0	0
820,000	0.55	0	0.35
610,000	0	0.63	1.55
460,000	1.84	2.8	2.06
340,000	3.88	-	-
170,000	10,32	-	-
90,000	13.65	-	-

Germination increased with decrease in spore concentration but only to a limited extent. Few spores germinated at concentrations greater than 610,000 spores/ml. and germination was low even at a concentration of 90,000 spores/ml.

Extending the period of incubation from 24 to 53 and 72 hours did not increase germination significantly.

(b) Effect of glucose, fructose and sucrose concentration on stimulation of spore germination at a high spore concentration

Spores were suspended in 1-6% concentrations of glucose, fructose and sucrose solutions at a concentration of 1,350,000 spores/ml. Percentage germination and average germ tube length after 24 hours is shown in Fig. 6 and Table 43 respectively.

Table 43 Effect of glucose, fructose and sucrose concentration on germ tube growth at a concentration of 1,350,000 spores/ml.

Medium	% Concn. of medium			
	1	2	4	6
	Average Germ tube length (μ)			
Glucose	28.5	40.0	33.8	59.3
Fructose	26.0	39.3	44.9	46.9
Sucrose	12.7	50.3	37.6	41.0
Water	16.0	-	-	-

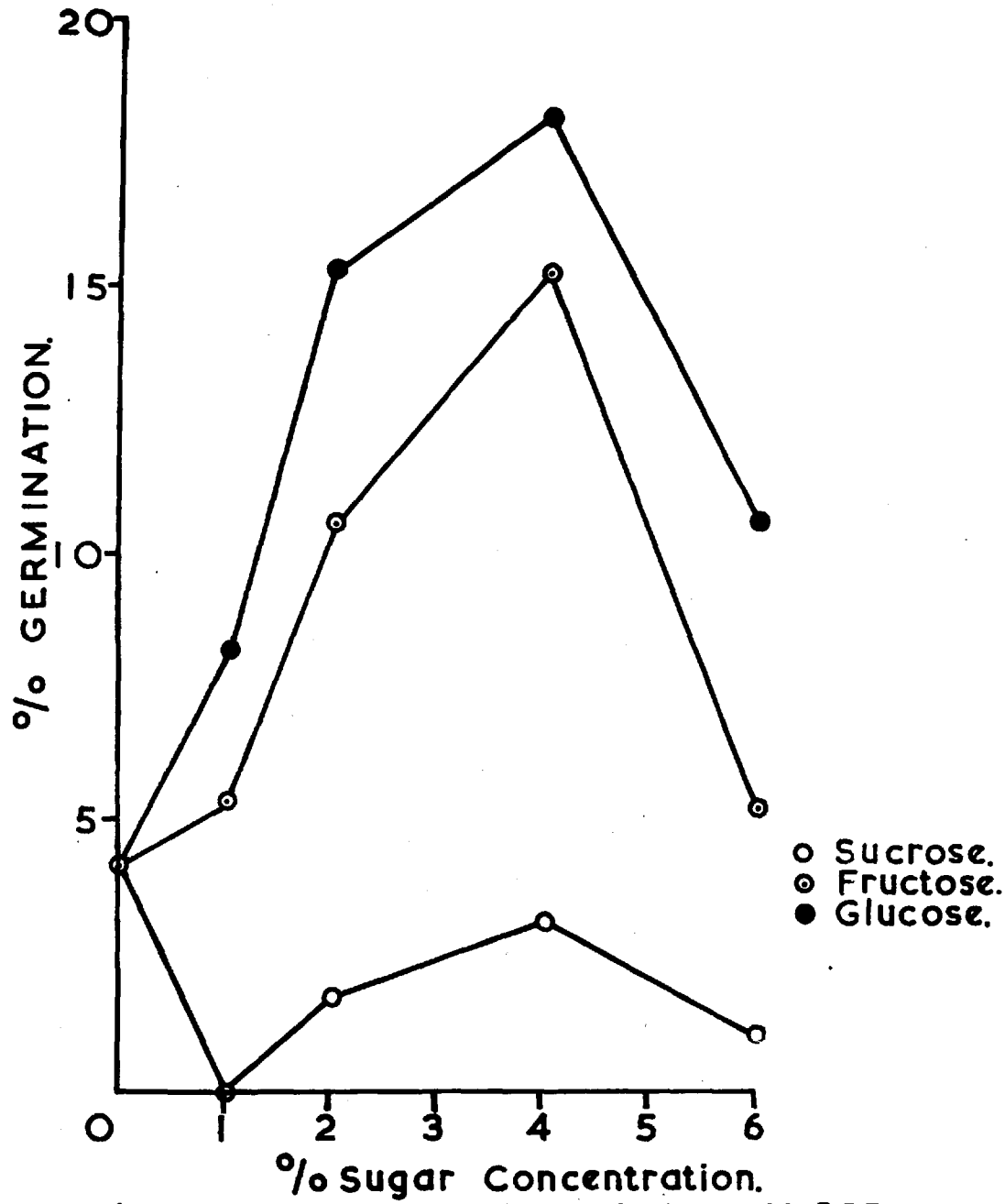

Fig. 6. EFFECT OF GLUCOSE AND SUCROSE CONCENTRATION ON GERMINATION OF SPORES AT A CONCENTRATION OF 1,350,000 SPORES/ML.

The optimum concentration of each sugar solution for percentage germination was 4%. Germination was best in glucose solutions at all concentrations tested. Germination in sucrose solutions was less than that obtained in the water controls.

Germ tube growth increased with the concentration of the sugar solutions.

It would appear that an increase in sugar concentration at high spore concentration does increase spore germination but only to a limited extent.

(c) Effect of spore concentration on germination of spores in 1% buffered glucose and water

Spore suspensions of different concentrations were prepared in 1% glucose containing 0.01M phosphate buffer (Sorensen's) at pH 5.2, and distilled water. The results expressed as percentage spore germination and average germ tube length after 24 hours are shown in Table 44.

Table 44. Effect of spore concentration on germination in 1% buffered glucose and distilled water

Spores/ml.	% Germination		Average germ tube length (μ)	
	Medium			
	1% Buffered glucose	Water	1% Buffered glucose	Water
1,330,000	2.68	0.11	79.7	17.5
660,000	12.4	0.06	104.2	17.5
330,000	82.53	0.82	120.0	40.0
170,000	81.90	0.76	136.0	64.7
80,000	89.22	0.75	169.3	84.0
40,000	90.57	6.67	91.5	95.4
20,000	86.52	2.35	155.3	44.3
10,000	53.75	6.10	164.3	126.0
5,000	95.35	8.89	192.3	61.6
3,000	85.11	11.1	139.3	80.5
1,000	93.48	-	243.9	-

Germination increased with decrease in spore concentration in both buffered glucose soln. and distilled water. The addition of Sorensen's phosphate buffer to 1% glucose soln. resulted in a marked stimulation of germination at concentrations less than 330,000 spores/ml. (see P.162). Germination in water though

comparatively low at all spore concentrations increased significantly at concentrations below 40,000 spores/ml.

In both buffered glucose and distilled water a decrease in spore concentration resulted in an increase in germ tube length. There were however, certain inconsistencies in this respect.

(d) Effect of buffers in 1% glucose on germination of spores

Spores were suspended in distilled water, 1% glucose and 1% glucose containing each of the following buffers at pH 5.2, 0.01M phosphate (Sorensen's), 0.015 M citrate, and 0.03M acetate. Concentration of spores was 420,000/ml. Percentage germination and average germ tube length after 24 hours are shown in Table 45.

Table 45, Effect of buffers in 1% glucose on spore germination

Medium	% Germination	Average germ tube length (μ)
Distilled water	0.6	50.0
1% Glucose	2.0	68.0
1% Glucose + phosphate buffer	45.0	96.4
1% Glucose + citrate buffer	62.9	233.5
1% Glucose + acetate buffer	1.8	125.0

The buffers differed in their ability to stimulate spore germination. This suggested that stimulation of germination in

1% glucose soln. buffered with phosphate in the previous experiment was not caused by a pH effect alone.

Although glucose + acetate buffer did not increase percentage spore germination it did promote germ tube growth as did the other buffers + glucose.

In another experiment the same buffers, in the absence of glucose, and at the same spore concentration as above, did not stimulate the germination of spores after 24 hours.

The stimulation of germination in glucose + buffer therefore appears to be due to both components in the mixture.

(e) Effect of different concentrations of glucose and buffer on spore germination at high spore concentration

In an earlier experiment it was seen that increase in glucose, fructose and sucrose concentration resulted in stimulation of spore germination at high spore concentrations. It was of interest to see whether a similar effect was observed when spores were suspended in phosphate buffered glucose of different concentrations.

Spores were suspended in water and 1-8% glucose solutions containing 0.01M phosphate buffer (Sorensen's) at pH 5.2. A concentration of 3,200,000 spores/ml. was used. Germination after 24 hours is shown in Table 46 .

Table 46. Effect of glucose concentration on spore germination

Medium	% Germination
Water + buffer	0.1
1% Glucose + buffer	3.25
2% " + "	0.95
4% " + "	1.25
6% " + "	3.64
8% " + "	3.49

No increase in germination was observed with increase in glucose concentration.

(f) Effect of buffered solutions of carbohydrates on spore germination

The stimulation of spore germination in a 1% glucose-phosphate buffer mixture led to an investigation on the effect of adding the same phosphate buffer at pH 5.2 to solutions of other carbohydrates.

Spores were suspended in buffered and unbuffered 1% solutions of different carbohydrates and buffer solution at a concentration of 220,000 spores/ml. All buffered solutions contained 0.01M phosphate buffer (Sorensen's) at pH 5.2. Percentage germination and average germ tube length after 24 hours are shown in Table 47.

Table 47. Effect of buffered solutions of carbohydrates on spore germination

Carbohydrate	% Germination		Average germ tube length (μ)	
	Buffered	Unbuffered	Buffered	Unbuffered
Xylose	67.5	5.2	29.5 (30)	103.3 (30)
Arabinose	18.8	2.2	64.0 (30)	224.0 (18)
Glucose	85.3	3.8	109.0 (30)	157.5 (27)
Fructose	70.0	1.6	93.4 (30)	22.1 (21)
Galactose	27.0	2.1	92.9 (30)	153.1 (21)
Sucrose	69.0	6.4	175.8 (30)	75.8 (30)
Carboxymethyl-cellulose	2.2	0.4	299.0 (12)	113.7 (6)
Pectin	0.3	0.6	161.9 (4)	62.1 (4)
Water	3.1	0.2	149.8 (19)	175.0 (5)

( ) Number of germ tubes measured.

Germination was stimulated by all carbohydrates except pectin in the presence of phosphate buffer. Germination was low in buffered pectin, carboxymethylcellulose, and all unbuffered solutions.

No consistent promotion of germ tube growth was observed in buffered solutions of carbohydrates and solutions of xylose, arabinose and galactose reduced germ tube growth.

(g) Effect of nitrogen compounds and vitamins in glucose on spore germination

Spores were suspended in distilled water; 1% glucose-nitrogen compound mixtures containing 0.00125M N; 1% glucose solution containing each of the following separately 0.025% Yeastrel, 0.02 p.p.m. biotin, and 0.2p.p.m. thiamin. Concentration of spores was 180,000/µl. Percentage germination and average germ tube length after 24 hours incubation are shown in Table 48 .

Table 48. Effect of nitrogen compounds, vitamins and Yeastrel in 1% glucose on spore germination

Medium	% Germination	Average germ tube length (µ)
1% Glucose	15.0	158.2
" + $NaNO_3$	12.7	146.6
" + NH_4NO_3	33.2	87.5
" + NH_4Cl	38.3	134.1
" + casamino acids (vitamin free)	78.4	274.4
" + 0.025% Yeastrel	82.1	287.5
" + thiamin	37.0	129.6
" + biotin	16.5	93.6
Water	0.4	-

Thiamin, Yeastrel and all nitrogen compounds except sodium

nitrate in the presence of glucose increased percentage spore germination.

Percentage germination and germ tube growth was best in Yeastrel and casamino acid - glucose mixtures.

Extension of the period of incubation to 40 hours in another experiment, using the same media and spore suspension yielded similar results.

(h) Effect of biotin, thiamin and Yeastrel on spore germination

Spores, 370,000/ml., were suspended in the following media:

(i) Biotin solution, conc. 0.02.p.p.m.

(ii) Thiamin (aneurine hydrochloride) solution, conc. 0.2 p.p.m.

(iii) Biotin + thiamin, conc. 0.02 and 0.2 p.p.m. respectively.

(iv) 0.025% Yeastrel.

(v) Water.

Results after 24 hours incubation are shown in Table 49 .

Table 49. Effect of biotin, thiamin and Yeastrel on spore germination

Medium	% Germination
Biotin	0.4
thiamin	3.0
Biotin + thiamin	0.3
0.25% Yeastrel+	2.3
Water	0.4

+ Yeastrel contains 11.2 p.p.m. riboflavin and 395.2 p.p.m. niacin.

Biotin, thiamin or Yeastrel did not stimulate spore germination significantly.

(i) Effect of nitrogen compounds and salts solution on spore germination

In this experiment spores were suspended in buffered and unBuffered solutions of nitrogen compounds containing 0.0025 M N, buffer soln., and distilled water and a salts solution. The pH of all solutions except distilled water was 5.2, and each buffered soln. contained 0.01M phosphate buffer (Sorensen's) at the same pH. Germination at a concentration of 300,000 spores/ml. after 24 hours is shown in Table 50.

Table 50. Effect of nitrogen compounds and salts solution on spore germination

Medium	% Germination	
	Buffered	Unbuffered
$NaNO_3$	1.0	0
NH_4NO_3	0	1.2
NH_4Cl	0.8	0.3
Casamino acids	1.8	0
Distilled water	0.4	0.3
Salts soln.+	0.3	0.4

+ Mineral soln. (Fergus, 1954) contained 3g. NH_4NO_3; 2.22g. $MgSO_4.7H_2O$; 2.59g. KH_2PO_4; 2.21g. K_2HPO_4; 1 l. distilled water. pH was adjusted to 5.2 with N HCl.

Spores were not stimulated to germinate by nitrogen or a mixture of nitrogen and salts.

(j) Effect of nitrogen compounds in pectin and carboxymethylcellulose on spore germination

In this experiment spores were suspended in buffer, water, and in buffered 0.5% solutions of pectin and carboxymethylcellulose containing 0.0025M N supplied by different nitrogen compounds. Each soln. was buffered with 0.005M phosphate buffer (Sorensen's) at pH 5.2, and contained 230,000 spores/ml. Percentage germination and average germ tube length after 24 hours incubation are shown in Table 51.

Table 51. Effect of nitrogen compounds in buffered pectin and carboxymethylcellulose (CMC) on spore germination

Medium	% Germination	Average germ tube length (μ)
0.5% Pectin	6.8	75.5
" + $NaNO_3$	15.9	135.4
" + NH_4NO_3	14.9	155.4
" + NH_4Cl	8.5	129.6
" + Casamino acids	18.1	151.7
0.5% CMC	2.2	75.4
" + $NaNO_3$	1.8	89.6
" + NH_4NO_3	2.9	131.5
" + NH_4Cl	2.3	97.3
" + casamino acids	3.7	133.5

Germination was low in solutions of pectin and carboxymethyl-cellulose but addition of nitrogen compounds to pectin resulted in increased germination.

Percentage germination and germ tube growth were best in a pectin-casamino acids solution.

2. Germination of spores in extracts of rind tissues

A study of the germination of spores in water extracts of rind tissues was made as the conditions for germination in such extracts were thought to closely resemble those existing for spores in water droplets in wounds on the fruit surface.

(a) Effect of spore concentration in complete rind extract on spore germination

Spore suspensions of varying concentration were prepared in a water extract of complete rind tissues. Percentage germination in each treatment after 24 hours is shown in Table 52.

Table 52. Effect of spore concentration in complete rind extract on spore germination.

Spores/ml.	% Germination
1,340,000	95.5
750,000	98.0
560,000	100.0
320,000	100.0
80,000	100.0

Good germination was recorded at high spore density and germination was not markedly decreased by increasing the spore concentration to 1,340,000 spores/ml.

(b) Effect of incubation time on germination of spores in rind extracts

In this experiment spores, suspended in water extracts of flavedo, albedo and complete rind tissues were incubated for various periods of time. Concentration of spores/ml. was 400,000. Percentage germination and average germ tube length for each period of incubation are shown in Figs. 7 and 8 respectively.

Germination as recognised by the production of germ tubes was first observed in the 3 extracts after 6 hours incubation and was almost completed after 10 hours incubation. Percentage germination (Fig. 7) and germ tube growth (Fig.8) was about the same in each of the three extracts.

The high germination in flavedo extract suggests that the inability of an inoculum in flavedo extract to promote infection through inter-vesicle flavedo wounds is obviously not caused by inhibition of germination in this extract.

(c) Effect of rind extract dilution on germination of spores

In this experiment spores were suspended in distilled water and flavedo, albedo and complete rind extracts at the following dilutions $^{N}/_{1}$, $^{N}/_{5}$, $^{N}/_{10}$, $^{N}/_{25}$, $^{N}/_{50}$ and $^{N}/_{100}$. The concentration of spores/ml. was 400,000. Percentage germination

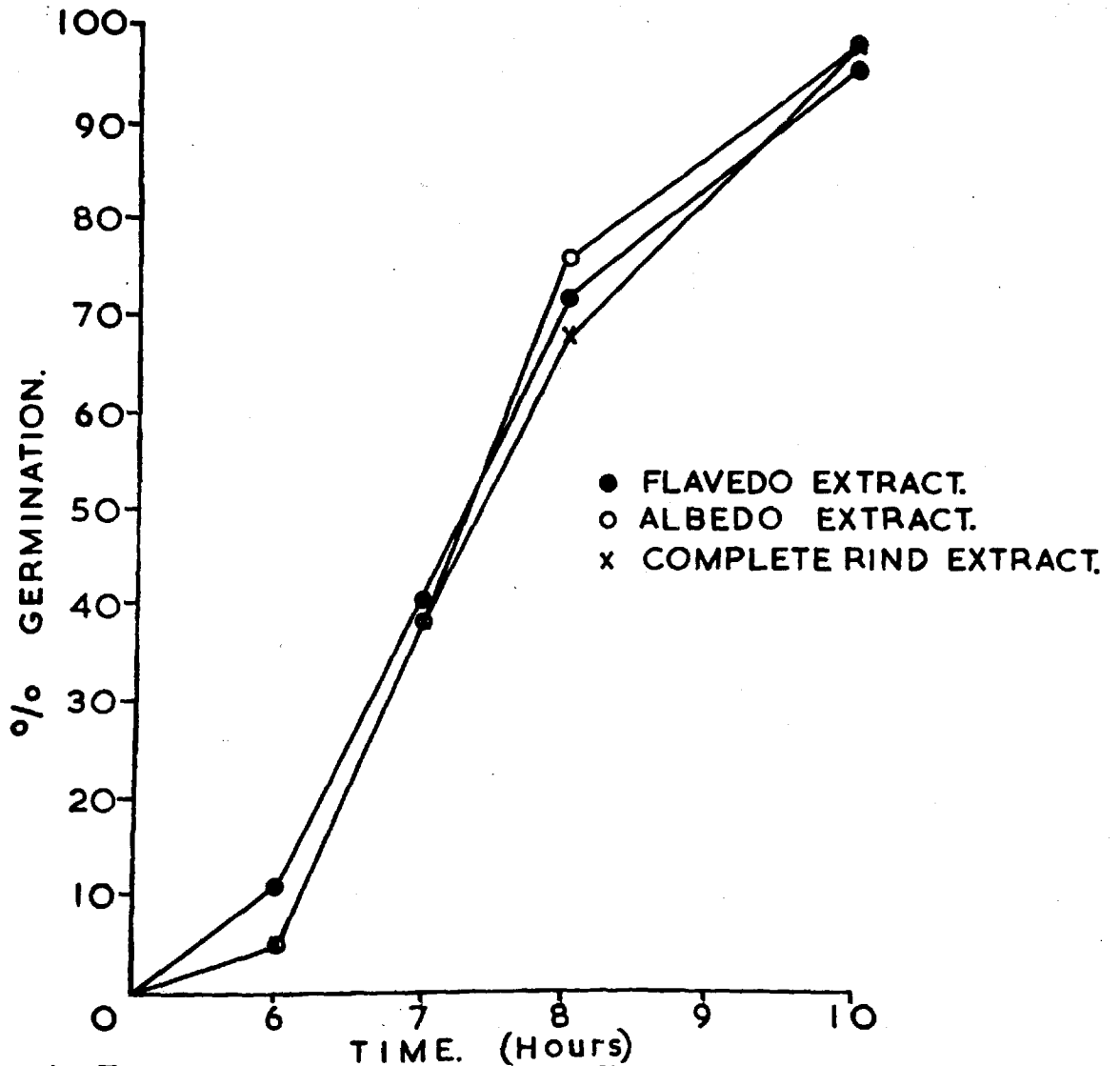

Fig. 7. EFFECT OF INCUBATION TIME ON PERCENTAGE SPORE GERMINATION IN RIND EXTRACTS.

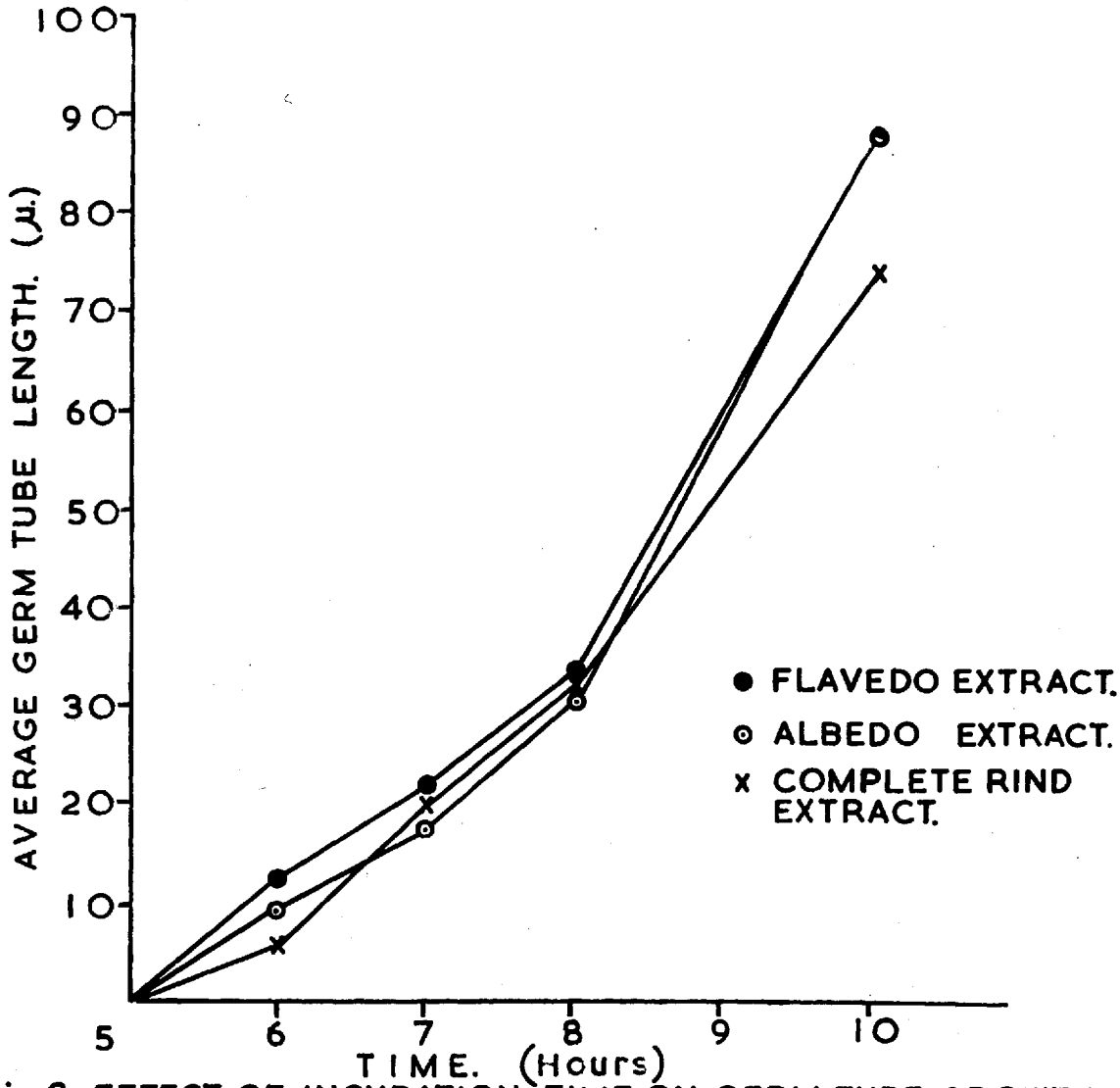

Fig.8. EFFECT OF INCUBATION TIME ON GERM TUBE GROWTH IN RIND EXTRACTS.

and average germ tube length after 12 and 18 hours incubation is shown in Table 53 .

Table 53. Effect of rind extract dilution on spore germination

Medium	Dilution	Incubation time (hours)				
			12	18	12	18
		mg.dry wt./100 ml. extract	% Germination		Average germ tube length (μ)	
Flavedo extract	N/1	740	93.9	100.0	224.3	Weft
"	N/5	148	88.3	90.1	185.5	341.7
"	N/10	74	56.6	65.3	102.9	185.9
"	N/25	29.6	36.2	33.4	50.0	167.2
"	N/50	14.8	23.4	28.6	34.2	111.9
"	N/100	7.4	11.6	25.8	25.2	75.8
Albedo extract	N/1	770	98.9	100.0	206.7	Weft
"	N/5	154	83.8	87.5	150.4	238.9
"	N/10	77	45.2	48.1	73.4	163.9
"	N/25	30.8	11.8	15.9	48.7	94.6
"	N/50	15.4	5.5	12.1	35.6	84.2
"	N/100	7.7	5.3	12.5	33.5	70.9
Complete rind extract	N/1	865	100.0	100.0	243.8	Weft
"	N/5	173	73.1	74.6	118.5	235.4
"	N/10	86.5	55.2	75.6	90.5	198.3
"	N/25	34.6	16.3	11.8	51.9	144.8
"	N/50	17.3	13.8	9.0	42.2	84.0
"	N/100	8.6	6.8	6.5	37.3	68.8
Water	-	-	0.2	0.3		

Spores germinated in all extracts diluted to N/100 indicating that very small amounts of stimulant in the extracts were necessary for germination.

3. Effect of emanations from P. digitatum on spore germination

Emanations from pure cultures of P. digitatum causing epinasty of potato plants were first reported by Miller et al. (1940). Growth of the fungus on potato dextrose agar produced emanations which induced epinasty in pea seedlings and also accelerated the respiration and ripening of lemon fruit (Biale and Shepherd, 1941).

Chemical proof that part at least of the fungus emanation is ethylene was provided by Young et al. (1951). Fergus (1954) overlooked this work but he and other workers studying this emanation assumed that the active emanation from the fungus was ethylene. This was based mainly on the fact that it is the only volatile chemical known to arise from biological processes and cause epinasty in plants.

The effect of emanations, from P.digitatum and other fungi causing wastage of Citrus fruit, on the germination of spores was studied in a number ofexperiments.

(a) Effect of emanations from P.digitatum on orange rind on germination of spores in water

Van Tieghem cells, each containing plugs of rind cut from Navel fruit, were inoculated with spores of P.digitatum. Controls composed of (i) cells + sterile distilled water and (ii) cells + rind

plugs + sterile distilled water, as described in Materials and Methods (P. 36) were used. There were 4 replicates/treatment and the concentration of spores in the hanging distilled water drop was 210,000/ml. Germination of spores after 24 hours incubation is given in Table 54 .

Table 54. Effect of emanations from P. digitatum on germination of spores in distilled water

Treatment	% Germination	Average germ tube length(μ)
Cell + rind + P.digitatum	20.5	29.6 (20)
Cell + rind + sterile distilled water	2.7	10.8 (20)
Cell + sterile distilled water	0	-

( ) Number of germ tubes measured.

Emanations produced as a result of growth of the fungus on orange rind stimulated germination of spores in water.

In another experiment carried out as above, the effect of emanations produced by the fungus on Valencia and Shamouti fruit on germination of spores in distilled water was investigated. Experimental conditions were identical with those of the preceding experiment except that the concentration of spores/ml. in the distilled water hanging drop was 170,000. Germination in each treatment after 24 hours is shown in Table 55 .

Table 55. Effect of emanations from P.digitatum on Valencia and Shamouti orange rind on germination of spores in distilled water

Treatment	Variety	% Germination	Average germ tube length (μ)
Cell + rind + P.digitatum	Shamouti	14.3	29.8 (87)
" + " + sterile water	"	1.0	15.9 (17)
Cell + rind + P.digitatum	Valencia	10.4	29.2 (58)
" + " + sterile water	"	0.5	10.4 (17)
Cell + sterile water	-	0	

( ) Number of germ tubes measured.

Emanations produced by the fungus on both varieties stimulated germination of spores.

Germination was lower than that in the preceding experiment and may have been influenced by the density of spores in the hanging drop. The effect of spore concentration in the hanging drop, in the presence of emanations on germination, is described later (P.183).

(b) Effect of emanations from P.digitatum on V8 juice agar on germination of spores in water

In this experiment Van Tieghem cells each containing a plug of V8 juice agar of similar volume to those used in preceding experiments were inoculated with P.digitatum. The concentrations of spores/ml. in hanging drops of distilled water were 170,000

and 220,000 in different treatments. Cells containing sterile water were used as controls for each spore concentration. There were 4 replicates/treatment. Germination after 24 hours incubation is shown in Table 56.

Table 56. Effect of emanations from P.digitatum on V8 juice agar on germination of spores in distilled water

Treatment	Spores/ml.	% Germination	Average germ tube length (μ)
Cell + V8 juice agar + P.digitatum	170,000	3.4	54.2 (37)
Cell + sterile water	170,000	0.1	-
Cell + V8 juice agar + P.digitatum	220,000	1.0	39.6 (5)
Cell + sterile water	220,000	0	-

( ) Number of germ tubes measured

Stimulation of germination by emanations produced by the fungus on V8 juice agar was not very marked. This may have been caused by a low yield of emanations by the fungus on this medium. This point was not investigated further.

(c) Effect of spore concentration in hanging drops in the presence of emanations from P.digitatum on spore germination

In this experiment Van Tieghem cells, each containing a plug of rind from Shamouti fruit, were inoculated with spores of P.digitatum. The concentration of spores in the hanging distilled

water drop in each treatment varied as below (Table 57). Controls composed of (i) cells + sterile distilled water and (ii) cells + rind plug + sterile distilled water were used only in that treatment in which spore concentration in the hanging drop was 170,000/ml. Germination after 24 hours is shown in Table 57 .

Table 57. Effect of spore concentration in hanging drops in the presence of emanations from P.digitatum on spore germination

Treatment	Spores/ml.in hanging drop	% Germination	Average germ tube length (μ)
Cell + rind + P.digitatum	1,220,000	72.7	20.7 (90)
Cell + rind + P.digitatum	690,000	36.0	22.4 (120)
Cell + rind + P.digitatum	170,000	14.3	29.8 (58)
Cell + rind + sterile water	170,000	1.0	15.9 (17)
Cell + rind + sterile water	170,000	0	-

( ) Number of germ tubes measured.

Germination of spores increased with increase in spore numbers in the hanging drop.

This result contrasts with that obtained with spores suspended in glucose and glucose-phosphate media where a decrease in percentage germination with increase in spore concentration was observed.

(d) Effect of emanations from various fungi on germination of spores in water

It was of interest to study the effect of emanations, from other fungi causing wastage of Citrus fruit, on germination of P.digitatum spores in distilled water.

In this experiment Van Tieghem cells, each containing a plug of rind from Navel fruit, were inoculated with the following fungi (i) P.digitatum (ii) P.italicum (iii) Trichoderma lignorum and (iv) Geotrichum candidum. Each of 3 replicates/treatment were inoculated with 0.2 ml. aqueous spore suspension containing 2×10^6 spores/ml. (approx.). Cells containing (a) sterile distilled water and (b) rind + sterile distilled water were used as controls. Concentration of spores/ml. in hanging distilled water drop was 220,000. Germination after 24 hours incubation is shown in Table 58.

Table 58.Effect of emanations from various fungi on germination of spores in distilled water.

Treatment	% Germination	Average germ tube length(μ).
Cell + rind + P.digitatum	17.2	66.0 (56)
" + " + P.italicum	1.0	37.3 (9)
" + " + T.lignorum	0.3	56.0 (2)
" + " + G.candidum	1.5	28.6 (11)
" + " + Sterile distilled water	0.5	35.0 (4)
" + sterile water	0	-

( ) Numbers of germ tubes measured.

Only emanations produced by P.digitatum on orange rind resulted in an increase in percentage germination of spores.

4. Germination of spores on fruit surface

(a) Germination of spores in the presence of minute wounds

Germination of spores in the presence of minute wounds on Valencia fruit was studied using the distilled water agar disk technique which is described in Materials and Methods (P. 38).

Two experiments were done and in each experiment 8 fruit from 2 different consignments of unwaxed Valencia fruit were used. Minute injuries, caused in the field or in packhouse treatment of fruit, were covered by an agar disk. The number of wounds covered by each disk was counted after the area covered by a disk was marked with a wax pencil prior to removal of the disks for germination counts. The minute injuried within each site were detected by staining with a soln. of 0.1% Light Green in 70% alcohol as described on P. 54 .

In the first experiment with fruit of consignment A, germination of spores after 24 hours incubation is shown in Table 59.

Table 59. Germination of spores in the presence of minute wounds on fruit from consignment A

Fruit	Number of wounds/site	% Germination	Average germ tube length (μ)
1	14	8.4	23.9 (18)
2	10	1.3	7.0 (3)
3	5	0.4	7.0 (1)
4	140	52.5	17.6 (30)
5	13	0	-
6	10	16.2	19.7 (30)
7	10	1.2	35.0 (4)
8	7	0.3	3.5 (1)
Plate control	-	0	-

Germination on the different fruit varied. This was caused partly by variation in the number and size of wounds at each site. Numerous small wounds causing high percentage germination on fruit 4 and few wounds causing low percentage germination on fruit 2 are shown in Plate 45 and 46 respectively.

The presence of wounds did not stimulate germination always as can be seen in fruit 5. Inhibition of germination on this fruit may have been caused by residual fungicide on the fruit surface after washing. Fruit of consignment A had been rinsed in 0.5% soln. of sodium ortho-phenyl-phenate (SOPP) as part of normal packhouse

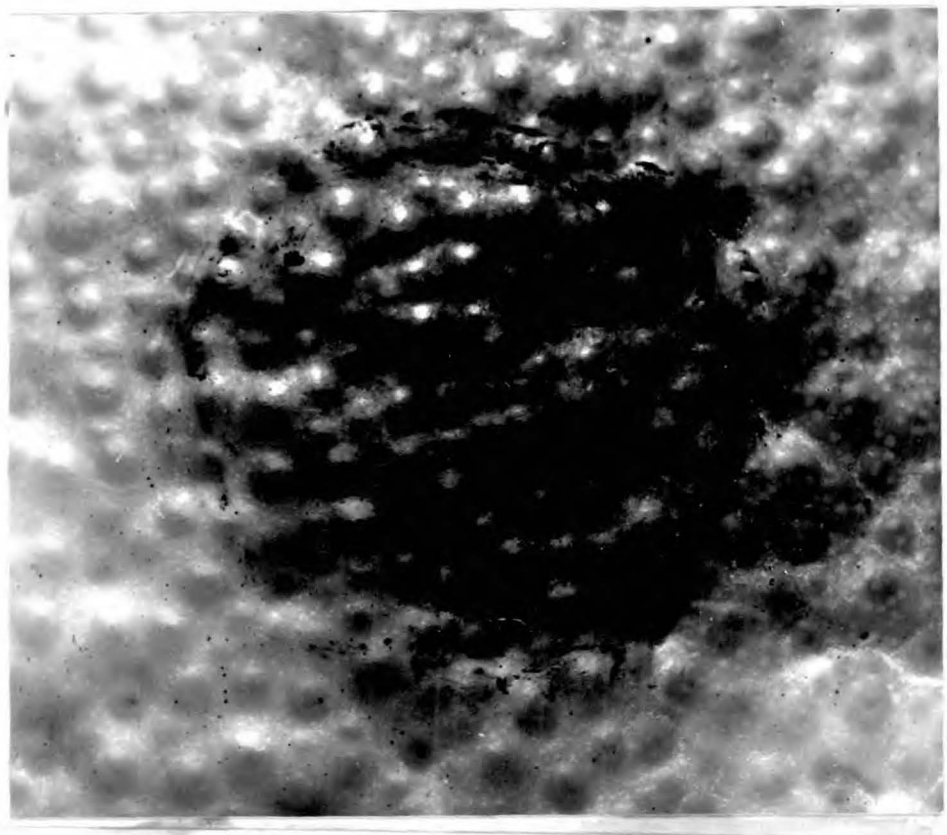

Plate 45. Wounds at inoculum site on fruit 4. X6.

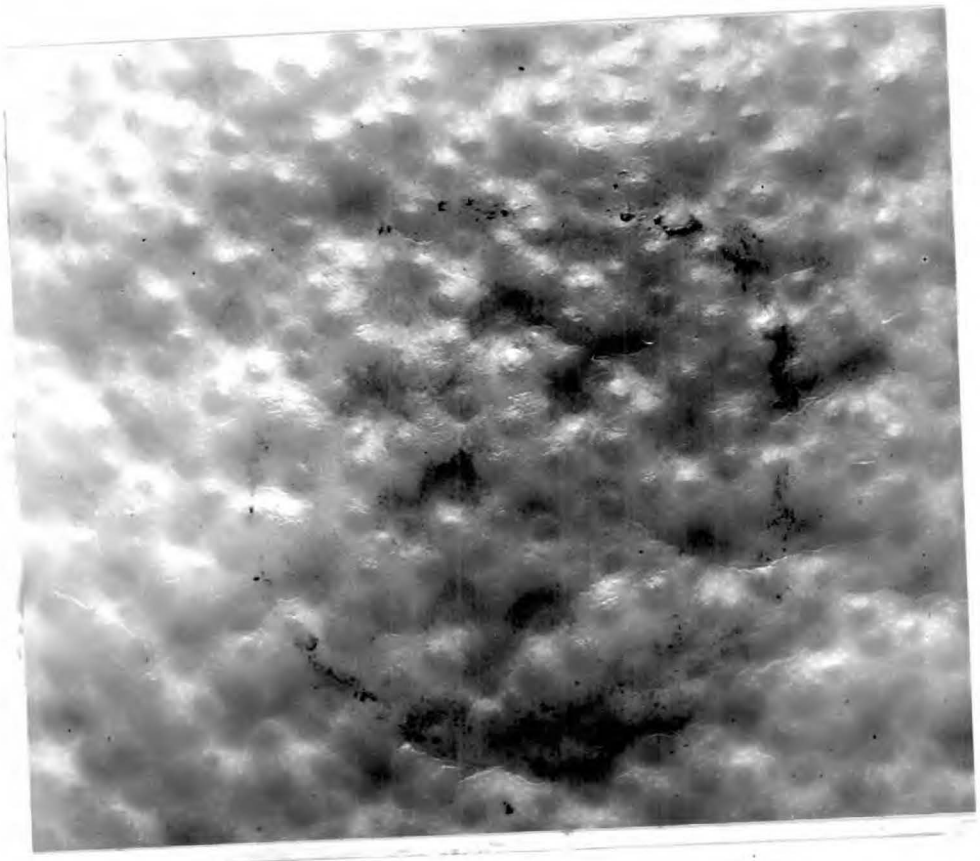

Plate 46. Wounds at inoculum site on fruit 2. X6.

procedure.

In the second experiment with fruit of consignment B, germination of spores after 24 hours incubation is shown in Table 60 .

Table 60. Germination of spores in the presence of minute wounds on fruit from consignment B

Fruit	Number of wounds/site	% Germination	Average germ tube length (μ)
1	34	99.4	98.9
2	12	80.8	74.6
3	24	97.3	184.5
4	17	98.2	252.5
5	58	97.6	122.6
6	70	99.7	74.9
7	52	98.1	106.3
8	82	97.8	123.8
Plate control	-	0.1	3.5

Germination was high on all fruit. Wounds on fruit from this consignment were in general larger and more numerous/site than found in fruit of consignment A. An example of such wounds as found on fruit 6 are shown in Plate 47 .

These two experiments illustrate the extent to which germination can vary on fruit of the same variety taken from different consignments.

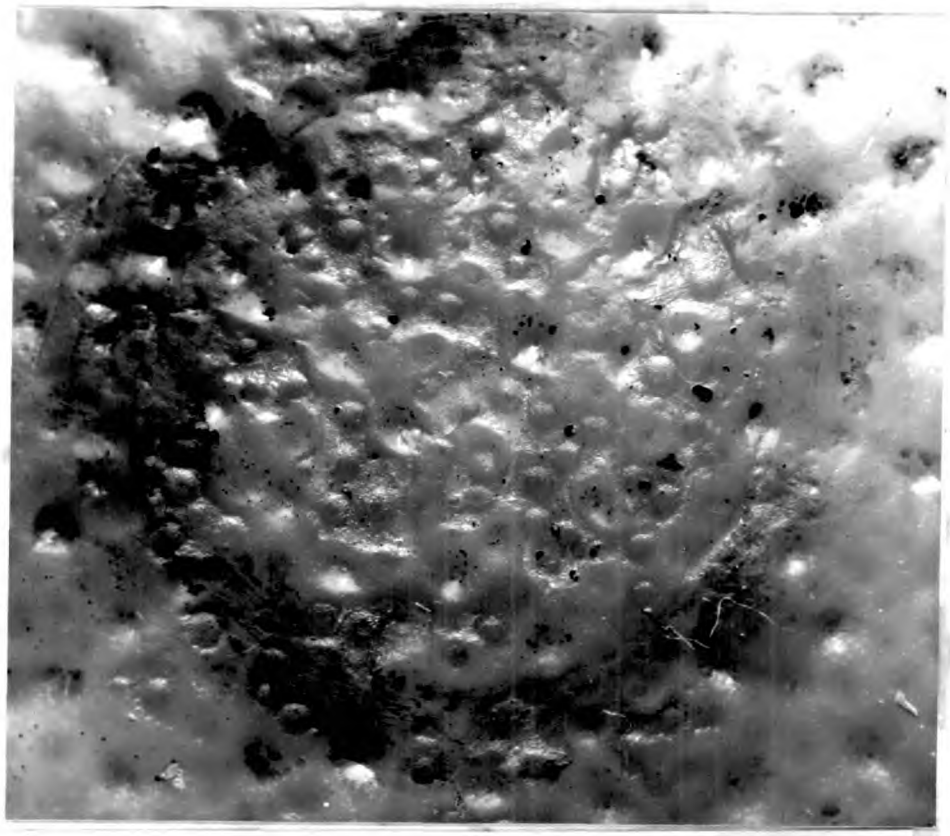

Plate 47. Wounds at inoculum site on fruit 6. X6.

Although small injuries, revealed by the presence of the dye, stimulated germination of spores this did not mean that these injuries were favourable infection courts for spores of the fungus. The number of injuries however do indicate the care in handling and the subsequent decay that may be expected.

(b) Effect of artificial wounds between and into oil vesicles on spore germination

Germination of spores was studied using the same technique as described in the previous experiment. Five Valencia fruit/treatment were used. Fruit wounded 3 times between the oil vesicles, and 3 times into the oil vesicles to depths of 1.0 and 3.0 mm. were used in each treatment. Controls consisted of 5 fruit that had not been wounded artificially, 1 plate control and 2 box controls as described on P. 38 .

Germination and average germ tube length after 8 hours incubation is shown in Tables 61 and 62 respectively.

Table 61. Effect of artificial wounds between, and into oil vesicles of Valencia fruit on spore germination.

Depth of prick (mm).	Between oil vesicles		Into oil vesicles	
	% Germination	Standard deviation	% Germination	Standard deviation
3.0	82.59	± 5.67	91.69	± 1.53
1.0	77.59	± 9.09	87.63	± 1.88
0	6.33	± 6.33	-	-
Box control	3.70	± 2.14	-	-
Plate control	0	-	-	-

Table 62. Effect of artificial wounds between, and into oil vesicles of Valencia fruit on germ tube growth

Depth of prick (mm)	Between oil vesicles		Into oil vesicles	
	Average germ tube length (μ)	Standard deviation	Average germ tube length (μ)	Standard deviation
3.0	17.29	± 3.25	24.22	± 0.88
1.0	18.34	± 3.88	24.70	± 1.21
0	8.33	± 1.47	-	-
Box control	7.70	± 4.21	-	-
Plate control	0	-	-	-

Table 61 shows that wounds into and between oil vesicles increase percentage spore germination and that germination in the presence of wounds into the oil vesicles was not significantly greater than in the presence of wounds between the oil vesicles.

Table 62 shows that wounds of both types increased the growth of germ tubes and that wounds into the oil vesicles promoted slightly better growth than wounds of comparable depth between the oil vesicles.

Another experiment investigating the stimulation of spore germination by wounds into and between oil vesicles of Navel fruit was carried out simultaneously and under the same experimental conditions as above.

Percentage germination and average germ tube length after 8 hours incubation are shown in Tables 63 and 64 respectively.

Table 63. Effect of artificial wounds between and into oil vesicles of Navel fruit on spore germination

Depth of prick (mm)	Between oil vesicles		Into oil vesicles	
	% Germination	Standard deviation	% Germination	Standard deviation
3.0	86.83	±7.26	77.57	±7.86
1.0	87.28	±4.09	85.51	±3.70
0	22.11	±17.24	-	-
Box control	4.93	±4.02	-	-
Plate control	0	-	-	-

Table 64. Effect of artificial wounds between and into oil vesicles of Navel fruit on germ tube growth

Depth of prick (mm)	Between oil vesicles		Into oil vesicles	
	Average germ tube length (μ)	Standard deviation	Average germ tube length (μ)	Standard deviation
3.0	23.66	±4.8	15.09	±4.88
1.0	20.23	±2.77	19.6	±5.87
0	7.28	±1.65	-	-
Box control	9.03	±2.67	-	-
Plate control	0	-	-	-

Tables 63 and 64 show that percentage germination and germ tube growth are increased in the presence of wounds of both types but that neither type of wound is significantly more stimulative than the other.

The results of these two experiments clearly indicate the response of spores to wounding in both Navels and Valencias is very similar.

The resistance of wounds, 1.0 mm. deep between the oil vesicles, to infection is therefore not caused by an initial inhibition of growth of hyphae in these wounds but results from some form of resistance becoming effective later.

L. ACTION OF ORANGE ESSENTIAL OIL

It was seen in an earlier experiment (P. 133) that fruit, pricked between the oil vesicles into the flavedo and inoculated with spores suspended in orange essential oil, were readily infected. This was not because growth of the germ-tubes was promoted by the oil, but was a result of the phytoxic effect of the oil on the exposed flavedo cells. This made them susceptible to infection. The following experiment demonstrated this directly.

1. Growth of spores in orange essential oil

Drops (0.02ml.) of spores suspended in orange essential oil were placed on glass slides and incubated for 24 hours as described for spore germination experiments (P. 35).

No germ-tubes were produced within this period of incubation.

2. Effect of irradiated essential oil on growth of mycelium

Zukerman (1951;1956) found that limonene, the main constituent of orange essential oil, after irradiation by exposure to sunlight inhibited growth of a number of fungi that cause wastage of Citrus fruit. P.digitatum was included amongst these. Zukerman suggested that the inhibition results from the formation of peroxides, especially the one formed by the oxidation of limonene.

In this experiment 5ml. orange essential oil contained in a quartz cell were irradiated in bright sunlight for 5 hours. The irradiated essential oil was then tested for its effect on the viability of P.digitatum spores.

To each of 3 petri dishes 0.1ml. irradiated oil was added, followed by 12ml. V8 vegetable juice agar which had been previously seeded with P.digitatum spores at 45°C. Controls with non-irradiated oil were used. The petri dishes were incubated at 22°C.

After a 5 day incubation period growth and sporulation was observed in all petri dishes.

It would therefore appear that orange essential oil irradiated as above was not toxic to the spores, or to mycelium.

In another experiment, the antifungal activity of irradiated and non-irradiated orange essential oil was tested with a technique similar to that used by Maruzella and Balter (1959), who found that a number of essential oils were toxic to certain phytopathogenic fungi.

Twelve ml. V8 vegetable juice agar were added to each of 8 sterile petri dishes. The solidified agar in each dish was seeded with a suspension of P.digitatum spores and to the centre of each dish a disk of Whatman No. 1 filter paper (6mm. diam.) that had just previously been soaked in either non-irradiated or irradiated essential oil (as described above), was added. There were 4 replicates per treatment. The petri dishes were incubated at 22-24°C. for 5 days.

No zones of growth inhibition around the filter paper disks were observed in either treatment.

These results show that although orange essential oil does not stimulate germination of spores or support growth of mycelium

of P.digitatum, neither does it show any marked toxicity to P.digitatum. Irradiated oil behaves in the same way.

M. PECTIC AND CELLULOLYTIC ENZYMES PRODUCED IN CULTURE

It was seen in earlier experiments in Section I (P.138) that an inoculum of spores in pectin broke down the resistance of 'inter-vesicle' flavedo tissue to infection in the presence of wounds. This suggested that infection may have resulted from the adaptive secretion of pectic enzymes in the inoculum drop.

1. Growth of the fungus on pectin, cellulose and glucose in liquid culture

Culture filtrates, used in the study of extracellular pectic and cellulolytic enzymes were prepared as described on P. 44, after the fungus had grown on a basal medium containing 1% pectin, glucose, NaPP, CMC and Solka Floc wood cellulose for 5 days in shake culture at 23°C. ± 1° (see P.43).

The effect of the different carbon sources on growth expressed as mg. dry weight mycelium after this period of incubation is shown in Table 65.

Table 65. Effect of different carbon sources on growth

Medium	Mean dry wt. mycelium (mg.) of 7 replicates
Basal medium + 1% Pectin	101.1 ± 6.0
" " + 1% NaPP	102.5 ± 10.3
" " + 1% CMC	42.3 ± 3.3
" " + 1% Wood cellulose	Sparse growth *
" " + 1% Glucose	207.9 ± 25.6

*Mycelium impregnated with fibres of wood cellulose.

Growth was best on glucose. Growth on pectin and NaPP while being less than on glucose, was much better than on CMC. Little growth was observed on wood cellulose.

2. Pectic Enzymes

(a) Pectin methylesterase (PME)

PME activity was measured by titration of liberated carboxyl groups with 0.01N NaOH as described in Materials and Methods (P.46).

(i) Effect of pH on PME activity

The effect of pH on the activity of PME in a culture filtrate of the fungus grown on basal medium containing 1% pectin as above is shown in Fig. 9.

PME activity was highest at pH 3.5.

This result contrasts with those of other workers who found that the pH optimum for activity of fungal PME occurs in the range pH 4.0 - 5.5 (see Kertesz, 1951, p.370).

(ii) Effect of carbon source on production of PME

The fungus was cultured on basal medium containing 1% pectin, NaPP, glucose, CMC and SOlka Floc wood cellulose as described on P.199.

The activity of PME in the different culture filtrates at pH 3.5 is shown in Fig. 10.

PME was produced in culture filtrates of the fungus grown on all carbon sources tested but the culture filtrate of the fungus grown on CMC was most active.

It therefore appears that PME is produced both adaptively and

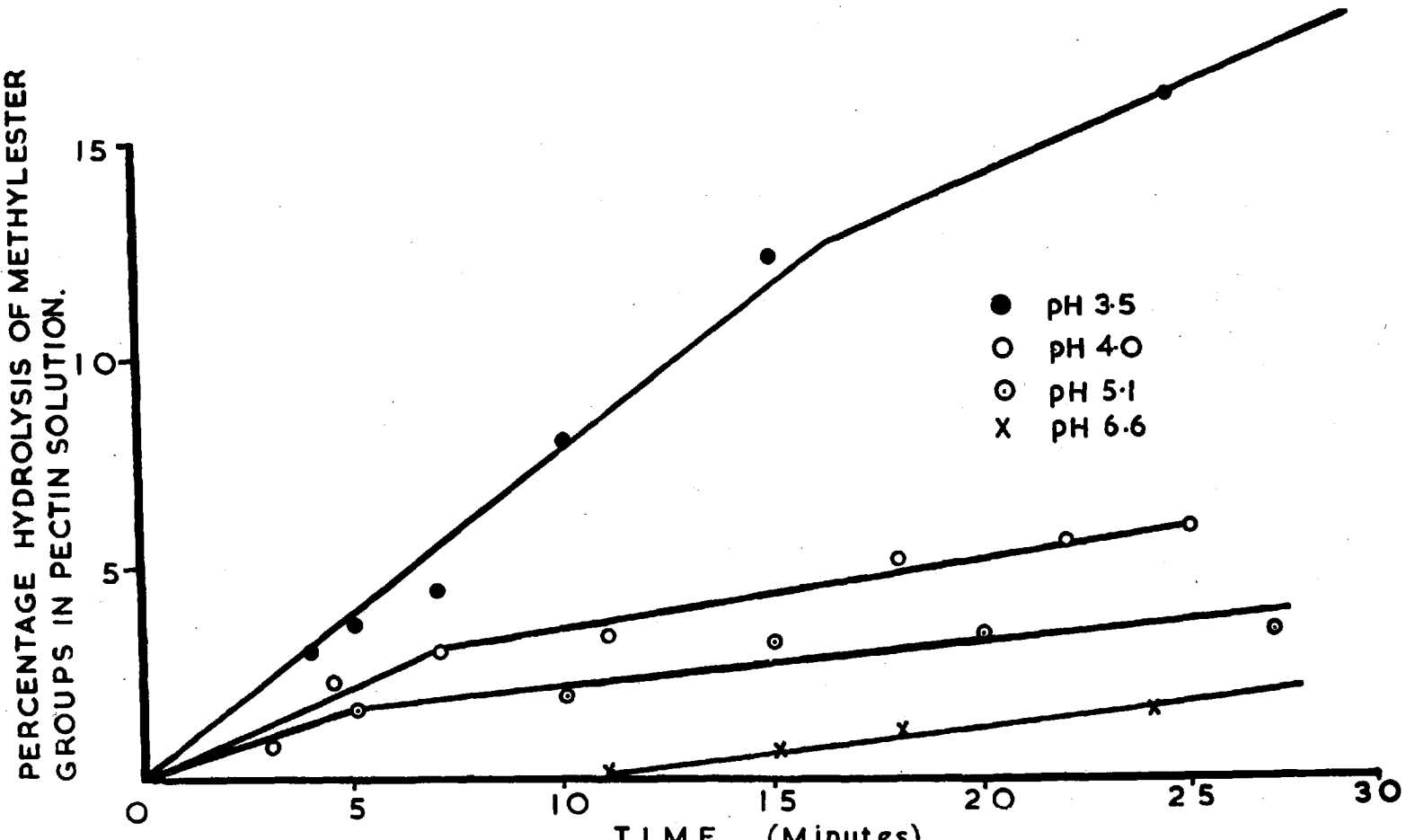

Fig. 9. EFFECT OF pH ON PECTIN METHYLESTERASE ACTIVITY OF CULTURE FILTRATES OF THE FUNGUS GROWN ON PECTIN.

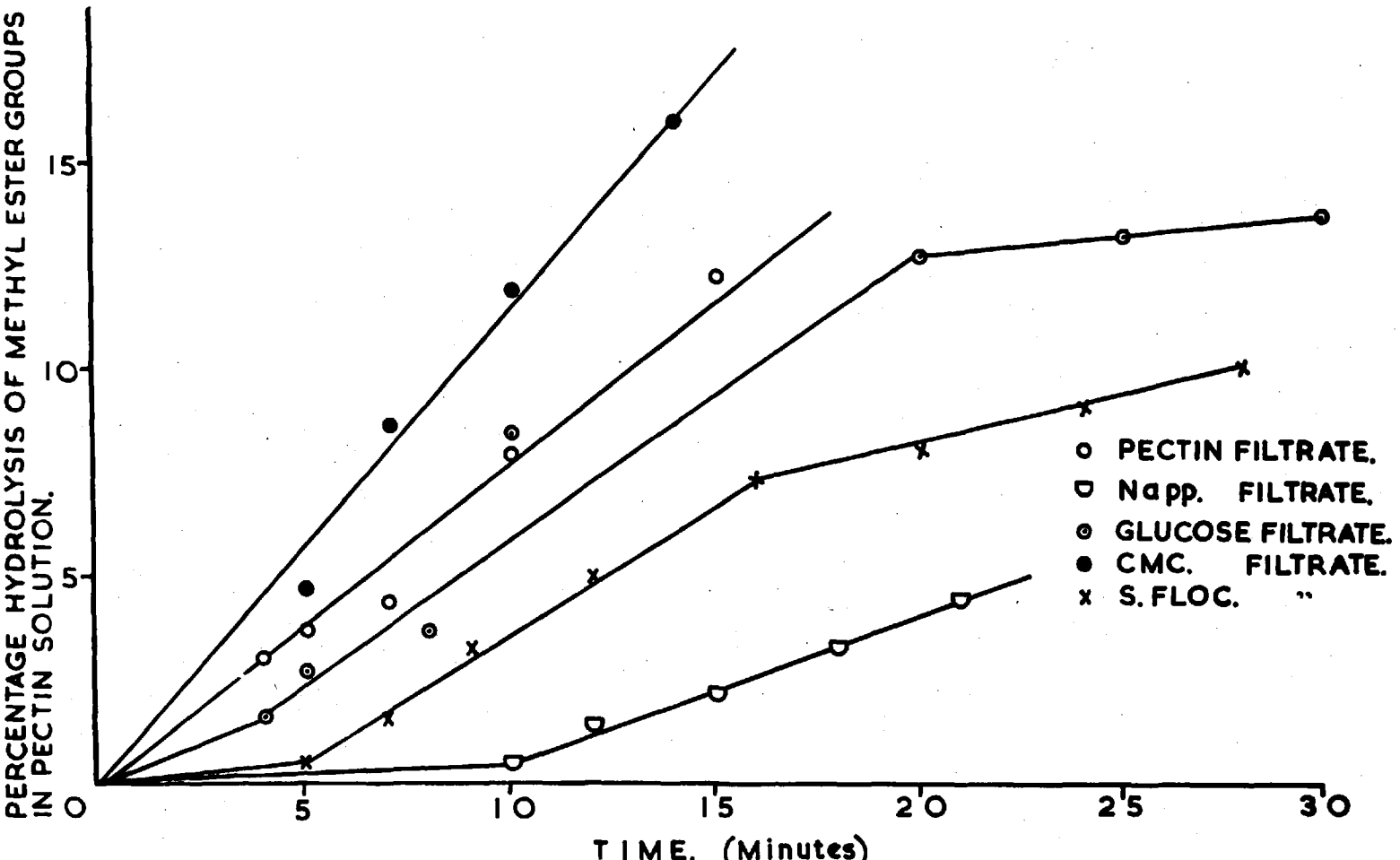

Fig. 10. PECTIN METHYLESTERASE ACTIVITY OF CULTURE FILTRATES OF THE FUNGUS GROWN ON DIFFERENT CARBON SOURCES.

constituitively.

(b) Polygalacturonase (PG).

PG activity was estimated by measuring the reduction in viscosity of pectin and NaPP solutions as described in Materials and Methods (P.45). Activity was calculated as $^{100}/_{t}$ where 't' is the time in minutes for 50% reduction in viscosity of pectin and NaPP solutions by culture filtrates of the fungus.

(i) Effect of pH on PG activity

Two experiments were carried out to study the effect of pH on PG activity.

In the first experiment, the PG activities of culture filtrates of the fungus grown on basal medium containing 1% pectin and 1% NaPP on pectin solution are compared at pH 4.5 - 6.0 (Table 66).

Table 66. Effect of pH on PG (visc. -P) activity of culture filtrates of the fungus grown in basal medium containing 1% pectin and 1% NaPP

Carbon source in basal medium	PG Activity			
	pH			
	4.5	5.0	5.5	6.0
Pectin	5.6	6.1	5.0	3.6
NaPP	10.8	12.3	8.3	6.9

The optimum pH for PG activity of both culture filtrates of the fungus was 5.0.

In the second experiment the PG activity of the same culture filtrates of the fungus as above was tested on NaPP solution at pH 4.5-6.0 (Table 67).

Table 67. Effect of pH on PG (visc.-NaPP) activity of culture filtrates of the fungus grown on basal medium containing 1% pectin and 1% NaPP

Carbon source in basal medium	PG Activity			
	pH			
	4.5	5.0	5.5	6.0
Pectin	5.9	3.7	3.4	2.4
NaPP	8.0	11.1	8.3	4.8

The optimum pH for PG activity of the fungus grown on pectin and NaPP was 4.5 and 5.0 respectively.

In both experiments PG activity of that culture filtrate of the fungus grown on NaPP was almost double that of the culture filtrate of the fungus grown on pectin. At optimum pH, culture filtrates of the fungus grown on pectin or NaPP were equally active in reducing the viscosity of pectin and NaPP.

(ii) Effect of carbon source on production of PG

In this experiment culture filtrates of the fungus prepared after 5 days growth on basal medium containing 1% pectin, NaPP, glucose, CMC and Solka Floc wood cellulose (P.199) were tested for PG activity on pectin and NaPP solutions at pH 5.0 (Table 68).

Table 68. PG activity of culture filtrates of the fungus grown on basal medium containing different carbon sources

Carbon source in basal medium	PG activity	
	Test Solution	
	Pectin	NaPP
Pectin	6.1	3.6
NaPP	11.1	8.7
CMC	2.3	3.0
Wood cellulose	2.0	1.9
Glucose	12.1	0

Culture filtrates of the fungus when grown on glucose and NaPP were most active when tested on pectin solution. That culture filtrate of the fungus grown on NaPP was most active when tested on NaPP solution. No PG activity was found in a culture filtrate of the fungus grown on glucose and tested on NaPP solution.

The results obtained with culture filtrates of the fungus grown on glucose were confirmed in another experiment.

(c) Cellulase (Cx)

Cx activity was estimated by the reduction in viscosity of CMC solution as described in Materials and Methods (P.45).

(i) Effect of pH on Cx activity

The effect of pH on Cx activity of culture filtrates of the fungus grown on basal medium containing CMC and Solka Floc-wood cellulose is shown in Table 69. Activity is expressed as $^{100}/_{t}$ where 't' is the time in minutes for 25% reduction in viscosity of CMC.

Table 69. Effect of pH on Cx (visc.-CMC) activity of culture filtrates of the fungus grown on basal medium containing 1% CMC and 1% wood cellulose

Carbon source in basal medium	Cx Activity			
	pH			
	4.5	5.0	5.5	6.0
CMC	2.9	3.3	3.2	2.7
Wood cellulose	0	0	0	0

The fungus grown on CMC produced a filtrate of low activity and on wood cellulose an inactive filtrate (see below). pH had little effect on activity.

Although from the above Table 69 it would appear that growth of the fungus on Solka Floc wood cellulose produced an inactive filtrate this was not so. A filtrate of very low activity was produced on this medium and activity at pH 4.5 - 6.0 is shown in Table 70. Activity is expressed as $^{100}/_{t}$ where 't' is the time taken in minutes for 20% reduction in viscosity of CMC solution.

Table 70. Effect of pH on Cx (visc.-CMC) activity of culture filtrate of the fungus grown on basal medium containing 1% wood cellulose

Carbon source in basal medium	Cx Activity			
	pH			
	4.5	5.0	5.5	6.0
Wood cellulose	-	-	0.9	1.6

Activity was greatest at pH 6.0.

N. PRODUCTION OF PECTIC AND CELLULOLYTIC ENZYMES IN VIVO

The production of pectic enzymes by the fungus when grown on pectin, NaPP and glucose *in vitro* suggested that the ability of the fungus to produce these enzymes *in vivo* might play a part in determining whether or not the fungus was pathogenic.

The production of pectic enzymes by the fungus *in vivo* was anticipated as the accumulation of pectin in the cell walls of the rind is a characteristic anatomical feature in the development of Citrus fruit (Bain, 1958). Orange rind contains comparatively large amounts of pectin and many references to the pectin content of this tissue are found in the literature, e.g. Samisch and Cohen (1949), Money and Christian (1950) and Rouse (1953).

The PME, PG and Cx activities in 0.2M NaCl extracts of healthy rind and rind infected by the fungus was investigated using Shamouti fruit. The preparation of extracts is described on P.47 .

1. Pectin methylesterase (PME)

Enzyme preparations of healthy and infected rind tissue were tested for PME activity as described on P.46 .

(a) Effect of pH on PME activity of 0.2M NaCl extract of healthy orange rind

Before investigating the PME activity of infected rind extract, the effect of pH on PME activity of healthy rind was investigated.

Fig.11 shows the relation between percentage hydrolysis of

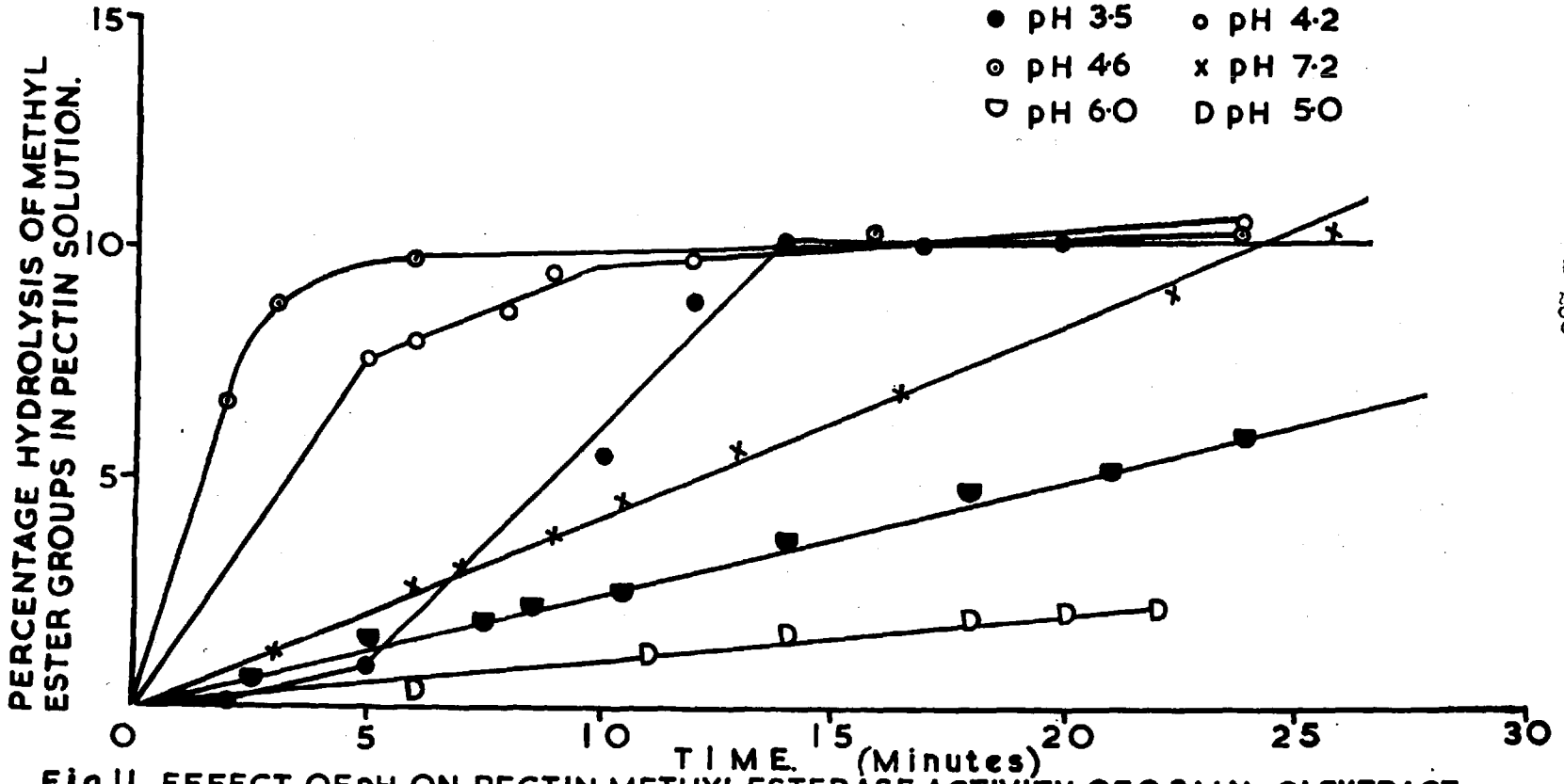

Fig.11. EFFECT OF pH ON PECTIN METHYLESTERASE ACTIVITY OF 0·2 M NaCl EXTRACT OF HEALTHY ORANGE RIND.

methyl ester groups with time at pH 3.5-7.2.

Initial rate of hydrolysis of methyl ester groups was greatest at pH 4.6 but the enzyme was inactivated after 10% methyl ester groups had been hydrolyzed. Inactivation of the enzyme also occurred at pH 3.5 and 4.2 when 10% of the methyl ester groups present had been hydrolyzed. At pH 5.0-7.2 activity bore a linear relationship with time and increased with pH.

The inactivation of PME at pH values below 5.0 was not observed by McDonnell et al. (1945). They assumed that rate of hydrolysis was constant until more than 50% of the methyl ester linkages had been hydrolyzed. This assumption was based on the work of Lineweaver and Ballou (1945) who studied the rate of de-esterification of pectin in 0.1M NaCl solution by pectinesterase from two sources, alfalfa and pea vines.

(b) Effect of pH on PME activity of 0.2M NaCl extract of infected orange rind

In this experiment the effect of pH on PME activity of 0.2M NaCl extract of infected rind was investigated. Fig.12 shows percentage hydrolysis of methyl ester groups with time at pH 3.7-6.8.

Within the 30 minute reaction period PME activity was greatest at pH 3.7 and decreased with increase in pH, little activity being observed at pH 6.8.

At pH 3.5 the enzyme was inactivated after 14.3% (approx.) hydrolysis of the methyl ester groups had occurred. A more gradual reduction in rate of hydrolysis with time was observed at other

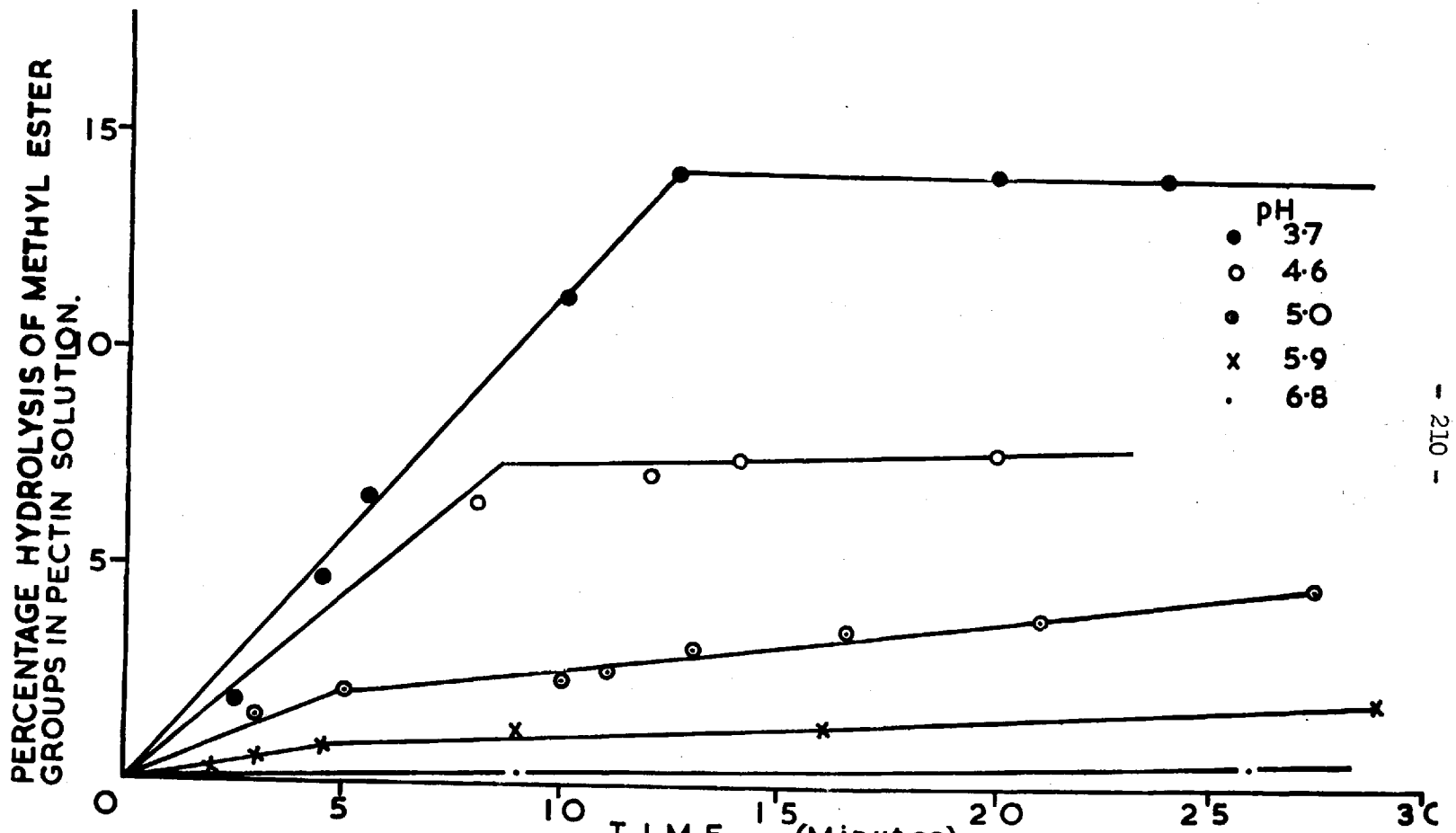

Fig. 12. EFFECT OF pH ON PECTIN METHYLESTERASE ACTIVITY OF 0·2 M NaCl EXTRACT OF INFECTED ORANGE RIND.

pH values.

(c) Comparison of PME activity of 0.2M NaCl extracts of healthy and infected orange rind

In this experiment the activity of PME in healthy and infected rind extracts was compared at pH 3.5 as this was very close to the pH of infected rind 3.3, (Table 71).

Table 71. Comparison of PME activity of 0.2M NaCl extracts of healthy and infected orange rind at pH 3.5

0.2M NaCl extract	PME activity expressed as % hydrolysis of methyl ester groups in 10 minutes
Healthy rind	6.0
Infected rind	11.0

PME activity is greater in the infected extract than in the healthy extract, so it would appear that PME is produced by the fungus in parasitizing the rind. It could also happen that the presence of the fungus in the rind tissues stimulates greater production of PME by those tissues. The former suggestion, however, seems more plausible as the production of PME by the fungus when grown in vitro has already been shown (P.200).

2. Polygalacturonase (PG)

PG activity of 0.2M NaCl extracts of healthy and infected rind tissues was estimated by measuring the reduction in viscosity

of pectin and NaPP solutions as described on P.45 . Activity was calculated as $100/t$ where 't' is the time in minutes for 10% reduction in viscosity of pectin and NaPP solutions.

(a) Effect of pH on PG activity of 0.2M NaCl extracts of healthy and infected orange rind

In this experiment PG activity in 0.2M NaCl extracts of healthy and infected rind was compared at pH 3.5-6.5.

No PG activity was observed in either extract at pH 3.5-6.5, when tested on NaPP solution.

PG activity in both extracts when tested on pectin solution is shown in Table 72.

Table 72. PG activity (visc.- P) of 0.2M NaCl extracts of healthy and infected orange rind at pH 3.5-6.5

0.2M NaCl extract	PG Activity (visc. -P)				
	pH				
	3.5	4.5	5.0	5.5	6.5
Healthy rind	0	0	0	0	0
Infected rind	0	2.2	4.0	4.5	1.3

No PG was detected in the healthy rind extract. Activity in the infected rind extract was very low having a pH optimum at 5.5. This activity is not thought to be of significance.

3. Cellulase (Cx)

Cx activity was estimated by the reduction in viscosity

of CMC solution as described on P.45 . Activity is expressed as $100/t$ where 't' is the time in minutes for 10% reduction in viscosity of CMC solution.

(a) Effect of pH on Cx activity

The effect of pH on Cx activity in 0.2M NaCl extracts of healthy and infected rind at pH 4.5-6.5 was investigated and the results are shown in Table 73.

Table 73. Effect of pH on Cx activity in 0.2M NaCl extracts of healthy and infected orange rind

0.2M NaCl extract	Cx Activity				
	pH				
	4.5	5.0	5.5	6.0	6.5
Healthy rind	0	0	0	0	0
Infected rind	1.5	2.1	2.2	2.8	1.0

No activity was observed in the healthy extract. Activity was very low in the infected extract and had a pH optimum at 6.0. The activity of Cx in the infected extract is not thought to be significant.

O. MACERATING ENZYMES PRODUCED IN VITRO AND IN VIVO

1. Activity of Macerating Enzymes in culture filtrates of the fungus grown on synthetic media and 0.2M NaCl extract of infected rind tissue

In this experiment the macerating activity of culture filtrates of the fungus grown on basal medium containing 1% pectin, NaPP, CMC, Solka Floc wood cellulose and glucose, and 0.2M NaCl extract of infected rind tissue were compared at pH 5.0 according to the method described on P.47. The results are shown in Table 74.

Table 74. Macerating Enzyme activity of culture filtrates of the fungus grown in synthetic medium containing different carbon sources and 0.2M NaCl extract of infected rind tissue

Medium	Macerating Activity
Basal medium + 1% Pectin	2.7 ± 0.9.
" " + 1% NaPP	3.1 ± 1.1.
" " + 1% CMC	1.9 ± 0.5.
" " + 1% wood cellulose	1.5 ± 0.6.
" " + 1% glucose	3.0 ± 1.1.
0.2M NaCl Extract of infedted rind	0.9 ± 0.1
Water	0

Macerating activity was greatest in culture filtrates of the fungus grown on pectin, NaPP and glucose whereas that of the infected rind extract was comparatively low.

Conclusions

From the results of this and the preceding experiments it appears that 0.2M NaCl extract of infected rind tissues contains only active PME. The activities of the other enzymes PG, Cx and Macerating Enzyme are so low that it is suggested that if they are important in pathogenesis, then they must have been inactivated in the rotted tissues after they had produced their effects.

The low Macerating Enzyme activity of infected extract is in agreement with the results of Miyakawa (1962), who found that "protopectinase" activities of both P.digitatum and P.italicum are much lower then those of less pathogenic species P.expansum and P.purpurogenum.

SECTION IV - DISCUSSION

The experimental results described above show clearly that uninjured oranges inoculated with an aqueous suspension of spores of P.digitatum do not become infected. This was found to be true for all parts of the fruit.

Bates (1936) found that Valencia fruit, inoculated with an aqueous suspension of spores, were infected at the stem ends in the absence of wounds. However, the exact point of entry was not determined. A relatively crude method was used for inoculation and Bates did not study the inoculated surface for the presence of wounds not visible to the naked eye. The method of inoculation used in the present work largely eliminated these deficiencies. Another report on the infection of uninjured fruit is that of Gioelli (1930) who stated that P.digitatum may penetrate the stem end of mandarin fruit growing under adverse conditions in the absence of wounds.

Uninjured Shamouti and Navel fruit were not infected when inoculated with spores in 1-3 per cent citric acid, orange juice, and other rind extracts although good growth of mycelium was observed in these media. This result was in contrast to that of Green (1932) who infected Spanish oranges with inocula of spores in 1-5 per cent citric acid and orange juice. It is suggested that the results of Green could have possibly been caused by the presence of minute wounds on the fruit surface or alternatively

could have been a characteristic of the fruit used by her.

The presence of wounds was a prerequisite for the infection of Navel, Valencia and Shamouti fruit by an aqueous suspension of spores. However, as had been observed by Bates (1936), not all wounded fruit inoculated in this way became infected. In the present work it has been found that Navel, Valencia and Shamouti fruit wounded into the flavedo between the oil vesicles, showed a marked resistance to infection from an aqueous suspension of spores.

A study of inoculated fruit showed that the fruit were resistant to infection although hyphae from germinated spores had penetrated the wounds. This resistance was characteristic of the flavedo cells and was less marked when deeper wounds into the albedo were made.

The mechanical barrier provided by the compact flavedo cells to penetration by hyphae may have played a part in this resistance. In contrast, it seems that the spongey albedo with its large intercellular spaces yields more readily to the fungus when this tissue is exposed by wounds between the oil vesicles. The fact that hyphae in wounds into the albedo between the oil vesicles did not penetrate the flavedo cells around the pricks on initial entry into the wounds, but instead grew down to the albedo layer supports this view.

The infection of fruit, wounded into the oil vesicles by an aqueous suspension of spores, which was observed by Bates (1933;1936)

was confirmed.

A microscopic study of the penetration of hyphae from germinated spores in wounds into and between the oil vesicles yielded useful information on the mode of entry of the fungus into the fruit through wounds.

Of most interest perhaps was the fact that hyphae, in wounds into the flavedo between the oil vesicles, did not penetrate the cells of the flavedo after an incubation period of 72 hours, and that when fruit were wounded between the oil vesicles into the albedo the hyphae penetrated the albedo before invading the flavedo tissue.

In fruit wounded into the oil vesicles the hyphae first penetrated the oil vesicle cover cells and then invaded the thin walled cells of the oil vesicles before penetrating the albedo. Only slight penetration of the flavedo by hyphae was observed on initial entry into the wounds and this was probably restricted to those cells killed by the essential oil which was released after the oil vesicles had been wounded.

These facts show clearly that the hyphae, coming from spores in water and in wounds into or between the oil vesicles, must reach the albedo tissue before lesions can develop in Navel fruit.

Navel, Valencia and Shamouti fruit, which were resistant to infection after being wounded and inoculated, frequently developed red spots around the pricks at inoculum sites. The red spots resulted from the loss in coherence of the sub-epidermal cells

around the pricks and this loss in coherence of cells appeared to result from substances produced by hyphae in the wounds. Whether in fact these spots are part of a mechanism of resistance of fruit to infection seems to be worthy of further investigation.

A study of the germination of spores in extracts of rind in vitro and in the presence of artificial wounds on the surface of Navel and Valencia fruit showed that germination was promoted by the presence of wounds, and that germination around wounds into the oil vesicles was not significantly greater than around wounds between the oil vesicles. Increasing the depth of wound did not result in a significant increase in germination. It would appear therefore that depth of wound probably affected infection of fruit not because the spores germinated better in deep wounds but because the deeper the wound the easier it was for the germinated spores to reach the more susceptible albedo tissue.

Differences in the resistance of Valencia and Navel fruit to infection could not be attributed to differences in the stimulation of germination of spores in wounds of similar depth in fruit of these two varieties.

The observation that healing of wounds in Shamouti fruit increased resistance to infection is of some practical importance because the incubation time necessary for the formation of lesions is markedly increased by prolonging the period between wounding and inoculation (i.e. the period of healing).

Green (1932) working with P.digitatum and P.italicum also

found that by leaving oranges, wounded to a depth of 3.5mm. to "dry out" (heal) for 7 days before inoculating with spores in water, a marked resistance to infection was acquired.

In similar work Bates (1936) used Navel oranges wounded to a depth of 3 mm. both into and between the oil vesicles, and then left them to "wilt" (heal) for 1, 4, 7 and 11 days. A very slight increase in the resistance to infection was apparent in fruit wounded between the oil vesicles after one day's wilting, but longer periods of wilting did not increase this resistance to any appreciable extent. Similar wounds into the oil vesicles, however, were more resistant to infection after wilting than were those between the vesicles of Navel fruit. In the present work it was found that Shamouti fruit, wounded to a depth of 2mm. into the oil vesicles and left to heal for 1-7 days, were more resistant to infection than Shamouti fruit, wounded between the oil vesicles to a depth of 4mm. and left to heal for the same period. Increasing the period between wounding and inoculation from 1 to 7 days did not make the fruit more resistant to infection.

In studying the resistance of the inter-vesicle flavedo tissues to infection it was found that Navel, Valencia and Shamouti fruit, pricked between the oil vesicles and into the flavedo, and then inoculated with spores suspended in albedo extract became infected whereas the same fruit wounded and inoculated with spores in flavedo extract became infected only to a slight extent. This was so although germination and growth of spores were equally good

in both extracts.

This suggests that some factor is produced by the growth of the fungus in albedo extract, which reduces the resistance of the inter-vesicle flavedo tissues to infection, and that this factor is less readily produced in flavedo extract. If this be so then the same or similar factor is also produced after growth in orange juice and citric acid at the site of wounds because spores suspended in these media also overcame the resistance of the flavedo to infection. It appears that good growth of the fungus at the inoculation site does not necessarily mean that the factor is produced in sufficient quantities to ensure infection. Once sufficient of the factor has been produced to ensure infection it can be assumed that it is produced cumulatively after growth on the dead tissues of the expanding lesion so that a progressive lesion is formed.

Orange essential oil did not promote germination or growth of spores but did promote infection through wounds into the inter-vesicle flavedo tissues presumably because it killed the flavedo and albedo cells, thus making them a suitable medium for growth of the fungus and so made it easier for the invading hyphae to reach the albedo tissue and hence form lesions.

It was of interest to note that uninjured Shamouti fruit and Shamouti fruit, wounded to a depth of 0.5mm. were not infected by spores suspended in essential oil. These fruit were not infected presumably because the deeper layers of the flavedo were not killed

by the oil.

The resistance of the inter-vesicle flavedo tissues to infection was also found in lemons and grapefruit. A similar observation had been made by Bates (1936).

The ability of spores suspended in buffered pectin to infect Navel, Valencia and Shamouti fruit, wounded into the inter-vesicle flavedo tissues, whereas spores in other carbohydrates did not was particularly interesting because pectin is one of the main components of orange rind. From work done on the germination and growth of spores in pectin and a study of the extracellular pectic and cellulolytic enzymes produced by the fungus in vivo and in vitro it is suggested that spores in pectin had this effect because of the production of some factor in this medium which reduced resistance to infection.

The possibility of the whole or part of this factor being enzymic seemed reasonable and was supported by the fact that PG and PME were produced in vitro in a medium containing pectin and NaPP as the carbon sources. It must be pointed out however that the PG activity of such culture filtrates in vitro was lower than expected should PG have been the only factor involved in overcoming resistance to infection.

PG and Macerating Enzyme activity was low in 0.2M NaCl extract of infected rind but PME was active. This then suggests that if PG and Macerating Enzyme are produced extensively in the production of lesions in fruit (this would seem to be a reasonable

assumption because of the high pectin content of rind) then they must be inactivated in some way after they have produced their effects.

From an abstract of work by Miyakawa (1963) it was learned that he found high polygalacturonase activity following invasion of Satsuma oranges by P. digitatum and P.italicum and 2 per cent of the acid was detected at an early stage of infection. Neither fungus produced titrable free D-galacturonic acid in culture media of peel extract unless pectin was added. The acid promoted infection of unripe fruits and this suggests like other acids, it destroys resistance to infection in the rind tissues,

The production of PG and PME in vitro by P.italicum (Blue Contact Mould of Citrus fruit) was shown by Collins and Sledjeski (1960) but the disorganisation of rind tissues by the fungus was not related to the production of these enzymes nor to the Macerating Enzyme activity of the fungus.

Macerating Enzyme activity was greatest in culture filtrates of the fungus grown in basal medium containing pectin, NaPP and glucose whereas that of the infected rind extract was comparatively low.

Miyakawa (1962) studying the decomposition and utilization of pectin by P.digitatum and P.italicum showed that protopectinase (Macerating Enzyme) was produced by both fungi but that activity of both species was much lower than those of the less pathogenic P.expansum and P.purpurogenum.

The observation that PG was active in that culture filtrate of the fungus grown in basal medium containing glucose suggests that PG is produced constituitively as well as adaptively. Macerating Enzyme activity was also comparatively high in this filtrate and it was of interest to note that Shamouti fruit wounded into the flavedo between the oil vesicles and inoculated with spores in this culture filtrate were more readily infected than similar fruit inoculated with spores in other culture filtrates.

The part played by the pectic, cellulolytic and Macerating Enzyme complex in pathogenesis needs to be studied further.

No evidence was found to support the view of Bates (1936) that scale infested fruit were more liable to infection than were those free from scale insects.

A study of the effect of adding a mixture of vitamins to a synthetic medium on the growth of mycelium supported the view of Fergus (1952) that such a mixture only allowed an earlier initiation of growth but did not increase the yield of mycelium. Wooster and Cheldelin (1945) on the other hand found that thiamin or the thiazole moiety of thiamin was required for growth.

As P.digitatum is almost specific to Citrus fruit it was thought that a study of the germination of spores in vitro might yield some useful information on the nature of the germination promoting stimulant found in the rind.

As had been observed by Dellerè (1953) spores do not germinate in distilled water. A suitable nutrient source was needed for good

germination and such a nutrient was supplied by a 1 per cent glucose soln. buffered with 0.1M phosphate (pH 5.2) when the spore concentration was 330,000 spores/ml. or less. Germination was also good in 1 per cent xylose, fructose and sucrose solns. buffered with 0.1M phosphate at pH 5.2.

The promotion of spore germination by a sugar-phosphate mixture was particularly interesting as it compared favourably with the work of Miyakawa (1958). He found that orange rind extract stimulated germination of spores and that the stimulating factor was readily extracted from the rind by water and absorbed on activated charcoal. The component of the rind extract absorbed by charcoal was released by 60-80 per cent acetone and the concentrated material from acetone extract stimulated germination only when added with the filtrate (charcoal treated extract) or with a very small amount of phosphate. Thus, the phosphoric compounds were found to activate the germination stimulant. From the work described here it would seem at least possible that the germination stimulant of Miyakawa could have been a sugar or a mixture of sugars.

The stimulating effect of emanations from rind infected with P.digitatum on the germination of spores in water was noted but the effect was not studied further.

From the many experiments carried out here it seemed that the rinds of Valencia fruit were tougher than those of the more susceptible Navel fruit generally. Data to support this view

however were not obtained.

A study of rind toughness i.e. finding the pressure required to wound the rinds of different fruit into and between the oil vesicles, together with experiments with the same fruit wounded and inoculated with an aqueous suspension of spores might result in a better understanding of the variation in resistance of fruit to infection.

Since it is clear from this work that wounds, especially wounds into the oil vesicles, are necessary for the infection of oranges by P.digitatum than it could happen that fruit, in which the oil vesicles are less frequent, could be more resistant to infection. The orchard conditions at time of harvesting which make the fruit more susceptible to wounding should be sought out and avoided if possible. Careful handling in harvesting and packhouse treatment of fruit and keeping the spore content of the air as low as possible, are essentials in the control of wastage. From in vitro experiments it would appear that healing (wilting or drying out) is a worthwhile practice and the suggestion that rind toughness may be a factor in disease resistance seems worthy of investigation.

SECTION V - SUMMARY

A. FORMATION OF LESIONS

1. The development of lesions in wounded Shamouti fruit, kept under controlled conditions, was studied. The first lesions were observed after 48 hours incubation.

B. INFECTION OF FRUIT THROUGH UNINJURED RIND

1. Uninjured Shamouti and Navel fruit were not infected by an inoculum of spores in water.

2. Uninjured Shamouti fruit, inoculated with spores in water extracts of rind tissues, orange juice or 1-3 per cent citric acid solutions, were not infected to a significant extent.

3. Spread of the fungus from Green Mould infected fruit to sound fruit was observed with Shamouti, Valencia and Navel fruit.

C. INFECTION OF FRUIT THROUGH WOUNDS BETWEEN THE OIL VESICLES

1. Shamouti, Navel and Valencia fruit, wounded into the flavedo and inoculated with an aqueous spore suspension, showed a marked resistance to infection.

2. Variation in the resistance of inter-vesicle flavedo tissues to infection was observed with Navel and Valencia fruit taken from different consignments.

3. The blossom and stem ends of Navel and Valencia fruit, wounded into the flavedo, were equally resistant to infection.

4. Grapefruit and lemons, which were wounded into the flavedo, were also resistant to infection.

5. The number of lesions and the rate at which they developed in Shamouti fruit, wounded to a depth of 4mm., increased with increase in spore load.

6. The greater the number of pricks/inoculum site the faster lesions developed in Shamouti fruit wounded to a depth of 4mm. One prick/inoculum site was sufficient for the formation of lesions.

7. Navel fruit wounded to a depth of 3.5mm. were inoculated by different methods. The results showed that the presence of water at the inoculum sites greatly increased the number and rate of development of lesions.

8. The resistance of Navel fruit wounded into the flavedo to infection was not reduced by emanations from fruit infected by P.digitatum.

9. Shamouti fruit, wounded to a depth of 4mm. and left to heal for 1 day, were more resistant to infection than were freshly wounded fruit.

D. INFECTION OF FRUIT THROUGH WOUNDS INTO THE OIL VESICLES

1. Valencia and Navel fruit were infected when pricked to a depth of 0.25mm. before inoculation.. The number of lesions formed increased with depth of prick up to a depth of 2mm.

2. The greater the number of pricks/inoculum site the more rapidly lesions developed in Navel fruit wounded to a depth of 4mm. Lesions always formed in Navel fruit wounded three times/inoculum site.

3. The number of lesions and the rate at which they developed in Navels, wounded to a depth of 4mm., increased with increase in spore load.

4. Four methods of inoculating Navel fruit wounded to a depth of 4mm. showed no marked differences in the number of lesions formed.

5. Shamouti fruit, wounded to a depth of 2mm. and left to heal for 1 day, were more resistant to infection than were freshly wounded fruit.

E. INFECTION OF FRUIT THROUGH INSECT WOUNDS

1. Scale infested Shamouti fruit were not more susceptible to infection than Shamouti fruit free from scale insects.

F. MICROSCOPIC STUDY OF THE PENETRATION OF HYPHAE IN WOUNDS INTO THE RIND TISSUES

1. The penetration of hyphae in wounds 0.25-4.0mm. deep into, and between the oil vesicles of Navel fruit after incubation periods of 24,48 and 72 hrs., was studied microscopically.

(a) Wounds between the oil vesicles

(i) In wounds up to 1.5mm. deep and after an incubation period of 72 hrs. hyphae penetrated the ruptured cells that resulted from wounding. They did not penetrate the compact flavedo cells or the cells of albedo.

(ii) In wounds 3 or 4mm. deep 48hrs. after inoculation hyphae penetrated the albedo near the pricks, but did not penetrate the flavedo on initial entry into the wounds.

(iii) Seventy two hours after inoculation hyphae in wounds 4mm. deep penetrated the flavedo to some extent and had grown extensively in the albedo.

(b) Wounds into the oil vesicles

(i) Twenty four hours after inoculation hyphae in wounds 0.25-1.5mm. deep had entered the cover cells of the oil vesicles and had also penetrated the thin walled oil vesicle cells to some extent.

(ii) Twenty four hours after inoculation, hyphae in wounds 3-4mm. deep had penetrated down the thin walled cells of the oil vesicles and had reached the albedo. Penetration of the flavedo was confined to those cells adjacent to the oil vesicles.

(iii) Forty eight hours after inoculation, hyphae in wounds 1-1.5mm. deep had penetrated the inter-vesicle tissues of the flavedo, and also the albedo.

After the same period, with wounds 3-4mm. deep hyphae had moved from the albedo into the flavedo which was extensively invaded, and the fungus was now sporulating on the surface of the fruit.

(iv) When wounds 0.25-0.5 mm. deep were inoculated, sporulating hyphae appeared on the fruit surface 72 hours after inoculation.

G. RED SPOTS

1. Navel, Valencia and Shamouti fruit that had been treated with fungicide chemicals and which were resistant to infection after being wounded and inoculated, frequently developed red spots at the periphery of the pricks at inoculum sites.

2. The formation of the red spots was associated with a lack of coherence of the sub-epidermal cells around the points of wounding.

3. Lemons and grapefruit resistant to infection when inoculated, after pricking between the oil vesicles to a depth of 1mm., also developed similar red spots.

4. Red spots developed in Navel fruit that had not been treated with chemicals and which had been inoculated after wounding between the oil vesicles to a depth of 1mm.

H. BREAKDOWN OF RESISTANCE OF FLAVEDO TO INFECTION

Resistance of the inter-vesicle tissues of the flavedo was studied by inoculating fruit, wounded to a depth of 1mm. (generally) between the oil vesicles, with spores suspended in different media.

1. Infection of Navel, Valencia and Shamouti fruit was promoted when spores were suspended in extracts of albedo, complete rind and flavedo, orange juice, orange essential oil and 1 per cent citric acid.

2. Flavedo extract was least effective in promoting infection of Navel, Shamouti and Valencia fruit. This extract became more efficient in promoting infection as the depth of the wound was increased.

3. Inocula of spores in orange essential oil were effective in promoting infection of Navel fruit, wounded between the oil vesicles, only when wounds deeper than 0.5 mm. were made.

4. The effect of different carbohydrate solutions on flavedo resistance in Navel, Valencia and Shamouti fruit to infection was studied. Only spores suspended in buffered pectin solution promoted infection consistently. Infection was promoted in buffered 0.1-1.0 per cent pectin solutions. All Navel fruit were infected when wounded between the oil vesicles to a depth of 0.5mm. and inoculated with spores in 0.5 per cent buffered pectin solution.

5. Culture filtrates of the fungus grown in a basal medium containing 1 per cent pectin, sodium polypectate, carboxymethyl-cellulose and Solka Floc wood cellulose as carbon sources did not affect flavedo resistance to infection in Shamouti fruit. However, a culture filtrate of the fungus with glucose as the carbon source did promote infection to some extent. An extract (0.2MNaCl) of infected rind did not promote infection.

I. SPORE GERMINATION

1. Spore concentration had a marked effect on germination in different media.

2. Increase in sugar concentration at high spore concentration only increased spore germination slightly.

3. Germination was good in 1 per cent glucose buffered with 0.01M phosphate (pH 5.2) and at a concentration of 330,000 spores/ml. or less.

4. Germination was also good in one per cent xylose, fructose, and sucrose solutions buffered with 0.01M phosphate (pH 5.2).

5. A mixture of 1 per cent glucose and 0.025 per cent Yeastrel extract or casamino acids, allowed good germination.

6. Different nitrogen compounds, a mineral salts' solution, and vitamins did not stimulate germination.

7. Spore germination was good in extracts of flavedo, albedo and complete rind.

8. In rind extracts, germ tubes were first observed after 6 hours and the production of germ tubes, was essentially complete 10 hours after incubation.

9. Emanations from rind of Navel, Valencia and Shamouti fruit infected with P.digitatum stimulated the germination of spores in water. Emanations from rind infected by certain other fungi did not stimulate the germination of spores in water.

10. Wounds into, and between the oil vesicles of Navel and Valencia fruit stimulated germination of spores on the surface. The effects of wounding between and into the vesicles were similar. Increase in depth of wound did not result in a significant increase in germination of spores.

J. ACTION OF ESSENTIAL OIL

1. Orange essential oil did not promote germination of spores.

2. Irradiated and non-irradiated essential oil was not toxic to P.digitatum.

K. GROWTH OF MYCELIUM IN CULTURE

1. Growth on different agar media was studied and was best

on V8 vegetable juice agar. The optimum temperature for growth varied with the different media but was always within the range 19.5-23°C.

2. Growth in liquid media containing rind extracts was best in flavedo extract. At a temperature of 25°C. maximum growth was produced in all media 10 days after inoculation.

3. Growth in Fergus' synthetic medium showed that addition of a mixture of vitamins did not increase growth significantly but that the addition of 0.1 per cent yeast extract added to this medium did.

4. A study of the effect of different carbon sources in a basal medium showed that growth was best with glucose and much less with pectin and sodium polypectate.

L. PECTIC AND CELLULOLYTIC ENZYMES PRODUCED IN VITRO

1. Pectinmethylesterase (PME)

(a) PME was produced in a basal medium containing 1 per cent pectin, sodium polypectate, carboxymethylcellulose or Solka Floc wood cellulose. PME was most active in the culture filtrate of the culture on carboxymethylcellulose.

(b) PME was most active at pH 3.5.

2. Polygalacturonase (PG)

(a) PG was most active in filtrates of cultures of the fungus grown in a basal medium containing 1 per cent glucose or sodium polypectate as carbon sources .

(b) PG was most active on pectin and sodium polypectate at pH 5.0.

3. Cellulase (Cx)

(a) Cx activity (visocosity-carboxymethylcellulose) was very low in a filtrate of cultures grown in a basal medium containing carboxymethylcellulose and almost absent from cultures grown in basal medium containing Solka Floc wood cellulose.

(b) Cx was most active at pH 5.0.

M. PECTIC AND CELLULOYLTIC ENZYMES PRODUCED IN VIVO

1. PME

(a) There was more PME in an 0.2MNaCl extract of infected rind than in a similar extract of healthy rind.

(b) The PME in an 0.2M NaCl extract of infected rind was most active at pH 3.7.

2. PG

(a) PG was not found in an 0.2M NaCl extract of healthy rind. In a similar extract of infected rind PG also showed very little activity.

(b) PG was most active at pH 5.5.

3. Cx

(a) Cx was not found in an 0.2M NaCl extract of healthy rind and Cx activity was very low in an 0.2M NaCl extract of infected rind.

(b) Cx was most active at pH 6.0.

N. MACERATING ENZYMES PRODUCED IN VITRO AND IN VIVO

1. Macerating Enzyme was most active in culture filtrates

of the fungus grown in basal medium containing 1 per cent pectin, sodium polypectate and glucose. There was little activity in an 0.2M NaCl extract of infected rind.

SECTION VI - REFERENCES

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PART II

SECTION 1 - INTRODUCTION

An account of transport experiments, which were part of a programme of the South African Co-operative Citrus Exchange Co. Ltd. to control wastage in oranges while in transport and storage, is given here. These experiments were carried out during the 1961 and 1962 packing seasons.

Dr.R.A. Christ, plant pathologist for the Citrus Exchange designed the experiments and supervised the treatment, packing and examination of fruit in South Africa (when necessary). The rest of the experimental work was done by me after the fruit had landed in the U.K.

A. Summary of experimental procedure from harvesting to shipping

Generally, the fruit were treated in the packhouse on the day of harvesting or one day later. Cleaning, sizing, grading, SOPP (sodium-o-phenylphenate) rinse, and waxing are included in packhouse treatment. Fruit were mainly packed in wooden packing cases but in some experiments telescopic cartons were used.

Fruit were then sent by rail to a sea port where they were inspected. In some experiments where wastage was high wasty fruit were removed and the sound fruit repacked at the port of departure. Dr. Christ examined the fruit at this point.

The fruit were then placed in a pre-cooler at 39°F. for 2 days before being transferred to a refrigerated ship (40°F. approx.), and transported to Southampton. After arrival at Southampton

the fruit were moved to Covent Garden, London, where they were examined by me.

B. Examination of fruit

Cases (or cartons) were placed on a flat table before examination of fruit. The lid was removed, the fruit were unwrapped layer by layer and the unwrapped fruit examined one by one for wastage. Unmarketable fruit are described as wasty. The wasty fruit from each case were put to one side, classified (see below) either as Green Mould, Water Rot, Blue Mould, Sour Rot or Brown Rot and the results recorded. The sound fruit were then repacked and the number of fruit/treatment was obtained by adding the wasty and sound fruit in each case.

C. Common post harvest diseases of oranges imported into the U.K. from South Africa

The more common fungal diseases found in oranges imported into the United Kingdom from South Africa are described below.

1. Green Mould

Green Mould caused by P.digitatum Sacc. is by far the most common cause of wastage in oranges. The disease symptoms are described on P. 14 .

2. Water Rot

Water Rot is the name given to small water soaked areas of rind, which have no mycelium on the surface. It is similar to the first stage of Green Mould infection and isolations made from fruit infected with Water Rot almost invariably yielded the Green

Mould fungus, P.digitatum. However, because the first stages of infection by P.italicum and other fungi are indistinguishable from Green Mould, the term Water Rot is used.

(3) Blue Contact Mould

Blue Contact Mould, caused by P.italicum Wehmer, results in much less wastage of fruit than does Green Mould. It starts from growth of spores at injuries or from contact of uninjured fruit with Blue Mould infected fruit. It is characterized by a blue powdery sporulating area, surrounded by a narrow white fringe, which in turn is surrounded by a halo of water soaked tissue. Under commercial conditions it is found that there is no marked tendency for the paper wrapper to adhere to the Blue Mould infected fruit as happens with Green Mould.

(4) Sour Rot

Sour Rot is a soft slimey decay caused by Geotrichum candidum (Ferr.) var. citri-aurantii. It produces a buff-coloured watery rot in which the white superficial wrinkled mycelium forms a skum under-moist conditions. Decomposed fruit flatten into a watery-mass which drips and results in spread by contact to other fruit. It usually has a putrid sour odour which is especially attractive to fruit flies. Mixtures of the Sour Rot fungus with other fungi, and in particular P.digitatum, greatly speed up decay. Injuries, defects and devitalized buttons are thought to contribute to ease of infection.

5. Trichoderma Rot

Trichoderma Rot is a firm pliable decay caused by Trichoderma lignorum (Tode) Harz. It is brown to cocoa brown in colour and under moist conditions meadow green spore masses are produced in clumps. The fungus itself usually has a coconut-like odour.

6. Alternaria Rot

Alternaria Rot or Black Rot of oranges occurs as a brownish or black slow decay at the stem end or the stylar end of fruit and is caused by Alternaria citri. Fruit affected by this disease often look sound on the exterior but when cut have a dry, black decayed area within. It is commonly found in Navels where infection occurs at the navel end.

7. Anthracnose Rot

Anthracnose Rot like Anthracnose Spot, which is a hard, firm brown rot usually affecting the rind only, is caused by Colletotrichum gloeosporioides. Anthracnose Rot may develop from an anthracnose spot inwardly but it usually appears as a firm to semi-pliable small brown area around the stem end. Later, the area enlarges and a soft pliable rot develops which may be dark brown or black in colour. The decay advances slowly along the core, inner rind and pulp. Anthracnose Rot is usually slow unless the fruit is overripe or of low vitality.

8. Diplodia Stem End Rot

Diplodia Stem End Rot is caused by Diplodia natalensis.

At first it develops as an almost imperceptible discolouration around the stem. This is soon followed by a leathery, pliable, brown discolouration of the rind. Later the decay usually becomes darker, externally and internally and develops quickly through to the stylar end where it shows on the surface before all the rind is affected. As it develops from the stem end it often shows wide bands on the surface corresponding to the divisions of the segments within.

9. Phomopsis Stem End Rot

Phomopsis Stem End Rot is caused by Diaporthe citri (Faw.) Wolf. It starts with very slight softening and an almost imperceptible discolouration around the stem button and later changes to brown and drab with greater pliability. Inside, the light-brown decay advances most rapidly along the centre and the inner portion of the peel. Internally the tissue does not discolour or darken so rapidly as in Diplodia Stem End Rot, which it closely resembles.

10. Black Spot

Black Spot usually appears as small reddish-brown to black spots on the surface of fruit and is caused by Guignardia citricarpa Kielly (Phoma citricarpa Kielly, Mc.Alp.). Although the spots are usually small they may spread up to 1 cm. in diameter and sometimes the spots may coalesce making the fruit unmarketable. As a spot matures a reddish-brown raised margin forms around the outer edges, while the centre sometimes becomes

depressed and assumes a light-tan colour. Lesions not only occur on the surface but also extend into the rind tissues from 1 to 2mm. Occasionally, where the spots coalesce the deeper layers of the rind may be affected.

SECTION 11 - TRANSPORT EXPERIMENTS, 1961

A. Effect of SOPP on the control of wastage

Variety: Midseasons (Dan Williams Seedlings).

Packhouse: White River Fruitgrowers' Co-op.

Examination: At London, 10-11 days after arrival at Southampton i.e. 43-44 days after packhouse treatment.

Replicates: 5 cases (count 216 i.e. 216 fruit/case) per treatment.

Wrappers: Sulphite, (plain).

Results are shown in Table 75.

Table 75. Effect of SOPP on the control of wastage in Midseasons (Dan Williams Seedlings, count 216)

Treatment	Rinsed after treatment	% Wastage					
		Total	Green Mould	Water Rot	Brown Rot	Sour Rot	Blue Mould
Water	No	3.2	3.1	0.1	-	-	-
0.15% SOPP	No	3.0	3.0	-	-	-	-
0.5% SOPP	No	2.7	1.6	0.4	0.3	0.4	-
1.5% SOPP	Yes	3.0	2.4	0.1	-	0.4	0.1

SOPP did not control wastage.

Green Mould was the most common rot found. Sour rot, Blue Mould and Brown Rot also occurred but to a much lesser extent. Brown Rot was caused by Trichoderma lignorum.

B. Effect of rinsing fruit in SOPP on day of harvesting and one day after harvesting on the control of wastage

Variety: Navel.

Packhouse: Hall and Sons:

Examination: At London, 10-11 days after arrival at Southampton i.e. 38-40 days after packhouse treatment.

Replicates: 3-4 cases (count 126) per treatment.

Wrappers: Sulphite.

Results are shown in Tables 76 and 77 .

Table 76. Effect of SOPP on the control of wastage in Navels (count 126) treated on day of harvesting

Treatment	Rinsed after treatment	% Wastage					
		Total	Green Mould	Water Rot	Blue Mould	Brown Rot	Sour Rot
Water	No	6.4	5.0	-	-	0.8	0.5
0.15% SOPP	No	5.3	4.2	0.3	-	-	0.8
0.5% SOPP	No	5.6	2.9	-	0.3	0.3	2.1
1.5% SOPP	Yes	5.4	3.4	-	-	0.2	1.8

Table 77. Effect of SOPP on the control of wastage in Navels (count 126) treated one day after harvesting

Treatment	Rinsed after treatment	% Wastage					
		Total	Green Mould	Water Rot	Blue Mould	Brown Rot	Sour Rot
Water	No	5.4	4.0	-	-	0.2	1.2
0.15% SOPP	No	6.0	4.4	0.4	-	0.2	1.0
0.5% SOPP	No	3.2	1.3	-	0.3	0.6	1.0
1.5% SOPP	Yes	3.0	1.6	0.2	-	0.4	0.8

Treatment with SOPP one day after picking was much more effective in the control of wastage. Treatment on the day of picking was almost ineffective. Wastage was controlled best by rinsing in 0.5 and 1.5% SOPP one day after harvesting.

Green Mould was the most common waste type. Sour Rot also occurred to a considerable extent and Blue Mould and Brown Rot less frequently. Brown Rot was caused by Trichoderma lignorum.

C. Effect of rinsing fruit in SOPP on day of harvesting and one day after harvesting on the control of wastage

Variety: Navel.

Packhouse: Hall and Sons.

Examination: At London, 12 days after arrival at Southampton. i.e. 46-47 days after packhouse treatment.

Replicates: 3-4 cases (count 126) per treatment.

Wrappers: Sulphite.

Results are shown in Tables 78 and 79 .

Table 78. Effect of SOPP on the control of wastage in Navels (count 126) treated on day of harvesting

Treatment	Rinsed after treatment	% Wastage				
		Total	Green Mould	Water Rot	Brown Rot	Sour Rot
Water	No	12.1	11.1	-	0.2	0.8
0.15% SOPP	No	7.7	6.5	0.2	0.2	0.8
0.5% SOPP	Yes	5.8	4.4	-	0.2	1.2
1.5% SOPP	Yes	8.6	6.3	0.4	1.4	0.4

Table 79. Effect of SOPP on the control of wastage in Navels (count 126) treated one day after harvesting

Treatment	Rinsed after treatment	% Wastage				
		Total	Green Mould	Water Rot	Brown Rot	Sour Rot
Water	No	6.2	5.4	0.4	-	0.4
0.15% SOPP	No	4.8	4.0	0.3	0.5	-
0.5% SOPP	Yes	4.6	3.6	0.2	0.4	0.4
1.5% SOPP	Yes	2.4	1.4	-	0.6	0.4

Treatment with SOPP one day after harvesting was much more effective in reducing wastage than treatment on the day of harvesting. Wastage was controlled best at 1.5% concn. one day after harvesting.

Green Mould was the predominant waste type. Sour Rot and Brown Rot were also found. Brown Rot was caused mainly by Trichoderma lignorum and to a lesser extent by Alternaria citri and Diplodia natalensis.

D. Effect of SOPP and diphenyl wrappers on the control of wastage

Variety: Navel.

Packhouse: Kiepersol Co-op.

Examination: At London, 11 days after arrival at Southampton i.e. 41 days after packhouse treatment.

Replicates: (a) 2-3 cases (count 126) per treatment.

(b) 2-3 cases (count 150) per treatment.

The wastage found in fruit of count 126 and 150 are shown in Tables 80 and 81 respectively.

Table 80. Effect of SOPP on the control of wastage in Navels (count 126)

Treatment	Rinsed after treatment	Wrapper	% Wastage				
			Total	Green Mould	Water Rot	Brown Rot	Sour Rot
Water	No	Sulphite	31.7	23.6	0.3	-	8.0
0.15% SOPP	No	"	16.7	11.9	0.4	0.4	4.0
0.5% SOPP	Yes	"	21.6	14.4	2.8	0.8	3.6
1.5% SOPP	Yes	"	34.1	23.7	8.8	0.3	1.4

Table 81. Effect of SOPP and diphenyl wrappers on the control of wastage in Navels (count 150)

Treatment	Rinsed after treatment	Wrapper	% Wastage				
			Total	Green Mould	Water Rot	Brown Rot	Sour Rot
Water	No	Sulphite	22.3	17.7	0.3	0.3	4.0
0.15% SOPP	No	"	22.1	16.3	2.5	0.4	2.9
0.5% SOPP	Yes	"	28.1	21.1	4.7	-	2.3
1.5% SOPP	Yes	"	19.7	15.4	3.0	0.3	1.0
Water	No	Diphenyl*	11.3	8.6	2.3	-	0.3
1.5% SOPP	Yes	"	12.0	9.8	1.8	0.4	-

* Diphenyl wrappers contained 45 mg. diphenyl per 100 sq.in.

Treatment of count 126 fruit with 0.15% and 0.5% SOPP reduced wastage but even with these treatments a considerable amount of wastage was still found. Control, however, was better at 0.15% concn. (without rinsing).

With count 150 fruit diphenyl wrappers reduced wastage considerably. Wastage was not reduced further by rinsing in SOPP prior to packing with diphenyl wrappers.

Green Mould was the most common cause of wastage in both count 150 and 126 fruit. Sour Rot was also fairly frequent. Brown Rots were caused by both Alternaria citri and Trichoderma lignorum.

E. Effect of SOPP and diphenyl wrappers on the control of wastage

Variety: Navel.

Packhouse: Kiepersol Co-op.

Examination: At London, 11 days after arrival at Southampton i.e. 39 days after packhouse treatment.

Replicates: 2 cases (count 126) per treatment.

The wastage found on examination is shown in Table 82.

Table 82. Effect of SOPP and diphenyl wrappers on the control of wastage in Navels (count 126)

Treatment	Wrapper	% Wastage				
		Total	Green Mould	Water Rot	Brown Rot	Sour Rot
None	Sulphite	61.8	56.6	0.8	4.0	0.4
None	Diphenyl (S.40)*	32.9	31.4	0.4	1.2	-
0.15% SOPP	Sulphite	21.1	19.9	1.2	-	-
0.15% SOPP	Diphenyl (S.40)	22.6	18.7	2.0	2.0	-
0.15% SOPP	Diphenyl[x] (A.35)	17.1	15.8	0.4	-	0.9

* Diphenyl (S.40) wrappers were ex Sweden, strip impregnated and contained 40 mg. diphenyl per 100 sq. in.

[x] Diphenyl (A.35) wrappers were ex Fruit Wrappers Ltd., (produced and whole surface impregnated in the U.S.A.) and contained 35 mg. diphenyl per 100 sq. in.

Both SOPP rinse and diphenyl wrappers used alone reduced wastage. Best control was obtained in fruit treated with 0.15% SOPP and wrapped in diphenyl (A.35) wrappers.

Green Mould was the predominant waste type. Sour Rot and Brown Rot were also found. Brown Rot was caused by Trichoderma lignorum

F. Effect of different fungicides on the control of wastage

Variety: Navel.

Packhouse: White River Fruitgrowers' Co-op.

Packhouse treatment: Fruit received full packhouse treatment (no SOPP or wax was applied). Fruit of all treatments except (g) (see below) were dipped into a suspension of Green Mould spores and left to dry.

The following treatments were then applied:

(a) 2% Potassium sorbate in Citrashine wax applied with normal wax applicator.

(b) 0.1% Pomarsol (TMTD) at 100°F., not rinsed after treatment.

(c) 0.1% R1526 (Riedel de Haën) at 100°F., not rinsed after treatment.

(d) 0.5% Ethyl-p-hydroxy benzoate at 100°F., not rinsed after treatment.

(e) 0.15% SOPP at 120°F., not rinsed after treatment.

(f) No treatment after dipping fruit in suspension of Green Mould spores.

(g) No treatment.

Replicates: 4 cases (count 126) in all treatments except (g) in which there were two.

Wrappers: Sulphite.

Examination: Two examinations were carried out:

(i) At Capetown 20 days after treatment. All cases were examined except one case in treatment (g). Wasty fruit were removed and untreated fruit were added to fill slack cases.

(ii) At London, 11-12 days after arrival at Southampton i.e. 47-48 days after treatment.

Wastage (expressed as % of fruit examined) found at Capetown is shown in Table 83 . Wastage (expressed as % of treated fruit arriving in London) is shown in Table 84 .

Table 83. Effect of fungicides on the control of wastage in Navels (count 126)

Examination at Capetown 20 days after treatment.

Treatment	Rinsed in suspension of Green Mould Spores	% Wastage *
2% Potassium sorbate	Yes	30.0
0.1% Pomarsol (TMTD)	Yes	22.4
0.1% R1526 (Riedel de Haën)	Yes	20.0
0.5% Ethyl p-hydroxy benzoate	Yes	30.2
0.15% SOPP	Yes	26.0
NONE	Yes	34.5
NONE	No	7.1

* Expressed as % of fruit examined.

Table 84. Effect of fungicides on the control of wastage in Navels (count 126) repacked after removal of wasty fruit 20 days after treatment

Examination at London, 27-28 days after repacking or 47-48 days after treatment.

Treatment	Rinsed in suspn. of Green Mould spores	% Wastage *				
		Total	Green Mould	Water Rot	Sour Rot	Brown Rot
2% Potassium sorbate	Yes	44.6	42.0	0.9	1.5	0.3
0.1% Pomarsol (TMTD)	Yes	24.5	22.4	-	2.1	-
0.1% R1526 (Riedel de Haën)	Yes	23.7	20.9	0.8	2.1	-
0.5% Ethyl p-hydroxy benzoate	Yes	41.0	39.3	0.3	1.4	-
0.15% SOPP	Yes	30.4	29.6	0.4	0.4	-
None	Yes	27.1	27.1	-	-	-
None	No	30.1	27.8	0.8	0.8	0.8

*Expressed as % of treated fruit arriving in London.

None of the treatments were really effective in reducing wastage. Wastage was however reduced slightly by R1526 and TMTD.

In addition to Green Mould, Sour Rot and Brown Rot were also found. The Brown Rots were mainly caused by Diplodia natalensis.

SECTION 111 - TRANSPORT EXPERIMENTS, 1962

1. Effect of spore concentration on the development of wastage

Variety: Navel.

Packhouse: Ngonini Estates, Swaziland.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP but no wax was applied. On the same day, the fruit were dipped in an aqueous suspension of Green Mould spores at the following concentrations:

(a) 10^6 spores/ml.

(b) 10^4 " / " .

(c) 10^2 " / " .

(d) Nil.

After dipping the fruit were left to dry, wrapped in plain wrappers and packed for export.

Examination: At London, 12 days after arrival at Southampton i.e. 37 days after dipping in spore suspension.

Replicates: 6 cases (count 150) per treatment.

Results are shown in Table 85 .

Table 85. Effect of spore concentration on the development of wastage in Navels (count 150)

Spores/ml.	% Wastage					
	Total	Green Mould	Water Rot	Brown Rot	Sour Rot	Blue Mould
10^6	6.6	5.6	0.4	0.1	0.3	0.1
10^4	3.6	3.2	0.2	-	-	0.1
10^2	3.1	2.3	0.4	-	0.3	-
Nil	2.1	1.7	-	0.3	0.1	-

Wastage increased with spore concentration.

In addition to Green Mould, Sour Rot and Brown Rot caused by Trichoderma lignorum also occurred.

B. Effect of spore concentration on the development of wastage

Variety: Valencia.

Packhouse: Ngonini Estates, Swaziland.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP but no wax was applied. One day later, the fruit were dipped in an aqueous suspension of Green Mould spores at the following concentrations:

(a) 10^7 spores/ml. (approx.).
(b) 10^5 " / " (").
(c) 10^3 " / " (").
(d) 10^1 " / " (").
(e) Nil.

After dipping the fruit were left to dry for 2-3hr., wrapped in plain wrappers and packed for export.

Examination: At London, 17 and 19 days after arrival at Southampton, i.e. 39 and 41 days after dipping in spore suspension.

Replicates: 5 cases (count 226) per treatment.

Results are shown in Table 86 .

Table 86. Effect of spore concentration on the development of wastage in Valencias (count 226)

Spores/ml. (approx.)	% Wastage				
	Total	Green Mould	Water Rot	Brown Rot	Sour Rot
10^7	8.4	8.3	-	0.1	-
10^5	3.0	2.8	0.1	0.1	-
10^3	1.8	1.6	0.1	0.1	-
10	2.7	2.3	-	0.2	0.2
Nil	2.5	1.9	0.3	0.3	0.1

Wastage was only markedly affected by spore concentrations greater than 10^3/ml.

In addition to Green Mould, Sour Rot and Brown Rot caused by Colletotrichum gloesporioides were also found.

C. Effect of spore concentration on development of wastage

Variety: Mediterranean Sweet.

Packhouse: White River Fruit Growers' Co-op.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.1% SOPP and waxing with Citrashine wax. Two days later, the fruit were dipped in an aqueous suspension of Green Mould spores at the following concentrations:

(a) Nil.
(b) 10 spores/ml. (approx).
(c) 10^2 " / " (").
(d) 10^3 " / " (").
(e) 10^4 " / " (").
(f) 10^5 " / " (").
(g) 10^6 " / " (").

After dipping the fruit were dried, wrapped in plain wrappers and packed for export.

Examination: Two examinations were carried out at London:

(i) 11-12 days after arrival at Southampton i.e. 34-35 days after "dipping" (All wasty fruit were removed and healthy fruit were wrapped and repacked).

(ii) 24 days after arrival at Southampton i.e. 47-48 days after "dipping".

Replicates: 4 cases (count 252) per treatment.

The wastage, expressed as % of treated fruit, found at the first and second examinations is shown in Tables 87 and 88 respectively.

Table 87. Effect of spore concentration on the development of wastage in Mediterranean Sweets (count 252)

Examination 34-35 days after "dipping".

Spores/ml. (approx.)	% Wastage				
	Total	Green Mould	Water Rot	Brown Rot	Blue Mould
0	1.7	1.3	-	0.4	-
10	2.1	1.5	-	0.6	-
10^2	2.5	2.1	0.1	0.1	0.2
10^3	2.7	2.6	-	0.1	-
10^4	4.5	4.4	-	0.1	-
10^5	10.0	9.8	-	0.2	-
10^6	11.0	10.3	0.3	0.4	-

Table 88. Wastage found in Mediterranean Sweets (count 252) 12-13 days after first examination

Spores/ml. (approx.)	% Wastage					
	Total	Green Mould	Water Rot	Brown Rot	Sour Rot	Blue Mould
0	1.8	0.8	-	0.4	-	0.6
10^1	2.3	1.6	-	0.2	-	0.5
10^2	2.3	1.5	-	0.5	0.1	0.2
10^3	4.9	3.5	-	1.0	-	0.4
10^4	6.3	6.0	-	0.1	-	0.2
10^5	8.2	7.5	0.1	0.2	-	0.4
10^6	11.4	10.5	0.3	0.3	-	0.3

A further development of wastage was observed after the first examination. At both examinations the amount of wastage increased with increase in spore concentration.

In addition to Green Mould, Blue Mould, Sour Rot and Brown Rot were also found. The Brown Rot was caused mainly by Diplodia natalensis and Colletotrichum gloeosporioides, and to a lesser extent by Trichoderma lignorum.

D. Effect of spore concentration and age of trees on the control of wastage

Variety: Navel.

Packhouse: Boschkom Estates.

Packhouse treatment: Fruit received packhouse treatment but no SOPP or wax was applied. After packhouse washing, fruit were treated as follows:

(a) Fruit (count 150), taken from trees 53 yrs. old, were dipped into a suspension of Green Mould spores, containing 10^6 spores/ml.

(b) Fruit (count 150), taken from trees 53yrs. old, were dipped into a suspension of Green Mould spores, containing 10^4 spores/ml.

(c) Fruit (count 150), taken from trees 53 yrs. old were not treated further.

(d) Fruit (count 126), taken from trees 5 yrs. old, were dipped into a suspension of Green Mould spores, containing 10^6 spores/ml.

(e) Fruit (count 126) taken from trees 5 yrs. old, were dipped into a suspension of Green Mould spores, containing 10^4 spores/ml.

(f) Fruit (count 126), taken from trees 5 yrs. old, were not treated further.

Examination: At London, 11 days after arrival at Southampton, i.e. 39 days after packhouse treatment.

Replicates: 3 cases (count 150 or 126) per treatment.

Wrappers: Plain.

Results are shown in Table 89 .

Table 89. Effect of rinsing fruit in Green Mould spores and age of trees on the development of wastage in Navels (count 126 and 150)

Fruit Count	Concn. of Green Mould spores	Age of trees (yrs.)	% Wastage			
			Total	Green Mould	Water Rot	Brown Rot
150	10^6/ml.	53	62.1	54.9	6.0	1.1
150	10^4/ml.	53	13.3	9.0	4.3	-
150	0	53	2.9	2.7	0.2	-
126	10^6/ml.	5	42.9	40.3	1.6	1.0
126	10^4/ml.	5	12.7	11.7	1.0	-
126	0	5	0.3	0.3	-	-

Fruit from old trees consistently appeared more susceptible to wastage than fruit from young trees. This is so irrespective of whether the fruit had been dipped in spore suspension or not. The more dense the spore suspension the greater was the amount of wastage found.

In addition to Green Mould, Brown Rots caused by Alternaria citri and Trichoderma lignorum were also found.

E. Effect of mouldy fruit in the orchard before harvesting on the development of wastage

Variety: Navel and Pineapple.

Packhouse: Hall and Sons, Mataffin.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP and waxing with Flavourseal.

Examination: At London, 11 days after arrival at Southampton i.e. 38-41 days after packhouse treatment.

Replicates: 5 cases per treatment, Navels and Pineapples were counts 150 and 216 respectively.

Wrappers: Plain.

Results are shown in Table 90 .

Table 90. Effect of mouldy fruit in the orchard before harvesting on the development of wastage in Navels (count 150) and Pineapples (count 216)

Variety	Orchard	No. of mouldy fruit/tree 6-7 days before harvesting	Age of tree (yrs.)	% Wastage				
				Total	Green Mould	Wither Rot	Sour Rot	Br. Rot
Navel	B.S.25	1.6	43	8.1	7.1	0.8	-	0.3
Navel	M.W.1A	1.2	53	6.5	5.5	0.8	0.3	-
Pineapple	M.W.31	0.2	30	2.7	2.7	-	-	-
Pineapple	B.W.104	1.2	21	0.8	0.5	0.1	0.1	-

Navels were more wasty than Pineapples. With Navels, the fewer the number of mouldy fruit in the orchard before harvesting the less was the wastage that developed subsequently.

The reverse held for Pineapples.

With both varieties Green Mould was the predominant waste type. Brown Rot and Sour Rot also occurred. Brown Rot was caused by Trichoderma lignorum and Alternaria citri.

F. Effect of mouldy fruit in the orchard before harvesting on the development of wastage

Variety: Mediterranean Sweet.

Packhouse: Hall and Sons, Mataffin.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP and waxing with Flavourseal.

Examination: At London, 10 days after arrival at Southampton, i.e. 41 days after packhouse treatment.

Replicates: 10 cases (count 216) per treatment.

Wrappers: Plain.

Results are shown in Table 91.

Table 91. Effect of mouldy fruit in the orchard before harvesting on the development of wastage in Navels (count 216).

Orchard (M.W.17)	No. of mouldy fruit/tree 8 days before harvesting	Age of trees (yr.)	% Wastage				
			Total	Green Mould	Water Rot	Sour Rot	Brown Rot
Sanitated	0.1	38	6.1	5.7	0.3	0.1	0.1
Non-Sanitated	18.5	38	6.3	5.6	0.4	0.2	0.1

The sanitary conditions in the orchard did not appear to affect the amount of wastage found after harvesting.

Green Mould was the most common cause of wastage. Brown Rot and Sour Rot also occurred. Brown Rot was caused by Trichoderma lignorum and Colletotrichum gloeosporioides.

G. Effect of mouldy fruit in the orchard before harvesting and diphenyl wrappers on wastage

Variety: Navel.

Packhouse: Hall and Sons, Mataffin.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP but wax was not applied. The fruit were then wrapped in plain or diphenyl wrappers, containing 21mg. diphenyl/100 sq.in.

Examination: At London, 11-12 days after arrival at Southampton i.e. 33-34 days after packhouse treatment.

Replicates: 6 cases (count 150) per treatment.

Results are shown in Table 92.

Table 92. Effect of mouldy fruit in the orchard before harvesting and diphenyl wrappers on the development of wastage in Navels (count 150)

Du Roi Orchard	No. of mouldy fruit/tree prior to harvesting	Wrappers	% Wastage				
			Total	Green Mould	Water Rot	Sour Rot	Blue Mould
Sanitated	0.05-0.1	Plain	1.8	0.8	-	0.7	0.3
Unsanitated	4.5	Plain	0.8	0.6	-	-	0.3
Sanitated	0.05-0.1	Diphenyl	0.9	0.7	-	-	0.2
Unsanitated	4.5	Diphenyl	1.9	1.2	0.3	0.3	0.1

The results were inconclusive.

H. Effect of SOPP on the control of wastage

Variety: Mediterranean Sweet.

Packhouse: Crocodile Valley Citrus Estates.

Packhouse treatment: The fruit received full packhouse treatment which included rinsing in 0.15% SOPP but no wax was applied.

On the same day the fruit received the following treatments in SOPP tank:

(a) Cold water at 60°F.

(b) Hot water at 110°F.

(c) Hot SOPP (approx. 0.15%) at 110°F.

Examination: At London, 10-11 days after arrival at Southampton i.e. 35-36 days after treatment.

Replicates: 10 cases (count 252) per treatment.

Wrappers: Plain.

Results are shown in Table 93 .

Table 93. Effect of SOPP on the control of wastage in Mediterranean Sweets (count 252)

Treatment	Temperature of dip.soln.	% Wastage				
		Total	Green Mould	Water Rot	Brown Rot	Blue Mould
Water	60°F.	5.2	5.0	0.1	-	-
Water	110°F	4.8	4.6	0.1	D.1	-
0.15% SOPP	110°F.	1.5	1.3	-	0.1	0.1

SOPP at 0.15% concn. and 110°F. was quite effective in the control of wastage.

Green Mould was the most common cause of wastage. Brown Rot and Blue Mould were also found. Brown Rot was caused mainly by Trichoderma lignorum.

I. Effect of SOPP pH on the control of wastage

Variety: Valencia.

Packhouse: White River Fruitgrowers' Co-op.

Packhouse treatment: The fruit were washed, graded and sized and received no SOPP or wax treatment. They were then passed through a suspension of Green Mould spores and after drying, were left for 2 days. After this time the following treatments were applied as rinses in a tank:-

(a) 0.5% Preventol Ue 5921 + 150p.p.m. Trilon B Powder (BASF) at pH 10.5,11.5 and 12.5 - measured at 48°C.

(b) 0.5% Sofonate + 0.25% Hexamine + 150p.p.m. Trilon B powder (BASF) at pH 10.6,11.5 and 12.5 - measured at 48°C.

(c) Control: Water at 45°C.

(a) and (b) are different SOPP - buffer formulations.

After treatment the fruit were dried, wrapped in sulphite wrappers and packed for export.

Examination: At London 6-7 days after arrival at Southampton i.e. 35-38 days after packhouse treatment.

Replicates: 3 cases (count 216) per treatment.

Wrappers: Sulphite.

Results are shown in Table 94 .

Table 94. Effect of SOPP pH on the control of wastage in Valencias (count 216).

Treatment	pH	% Wastage					
		Total	Green Mould	Water Rot	Blue Mould	Brown Rot	Sour Rot
0.5% Preventol Ue 5921 150p.p.m. Triolon B Powder (BASF)	10.5	12.4	10.1	0.8	0.2	0.5	0.9
"	11.5	19.7	16.7	2.3	-	0.2	0.5
"	12.5	17.7	17.1	0.3	-	0.2	0.2
0.5% Sofonate +0.25% Hexamine + 150p.p.m. Trilon B Powder (BASF)	10.6	17.5	15.9	0.3	-	0.5	0.8
"	11.5	15.6	14.2	0.5	-	0.3	0.6
"	12.5	15.9	14.1	0.9	-	0.8	0.2
Water	-	34.3	32.9	1.1	-	0.2	0.2

Both Preventol Ue 5921 and Sofonate-Hexamine were effective in the control of wastage.

The pH of Preventol Ue 5921 Solutions had an effect on the efficiency of this fungicide. Least wastage was found in those fruit treated with this soln. at pH 10.5.

Varying the pH of Sofonate-Hexamine did not have a marked effect on the wastage found.

As expected Green Mould was the most common cause of wastage. Blue Mould, Sour and Brown Rots were also found. Brown Rots were caused by Phomopsis citri (Stem End Rot), Trichoderma lignorum, Alternaria citri and Guignardia citricarpa (Black spot).

J. Effect of SOPP and TMTD on the control of wastage

Variety: Navel.

Packhouse: Hall and Sons, Mataffin.

Packhouse treatment: Fruit received full packhouse treatment (no wax was applied). On the same day fruit were treated as follows:

(a) Not dipped.

(b) Dipped in water at 120°F. for 3 min. and rinsed.

(c) " " 0.15% SOPP at 120°F. for 3 min.

(d) " " 1.5% SOPP at 120°F. and rinsed.

(e) " " 0.1% TMTD at 120°F. and rinsed.

(f) Sprayed with a nutrient soln., composed of 200ml. orange concentrate, 50g. sucrose, and 2g. $(NH_4)_2SO_4$ in 2 l. water, kept moist for 2 days and then dipped in 1.5% SOPP for 3 minutes and rinsed (i.e. treated with SOPP after pregermination of spores).

Examination: At London, 11 days after arrival at Southampton i.e. 41 days after packhouse treatment.

Replicates: 5 cases (count 150) per treatment.

Wrappers: Plain.

Results are shown in Table 95 .

Table 95. Effect of SOPP and TMTD on the control of wastage in Navels (count 150)

Treatment	% Wastage				
	Total	Green Mould	Water Rot	Brown Rot	Sour Rot
None	3.2	2.9	0.1	-	0.1
Water + Rinse	5.1	4.4	0.3	0.3	0.1
0.15% SOPP	1.7	1.5	-	0.3	-
1.5% SOPP + Rinse	2.4	2.3	-	-	0.1
0.1% act. TMTD + Rinse	2.3	2.1	0.1	-	-
1.5% SOPP + Rinse after pregermination	2.3	1.7	-	-	0.5

Wastage was controlled most effectively by rinsing in 0.15% SOPP. Pregermination of spores followed by rinsing in 1.5% SOPP was only slightly more effective in controlling wastage than rinsing in 1.5% SOPP only.

Green Mould and to a lesser extent Brown Rot and Sour Rot were the causes of wastage.

K. Effect of SOPP and nickel sulphate on the control of wastage

Variety: Mediterranean Sweet.

Packhouse: Hall and Sons, Mataffin.

Packhouse treatment: Fruit received packhouse treatment but no SOPP or wax was applied. Fruit were treated as follows on the same day:

(a) Not dipped.

(b) Three min. dip in tank + water at 120°F.

(c) Three min. dip in tank + 0.15% SOPP at 110°F.

(d) Three min. dip in tank + 1.5% SOPP at 120°F. then rinsed.

(e) Three min. dip in tank + 0.1% $NiSO_4$ at 120°F. then rinsed.

(f) Sprayed with a nutrient soln., composed of 200ml. orange concentrate, 50g. sucrose, and 2g. $(NH_4)_2SO_4$ in 2 l. water, kept moist for 2 days, then dipped for 3 min. in tank containing 1.5% SOPP at 120°F. and then rinsed.

Examination: At London, 10-11 days after arrival at Southampton i.e. 40-41 days after packhouse treatment.

Replicates: 5 cases (count 216) per treatment.

Wrappers: Plain.

Results are shown in Table 96.

Table 96. Effect of SOPP and nickel sulphate on the control of wastage in Mediterranean Sweets (count 216).

Treatment	Temp. of dip. soln.	% Wastage					
		Total	Green Mould	Water Rot	Brown Rot	Sour Rot	Blue Mould
None	-	17.3	14.8	1.1	0.1	0.3	1.0
Water	120°F	8.2	7.9	0.3	-	0.1	-
0.15% SOPP	110°F	3.9	3.6	0.1	0.1	0.1	-
1.5% SOPP	120°F	6.9	5.4	0.5	0.1	0.9	-
0.1% $NiSO_4$	120°F	15.4	12.4	0.6	0.4	1.6	0.5
Nut. Soln. + 1.5% SOPP	120°F	9.0	6.0	0.8	0.3	1.9	-

Only 0.15% SOPP was effective in the control of wastage.

Green Mould was again the most common cause of wastage. Brown Rot, Sour Rot and Blue Mould were also found but less frequently. Brown Rot was caused by Trichoderma lignorum and Colletotrichum gloeosporioides.

L. Effect of different fungicides on the control of wastage

Variety: Navel.

Packhouse: White River Fruitgrowers' Co-op.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP but no wax was applied. On the following day, the fruit, except those in treatment (g), were dipped in a suspension of Green Mould spores (10^6 spores/ml.) and left to dry.

On the same day the fruit were treated as follows:-

(a) No treatment.

(b) Water at 48°C.

(c) 0.1% $NiSO_4$ (i.e.45.5g.$NiSO_4.7H_2O$ in 25l. water) at 48°C.

(d) 0.1% $HgCl_2$(i.e. 25g.$HgCl_2$in 25 l. water) at 48°C.

(e) 1.5% Preventol-Hex-NaOH (SOPP)(i.e. 1875 ml. Preventol Hexamine + 100g.NaOH) at 48°C.

(f) 0.15% SOPP at 48°C. in tank.

(g) No treatment (no spores).

Examination: At London, 11-12 days after arrival at Southampton i.e. 41-42 days after dipping in spore suspension.

Replicates: 5 cases (count 150) per treatment.

Wrappers: Plain.

Results are shown in Table 97 .

Table 97. Effect of different fungicides on the control of wastage in Navels (count 150)

Treatment	Rinsed in suspension of Green Mould spores	% Wastage				
		Total	Green Mould	Water Rot	Brown Rot	Blue Mould
None	Yes	25.2	22.8	2.3	-	-
Water	Yes	14.5	13.2	1.3	-	-
0.1% $NiSO_4$	Yes	14.8	14.4	0.4	-	-
0.1% $HgCl_2$	Yes	3.7	3.5	0.3	-	-
1.5% SOPP	Yes	4.0	3.6	0.3	-	0.1
0.15% SOPP	Yes	9.9	9.7	0.1	-	-
None	No	1.7	1.5	0.1	0.1	-

Both $HgCl_2$ and 1.5% SOPP were effective in the control of wastage.

In addition to Green Mould, Brown Rot and Blue Mould were also found but infrequently.

M) Effect of diphenyl wrappers on the control of wastage

Variety: Navel.

Packhouse: Sundays River Co-op.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP but no wax was applied. This was followed by dipping the fruit into a suspension of Green Mould spores, containing 10^5 spores/ml. and then wrapping as follows:-

(a) In sulphite (plain) wrappers.

(b) In diphenyl (Sundays River Co-op.) wrappers, containing 54 mg. diphenyl/100 sq.in.

(c) In diphenyl ("Sax")* wrappers, containing 55 mg. diphenyl/100 sq. in.

*"Sax" is a brand name for paper.

Examination: At London, 12 days after arrival at Southampton i.e. 36 days after packhouse treatment.

Replicates: 5 cases (count 150) per treatment.

Results are shown in Table 98 .

Table 98. Effect of diphenyl wrappers on the control of wastage in Navels (count 150)

Wrapper	% Wastage				
	Total	Green Mould	Water Rot	Brown Rot	Sour Rot
Sulphite	9.5	8.8	0.3	0.3	0.1
Diphenyl (S.R.C.) 54 mg./100 sq. in.	3.9	3.9	-	-	-
Diphenyl (Sax) 55 mg./100 sq.in.	7.3	6.3	0.8	0.1	0.1

Only S.R.C. diphenyl wrappers controlled wastage effectively.

In addition to Green Mould, Brown Rot, caused mainly by Colletotrichum gloesporioides, and Sour Rot were also found.

V. Effect of diphenyl wrappers on the control of wastage

Variety: Midesason Bailidge Early.

Packhouse: Sundays River Co-op., Hermitage.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP and waxing with Flavourseal (no bleach was applied). On the same day or one day later all fruit were dipped in an aqueous suspension of Green Mould spores, containing 150 mg. spores in 30 l. water, left to dry for 2-4 hr. and then wrapped in wrappers of the following types before packing:-

(a) Plain wrappers.

(b) "T5" * diphenyl wrappers, containing 60 mg. diphenyl/ 100 sq. in.

(a) "Sax"* diphenyl wrappers, containing 60mg.diphenyl/ 100 sq. in.

(d) SOPP wrappers.

* "T5" and "Sax" are the brand names for paper of different manufacturers.

Examination: At London, 10 days after arrival at Southampton i.e. 40-41 days after dipping in spore suspension.

Replicates: 4 cases (count 252) per treatment.

Results are shown in Table 99 .

Table 99. Effect of diphenyl wrappers on the control of wastage in Midseasons Bailidge Early (count 252)

Wrapper	% Wastage		
	Total	Green Mould	Water Rot
Plain	3.4	3.3	0.1
T5 Diphenyl (60 mg./100 sq.in.)	1.6	1.6	-
Sax Diphenyl (60 mg./100 sq.in.)	0.7	0.6	0.1
SOPP	1.3	1.2	0.1

Sax wrappers were the most effective in the control of wastage.

O. Effect of wrappers and inserts in cartons on the control of wastage

Variety: Midseason Bailidge Early.

Packhouse: Sundays River Co-op., Hermitage.

Packhouse treatment: Fruit received full packhouse treatment, which included rinsing in 0.15% SOPP and waxing with Flavourseal. On the same day or one day later all fruit were dipped in an aqueous suspension of Green Mould spores, containing 150 mg. spores in 30 l. water, left to dry for 2-4 hrs. and then with or without wrappers were placed in cartons as follows with :-

(a) Plain wrappers,

(b) "T5"* diphenyl wrappers, containing 60mg. diphenyl/ 100 sq. in.

(c) "Sax"* diphenyl wrappers, containing 60 mg. diphenyl/ 100 sq. in.

(d) SOPP wrappers.

(e) No wrappers.

(f) "Sax" tissues between layers.

(g) Diphenyl pads.

(h) SOPP strips.

(i) DBTCE (di-bromo-tetra-chlor-ethane) strips.

*"Sax" and"T5" wrappers are produced by different paper manufacturers.

Examination: At London, 10-11 days after arrival at Southampton, i.e. 40-42 days after dipping in spore suspension.

Replicates: 8-9 cartons (125 fruit/carton) per treatment.

Results are shown in Table 100.

Table100. Effect of different wrappers and inserts on the control of wastage in Midseasons Bailidge Early.

Treatment	% Wastage				
	Total	Green Mould	Water Rot	Brown Rot	Blue Mould
Plain wrappers	2.8	2.8	-	-	-
T5 diphenyl wrappers	0.8	0.6	0.2	-	-
Sax diphenyl wrappers	1.2	1.2	-	-	-
SOPP wrappers	1.3	1.2	-	0.1	-
No wrappers	8.9	8.6	0.2	0.1	-
Sax tissues between layers	2.1	2.1	-	-	-
Diphenyl pads	4.0	3.8	0.1	0.1	-
SOPP strips	6.4	6.0	0.1	0.1	0.2
DBTCE strips	2.1	2.0	-	0.2	-

Wrappers resulted in a marked reduction in wastage. Best control was found with T5 diphenyl wrappers.

With the unwrapped fruit best control was obtained with Sax and DBTCE tissues. The control obtained by these was not as good as when fruit were wrapped. The main effect of wrapping was to prevent spread of Green Mould by 'contact'.

Green Mould was the most common cause of wastage although Blue Mould and Brown Rot also occurred. Brown Rot was caused by Colletotrichum gloeosporioides mainly.

P. Effect of different wrappers on the control of wastage

Variety: Navel.

Packhouse: White River Fruit Growers' Co-op.

Packhouse treatment: Fruit received full packhouse treatment which included rinsing in 0.15% SOPP but no wax was applied. On the following day the fruit, except those in treatment (g), were dipped into a suspension of Green Mould spores (1.5×10^6 spores/ml.), dried and wrapped in the following types of wrapper:

(a) Sulphite wrappers (plain).

(b) Diphenyl wrappers containing 12 mg. diphenyl/100 sq. in.

(c) Diphenyl wrappers, "Zebediela" containing 59 mg. diphenyl/100 sq. in.

(d) Diphenyl wrappers, "Sax"* strip impregnated, containing 53 mg. diphenyl/100 sq. in.

(e) SOPP wrappers (plain wrappers impregnated with SOPP).

(f) Permachem* wrappers, (plain).

(g) Sulphite* wrappers (plain).

+Diphenyl wrappers, "Zebediela", are ex Zebediela Citrus Estates.

x"Sax" "Permachem" and Sulphite are brand names for paper of different manufacturers.

Examination: At London, 12-13 days after arrival at

Southampton i.e. 43-44 days after dipping in spore suspension.

Replicates: 5 cases (count 150) per treatment.

Results are shown in Table 101.

Table 101. Effect of different wrappers on the control of wastage in Navels (count 150)

Wrapper	Rinsed in suspension of Green Mould Spores	% Wastage			
		Total	Green Mould	Water Rot	Brown Rot
Sulphite (plain)	Yes	41.7	40.1	1.3	0.3
Diphenyl (12mg./100 sq. in.)	Yes	16.0	15.1	0.9	-
Diphenyl (Zeb., 59mg./ 100 sq.in.)	Yes	6.2	6.0	0.2	-
Diphenyl (Sax, 53mg./ 100 sq. in.)	Yes	5.5	5.1	0.3	0.1
SOPP	Yes	11.9	11.3	0.4	0.1
Permachem (plain)	Yes	32.3	30.4	1.6	0.3
Sulphite (plain)	No	2.8	2.7	-	0.1

Zebediela and Sax diphenyl wrappers were quite effective in the control of wastage, this was so because of the high amount of diphenyl in them.

As expected Green Mould was the predominant fungus causing wastage. The Brown Rots were most commonly caused by Colletotrichum gloeosporioides, with Alternaria citri, Trichoderma lignorum and Diplodia natalensis occurring less frequently.

Q. Effect of Flavourseal wax, SOPP and diphenyl on the control of wastage

Variety: Valencia.

Packhouse: Fruit were ex White River Fruitgrowers' Co.-op., where they were washed, graded and sized. No SOPP or wax was applied.

Packhouse treatment: Fruit on arrival at Nelspruit Research Station were rinsed in a suspension of Green Mould spores in 2% citric acid. After drying the fruit were left until the following day when the waxing treatments were applied as follows:-

(a) Flavourseal.*

(b) Flavourseal D.1. (incorporating SOPP).

(c) Flavourseal D.D. (incorporating SOPP and dephenyl).

* Flavourseal is wax; SOPP and diphenyl are the active fungicides.

Replicates: 5 cases (count 216) per treatment.

Wrappers: Sulphite.

Examination: Two examinations were carried out:

(i) At Capetown, 9-10 days after treatment. One case of treatments (a) and (c) were examined by Dr. R.A. Christ and the wasty fruit removed.

(ii) At London, 6-7 days after arrival at Southampton i.e. 36-38 days after treatment.

Wastage (expressed as % of fruit examined) found at Capetown is shown in Table 102. Wastage (expressed as % of treated fruit arriving in London) is shown in Table 103.

Table 102. Effect of Flavourseal wax, SOPP and diphenyl on the control of wastage in Valencias (count 216)

Examination at Capetwon 9-10 days after treatment.

Treatment	% Wastage *
Flavourseal	9.3
Flavourseal D.1. (incorporating SOPP)	Not examined
Flavourseal D.D. (incorporating SOPP + diphenyl)	8.6

* Expressed as % of fruit examined.

Table 103. Effect of Flavourseal wax, SOPP and diphenyl on the control of wastage in Valencias (count 216)

Examination at London, 36-38 days after treatment.

Treatment	% Wastage *					
	Total	Green Mould	Water Rot	Sour Rot	Brown Rot	Blue Mould
Flavourseal	26.3	24.6	1.0	0.3	0.4	-
Flavourseal D.1. (incorporating SOPP)	30.8	28.5	1.0	0.7	0.5	0.3
Flavourseal D.D. (incorporating SOPP + diphenyl)	23.8	21.8	0.8	0.5	0.4	-

* Expressed as % of treated fruit arriving in London.

Only Flavourseal wax with SOPP and diphenyl incorporated reduced wastage to a very slight extent.

In addition to Green Mould, Sour Rot, Blue Mould and Brown Rot were also found. Brown Rot was caused by Phomopsis citri and Trichoderma lignorum.

SECTION IV - REFERENCES

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ACKNOWLEDGEMENTS.

I wish to express my gratitude to Professor R.K.S. Wood for suggesting the problem for research, for his guidance throughout the work and for his helpful criticism during the preparation of the manuscript.

I wish to thank Mr. R. Adams for his generous help in technical problems and for the text figures prepared by him. I am also grateful to Mr. A. Horne for his photographic work and to the other members of the Botany Department for their help in so many ways.

My sincere thanks is also expressed to the staff of the South African Co-operative Citrus Exchange Co., Ltd. and in particular to Mr. R.A. Matthew for his assistance in the work.

I wish to thank my wife Pat for her stencil cutting and for her helpful suggestions during the preparation of the thesis.

I also wish to thank the National University of Ireland for the award of a Travelling Studentship Bursary which made the work possible.