THE ESTIMATION OF LATENT INFECTION IN ORANGES*

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(Plate 1)

Summary

The main objective of work described in this paper was to find out what differences occurred in the degree of latent infection of Washington Navel oranges grown under different circumstances and treated in various ways. Observations were also made of fungi occurring as latent infections and on the conditions for, and mode of, infection by *Colletotrichum gloeosporoides*, the principal form concerned.

Estimates of the degree of infection were based on results obtained by plating pieces taken from the rind of apparently sound oranges. The methods used are described. *Colletotrichum gloeosporoides* was easily the most common and widespread form found. *Alternaria* spp. also occurred generally but much less frequently. *Phoma citricarpa* and *Septoria citricola* were characteristic of oranges from certain places.

With respect to infection by *Colletotrichum* it was found that significant differences may occur between different parts of the orange, between oranges from different trees in the same grove, and between oranges from different districts. Significant differences may also occur between oranges taken in successive years from the same trees.

The question of the time that infection by *Colletotrichum* occurs was examined under natural conditions and under conditions where the inoculation was controlled. With natural conditions in South Australia, the first significant increase in the degree of infection occurred in April or May and was often followed by further significant increases during the winter months. In experiments where inoculation was controlled, infections occurred as the result of inoculating oranges in January; however, they were more frequent with inoculations in March and later months.

Infections with *Septoria citricola* were also established by using appropriate spore suspensions. Here, inoculations made in April were the most successful. Observations on the time required by *Colletotrichum* to establish itself within the rind tissues revealed that in a humid atmosphere at 25°C. relatively little infection occurs in less than 24 hours, a fact which no doubt accounts at least in part for the freedom from infection of South Australian oranges during the hot dry weather that is normal for the period October–April/May.

* The work described in this paper was commenced in 1939. The four junior authors were each associated with it in turn for about twelve months.

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Applications of Bordeaux mixture reduced latent infection to a degree that varied according to the times of application. The effect of curing or "sweating" oranges was not consistent from season to season. During two seasons a relatively small but significant reduction was effected but this did not occur in the third season.

Storage of oranges at 4.5°C. for six weeks had no significant effect on their degree of infection.

The microscopic features of the infection process are discussed briefly and agree substantially with those described for tropical fruits by Simmonds (1941).

I. Introduction

Interest in the circumstances of infection of citrus and other fruits by *Colletotrichum gloeosporoides* and by other fungi has revived in recent years. This is evident from contributions by Bates (1936) from Southern Rhodesia, Baker and Wardlaw (1937) and Baker, Crowdy, and McKee (1940) from Trinidad who considered the problem in citrus fruits particularly. Simmonds (1941) extended observations to the similar phenomenon in various tropical fruits, especially the banana.

Shear and Wood (1913) were perhaps the first investigators to draw attention to the fact that *Glomerella cingulata* (Stonem.) Sp. and von S. and homologues referable to the form genera *Colletotrichum* and *Gloeosporium,* was able to remain in a dormant or quiescent condition in the leaves, stems, and fruits of a wide range of host plants. In drawing attention to this behaviour as a feature of infection by *Glomerella* they suggested that the quiescent state remains "until the host becomes weakened in some way or until specially favourable conditions for the fungus occur." They also pointed out that "in many cases the fungus never develops until the infected part of the host dies."

Our interest in the latent infection of oranges arises from the fact that after a period of cool storage citrus fruits, especially grape fruit and Washington Navel oranges. develop a disorder of the pericarp commonly described as "storage spot" or "pitting." Opinions concerning the etiology of this disease vary. According to one view, the spotting is due to a disturbed metabolism in orange pericarp occasioned by the circumstances of storage, whilst another view ascribes some role to the presence of pathogenic organisms in the tissues of the pericarp. In the present account, however, we do not consider these differences of viewpoint and are simply concerned with the question of the extent to which latent infection by potentially pathogenic fungi occurs.

Latent infections in fruits may be studied by either one of two methods. They may be studied directly, in microscopic preparations, as was done by Simmonds (1941), or less directly from results obtained by plating treated pieces of apparently healthy orange rind tissues on media suitable for the growth of the organism sought. This latter method, used by Baker and Wardlaw (1937) and others, is advantageous in that it is possible to examine numerous pieces of tissue either from a single orange or from several oranges which may have come from populations with different histories. The method therefore provides a means for considering data quantitatively.

Most of the results described below arose from the application of methods involving the plating of pieces of orange rind, but some consideration was also given to the development of infection under controlled conditions of inoculation and to the microscopic details of infection.

II. Experimental Procedure

(*a*) *Estimating the Degree of Latent Infection*

(i) *Isolation Methods.*—The method of disinfecting the surfaces of apparently healthy oranges from which isolations were to be made was essentially that used by Baker and Wardlaw (1937) with the difference that, Agral 2 (a sulphonated organic wetting agent) was incorporated with the disinfectants used. This was done because preliminary trials revealed that the addition of Agral 2 served to eliminate practically all the few miscellaneous fungi which, it was suspected, were present as surface contaminants rather than as latent infections.

The following standard procedure was adopted. The oranges to be disinfected were:

(1) Immersed for ten minutes in a saturated solution of borax + 0.45% Agral 2 at 45°C.

(2) Washed in two changes of sterile water.

(3) Immersed for ten minutes in 0.1% mercuric chloride + 0.45% Agral 2 at room temperature.

(4) Washed in two changes of sterile water.

Subsequent to surface disinfection small round pieces of rind tissue were removed with a sterile cork borer (5 mm.) and planted on malt agar plates (25 g. malt extract, 22 g. agar to 1 l. water).

The number of pieces taken from each orange varied from five, in the earlier stages, to fifteen later on. The pieces were selected at random, either from the orange as a whole or from a particular region such as the "stem end," defined as a region extending for about two inches from the stem connexion. With the exception of some oranges sampled after cool storage the oranges were selected for freedom from visible defects. Special precautions were taken to minimize the chances of contamination of plates from external sources.

The value of any isolation method in establishing the existence of latent infection turns on the question whether fungi developing on the isolation plate have developed from hyphae within the tissues or from spores or other fungal structures on the surface of the orange. Early in the investigation the following experiment was undertaken to throw more light on this point. Twenty-four fully grown oranges were selected from a sample which, when examined by the isolation method described, showed no evidence of latent infection. Half of the surface of each of these oranges was inoculated with a spore suspension of *Colletotrichum gloeosporoides* applied with an atomizer and allowed to dry immediately. Three days after inoculation twelve of the oranges were

disinfected by the standard procedure and the remainder left to be used as controls. Six pieces, selected at random, were plated from each half of the twenty-four oranges. Forty-seven pieces of the 72 pieces, i.e. 65 per cent. of those plated from the inoculated halves of the non-disinfected oranges, yielded *Colletotrichum* on culturing but none of the pieces from either half of the surface disinfected oranges or from the uninoculated halves of the non-disinfected oranges yielded any growth of fungus. Obviously the disinfection process employed completely inhibited growth from surface borne spores of *Colletotrichum*. Further observations on infection processes are reserved for discussion later.

(ii) *Statistical Considerations.*—In the sequel it will be seen that populations of oranges, whether they be from different trees, different districts, or be subject to different treatments, vary in the number of pieces per orange yielding a fungous growth, and an estimate of the significance of these differences was desired. The matter was examined in a preliminary experiment. One

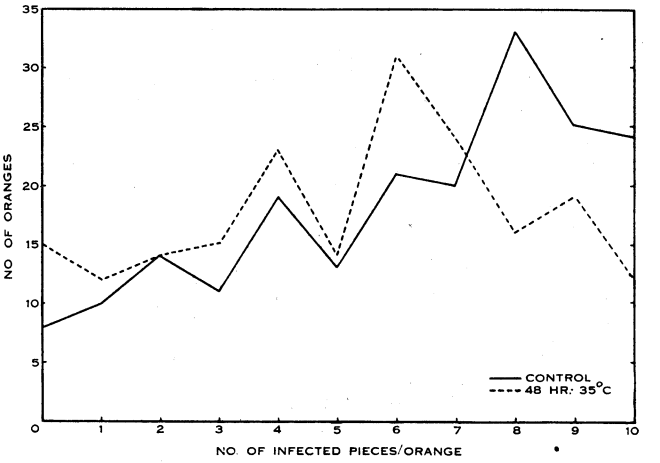

Fig. 1.—The distribution of two populations of oranges in terms of the number of infected pieces (out of ten) per orange.

hundred and thirty-four oranges were picked from each of three trees, divided equally into two groups and then bulked to give lots of 201 oranges. One of these lots was cured or "sweated" by keeping the fruit in a thermostatically controlled cabinet at 35°C. for two days. Each lot was examined for latent infection as soon as possible after picking or after the curing treatment. Ten pieces were plated from each orange, five at random positions from the "stem end" and five from random positions on the remainder of the orange. When examined subsequently, practically the only fungal growth present was that of *Colletotrichum*. The distributions of the two orange populations in terms of numbers of pieces yielding *Colletotrichum* are shown in Figure 1.

Consideration of the evidence regarding the effect of the sweating treatment is reserved till later when other evidence concerning such treatments is given.

On the statistical problem which this experiment was designed to elucidate, E. A. Cornish* kindly offered the following comment. "The method of determining the latent infection present provides a discrete variable of finite range. For a variable of this nature the binomial distribution might be considered to provide a suitable basis for testing the significance of treatment difference but it cannot be used because every piece of rind of specified size has not an equal and independent chance of becoming infected. In seeking an alternative method of analysis, we may note first that the score assigned to any orange, viz. the number of pieces out of ten plated, can be taken as a sampling estimate of the amount or degree of infection on that orange. In the second place any correlation which may exist between observations on the same orange does not invalidate the score assigned to that orange as a variable for estimating the degree of infection or for use in an analysis.

"The discontinuity of the variable will to some extent affect the analysis based on the normal distribution, but even with a range as small as that used this effect will not be great. It might be reduced if the number was increased to say fifteen" (a procedure adopted in later work). "The skewness of the distribution probably influences tests of significance more than the discontinuity of the variable but provided that counts are distributed over the greater part of the range as they are in this case they will not affect the result appreciably."

In cases then where results were similar in character to the above it has been assumed for purposes of analysis that distribution was normal.

(*b*) *Inoculation Experiments*

It was suspected that the extent of infection with *Colletotrichum* or other fungi which can establish latent infection under natural conditions was related to the incidence of rainfall. If trees could be protected from rain the infection of oranges would be minimized. To accomplish this a glass roof was erected over four fully grown Washington Navel orange trees. The sides of this structure were equipped with roller blinds which could be let down when rain occurred. For the first two years this structure was located in a private orange grove at Mypolonga, South Australia, and for the third season in the Government orchard at Fullarton. The structure was generally effective for the purposes intended but the occurrence of light, driving rain entering from the sides could not always be anticipated. The trees grew well, bore normal crops of oranges, and did not appear to be affected in any way by the covering. They were watered by furrow irrigation.

Inoculation was effected by atomizing spore suspensions on to the uninjured surface of unblemished oranges, and, to overcome possible variations in the virulence of different strains, mixtures of several single spore isolates were used. The maintenance of moist conditions in the infection court for a sufficient period for infection to occur was difficult with inoculations effected during hot dry

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weather. To achieve this in later experiments, the following procedure was adopted. After applying the spore suspension, the droplets were first allowed to dry and then the oranges were covered with a thin pad of cotton wool wetted with sterile water and fairly well wrung out. This was then enclosed in closely fitted tinfoil and finally in paraffin-treated paper bags tied securely around the stem. Evidence of infection was secured by plating pieces of tissue from inoculated oranges by methods previously described.

(*c*) *Study of Microscopic Features of Infection*

Oranges protected from natural infection were picked during May and June and their surfaces disinfected by immersion in 70 per cent. alcohol. Defined areas were inoculated with a heavy spore suspension of *Colletotrichum* and maintained for periods of 12-143 hours in a moist atmosphere at 25°C., after which hand or microtome sections were cut. For embedding, pieces of the rind were fixed in formalin-aceto-alcohol, while for dehydration tertiary butyl alcohol was used (Johansen 1940). The cotton blue-lactophenol combination used by Simmonds (1941) was not found very satisfactory for tracing hyphae below the cuticle. Better results were obtained with the following schedule:

(1) Stain with 0.1% thionin in 5% aqueous phenol for one hour.

(2) Counterstain with orange G in absolute alcohol or clove oil for 30-60 seconds.

(3) Clear with methyl salicylate; mount in Canada balsam.

The use of thionin in this way (Johansen 1940) stains the delicate intercellular hyphae blue but the material in which they are embedded appears reddish owing to the metachromatic property of thionin. Care with the period of counterstaining was the principal precaution required in the use of this procedure.

III. Results

The main part of the investigation was concerned with estimating the latent infection of oranges growing naturally in different districts or subjected to different treatments and the data obtained can be considered most conveniently in the series of subsections which follow.

(*a*) *Fungi Present as Latent Infections*

Colletotrichum gloeosporoides was easily the most common species isolated from the rind of apparently sound Washington Navel oranges grown in districts mentioned later. Its prevalence may be illustrated by reference to an experiment where freshly picked oranges from six districts, three in New South Wales, two in South Australia, and one from Victoria were sampled. Six hundred and sixty-five oranges, drawn almost equally from these districts, were sampled by plating twelve pieces from each orange. Three thousand and twenty-two of the pieces (38.6 per cent.) plated yielded some fungal growth and of these

89.4 per cent. were identified as *Colletotrichum gloeosporoides*. Furthermore, under circumstances where the number of infected pieces was high, a matter dealt with later, the proportion of isolates identifiable as *Colletotrichum gloeosporoides* was also high. The point may be illustrated by reference to the sampling of oranges from Griffith, New South Wales. From 64 per cent. of the pieces plated fungi grew and of these 97 per cent. were identified as *Colletotrichum*.

Of other fungi developing on the isolation plates, *Phoma citricarpa, Septoria citricola,* and *Alternaria* spp. were most noteworthy. *Phoma* and *Septoria* only occurred on a small proportion of the pieces plated, e.g. 1.8 and 2.3 per cent., though the number of oranges (18-21 per cent.) from which such pieces came was quite significant in some consignments. The point of special interest about these fungi was their restricted distribution. *Phoma citricarpa* occurred only in consignments from the Gosford district whilst *Septoria* was found only in consignments from the Murrumbidgee Irrigation Area and from South Australia. Both these findings agree with what is known about the distribution of "spot" diseases caused by these fungi and it is evident that like *Colletotrichum* they may occur as latent infections (Kiely 1948).

The fungi identified as *Alternaria* spp. were very variable in their growth characteristics; some agreed quite closely with descriptions of *A. citri* but specific identification was not generally pursued. *Alternaria* like *Colletotrichum* was found (though usually in much lower numbers) in oranges from all districts. Occasionally, however, where infection with *Colletotrichum* was low, as was generally the case with oranges from the Upper Murray Irrigation Areas of South Australia, the proportion of isolates identifiable as *Alternaria* might be quite high; in one sampling of 150 oranges from Waikerie, South Australia, 5.5 per cent. of the pieces yielded *Colletotrichum* and 6.4 per cent. *Alternaria*.

(*b*) *Variations in Latent Infection in Oranges from Different Districts, in Oranges from Different Trees in One District, and in Different Parts of a Single Orange*

Samples of five sound oranges taken at random from each of twelve healthy trees growing in eight districts were obtained in 1939 and examined by methods previously described. Twelve pieces were plated from each orange, six from the "stem end" and six from the remainder of the orange. Twelve hundred pieces were therefore examined from each grove. The results showed that significant variations occurred between oranges from different districts, from different trees in each grove, and from different parts of the orange. The degree of infection found in oranges from different districts is given in Table 1 where, for the sake of facilitating comparison with data in other tables, the number of infected pieces is expressed as a percentage of all the pieces plated.

The higher degree of latent infection in the oranges from New South Wales is quite evident. It will be noted that these oranges were the first lots picked but this fact is unlikely to account for the differences since later data show that if there is a significant change in the degree of latent infection it increases in later pickings.

Differences in infection between oranges from different trees in one grove and between different parts of a single orange were evident in oranges from all districts but most marked in oranges where latent infection was generally high.

TABLE 1
DEGREE OF INFECTION IN ORANGES FROM DIFFERENT DISTRICTS 1939

District	Picking Date	Pieces with *Colletotrichum* (%)
Griffith, N.S.W.	April 24	62
Somersby, Gosford, N.S.W.	May 8	54
Mangrove Mountain, Gosford, N.S.W.	May 8	49
Mildura, Vic.	May 29	Trace
Berri, S.A.	June 18	3
Waikerie, S.A.	June 21	1
Mypolonga, S.A.	July 17	3
Torrens Valley, S.A.	July 29	14

The differences between oranges from different trees may be illustrated by the data with oranges from Mangrove Mountain, Gosford. In this case where the mean infection of all oranges was 49 per cent. the degree of infection of oranges from tree to tree varied from 8.92 ± 8.1 per cent. Actually, on the basis of the latent infection of the oranges they bore, the trees fell into three groups of almost equal numbers with infections of 89 ± 6.8, 36 ± 6.8, and 19 ± 7.4 per cent. respectively. It might have been expected that differences of this order would be reflected in the health and vigour of these trees since small dead twigs in unthrifty trees often carry the fructifications of *Colletotrichum* but neither in these trees nor in others where similar differences occurred could the trees be

TABLE 2
MEAN PERCENTAGE LATENT INFECTION IN DIFFERENT PARTS OF ORANGES FROM NEW SOUTH WALES 1939

District	Infected Pieces "Stem End" (%)	Infected Pieces Remainder of Orange (%)	Standard Error (±)
Griffith	84	41	2.5
Somersby	68	39	2.4
Mangrove Mountain	59	40	2.6

differentiated on their appearance in the orchard. Actually, this was to be expected since in the first instance the trees used had been selected for uniformity and thriftiness.

Differences in infection in different parts of the orange are again most evident in the more heavily infected oranges and may be illustrated by reference to the results obtained with the oranges from New South Wales (Table 2).

The "stem end" is here defined as an area with a radius of about two inches from the "button" or stem connexion. In some preliminary work, samples were taken from the "stem end," the navel end, and other parts of the orange which might be defined but the results suggested that the most useful distinction was that referred to in Table 2. It was used throughout the investigations and provided results consistent with those quoted above.

(c) *When Does Infection Occur?*

(i) *Under Natural Conditions.*—Estimates of latent infection at intervals subsequent to the time that the fruit "sets" were obtained for oranges from two districts over two years. For each occasion the estimate was obtained by sampling 30 oranges drawn in equal numbers from the same three trees. In the first season ten pieces and in the second season fifteen pieces taken at random from the orange were examined in the usual manner. The results of these examinations are given in Table 3.

TABLE 3

MEAN PERCENTAGE LATENT INFECTION IN ORANGES SAMPLED AT INTERVALS FROM "FRUIT SETTING" TO MATURITY

District	Month of Sampling								
	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
Athelstone 1940-41	0.7*	1.0*	2	3	**37**†	25	38	**63**	70
Athelstone 1942	—	0.7*	0	0.7	0.5	**32**	**52**	**70**	—
Waikerie 1940-41	0.7*	0.3*	7	8	6	**26**	29	25	25
Waikerie 1942	—	0.7*	0.2*	0.2	1.3	**52**‡	55	62	—

* These figures refer to the presence of *Alternaria* spp.; there were no colonies of *Colletotrichum*.

† Figures in bold type are significantly greater than those for preceding dates of sampling.

‡ This sample was actually taken early in June, six weeks subsequent to the previous sample and a fortnight prior to the next one.

From Table 3 it can be seen that for these two South Australian districts the first significant increase of infection occurred in April or May. The end of April and May are the times when the autumn rains, characteristic of the South Australian climate, usually commence but no clear correlation between rainfall and degree of infection can be established with the limited data available. Rainfall, however, may not be the only important element in promoting infection. In 1941 abundant rains fell in South Australia at the end of January and again at the beginning of March but neither of these occasions led to significant changes in the infection of oranges sampled shortly afterwards. Air temperature, humidity, and wind in their effect on the persistence of moisture on the surface of oranges might all be important but the limited information available does not warrant further pursuit of the subject here.

Two further points about the data in Table 3 may be noted. At Athelstone, close to Adelaide where rainfall is considerably greater than at Waikerie, the degree of infection increased significantly later in both seasons and by July had

reached a fairly uniform and high figure. At Waikerie no second significant increase occurred but there was a significant difference between infection in the two seasons. Evidence confirmatory of this variation from season to season was obtained from a grove at Mypolonga where, over a four year period, in oranges sampled at closely corresponding dates, latent infections of 43.2, 3.3, 18.6, and 43.9 per cent. respectively, were found in successive years.

(ii) *Under Controlled Conditions.*—These experiments were conducted over a three year period with oranges grown on trees under the cover described earlier. Each season oranges on two of the trees were inoculated with a spore suspension of *Colletotrichum* while those on the other two trees were inoculated with *Septoria.* Fifteen to twenty oranges on each tree were inoculated on each occasion but for various reasons not all these were available on the date when all oranges were picked for examination. Controls in the experiment were provided by oranges borne on the same trees as those inoculated; they differed only in that they were not atomized with the appropriate spore suspension.

TABLE 4

INFECTION OF ORANGES INOCULATED ON VARIOUS DATES WITH *COLLETOTRICHUM*, MYPOLONGA 1941

Date of Inoculation	Oranges Examined (No.)	Pieces Yielding Growth (%)	Oranges Infected (No.)
March 4	19	45.0	17
April 8	20	55.0	19
July 22	28	43.0	26
Uninoculated Group A*	39	10.3	10
Uninoculated Group B	146	1.1	11

* This group was taken from the two trees on which other oranges were inoculated with *Colletotrichum.* The two trees were both on the western side of the enclosure and thus more exposed to risks from light, driving rain entering from the side. Group B oranges were taken from trees on which other oranges had been inoculated with *Septoria.* In their position they were also less exposed to the risks of wetting by rain.

In 1939-40, a total of 116 oranges inoculated in groups on seven occasions extending from November 7 to May 25 were examined in July for evidence of infection but the highest percentage of infected pieces secured, the result of inoculation on February 22, was only 6.2 per cent. The position with *Septoria* where 98 oranges in all were examined, was similar; only 1.2 per cent. of infected pieces were obtained as the result of inoculations on the same date. Of 53 oranges serving as controls in this experiment, none yielded any evidence of infection with either fungus.

In the following season more care was taken to ensure that adequate moisture on the orange surface was maintained during the period infection was likely to occur. These steps have been described and apparently they contributed to the more satisfactory results for inoculation with *Colletotrichum* shown in Table 4.

With *Septoria,* infections arising from inoculation were again infrequent. Forty oranges were inoculated on each of the three occasions mentioned in Table 4 but only two of the oranges inoculated on March 4 and one inoculated on July 22 yielded evidence of infection. No isolations of *Septoria* were obtained from any of the oranges serving as controls.

For the experiments in 1942 the glass roof was moved to the Government orchard at Fullarton. The rainfall there was heavier and more frequent than at Mypolonga and the effective protection of trees from driving rain more difficult. As in previous years oranges on one pair of trees were inoculated with *Colletotrichum;* while those on another were inoculated with *Septoria.* Results obtained with these inoculations are given in Table 5.

TABLE 5

INFECTION OF ORANGES INOCULATED ON VARIOUS DATES WITH EITHER *COLLETOTRICHUM* OR *SEPTORIA,* FULLARTON 1942

Date of Inoculation	*Colletotrichum*			*Septoria*		
	Oranges Examined (No.)	Pieces Yielding *Colletotrichum* (%)	Oranges Infected (No.)	Oranges Examined (No.)	Pieces Yielding *Septoria* (%)	Oranges Infected (No.)
January 20	15	10.9	10	17	0.7	1
April 24	17	84.4	17	17	24.6	14
June 29	16	61.3	15	17	3.7	5
Uninoculated "controls"	56	5.2	9	43	1.2	3

(iii) *Time Required to Establish Infection.*—The difficulty of maintaining a film of moisture around artificially inoculated oranges prompted the making of a small experiment to ascertain the period necessary to establish infection. Fifteen oranges grown on one of the protected trees were selected and examined for evidence of natural infection by plating five pieces from each orange. They were then inoculated with a heavy suspension of *Colletotrichum* spores, divided into three lots of five, and kept in large Koch dishes at 25°C. with ample moisture. After 24, 48, and 72 hours respectively, one of the lots was removed and the oranges examined by plating five pieces before, and five pieces after, surface disinfection. The results of this experiment are given in Table 6.

The oranges used in this experiment were practically free from infection prior to their inoculation since *Colletotrichum* grew from only one of a total of 75 pieces plated from the oranges at this stage of the experiment. The figures in the last column of Table 6 reveal the degree of infection obtained with each period of incubation. Infection after 72 hours' incubation was close to the maximum possible under the conditions of the experiment. It was significantly greater than that obtained with 48 hours' incubation which, in turn, was signifi-

cantly greater than that with oranges incubated for 24 hours. The data in the third column can be taken as a measure of the potency of the inoculum used. The high, uniform figures for each group were contributed partly by the number of infections and partly by viable spores or young fungal growths which had not yet established themselves as infections, i.e. had not yet grown beyond the inhibitory action of the disinfectants used. The contribution of these two elements to the situation can be seen most clearly with oranges incubated for 24 hours.

TABLE 6

NUMBER OF PIECES (OF A POSSIBLE 25) YIELDING *COLLETOTRICHUM* BEFORE AND AFTER INOCULATION; INOCULATED ORANGES INCUBATED AT 25°C.

	Isolations of *Colletotrichum*		
		After Inoculation	
Incubation Period (hr.)	Before Inoculation (No.)	Before Disinfection (No.)	After Disinfection (No.)
24	0	23	3
48	1	21	13
72	0	23	21

Here the difference in numbers before and after disinfection was highly significant. The result obtained before disinfection was no doubt contributed to by surface borne spores and appressoria since these could be observed in hand sections made from these oranges. That these structures scarcely contributed to the figures in the last column, those on which the degree of infection is based, was confirmed by a later experiment. In this case, oranges free from evidence of infection were inoculated and incubated for a 13 hour period at 25°C. Hand sections revealed that spore germination and appressorial formation had occurred freely but after surface disinfection no pieces yielded *Colletotrichum.*

It is evident that hyphae etc. produced from these spores had not yet passed beyond the inhibitory effect of the disinfectant, a fact which was interpreted to mean that, while spore germination had occurred after 13 hours, infections were not then established. This experiment also throws further light on the minimal period required for infection to occur at 25°C. It lies between 13 and 24 hours.

(*d*) *Effects of Spray Treatments on Latent Infection*

Spraying trials conducted by the Department of Agriculture afforded an opportunity to study the effects of spray treatments on the degree of latent infection.

In 1939-40 Bordeaux mixture (3:3:100) was applied either once or twice to blocks of five trees at Mypolonga during the months shown in Table 7. When

the fruit was mature at the end of August, 45 oranges were drawn from each block and sampled for infection in the usual way. The results are summarized in Table 7.

TABLE 7

RESULTS OF TRIAL AT MYPOLONGA 1939-40 SHOWING THE LATENT INFECTION OF ORANGES FROM TREES SPRAYED WITH BORDEAUX MIXTURE (3:3:100) AT VARIOUS TIMES

Months When Spray Applied	A Dec. only	B Dec. Jan.	C Dec. April	D Dec. May	E April only	F No Spray
Latent infection (%)	4.25	0.2	0.2	8.3	0.7	18.6

The degree of infection found, even in unsprayed oranges, was low and there is some doubt about the validity of the usual test for significance. An alternative test devised by Pitman (1937) was therefore used. By using this test we found that the following comparisons were significant at $P \leqslant 0.01$, viz.:

(1) Less latent infection occurred in oranges from sprayed trees.

(2) Sprays applied in December only or in December and May were less effective than the other applications employed.

It should also be noted that there was no significant difference between oranges receiving a single application in April and those which, in addition, had been sprayed in December.

In this and other experiments where deposits of spray occur on some oranges, it might be suggested that the presence of copper would tend to inhibit the growth of *Colletotrichum* if it were present as a latent infection. The point was examined but it is not necessary to give details, for no evidence of any such inhibitory action was found even in cases where the deposit was fresh and heavy.

TABLE 8

RESULTS OF SPRAYING TRIALS AT MYPOLONGA AND WAIKERIE 1941

District	Date Of Spray Application	Sampling Date	Mean Percentage Latent Infection		Standard Error (±)
			Sprayed	Unsprayed	
Mypolonga	February 21	June 28	18.5	43.9	3.93
Mypolonga	April 24	Sept. 5	24.4	46.9	3.60
Waikerie	February 27	June 20	3.1	17.5	2.0
Waikerie	March 27	July 25	7.3	40.3	3.3

In the following season, observations were extended to include a spray trial on trees at Waikerie as well as at Mypolonga. Comparisons were limited to two groups of trees; the first sprayed twice with Bordeaux mixture 3:3:100 on the dates shown in Table 8 while a second group was not sprayed. The oranges from both places were sampled twice; each sample consisted of 50 oranges taken in equal numbers from five trees of each group. The results are summarized in Table 8.

In all cases the effects of spraying on latent infection were significant but it will be noted that infection of oranges on the sprayed trees, especially those at Mypolonga, reached a comparatively high figure. The spray schedule employed was not efficient when circumstances favoured a high degree of infection. The results from the second sampling confirm those of the first and in their general increase accord with other observations. The significant increase in infection at the July picking of the unsprayed oranges from Waikerie should also be noted.

(e) Effect of "Sweating" Treatments on the Latent Infection of Oranges

Since storage trials revealed that "sweating" treatments applied to oranges prior to their cool storage may reduce the subsequent development of storage spot (Huelin 1942) we were interested in the question of the effects of such treatments on the degree of latent infection. Figure 1, referred to previously, deals with two groups of oranges which differ from one another only in that one group had been "sweated" at 35°C. for 48 hours prior to sampling. The mean infection determined for the untreated sample was 50.8 per cent., while that for the sweated oranges was 7.2 per cent. less – a difference which is significant.

TABLE 9

EFFECTS OF VARIOUS "SWEATING" TREATMENTS WITH ORANGES GROWN AT ATHELSTONE, PICKED JUNE 24, 1940

Sweating Treatments	Latent Infection (%)	Standard Error (±)
16 hr., 49°C.	44.6	3.75
48 hr., 40.5°C.	38.0	4.11
6 days, 32°C.	40.2	4.33
Untreated	65.3	3.75

In 1940 more comprehensive observations were made. They took into account a wider variety of "sweating" treatments and in sampling more attention was given to the various factors already considered which contribute to the variability of latent infection. The results of these observations are summarized in Table 9.

Each of the treatments referred to in Table 9 effected a significant reduction in latent infection when compared with that for the untreated oranges but there was no significant difference between the various treatments used.

Oranges from the same group of trees were examined again in the following season for the effects of sweating treatments. They were picked for treatment on three occasions: in May, June, and July. The degree of infection in untreated oranges was estimated at 28.9, 31.9, and 62.0 per cent. respectively but on no occasion did sweating treatments at 29.5°C. for 7 days or 32°C. for 2, 5, 8, and 11 days respectively, effect a significant reduction in latent infection. "Sweating" then was not consistent in its effects; on some occasions it reduced, on others it had no significant effect on, the degree of latent infection.

(f) Does the Degree of Infection Increase if Oranges are Stored?

Many observations have been made on the extent of latent infection in oranges before and after a period of cool storage and they all support the same conclusion, viz. that during a storage period there is no overall increase in the degree of infection. Only two cases supporting this view need be quoted. In the first case a sample of 66 oranges taken from a larger batch which had been sweated at 35°C. for two days was examined for latent infection in the usual way by plating ten pieces from each orange. The remaining oranges were then stored at 4.5°C. for a period of six weeks. On removal, 5 per cent. had developed storage spot. Sixty-six of these oranges were again examined by plating ten pieces taken from positions determined by a method of random selection which took no account of whether a lesion was, or was not, present at the point of sampling. The estimate of infection prior to storage was 45.1 ± 3.4 per cent., that subsequent to storage 44.1 ± 3.1 per cent.; an insignificant difference.

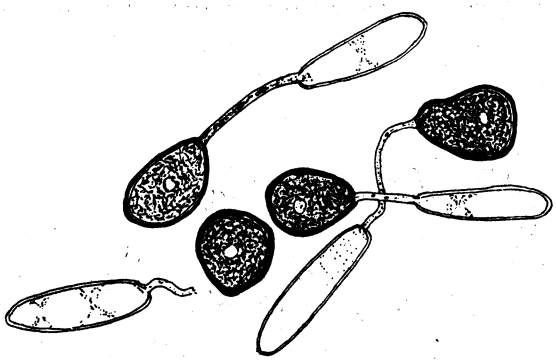

Fig. 2.—The appressoria of *Colletotrichum gloeosporoides*. x 1000.

In the second case, 30 oranges were taken from a larger group of similar oranges and examined for latent infection, immediately after picking, by plating 15 pieces from each orange. The remainder of this group was stored at 4.5°C. for six weeks during which time a proportion developed "storage spot." Twenty of these spotted oranges were selected for examination and 15 pieces were plated from apparently healthy areas on them. In addition, one piece was plated from each lesion which meant that from 19-30 pieces were plated from each orange. We are not concerned here with differences between apparently healthy and lesioned tissues in the latter group of oranges but only with estimates for the whole orange so as to compare them with similar oranges prior to storage. The estimate prior to storage was 62.0 ± 4.7 per cent.; that after storage 56.0 ± 19.0 per cent.; again the difference is not significant.

(g) Microscopic Features of the Latent Infection of Oranges

A full account of the histological structures associated with the latent infection of oranges would vary but slightly from the excellent account of

Simmonds (1941) concerning similar structures associated with the latent infection of tropical fruits. In briefly describing the microscopic features then it is only necessary to stress such differences as appeared in our preparations.

Soon after germination the spores of *Colletotrichum* develop a characteristic organ – the appressorium (Fig. 2). This is brown in colour and borne at the end of a short germ tube. Appressoria vary greatly in shape, a feature which seems to be determined by the surface on which they develop and to which they are very closely applied. A distinct pore, seen best when viewed from above, occurs on the appressorial wall in contact with the host. Simmonds describes and illustrates a peg-like structure in this position but our observations suggest that it is a much broader structure than the term peg suggests. It seems better described as a thickening of the lip of the pore. It projected from the main body of the appressorium and, in many cases, was partly sunken in the cuticle. Its function in attachment seemed clear. We could see no evidence of the presence of mucilaginous substances which, it has been suggested, serve a similar function.

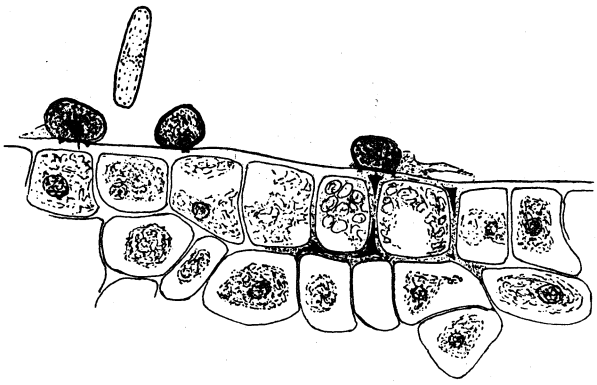

Fig. 3.—Penetration of pericarp tissues by hypha developing from an appressorium of *Colletotrichum*. x 800.

The next stage in the infection process, the penetration of the cuticle, was observed only after prolonged search of sections stained with lactophenol-cotton blue. A fine infection thread, staining light blue and proceeding from the appressorial pore passes directly through the cuticle. After its passage the hypha, though not appreciably thicker, stains more sharply and its appearance suggests that during passage of the cuticle it lacked a cell wall. From its subcuticular position immediately beneath the appressorial pore, the fine filament, if it is not directly over the junction of two cells, grows just beneath the cuticle to a position where two epidermal cells meet. In this region it tends to enlarge and from there grows downwards, still in the intercellular region (Fig. 3). Growing in this way, the greatest depth to which hyphae have been traced (in material fixed 143 hours after inoculation) was an angular intercellular space three cells below the cuticle.

The description just given agrees with that of Simmonds in that the infecting hyphae are intercellular but it differs in the extent to which they spread. In the fruits which Simmonds investigated, the banana, mango, and pawpaw, infection appears to have been confined to the development of sub-cuticular hyphae which, in some cases, were relatively large. The differences observed in the two cases may of course be due simply to the differences in the nature of the hosts studied.

So far as the effect of infection on the host tissue was concerned, there was no evidence of any discolouration of cells or cell walls in unstained preparations. Changes, however, were evident when the thionin-orange G stain was used. In addition to its advantage of staining the hyphae blue in a background of pinkish coloured intercellular material, it also stained blue the granulated and somewhat disorganized cells in the invaded region. On the other hand, healthy uninvaded sections took up the counterstain orange G in such a way as to define the two regions clearly (Plate 1).

IV. Discussion

The objective of a wider investigation, of which this is the initial part, is to elucidate the role which infection by *Colletotrichum* plays in the development of the spotting which occurs in oranges after a period of cool storage. In the first instance, we wanted to ascertain the extent to which different parts of the orange or different groups of oranges might vary in respect to their infection, a fact which is complicated in oranges by the phenomenon of latency.

The results reveal that considerable variations in the degree of infection may occur and that it is possible to give a numerical expression to the prevalence of infection in different parts of the orange, in oranges from different trees and from different districts, and in oranges from the same trees in different seasons.

The significant differences in the infection of oranges picked at the same time from different trees in the same orchard suggest either that inoculum is more abundant on some trees than others or that oranges from some trees are more susceptible than those from others. At this stage it is not possible to present clear-cut evidence in favour of either one of these alternatives but for our purposes the material fact lies in the existence of the differences.

Considerable differences in infection may also occur between oranges taken from the same trees in different seasons, in oranges from different districts, and according to the time when oranges are picked for sampling. These differences, coupled with the absence or low degree of infection occurring in oranges, protected from rain, all point to the importance of weather in determining the degree of infection. However, there is not sufficient data available to indicate the relative importance of the various elements that together constitute the weather. In South Australia it is to be noted that the first significant increase in infection occurs naturally in April or May when the rains of autumn usually commence.

Spray treatments with Bordeaux mixture reduce the degree of infection but the extent of reduction is dependent on times of application. Information was gained from trials where only a limited number of the possible times of application were used. Where the degree of infection was light a single application in April was relatively effective but where conditions for infection are more favourable additional later sprays appear to be necessary for really efficient control.

V. Acknowledgments

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We are also grateful to E. A. Cornish for the considerations referred to earlier and for help generally on statistical problems which arose in the course of the investigation.

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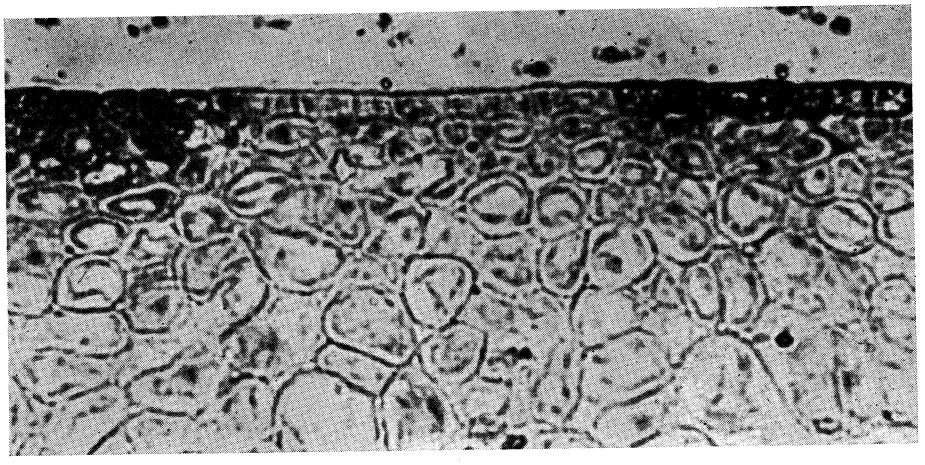
Explanation of Plate 1

Staining reaction of tissues invaded by *Colletotrichum*. The darker regions are places at which penetration has occurred. x 450.



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