THE PERENNATION OF *SPHAEROTHECA MORS-UVAE* AS CLEISTOCARPS WITH PARTICULAR REFERENCE TO MICROBIAL ACTIVITY.

by

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ABSTRACT

Less than 1% of overwintered cleistocarps of Sphaerotheca mors-uvae (Schw.) Berk. discharged ascospores in the spring. This investigation was concerned with the factors responsible for this low survival rate.

The time at which cleistocarps were formed on the black currant bush had little effect on their subsequent survival though cleistocarps formed in late summer were potentially the best source of infective ascospores.

Observations on the glycogen content of asci during the winter suggested that degeneration within the ascus could result from a depletion of this reserve material and, additionally, that a deficiency of osmotically-active substances in the ascus could lead to a failure in the dehiscence mechanism.

The development of micro-organisms on black currant leaves treated with urea was studied in relation to cleistocarp survival. The changes in the bacterial, fungal and actinomycete populations were relatively short-term and did not result in any appreciable degeneration of asci within cleistocarps though certain micro-organisms, notably actinomycetes, were closely associated with the cleistocarps and some were shown to produce chitinases and glucanases in vitro.

Bacteria and fungi were also found in ultra-thin sections of overwintered cleistocarps viewed in the transmission E.M. but there was little evidence of degradation of the cleistocarp wall. These studies together with chemical analyses suggested that the chitin and glucan components of the wall were protected from enzyme hydrolysis by substantial deposits of melanin.

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INTRODUCTION

Powdery mildew, caused by Sphaerotheca mors-uvae (Schw.) Berk. has been a serious disease of gooseberry in England since it was first reported by Salmon (1908) and more recently has been troublesome on black currant (Corke and Jordan, 1964). The perennation of the fungus on both hosts has been studied in detail by Merriman and Wheeler (1968) and on black currant by Jordan (1966, 1967, 1968). It is now known that cleistocarps borne on black currant leaves and stems, but not those formed on gooseberry, overwinter and discharge viable ascospores in spring. However, the numbers of cleistocarps that successfully survive the winter period is considered extremely small.

The primary aim of this investigation was to study factors which might account for this low survival.

I. OVERWINTERING OF CLEISTOCARPS

Review of literature.

There is good experimental evidence that cleistocarps of many powdery mildews function as overwintering structures, for instance, Erysiphe graminis on wheat (Marchal, 1903), E. cichoracearum on Arctium lappa (Cook and Wheeler, 1967), E. polygoni on a number of hosts (Smith and Wheeler, 1969), Phyllactinia moricola on mulberry (Itoi, Nakayama and Kubomura, 1962), Uncinula salicis on Salix discolor and Microsphaera penicillata on Lathyrus ochroleucus (Smith, 1971). In these instances, the cleistocarps survived the winter under natural conditions and discharged ascospores in spring which were capable of infecting their respective hosts. This was also demonstrated for cleistocarps of Sphaerotheca mors-uvae on leaves of black currant by Merriman and Wheeler (1968) and similarly for cleistocarps on black currant stems by Jordan (1966, 1967). These workers showed that the ascospores which were discharged from the overwintering cleistocarps could infect young leaf tissue at a time when buds were breaking in the black currant plantations. However, Merriman and Wheeler (1968) trapped few ascospores in laboratory dehiscence tests. Only one spore was obtained from a total of c. 17000 cleistocarps tested during the spring from leaves that had overwintered on the soil. More spores were trapped from cleistocarps on leaves that had been placed in a Terylene net bag and suspended from a wall throughout the winter. It was suggested that the leaves on the soil remained wet for relatively longer periods and in this situation the leaf microflora affected the survival of cleistocarps during the winter. Other workers have also implied that survival of powdery mildew

cleistocarps is influenced by microbial activity. Rasulev (1965) suggested that soil micro-organisms reduced the viability of Uncinula necator cleistocarps overwintering on vine debris. He found that whereas cleistocarps taken from plant tissue and placed on or in the soil overwintered successfully, those associated with the plant did not; this difference he ascribed to saprophytic microbial organisms especially fungi developing in response to the plant tissues and either directly or indirectly causing degeneration of asci and ascospores. Smith (1971) found that E. polygoni overwintered in England had a much less stable ascospore content during winter months compared to other powdery mildews surviving in the much colder Canadian winters. He considered that one possible reason for this was the much greater microbial activity during the English winter causing degeneration. Similarly, Smith and Wheeler (1969) suggested that micro-organisms were implicated in the generally earlier decline of ascospore content of E. polygoni cleistocarps overwintered on the soil compared to those in a drier environment i.e. suspended in bags on a wall. Salmon (1914a), however, found that total degeneration occurred also in cleistocarps of S. mors-uvae that had overwintered on the shoots of gooseberry plants but that samples taken from the bushes in autumn and kept dry in the laboratory dehisced when moistened in the following spring. He concluded that degenerated cleistocarps "had not reached that stage of development at which they can remain alive through the winter". He added that either the different weather conditions of this country or differences of a varietal nature were affecting the fungus which had recently been introduced to this country. Also, he thought it possible that only cleistocarps formed early in the season and reaching maturity

in July or August were able to provide viable spores the next year. Salmon (1914b) also observed that many cleistocarps which formed on the gooseberry shoots fell to the soil in autumn and as the initial infections were always found on leaves in the upper branches of the bush in the spring, he deduced that those cleistocarps in the soil did not produce viable spores. Merriman and Wheeler (1968) have also shown that total degeneration takes place in cleistocarps remaining on gooseberry bushes.

The time of formation of ascospores within the ascus is another aspect of the overwintering of cleistocarps in which conflicting results have been obtained. Many workers have demonstrated a slow differentiation of ascospores within cleistocarps of some powdery mildews during the winter. Laboratory experiments have shown that ascospore differentiation takes place within a few days if cleistocarps of Erysiphe graminis are immersed in water or alternately wetted and dried at various temperatures (Wolf, 1878; Graf-Marin, 1934; Cherewick, 1944; Moseman and Powers, 1957). Under natural conditions ascospores are differentiated in the spring in U.S.A. By contrast, Turner (1956) found that ascospores of E. graminis were released from July to late September in Britain during periods of high relative humidity, i.e. in the same year of formation. She found no evidence that ascospore formation increased following a prechilled water treatment (c.f. Graf-Marin, 1934 in the U.S.A.) but differentiation of ascospores within the asci was closely connected with the availability of water. Jordan (1966,1967), in his examination of cleistocarps of S. mors-uvae formed on shoots of black currant, found that maturation of ascospores progressed steadily during the spring and dehiscence occurred during April, May and June. However, Merriman and Wheeler (1968) found that

differentiation of ascospores began in August within cleistocarps formed in that season and was almost complete by the end of September. They tentatively suggested that the differences between their findings and those of Jordan (1966,1967) might be attributed to local climatic factors or to the different varieties of black currant which were involved at the two localities. Price (1969) found that some differentiation took place in cleistocarps of S. pannosa on rose thorns placed on soil from October to March. Here more asci and ascospores were observed in January but this was followed by complete degeneration and no ascospores were released. Kulikov (1953) looked at differention of cleistocarps of S. mors-uvae on gooseberry by counting the percentage of immature (white) cleistocarps in autumn and then again in the spring after the samples had overwintered. He found no difference in the nature/immature ratio and concluded that no differentiation or maturation took place of either the cleistocarp wall or ascospores throughout the winter.

There are relatively few observations on ascospore discharge within the powdery mildews other than brief statements that dehiscence occurs. Smith (1968) found that most ascospores were discharged from cleistocarps of E. polygoni from Heracleum sphondylium during the first 12h of a 5-day test period. In a more detailed analysis of dehiscence of E. cichoracearum on Arctium lappa Cook and Wheeler (1969) found that discharge was greatest 6-24h after wetting the plant material and cleistocarps. The percentage of cleistocarps which dehisced was estimated from the time cleistocarps were placed on the soil until the end of March and it was found that a total of 74% discharged ascospores. Cutter and Wheeler (1968) analysed the dehiscence of E. cichoracearum

cleistocarps in relation to field temperatures; discharge was apparently related to the number of hours above 5° in any period of observation. This was supported by laboratory tests: relatively few ascospores were discharged at 4° but progressively more spores were discharged at 10, 15 and 20°. Few experiments have been carried out on the dehiscence of S. mors-uvae cleistocarps. Salmon (1914b) examined dehiscence of gooseberry cleistocarps that had 'overwintered' in the laboratory. He placed some samples outside in February, and transferred others to 15° and 27°. Those outside continued to dehisce over a period of 15 days with temperature variations from 15.5° to -1°, but in the sample, as at 15°, dehiscence was greatest after 24h from the time of wetting. At 27° fewer spores were released but the maximum discharge occurred after 1½h. Merriman (1968) conducted tests at 20° and found that once wetted cleistocarps of S. mors-uvae reached a peak of ascospore discharge after 6h, although only a relatively small number of spores were released.

EXPERIMENTAL

1. Survival of cleistocarps in relation to time of formation.

(a) Sampling of leaves from black currant bushes.

Leaves with cleistocarps were taken from a planting of the cultivar Wellington at Southmoor, Oxon, at intervals from July to September 1969. Powdery mildew had been severe in this plantation in 1968 but no spraying was carried outin 1969. The first infections of 1969 were observed at the beginning of June. There was a period of high rainfall in mid-June but by the beginning of July powdery mildew was well established and many young leaves were covered with the primary mycelium of S. mors-uvae.

In all, six samples were taken. These were:

Sample	Cleistocarps initiated in -	Leaves detached at end of -
J	July	July
A	August	August
S	September	September
J/A	July	August
A/S	August	September
JAS	July	September

This sampling was based on the assumption that production of leaf tissue and formation of cleistocarps occurred approximately in 4-5 week cycles during July, August and September. This was derived from two observations: (1) on potted black currant plants at 15° cleistocarps were initiated 5-8 days after inoculation with S. mors-uvae and ascospores were differentiated after 14 days, (2) Merriman (1968), in mapping disease development in a plantation, noted that there was a rapid

increase in the colonisation of leaves by S. mors-uvae during July leading to defoliation and that subsequently new foliage was produced at the beginning of August.

On 1 July, 1000 leaves bearing well-developed mildew colonies but no cleistocarps were marked with no. 25 H string tags (Tag Craft) round the petioles. Of these, c. 200 leaves were lost through premature defoliation as a result of mildew. Other, similar leaves were tagged on 1 August and 1 September but at these times fewer leaves were available and many of those tagged either abscissed prematurely or did not bear cleistocarps. Thus the A, S and A/S samples totalled only 140, 128 and 200 leaves respectively compared to 240 leaves in each of the other samples.

The leaves were placed in bags (15 cm^2) of Terylene net (9.8 mech/cm^2), ten leaves per bag, which were then pinned to battens at 15 cm spacing on a frame (1 m^2) made from 2 cm^2 untreated wood. This frame was pegged to freshly-dug soil in the walled garden at Silwood Park.

(b) Sampling leaves on the soil.

The J, JA, AS, JAS collections were sampled at approximately 2 week intervals and the smaller A and S collections monthly until January 1970 when they also were sampled every 2 weeks. Six leaves, chosen at random, were taken from each bag. The leaf material and cleistocarps were allowed to imbibe water for 30 min (Merriman and Wheeler, 1968) the cleistocarps with associated secondary mycelium were stripped from the lamina, mounted in cotton-blue lactophenol and examined microscopically after crushing beneath a cover-slip. The proportion of cleistocarps containing asci and /or ascospores was then determined. Although, the numbers of

cleistocarps varied on different leaves, usually 500-1000 from each leaf were taken for these estimations. The state of the leaf tissue, the loss of secondary mycelium and cleistocarps from the leaf and the insects associated with these overwintering leaves were also recorded.

(c) Tests of cleistocarp dehiscence and ascospore viability.

The ability of cleistocarps on the leaf samples to dehisce and to discharge ascospores was tested by a method similar to that of Smith and Wheeler (1969). Cleistocarps embedded in secondary mycelium were placed on moistened filter paper in the bases of small transparent polystyrene boxes, 5.7 x 3.7 x 2.2 cm (Stewart Plastics Ltd., Surrey). Glass slides, cut to appropriate size, were placed in the lids of the boxes and secured with Plasticine. The boxes were inverted, some were placed outside at field temperatures, others at 15° and counts made of the numbers of ascospores trapped on the slide after 24, 48 and 72h using a binocular microscope (x100).

The viability of the discharged ascospores was examined in a similar manner using young black currant leaf tissue (cv. Wellington). In these tests pieces of secondary mycelium containing cleistocarps were placed on filter paper in the lid of each box and allowed to discharge spores onto the abaxial surfaces of six black currant leaf discs ($2cm^2$) floating on distilled water plus 20 p.p.m. kinetin in the bottom of the box. The boxes were kept outside for 3 days, the cleistocarps were then removed and the leaves incubated at 20° until sporulating colonies of mildew were observed. Dehiscence was assessed from samples of c. 1000-3000 cleistocarps every 2-4 weeks until January and then weekly in all six collections. Other samples were incubated at 15° to measure

the potentiality of any cleistocarps to dehisce after varying times on the soil.

Results.

(a) Comparison between treatments.

All collections (J,A,S,J/A, A/S, JAS) contained cleistocarps that had the ability to survive the winter and discharge ascospores that were able to infect leaf tissue at a time when leaves were being produced on the bushes. In 1970 bud burst occurred during early March. The results of three infection tests are given in Table 1 .

Table 1 . Number of colonies developing from ascospore infection tests.

Date	No. colonies per 6 leaf discs.					
	J	A	S	JA	AS	JAS
21.1.70	4	0	0	1	0	2
20.2.70	0	6	5	1	3	2
24.3.70	4	2	2	10	4	2

Figs 1a, b &c show that natural dehiscence occurred over a long period and in mid-February was detected in all collections. There was some suggestion that cleistocarps placed on the soil early in the autumn eg. collections J, J/A, were able to dehisce a large percentage of their spores somewhat earlier in spring than those set out later in the autumn. Indeed, many spores were discharged before leaf material was available for infection.

Fig.1a. Overwintering of cleistocarps 1969-70

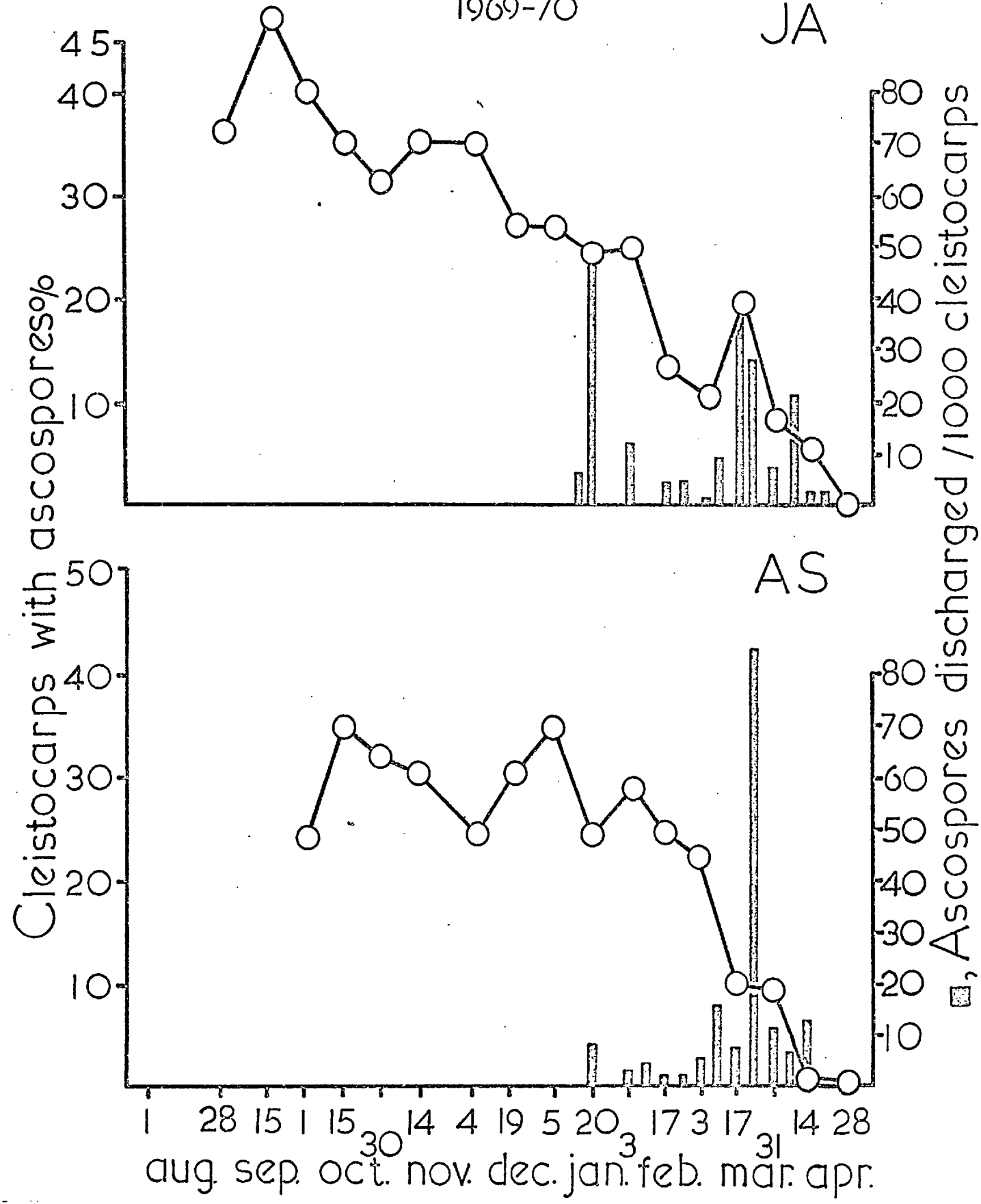


Fig.1b. Overwintering of cleistocarps

1969-70

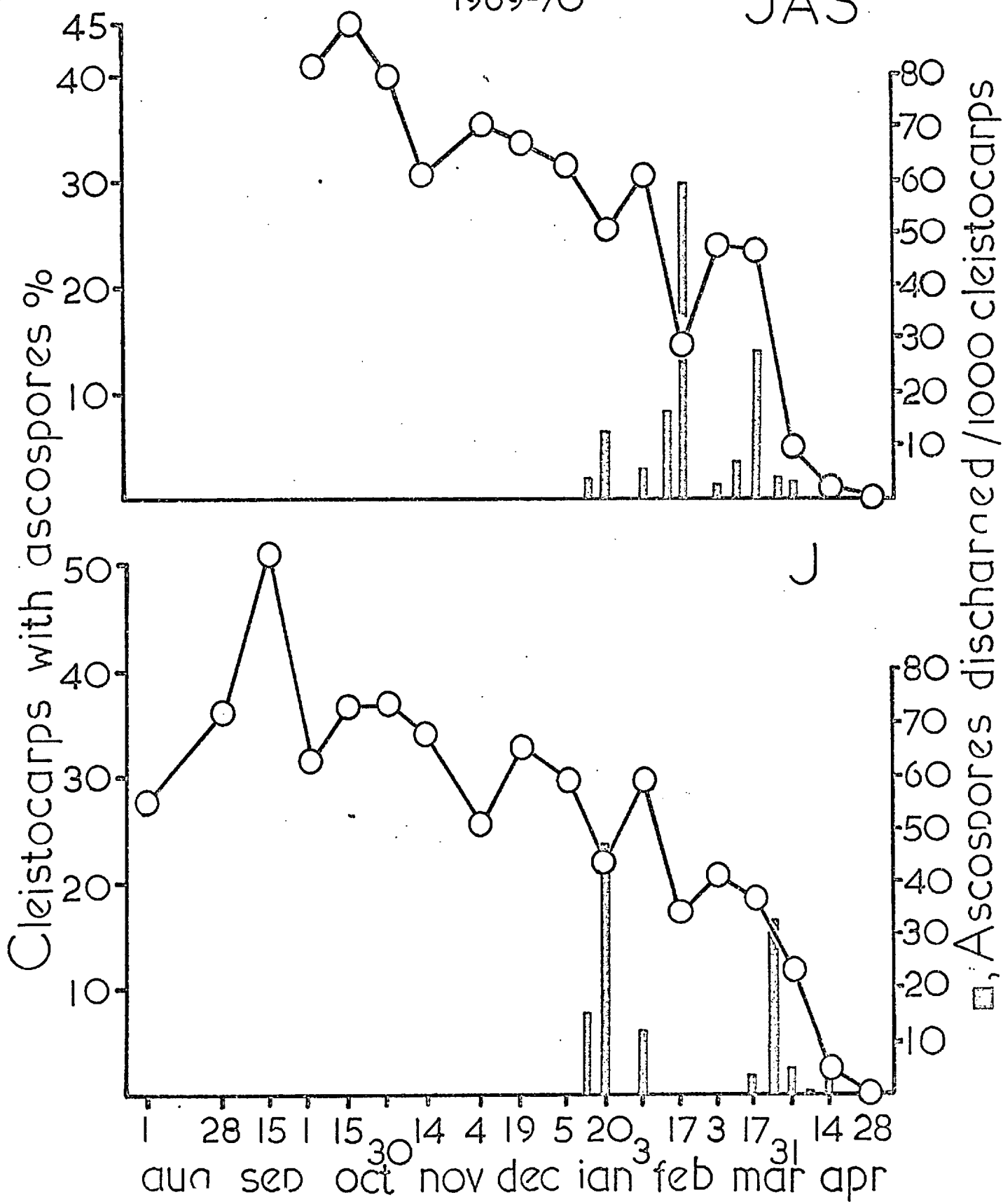
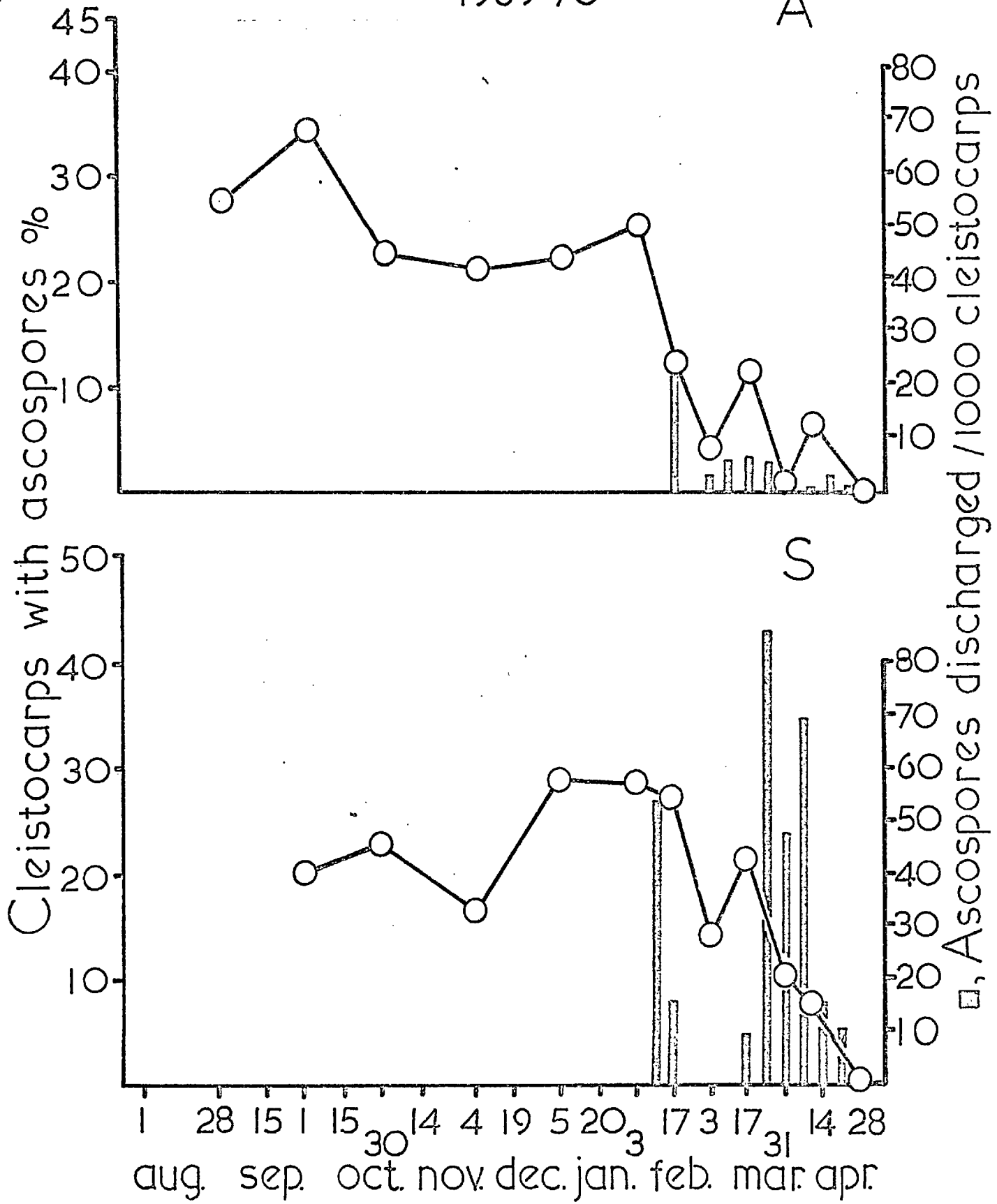


Fig. 1c. Overwintering of cleistocarps 1969-70



Inspection of the leaves in the J collection on 14 November i.e. c. 4 months after placing the leaves on soil, showed that about twenty cleistocarps had dehisced per sample of ten leaves. This suggested that substantial dehiscence had occurred during the unusually warm weather. However, not until 19 December were spores trapped in a dehiscence test. There were subsequently two peaks of ascospore discharge, in mid-January and mid-March; few spores were discharged in the intervening period. The reason for this apparent break in dehiscence is not clear. There were particular difficulties with the sample at this time which could have influenced these results. For example, few cleistocarps remained on the leaves and those present were without secondary mycelium so pieces of leaf material with cleistocarps were used in dehiscence tests instead of mycelial felts plus cleistocarps, with the disadvantage that spores were obscured by the distorted nature of the leaves.

Removal of secondary mycelium and cleistocarps occurred in other samples, but less severely, mainly in September and October. Leaves that had been covered by a dense mycelial felt were often stripped bare except for small patches between the angles of the leaf veins. On some leaves there was evidence of selective removal of mycelium and immature cleistocarps, the mature (brown) cleistocarps being left intact. Some losses of fungal material were probably caused by psocids which were seen on the J and J/A collections and which were observed to feed on mycelium and cleistocarps in laboratory experiments, but faecal pellets of other insects were also present on the leaves and these contained many immature cleistocarps. For example, six pellets sampled on 15 October

contained 439 cleistocarps, 132 with asci and sixty-two with fully differentiated ascospores. Fig. 2 shows cleistocarps within faeces.

In contrast to the J collections, S collections released the majority of their spores in late March - early April. Also, these samples were less attacked by psocids and other insects and possibly by micro-organisms. Although the total number of cleistocarps was small, survival can be considered more successful in that a higher percentage of cleistocarps which survived discharged their spores at a time when there was abundant leaf tissue on black currants in the field .

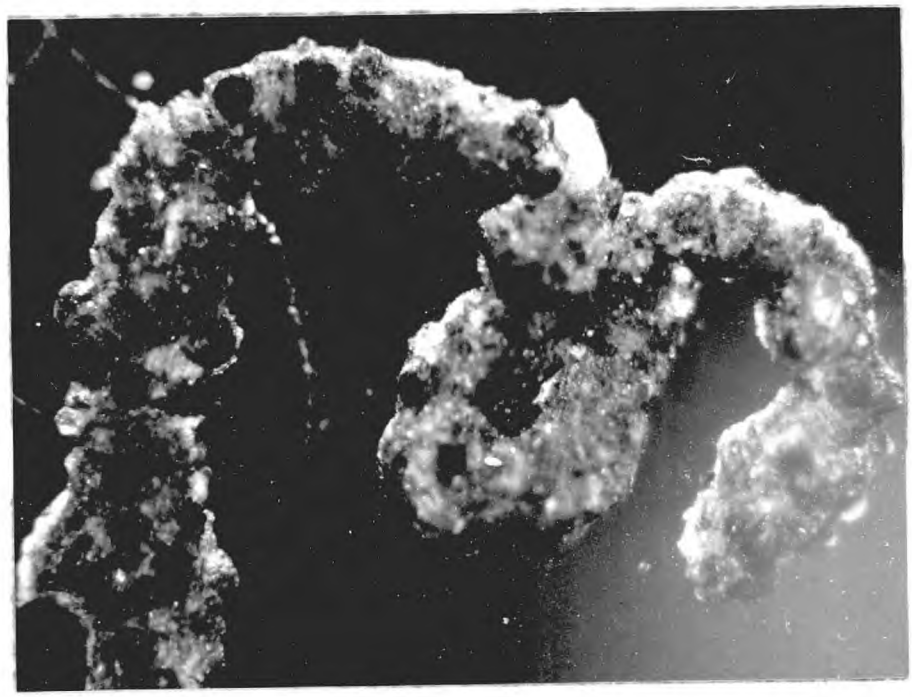
JAS cleistocarps survived poorly in comparison to those in the J/A and S collections. From the time of initial sampling on 1 October some of the cleistocarps in this collection contained degenerating spores and although the percentage of ascospores remained high throughout the winter the total number of spores released was comparatively small. As the percentage of cleistocarps with ascospores was no greater than J samples the result indicates that differentiation of ascospores did not take place after the end of July and the cleistocarps remaining on the leaf on the bush throughout the summer may infact have begun to degenerate before leaf-fall. There was little indication of possible causes of this poor survival.

(b) General observations on perennation on the soil.

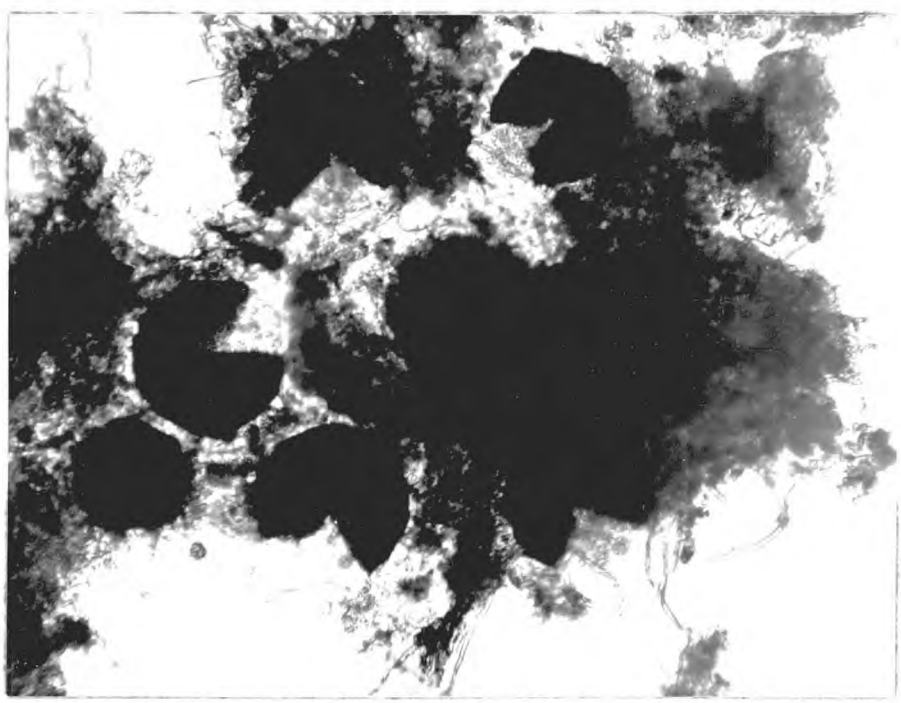
In all samples there were marked fluctuations in the percentages of cleistocarps with ascospores although each observation was based on 5000-10000 cleistocarps. There was no evidence of differentiation of asci of ascospores. The fluctuations apparently reflected the different

Fig. 2 . Faecal matter containing cleistocarps of S. mors-uvae recovered from black currant leaves on the soil.

(a) Whole faeces with cleistocarps (x110)



(b) Cleistocarps broken to show some with an ascus and ascospores (x250)



states of maturity of cleistocarps on each leaf and the degeneration of ascospores which occurred from the time the leaves were laid on the soil.

Degeneration was first seen in ascospores and then asci. The stages were:-

i) The appearance of small areas of fat in ascospores (Fig. 3).

ii) Shrunken ascospores without cytoplasm with fat alone remaining within the ascospore cell membrane.

iii) Asci containing less than eight ascospores.

iv) Asci unable to imbibe water; ascospores completely broken down.

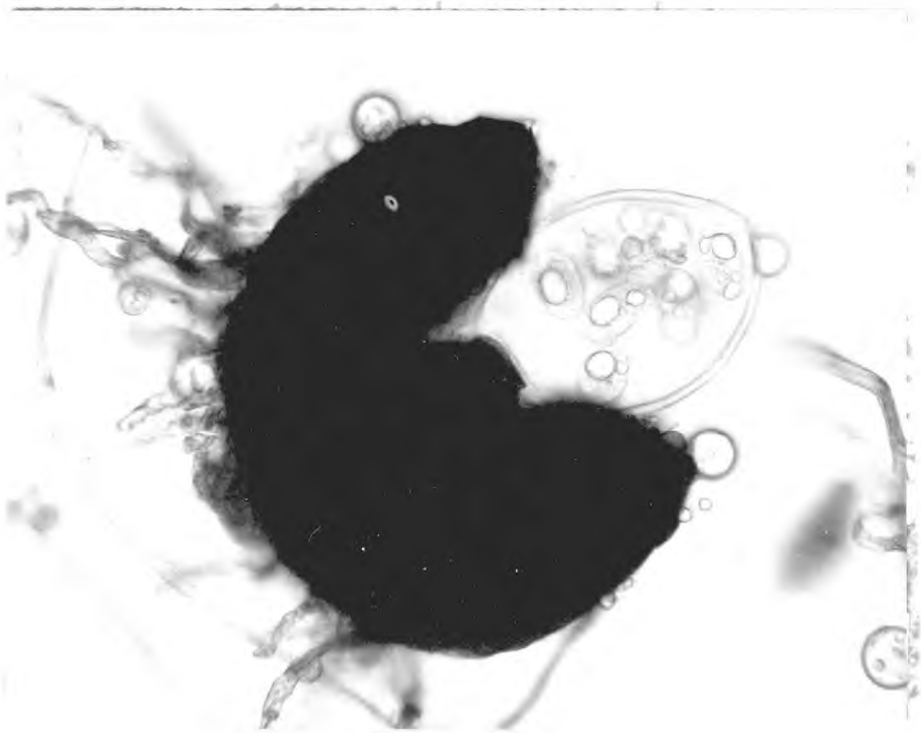
v) Asci absent. Usually this was accompanied by appearance of large quantities of oil droplets in the cleistocarps.

Later, especially in February and March, degeneration occurred on a large scale and little of the decline in percentage cleistocarps with ascospores shown in Figs. 1a, b, c can be ascribed to dehiscence because observation at this time showed that very few cleistocarps had dehisced. Where dehiscence had occurred cleistocarps appeared either with split walls showing clearly the line of rupture or with the lower portion of the wall only remaining as a shallow cup with all contents removed. The number of cleistocarps dehisced, in each sample, is given in Table 2. It can be seen that dehiscence was similar in all collections, whether placed at 15° or at field temperatures, with only one or two cleistocarps per 1000 discharging spores. Full results are given in appendix II Tables 1&2

Considering that in all samples much of the dehiscence occurs before the black currant bushes are in full leaf the numbers of cleistocarps producing potentially infective inoculum was extremely small.

Fig. 3 . Degeneration of S. mors-uvae ascospores.

(a) Appearance of fat droplets in ascospores and outside ascus. (x600)



(b) Appearance of ascus and ascospores without signs of degeneration. (x350)

Table 2. Numbers of cleistocarps dehiscing at 15° and field temperatures.

Sample	Temp.	No. cleistocarps	No. dehisced	No. dehisced/ 1000 cleistocarps
J	15	26,525	26	0.98
	Field	27,793	43	1.55
A	15	30,866	23	0.75
	Field	28,120	25	0.89
S	15	15,384	32	2.09
	Field	14,743	41	2.78
JA	15	32,884	48	1.46
	Field	35,208	63	1.79
AS	15	33,930	47	1.39
	Field	34,915	59	1.69
JAS	15	35,488	25	0.70
	Field	36,864	41	1.11

The maximum number of spores released was closely related to the field temperatures and it was apparent that when free water was present and the asci were fully turgid dehiscence generally took place at temperatures in excess of 5°. But as the total number of ascospores trapped was small a more detailed assessment of dehiscence in terms of temperature was not possible. Table 3 shows that in all samples dehiscence was greatest in the first 24h.

Table 3 . Dehiscence of samples at 24, 48 and 72h.

Sample	24h	48h	72h
J	156	121	9
A	106	27	4
S	154	89	17
JA	273	131	27
AS	317	59	33
JAS	168	89	16
Totals	1,174	286	106

Observations of the cleistocarps indicated that when they reached a state (usually 4-5 months after being put in soil) when dehiscence became possible the slightest external pressure on the wall caused it to rupture and the ascus swelled if the cleistocarp was wet. This change in the ability to dehisce compared to samples taken in autumn could reflect a weakening of the cleistocarp wall or a change in the osmotic pressure within the ascus increasing the internal stress on the wall and so rupturing it.

Ability of some insects to feed on S. mors-uvae mycelium and cleistocarps.

Three black currant leaves c. 1 cm long, each entirely covered with a thick secondary mycelial felt of S. mors-uvae containing cleistocarps were placed in a perspex box (5 cm diameter, 2.5 cm deep) having a solid charcoal base (0.7 cm deep) which was saturated with water. Ten psocids Ectopsocus briggsi and ten collembola (Entomobrya intermedia) were collected from a sample of leaves overwintering on the wall on 4 February

1970 and placed in two boxes. All collembola were adults but of the ten psocids three were winged adults and the remainder nymphs. The boxes were incubated at 10° under a low light intensity. At intervals, the insects were observed and the disappearance of the secondary mycelium and cleistocarps was recorded.

After 10 days the psocids had entirely stripped the leaves of mycelium and cleistocarps and the faeces that were examined contained fragments of cleistocarps wall. This was not so with the collembola. They were seen to feed on the mycelium but this disappeared from parts of the leaves only; they did not feed on the cleistocarps. When saprophytic fungi developed on the leaves in these conditions of high relative humidity they were consumed in preference to the mycelium of S. mors-uvae.

2. Artificial dehiscence and histochemical studies of cleistocarps overwintering on the soil.

The potential dehiscence of cleistocarps was examined and related to natural dehiscence of samples overwintering on the soil. The changes in reserve materials within the ascus and ascospores were also studied.

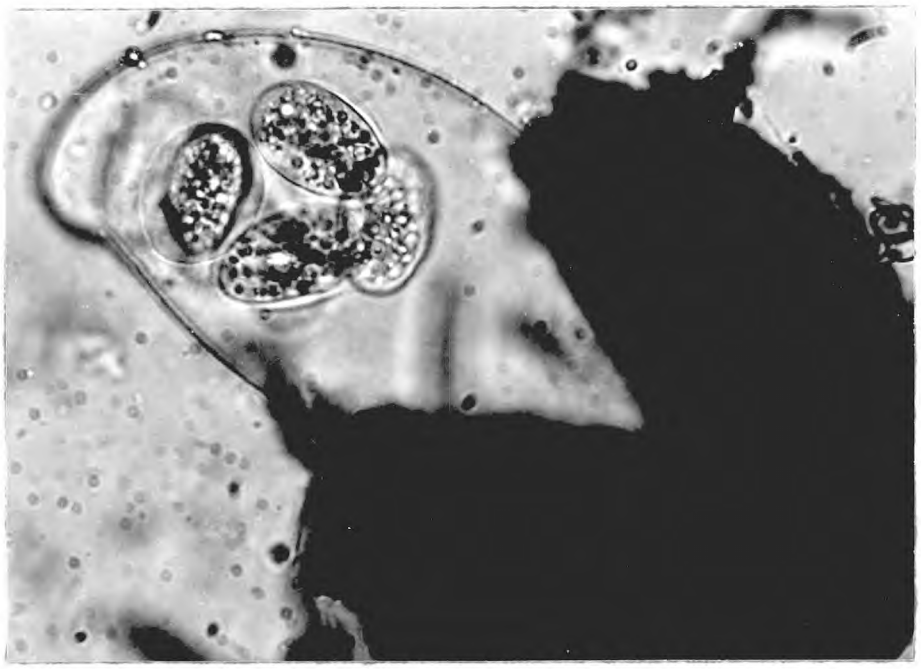
Methods.

Six leaves with cleistocarps were taken every 10-15 days from a collection placed on soil on 30 September 1970 and the state of the asci and ascospores was recorded. Leaves were soaked for 1h in tap water, small pieces of mycelium and cleistocarps were detached and carefully teased apart to separate the cleistocarps. The cleistocarps (200-400/sample) were mounted in distilled water under cover-slips and the walls broken with gentle pressure. The number of asci that swelled and those discharging ascospores were counted after 10 min and 1h respectively. A control sample of cleistocarps was taken from leaves kept dry at 10°.

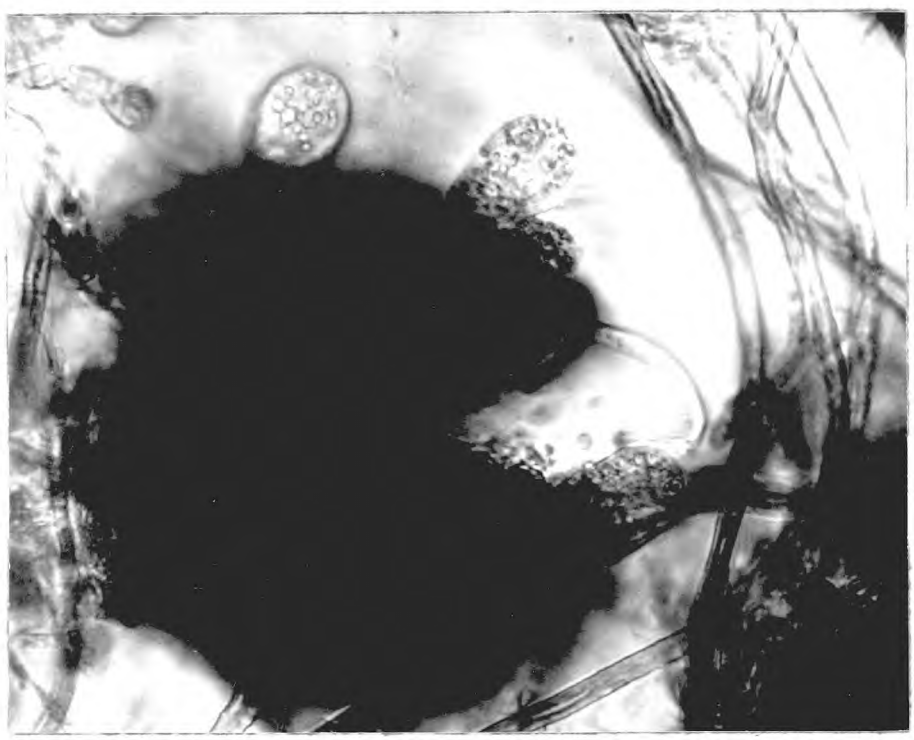
In histochemical studies Lugal's iodine (C.M.I. Plant Pathologist Pocket Book, 1968) was used to detect glycogen and Sudan IV to detect fat within the asci and ascospores. Cleistocarps were soaked for 24h in 1% gum arabic before sectioning with a freezing-microtome (Cambridge Instrument Co. Ltd.). Sections were cut 5-10 μ thick. Fig. 4 shows cleistocarps with a swollen ascus and an ascus with discharged ascospores.

Fig. 4 . Slide dehiscence of S. mors-uvae cleistocarps.

(a) Swollen ascus. (x800)



(b) Ascus with discharged ascospores. (x700)



Results.

Microtome sections of mature cleistocarps showed 3-4 layers of thin-walled cells immediately beneath the pseudoparenchymatous cells of the wall. The contents of these stained heavily with Sudan IV, indicating very large accumulations of fat. Fat was also present in the ascospores. Initially it was distributed throughout the cytoplasm but in later samples, at a time when dehiscence occurred, many ascospores contained fat in bodies either one positioned centrally or two, one at each pole. In this case there was little fat in the cytoplasm of the cell outside these areas. Often the central area of fat appeared as a thin plate running almost the entire length of the cell along the equatorial plane. This change in accumulation or distribution of fat was often seen in early spring but as it was impossible to detect ascospores which were viable and would be dehisced the contents of these spores might be indicative of degeneration and not processes of maturation. This view is supported by observations made on cleistocarps overwintered on a south-facing wall. After staining dehisced ascospores with Sudan IV the fat was seen to be in very small bodies distributed throughout the cell's. Few of these spores showed large accumulations of fat as described above but they were seen in many of the ascospores from cleistocarps that had not dehisced in these tests. Also as it has been shown that some of the cleistocarps contain infective ascospores when tested at leaf fall (page 59) and as these spores do not show fat accumulation in descrete areas it would seem unlikely that the observations noted have any significance in

ascospore differentiation.

The significance of the fat in cells beneath the cleistocarp wall also remain unresolved. Little change took place in these cells throughout the time the cleistocarps were on the soil until the spring when degeneration within the cleistocarps took place and oil droplets appeared, presumably derived from breakdown of these cells.

More substantial evidence for a process of differentiation came from a study of the glycogen content of the asci through the overwintering period. Staining with Lugal's iodine showed that when ascospores were first formed, the asci contained high concentrations of glycogen, developing a deep wine colour with iodine. However, it was found that when cleistocarps walls were split in water beneath a cover-slip some asci were able to swell and these only stained faintly yellow-red or not at all in iodine. All cleistocarps tested that had ascospores but whose asci did not swell contained large amounts of glycogen. If glycogen was being converted into simple sugars and increasing the osmotic potential of the ascus then the process of dehiscence within an intact cleistocarp could be associated with this increased ability to imbibe water and cause mechanical rupturing of the pseudoparenchynatous wall.

Each sample taken from the population of cleistocarps on the soil contained asci without glycogen that could imbibe water and swell. Although the numbers fluctuated (see Fig.5a) there was no evidence for an increase in the number of asci capable of swelling or dehiscing over the winter months even during periods when temperatures were relatively high and rainfall was sufficient to allow metabolic processes to take place within the asci. In fact a marked decrease in the numbers swelling

Fig.5a. Artificial dehiscence of c'carps

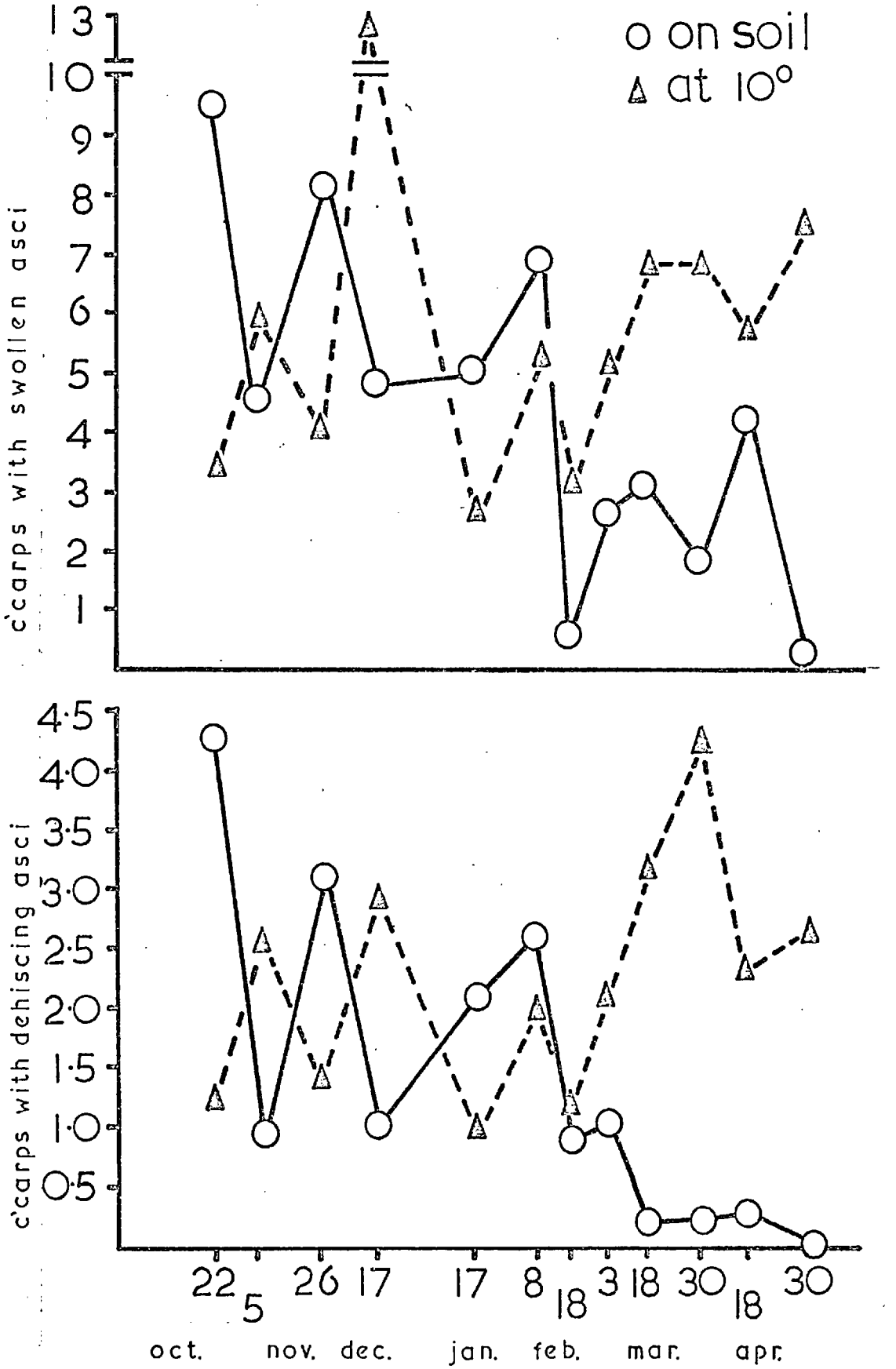
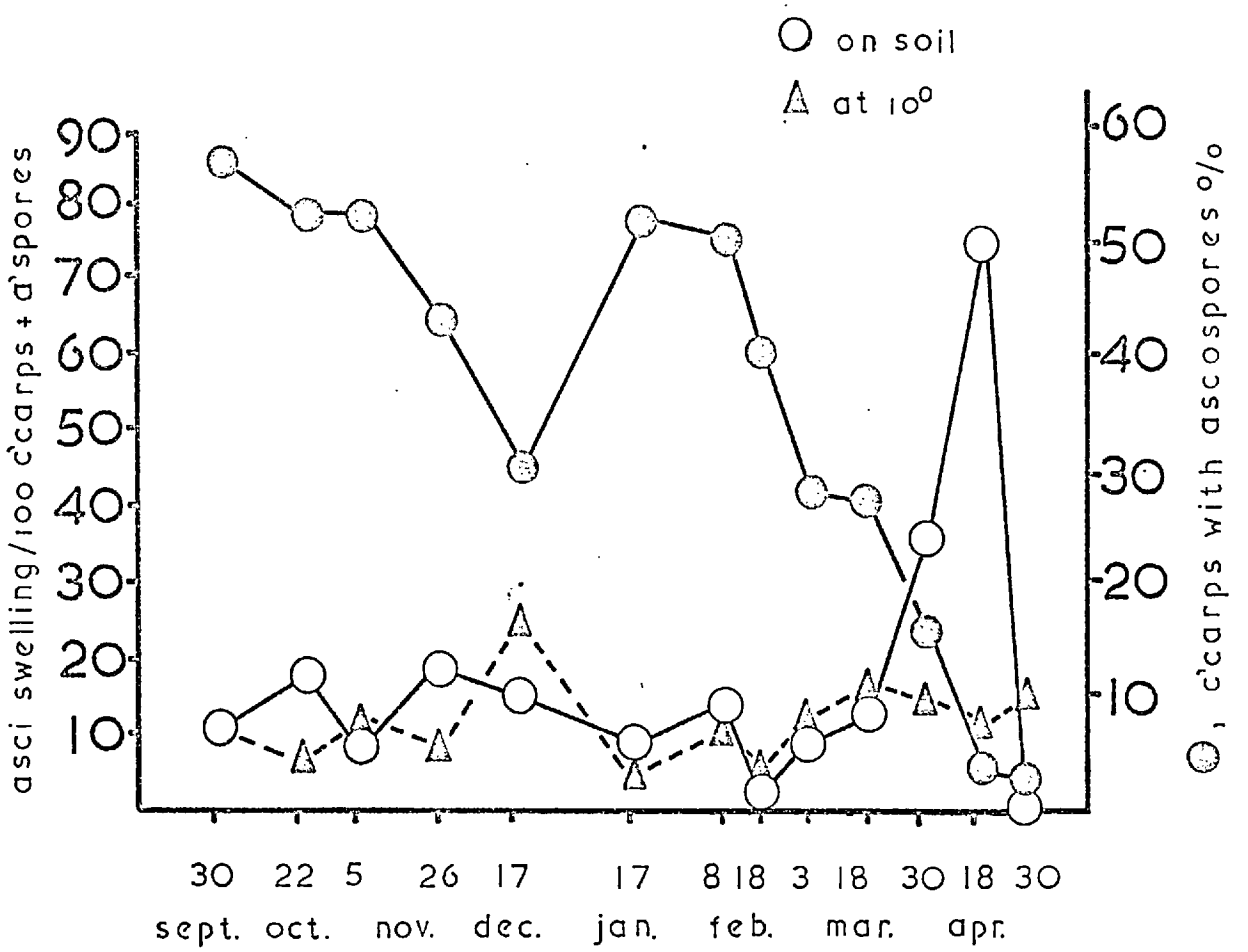


Fig.5b. Artificial dehiscence of c'carps 1970-71



and discharging spores occurred in the dehiscence tests in early February when there was a similar decline in cleistocarps with ascospores (Fig. 7). In Fig. 5b the numbers of asci swelling are related to the numbers of cleistocarps with ascospores. The proportion of asci capable of imbibing water remained fairly constant throughout the winter and early spring then rose sharply prior to total loss of cleistocarp contents. This result was similar to that obtained for the discharge of spores in samples overwintered on the wall (Fig.8(ii)).

It was apparent from observation (in mid-April) on the artificial dehiscence of samples overwintering on the soil that although asci imbibed water and protruded from the cleistocarp the size of the swollen ascus was considerably smaller than in comparable samples kept dry at 10°. These partially swollen asci were not stained by iodine. It is possible that discharge of ascospores did not take place in these asci because their ability to imbibe water was not sufficient to rupture the cleistocarp wall. Similarly, in normal dehiscence tests for samples on the soil cleistocarps were seen to split and protrude an ascus which reached little more than 50% of its potential size and often, after a short time receded into the cleistocarp wall.

Occassionally in tests where the cleistocarp was artificially broken, asci with spores showing partial degeneration became swollen and discharged these spores which were nothing more than membranes completely filled with fat. This was also seen in dehiscence tests in the polystyrene boxes. Thus, the ability of an ascus to swell and discharge

spores may not be related to the viability of the contained ascospores. Also in the slide dehiscence studies not all eight spores were dehisced, often some spores failed to move to the apex when the ascus swelled and remained in the ascus after dehiscence.

Natural dehiscence of cleistocarps involves basically two events - the swelling of the ascus (by the imbibition of water) and the rupturing of the ascus wall.

In the sense that swelling of asci is an essential first step in dehiscence it can be considered to indicate the potential for dehiscence. It is of interest to compare the potential for dehiscence as observed in those tests where the cleistocarp wall was ruptured by pressure with the actual dehiscence obtained in tests with slide chambers. Table 4 shows the proportions of asci swelling and discharging spores in material kept on soil and in material kept in the laboratory at 10° (potential dehiscence) together with the dehiscence obtained (actual dehiscence) in the slide chambers from material kept on soil and hung on a wall.

Table 4. Potential and actual dehiscence of cleistocarps in different situations.

	Situation	Nos. dehiscing/1000 cleistocarps with ascospores
* Potential dehiscence	Laboratory 10°	44.1
	On soil	48.1
+ Actual dehiscence	On wall	40.6
	On soil	4.2

* The average of dehiscence throughout winter of 1970-71.

\+ The average of dehiscence from February 1970.

There are two main points of interest:

(1) The potential dehiscence of cleistocarps on soil (48.1) was similar to that of cleistocarps stored dry at 10° (44.1). It could be argued from these data that potential dehiscence is already determined at leaf fall and this is the simplest explanation. The possibility is not excluded, however, that in the sample on soil some cleistocarps actually dehisced and the percentage of swelling asci (potential dehiscence) was only maintained at 48.1 by maturation in other cleistocarps.

(2) The actual dehiscence of cleistocarps on soil (4.2) was much lower than might be expected from the figures for potential dehiscence whereas that of cleistocarps on the wall (40.6) corresponded fairly closely to them. The low figure for the cleistocarps on soil could be explained in terms of the depletion of osmotically active materials and thus inability of the asci to rupture the wall. During the mild, wet winters

of 1968, 1969 and 1970, temperatures were above 5° for relatively long periods. During these periods, glycogen and perhaps other materials may have been catabolised to meet the requirements of the living cell and thus were no longer available at the critical time for dehiscence. By contrast the samples suspended on the wall would have kept much drier and this would act against premature depletion of food reserves in this way.

3. Survival of cleistocarps in different environments.

Experiments were undertaken to observe the survival of cleistocarps in immediate contact with the soil. By so doing it was thought possible to subject cleistocarps to maximum microbial activity and also to longer periods of moisture than those overwintering on leaves on or above the soil.

Methods.

Experiment (i).

Soil was collected from black currant plantation at Silwood Park, air-dried for 3 days in the laboratory and sieved through 2000 μ mesh. Six grams of this soil were then put in to each of forty-eight glass tubes. Each tube was 10 cm long with a top diameter of 1.5 cm with the lower 3 cm of the tube restricted to 3 cm diameter. A small pad of glass wool was inserted into the constricted end to act as a drainage plug and thus minimise waterlogging of the soil.

Leaves infected with S. mors-uvae were collected from Fernhurst, Kent, in September 1968. These were soaked in water for 1h and the cleistocarps plus secondary mycelium were then removed. Pieces of mycelium with about 1500-2000 cleistocarps were then placed in each of twenty-four tubes and mixed with the top 2 cm of soil. The tubes were covered with Terylene net (1600 mesh/cm) to prevent entry of earthworms and other fauna and then placed in soil so that they protruded 1 cm above the soil level.

A leaf disc (1 cm diameter) with a similar quantity of cleistocarps was placed, abaxial surface uppermost, in each of the remaining twenty-four tubes. This set of tubes was placed in soil like the others. Both sets were inserted in soil on 5 October 1968. Subsequently at monthly intervals three tubes were taken from each set. Two methods were used to recover cleistocarps. In the first three months the soil was air-dried for a few hours and then placed in a 100 ml measuring cylinder with water. With gentle stirring the cleistocarps and secondary mycelium, in which air was entrapped, floated to the surface together with plant debris. The sample was then sorted to collect the cleistocarps. In later samples small quantities of soil were taken and the cleistocarps removed using mounted needles; the sorting was carried out under a binocular microscope (x20). The percentage of cleistocarps with ascospores were assessed and dehiscence tests carried out by the methods already described (pp. 15 & 16).

Experiment (ii).

Leaves with cleistocarps were collected from Southmoor, Oxon, on 14 October. Secondary mycelium and cleistocarps were removed as described for expt. (i) but to facilitate recovery they were enclosed in Terylene net bags (1600 mesh/cm^2). Samples of 250 leaves or cleistocarps taken from 250 leaves were placed in four situations;-

i. On a south-facing wall, 2m above soil level, on leaves.

ii. On the soil, on leaves.

iii. In the soil on the leaf at 2 cm depth.

iv. In the soil without leaves.

Cleistocarps were sampled at intervals of 2 weeks and dehiscence tested at 10-day periods from 13 February 1971 until total loss of ascospores.

Results.

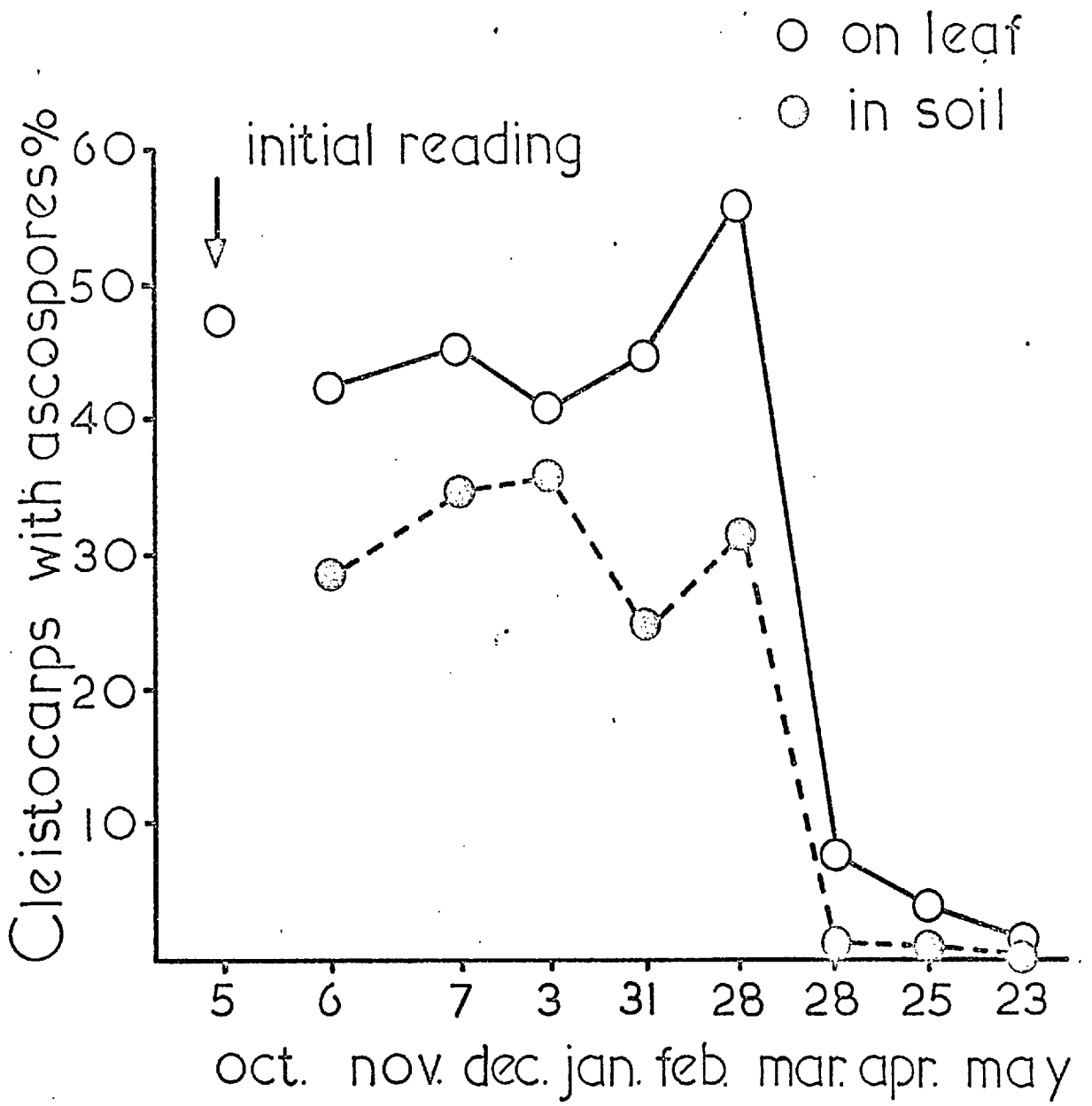
The results of experiment (i) are given in Fig. 6 and Table 5 . The ascospore content of cleistocarps placed in soil without leaf tissue was significantly lower ($p < 0.01$) than that of cleistocarps remaining on the leaf discs. Additionally, there was no dehiscence from cleistocarps which had been buried in soil.

Table 5 . Ascospore discharge on and in soil 1968-69.

Date	On Soil			In Soil		
	No. spores	No. c'carps	Spores/ 1000 c'carps	No. spores	No. c'carps	Spores/ 1000 c'carps
13.2.69	24	2,323	10.3	0	2,871	0
7.3.69	61	3,098	19.7	0	3,742	0
23.3.69	26	2,515	10.3	0	2,160	0
9.4.69	9	2,748	3.3	0	2,628	0
Totals	120	10,684	11.2	0	11,401	0

The difference in ascospore content between the two samples may have resulted in part from the sampling techniques for both methods favoured the selection of the larger, more mature, cleistocarps with fully differentiated ascospores and thus the figures for cleistocarps in the soil may have been exaggerated. It was not apparent whether the smaller, lightly pigmented cleistocarps were not recovered because they were being destroyed by soil micro-organisms or because they were extremely

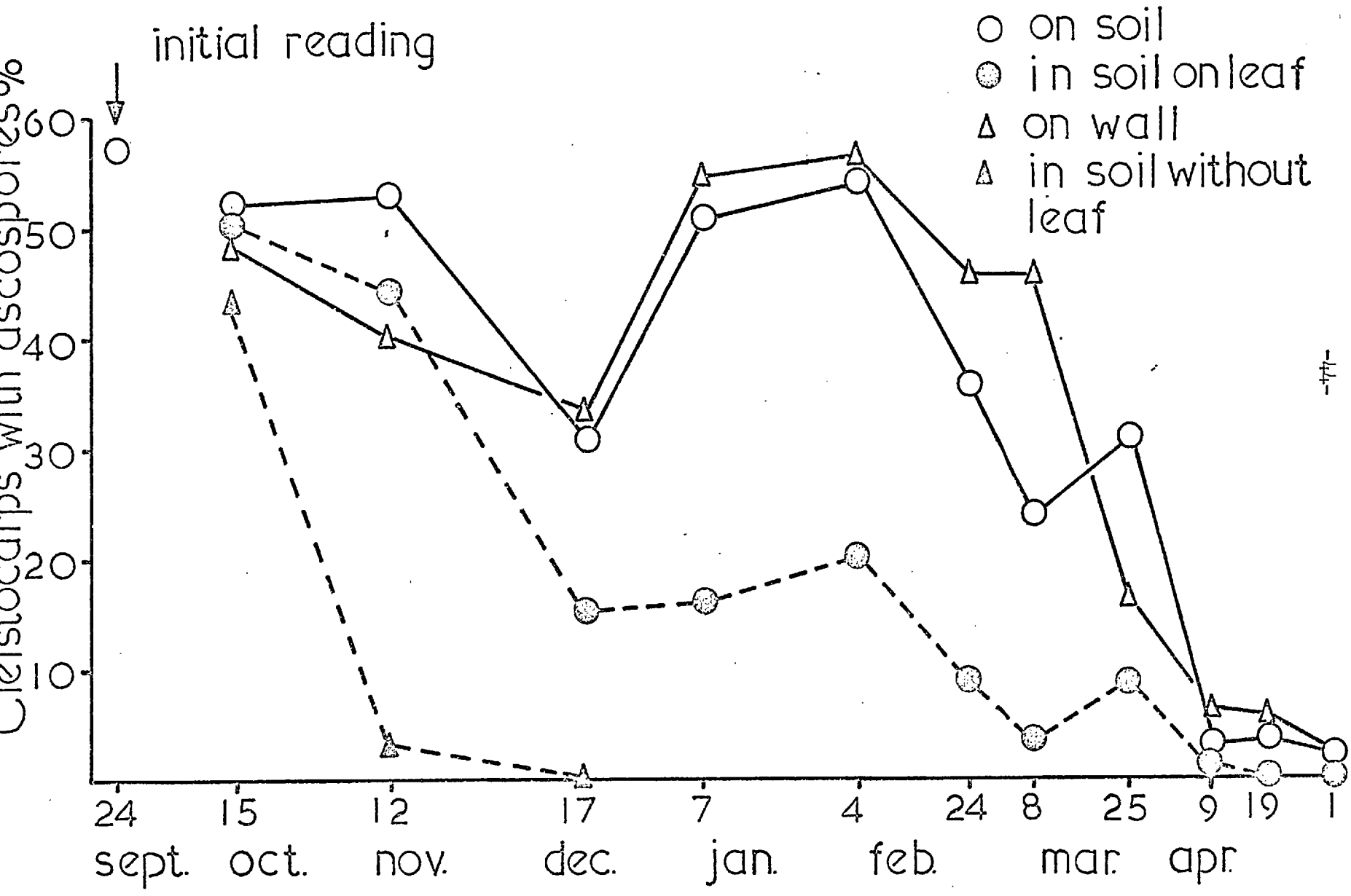
Fig.6. Overwintering of cleistocarps 1968-69



difficult to sort from soil particles. There was, however, evidence that the secondary mycelium was being degraded and by early February little of the mycelium or the appendages of cleistocarps remained. There was a sharp decrease in percentage of ascospores in the soil sample in autumn and early spring but it was not possible to relate this either to microbial activity or to higher moisture levels causing a depletion of reserve materials in the ascus. Certainly the decline in spring was not due to dehiscence. The samples taken were small but in total over 1000 cleistocarps were looked at and none showed signs of dehiscence.

The results of experiment II are given in Fig 7 . There was little difference in the ascospore contents of cleistocarps within the samples on the wall and on the soil. However, there were obvious differences in this respect between these samples and those placed in the soil. Cleistocarps on leaves in soil (ISL sample) contained ascospores until early April despite considerable degeneration in November and December. The leaves themselves began to be severely attacked by micro-organisms in October shortly after being placed in the soil; much of the epidermal tissues were completely lost and with them the attached cleistocarps. The areas of the leaf bearing cleistocarps were extensively colonised by bacteria and slime moulds in particular. In samples taken in January actinomycetes were seen on all the leaves and were readily isolated when pieces of mycelium with cleistocarps were plated onto chitin agar (appendix page 245). These actinomycetes were not confined to areas of the leaf with secondary mycelium so they may have been a consequence of the increased bacterial populations rather than the

Fig.7. Overwintering of cleistocarps 1970-71



presence of S. mors-uvae. Nevertheless, there was a marked loss of much of the mycelium and cleistocarps appendages. Even though 15% of the cleistocarp at this time contained ascospores very few gave a positive test for glycogen; mostly a yellow-red colour resulted after mounting in iodine. Similarly it was rare for any of the asci to imbibe water when the cleistocarp wall was split after samples had been mounted in water on a glass slide.

The number of actinomycetes in the four situations was assessed on 18 January by taking c. 20-50 cleistocarps, plating them on 1% chitin agar and incubating at 10°. The results are given in Table 6. The time taken to sporulation was used as an indication of the growth of the actinomycetes before they were placed on the agar. It would seem that they were present in all four situations but those developing cleistocarps on leaves in soil (OSL) were likely to have developed from mycelium already actively growing and those developing from the other samples probably grew from spores present on the mycelium or cleistocarps. The rather low number of actinomycetes developing from cleistocarps in the soil without leaves (IS) was in keeping with observations on these samples; comparatively little bacterial colonisation was seen and no actinomycetes observed growing in association with the secondary mycelium.
Species of Cephalosporium, Penicillium and Fusarium grew most abundantly when pieces were plated on chitin agar. The IS cleistocarps degenerated almost completely by mid-November, only 7 weeks after being placed in the soil. The few remaining asci at this time showed complete absence of glycogen and they did not imbibe water. The secondary mycelium

of this sample began to disintegrate after 4 weeks in the soil but there was little obvious loss or destruction of the immature cleistocarps.

Table 6. Actinomycetes colonies developing from cleistocarps above, on and in soil.

Situation	No. samples	No. act. developed	Nos. with chitinase	Days to sporulation
On wall (OW)	20	3	2	c.15
On soil (OS)	20	13	10	c.15
In soil on leaf (ISL)	20	16	14	5
In soil (IS)	20	7	5	c.15

In all samples there were cleistocarps present with an almost complete absence of pigmentation and these remained. Thus, it would seem unlikely that the loss of ascospores occurring here was caused by micro-organisms weakening the cleistocarp wall itself. Certainly soil micro-organisms could be implicated in the ISL samples were a rapid rise in bacterial populations corresponded to a fall in the percentage of cleistocarps with ascospores. A noticeable difference between the collections was the large number of nematodes among cleistocarps of the sample ISL; these were found in large numbers from the first sampling date. The majority belonged to the bacteria and debris feeding nematodes of the family Rhabditidae which possess sucking mouth parts and lack the stylets normally associated with nematode feeding on living fungal cells.

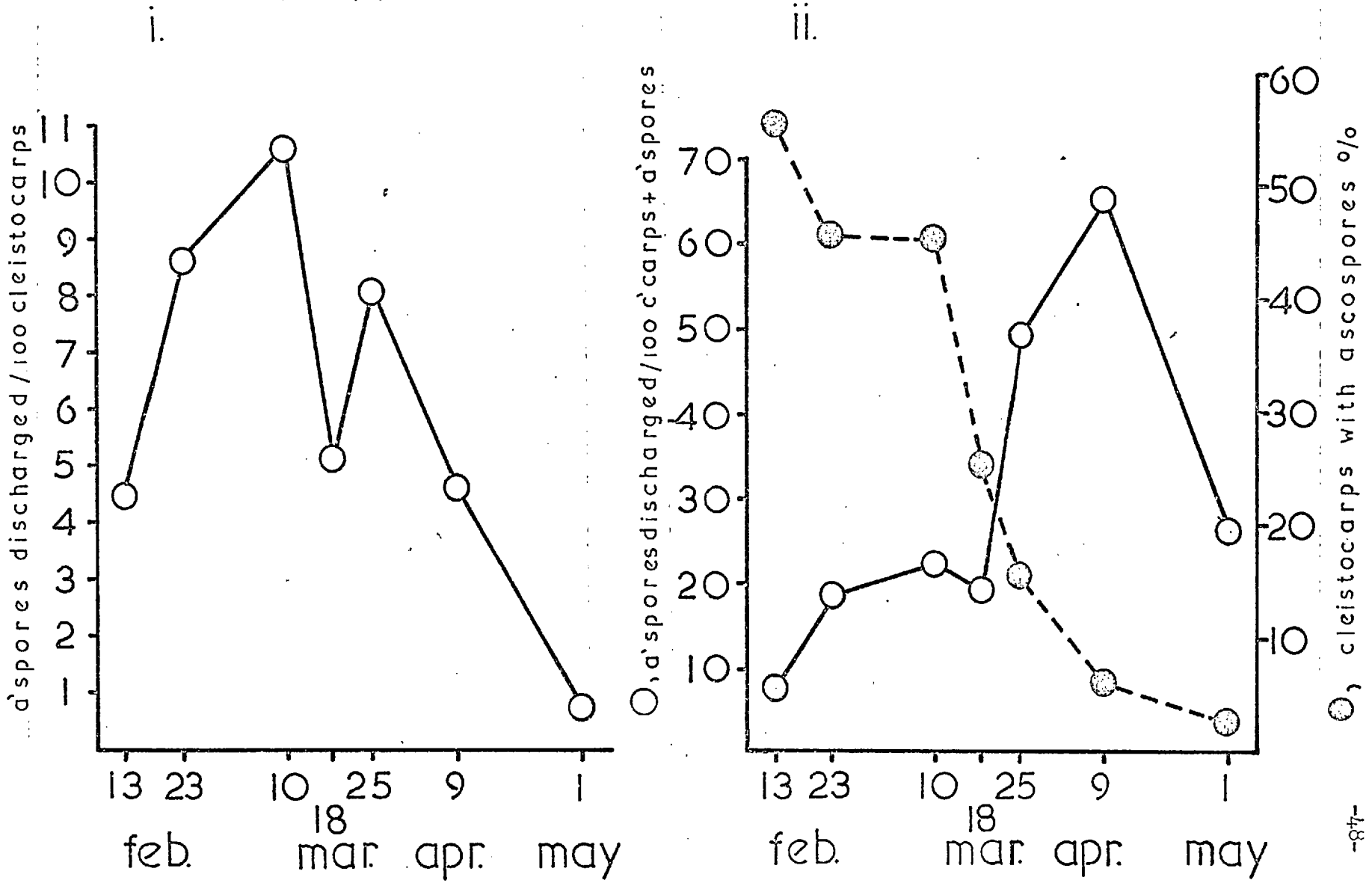
The results of dehiscence tests on the samples are given in Table 7 and in Fig 8. No dehiscence tests were carried out on the IS samples

Table 7. Dehiscence of cleistocarps on, in and above soil 1971.

Date	Above soil on wall					On soil on leaf					In soil on leaf				
	No. ccarps	No. aspores	Spores/ 1000 c'carps	(a)	No. swollen	No. ccarps	No. aspores	Spores/ 1000 c'carps	(a)	No. swollen	No. ccarps	No. aspores	Spores/ 1000 c'carps	(a)	No. swoll
13.2.71	19,586	891	45.5	80.3	5	19,536	18	0.92	1.7	0	18,744	13	0.7	2.9	0
23.2.71	20,200	1756	86.9	186.6	6	17,073	0	0	0	0	16,541	0	0	0	0
10.3.71	16,725	1785	106.7	228.4	5	15,015	8	0.5	2.2	0	15,004	0	0	0	0
Bud burst															
18.3.71	23,728	1215	51.2	196.9	6	17,558	30	1.7	0.7	1	18,927	0	0	0	0
25.3.71	23,648	1917	81.1	491.3	10	17,081	49	2.9	0.9	0	15,072	0	0	0	0
9.4.71	12,501	580	46.4	664.8	7	12,795	16	1.3	29.8	0	14,121	0	0	0	0
1.5.71	17,554	135	7.7	293.5	2	16,160	91	5.6	169.6	0	15,644	0	0	0	0
Totals	133,942	8,279	61.8	306.0	41	115,198	212	1.8	29.2	1	114,053	13	0.1	0.4	0

(a) Ascospores released/1000 cleistocarps with ascospores.

Fig.8. Ascospores discharged: c'carps wintered on a wall
1970-71



because all ascospores had degenerated by 17 December. The ISL samples contained very few ascospores in viable condition and of c. 114000 cleistocarps tested only 13 spores were caught and all these in the first dehiscence test on 13 February. There was a marked difference in dehiscence of cleistocarps on leaves kept on soil and that of leaves suspended on a wall. Considering that the percentage of cleistocarps with ascospores were almost identical in these two samples throughout the winter this difference must reflect differences in the ability of asci to swell and discharge the spores.

In other aspects there were minor differences between these two samples. There was considerable loss of mycelium from leaves on soil but, apart from damage by psocids and collembola (p.27), there was little loss of secondary mycelium and cleistocarps from leaves on the wall. In both samples there was apparently little change in the glycogen content of asci until January when many asci with ascospores stained yellow-red. Subsequently there was a rapid decrease in the numbers of asci containing glycogen in cleistocarps on the soil and by 25 March none were found. Asci in cleistocarps on the wall also showed a loss of glycogen but this depletion was relatively slow and not until the final dehiscence test on 1 May were no asci found giving a positive reaction. More accurate assessment of the dehiscence of S. mors-uvae was possible with the sample on the wall where large numbers of spores were trapped (Fig.8(i)). Most spores (per 1000 cleistocarps) were released when 45% of the cleistocarps still contained ascospores. This agrees with the many other observations on dehiscence that maximum discharge

occurs before the rapid decline in percentage cleistocarps with ascospores (p. 24). When the results are recalculated to take into account dehiscence and degeneration in the sample under test and expressed as spores dehisced per 1000 cleistocarps with ascospores then it can be seen that a maximum number of cleistocarps discharged spores 4 weeks later in mid-April (Fig. 8(ii)). This might simply be owing to more favourable temperatures for dehiscence but it could indicate also an increase in the maturity of the remaining cleistocarps. The number of cleistocarps that dehisced from this sample was 0.8% and in terms of cleistocarps with ascospores 4.2%. In contrast of the 115198 cleistocarps tested from the OS sample the figures were 0.02% and 0.36% respectively, but almost half the number of spores were trapped in one dehiscence test on 1 May.

4. Survival of cleistocarps on gooseberry.

The perennation of cleistocarps on leaves of gooseberry was examined in one experiment and that of cleistocarps on shoots of gooseberry in another.

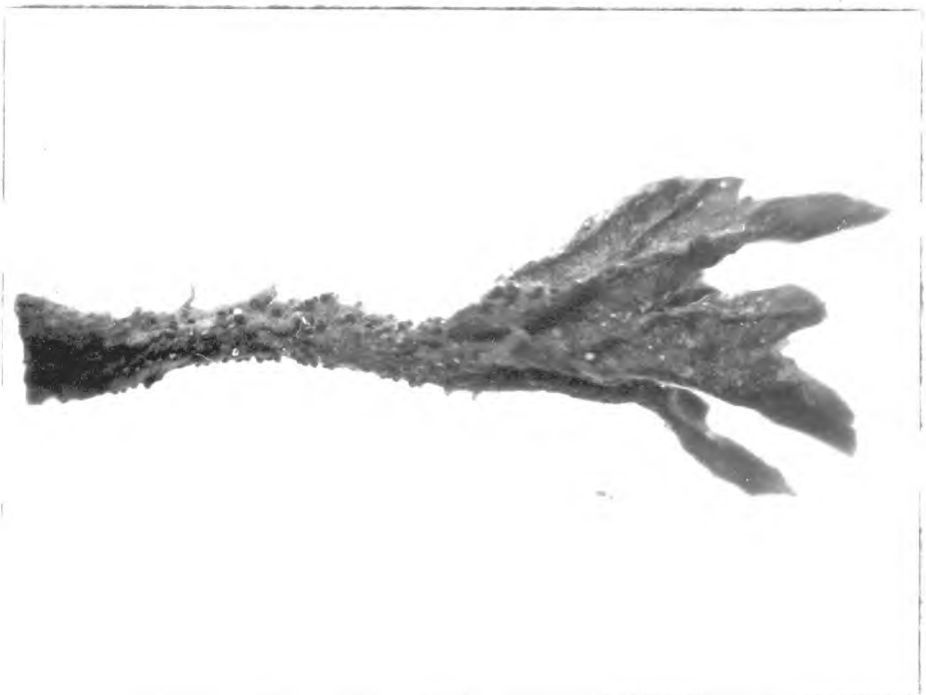
Previous work (merriman and Wheeler, 1968) indicated that cleistocarps on shoots did not survive the winter but degenerated shortly after leaf fall.

Experiment i.

Approximately 250 leaves were taken from a planting of the cultivar Careless at Southmoor, Oxon, on 8 September 1969. The samples was placed on the soil in the walled garden at Silwood Park in a Terylene bag (1600 mesh/cm^2) as previously described. (The fine mesh bag was required as the maximum size of these very stunted leaves was only 0.5 cm.) The ascospore content of cleistocarps and the ability of cleistocarps to dehisce were estimated on samples of ten leaves, taken first on 9 September 1969 when the bag was pegged to soil, then at intervals of 2-3 weeks until 26 February 1970 and subsequently at 10-day intervals. Fig. 9 shows distribution of cleistocarps on the leaves.

Additionally in this experiment the infectivity of ascospores was tested on young gooseberry leaves and on leaf discs (2 cm^2) of black currant. The ability of conidia from colonies initiated by ascospores on the two hosts to infect both gooseberry and black currant was further tested by transferring conidia with a camel-hair brush from infected to uninfected leaf tissue. The whole leaves or discs were incubated in polystyrene boxes at 20°.

Fig. 9. Gooseberry leaf bearing cleistocarps of S. mors-uvae. Note portion of cleistocarps on petiole and at base of lamina. (x20)



Experiment II.

Sixty-six stems bearing secondary mycelium and cleistocarps of the current seasons infection were tagged within a mixed planting of the gooseberry cultivars, Careless, Lancashire Lad and Whinnam's Industry at Silwood Park on 6 October 1970. No distinction was made between these cultivars because of the very small number of infected stems available. Thirty-three tagged stems were then cut at random from the bushes and pieces c. 2 cm long with 200-300 cleistocarps were placed in a small Terylene bag and laid on the soil. The remaining thirty-three tagged stems were left on the bushes. The ascospore content of cleistocarps was assessed at 3 weekly intervals and the ability of cleistocarps to dehisce tested on six occassions between 18 February and 26 April 1971.

Results.

The results of Expt (i) and Expt (ii) are given in Figs. 10 and 11 respectively. Initially in all the samples there were cleistocarps with fully differentiated ascospores, although the surrounding mycelium was still a very light brown, and there were others in which the ascospores were obviously degenerating. Both this initial degeneration and the fluctuations in ascospore content of successive samples (e.g. Fig.10, between 25 November and 22 December) were also observed with collections from black currant where they appeared to result from the substantial differences in the maturity of cleistocarps when collected (p. 22). The decline in glycogen content of the asci was also similar to that of cleistocarps from black currant that were placed on

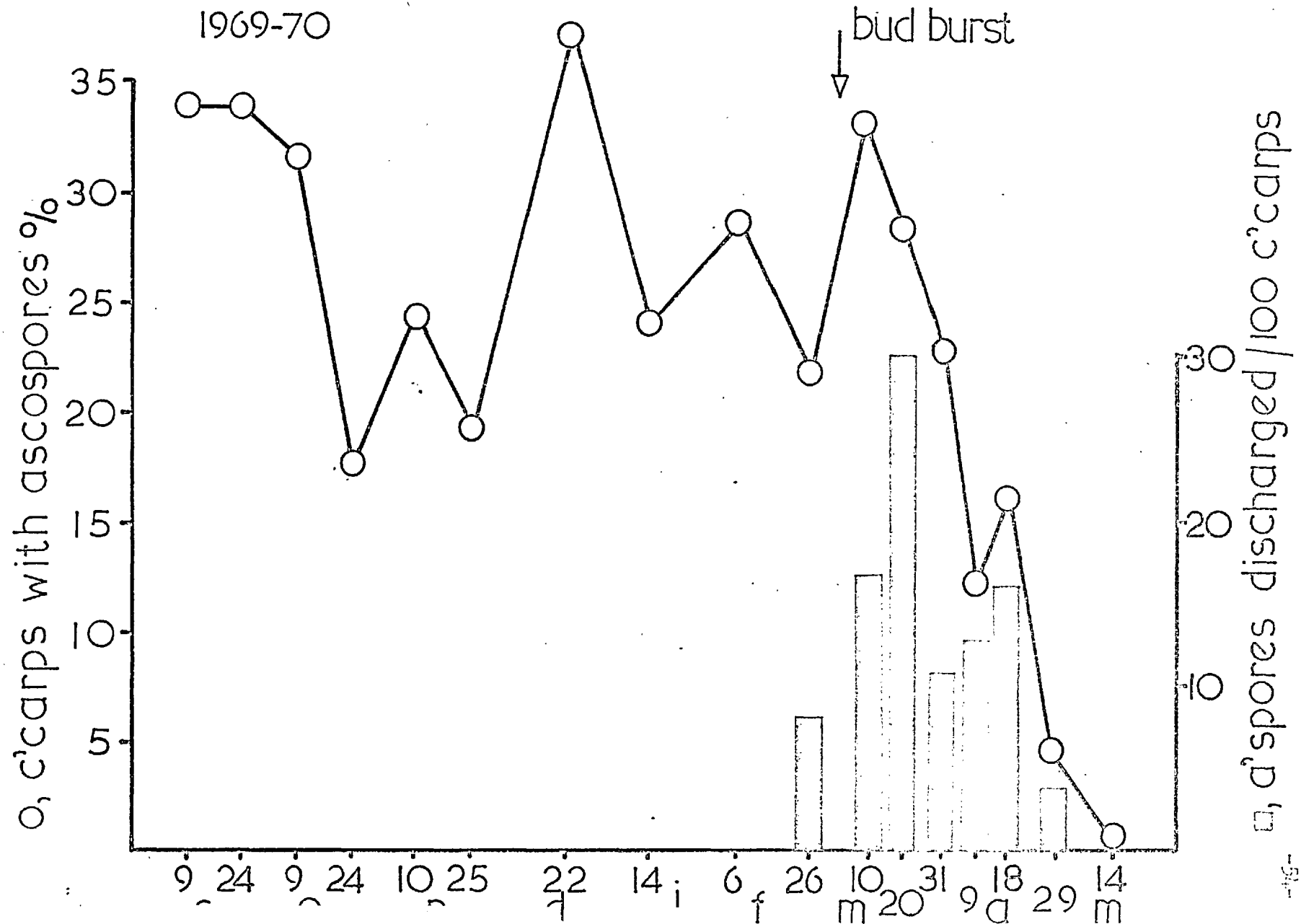


Fig.10. Overwintering of c'carps. Gooseberry leaves
1969-70
bud burst
o, c'carps with ascospores %
□, a'spores discharged / 100 c'carps
35
30
25
20
15
10
5
30
20
10
9 24 9 24 10 25 22 14 i 6 f 26 10 m 20 31 9 a 18 29 14 m

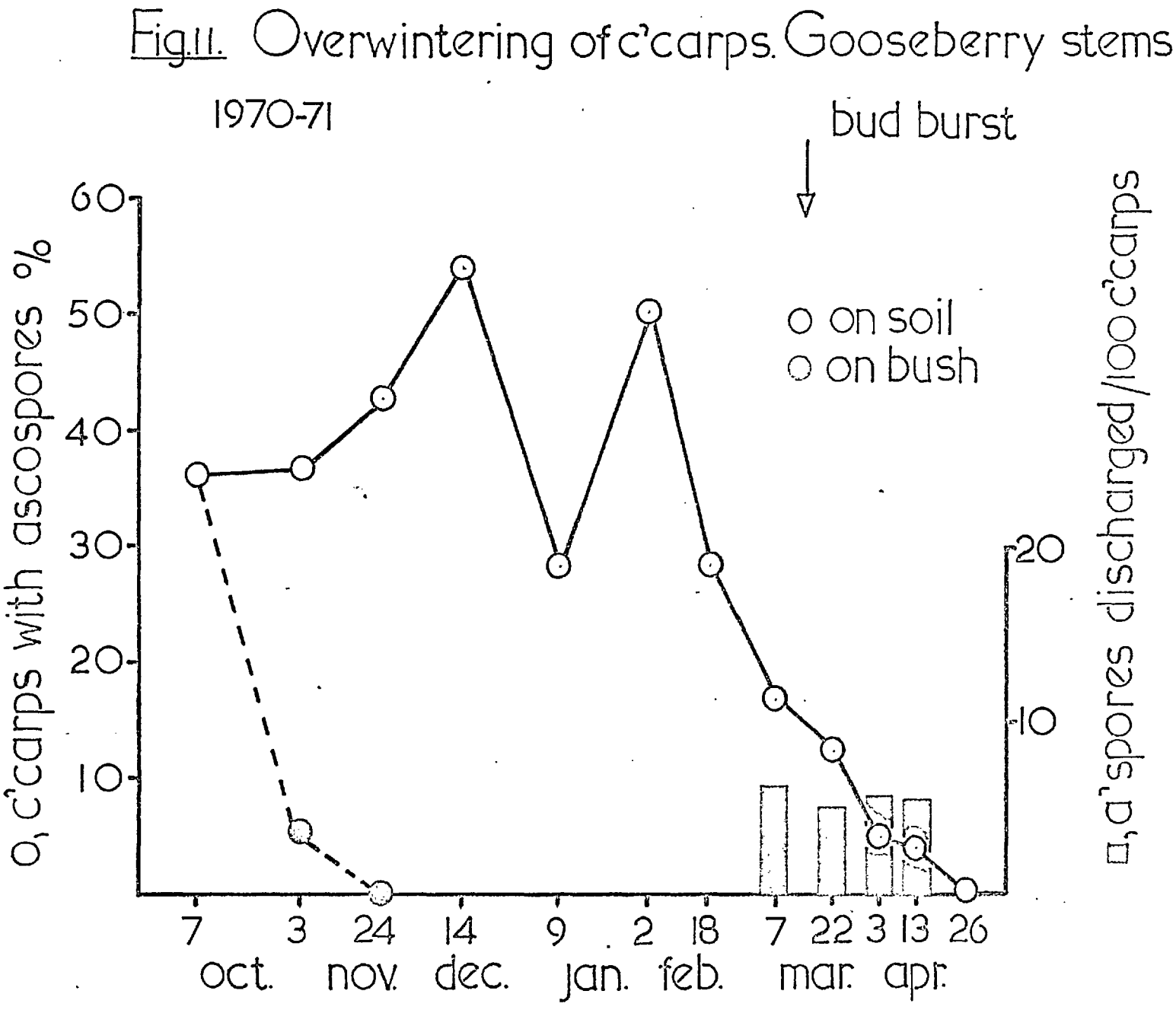


Fig.11. Overwintering of c'carps. Gooseberry stems
1970-71
bud burst
o on soil
o on bush
o, c'carps with ascospores %
□, a'spores discharged/100 c'carps
60
50
40
30
20
10
20
10
7 3 24 14 9 2 18 7 22 3 13 26
oct. nov. dec. jan. feb. mar. apr.

soil (p.49).

Cleistocarps on both stems and leaves that were overwintered on soil contained asci, which in dehiscence tests in early spring, possessed the ability to discharge spores. This was at a time when leaves of gooseberry and also of black currant was available for natural infection in the field. In 1969, bud burst of gooseberry began towards the end of February and in 1970 during the second week of March. An increase at this time in the numbers of cleistocarps with split walls or with only the basal portion remaining indicated natural dehiscence in these samples. Additionally, tests at field temperatures showed that other cleistocarps in the samples contained viable ascospores that could infect leaf material of the gooseberry cultivar Careless and the black currant cultivar Wellington and Amos Black. Conidia from these ascospore-initiated colonies on gooseberry infected black currant and conidia from black currant, similarly derived, infected gooseberry.

In marked contrast to the successful perennation of cleistocarps on the soil those remaining on the stems on the bush degenerated completely. By 24 November 1970, 6 weeks after the beginning of the experiment, no ascospores remained except as cells filled with fat. After only 4 weeks many asci had lost the ability to imbibe water and spores contained large accumulations of fat. No asci were stained by iodine and none released spores in dehiscence tests because of this degeneration. Many cleistocarps were observed in late November and early December and it was apparent that this phenomenon was total throughout the plantation irrespective of cultivar.

There was little evidence to suppose that this total and early

degeneration was brought about by micro-organisms on the stems attacking the cleistocarp wall. Even through the mycelium bearing the cleistocarps was only partially thickened few bacteria and actinomycetes possessing chitinase enzymes were found associated with the sample. From forty pieces of mycelium placed on 1% chitin agar only five actinomycetes colonies and only three bacterial colonies that cleared this agar were found after 10 days incubation at 10°. Species of Penicillium, Alternaria, Cladosporium and Epicoccum developed from most pieces of the S. mors-uvae mycelium but none of these fungi was observed to be actively growing on the cleistocarps in the dry environment of the stems.

The number of cleistocarps that dehisced within the leaf and stem samples of gooseberry on the soil, although small, was comparatively larger than that of samples on black currant leaves (p.50). From gooseberry leaves, 6676 cleistocarps were tested and 839 ascospores released; this represents discharge from some 105 cleistocarps or 2% of the sample. In tests on stem material 3060 cleistocarps discharged 149 spores equivalent to 17 cleistocarps or 0.7% of sample. Recalculating these figures in terms of the number of cleistocarps with ascospores, figures of 8% and 6% dehiscence are obtained for cleistocarps on leaves and stems respectively. The variations between the individual samples on which these figures are based is given in Table 8 . As in other cleistocarp dehiscence assessments there was an increase in the dehiscence of cleistocarps with ascospores later in the spring. But it was unfortunate that a very small amount of infected leaves and stems was available initially; thus the results must be assessed in relation to this fact.

Table 8. Ascospore discharge, gooseberry cleistocarps, 1970 and 1971.

a) Cleistocarps on leaves.

Date	No. of c'carps	No. a'spores	C'carps with a'spores (%)	A'spores released/1000 c'carps with a'spores
6.2.70	246	0	23.5	0
26.2.70	1147	53	21.8	212.4
10.3.70	1167	165	33.3	425.3
20.3.70	1299	384	28.3	1046.0
30.3.70	762	81	22.5	472.3
9.4.70	615	78	12.0	1056.9
18.4.70	450	72	16.0	1000.0
29.4.70	395	6	4.5	337.1
9.5.70	598	0	0	0

b) Cleistocarps on stems.

Date	No. of c'carps	No. a'spores	C'carps with a'spores (%)	A'spores released/1000 c'carps with a'spores
13.3.71	1229	75	17.1	356.8
22.3.71	701	36	12.2	420.1
30.3.71	401	22	5.5	995.5
13.4.71	301	16	3.9	134.9
26.4.71	428	0	2.0	0

5. Further experiments with cleistocarps from black currant.

i. Infection of black currant with ascospores from non-wintered cleistocarps.

Previous observations (p.32) indicated that a few cleistocarps contained asci capable of imbibing water, swelling and discharging ascospores at, or soon after, leaf fall. The infectivity of the ascospores released from these asci which had not been subjected to overwintering was examined.

Leaves, collected from black currant bushes on 7 October 1970, were dried in the laboratory for 4 days and then kept at 10° until 29 December when a few were immersed in water for 1h so that the mycelium could easily be removed. Pieces of this mycelium with apparently mature cleistocarps (as judged by size, shape and colour) were selected and these were placed on moistened blotting-paper in the lid of small polystyrene boxes. The walls of the cleistocarp were split either by gentle tapping with a mounted needle or by pressing the cleistocarp, carefully, beneath a small coverslip. When asci emerged the lid of the box was placed in position so that ascospores released could be deposited on black currant leaves floating on distilled water in the base of the box. After 30-60 min the cleistocarps were inspected for discharge of ascospores. Two boxes were set up as described with c. fifty cleistocarps split by these methods per box. Other boxes contained leaf material only as a control, to ensure that leaves taken from potted plants in the greenhouse were free from conidial infections. The boxes were incubated at 15° and observed over 15 days for sporulating colonies.

Although most of the asci that swelled subsequently retracted into the cleistocarp without dehiscing some asci did in fact discharge spores that were infective. After 10 days two sporulating colonies were seen on leaves in one box and 2 days later a single colony developed in the second box. This showed that at least a small percentage of asci contained viable spores. The role of these particular ascospores remains in doubt. They could be from cleistocarps that normally discharge spores in the spring or from those that dehisce very early in the winter in November or December when occassionally temperatures and moisture requirements are satisfactory for dehiscence.

ii. Viability of ascospores at low temperatures for long periods.

Leaves collected from Fernhurst on 26 September 1968 were stored dry at -18°. The ascospore content of cleistocarps was examined on the dates shown in Table 9 .

There was no evidence from the results (Table 9) to indicate that ascospores degenerate at -18°. After the material thawed, the asci readily imbibed water, and the ascospores appeared unharmed.

Table 9 . Contents of cleistocarps stored at -18° for 3 years.

Date	No. cleistocarp	No. asci	% asci	No. ascospore	% ascospore
10.10.68	9,709	4,562	46.9	3,825	39.4
17.10.69	5,266	2,831	53.8	2,681	50.9
10.10.70	7,827	4,999	63.9	3,845	49.1
6.2.71	6,080	4,112	67.6	3,060	50.3

On 6 February cleistocarps were dehisced artificially by first splitting the wall and ascospores were allowed to discharge onto leaf discs of black currant. (Table 10).

Table 10. Artificial dehiscence of cleistocarps kept at -18° for 3 years.

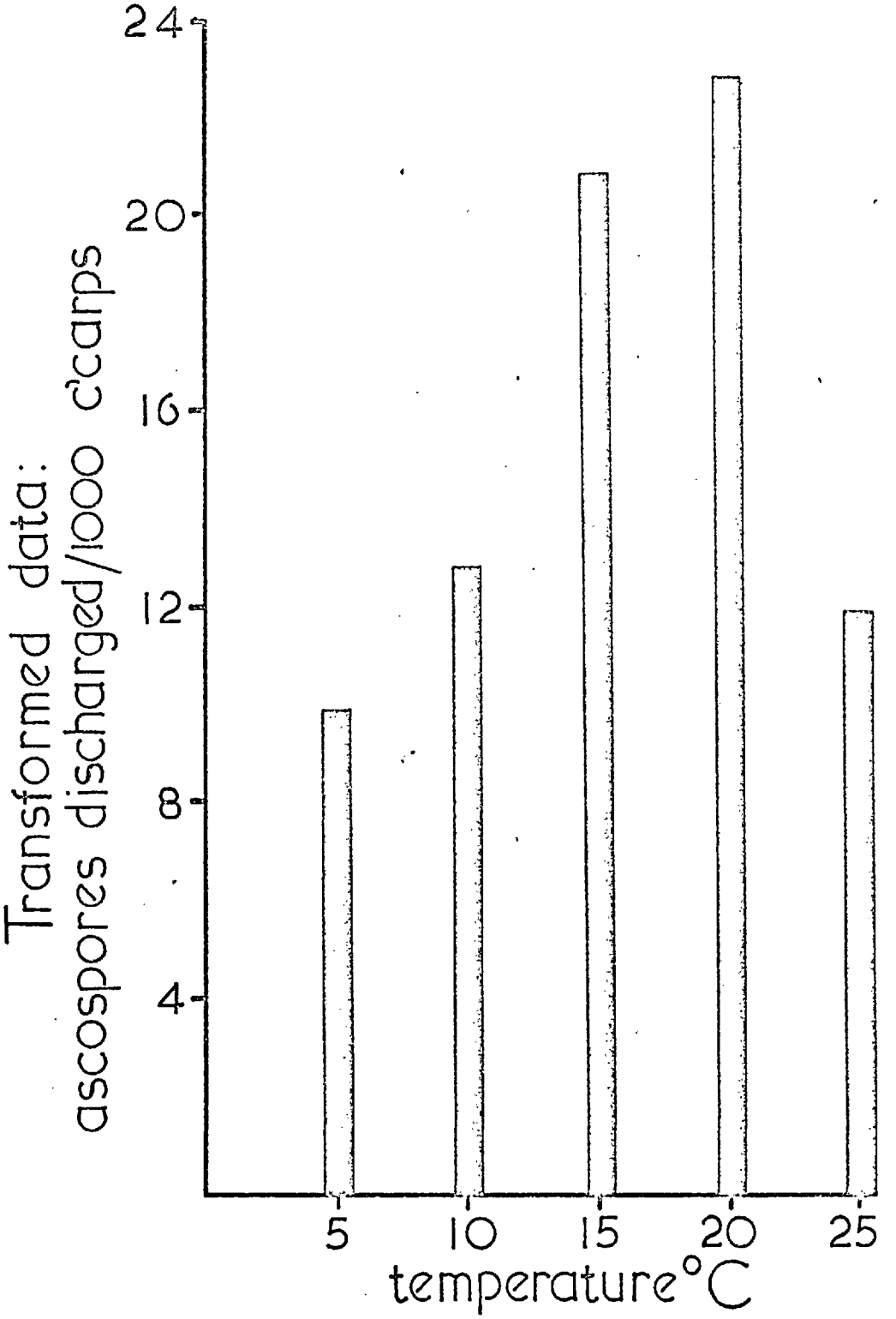
No. cleistocarps	No. swelling	%	No. dehiscing	%
146	3	2.1	2	1.4
250	25	10.0	10	4.0
319	17	5.3	6	1.9
715	45	6.3	18	2.5

Some ascospores were viable and produced sporulating colonies of S. mors-uvae. Cleistocarps may therefore provide a means of maintaining cultures of S. mors-uvae over long periods of time and thus obviate the repeated conidial inoculations onto leaf discs or potted plants.

iii. Ascospore discharge in relation to temperatures.

The effect of temperature, in the range 5-25°, on cleistocarp dehiscence was examined on three occassions using black currant leaves overwintered on the wall (p. 40). On each sampling date c. 5000-6000 cleistocarps were placed in each of fifteen dehiscence chambers (p. 16) and three such chambers were incubated at 5, 10, 15, 20 and 25° for 24h. The number of discharged ascospores were then counted and also the number of asci which had swollen but had not dehisced. The full results are

Fig.12. Effect of temperature on a'spore discharge



given in appendix table 2 and these are summarised in Fig.12, where each observation is based on dehiscence from 15000-20000 cleistocarps. An analysis of the results showed that the differences in the treatments, as a whole, were highly significant ($p < 0.001$); the figures for 5, 10 and 25° were not signigicantly different from one another nor did those for 15° differ significantly from those at 20° though there was a significant difference between those two grouping ($p < 0.001$). It is likely that this reflects the heterogeneity of the sample of cleistocarps and the difficulty in selecting cleistocarps with similar percentages of viable asci and ascospores. But it is apparent that temperature markedly affects the ability of cleistocarps to discharge spores in a range 15-20°. The relatively low discharge at 25° was of special interest as degeneration of ascospores at this temperature had been observed in other experiments when cleistocarps remained wet for a short period. However, it was only at this temperature that many ascospores were found to have germinated after 24h.

The number of asci that imbibed water but did not dehisce at the time of counting is given in Table 10. Observations of this were difficult because of the tendency for these swollen asci to dehisce or for others to rupture the cleistocarp wall and swell when brought to laboratory temperatures; this was especially so for 5° and 10° samples.

From this result it may be inferred that temperature affects the processes of dehiscence in two ways: 1) influencing the ascus to swell within the cleistocarp wall and to rupture it, 2) to influence the capability of the ascus once swollen to discharge spores.

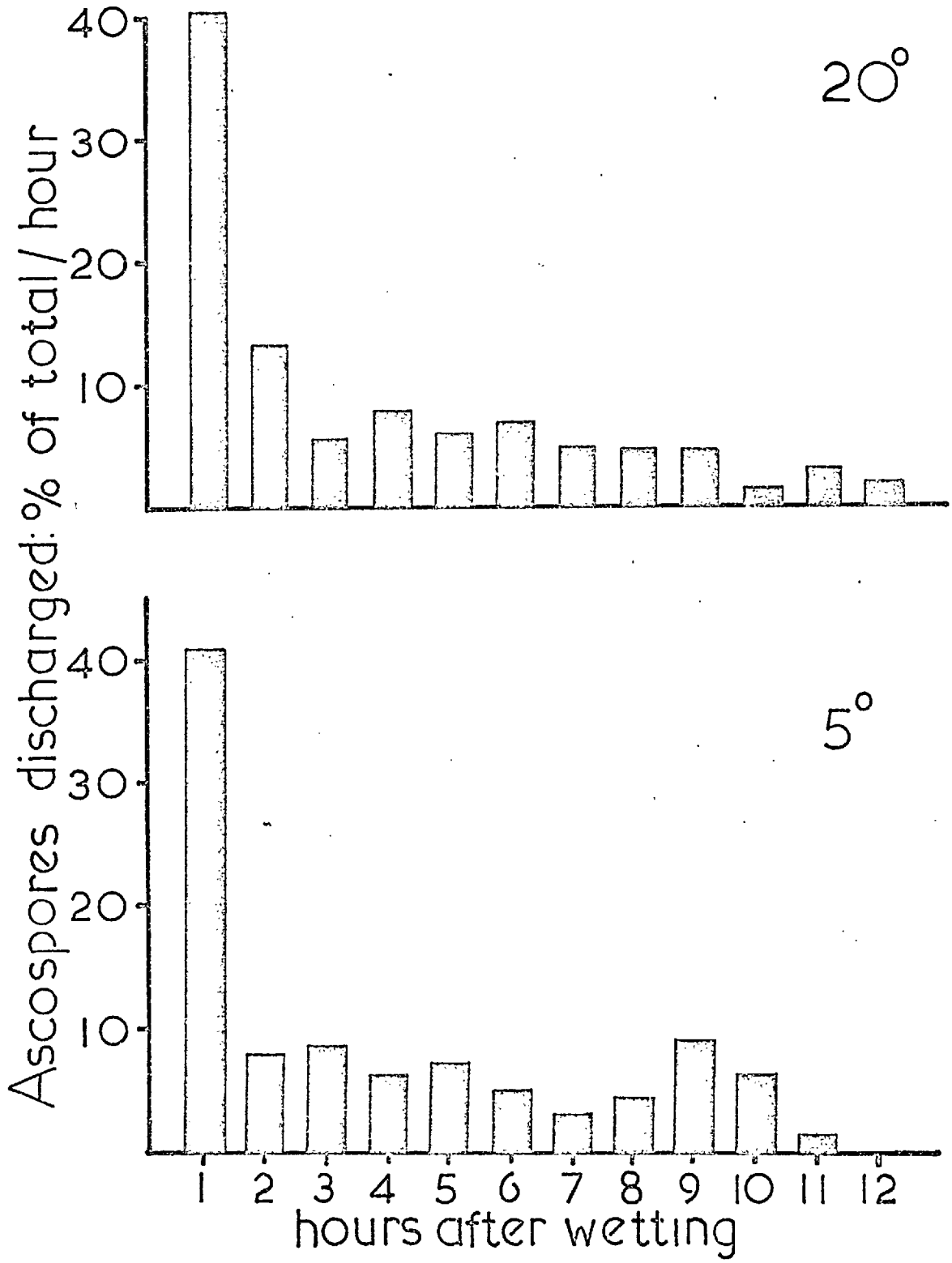
Table 10. Numbers of asci swollen at different temperatures after 24h.

Temperature °C	Nos. of asci swollen
5	130
10	25
15	12
20	11
25	2

iv. Ascospore discharge in relation to time of wetting cleistocarps.

On 7 April 1971 the number of ascospores released at each hour after wetting samples of cleistocarps were estimated at 5° and 20°, using samples overwintered above soil level. So that cleistocarps did not discharge spores before being incubated at the two temperatures leaves were initially soaked in iced tap water before the mycelium and cleistocarps were removed. Any cleistocarps that had asci protruding from the split walls were removed or the asci were ruptured with a mounted needle. Each treatment consisted of three dehiscence boxes containing c. 5000 cleistocarps. The results are given in full in appendix table 3 and summarised in Fig. 13. Although there was more than a three fold difference between the total number of spores released from approximately similar numbers of cleistocarps at the two temperatures there was no difference in the pattern of spore discharge per hour. Both at 5° and 20° most spores (40%) were trapped within the first hour and subsequently the numbers declined over the 12h period.

Fig.13. Ascospore discharge at 5° & 20° at hourly intervals

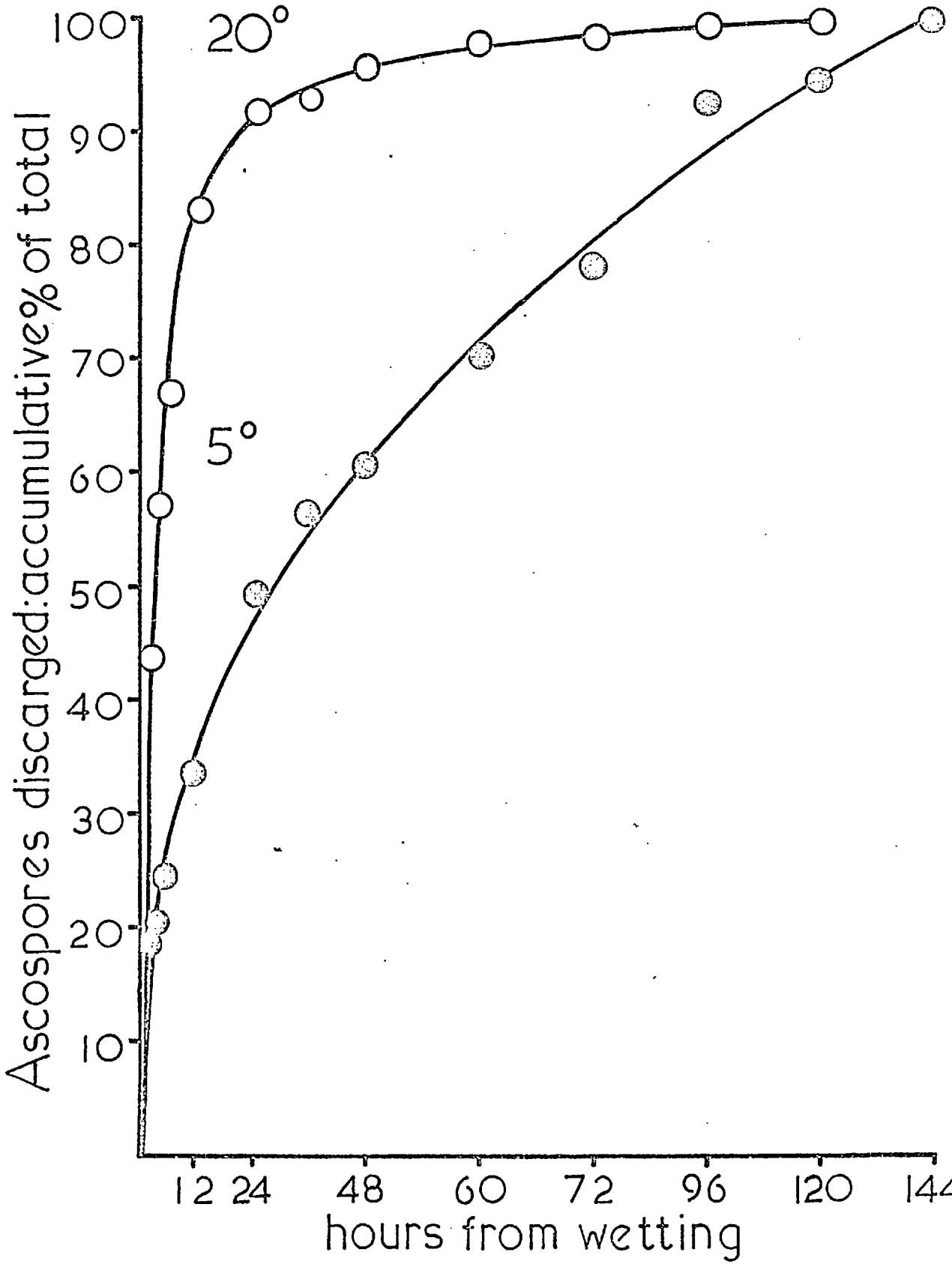


v. Rate of ascospore discharge during prolonged incubation.

The discharge of ascospores from cleistocarps at 5° and 20° over 5 days was examined in an experiment similar to that just described. Counts of ascospores were made after 2, 4, 6, 12h and thence at 12h intervals up to 5 days after initial wetting. The results are given in appendix table 4 and Fig.14.

The number of ascospores released over the initial 2h period, as a percentage in the first 12h, was similar at both temperatures (i.e. 54.5% and 52.4% respectively). However, after 12h the rate of release at 20° declined rapidly to almost zero. At 5° spore release was low and inconsistent in one sample but in general discharge after 12h continued at a low but steady rate. This could reflect a slow maturation of the ascus at this temperature whilst continuously wetted, or the release of spores from asci which had swollen but required longer periods for spores to be discharged. It was not possible to count swollen asci at each of the sampling times as the sudden rise in temperature would have caused dehiscence when the samples were brought into the laboratory. To observe this would necessitate discharge and observation of cleistocarps at 5° constant temperature.

Fig.14 Ascospore discharge at 5° & 20° over 5 days



II. MICROBIAL ACTIVITY ON HOST TISSUE INFECTED WITH S. MORS-UVAE.

Review of Literature

Much of the work on the use of nitrogenous compounds to prevent the perennation of plant pathogenic fungi has been concerned with their effect on apple leaves infected with the apple-scab fungus Venturia inaequalis. The fungus slowly forms perithecia on abscissed leaves during the winter months from which ascospores are discharged after periods of rain in the following spring.

Keitt and Palmiter (1937) found that a saturated solution of ammonium sulphate applied as a nitrogenous fertiliser to the orchard floor in spring, killed ascospores that were mature and prevented maturation of others. Palmiter (1946) followed up these results with an extensive screening programme over several years and found that 12% solutions of sodium nitrate and ammonium sulphate or a 0.5% solution of Elgetal(22% dinitro-ortho-cresol) killed or prevented 90-100% of ascospores from discharging. These solutions were applied to the orchard floor when the trees were in the green bud growth stage in the spring and at a time when ascospores were ready to be released. Similar results were obtained by Hirst and Stedman (1962) with ammonium sulphate applied 3 weeks before bud burst.

Recently workers at East Malling Research Station have shown that urea solutions, applied at leaf fall to leaves infected with V. inaequalis, cause considerable reduction in the number of ascospores released from those leaves in the spring. Their experiments have been concerned especially with the direct and indirect effects of urea on the formation and survival of the perithecia, in particular, with the role

of the stimulated microflora, the high leaf nitrogen content and the rate of decomposition of the lamina after treatment with urea. They found that the bacterial populations of leaves treated with urea increased rapidly during the first 20-30 days and continued to be significantly higher than water treated controls until spring (Burchill, Hutton, Crosse and Garrett, 1965; Crosse, Garrett and Burchill, 1968; Burchill and Cook, 1971). Urea was found to cause a selective stimulation of bacteria with higher than average multiplication rates in the initial phase after treatment and resulted in a major shift towards a predominantly gram-negative and non-chromogenic population. There was a relationship between the numbers of gram-negative organisms and suppression of ascospore release . Some bacteria, in particular, pseudomonads inhibited the growth of V.inaequalis in in vitro tests and caused a partial reduction in perithecial development when they were applied to leaf discs within one month of inoculation with V.inaequalis (Crosse et al, 1968; Ross and Burchill, 1968). Those perithecia that did develop were slower to mature and noticeably smaller.

Urea was found also to promote the growth of pseudomonads that were not only antagonistic to V.inaequalis but also possessed macerating or pectolytic enzymes. Thus, the leaves were not only decomposed rapidly after treatment, but they had a soft and leathery texture for which slugs and earthworms showed a particular preference. Similarly, on urea treated leaves there were changes in the composition and concentration of fungal populations which were also implicated in the reduced development of perithecia (Burchill and Cook, 1971; Cook, 1969). Species of Cladosporium, Fusarium, Gonatobotrys and Epicoccum dominated the

microflora following a 5% urea dip and after 4-6 weeks many perithecia were invaded by alien fungal hyphae. It was not possible, however, to determine whether this invasion was a result of parasitism or saprophytism (Burchill and Cook, 1971). The incidence of both Fusarium sporotrichioides and F.avenaceum (Cook, 1969) were especially increased and these caused suppression of perithecial development when inoculated onto leaf discs.

There are also indications that urea can act directly on the survival of V.inaequalis. Ross and Burchill (1968) found that urea killed both mycelium and perithecia of V.inaequalis when applied to sterile leaf discs on which the fungus had been cultured and allowed to attain various stages of perithecial development. However, when urea was applied to the leaf discs first and then they were inoculated with V.inaequalis the mycelium grew but no perithecia developed. Thus, urea was acting directly on V.inaequalis in two ways. Firstly it increased the nitrogen level within the leaf which became inhibitory to early stages of perithecial development. Secondly, it caused a fungitoxic effect which resulted in death of the fungus irrespective of its state of development. This direct effect was seen in field experiments where a pre-bud burst spray resulted in mature perithecia containing asci and ascospores without the ability to release spores (Burchill, 1968). At this time a very low concentration of urea (0.5%) was effective in reducing the numbers of ascospores released from leaves not treated the previous autumn.

Experimental

1. Effects of urea on microbial populations and on survival of cleistocarps.

The experiments with S. mors-uvae described in Section I have shown that in the soil cleistocarps degenerate rapidly either because their food reserves become depleted or because they were adversely affected by other micro-organisms. No absolute distinction could be made between these two possibilities. Urea was used, therefore, on leaves bearing cleistocarps placed on the soil for two reasons: 1) to increase the associated microflora to levels which could be detrimental to the survival of the cleistocarps long before the normal degeneration occurred in spring, 2) to test whether it was directly toxic to survival of cleistocarps. There is, however, a fundamental difference between applying urea to apple leaves bearing V.inaequalis and black currant leaves with S. mors-uvae. In the former case the urea was used to prevent the thin walled mycelium from forming perithecia during the winter months. In the latter the thick walled overwintering cleistocarps have already formed and reached structural maturity at leaf fall. Apparently no further development of the cleistothecia takes place once the leaf becomes detached from the plant.

Methods.

In the winters 1968-69 and 1969-70 four experiments were carried out in which leaves of black currant were treated at leaf fall and at pre-bud burst.

Experiment i. 1968-69.

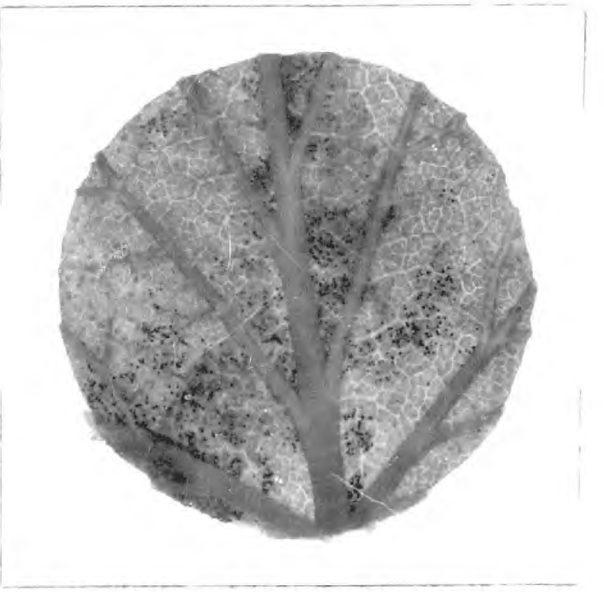
On 26 September 1968 leaves bearing a thick felt of secondary mycelium with cleistocarps were taken from bushes within a planting of the cultivar Wellington at Fernhurst, Kent. The leaves were soaked in water for 30 minutes to observe the number of cleistocarps present and then discs (2 cm^2) were cut from the petiole end. These were divided into four groups, each of 200 discs, based on a subjective estimate of (i) 500-100 (ii) 1000-2000 (iii) 2000-3000 (iv) 3000-4000 cleistocarps per disc (Fig. 15). The discs were allowed to dry at room temperature until no free water remained visible and samples were dipped momentarily in either 5% urea + 0.01% spreadite (Murphy Chemicals Ltd.) or distilled water + spreadite, and then dried. The discs were placed in Terylene net bags (9.8 mesh/cm^2) 9 x 12 cm which were each divided into two compartments; each bag contained two sets of four discs, one set consisting of a disc from each of the four cleistocarp categories listed above. The bags, with the discs abaxial surface uppermost, were pinned to a wooden frame (see p. 15 and Fig. 16) which was placed on newly dug soil in the walled garden at Silwood Park on 1 October 1968.

A further sample of non-treated leaves was placed on the soil at the same time and on 14 January 1969 leaf discs were cut from these and treated with urea as above to investigate the effect of a spring application. In this instance the leaf discs (2 cm^2) were placed in twenty-one bags with eight discs in each: they were not divided into the four cleistocarp categories listed above.

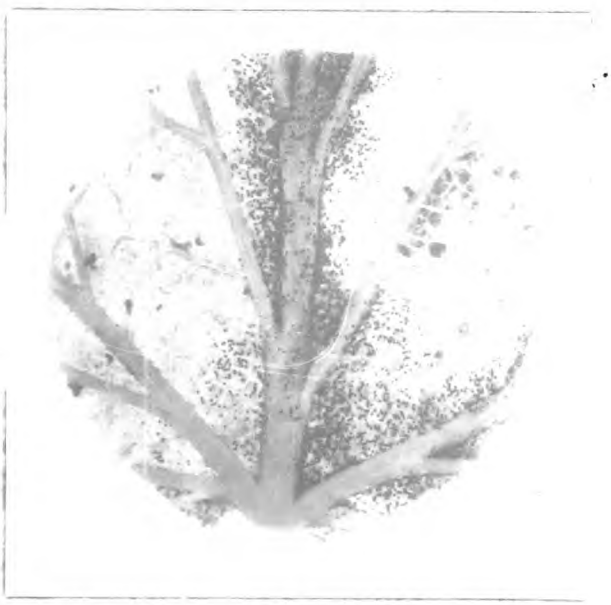
Fig.15. Four categories of 2 cm^2 leaf discs bearing cleistocarps of *S. mors-uvae* used in autumn urea experiment 1968-69.

(a) 500-1000 cleistocarps

(b) 1000-2000 cleistocarps



(c) 2000-3000 cleistocarps



(d) 3000-4000 cleistocarps

Fig. 16. Terylene bags containing 8 leaf discs with cleistocarps of *S. mors-uvae* pinned to wooden frame and laid on the soil; autumn urea experiment 1968-69.

Recovery of micro-organisms.

At intervals during the winter one set of eight leaf discs from the autumn treatment and two sets of four discs from the spring treatment were sampled at random. There was little decomposition of tissue in these experiments and discs were always present without appreciable destruction of the lamina. The discs were placed in 10 ml sterile water and macerated in an M.S.E. micro-homogeniser for 4 min. After allowing debris to settle aliquots of the suspension were taken and dilutions made to give c. 30-100 colonies per agar plate.

Enumeration of micro-organisms.

Total bacteria and yeasts were estimated by mixing 1 ml diluted supernatant with 10 ml nutrient-broth yeast agar (Crosse et al, 1968) (appendix p.244). Total fungi were estimated similarly on plates containing Czapex-DOX yeast agar (appendix p.244). All plates were incubated at 25^{0}. The bacteria were counted 5-6 days and fungi 7-8 days after plating.

Observations on cleistocarps.

Percentage cleistocarps with asci and ascospores and the ability of cleistocarps to dehisce were determined on four leaf discs by methods already described (p.15&16).The state of the leaf tissue was also recorded by comparing the ease with which the leaves were teased apart with mounted needles after soaking in water for 30 min. The number of cleistocarps sampled on the eight leaf discs in the autumn treated leaves varied from 16,000 initially to 5,000 in the spring. With spring treated

discs smaller numbers (4000-7000) were assessed per sample.

Experiment ii (1969-70).

For the 1969-70 experiments leaves were collected from Southmoor, Oxon on 10 October 1969. No selection was made in relation to cleistocarp numbers. A sample of 150 leaves from this collection was placed in a Terylene net bag on soil for use in the spring of 1970, The remaining leaves were divided amongst three Terylene bags (36 cm^2) each with two compartments so that each compartment contained c. 250 leaves. The bags were placed on the soil on 22 October and immediately sprayed with the following solutions:

Bag 1. 5% urea + 5% fructose + 0.05% Lissapol NX

Bag 2. 5% fructose + 0.05% Lissapol NX

Bag 3. 0.05% Lissapol NX.

The sprays were applied with a Shandon laboratory spray gun until the leaves were completely wetted. The same spray treatments were repeated on 27 November 1969 and 26 February 1970. The leaves were sampled immediately after spraying on 22 October 1969, 4 days later and then at intervals of 10-28 days.

In the spring experiment of 1970 the sample of 150 leaves set out on soil (on 22 Oct 1969) were divided on 26 February 1970 into three Terylene bags at random. The bags were placed on soil and sprayed as before with the following:

Bag 1. 5% urea

Bag 2. 10% urea

Bag 3. Distilled water.

Recovery of micro-organisms.

Five leaves were taken from both sets on each sampling occasion and 1 cm^2 discs cut from the petiole region or from areas containing maximum amount of mycelium and cleistocarps. The ten discs were placed in 100 ml 0.9% sterile saline with 0.001% Tween 80 and shaken for 30 min on a Griffin flask shaker. The final supernatant was diluted and aliquots incorporated into various media.

Total bacteria and yeasts and total fungi were estimated as in (i) above. Fungi, bacteria and actinomycetes capable of degrading chitin, (chitinoclastic organisms) were estimated by plating 0.2 ml of the diluted leaf washings on to 5 ml solidified 0.2% chitin agar (appendix p.245) poured over a basal layer of distilled water agar. The plates were dried by exposure at 37° for 4 h or by storing at 25° for 4 days before use. The chitin was obtained as a partially purified product from shrimp exoskeletons (Sigma Chemicals Ltd.) and was purified by the methods of Lingappa and Lockwood (1962) (p. 117) and balled-milled by the method of Baxby and Gray (1968). Antibiotics in the following combinations (Baxby and Gray, 1968) were added to the chitin media to select bacteria and actinomycetes:

1) bacteria, nystatin (50 µg/ml) + actidione (5 µg/ml);

2) actinomucetes, nystatin (50 µg/ml) + actidione (50 µg/ml) + polymixin B sulphate (5 µg/ml) + sodium penicillin (1 µg/ml). The antibiotics were sterilised for 24 h using propylene oxide before in corporation into the agar at 45°.

Gurr's crystal violet (2 ppm) was incorporated into the nutrient-broth yeast agar to inhibit gram-positive organisms (Crosse et al, 1968). The numbers of pigmented bacteria growing on the plates used for total bacteria and yeast estimations were counted separately into three categories: yellow, orange and pink.

Estimations of urea on leaves.

The amount of urea was estimated on leaf discs 4 h, 4 days and 11 days after spraying by the colorimetric method of Archibald (1945) using ∝-isonitropropiophenone (INPP) as reagent. The 1 cm^2 leaf discs were taken from the samples on the soil and shaken for 30 min in 100 ml distilled water. To 7 ml of the urea solution 5 ml of sulphuric acid/phosphoric acid/water (1:3:1) was added and 0.4 ml alcoholic ∝-INPP (4g in 100 ml ethanol). After mixing the solution was boiled for 1 h exactly, cooled and placed in a water bath at 20° for 15 min. Readings were taken at 540 mμ in a Beckman DB spectrophotometer.

Laboratory experiments on the effect of urea on cleistocarps.

On 16 November 1968 leaf discs 2 cm^2 were cut from leaves collected from Fernhurst, Kent on 26 September 1968 that had been soaked in water for 2 h. They were allowed to dry and one half of the sample was dipped momentarily in 5% urea + 0.01% spreadite, the remainder in spreadite alone. The discs were plated in Van Teigan cells fixed, three to a glass slide, with 'Araldite'. The discs rested in the cells on small circles of filter paper which were kept permanently moistened with distilled water. The slides with leaf discs were placed in 9 cm Petri dishes which

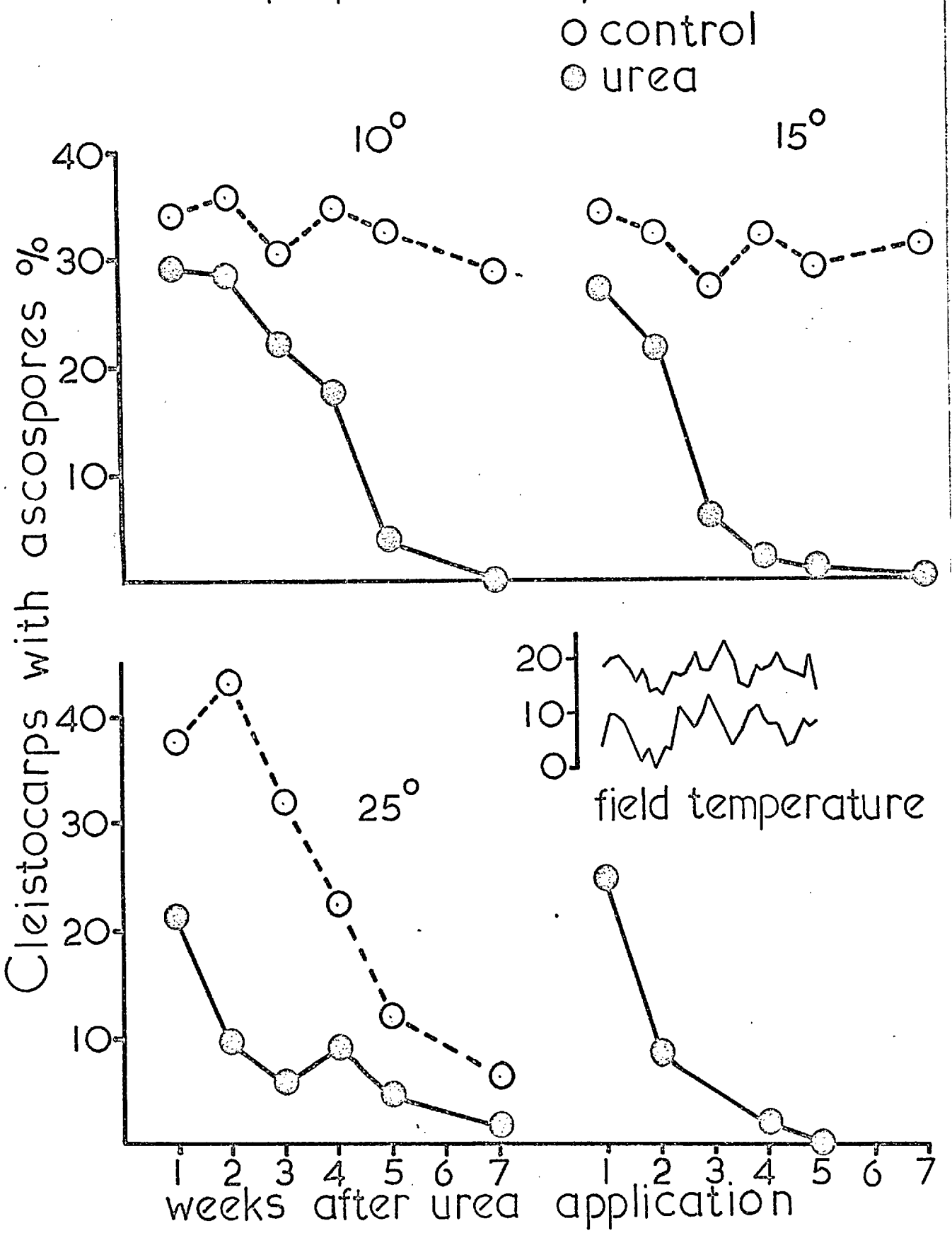
were kept in large perspex boxes 38 x 24 x 8.5 cm to retain moisture and incubated at 10°, 15° and 25° and at field temperatures. Five Petri dishes containing fifteen leaf discs were incubated at each temperature. The leaf discs were sampled for ascospore content of cleistocarps initially and at 7 day intervals for 7weeks. Observations were also made on the effect of the treatments on the leaf tissue and the secondary mycelium.

Results.

1. Laboratory experiments.

Preliminary laboratory tests on the effect of urea on cleistocarps showed that, at the four temperatures (10°, 15°, 25° and field) urea caused rapid degeneration of the asci and ascospores. The control leaves dipped in the spreadite solution were little effected except those incubated at 25° (Fig. 17). There was, however, considerable variation in the extent of degeneration within each of the treatments, especially in later samples. Some leaves were relatively undecomposed even after 3-4 weeks from dipping in the urea solution and these bore cleistocarps containing normal ascopores whose asci were able to imbibe water and swell in a slide dehiscence test (p. 29). For instance, in the 25° samples taken at the fourth week one disc contained cleistocarps with 21.3% ascospores and the following week a disc with 35.4% was sampled. At the latter time, however, none of the asci were able to imbibe water. This was seen to a lesser extent in the samples at the other temperatures and may have resulte from variations in uptake of urea or a difference in response of the bacteria present to urea. Some leaves at the time of dipping showed little damage of the

Fig.17 Effect of urea on cleistocarps kept permanently turgid



tissue following mildew colonisation, in others the mildew or secondary saprophytes had caused considerable decay. The variation in ascospore degeneration could, therefore, reflect the difference in the build up of bacteria on these leaves which had different initial populations of micro-organisms. However, although decomposition was greatest on the urea-treated leaves bacteria were present in large numbers on the control leaves and the very large difference in decline of ascospores was unlikely to have been caused solely by number s of micro-organisms. The inability of asci to imbibe water in those cleistocarps beginning to degenerate could have resulted from direct damage of the ascus membrane by urea or its breakdown products.

Observations on the urea treated secondary mycelium showed that it was more easily fragmented into small segments when removed with cleistocarps at sampling, but there was no indication that the amount present was reduced or the number of immature cleistocarps declined over the seven weeks of the experiment. The instability of the control samples at 15° and especially at 25° may have been caused by the inability of the ascospores to metabolise at these higher temperatures when kept moist for long periods. Similar results were recorded by Merriman (1968) for cleistocarps of S. mors-uvae kept permanently moistened at 20°.

The experiments showed that at a range of temperatures urea could cause degeneration in a very short period, if the cleistocarps were kept continuously moist, although the exact cause for this was unknown. Thus, field experiments were carried out to evaluate the possibility of causing degeneration within cleistocarps under natural environmental conditions.

2. Field experiments.

Experiment i (1968-69).

The first experiment showed little evidence, except during the first 30 days, that urea was effecting the bacterial populations on the leaves Fig. 18 A and appendix tables 536). During the initial period the urea-treated tissue supported twice the number of bacteria compared to the control but this stimulation was soon lost and from the fourth sample on 12 November until the experiment ended in May there was no consistent difference between the total number of micro-organisms in the two treatments.

The fungal population (Fig. 18 B) similarly showed no effect of urea and in contrast to the bacteria there was no initial difference in the fungal populations between urea and control leaves. Yeasts were found in the first samples but these were rapidly replaced by species of Fusarium, Alternaria, Cladosporium, Epicoccum, Aureobasidium and Penicillium. There was no obvious difference between the incidence of any one of these species in the two treatments. Many fungi colonised the leaf tissue and grew over the mycelium of S. mors-uvae but few were found growing on the cleistocarps themselves. However, a Cephalosporium sp. and a Fusidium sp. were often found growing on the cleistocarps, the Cephalosporium being quite specific to the cleistocarps and sporulating freely on them. These two fungi were, however, rarely isolated by the dilution plate method.

It is likely that the high rainfall during the first 2 weeks leached the urea or is breakdown products from the leaves and resulted in

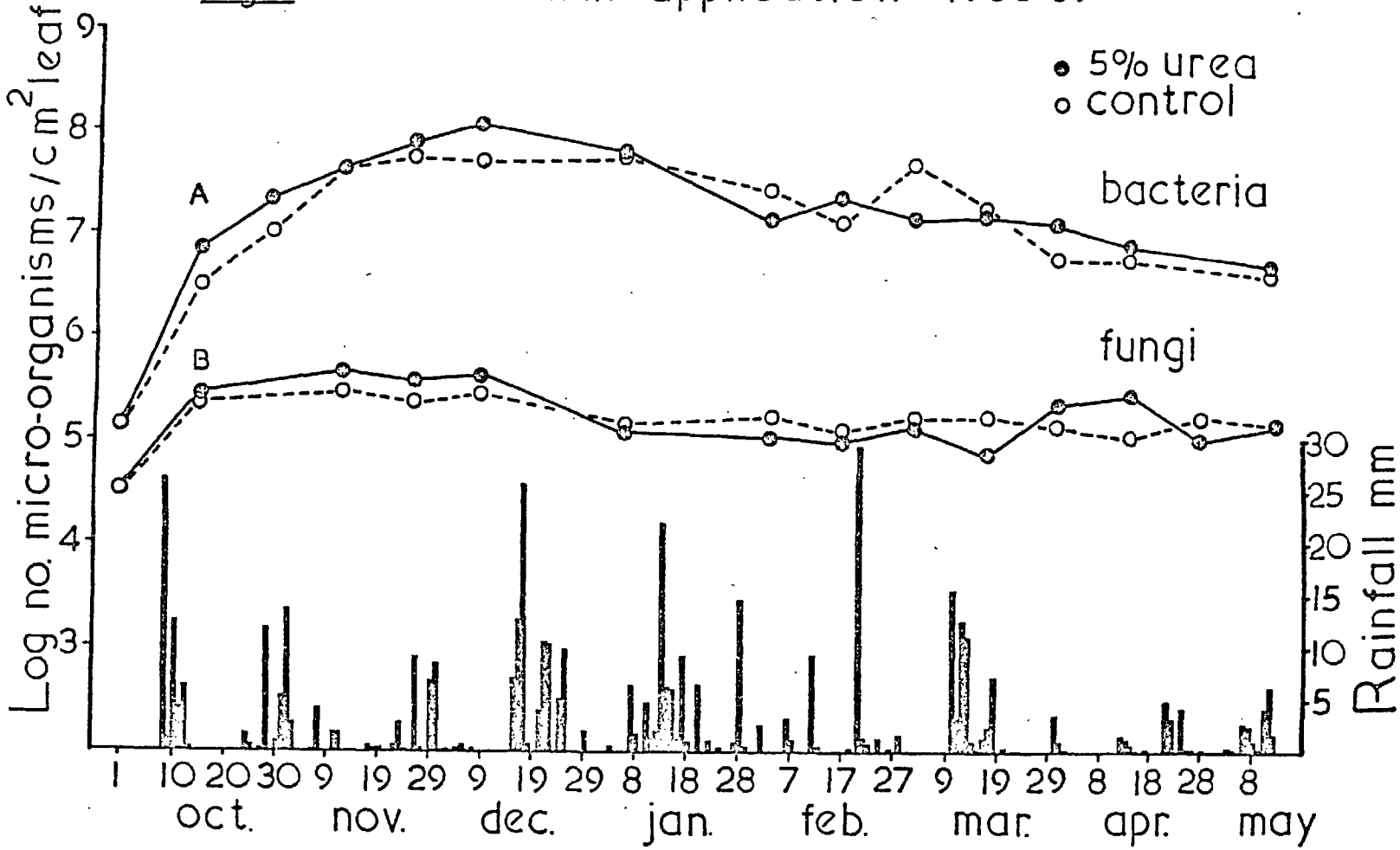


Fig18. Urea: autumn application 1968-69
5% urea
control
bacteria
fungi
A
B
Log no. micro-organisms/cm² leaf
Rainfall mm
1 10 20 30 9 19 29 9 19 29 8 18 28 7 17 27 9 19 29 8 18 28 8
oct. nov. dec. jan. feb. mar. apr. may

Table 11. Ascospore discharge - autumn urea treatment 1968.

Date	5% UREA			CONTROL		
	No. spores	No. c'carp	Spores/ 1000 c'carp	No. spores	No. c'carp	Spores/ 1000 c'carp
6.1.69	3	3957	0.76	0	2878	0
20.1.69	10	4028	2.48	7	5457	1.28
3.2.69	2	3197	0.63	0	2572	0
17.2.69	17	5609	3.03	58	3152	18.40
3.3.69	26	2577	10.09	43	3738	11.50
17.3.69	76	3081	24.67	9	2199	4.09
31.3.69	27	2389	11.30	14	4297	3.26
7.4.69	25	2887	8.66	41	6126	6.69
14.4.69	2	2744	0.73	2	5613	0.36
28.4.69	0	4462	0	0	3809	0
Totals	188	34,931	5.38	174	29,841	4.37

Table 12. Ascospore discharge - spring treatment 1969.

Date	5% UREA			CONTROL		
	No. spore	No. c'carp	Spores/ 1000 c'carp	No. spore	No. c'carp	Spores/ 1000 c'carp
11.2.69	20	2482	8.06	32	2821	11.34
25.3.69	23	2146	10.72	18	1901	9.47
15.4.69	2	1564	1.28	0	1728	0
Total	45	6,192	7.27	50	6,450	7.75

the similarity of the populations. There can be little doubt that some urea was taken up by the leaves, although no urea or nitrogen determinations were made, but the difficulty of wetting the discs covered on both surfaces with a thick felt of mycelium may have restricted the amount absorbed.

In the spring treatment in 1969 the total numbers of bacteria (Fig. 20 b appendix Table 7) were found to be substantially increased after the urea application. After 4 weeks the populations of bacteria and yeasts were x7.5 greater than those on the control leaves. During the first 10 days after placing the leaves on the soil they were protected from the direct effects of rainfall by a polythene sheet placed 15 cm above soil level over the bags. It is likely that this prevented leaching of the urea-N sufficiently to allow a stable bacterial population to develop in response to it. However, in this experiment as in the autumn treatments the ascospore content of cleistocarps was not effected (Fig. 19 & 20A) and spores were released in dehiscence tests carried out in the spring (Tables 11&12). In both experiments the ascospores were proven to be viable in infection tests on fresh leaf tissue.

In the autumn experiment a rough estimate was made of the numbers of cleistocarps remaining on the leaf discs during the 7 months on the soil. Table 13 shows that the totals, for urea-treated and control leaf discs, at each sampling date were similar. In autumn a set of leaf discs bore 16,500-20,000 cleistocarps, (referring to the categories given on p.72), this was an average of 16,750 cleistocarps. In both treatments this initial number declined to about 5000 in May when two thirds of the cleistocarps had been lost from the leaves.

Fig.19. Urea: autumn application. 1968

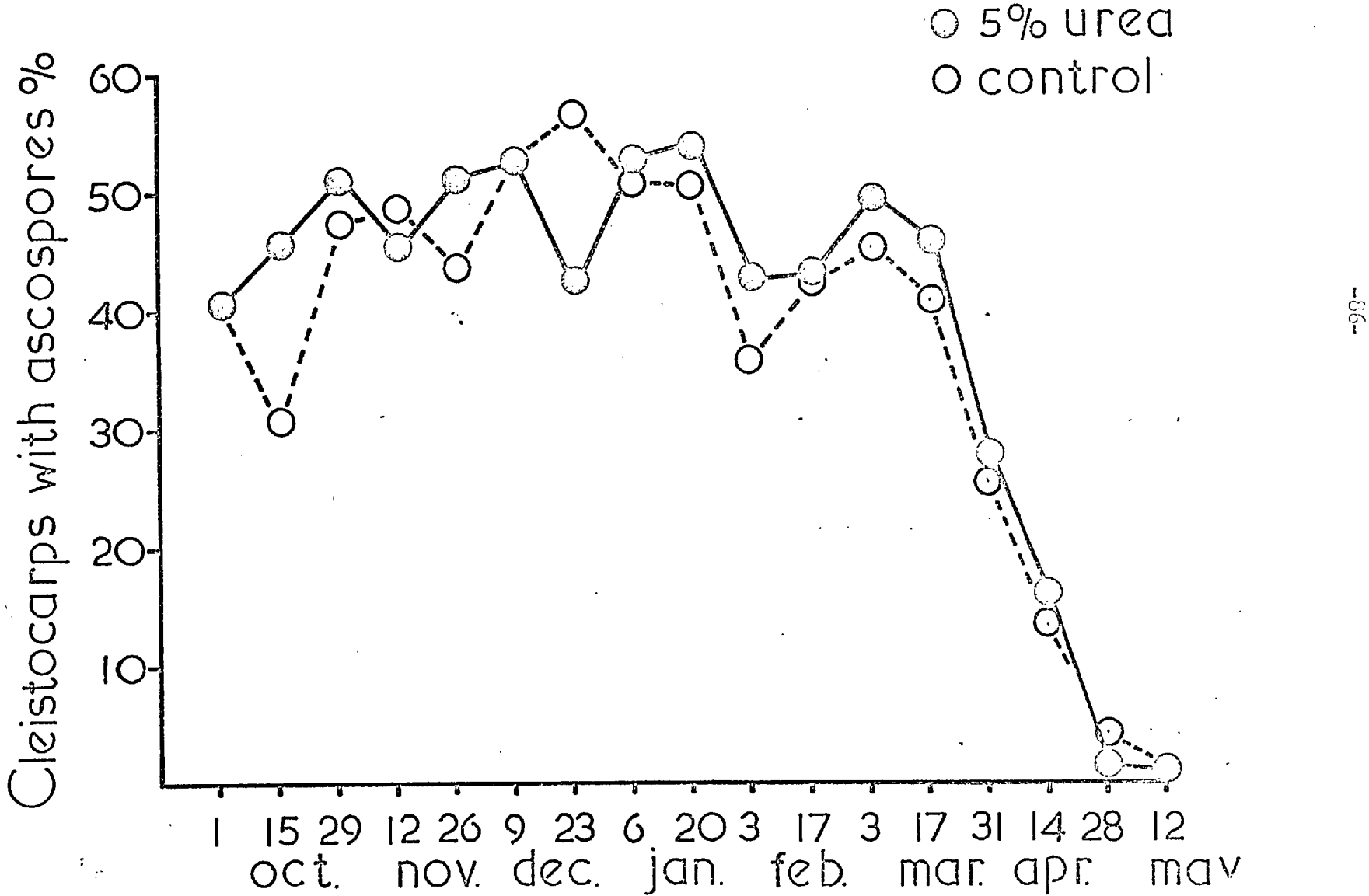
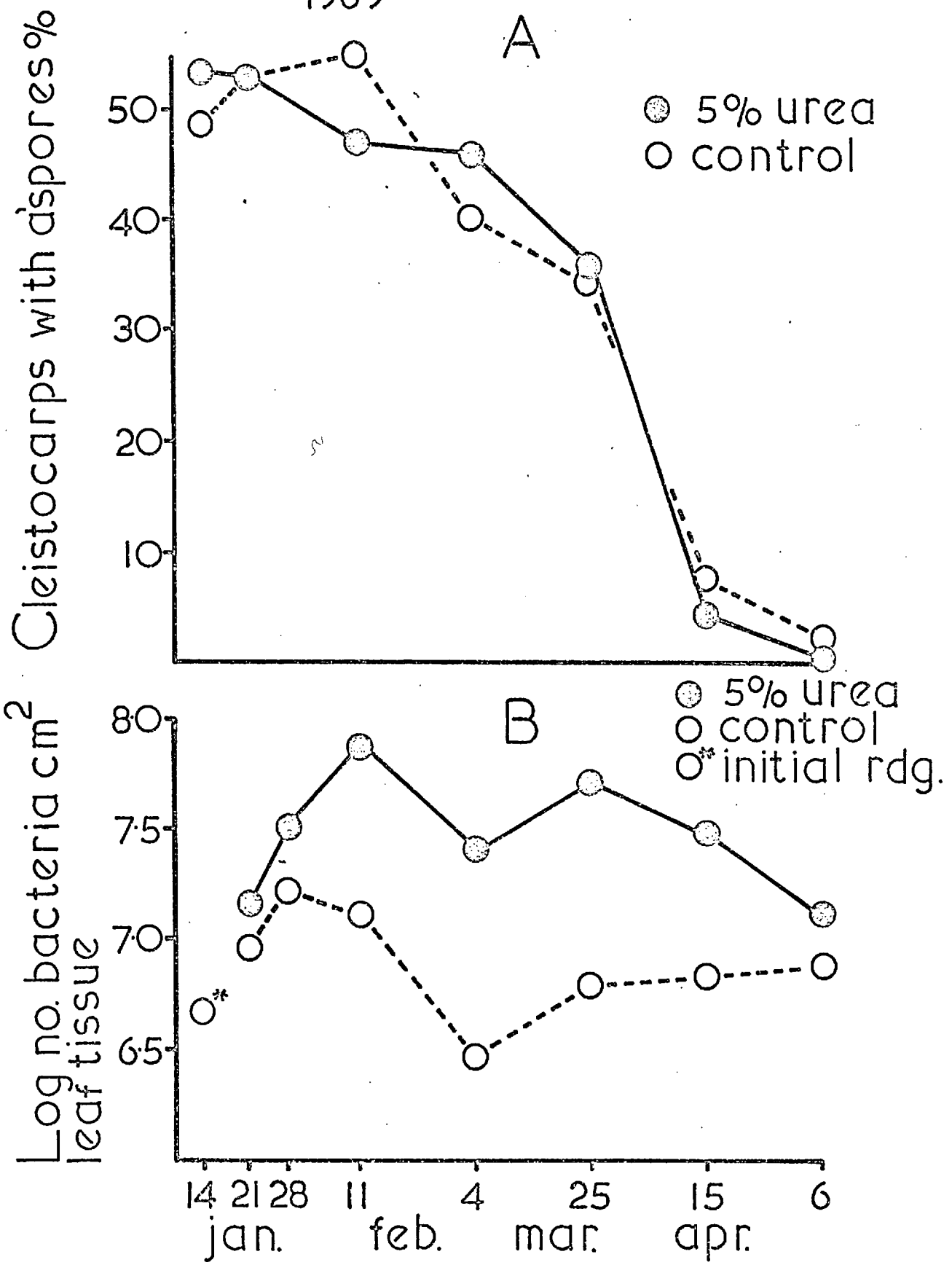


Fig.20. Urea: spring application 1969



Thus, in contrast to laboratory experiments there was no evidence that urea caused a weakening of the mycelium which on the soil would free the cleistocarps from their attachment to the leaf surface.

Table 13. Loss of cleistocarps from leaves on soil - 1968-69.

Date	CONTROL	5% UREA
	No. c'carps/8 leaf discs	No. c'carps/8 leaf discs
1.10.68	16,750	16,750
15.10.68	15,941	16,685
30.10.68	17,334	12,371
12.11.68	16,070	18,001
26.11.68	15,012	13,388
10.12.68	11,338	14,154
27.12.68	12,503	7,282
6.1.69	10,185	11,412
20.1.69	8,621	12,011
3.2.69	5,277	5,104
17.2.69	10,391	9,375
3.3.69	7,262	10,094
17.3.69	8,439	8,351
31.3.69	5,267	5,923
14.4.69	6,148	5,864
28.4.69	7,809	5,029
12.5.69	4,679	5,641

The leaf discs remained virtually intact throughout the time they were on the soil; they turned brown and became brittle when dry. But there was no greater decomposition of urea-treated leaves and these were still structually intact at the termination of the experiment in May.

Experiment ii (1969-70).

The apparent failure of urea to affect the survival of cleistocarps by promoting a high microbial population was investigated further. In the first experiment an autumn spray in 1969, leaves were sprayed to run-off on 22 October when placed on the soil and twice more when bacterial populations declined to numbers similar to those of the control leaves. In these tests 5% fructose was used as a sticker and Lissapol NX (Imperial Chemical Industries Ltd.) as a wetting agent, in the hope that urea would be taken up and remain in the leaves for a greater period than in the 1968-69 experiments. Because of the reported role of chintoclastic organisms (e.g. Lockwood, 1959; Lloyd and Lockwood, 1966), especially actinomycetes in breaking down fungal mycelium counts were also made of these organisms associated with the mycelium of S.mors-uvae.

In the spring experiment of 1970 dehiscence tests were carried out on the leaves before spraying with urea so that treatment occurred at time of proven natural discharge of spores from these leaves. Smaller samples of cleistocarps were taken for assessments; six leaves were selected and from these c. 500-1000 cleistocarps taken per leaf. No assessments were made of micro-organisms in the spring treatment.

The results of the autumn treatment of 1969 on microbial populations are given in Figure 21 and appendix Tables 8 & 9 . Fungi were little effected and although there was a statistical difference between the water control and the other two treatments the difference in terms of fungal propagules was small and thought unlikely to have affected the viability of the cleistocarps. Those fungi commonly found on the dilution-plates were the same types as noted in experiment (i) above and there was no evidence that their respective numbers differed between the treatments. The bacteria/cm^2 leaf tissue increased rapidly from 1.2×10^6 when laid on the soil to 6.1×10^7, 7.7×10^7 and 9.8×10^7 after 4 days for water, fructose and urea samples, respectively. However, the urea populations were maintained at this level for 2 weeks compared to a sharp decline in the controls. But after a further 2 weeks there was also a drop in the urea populations although they remained three times higher than the water control. After spraying on the second occassion the populations again rose in all three treatments and it was probable that lissapol NX had a stimulatory effect on the micro-organisms at the concentration used. Nevertheless, the urea populations were five times the lissapol control. This difference was maintained but there was a slow decline in total numbers of bacteria in all treatments until the end of the experiment in April. Fructose alone had little effect on prolonging a high microflora and after the second spray there was a fall in the populations similar to that of the water control. Failure to increase the populations after the third spraying on 26 February probably resulted from the low temperatures at this time.

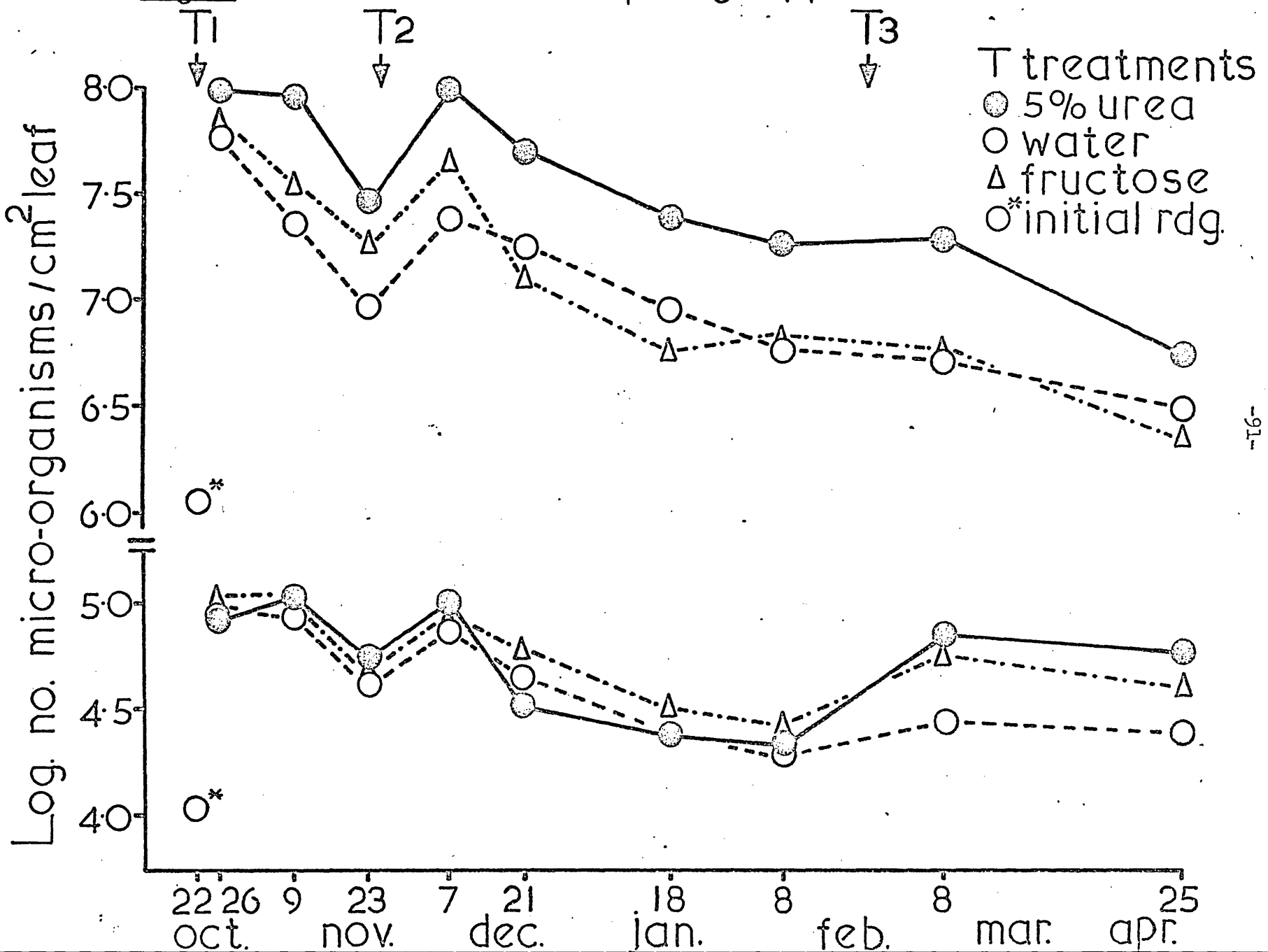


Fig.21. Urea: autumn-spring application 1969-70
T1
T2
T3
T treatments
5% urea
water
fructose
initial rdg.
Log. no. micro-organisms/cm² leaf
8·0
7·5
7·0
6·5
6·0
5·0
4·5
4·0
22 oct.
26
9 nov.
23
7 dec.
21
18 jan.
8 feb.
8
25 mar. apr.

Urea failed to change the composition of bacteria in terms of gram-negative and chromogenic organisms (Tables 14:15). There was a rise of 10% in the numbers of gram-negative bacteria and a corresponding 20% reduction in the numbers of chromogenic bacteria from the initial populations on the leaves to those estimations made 4 days after spraying with urea.. But there was little difference between the treatments after this time or following the reapplication of the test solutions. The means for all the samples for the gram-negative bacteria were 32.2%. 28.6%, 28.4% and for chromogenic bacteria 57.6%. 62.7% and 63.5% for leaves treated with urea, fructose and Lissapol NX respectively.

Table 14. Percentage gram-negative bacteria, autumn applications 1969.

Date	0.05% Lissapol NX	5% Fructose + liss.	5% Urea fructose + lisspol
22.10.69	25.76*		
26.10.69	23.72	25.90	36.45
9.11.69	26.74	35.54	26.66
23.11.69	30.60	40.64	38.84
† 27.11.69			
7.12.69	38.59	36.09	41.28
21.12.69	25.85	33.07	39.57
18.1.70	20.25	20.60	35.10
8.2.70	29.63	22.75	29.32
† 26.2.70			
8.3.70	20.25	18.46	22.41
25.4.70	28.42	24.66	19.86

* Initial population † Date of reapplication of sprays.

Table 13. Percentage chromogenic bacteria, autumn applications 1969.

Date	0.05% Lissapol NX	Fructose + liss.NX	Urea fructose + lissapol NX
22.10.69	75.13*		
26.10.69	65.02	55.69	54.16
9.11.69	66.19	71.99	62.89
23.11.69	63.15	59.42	61.53
† 27.11.69			
7.12.69	59.24	63.54	56.90
21.12.69	77.10	63.61	57.73
18.1.70	66.85	64.80	62.57
8.2.70	63.37	63.56	58.34
† 26.2.70			
8.3.70	56.61	61.73	60.84
25.4.70	53.85	59.88	42.84

* Initial population. † Date of reapplication of sprays.

Chitinoclastic organisms were difficult to isolate due to the large numbers of bacteria developing on the plates, even in the presence of antibiotics. Preliminary experiments with 0.2% chitin without inorganic salts (Lingappa and Lockwood, 1962) gave no appreciable reduction in bacteria and fewer actinomycetes were isolated.

The number of actinomycetes and bacteria recorded on the chitin plates are given in Figure 22 A & B (and appendix Tables 10 & 11). In both cases urea-treated leaves contained a significantly higher population of

chitin degrading organisms, although the difference between treatments was relatively small. Also, although, there was a relatively large increase in the number of chitinoclastic bacteria the degree of clearing of these isolates was often poor and the zone of partial clearing extended for only 1-2 mm from the edge of the colony after 14 days incubation at 25°C. Fig. 22 shows that there was a marked decrease in the number of actinomycetes on the leaves after the first spray which resulted in a change from a population of non-sporing actinomycetes with a much branched mycelium to streptomycete colonies probably derived from the soil populations; the latter, without exception possessed chitinase enzymes. Chitinoclastic fungi were not found on the selective media plates and estimates of these were abandoned after the fourth sampling. Commonly Fusarium sp., Fusidium sp., Cephalosporium sp. and Cephalothecium sp. and also species of Alternaria and Cladosporium were isolated. None of the isolates contained chitinases, although the Fusidium, Fusarium and Cephalosporium grew well on the chitin agar plates.

Estimation of urea on sprayed leaves.

Urea was estimated to ensure firstly that it had been taken up by the leaf tissue after application and secondly to determine its rate of loss from the leaves. Results are given in Tables 16 i & ii.

In all instances more than 80% of the initial amount of urea absorbed was lost in 4 days and none remained after 9 days from spraying. It would seem that the stimulation of bacterial populations lasted only as long as urea was present and that these populations declined rapidly following urea breakdown.

Fig.22. Chitinoclastic organisms 1969-70

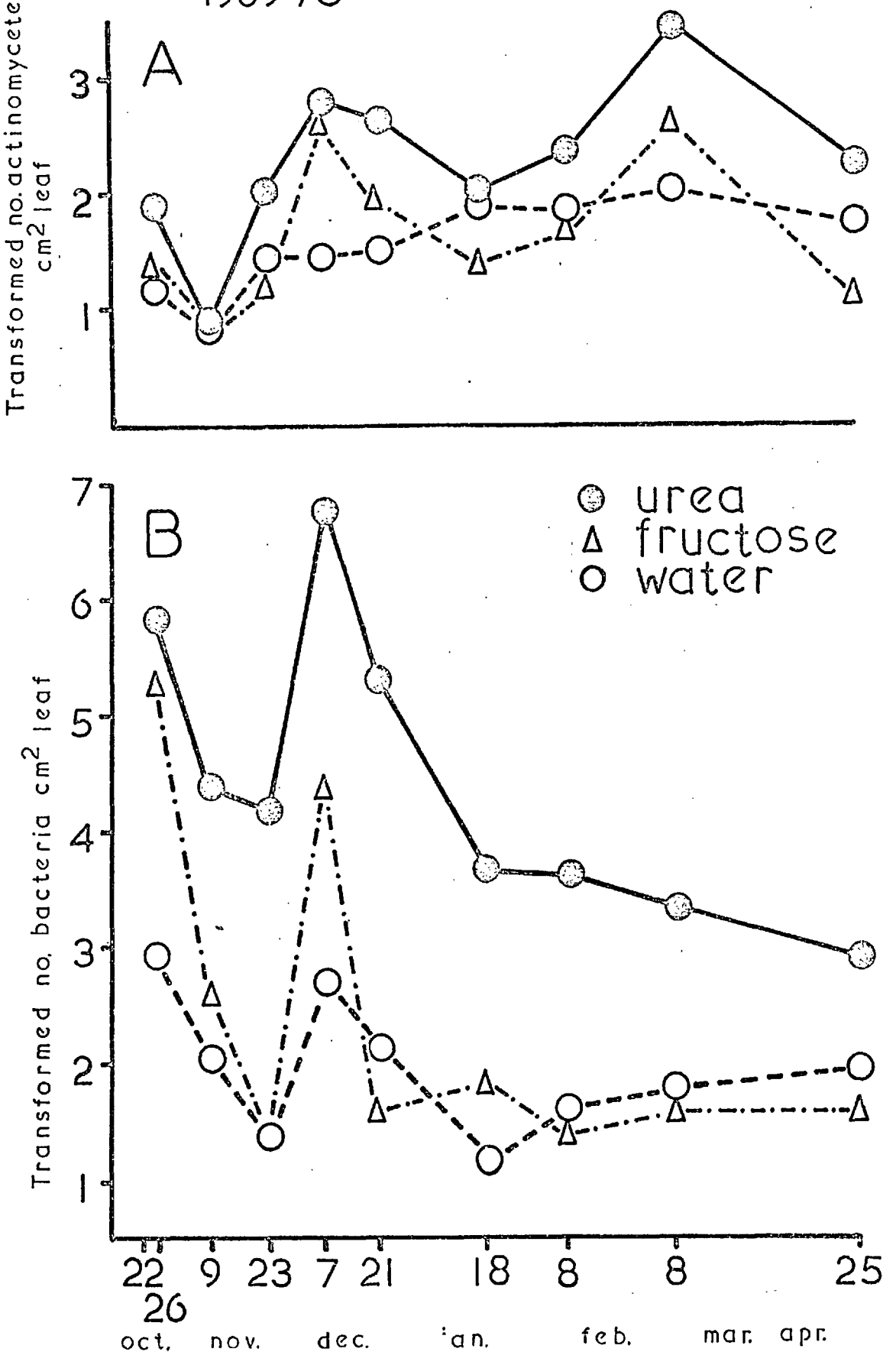


Table 16. Estimations of urea taken up by leaves after spraying.

i) Autumn spray 1969-70.

1			2			3		
Date	Urea mg/ cm^2 leaf*	% Loss	Date	Urea mg/ cm^2 leaf	% Loss	Date	Urea mg /cm^2 leaf	% Loss
22.10.69	2.354		27.11.69	1.391		26.2.70	2.782	
26.10.69	0.064	97.28	7.12.69	0	100	2.3.70	0.471	87.08
31.10.69	0	100				7.3.70	0	100

* Estimations were made initially 4h after spraying leaves on the soil.

ii) Spring application 1970.

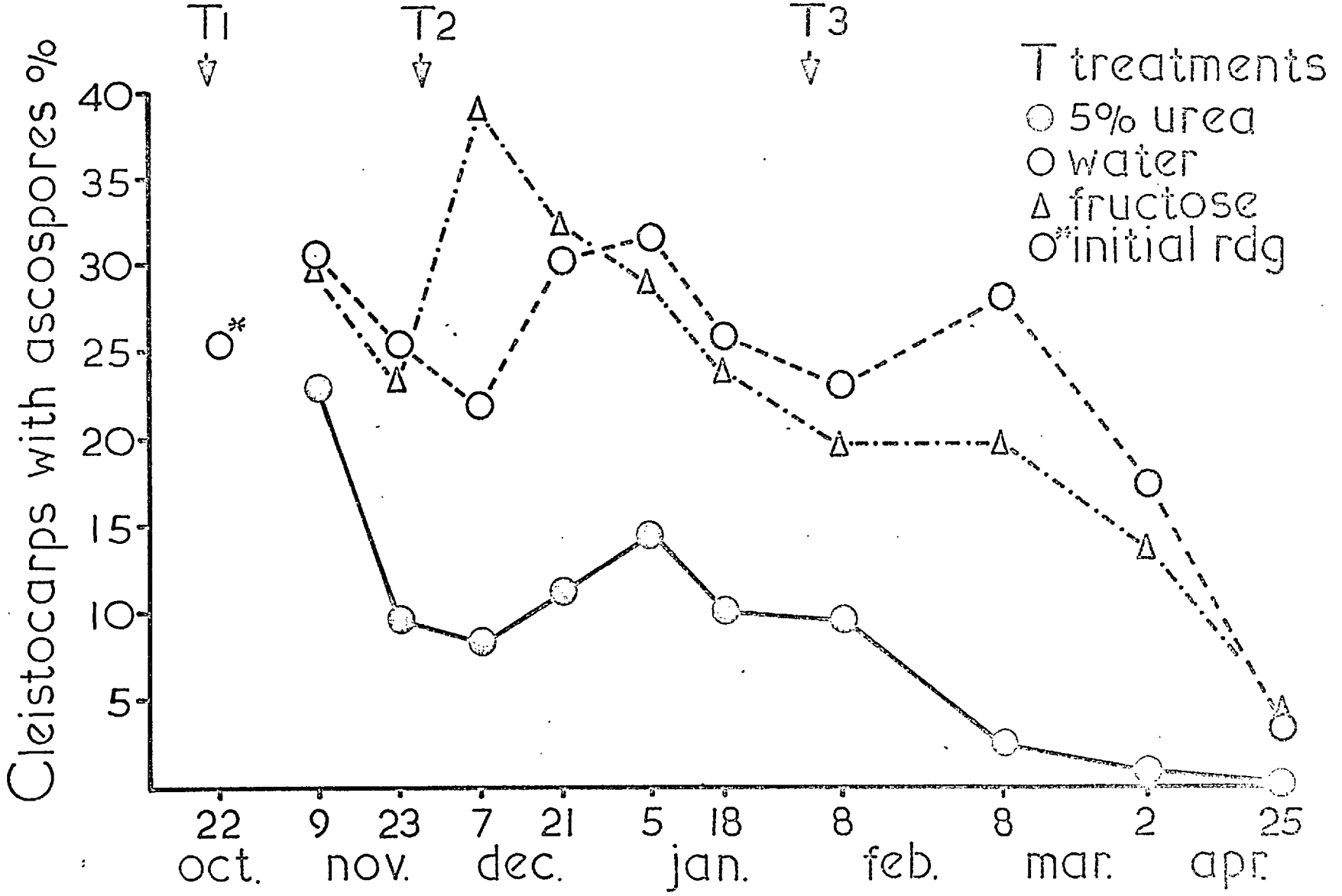
Date	5% UREA		10% UREA	
	Conc. mg/cm^2 leaf	% Loss	Conc. mg/cm^2 leaf	% Loss
26.2.70	1.359		5.350	
2.3.70	0.214	84.22	1.123	79.25
9.3.70	0	100	0	100

This suggests that nitrogen levels were not increased significantly for long periods. The difference between the urea-treated populations and the water control was probably maintained by the relatively lower temperatures in the first four months of 1970.

Effect of urea on cleistocarps.

In both experiments urea had a pronounced effect on the viability of asci and ascospores. In the autumn treatments (Fig. 23) there was little noticeable difference until late November when degeneration of the

Fig.23 Urea: autumn-spring application 1969-70



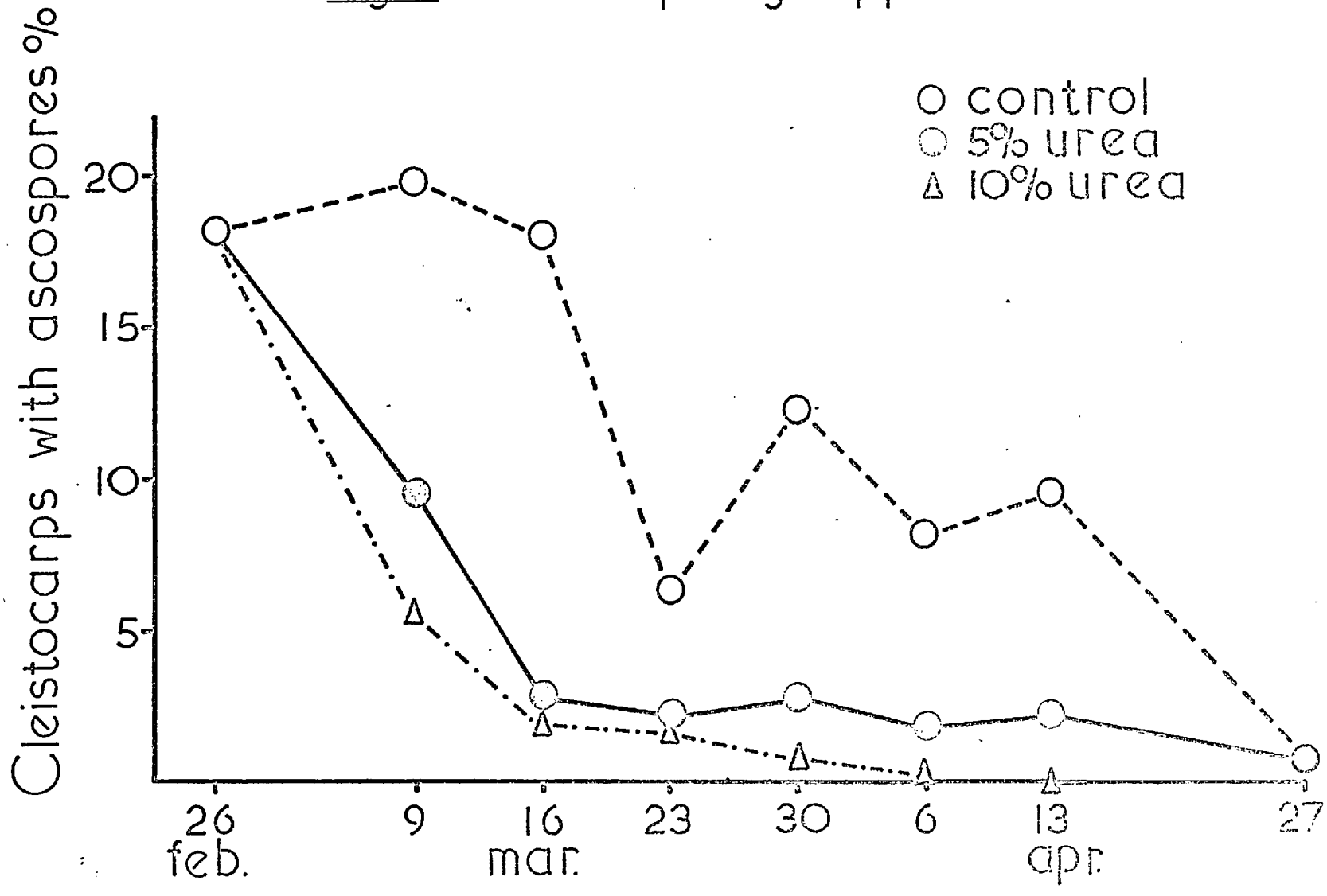
ascospores began to take place in urea-treated samples. Degeneration, however was not uniform; some cleistocarps taken at this time and in samples taken in mid-December still contained ascospores which were stained with neutral-red and whose asci imbibed water and discharged spores in slide-dehiscence tests. But many cleistocarps were without contents and filled with large quantities of fat, and after mid-January few remained whose asci were able to swell and discharge spores. The degeneration within the cleistocarps was similar to the pattern noted in other experiments (p. 24) except that following urea treatment the ascus was unable to imbibe water whilst still containing ascospores which appeared normal. The inner ascus membrane was drawn away from the ascus wall grouping the eight spores into a small area, an appearance similar to that of a plasmalysed cell. Results of dehiscence tests (Table 17) showed that no spores were released from cleistocarps treated with urea whereas from the fructose and water controls 369 and 581 spores where trappe respectively from a similar number of cleistocarps.

In the spring application the degeneration with both 5% and 10% urea was extremely rapid (Fig. 24) at a time when ground temperatures were relatively low and bacterial growth thought to be minimal. Although bacterial and fungal populations were not estimated reference can be made to the autumn-spring treatment of experiment (i), in which the third spray was on 26 February coinciding with the spring application of experiment (i) (Fig. 21). In the autumn-spring experiment there was no stimulation of bacteria although the fungal populations rose immediately after spraying. But as the increase in fungal populations of the urea

Table 17. Ascospore discharge, autumn area treatment 1969.

Date	TREATMENTS											
	5% urea+Fructose+Lissapol				Fructose+Lissapol				Lissapol			
	No. spores	No. c'carps	Spores/ 1000 c'carps	Asci swollen not dehisced	No. spores	No. c'carps	Spores/ 1000 c'carps	Asci swollen not dehisced	No. spores	No. c'carps	Spores/ 1000 c'carps	Asci swollen not dehisced
1 Mar.	0	2831	0	1	241	3183	7.57	0	330	3758	8.78	4
1 Mar.	0	2944	0	0	94	3061	3.07	0	121	2684	4.51	3
2 Apl.	0	2314	0	0	28	2278	1.23	0	122	4720	2.58	21
6 Apl.	0	2745	0	0	6	2278	0.26	0	8	3406	0.23	0
Totals	0	10,834	0	1	369	10,800	3.42	0	581	14,568	3.99	28

Fig.24. Urea: spring application. 1970



and fructose treated leaves was similar and as cleistocarps on the fructose sprayed leaves remained viable this increase after spraying on 26 February probably had little influence on the survival of the cleistocaprs. Therefore, the rapid degeneration occurring after treatment with 5% and 10% urea in spring was unlikely to have been caused by the activity of micro-organisms.

The first signs of degeneration in the spring application was again an effect on the ascus with the innter wall membrane being drawn away from the ascus wall. Fig. 25 a & b shows this effect on treated and nontreated cleistocarps.

Cleistocarps sprayed with 10% urea degenerated slightly more rapidly than those treated with 5% urea and this was reflected in the dehiscence tests (Table 18); twice as many spores were released from cleistocarps on leaves treated with 5% urea compared with those treated with 10% urea. However, the number of spores released was extremely small compared to the water control. No tests of infectivity were carried out on the spores released from urea-sprayed leaves owing to the very low sumber discharged.

Effect of urea on leaves.

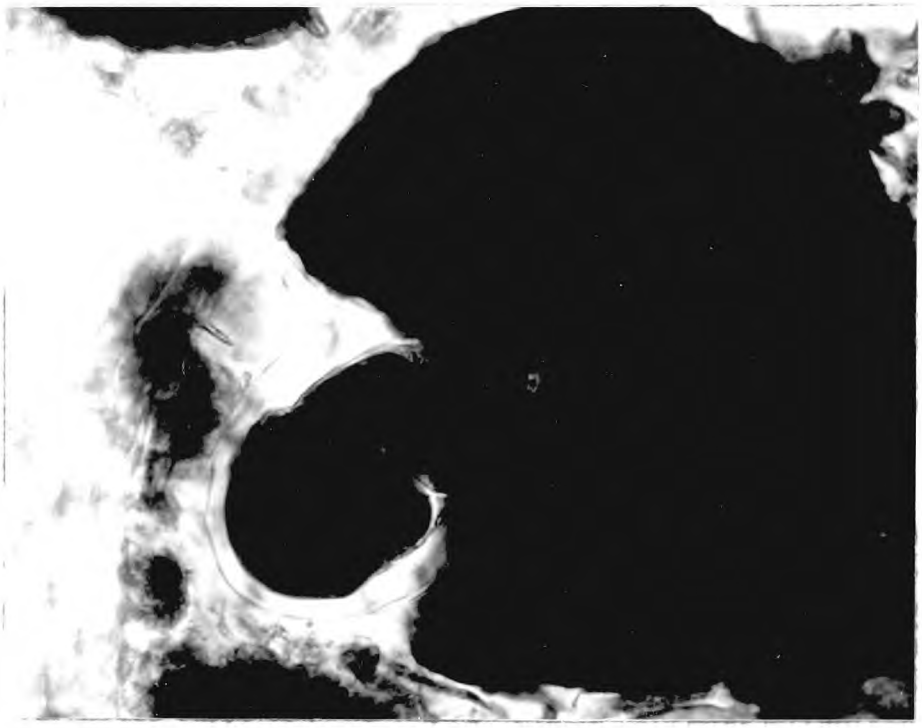
There was little difference in these experiments in the breakdown of urea-treated and the control leaves. In very few of the leaves was there extensive loss of limina tissue. Most leaves with secondary mycelium and cleistocarps were considerbly thickened and distorted in response to the fungal colonisation and these leaves were found to be very resistant to decomposition. Only in the last samples taken were

Table 18. Ascospore discharge, spring application 1970.

Date	WATER CONTROL			5% UREA			10% UREA		
	No. spores	No. c'carps	Spores/1000 c'carps	No. spores	No. c'carps	Spores/1000 c'carps	No. spores	No. c'carps	Spores/1000 c'carps
26 Feb.	0	1,652	0	0	1,621	0	3	1,404	0.214
2 Mar.	24	1,985	1,209	0	2,124	0	0	2,859	0
9 Mar.	23	1,985	1.159	1	3,055	0	0	3,773	0
16 Mar.	229	3,325	6.887	0	2,562	0	7	1,291	0.542
23 Mar.	88	2,696	3.264	7	3,061	0.229	0	3,366	0
30 Mar.	92	4,782	1.924	11	3,417	0.322	0	2.359	0
6 Apl.	58	4,017	1.444	0	4,500	0	0	2,794	0
13 Apl.	16	3,637	0.440	0	2,526	0	0	1.854	0
27 Apl.	8	2,997	0.267	0	1,676	0	0	2,027	0
Totals	538	27,012	1.992	19	24,542	0.077	10	21,727	0.046

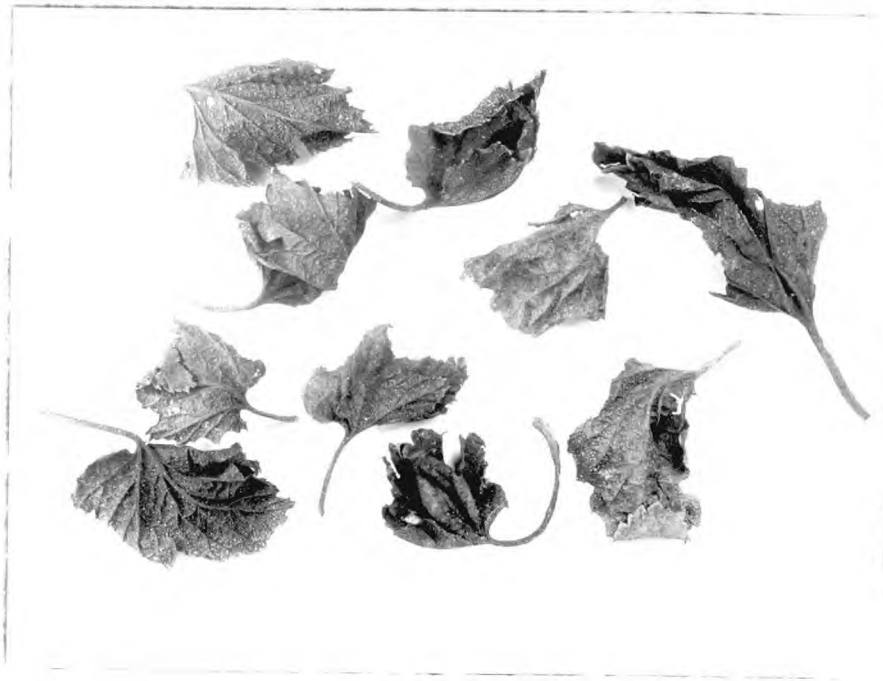
Fig. 25. Appearance of ascus and ascospores of *E. morr-uvae* after treatment with urea; spring application, 1970. Ascospores stained with cotton-blue in lactophenol.

(a) Urea treated cleistocarp (x900)



(b) Water treated cleistocarps (x700)

Fig.26. Appearance of leaves used in autumn-spring urea experiment 1970-71 after remaining on the soil for 20 months. (x1)



there signs of destruction to epidermal tissue and this was relatively slight. Destruction of tissue did take place however in leaves infected with mildew when leaf expansion was almost complete. Little of the tissue between the veins remained after the leaves had remained for 5-6 months on the soil following the urea treatment. The leaves treated with the three test solutions were left on the soil after the completion of the ascospore counts and microbial estimations and even after 20 months the smaller leaves were still intact, although, they were completely stripped of their secondary mycelium and cleistocarps.(Fig. 26).

2. Effect of certain actinomycetes on cleistocarp survival.

Two streptomyses spp. were isolated from the soil dilution-plate assessments of actinomycetes associated with secondary mycelium and cleistocarps on leaves (p. 89). Both produced large zones of clearing on 0.2% chitin agar and on a secondary mycelium/cleistocarp agar prepared from the unpigmented mycelium of S. mors-uvae (p.143). Their ability to cause degeneration was tested by inoculating cleistocarps with spore suspensions of the isolates.

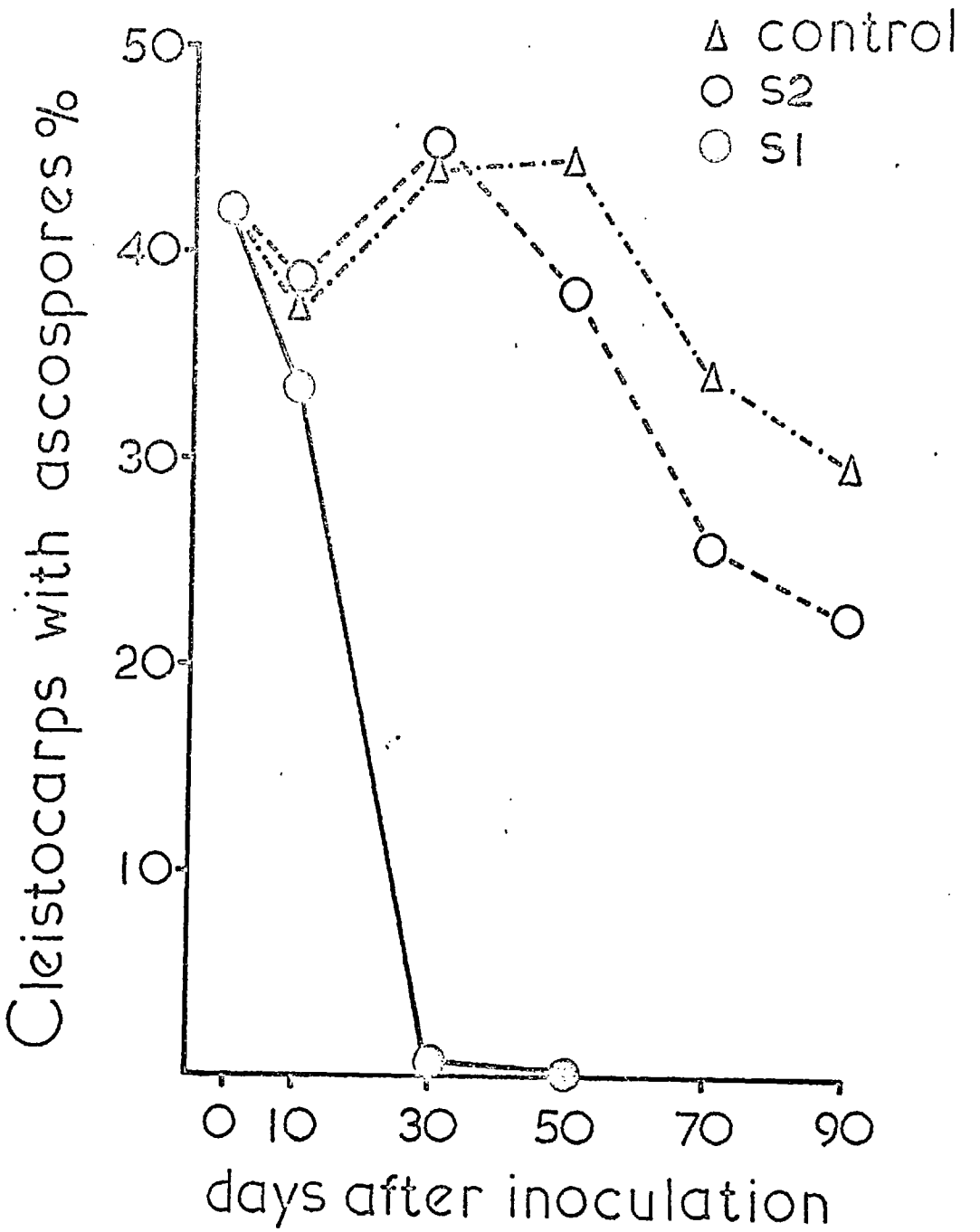
McCartney tubes (50 ml) were filled with 15 g soil, previously sieved through 2000 μ mesh and dried at 60° for 1 h. The tubes and soil were autoclaved for 1 h. at 120°. Approximately 5000 cleistocarps taken from mature leaves were placed, with secondary mycelium, on the soil surface in nine tubes. Spore suspensions of the streptomycetes were prepared by adding 3 ml sterile 0.9% saline to 10-day-old cultures grown on nutrient-agar slopes. Soil tubes were inoculated by adding 1 ml

suspensionsto each. Three tubes were inoculated with each isolate and 1 ml sterile saline added to the remaining three tubes for controls. Subsequently 3 ml of sterile distilled water was added to each tube, an amou sufficient to moisten the soil. No effort was made to standardise the concentration of spores from the two cultures. Tubes were incubated at 10° and sampled first after 10 days and subsequently at 20-day intervals by asceptically taking small pieces of mycelium and estimating the percentage cleistocarps with ascospores.

The results are given in Figure 27 and appendix Table 12. One actinomycete., S_1, caused total degeneration of the ascospores after 30 days, where as there was little degeneration in cleistocarps inoculated with S_2 or in the control samples until 50 days. Even at this time degeneration was slight and after 90 days incubation asci with ascospores still contained large accumulations of glycogen and some asci swelled and discharged spores in the slide dehiscence test. There was no statistical difference between the percentage of ascospores of S_2 and control samples (appendix p.272).

Microscopic examination of S_1 samples showed mycelial development after 10 days and considerable growth and sporulation at 30 days; here the mycelium had colonised the entire cleistocarp sample. But there was little evidence of penetration of either the cleistocarp wall or the secondary mycelium. It was evident that the streptomycete had grown into the fractured ends of the secondary mycelium hyphae but was not seen to penetrate it or to grow directly from the cleistocarps. Degeneration of these cleistocarps was accompanied by large release of fat globules from

Fig.27 Effect on cleistocarps by two Streptomyces spp



the breakdown of the thin walled fat-laden tissue beneath the cleistocarp wall. In contrast to this colonisation by S_1, isolate S_2 took appreciably longer and was comparatively short lived; the isolate was not recovered after 50 days from inoculation.

It, therefore, seemed unlikely that S_1 had a direct effect on the cleistocarps by penetrating the wall and destroying the ascus. If S_1 did cause lysis of the mycelium it was slight and probably confirmed to the partially thickened mycelium bearing immature cleistocarps. It is likely that S_1 was supported by the large number of bacteria associated with the cleistocarps and its colonisation of the samples for a long period may have been aided by the production of metabolic products antagonistic to competitors. These toxic substances, perhaps antibiotics may have been taken up by the cleistocarps causing breakdown and lysis of the asci and ascospores.

III. STRUCTURE AND COMPOSITION OF CLEISTOCARPS.

Review of literature.

There is much evidence to suggest that enzymes of soil micro-organisms can readily lyse hyaline mycelium of fungi, but whether they are the primary cause of lysis of living fungal walls has been the subject of much discussion and several workers (Carter and Lockwood, 1957; Lloyd, Noveroske and Lockwood, 1965; Lloyd and Lockwood, 1966) have suggested that nutritional deficiencies and the action of antibiotics induce autolysis of the living mycelium and only the final dissolution of cell walls occurs by the action of extra-cellular enzymes from other micro-organisms. Nevertheless, in vitro studies have shown that bacteria (Park, 1956; Michell and Alexander, 1963), fungi (Jones and Webley, 1967; Jones and Watson, 1969) and actinomycetes (Garcia Mendoza and Villanueva, 1962; Garcia-Acha and Villanueva, 1963; Skujins et al., 1965; Jones, Bacon, Farmer and Webley, 1968) have enzyme systems capable of hydrolysing cell wall preparations. Organisms grown on cell walls or killed mycelia of fungi, as sole carbon sources have been found to produce chitinases and glucanases in high concentrations. These enzymes in particular have further been implicated in processes of cell wall lysis by their ability to cause almost total hydrolysis of cell walls in incubation experiments and from the results of chemical analysis of mycelium where their specific substrates, namely chitin and glucans, have been found as major components (Phaff , 1963; Horikoshi, Koffler and

Arima, 1963; Skujins et al, 1965; Potgieter and Alexander, 1966; Jones, 1970).

In contrast to hyaline cell walls, pigmented cell walls survive for long periods in the soil (Lloyd and Lockwood, 1966; Linderman and Tousson, 1966) and they resist enzyme hydrolysis in controlled incubation experiments. Although the soil contains organisms able to lyse cell-wall agar preparations from hyaline fungi no organism has been isolated capable of producing a similar effect on pigmented cells. For example, Potgieter and Alexander (1966) found that Rhizoctonia solani was completely resistant to attack. Likewise no organisms were found lysing this fungus or Cladosporium spp. by Broomfield and Alexander (1967). Lockwood (1959, 1960) similarly reported that R. solani and Helminthosporium sativum were completely resistant to lysis when agar plates containing 4 day cultures were buried beneath samples of Michigan soil for 14 days. Hyaline fungi, in constrast, were completely destroyed after this time. In further experiments Lloyd and Lockwood (1966) found that the dark pigmented conidiophores and the older mycelium of Helminthosporium victoriae resisted lysis when placed on the top of soil in Petri dishes as did species of Rhizoctonia.

Potgieter and Alexander (1966) found that cell walls of R. solani with abundant melanin were comparatively unaffected by chitinase and glucanase enzymes whereas Skujins et al (1965) had earlier reported that the two enzymes digested 94% of the cell-wall material of Fusarium solani. With R. solani n-acetyl glucosamine was released only when cell walls were incubated with both chitinase and glucanase; no n-acetyl glucosamine

was released with chitinase alone. Similarly, Broomfield and Alexander (1967) found that the hyphal wall of both Aspergillus phoenicis and Sclerotium rolfsii were digested by chitinase and glucanase where as the conidial walls of A. phoenicis and the outer sclerotial walls of S. rolfsii, both containing melanin, were resistant to enzyme hydrolysis. Jones (1970) incubated a Streptomyces sp. in a medium containing the pigmented rind cells of Sclerotinia sclerotiorum as carbon source and found that it grew poorly, although some laminarase (β-(1-3) glucanase) activity was present in the culture filtrate. In similar incubation experiments a Streptomyces sp. also grew poorly on the sporangiophore walls of Mucor ramannianus, in which the presence of melanin was suggested by infra-red spectroscopy, but grew well on and completely hydrolysed the hyphal and arthrospore cell walls of this fungus (Jones et al, 1968).

The implication that melanin in cell walls inhibits enzyme hydrolysis was further studied by Kuo and Alexander (1967) with an albino mutant of Aspergillus nidulans. Whereas the hyaline mutant was readily lysed by enzymes, the pigmented walls were resistant. This study was supported by the work of Old and Robertson (1970b) who showed that the effects of soil and lytic enzymes (snail-gut and chitinase) on two wild type and two hyaline isolates of Cochliobolus sativus were quite different and that no lysis occurred in pigmented conidia compared to lysis after 3 h with hyaline spores. They produced electron micrographs (Old and Robertson, 1970a) showing evidence of erosion of conidial walls by micro-organisms after the spores were placed in soil. Bacteria were found inside both hyaline and pigmented conidia but pigmented conidia resisted lysis for

more than 2 weeks in soil. This resistance was thought to be associated with an electron-dense surface layer found only in pigmented spores; a layer which they suggested was mainly melanin.

Melanins are not the only compounds thought to confer resistance to enzyme hydrolysis. For example, Ballesta, Uruburu and Villanueva (1969) have suggested that an external layer of xylan or xylose prevents hydrolytic enzyme systems from attacking the glucan layers in the walls of Torulopsis aeris.

The resistance of cleistocarps of the Erysiphales to soil or leaf litter micro-organisms has not been investigated, nor generally has the chemical composition of the cleistocarp wall and that of the secondary mycelium. The only report appears to be by Gastaud (1944) who examined histochemically the pigment in the cleistocarps of Sphaerotheca humuli; he suggested this was a melanin-type compound. The resistance of cleistocarps of S. mors-uvae subjected to high microbial populations on leaves of black currant was therefore investigated together with the chemical composition of the cleistocarp wall.

Materials and Methods

1. Assay Procedures.

Total non-nitrogenous carbohydrates were determined by the Anthrone reagent (Hewitt, 1958). To 0.3 ml sample, 3 ml of Anthrone reagent were added and heated over a boiling water bath followed by immediate cooling. Readings were taken in a Beckman DB spectrophotometer at 620 mμ and compared to a standard curve prepared with glucose. In this procedure hexosamine gives no significant colour (Bartnicki-Garcia and Nickerson, 1962).

Reducing sugars were determined on 1 ml aliquots of neutralised and deionised acid hydrolysates by the method of Nelson (1944) as modified by Somogyi (1952). The colour reaction was measured at 540 mμ against a water blank and the values were expressed in terms of glucose.

Glucose was determined by the glucose oxidase method of Marks (1959), a modification of that of Middleton and Griffith(1957) . 'Fermcozyme' - a stable liquid preparation of glucose oxidase containing 750 μg/ml was obtained from Hughes and Hughes Ltd., London. The glucose oxidase reagent was prepared by adding 0.5 ml Fermcozyme to about 80 ml of 0.15M acetate buffer, pH 5.0. To this was added 5 ml of peroxidase solution (20 mg peroxidase/100 ml acetate buffer) and 1 ml of O-tolidine (1% in absolute ethanol). The solution was made up to 100 ml with buffer and stored at 2° in a dark bottle. Generally 0.5 ml samples were added to 3 ml of reagent, mixed gently and the colour read at 625 mμ after exactly

10 min. Glucose standards were prepared in saturated benzoic acid.

Chitin in the cell-walls of cleistocarps was determined by the histo-chemical method of Vouk (1915) modified by Hopkins (1929). Purified chitin and also chitosan (de acetylated chitin) were used for reference in this test. Chitosan was prepared by treating purified chitin with hot concentrated KOH (120 g/100 ml) at 160° according to the method of Bartnicki-Garcia and Nickerson (1962). A commercial preparation of chitosan was obtained from Sigma Chemical Co.

Chitin was determined quantitatively by hydrolysing samples of cell wall material with 6 N HCl at 100° for 6 h (Smithes, 1952) in 5 ml (freeze-drying) ampoules sealed under an atmosphere of oxygen-free nitrogen. The hydrolysate was centrifuged and aliquots of the supernatant were immediately lyophilised. The residue was taken up in distilled water and total hexosamine measured by the method of Levvy and McAllan (1959) using the p-dimethylaminobenzaldehyde reagent (DMAB) of Reissig, Strominger and Leloir (1955) obtained as a purified preparation from British Drug Houses Ltd. The DMAB reagent was prepared by adding 10 g to 100 ml of analytical glacial acetic acid containing 12.5% (v/v) 10 N HCl. To 0.6 ml of hexosamine solution (hydrochloride) was added 0.1 ml of 1.5% (v/v) acetic anhydride in acetone (prepared daily), followed by 0.5 ml of 0.7M potassium tetraborate (Sigma Chemicals Ltd.); this was adjusted to pH 9.2 with HCl. The tubes were sealed with a glass marble and treated in a boiling water bath for exactly 3 min. After cooling, 6 ml of the DMAB reagent were added and the contents of the tubes mixed. Colour development was carried out in a water bath at 37° for 20 min. and readings taken at 585 mμ against a reagent/distilled water blank.

Hexosamine was calculated using glucosamine hydrochloride (Sigma Chemical Co.) as standard.

N-acetyl hexosamine was estimated by the method of Reissig et al. (1955) by adding 0.1 ml of a 0.7M potassium tetraborate solution to 0.5 ml sample, boiling for 3 min. and after cooling adding 3 ml DMAB. The colour was read as above in the glucosamine hydrochloride determination. A purified preparation of N-acetyl-D-glucosamine was obtained from Koch-Light Laboratories Ltd., and used as standard.

Monosaccharides were liberated from cell wall preparations by placing c. 10 mg samples in 72% H_2SO_4 (1.84 g/ml) at room temperature for 12 or 24 h; they were then hydrolysed in H_2SO_4 for 12 or 24 h at 105°. Monosccharides were also analysed from a 6 h, 6N HCl hydrolysis of cell walls. The acid was removed in vacuo over P_2O_5 and the residue taken up in distilled water and deionised. All hydrolyses were carried out under an atmosphere of oxygen-free nitrogen in a 5 ml freeze-drying ampoule. The 1N H_2SO_4 hydrolysates were neutralised with $BaCO_3$ and the $BaSO_4$ formed, together with the excess $BaCO_3$ were centrifuged and discarded. A sub-sample of the supernatant was shaken on a Griffin flask shaker for 30 min. with a small amount of Bio-Deminrolit (Permutit Co. Ltd.). This deionised sample was lyophilised and kept at -18° until required for analysis.

Monosaccharides were characterised by gas-liquid chromatography using a Pye 104 series analytical chromatograph. The model was equipped with a hydrogen flame ionization detector and a coiled 1.5m by 4 mm (internal diameter) glass column containing 6% SE-52 as stationary phase on CQ72-85 as support. The imput pressure of nitrogen was 10 p.s.i.

Monosaccharides were converted to their trimethylsilyl (TMS) derivatives by the method of Sweeley, Bentley, Makita and Wells (1963). The sample was taken up in 1 ml pyridine (kept over KOH pellets in a desiccator) and converted to the TMS derivatives by the addition of 0.2 ml hexamethyldisilazane and 0.1 ml trimethylchlorosilane at room temperature. The reaction was carried out in a small plastic-stoppered vial which was shaken for 30 sec then allowed to stand for 5 min at room temperature. The heavy precipitate formed was not removed. As a routine measure a duplicate sample was taken up in pyridine, the reagents added and after shaking, warmed at 80° for 3 min. Manual injections of 2-4.5 µl were made of the reaction mixture using 1 µl hexane (A.R.) as a front marker. With the carrier gas flow as stated and an oven temperature of 150° the retention times for α-glucose and β-glucose were 22.3 and 38.3 min respectively.

Identification of peaks was made either by comparison of the retention times relative to α-glucose or by an increase in the peak heights after addition of a purified commercial preparation of the suspected sugar to the cell wall extract. Mannose and galactose were obtained from B.D.H. Chemicals Ltd., β-glucose from Sigma Chemical Co. and the α-glucose from Hopkin and Williams Ltd.

Melanin was extracted from the cell-wall preparations of the mature secondary mycelium and cleistocarps by the methods of Nicolaus, Piattelli and Fattorusso (1964). The sample was continuously extracted for 36 h and 40 h in petroleum-ether and ethanol respectively. After air-drying at room temperature the sample was placed in concentrated HCl of 14 days,

followed by boiling with 5 N HCl for 60 h. The sample was then washed twice in distilled water, twice with ethanol and continuously extracted with ethanol for 60 h, then washed four times with water and twice with 5 N HCl. The cell walls were again continuously extracted with 5 N HCl for 72 h, washed four times with water and twice with tetrohydrofuran (Bonner and Duncan, 1962). The sample was boiled with tetrahydrofuran for 12 h, centrifuged and the residue taken up in hot 0.5 N NaOH for 1h, the supernatant acidified and the precipitate centrifuged, washed twice with water and finally with acetone. The product was dried at 50° and weighed. Melanin was characterised by the procedures of Lingappa, Sussman and Bernstein (1963). Protein content of the malanin complex was measured by the method of Folin and Ciocalteu (1927).

2. Substrates for production of enzymes.

A β-(1-3)glucose was obtained from Dr.B.Larsen of the Norwegian Institute of Seaweed Research, Trondheim, this had been prepared from Laminaria hyperborea and constituted the 'insoluble' form of laminarin. A 'soluble' product was obtained from Koch-Light Laboratories Ltd. A β-(1-6)glucan (lutean) was provided by Dr.D.Jones of the Macaulay Institute for Soil Research, Aberdeen, Scotland. Chitin was obtained as an impure coarsely ground flake from B.D.H. Ltd. The method of purification followed that of Lingappa and Lockwood (1962). The preparation was washed alternately for 24 h in 1N NaOH and 24 h in 1 N HCL (six times)followed by washing with 95% ethanol (three times). This removed almost all of the impurity and gave a pink-white flake. About 15 g of the chitin was moistened with acetone and dissolved in 100 ml of cold (2°)

concentrated HCl by stirring continuously for 20 min in an ice-bath. The thick viscous liquid was filtered through a thin-glass wool pad under pressure into a Büchner flask containing 500 ml of iced distilled water. The chitin dissolved by the acid was precipitated as a fine colloidal suspension. The remaining residue was redissolved in acid and filtered; this procedure was repeated three times. The suspension of chitin was allowed to stand, the supernatant discarded, and distilled water added. This was repeated until the suspension was pH3. The preparation was stored at 2°. An alternative method of removing the acid and washing was to centrifuge the suspension combining the precipitates and washing six times until the final supernatants were pH6. The chitin was lyophilise and stored over P_2O_5.

3. Enzyme preparations.

For the comparison of enzymes induced by the cell-wall preparations, isolates were grown on the basal liquid medium of Skujins et al. (1965) (appendix p. 246), 10 ml/50 ml flask, to which cell-walls were added as a sole carbon source at a concentration of 1 mg/ml. This medium was previously autoclaved at 105° for 15 min. The cultures were grown on a shaker (120 cycles/min) at 25°.

The streptomycete, S_1, was maintained on a glycerol-asparagine agar (appendix p. 246) and a 0.5 cm agar disc, taken from a sporulating colony, was used as inoculum. Flasks were inoculated with Penicillium javanicum by taking a similar size disc from a colony grown of V8 juice agar (appendix p. 247).

A chitinase preparation was obtained from Koch-Light Laboratories Ltd. which had been derived from an unidentified actinomycete. This commercial source was slightly active against laminarin but completely

without activity against lutean (c.f. Bacon, Farmer, Jones and Taylor, 1969). A second chitinase was prepared from the culture filtrate of an unidentified bacterial isolate (B9) grown on chitin; large clear zones developed around colonies of this isolate when it was grown on an agar plate containing the unpigmented cell walls of S. mors-uvae. The bacterium was grown in the basal liquid medium of Skujins et al. (1965) to which was added 0.1% purified chitin. The cultures were incubated at 29° on a shaker and the culture filtrate was collected after 4 days. Ammonium sulphate was added to the filtrate to 0.7 saturation, the precipitate collected after standing at 2° for 12 h and taken up in distilled water and dialysed, with several changes, against distilled water for 12 h at 4°. The preparation was stored at -18°.

A β-(1→3)glucanase was obtained by growing a Micromonospora sp. (M3) (isolated from leaves bearing cleistocarps) in the basal medium containing 0.05% laminarin. For a highly active enzyme preparation of a β-(1-3)glucanase, Penicillium javanicum was inoculated into 100 ml flasks containing the medium of Reese and Mandels (1959) (appendix p.247). Two volumes of acetone were added to the culture filtrates and the resulting precipitate dissolved in 50% alcohol, centrifuged and taken up in 75% alcohol. The preparation was lyophilized and stored at -18°.

Chitinase activity of the culture filtrates was estimated by the release of n-acetyl hexosamine at 37° in a 5 ml reaction mixture containing 1 ml chitin suspension (5 mg/ml), 3 ml phosphate-acetate buffer (pH 5.5) and 1 ml of the enzyme preparation. The buffer was prepared by titrating 0.05 M Na_2HPO_4 solution to pH 5.5 with glacial acetic acid (Skujins et al.

1965). Glucanase activity was measured by determining the formation of glucose at 37° in a reaction mixture of 1 ml of the particular glucan, 3 ml McIlvaine's citrate-phosphate buffer (pH 5) and 1 ml of the enzyme solution. Laminarin was dissolved by heating the suspension at 100° for 15 min before adding to the buffered enzyme solution (Jones and Webley, 1967).

Similarly, the effect of the enzyme preparations either individually or combined in releasing n-acetyl hexosamine and glucose was estimated in a 5 ml reaction mixture with 1 ml cell walls (5 mg/ml). Toluene was added if the assay continued for longer than 4 h, to prevent microbial contamination.

Electron microscopy.

Leaves bearing cleistocarps were collected and prepared immediately for electron microscopy. Immature and mature cleistocarps were selected and fixed in September 1970 and mature cleistocarps wintered until the following April were treated similarly.

Cleistocarps were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7 for 4 h at room temperature. They were placed in a vacuum where direct impregnation was slow. Afterwards they were washed in buffer three times for 10 min and treated with OsO_4 (1% w/v) in cacodylate buffer for 1 h at room temperature.

Graded concentrations of ethyl alcohol were used for dehydration and four changes of absolute alcohol were made before infiltrating with Epon using epoxy-propane as a link reagent. The resin was changed several times during soaking for 3-12 days at room temperature and finally

polymerised at 60° for 2 days.

Sections were mounted on formvar-coated grids and stained with uranyl acetate (1% aqueous, for 20 min at 60°) and the lead stain of Reynolds (1963) (5 min at room temperature).

Experimental

1. Preparation of cell-wall material.

As cleistocarps of S. mors-uvae were not easily separated from the mycelium in which they formed cell-walls were prepared from cleistocarps, their appendages and the secondary mycelium.

A. Immature secondary mycelium and cleistocarps.

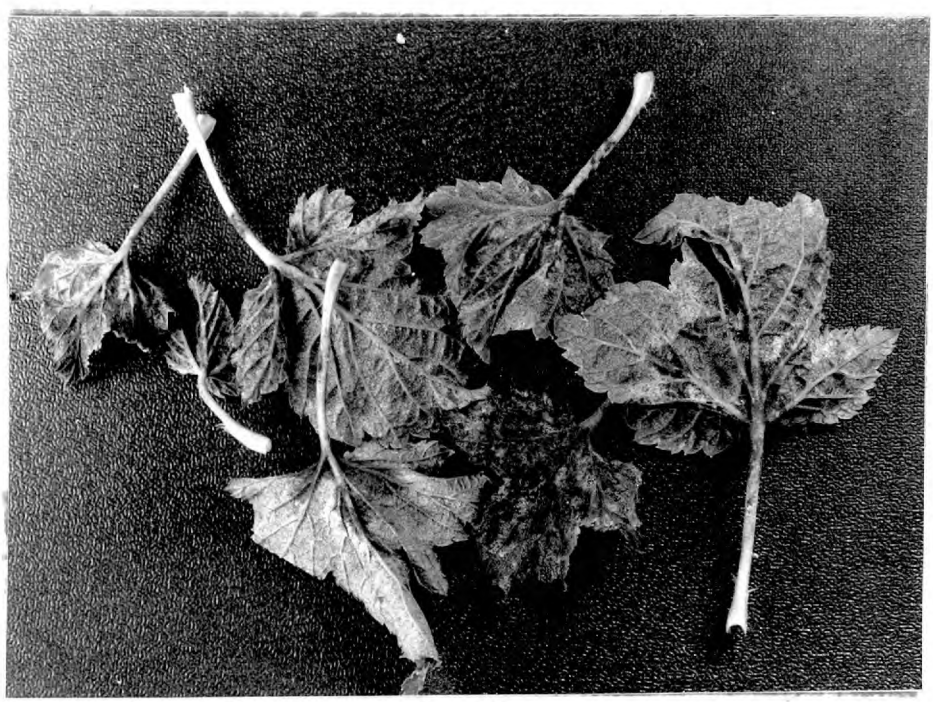
It was not possible to collect sufficient primary mycelium from black currant leaves bearing S. mors-uvae as the amount was small even on heavily infected leaves. Additionally, attempts to remove this sparse mycelium resulted in the removal of large amounts of leaf material, especially scent glands which are extremely numerous on the abaxial surface. However, when the level of S. mors-uvae in a plantation was high the adaxial surface of some leaves became infected with a heavy inoculum as they unfurled . After 2-3 weeks from infection, sporulation ceased and secondary thickening of the mycelium took place. Within this, large numbers of cleistocarps were formed and provided a sample of mycelium that was light brown and in which pigmentation was beginning. These samples were freed of conidia by prolonged washing of the leaf surface and the mycelium was removed with a minimum amount of leaf tissue. After washing the leaves the mycelium was removed using mounted needles and stored at -18°. Fig 28a shows leaves bearing the immature mycelium.

Sub-samples of c. 0.2 g (fresh weight) were thawed at room temperature, washed in distilled water and centrifuged five times. Both the mycelium

Fig. 28. Black currant leaves bearing immature and mature cleistocarps and secondary mycelium of *S. mors-uvae*.

(a) Immature (x 2)

(b) Mature (x1)



and cleistocarps were extremely resistant to usual breakage procedures and treatments in a Sorvall Omnimix at 16000 r.p.m. and in a micro-attachment at 50000 r.p.m. failed to rupture the mycelium or cleistocarps. Similarly, treatment in an M.S.E. 100 watt ultrasonic disintegrator (U.S.D.) had little effect on the mycelium. Satisfactory breakage of the cell walls was achieved, however, using Ballotini No. 13 glassbeads (80-120 μ diameter) in the U.S.D. working at a maximum speed of 4-5 microns on the amplitude meter.

The washed fungal mycelium was placed in the glass vessel with a minimum quantity of distilled water and beads added to form a thick slurry with a thin waterfilm on the surface (Bartnicki-Garcia and Nickerson, 1962). Direct treatment of the sample in the U.S.D. resulted in partial breakage of mycelium with the cleistocarps completely homogenised. This differential breakage was avoided by initially grinding the sample in a pestle and mortar with a small amount of iced distilled water. This shredded the mycelium to lengths of c. 0.5 mm and gave more uniform breakage in the U.S.D. Also the preliminary treatment released leaf debris, pollen and spores, especially of Cladosporium and Alternaria, from within the mycelium into the supernatant liquid. After grinding the samples were centrifuged and washed with cold distilled water (five times) and transferred to the U.S.D. where one treatment of c. 20 min was sufficient to reduce the mycelium to lengths of 0.01 mm and to fragment the cleistocarp walls similarly. During the grinding the vessel was enclosed in an iced water-bath to reduce the temperature. The disintegration of the cells was examined by taking samples, mounting in Lugol's Iodine solution and observing breakage microscopically.

The cytoplasm of either the mycelium or the thin walled cells beneath the cleistocarp wall stained bright yellow. The treatment was continued until this cytoplasm was removed.

The glass beads were allowed to settle and the supernatant containing the cell walls was collected. This was repeated about fifteen times until a microscopic examination showed that the sample was free of whole beads. The remaining small broken pieces of beads represented only a small fraction of the final preparation and as it was impossible to remove them without loss of cell-wall material their weight was estimated by digesting a small sample with concentrated H_2SO_4 and 30% H_2O_2 (9:1, v/v) at 100° (Bartnicki-Garcia and Nickerson, 1962). The final preparation was lyophilised and stored over P_2O_5 until required.

From the original collection of 1000 leaves bearing immature mycelium 100 mg of the final cell-wall material was prepared.

B. Secondary mycelium and mature cleistocarps.

Leaves were collected from the plantation when they bore a pigmented mycelium and fully differentiated cleistocarps. The mycelium was contaminated much more than in the immature sample and so the leaves were washed in a continuous stream of water until observations under a binocular microscope (x60) showed that foreign debris had been removed. The mycelium was then removed from the leaves and ground in iced water in a pestle and mortar. In this sample, in contrast to the immature cell-walls, the cleistocarps were more refractory than the secondary mycelium and were only fragmented by prolonged gentle grinding (c.60-90 min) in a pestle and mortar. Preliminary experiments showed that without this

initial treatment ultra sonic disintegration produced a sample with satisfactory breakage of cleistocarp walls but with the mycelium homogenised to a structureless matrix. Grinding was followed by two 20 min treatments in the U.S.D. which reduced the cell walls to lengths of 0.01 mm.

It was apparent from observation on the final washed cell-wall pellet that not only were cell walls of contaminant fungi present but a proportion of the walls was unpigmented and only partially thickened. As the transition of the mycelium from a primary to a secondary state is a comparatively slow process and one that does not take place uniformly throughout the whole mycelium it was to be expected that the process would, for parts of the mildew colony be incomplete at the time of leaf senescence. In this respect, although leaves were always chosen bearing a dark brown mycelium with dark brown/black cleistocarp (Fig. 28B), it is probable that no sample of mycelium contained only cells with thickened or pigmented walls. In addition the mycelium nearest the leaf surface remained unthickened on all leaves.

2. Major components of cell walls.

A. Monosaccharides.

Direct measurement of carbohydrates by placing cell walls in Anthrone reagent revealed that 25.8% of the mature cell walls (secondary) and 34.6% of the immature cell walls (primary) was hexosan. A small black residue formed during analysis of secondary walls was removed by centrifuging before readings were taken.

Preliminary experiments hydrolysing the samples in a sealed ampoule without nitrogen with 1 N H_2SO_4 yielded only 5-6% Anthrone positive substances but after treating the cell walls under oxygen free nitrogen breakdown of carbohydrate was reduced and values of 24.1% and 26.7% were obtained for secondary and primary walls respectively. These values were based on acid hydrolysates including the residual material. The quantities of carbohydrate material in the neutralised and deionised hydrolysates are given in Table 19.

Three points are of interest from these results:

i. There was incomplete release of secondary wall carbohydrate from a 12 h compared to a 24 h hydrolysis, i.e. 24.1% before and 22.7% after neutralisation and deionisation.

ii. There was complete release of wall carbohydrates from the primary wall samples and considerable breakdown was indicated by the difference between the direct Anthrone result (34.6%) and that after hydrolysis (26.7%); a difference of 7.9%. For the secondary walls this difference was only 3.1%.

iii. The susceptibility of the carbohydrate in the walls to acid

hydrolysis was indicated by the 24/24 h treatment which resulted in a total recovery of only 16.1% from the secondary cell walls.

Table 19. Carbohydrate components in cell walls of S. mors-uvae.

Component	% 2° walls*	% 2° walls+	% 1° walls+
Anthrone before neutralisation	16.1	24.1	26.7
Anthrone after neutralisation	16.1	22.7	26.7
Reducing sugars	15.4	21.2	25.8
Glucose	13.6	18.5	22.4

* 24 h 72% H_2SO_4, 24 h 1 N H_2SO_4

\+ 12 h 72% H_2SO_4, 12 h 1 N H_2SO_4

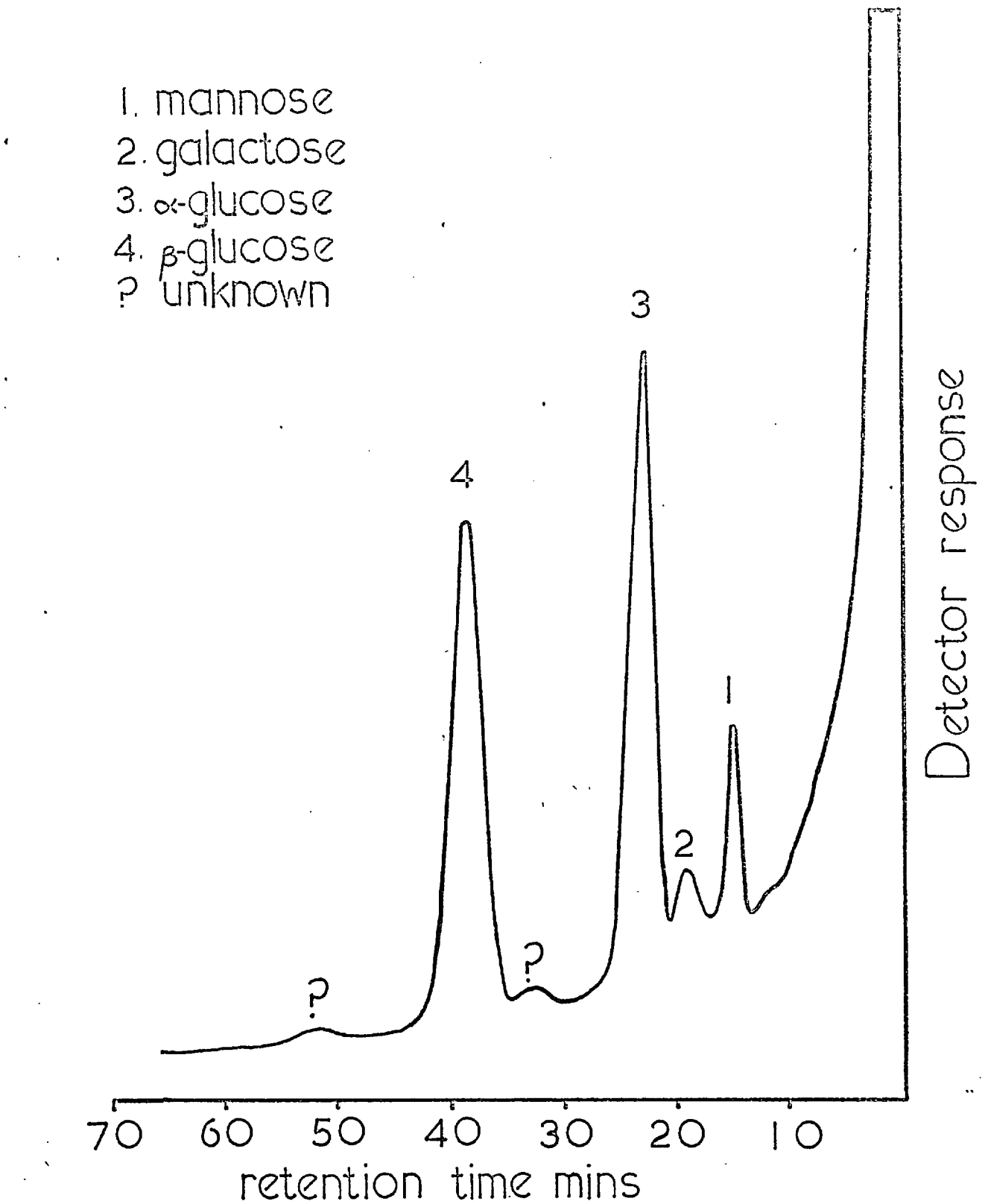
2° secondary walls

1° primary walls.

Gas-liquid chromatography (GLC) of neutralised and deionised extracts of both secondary and primary cell walls revealed that glucose, galactose and mannose were present in the 1 N H_2SO_4 hydrolysates. Fig. 29 was typical of the results obtained; that for the primary cell walls differing only in the relative amounts of the three sugars present. Digests of the samples were also made with 6 N HCl as it has been reported that some monosaccharides are completely destroyed by H_2SO_4 (Hamilton and Knight, 1962). The yield of carbohydrate was low, only 6% of the wall weight, and no other monosaccharides were revealed by GLC.

Table 20 shows that there was fairly close agreement between the total amounts of the three monosaccharides determined by GLC and the

Fig.29 Examination by GLC of sugars in secondary cell walls



values for reducing sugars given in Table 19 especially for the primary cell wall sample. The GLC results for the secondary walls were slightly higher.

Table 20. Estimation by gas-liquid chromatography of monosaccharides in cell walls: H_2SO_4 hydrolysates.

Component	% 2° walls*	% 2° walls+	% 1° walls+
Glucose	14.0	20.3	23.0
Galactose	1.1	1.2	1.0
Mannose	2.2	2.4	2.6
Totals	17.3	23.9	26.6

* 24 h 72% H_2SO_4, 24 h 1 N H_2SO_4

\+ 12 h 72% H_2SO_4, 12 h 1 N H_2SO_4

2° secondary walls

1° primary walls.

The quantities of mannose and galactose revealed by GLC were similar for 24/24 h and 12/12 h hydrolysates and may indicate that these monosaccharides are relatively resistant to acid hydrolysis compared to glucose. Thus, acid hydrolysis probably gave low values for total carbohydrate compared to the direct Anthrone because of destruction of the glucan fraction of the cell wall whilst leaving the mannan and galactan components in the form of their monomers without significant breakdown. On this basis, the total glucan content of the secondary walls was estimated to be 22.2% and that of the primary wall 31.0%, by substracting the mannose and galactose content from the direct Anthrone values

(i.e. 25.8-1.2-2.4 and 34.6-1.0-2.6).

As there was incomplete agreement between the Anthrone and reducing sugar values of Table 19 it is probable that the figures for glucan given above are in excess by c. 1% of the true amounts. GLC examination of hydrolysates revealed two unidentified peaks with retention times of 1.42 and 2.29 (relative to ∝-glucose); these could be non-reducing sugar components of the cell wall but as they were present in small amounts they were not identified.

B. Hexosamine.

Total hexosamine released from 6 N HCl hydrolysis of the secondary and primary cell walls was 10.2% and 12.7% respectively. The hexosamine was identified as glucosamine by GLC.

The hexosamine components were further investigated for secondary cell-wall samples by the method of Blumenthal and Roseman (1957) (Procedure I. Table 21). Extraction in 10% NaOH was carried out at 100° for 30 min and in 2% HCl at room temperature for 1 h. The final pellet of wall material was hydrolysed with 6 N HCl under nitrogen.

Samples were also treated by a slight modification of the methods of Barthnicki-Garcia and Reyes (1964). Two treatments were carried out: 1) extraction with cold 1 N KOH, hot 1 N KOH and hot 1 N HCl, 2) cold 1 N HCl, hot 1 N HCl and hot 1 N KOH (procedure II and III. Table 22). Both hot acid and alkali treatments consisted of boiling at 100° in 3,3 and 2 ml of each solvent for 30 min. The cold extractions were at room temperature .

Table 21. Hexosamine components of cell walls: Procedure I.

Treatment	% glucosamine
10% NaOH (hot)	2.48
2% HCl (cold)	0
10% NaOH (hot)	1.17
Residue	6.67
Total	10.32

Table 22. Hexosamine components of cell walls: Procedures II and III.

II

Treatment	% glucosamine
1 N HCl (cold)	0
1 N HCl (hot)	4.58
1 N KOH (hot)	0.83
Residue	5.49
Total	10.90

III

Treatment	% glucosamine
1 N KOH (cold)	0.70
1 N KOH (hot)	2.73
1 N HCl (hot)	2.62
Residue	4.52
Total	10.57

The residues in procedures II and III were assumed to be n-acetyl glucosamine (chitin) which has the property of being insoluble in both dilute alkali and acid, whereas the alkali-insoluble, acid-soluble fraction of procedures II and III was likely to be chitosan, the deacetylated product of chitin. Thus, the higher residue of procedure I not only contained chitin but also chitosan.

However, during these extractions considerable quantities of the pigment was dissolved by KOH and a lesser amount by the acid treatment. This pigment was later characterised as melanin (p.135). Complexes between melanin and glucosamine giving them the property of being soluble in both acid and alkali have been found in other fungal walls (Bartnicki-Garcia and Reyes, 1964; Broomfield and Alexander, 1967). It was, therefore likely that a portion of the glucosamine in the 1 N HCl extract was bound to melanin. A 4 ml sample of the acid extract was neutralised with KOH to precipitate the pigment which was washed twice with water and hydrolysed with 6 N HCl for 8 h. It was found that c. 1-2% of the glucosamine of the cell walls was attached to melanin.

The amount of deacetylated glucosamine could not be estimated directly as acid extracts contained not only the glucosamine associated with melanin but also that fraction which was soluble in alkali as well as in acid. A determination was made on a larger cell wall sample of the glucosamine attached to melanin (p.136) and subtracting this from the alkali soluble fraction of procedure III gave an estimated 1.69% for glucosamine soluble in alkali alone. Subtracting this value from the acid and alkali soluble fraction of procedure II gave an approximation of the deacetylated

glucosamine present, c. 4%. Table 23 summarises the hexosamine components of the cell wall.

Table 23. Hexosamine components of secondary walls, i.

Component	% cell walls
alkali soluble glucosamine	1.69
deacteylated glucosamine	3.72
n-acetyl glucosamine	5.49
Total	10.81

During further experiments on the analysis of the hexosamine components, it was found that the acid treatments did not only remove deacetylated glucosamine from the cell walls. Separate assays made on acid hydrolysates after removal of the HCl showed that a significant part of the glucosamine was acetylated. None was found, however after assaying the alkali extracts. It remains for further experiments to show whether the acetylated hexosamine was bound to the melanin or was brought into solution directly by the acid treatment. Table 24 gives the recalculated components of the secondary cell walls.

It also remains to be determined if a deacetylated glucosamine component exists in the cell walls other than in a complex with melanin. The quantity calculated in Table 24 was very small and it is conceivable that it is only an analytical error because it was impossible to determine deacetylated glucosamine directly.

Table 24. Hexosamine components of cell walls, ii.

Component	% cell walls
alkali soluble glucosamine	1.69
deacetylated glucosamine	0.37
glucosamine with melanin	1.74
n-acetyl glucosamine	7.09
Total	10.89

C. Analysis of pigment in the secondary mycelium and cleistocarps.

The dark brown pigment of the secondary cell walls was estimated, by the methods of Nicolaus et al,(1964), to constitute 18.9% of the wall weight. The final product was characterised as melanin by the criteria used by Lingappa et al. (1963) for a pigment extracted from Aureobasidium pullulans. These were:

i. Insolubility in alcohol, ether, chloroform and acetone.

ii. Solubility in 0.5 N NaOH and cold 1 N Na_2CO_3.

iii. Formation of a heavy brown precipitate with a small amount of Fe_2CO_3; the precipitate disappearing upon addition of more of the salt.

iv. Bleaching by H_2O_2 and 2% $KMnO_4$.

v. The absorbance curve between 400-600 mμ.

A straight line relationship was obtained by plotting the logarithm of absorbancy against wavelength. Melanin (1 mg) was dissolved in either 10 ml 0.5 N NaOH or 1 N KOH and the absorbance determined. The line obtained had a slope of -0.0029.

During the extraction procedure a portion of the pigment was found to be soluble in acid and a separate determination was made to estimate this and to define its chemical composition.

A sample of secondary mycelium and cleistocarps was ground for three, 20 min periods in the USD until the cell walls of both cleistocarps and mycelium were completely homogenised. After removal of glass beads the sample was washed and lyophilised and a 100 mg sub-sample hydrolysed with 1 N KOH and 1 N HCl at 100° for 10 min periods. Six treatments of alkali and two treatments of acid completely removed the pigment and left the fungal material a dark grey. The acid and alkali extracts were combined and centrifuged to remove residual wall material. The melanin complex was precipitated by bringing the hydrolysate to pH 6.8 and washed twice with distilled water. This procedure was repeated three times and the precipitates dried overnight at 70° and weighed. The melanin complex accounted for 26.2% of the secondary cell walls.

A portion of the melanin complex was hydrolysed with 6 N HCl for 8 h at 105° in a sealed ampoule under nitrogen and analysis revealed that 5.96% was glucosamine and 12.67% protein. Thus, the glucosamine and protein of the complex in terms of cell wall weight was estimated to be 1.74% and 3.8% respectively. As the weight of the melanin complex hydrolysed was small (15.62 mg) the melanin content of the cell walls was estimated by subtracting the values obtained for glucosamine and protein from the weight of the original melanin complex, this gave a value of 20.6% of the cell wall weight and was similar to the 18.9% obtained (p.135) by the method of Nicolaus et al. (1964) The difference

reflected, perhaps, the maturity difference of the fungal cell walls of the two separate samples from different collection of leaves.

3. Isolation of micro-organisms with lytic activity.

The difficulties involved in collecting large amounts of mycelium and cleistocarps from leaves precluded the use of standard techniques of isolating organisms capable of lysing S. mors-uvae mycelium (cf. Mueller and Durrell, 1957; Salton, 1955). Similarly, the enrichment techniques of incubating leaf or soil washings with mycelium of S. mors-uvae in liquid media was possible only for the mature secondary mycelium and cleistocarps where larger quantities could be collected from black currant leaves.

A. Isolation of organisms capable of lysing mature mycelium.

Three methods were employed:

i. Enrichment methods using soil and mature mycelium.

ii. Inoculating plates of cell-wall agar with leaf washings.

iii. Inoculating plates as in ii. with pure cultures of bacteria and actinomycetes.

Results.

i. Mature mycelium taken from leaves was washed several times and ground in a pestel and mortar to disentangle and break the mycelium into short lengths. All cleistocarps walls were fragmented, and after washing to remove cytoplasm and asci, the mycelium was added to the basal inorganic media of Potgieter and Alexander (1966) at a concentration of 0.15% (w/v). To a duplicate 100 ml of the medium 0.001% yeast extract was added. The flasks containing the media were autoclaved at 110° for 20 min and

after cooling 1 g of soil was added to each flask. The flasks were then incubated at 29° on a rotary shaker at 125 cycles/min for 2 weeks. Agar plates were prepared containing 0.15% (w/v) of purified cell walls (appendix p.246), and the artificial compounds nystatin and actidione (50 ug/ml). Plates were inoculated with 0.2 ml of a suitably diluted sample of the soil enrichment medium or with a loopful of undiluted culture filtrate streaked over the agar surface. The plates were incubated at 25° and observed for 3 weeks.

None of the bacteria or actinomycetes produced zones of clearing on the cell wall agar. The results of the number of organisms isolated is given in Table 25 .

Table 25. Colonies developing on cell wall agar (enrichment media diluted 10^{-6}).

	Replicates	Bacteria	Actinomycetes	Total/plate
With yeast extract	1	328	0	328
	2	330	1	331
	3	360	3	363
	4	434	3	437
	Total	1452	7	1459
Without yeast extract	1	270	4	274
	2	230	5	235
	3	237	3	240
	4	226	5	231
	Total	963	17	980

More bacteria were isolated when yeast extract was present in the soil media and there were fewer actinomycetes than when yeast was absent; without yeast extract more than twice the number of actinomycetes developed on the plates.

The result of plating the supernatants on to chitin agar is given in Table 26.

Table 26. Clearing of chitin by soil micro-organisms (enrichment media diluted 10^{-6}).

	Replicates	Bacteria	Actinomycetes	Total bacteria per plate
With yeast extract	1	10	2	246
	2	7	1	259
	3	7	2	188
	4	9	0	240
	Totals	33	5	933
Without yeast extract	1	13	4	165
	2	17	4	189
	3	15	2	172
	4	13	2	194
	Totals	58	12	720

Similarly more organisms were isolated from enrichment cultures supplied with yeast extract but an average of 70 organisms per plate were isolated that cleared chitin agar from flasks where yeast extract was absent compared to 38 with yeast present.

Actinomycetes grew well and sporulated freely on the cell-wall media but no clearing resulted. This experiment was repeated with similar results and no organisms were isolated that produced zones of clearing on the cell-wall agar.

ii. Ten leaves containing secondary mycelium were taken from a collection which had been placed on the soil for 3 months. Discs 1 cm^2 were cut from the leaves and placed in 100 ml of 0.9% saline containing 0.001% Tween 80 and shaken for 15 min in a Griffin flask shaker. Aliquots (0.2 ml) of the diluted washings were plated onto the cell-wall agar.

No organisms produced clearing but actinomycetes were the dominant organisms isolated suppressing bacterial growth at lower dilutions.

iii. Fourteen isolates of bacteria and eleven actinomycetes previously isolated from leafwashings (p. 77) were inoculated onto plates of cell-wall agar without antibiotics. All were able to use chitin as a sole carbon source and several produced glucanases (see Table 27) . The growth of the actinomycetes was good with extensive sporulation, especially of the streptomycetes but, as with the bacteria tested there was no clearing of the cell-wall agar.

B. Isolation of organisms lysing immature mycelium.

The bacteria and actinomycetes that were isolated from leaf washings (p. 77) were streaked onto plates of 0.15% immature cell wall agar. Plates were incubated at 25° and observed over 21 days.

Ten bacterial and ten actinomycete isolates cleared the agar but none completely lysed the fungal cell walls. The degree of clearing, based on the extent of the clear zone and the quality of clearing within this zone, is indicated in Table 27. Isolates differed in these two

aspects of clearing. Thus isolate S_1 produced an almost completely clear zone of 12 mm diameter in contrast to S_2 where the diameter of the clear zone was greater but the cell walls were only slightly reduced in size.

Table 27. Clearing by micro-organisms on cell-wall and chitin agar.

Bacteria	Isolate no.	Diam. zone of clearing mm.	Degree of clearing *	No. days to initial clearing	Clearing on chitin agar	Glucose from laminarin
	1	1.0	+	3	(++)	-
	2	4.0	++	3	(+)	+
	3	<1.0	+	17	(++)	-
	4	2.0	++	3	(++)	+
	5	<1.0	+	17	(++)	-
	6	17.0	+++	3	(+++)	-
	7	4.0	+	6	(++)	-
	8	90.0	+++	2	(++++)	-
	9	90.0	+++	2	(++++)	-
	10	90.0	+++	2	(++++)	-
	11	0	-	-	(++)	-
	12	0	-	-	(++)	-
	13	0	-	-	(+++)	N.T.
	14	0	-	-	(+++)	N.T.

Actinomycetes Isolate no.	Diam. zone of clearing mm.	Degree of clearing*	No. days to initial clearing	Clearing on chitin agar	Glucose from laminarin
S_1	12.0	++++	3	(++)	+
S_2	34.0	++	3	(+++)	-
M_1	14.0	++++	6	(++)	+
M_2	18.0	+++	6	(++)	N.T.
S_3	18.0	+++	3	(+++)	-
S_4	18.0	++	6	(++)	N.T.
S_5	20.0	++	6	(+)	N.T.
S_6	12.0	+	6	(+++)	N.T.
S_7	26.0	+++	3	(+++)	-
S_8	12.0	+	6	(++)	N.T.
M_3	0	-	-	(++)	N.T.

* Degree of clearing: ++++ good; little cell wall remaining

+++ moderate

++ moderate-poor

\+ poor

\+ Clearing on chitin: (++++) entire plate

(+++) several mm

(++) 1-2 mm

(+) restricted to area of colony.

N.T. Not tested.

With the bacterial isolates there was a stronger relationship between area cleared and degree of clearing. The ability of isolates to clear a 0.2% chitin agar over 21 days was also investigated and these data are also given in Table 27. With bacterial isolates the degree of clearing of the two media was similar, but with four isolates (nos. 11-14) there was the suggestion that chitinase was not the only enzyme responsible for the clearing of the cell-wall agar. This was supported by similar data for the actinomycetes. For example, isolate S_1 with limited chitinase activity produced a zone of clearing on cell-wall agar that was superior to isolates with greater chitinase activity. As this isolate and the monomicrospora sp. (M_1) both hydrolysed laminarin (a β-(1-3)glucan) it was likely that the superior degree of clearing resulted from the activity of enzymes on both the chitin and glucan components of the cell walls.

4. Characterisation of lytic enzymes and their activity on cell-wall material.

A. Preliminary experiments with a commercial chitinase preparation.

Chemical analyses indicated that both glucan and hexosamine were major components of cell walls. But whereas clearing resulted when actinomycetes and bacteria producing chitinase (and glucanases) were grown on the primary cell walls no clearing occurred on the secondary mycelium containing melanin. The resistance of the mature cell walls to enzyme breakdown was investigated in three experiments using a commercial preparation of chitinase on whole mycelium and cell walls.

Methods.

i. The enzyme preparation was used at a concentration of 1 mg/ml (equivalent to the release of 15 µg of n-acetyl glucosamine per hour at 37° from purified chitin) in a 5 ml reaction mixture containing 1 mg/ml cell walls (material and methods p. 119). Production of n-acetyl glucosamine was followed over a 4 h period.

ii. The enzyme preparation was used as in (i) on whole, primary and secondary mycelium and cleistocarps (instead of cell walls). The mycelium was removed from the two collections of leaves, washed and lyophilised as previously described (p.122). Release of n-acetyl glucosamine followed over a 12 h period.

iii. Cell walls were incubated in a 3 ml reaction mixture containing 1 ml cell walls (5 mg/ml); 1 ml phosphate-acetate buffer and 1 ml chitinase (5 mg/ml) at 37° for 48 h. At these concentrations the commercial

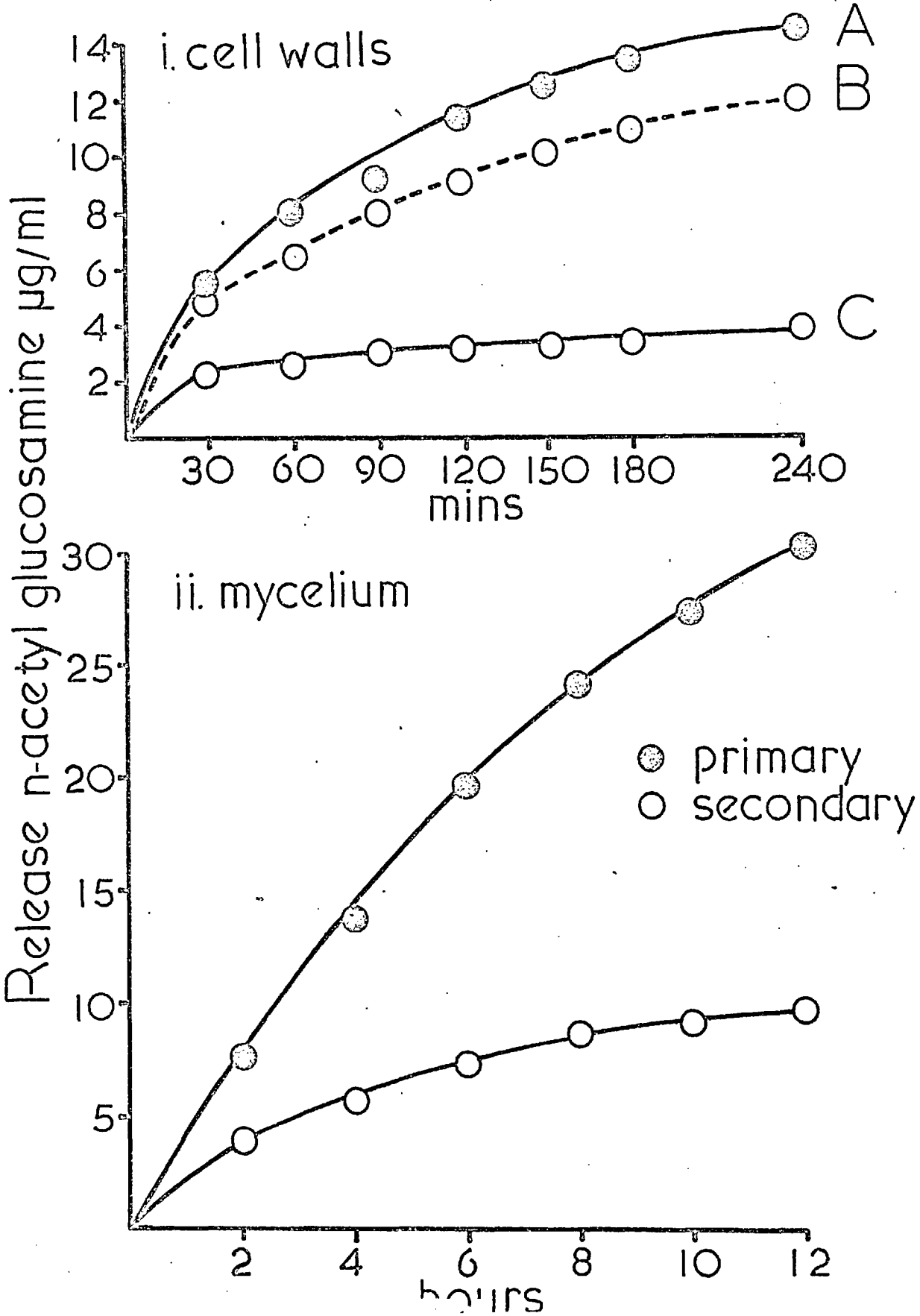
preparation released 50 µg of n-acetyl glucosamine and 42.5 µg of glucose per hour at 37° and pH 5.5 from purified chitin and laminarin respectively. After 24 h the reaction mixture was centrifuged and n-acetyl glucosamine and glucose assessed on the supernatants. The cell walls were washed three times with distilled water and reincubated for a further 24 h. with the enzyme solution and buffer. Toluene was added to prevent microbial contamination.

Results.

i. The release of n-acetyl glucosamine from the cell-walls is given in Fig. 30 i A & B. There was a considerable difference in the hydrolysis of the two samples. More than three times the quantity of n-acetyl glucosamine was released from primary compared to the secondary cell walls (72.5 µg and 20.6 µg per 5 ml reaction mixture respectively). Whereas n-acetyl glucosamine was released from the mature cell walls only during the initial phase of incubation hydrolysis of primary cell walls continued progressively over the 4 h period. N-acetyl glucosamine released represented 1.45% and 0.41% of the primary and secondary wall weight.

Chemical analysis of the cell walls had shown that the percentage of hexosamine was similar in both wall samples (p.131). But because of the difficulty in obtaining large quantities of the primary mycelium no estimation was possible of the chitin in the cell wall. Therefore, the greater release of n-acetyl glucosamine from the primary sample, in the experiment above, could have been caused by a higher percentage initially present compared to that contained in secondary walls. To determine if this was so, use was made of the fact that when samples of the secondary

Fig.30. Hydrolysis of cell walls by chitinase



cell walls were ground in the USD for long periods the supernatant contained a black suspension which satisfied the tests for melanin proposed by Lingappa et al. (1963). Therefore, a sub-sample of 50 mg of secondary cell walls from that used in the enzyme incubation experiment above was ground for three 20 min periods until the mycelium and most of the cleistocarp walls were completely homogenised. This sub-sample was washed, lyophilised and subsequently treated with the commercial enzyme preparation as described above.

The release of n-acetyl glucosamine from this sample (Fig. 30 i. c) over the 4 h period showed it to be only slightly less than that from the primary cell walls and indicated that enzyme hydrolysis of the secondary walls was not restricted by the concentration of substrate present but more probably by its availability to enzyme action.

ii. The action of the enzyme preparation onwhole mycelium and cleistocarps is given in Fig. 30 ii. The amounts of n-acetyl glucosamine released from the primary and secondary mycelium after 12 h incubation were 3.0% and 0.95% of the wall weight respectively. N-acetyl glucosamine released during the first 4 h of incubation was similar to that released when cell walls were used. This confirmed that the grinding methods used in cell-wall preparations had not altered the susceptibility of the walls to enzyme lysis and that the resistance to chitinase of the mature cell walls (experiment i) was also characteristic of intact mycelium and whole cleistocarps.

iii. The results of the 24 h and 48 h incubation are given in Table 28, and show that twice the amount of n-acetyl glucosamine and about three times the quantity of glucose was released from primary cell walls compared

to the secondary sample. In both incubations slightly more glucose was released in the second incubation period than during the initial 24 h in contrast to n-acetyl glucosamine release which was much lower during the second 24 h period.

Table 28. Release of n-acetyl glucosamine and glucose during a 48 h incubation.

Time (h)	Sample	µg/ml n-acetyl glucosamine released	µg/ml glucose released
24	1°	57.5	36.5
	2°	25.0	13.0
48	1°	12.5	45.0
	2°	10.0	17.5

1° primary 2° secondary

Possibly chitin was initially shielding the glucan of the cell wall and thus protecting it from the β-(1-3) glucanase. Once a significant proportion of the chitin was hydrolysed (eg. after 24 h incubation) glucan was exposed to enzyme action and so more glucose was released during the second incubation period. The figures in Table 28 represent a release in 48 h of n-acetyl glucosamine and glucose equivalent to 2.10% and 1.83% in terms of secondary wall weight. In contrast 4.20% and 4.89% of the chitin and glucan of primary cell walls was hydrolysed in 48 h.

In the absence of a β-(1-4) and β(1-6) glucanase in the commercial enzyme preparation the experiment indicated that at least a part of the glucan of cell walls had β-(1-3) linkages.

B. Effects of chitinase and glucanase on cell walls.

i. Production of a β-(1-3) glucanase from Micromonospora, M1.

The Micromonospora sp. produced the highest degree of clearing on the unpigmented cell-wall agar of S. mors-uvae and released glucose when grown in a liquid medium containing 0.05% laminarin. This isolate was examined as a possible source of β-(1-3) glucanase. It was grown on the media of Reece and Mandels (1959) and Skujins et al (1965) and glucanase activity assessed at 24 h intervals after inoculation. On both media growth was slow but maximum release of glucose occurred after 6 days with complete disappearance of glucose after 10 days. However, no enzyme was found when 1 ml aliquots of culture filtrates were incubated in a 5 ml reaction mixture with insoluble laminarin (5 µg/ml) nor was there a release of the enzyme into the culture liquid during a period of 10 days after glucose was depleted in the medium. This suggested that β-(1-3) glucanase was not repressed by glucose to a basal level as found in Pyrenochaeta terrestris (Horton and Keen, 1966) where cellulose synthesis was affected by the concentration of glucose in the media. Nor could glucose be implicated in the release of the enzymes into the culture medium as indicated in other works. For example Bemiller, Tegtmeier and Pappelis (1969) found that a cell bound cellulose of Diplodia zeae was detected only after cellulose and glucose disappeared and they suggested that glucose regulated the release of superficial cellulolytic enzymes into the culture medium rather than their synthesis. Further, they suggested that the release was a function of age or condition

of the culture and occurred after the growth period. Several other workers have reported liberation of cellulose into the culture medium after the depletion of substrate and following the growth period (eg. Moreau and Trique, 1966; Deshpande and Deshpande, 1966). With isolate M_1, however both laminarin and glucose disappeared from the culture medium but no enzyme was detected. This suggested that the glucanase was present but bound firmly to the surface of the mycelium.

This possibility was tested by incubating washed mycelium of M_1 (from 60 ml of a 6 day culture) in a mixture of 1 ml water, 1 ml citrate-phosphate buffer (pH 5) and 1 ml laminarin (5 mg/ml). Glucose was released (Table 29) yet tests carried out on the supernatant of the reaction mixture showed no enzyme was present.

Table 29. Release of glucose from laminarin incubation with M_1.

Incubation (h)	µg/ml glucose
1	35.5
3	86.0
6	158.0

To determine that the glucose was being produced by the action of extracellular enzymes bound to the actinomycete mycelium 30 ml samples from a 5 day culture were incubated in a 3 ml reaction mixture as follows:

i. Washed mycelium + laminarin + buffer.

ii. Mycelium previously treated for 60 min at 37° in 1% merthiolate + laminarin + buffer.

iii. Mycelium treated with 1% merthiolate + water + buffer.

The results of a 6 h incubation are given in Table 30.

Table 30. Release of glucose by living and killed mycelium of M_1.

Time (h)	µg glucose released		
	i	ii	iii
½	12.0	18.6	2.5
1	25.0	31.5	3.0
3	56.0	66.0	4.0
6	100.0	115.0	4.5

The result of the experiment showed that 1% merthiolate killed the mycelium but had no effect on enzyme activity. Further, it showed that the glucose released came from the enzyme hydrolysis of laminarin and was not leached out of the actinomycete mycelium after it had been killed. The enzyme was not removed from the mycelium after washing with 0.1% NaCl at pH 7 or pH 8.5, or by 0.15 M acetate buffer (pH 5) or 0.15 M citrate-buffer (pH5). However, after a 250 ml sample from a 10 day culture was homogenised in the USD for 20 min periods glucanase activity was detected in the supernatants when incubated with laminarin in a 3 ml reaction mixture at 37° for 1 h (Table 31).

The effect of the cell-bound enzyme on the cell wall preparations was examined using 60 ml samples of M_1 from 6 day cultures. The mycelium was washed and placed in 1% merthiolate for 1 h at 37°. Incubation mixtures consisted of 1 ml cell walls (5mg/ml) 1 ml citrate-phosphate buffer (pH 5) and 1 ml M_1 mycelium. The results are given in Table 32.

Table 31. Release of β-(1-3) glucanase from M_1 after USD treatment.

No. of 20 min USD treatments	Activity of supernatant after treatment μg/ml glucose released
1	10.0
2	18.5
3	28.5
4	35.0

Table 32. Release of glucose from cell wall preparations by M_1.

Time (h)	μg/ml glucose released		
	1° walls	2° walls	Laminarin
1	6.0	6.0	48.5
3	12.0	6.0	112.0
6	19.0	6.0	196.0

Three times the quantity of glucose was released from the primary compared to the secondary cell walls after 6 h but because of the difficulty of an enzyme bound to the cell wall of M_1 coming into direct physical contact with the glucan molecules of the fungal wall preparations the amount released in the 6 h period was small compared to that from laminarin.

In view of the difficulty of obtaining an active preparation of a β-(1-3) glucanase from M_1 a known producer of the enzyme, Penicillium javanicum was grown in three 250 ml flasks containing 100 ml of the medium of Reece and Mandels (1959) with insoluble laminarin as sole

carbon source. Fig. 31 shows β-(1-3) glucanase activity of the culture filtrate over a 7 day period. Activity was expressed in terms of the release of glucose from an incubation mixture containing 0.65 ml culture filtrate, 0.15 ml citrate buffer (pH 5) and 0.25 ml laminarin (10 mg/ml).

The enzyme activity increased rapidly after the second day when glucose disappeared from the medium. The culture filtrates were collected after 4 days and the enzyme partially purified (p.119) and stored at -18°. The preparation was incubated with chitin and carboxymethyl cellulose and found to have no activity. However, when incubated with lutean (a β-(1-6) glucan) the enzyme was found to contain activity equivalent to the release of 80 µg/ml of glucose in a 5 ml reaction mixture with 1 ml lutean (5 mg/ml).

ii. Production of chitinase.

Chitinase was prepared from the bacterial isolate B_9 grown in 100 ml of the medium of Skujins et al. (1965) with 0.1% chitin as carbon source. No enzyme was found in the culture medium until 60 h after inoculation following the disappearance of n-acetyl glucosamine produced from the hydrolysis of chitin (Fig. 32). The enzyme was found to be relatively unstable in the culture medium and after 96 h little remained. The chitinase was partially purified using ammonium sulphate, dialysed and kept at -18° until required (p.119).

iii. Effect of chitinase and glucanase on cell walls.

Primary and secondary cell walls were incubated with laminarase and chitinase whose activities were equivalent to the release of 590 ug/ml

Fig.31. Production of β(1-3) glucanase by Penicillium javanicum

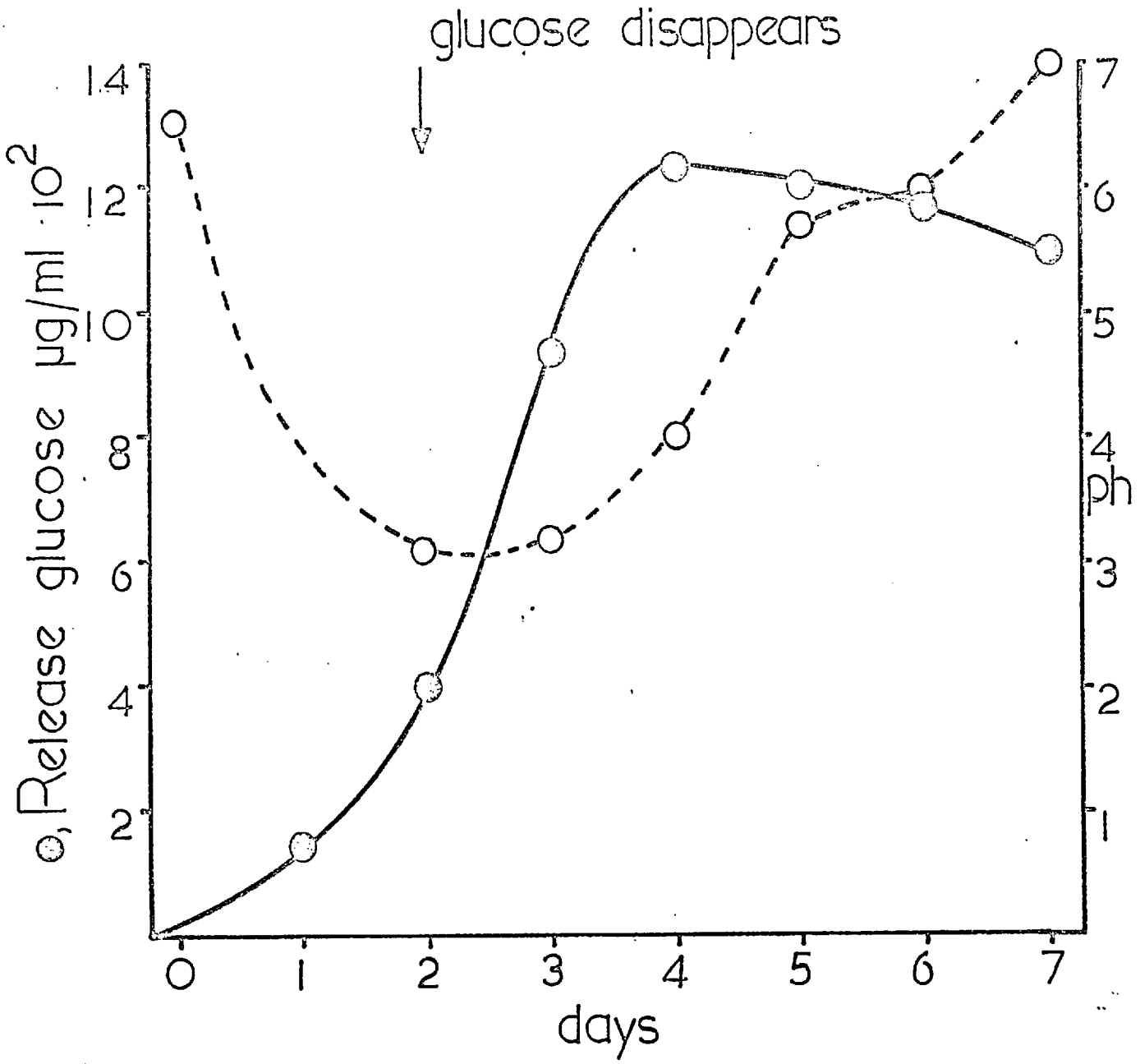


Fig.32. Production of chitinase by B_9

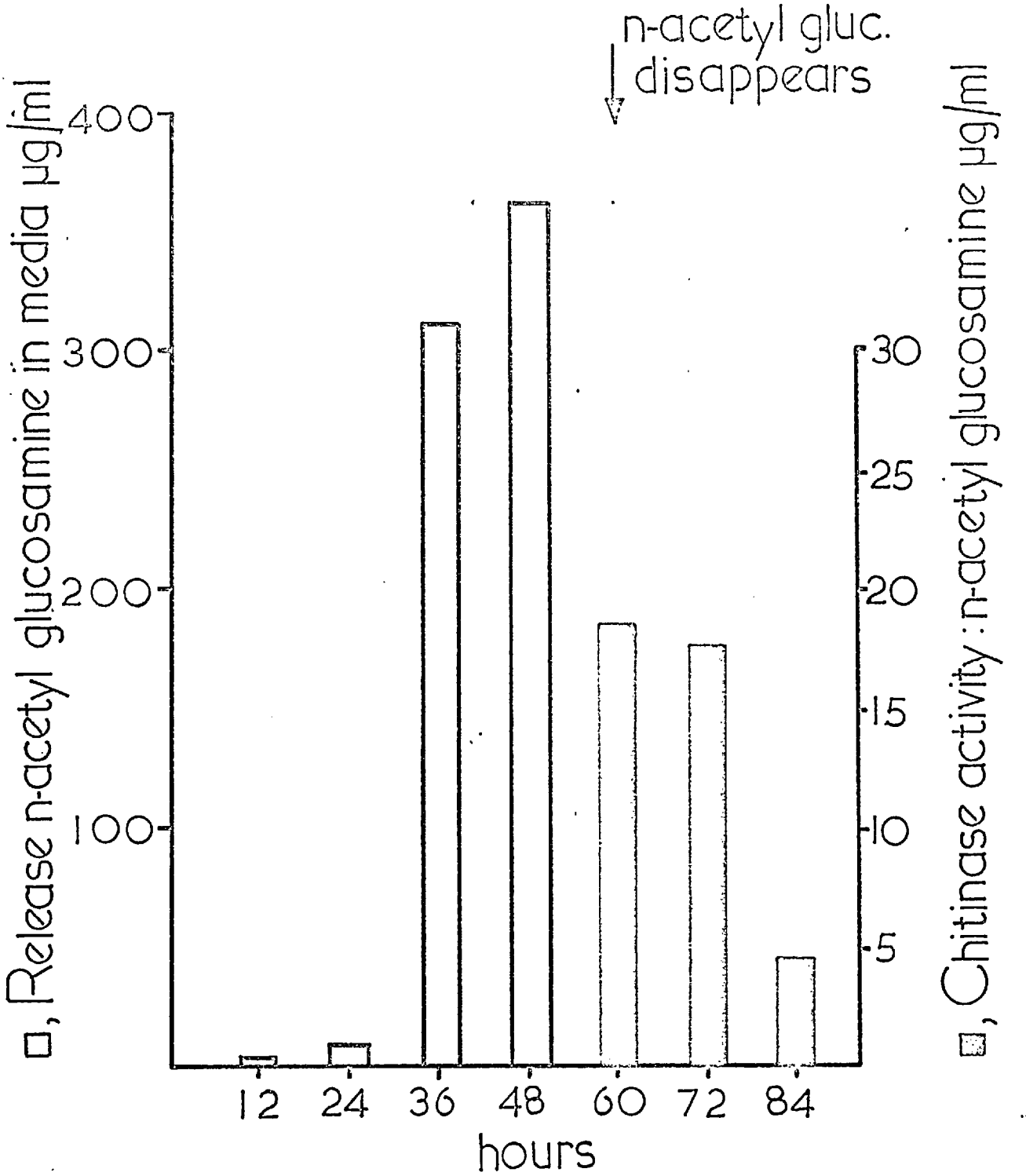
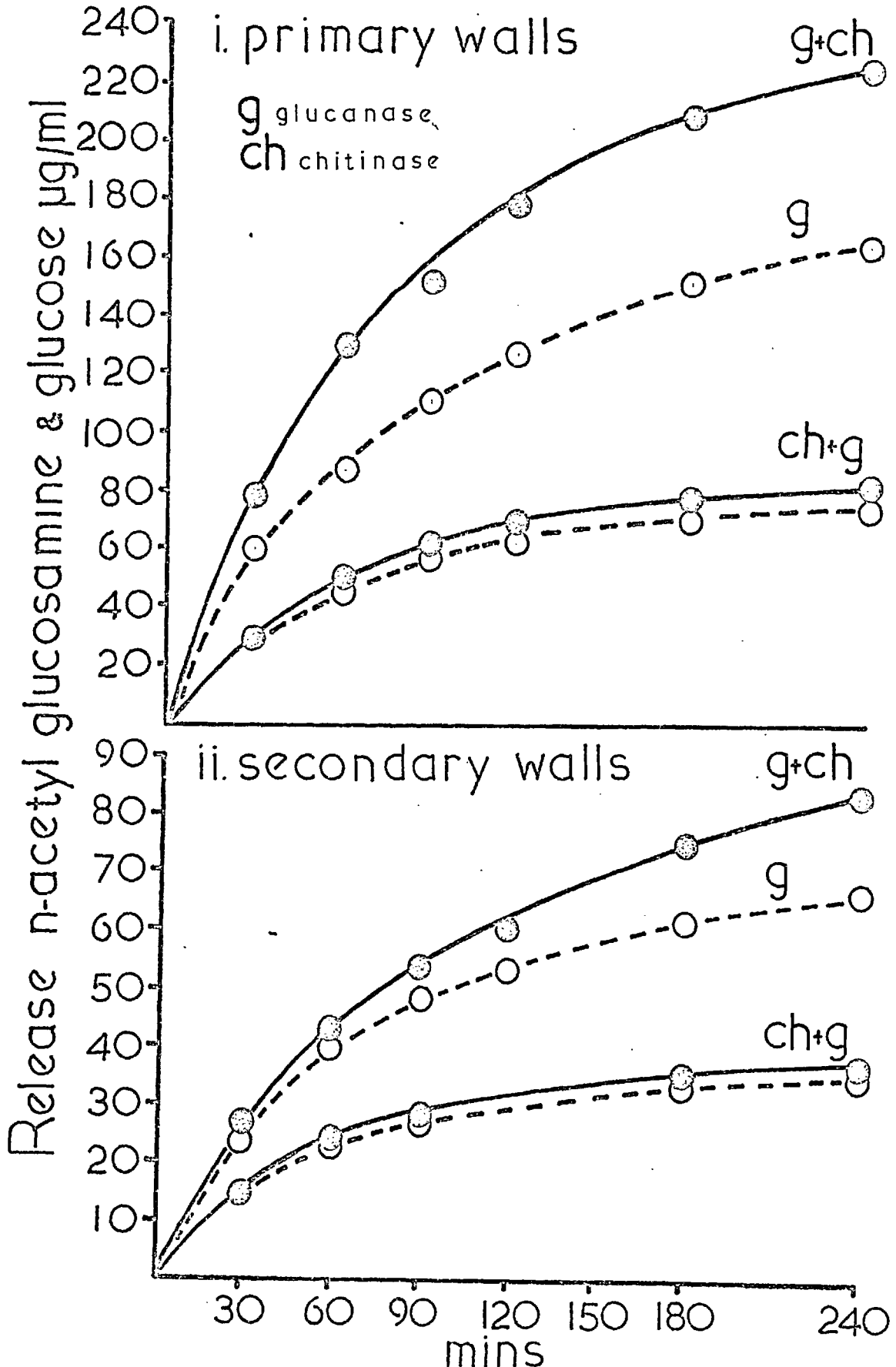


Fig.33. Hydrolysis of cell walls



glucose and 35 ug/ml n-acetyl glucosamine respectively in a 5 ml reaction mixture. The glucanase was incubated alone and together with the chitinase at the optimum pH for bothe enzymes i.e. pH 5 and pH 5.5.

Hydrolysis was followed over a 4 h incubation period at 37°; toluene was then added and the cell walls were re-incubated for a further 20 h. Chitinase was not incubated without glucanase; this had been done with the commercial preparation (p.145) in which β-(1-3) glucanase was present though at a low concentration.

The results are given in Fig. 33 i & ii. Similar quantities of glucose was released at pH 5 and pH 5.5 when the glucanase was incubated with chitinase whereas there was a slight increase in n-acetyl-glucosamine released with chitinase and glucanase at pH 5.5. The amounts of glucose and n-acetyl glucosamine liberated from the cell walls after 4 h and 24 h are given in Table 33. There was no increase in the amounts liberated during further incubation under these conditions.

Table 33. Hydrolysis of cell walls after 4 h and 24 h incubation.

Incubation time (h)	Sample	Glucose released with a.	Glucose released with a + b.	N-acetyl glucosamine released with b + a.
4	1°	16.3	22.5	8.2
	2°	6.7	8.4	3.7
24	1°	25.4	36.0	11.0
	2°	11.3	16.0	4.7

1° primary walls 2° secondary walls

a. β-(1-3) glucanase b. chitinase

From Fig. 33 and Table 33 several points are of interest:

(1) Considerably more glucose was liberated from both primary and secondary cell walls when the β-(1-3) glucanase was present with chitinase.

(2) Hydrolysis of glucose and n-acetyl glucosamine in 24 h from primary cell walls corresponded closely to estimations made from chemical analysis (p. 130). However, considerably less glucan and hexosamine was hydrolysed from secondary walls compared to the calculated amounts available.

(3) The majority of the glucan of the cell walls was probably a β-(1-3) linked glucose polymer but because of the presence of β-(1-6) glucanase it was not certain whether the entire cell wall glucan was β-(1-3) linked or whether both were present.

(4) There was evidence that the hexosamine of the primary cell walls was completely acetylated. Chemical analyses revealed 12.7% total hexosamine and enzyme hydrolysis indicated that 11.0% of the wall was n-acetyl glucosamine.

C. Induction of enzymes by micro-organisms grown on cell walls.

Penicillium javanicum and the streptomycete isolate S_1 were grown on the primary and secondary cell-wall preparations and the cultures assayed for production of glucanases and chitinase by incubating filtrates with purified substrates in the incubation mixtures described previously (p.119).

Both isolates produced chitinase and glucanases when grown on the cell walls of primary and secondary cell walls. A higher concentration of β-(1-3) glucanase was produced by growth of P. javanicum on primary walls but the difference between the activity of the primary and

secondary filtrates was small (Table 34). Comparison of the activity of chitinase in the culture filtrates showed that primary wall filtrates were more than twice as active as those from the mature cell walls.

Table 34. Relative activity of β-(1-3) glucanase and chitinase from P. javanicum.

Incubation (days)	µg/ml glucose and n-acetyl glucosamine released in incubation mixture/24 h.			
	Primary walls		Secondary walls	
	Glucose	N.A.G.	Glucose	N.A.G.
3	166	113.5	122	46
6	452	165	427	65
10	816	222.5	691	105
13	706	N.T.	601	N.T.

N.T. Not tested.

A β-(1-6) glucanase was induced by growth of P. javanicum on cell walls and its activity was assayed after 10 days. There was no difference in the enzyme activity of the filtrates from the two wall samples and 138 µg/ml glucose was released from lutean in a 24 h 5 ml incubation mixture from both primary and secondary filtrates; this was considerably lower than the concentration of β-(1-3) glucanase produced after 10 days (Table 34).

The results of similar incubations for culture filtrates of Streptomyces S1 are given in Table 35. The concentrations of the enzymes in the culture filtrates from primary and secondary walls differed considerably. The β-(1-3) glucanase activity was about three times

higher in the primary cell-wall filtrates and chitinase activity twice that of the secondary wall culture filtrates. However, β-(1-6) glucanase was the major glucanase produced by S_1 on both primary and secondary cell-wall preparations and from the primary and secondary filtrates 330 and 224 ug/ml glucose was released from lutean respectively in the incubation mixtures. Thus, the activity of the β-(1-6) glucanase was several times that of β-(1-3) glucanase induced by the cell-wall preparations.

Table 35. Relative activity of β-(1-3) glucanase and chitinase from Streptomyces S_1.

Incubation (days)	ug/ml glucose and n-acetyl glucosamine released in incubation mixture/24 h.			
	Primary walls		Secondary walls	
	Glucose	N.A.G.	Glucose	N.A.G.
6	96	13	33	7.5
10	133	18	40	8.5
13	106	18	46	7.5

Observations on the cell walls.

Both primary and secondary walls of the samples inoculated with P. javanicum retained their integrity and showed only slight reduction in the thickness of the cell walls. In contrast the Streptomyces S_1 brought about complete breakdown of the primary cell walls after 6 days, reducing the sample to a structureless mass. The cell walls of the mature mycelium were also homogenised although the cleistocarps cell walls,

being very much thicker remained without obvious signs of hydrolysis when observed under the light microscope (x400) up to 13 days after inoculation when the experiment was terminated. Clearly for complete hydrolysis of cell walls chitinase, β-(1-3) and β-(1-6) glucanase are not adequate alone although they substantially reduce the cell wall thickness.

Potgieter and Alexander (1965) found that prolonged incubation of Neurospora crassa with chitinase and a β-(1-3) glucanase left the hyphal walls thinned but intact. Possibly in S. mors-uvae protease activity is required attacking the protein molecules of the melanin complex before complete dissolution of the walls is achieved.

5. Electron microscopy of S. mors-uvae cleistocarps.

A study was made of the structure of the cleistocarps from the two samples of S. mors-uvae (p. 122) used in the chemical analysis and enzyme work. Sections were made of the immature and mature cleistocarps and their mycelia and of the mature cleistocarps after they had overwintered on the soil in the walled garden at Silwood Park to find if micro-organisms had colonised and degraded the cleistocarp walls.

A. Immature (primary) cleistocarps.

A section through the cleistocarp reveals two distinct areas (Fig. 34); a central region in which the ascus and ascospores are differentiated and a multi-layered perioheral tissue in which two regions can be distinguished.

i. The central region.

The central region is bounded by a wall similar in thickness (c. 0.2 μ) to those of adjacent isodiametric cells (Fig. 34). The wall does not show any discontinuity; suggesting previous cell fusion, and encloses an area with a nucleus and in which large vacuoles, mitochondria and lipid are present.

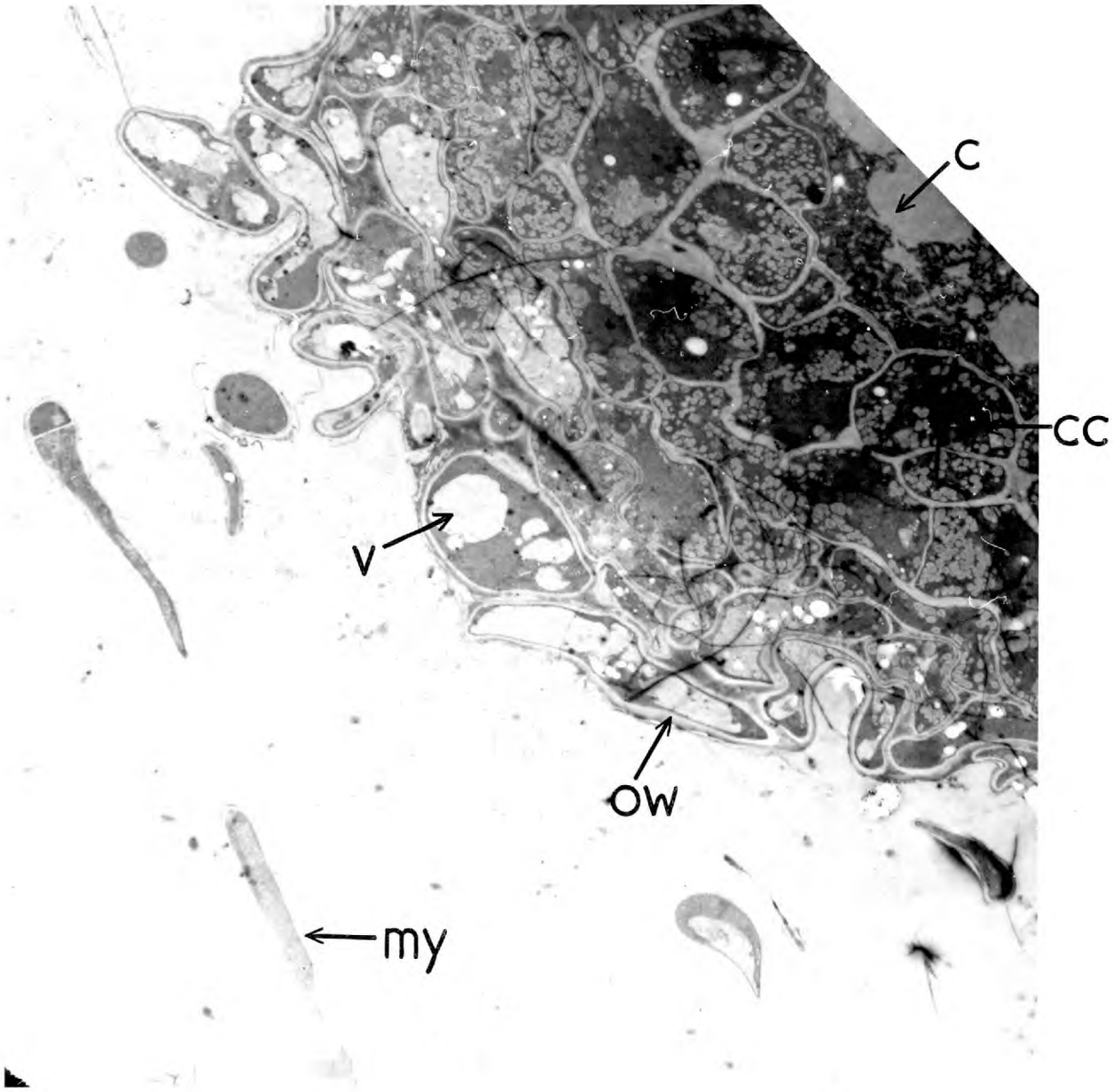
ii. Peripheral layers.

a) Inner cell wall layers or centrum cells.

Outside the central area are three to four layers of isodiametric cells. Although these layers (centrum cells) may be a tissue which support the differentiation and maturation of the ascus and ascospores and the region may be distinct in function from the wall cells, in early cleistocarp stages (Fig. 34) the two regions are morphologically indistinct.

Fig. 34. Section through an immature cleistocarp showing central region (c), peripheral layers and mycelium (my). Note transition between isodiametric centrum cells (cc) and the elongated cells of the outer wall layers (ow). There is an increase in size of the vacuoles (v) and loss of lipid towards the boundary of the cleistocarp wall. (x 2,850)

Fig. 34.



The cell walls of this region have a fibrillar structure (Fig. 35). Electron-dense extra-cellular material is present along the junction of adjoining cell walls and especially at the corners. Where a nucleus is seen it is single in these cells. Mitochondria and large vacuoles with associated concentric membrane structures are common. A prominent feature of these cells is the large amount of lipid which distinguishes this layer from the cell of the outer cleistocarp wall.

These layers around the ascus seem to correspond to the centrum cells of other members of the Erysiphaceae described by Gordon (1966). These cells initially filled the central area of the cleistocarp and he considered some of them were absorbed in the ascus formation.

b. Outer wall of cleistocarp.

Passing from the centrum cell towards the periphery of the cleistocarp there is a gradual change of cell shape from the inner isodiametric centrum cells to cells whose periclinal walls become progressively more elongated and the anticlinal walls shortened. This region of approximately three or four layers is distinguished as the outer cleistocarp wall (Fig. 34).

In the outer wall layers the inner region of the cell wall is transparent and fibrillar (Fig. 36), as in the centrum cells. Beyond this layer electron dense material is extensively developed at the anticlinal wall junctions and between the periclinal walls emphasising their fibrous structure.

The walls of these cells, therefore, appear two layered although the outer wall layers of adjoining cells are continuous. The cell walls

Fig. 35 . Cytoplasmic contents of centrum cells of immature cleistocarp showing a nucleus (n), mitochondria (m), vesicle (v), lipid (l), concentric membrane structures (cm), the fibrous cell wall (fw), electron-dense material between adjacent walls (em). (x 17,500)

Fig. 35.

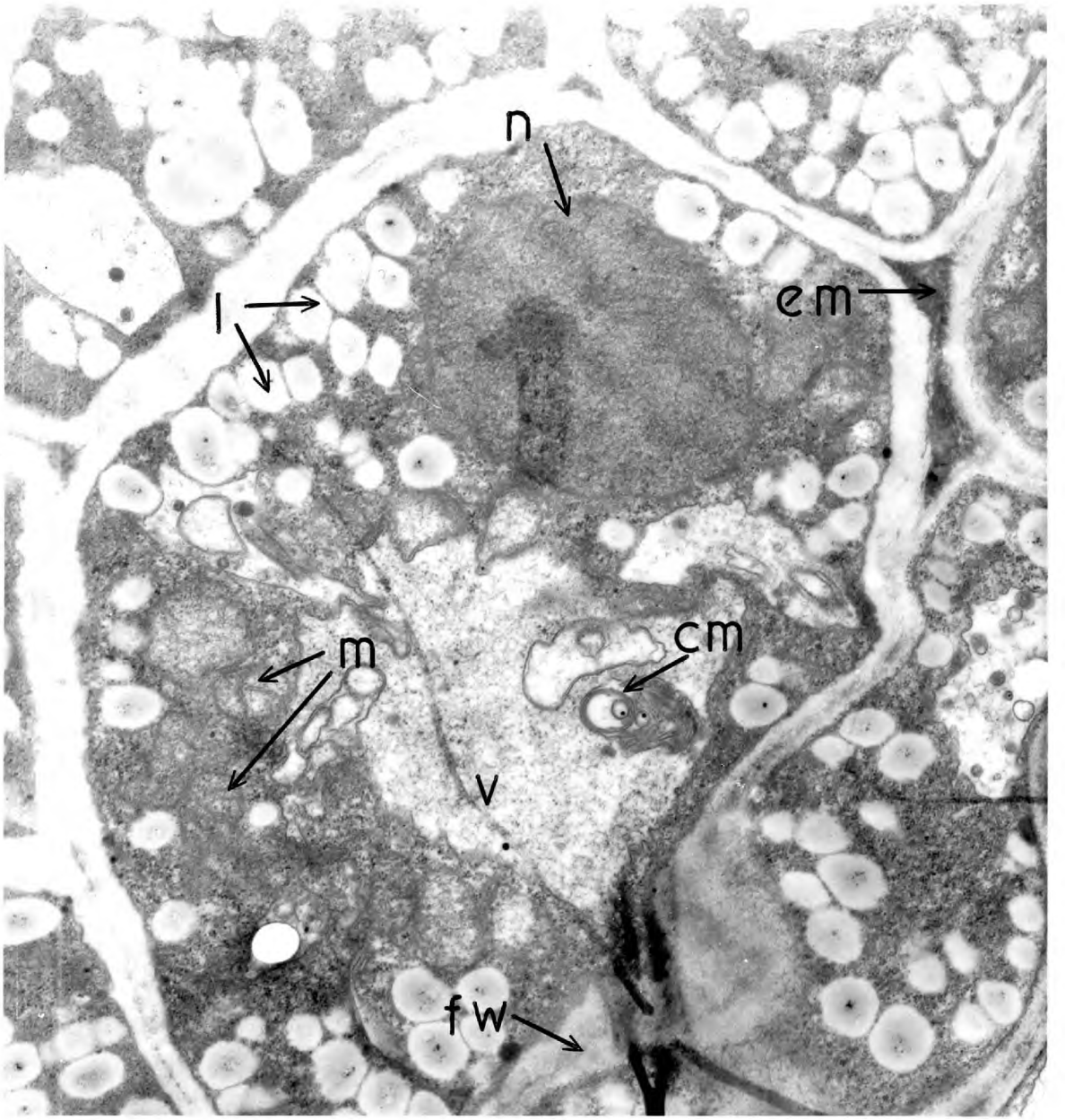


Fig. 36. The outer wall cells of an immature cleistocarp showing fibrous walls (fw), the electron-dense material between anticlinal walls extending between the fibrous layers of the wall(em).A thin electron-dense cuticle (cu) is shown. The cytoplasmic contents of the wall cells are indicated: nucleus (n), mitochondrion (m), vesicle (v) and lipid (l). (x 18,000)

Fig. 36.

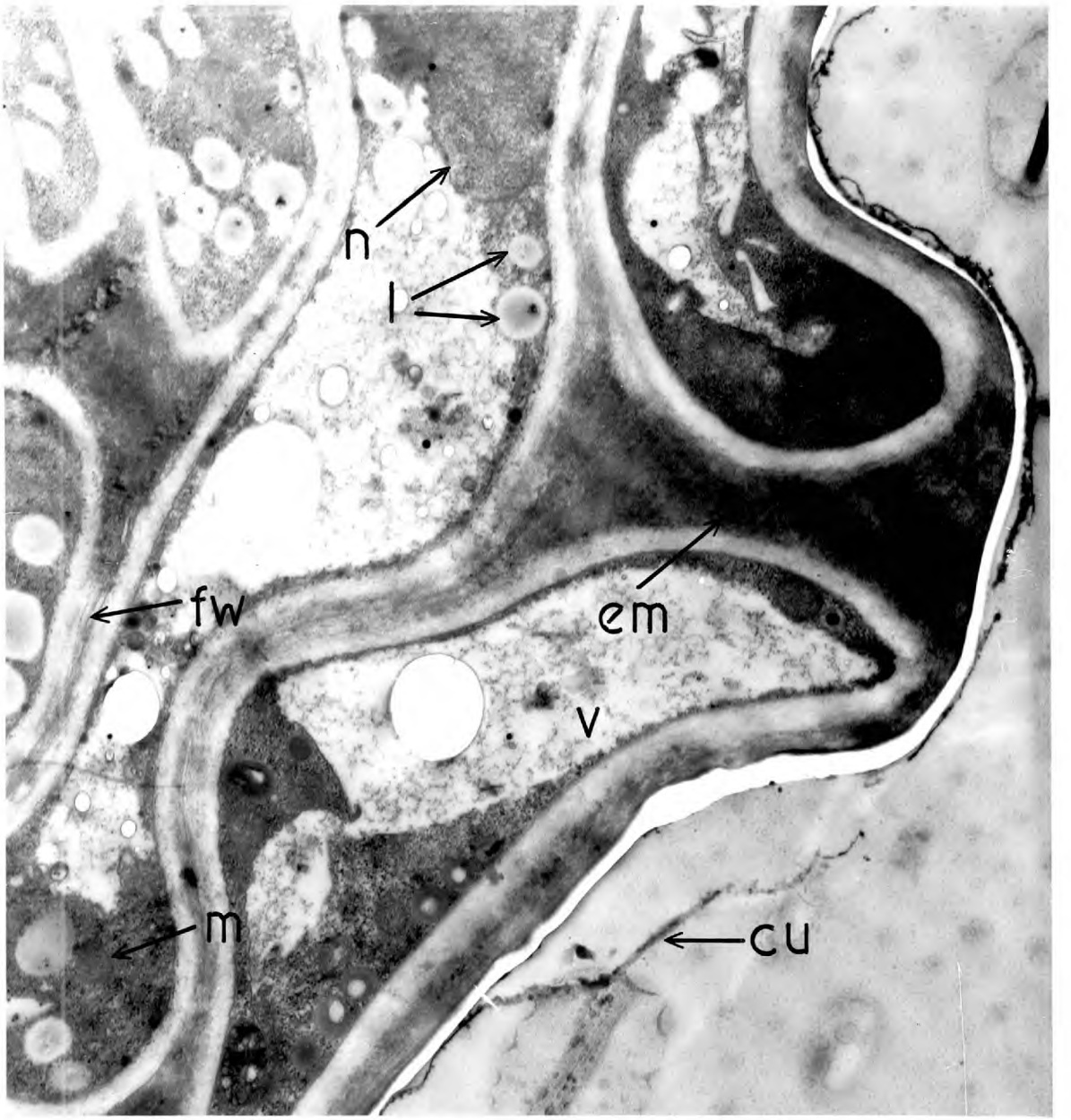
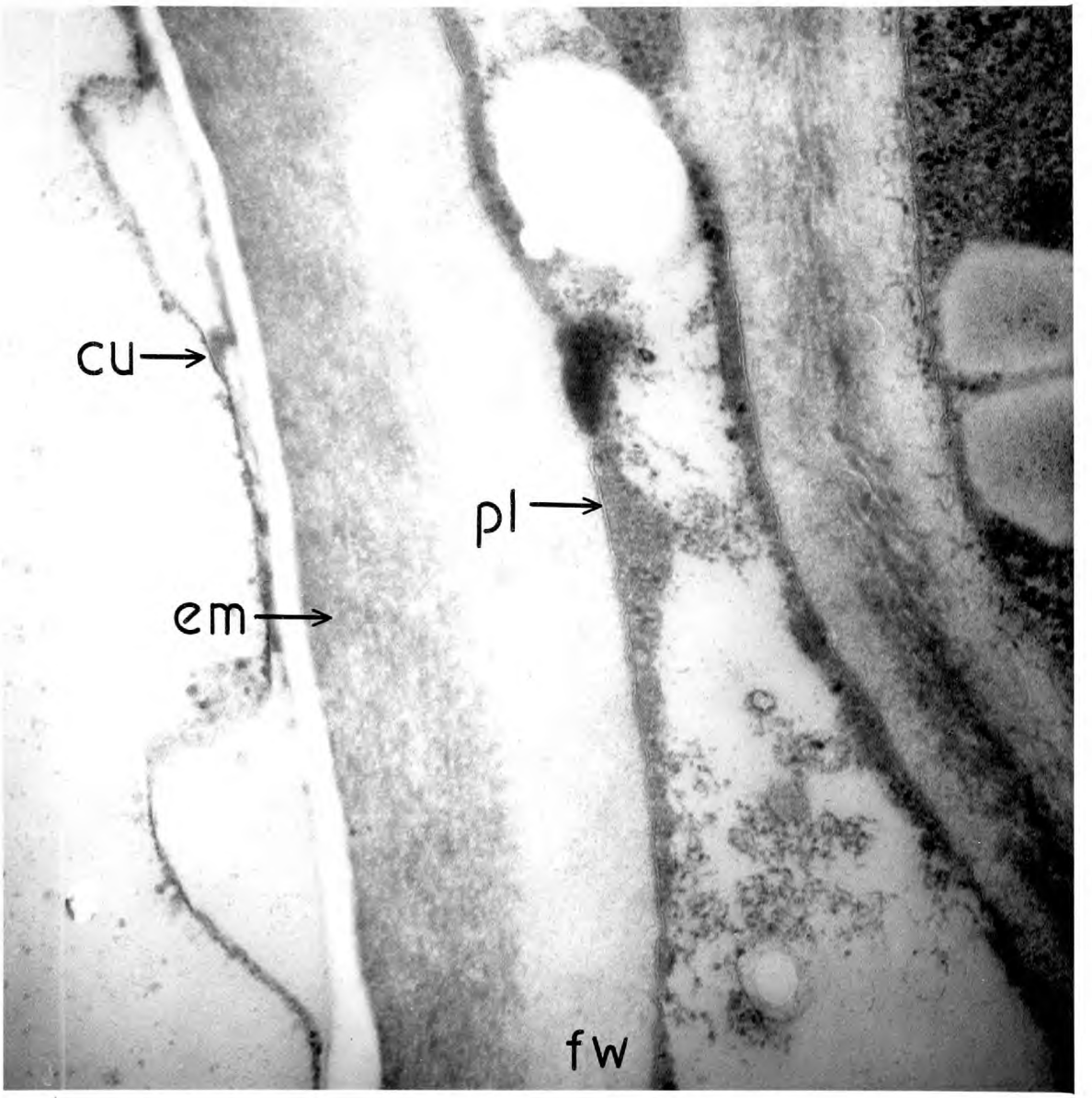


Fig. 37. Showing the two regions (em,fw) of a marginal cell of the outer wall of an immature cleistocarp bounded by the cuticle (cu). A plasmolemma (pl) enclosing the cytoplasm of the cell is shown. (x 64,500)

Fig. 37.



increase in thickness towards the periphery of the cleistocarp where the electron-dense material is most fully developed (Fig. 37) and forms a darkly stained surface layer.

Outside this dark layer a thin cuticle is present (Figs. 36, 37 & 38). Within these cells the vacuoles are large compared to the cell profile area (c. 50%) and the lipid droplets are fewer than in the centrum cells Fig. 36 & 38). But as in the centrum cells nuclei, mitochondria and concentric membrane structures are present. Some of the outer cells of this layer are extended as appendages (Fig.38).

The structure of the hyphae associated with the cleistocarps (Fig.34) is similar to marginal cells of the cleistocarp.

B. Mature (secondary) cleistocarps.

i. Central region.

The central region (Fig. 39) is shown containing an ascus with a thick wall (4-5 μ) which has several layers. In some regions outside the ascus wall (Fig.40) the cytoplasm is similar to the contents of the surrounding centrum cells. It is likely that the boundary wall of the central area had formed the ascus wall and one or some of the inner centrum cells has lost its wall leaving the contents in direct contact with the ascus.

ii. Peripheral layers.

a. Centrum cells.

In the mature cleistocarps the centrum cells are clearly distinguishable from the outer cleistocarp cells. These two to three layers have thin

Fig. 38. A section through the outer wall of an immature cleistocarp showing the distribution of the electron-dense material (em) between cell walls and forming a boundary layer to the cleistocarp. Note the cuticle (cu) is continuous around the marginal cells of the cleistocarp wall and the appendage cell (a). (x 9,000)

Fig. 38.

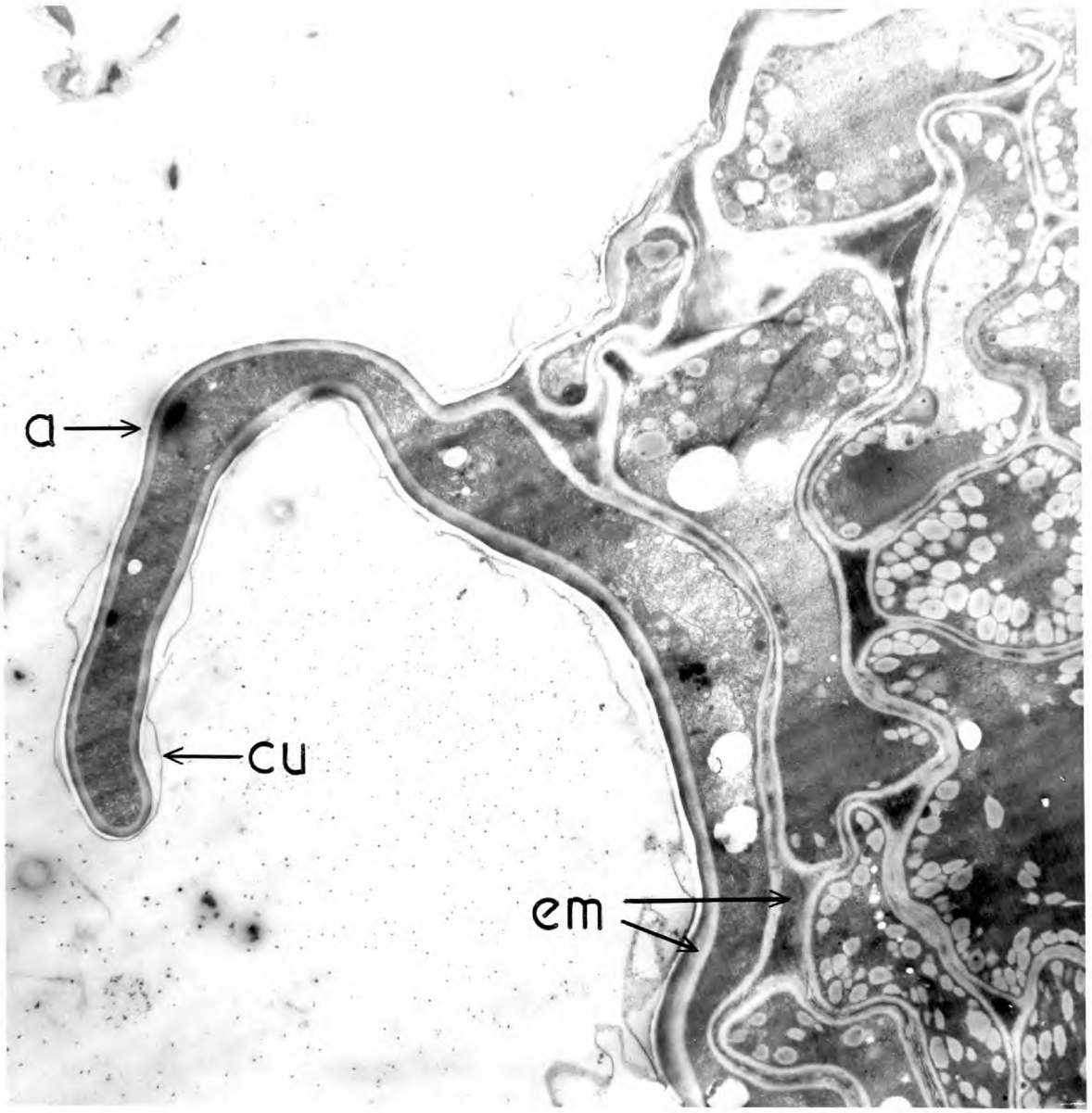


Fig. 39. Section of mature cleistocarp showing the distinction between the central region containing an ascus with thick wall (aw), the centrum cells (cc) and the cells of the outer wall of the cleistocarp (ow). Note the loss of cytoplasmic contents of the outer wall cells, the presence of osmiophillic lipid (ol) of the centrum cells and the increase in the thickness of the cell walls towards the periphery of the cleistocarp. (x 2,000)

Fig. 39.

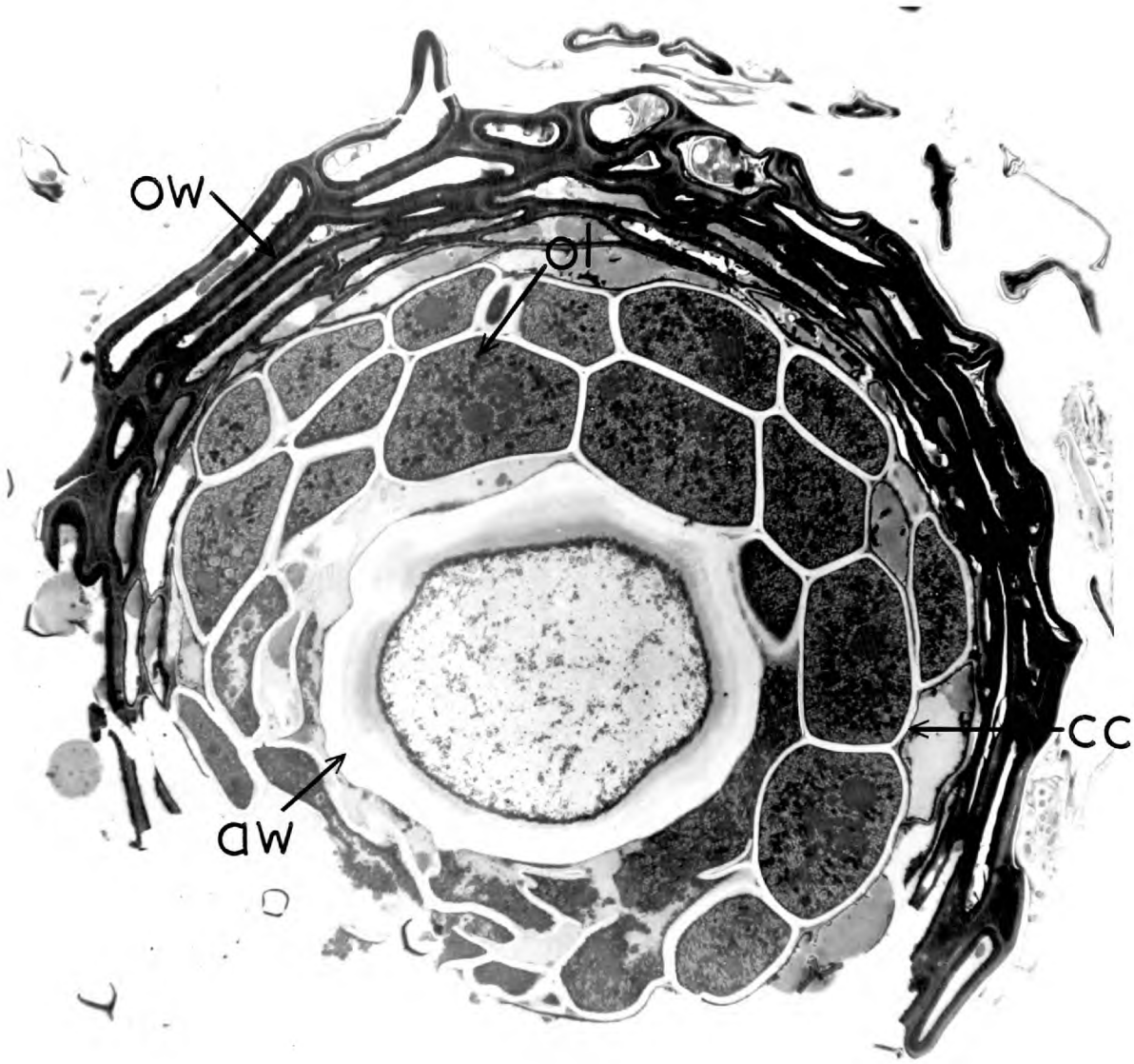
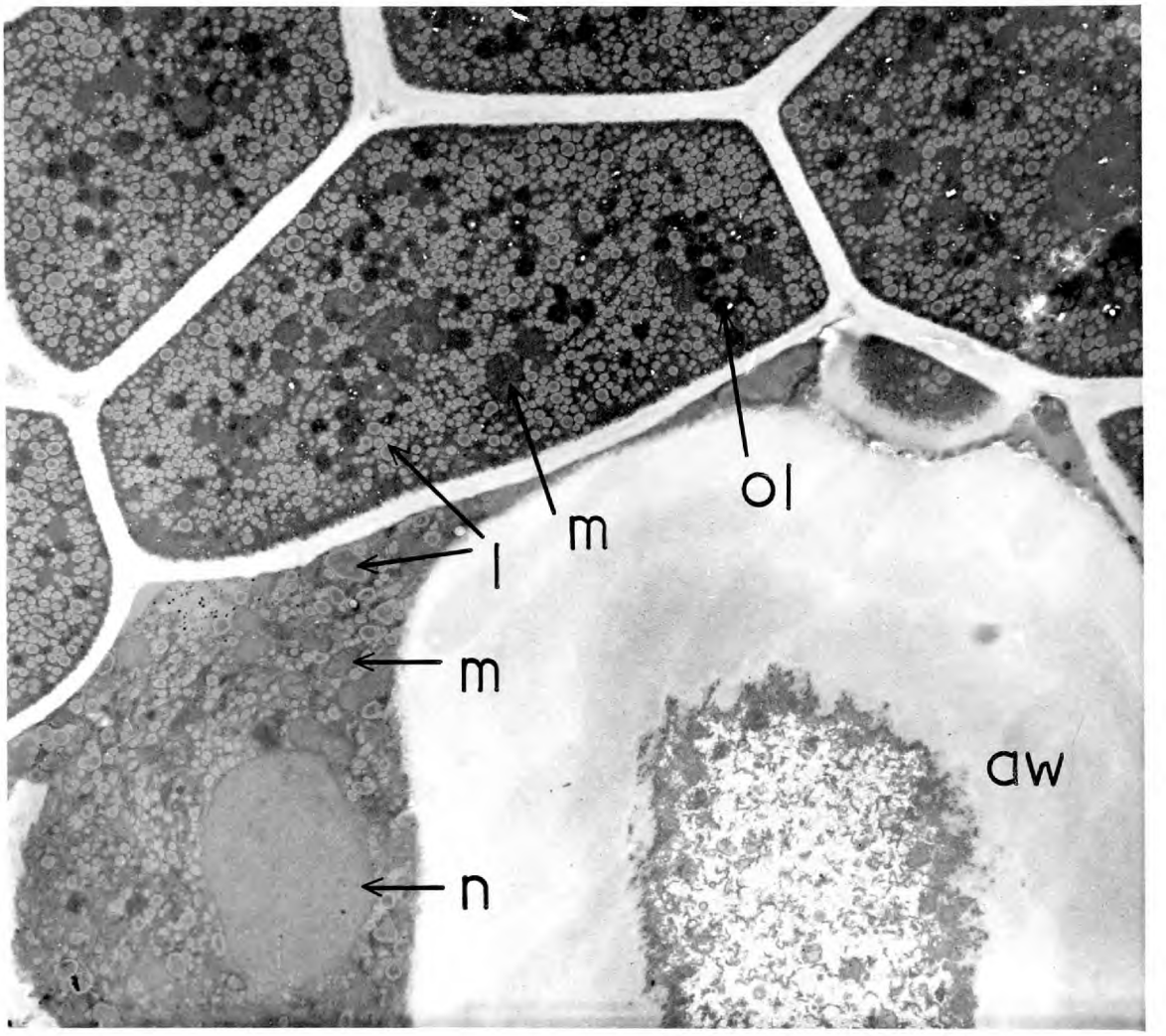


Fig. 40. Section through a mature cleistocarp showing the central region containing an ascus with thick wall (aw) outside which the cytoplasm, not enclosed by a cell wall, contains a nucleus (n), mitochondria (m) and lipid (l). The centrum cells are shown with osmiophillic lipid (ol). Note lack of vacuoles compared to immature centrum cells. (x 5,400)

Fig. 40.



cell walls. They are similar in shape and content to the centrum cells of immature cleistocarps except for the apparent lack of vacuoles and the addition of osmio-phillic lipid (Figs. 39 & 40). The concentric membrane structures formally associated with vacuoles are absent.

b. Outer wall of cleistocarp.

The boundary between the centrum cells and the outer cleistocarp wall is well marked at this stage. The outer wall layers have thickened cell walls and cell contents are absent (Fig. 41). The thickness of the successive cell layers towards the periphery show increased thickening of their walls. The cells of these layers (Figs. 39 & 41) have an electron-dense layer around the cell lumen. There is also an increase of the darkly stained material around the anticlinal and periclinal walls.

Measurements were taken of the total wall thickness and the thickness of the fibrillar, transparent, layer of the immature and mature cleistocarps. Table 35 shows that there was a significant increase in the total thickness of the cell wall of mature cleistocarps ($p < 0.001$). However, the increase in total wall thickness accompanied a decrease in the depth of the transparent zone of the cell wall. In the immature cell wall the transparent layer was statistically greater ($p < 0.001$).

Thus, it is probable that the electron-dense material bordering the cell lumen partly represents a deposit of new wall material and also infiltration into the existing transparent layer.

The mature cleistocarps have a cuticular layer similar to that of the immature samples. The secondary mycelium (Fig. 42) appears to be of similar structure to the outer cleistocarp cells.

Table 36. Measurements of total thickness and the thickness of the fibrous, transparent, layer of immature and mature cleistocarp.

Readings taken on the outermost cell wall of the cleistocarp.

	Entire cell wall (μ)		Fibrous layer of wall (μ)	
	immature	mature	immature	mature
	0.61	0.58	0.24	0.28
	0.67	0.65	0.27	0.29
	0.53	0.72	0.27	0.29
	0.56	0.83	0.24	0.15
	0.36	0.97	0.22	0.13
	0.41	0.93	0.15	0.13
	0.32	1.00	0.20	0.12
	0.50	1.00	0.22	0.16
	0.52	0.99	0.25	0.10
	0.68	0.95	0.30	0.15
	0.47	0.86	0.25	0.15
	0.49	0.80	0.30	0.23
	0.75	0,76	0.30	0.16
	0.66	0.65	0.29	0.15
	0.68	0.94	0.25	0.13
	0.62	0.58	0.33	0.10
$\bar{x}$	0.55	0.83	0.26	0.20

Four readings were taken at random on four marginal cells of immature and mature cleistocarps.

Fig. 41. Mature cleistocarp wall showing cytoplasmic contents of centrum cells with sharp boundary between these and the outer wall cells. Note general lack of contents of outer wall cells; some show remains of membrane system (ms). Note also the progressive thickening of the periclinal cell walls of the outer wall layers of the cleistocarp towards the periphery. Breaks in these wall cells are indicated (br). (x 11,400)

Fig. 41.

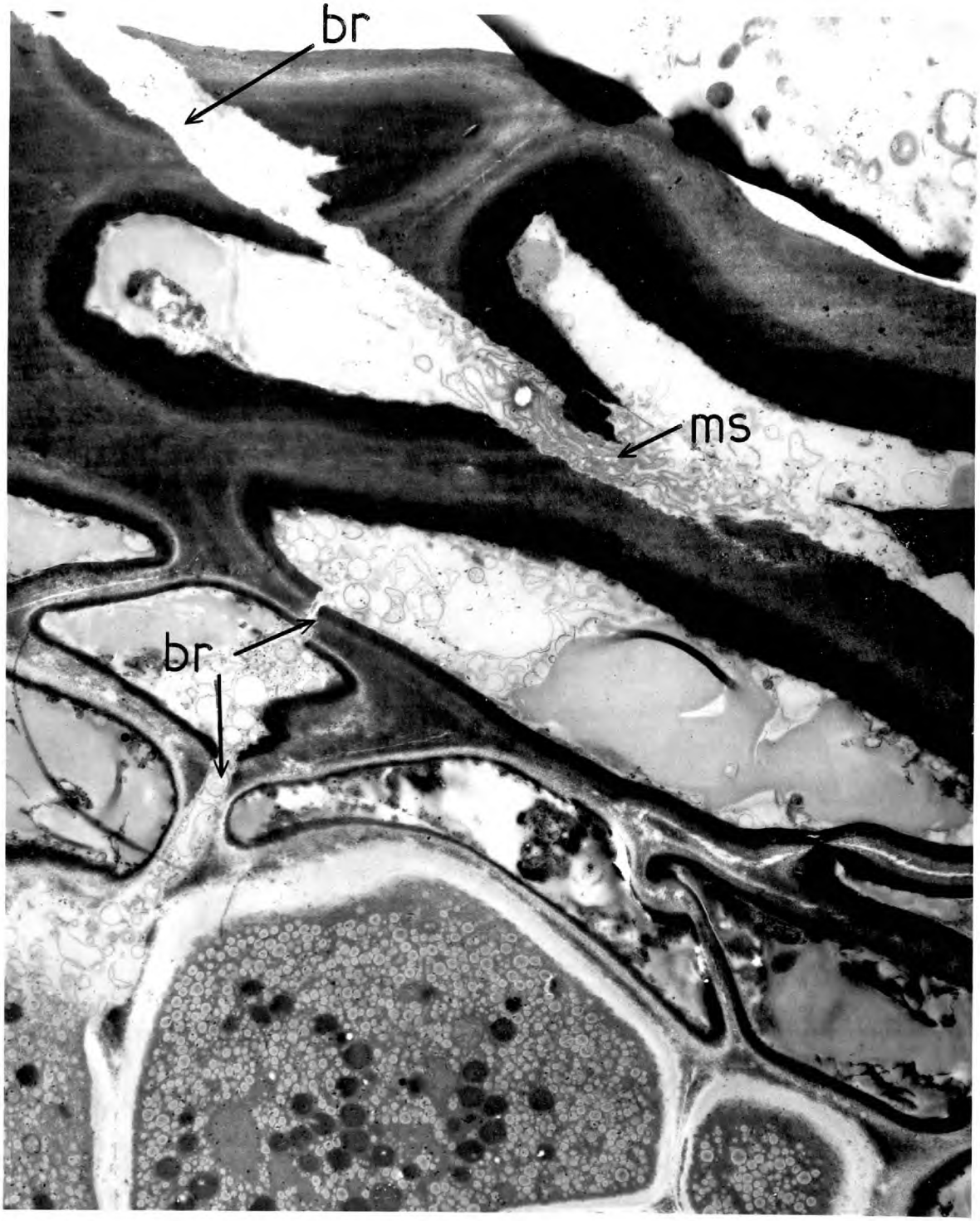


Fig. 42. Section through a mature cleistocarp wall showing the presence of a cell within the lumen of an outer-wall cell. The cytoplasm is bounded by a cell wall (cw) inside which a plasmolemma (pl) is indicated. Note also the similarity of the wall of the secondary mycelium (sm) and the cleistocarp. (x 23,700)

Fig. 42.

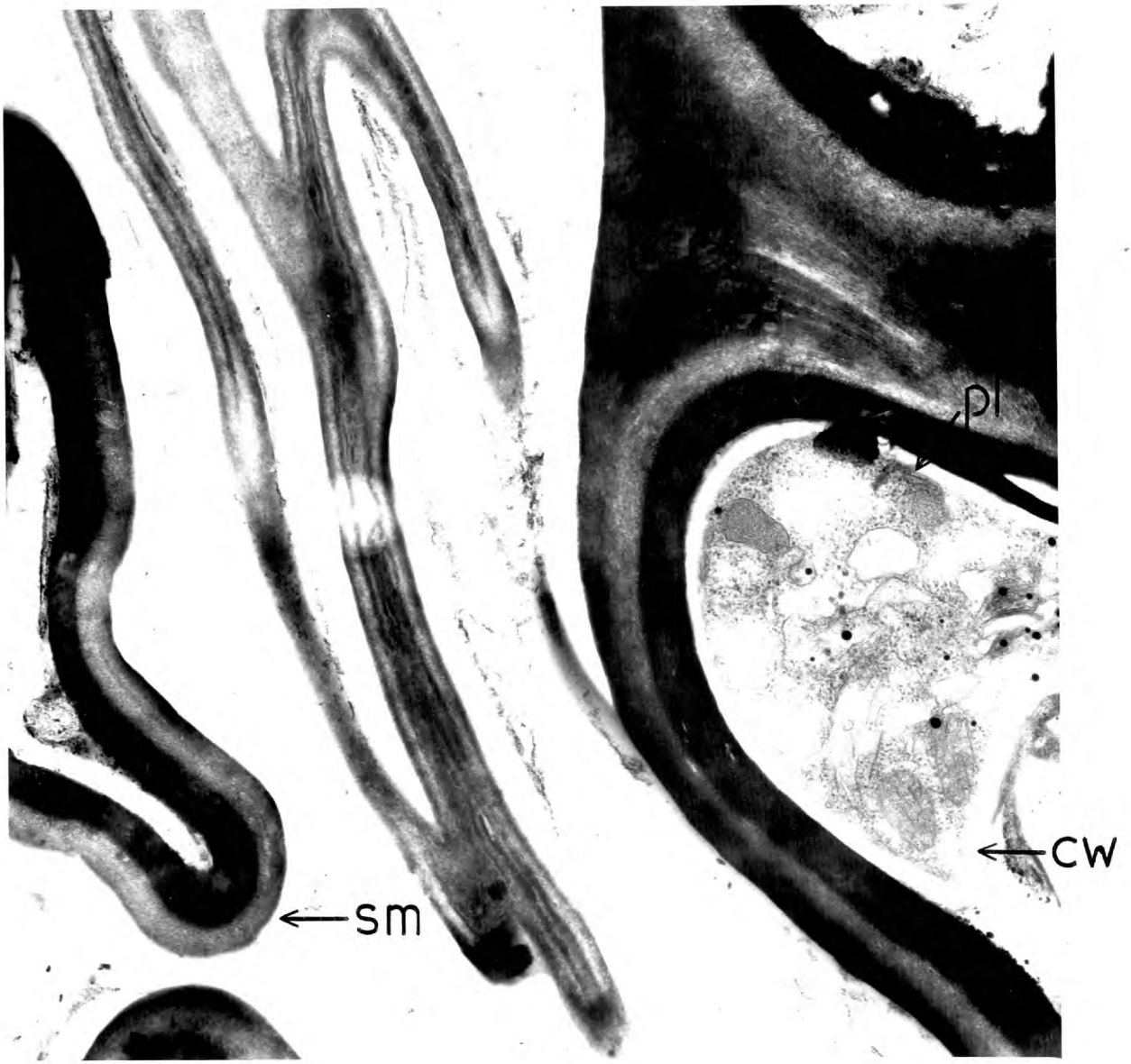
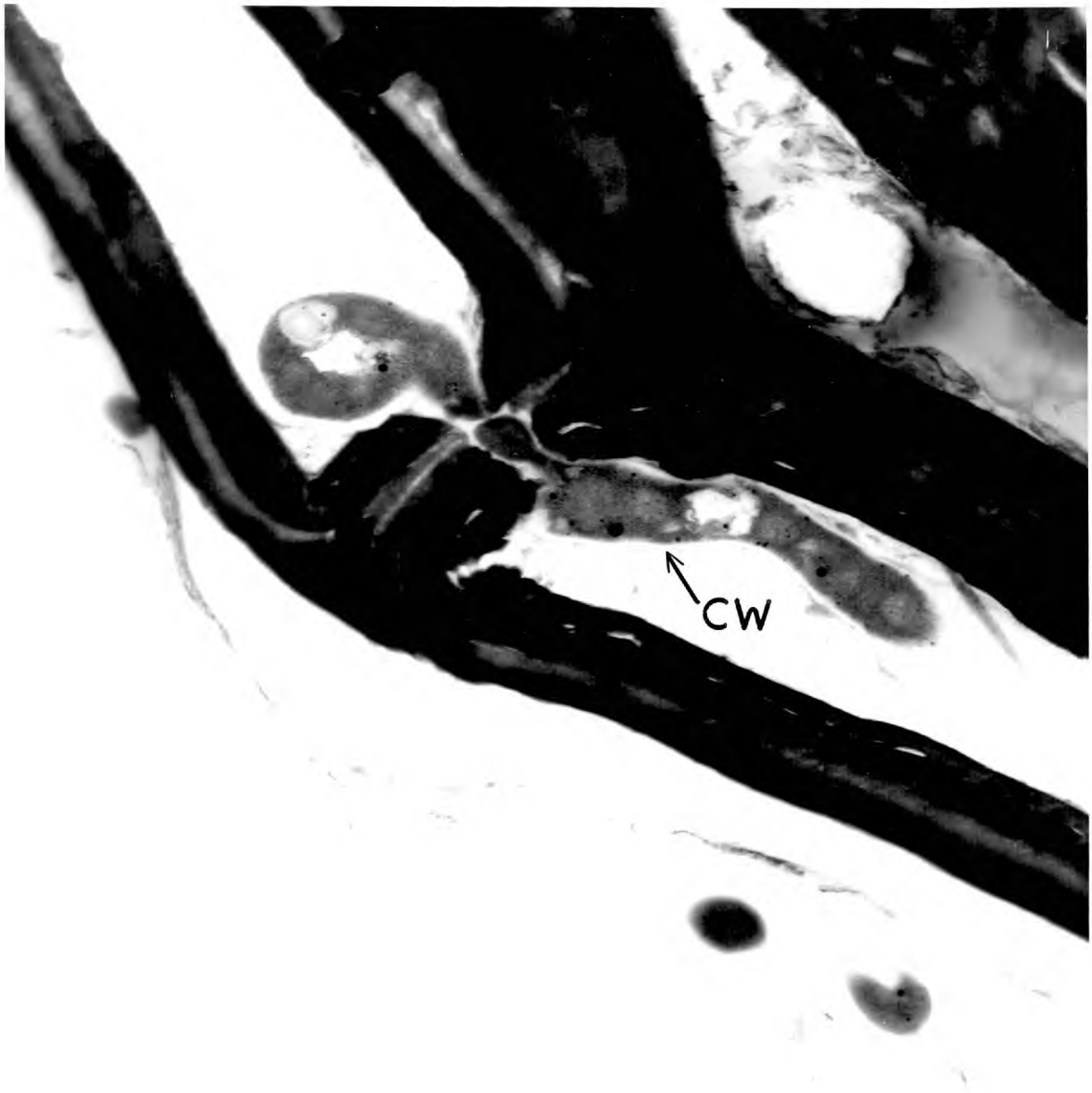


Fig. 43. Mature cleistocarp with a fungal cell passing through an anticlinal wall between two marginal outer-wall cells. Note the organised cytoplasm of the fungal cell compared to the outer-wall cells and the presence of a wall (cw) around the cytoplasm. (x 16,200)

Fig. 43.



Figs.41,42 &43 suggest that the wall of some cleistocarps is broken even before they are wintered and fungi are seen in the lumen of intact marginal cells of these cleistocarps. Structures identifiable with sections of fungal hyphae are seen in the lumen of cleistocarp cells (Figs.42 & 43) where the cytoplasmic contents are bounded by a plasmolemma and cell wall. Fig.43 shows a structure which has passed through a septal pore between cell walls or penetrated directly through an anticlinal wall. However, septal pores were not seen between other noninvaded cells. It is probable that colonisation of outer cleistocarp cells takes place while the cleistocarps are on the leaf attached to the black currant bush.

Overwintered cleistocarps.

Centrum cells and asci are generally absent from cleistocarps overwintered on the soil. Fig. 44 shows large numbers of bacteria and fungal cells present within the cleistocarp although there was no obvious degradation of the outer cleistocarp walls. Bacteria are present in the cleistocarp wall cells and also within the area previously occupied by the centrum cells and ascus (Fig.45). The absence of internal membranes and the presence of a wall and an unstained area probably containing DNA provides evidence that these structures are bacteria. In Fig.45 a bacterium is shown within the wall surrounded by a transparent area which suggested bacterial lysis of the cell wall. The presence of profiles identified with fungi are equally numerous and Fig. 46 shows a fungal cell with elipsoidal bodies within the cytoplasm. These structures have previously been recorded only in the fungal component of lichens (Brown and Wilson, 1968) and have more recently been renamed 'concentric bodies' by Griffiths and Greenwood (unpublished).

Fig. 44. Glancing section through an overwintered cleistocarp showing bacteria (b) and fungal cells (f) within the outer-wall cells. Note the breaks (br) in the wall and numerous cells without cytoplasm. (x 10,700)

Fig. 44.

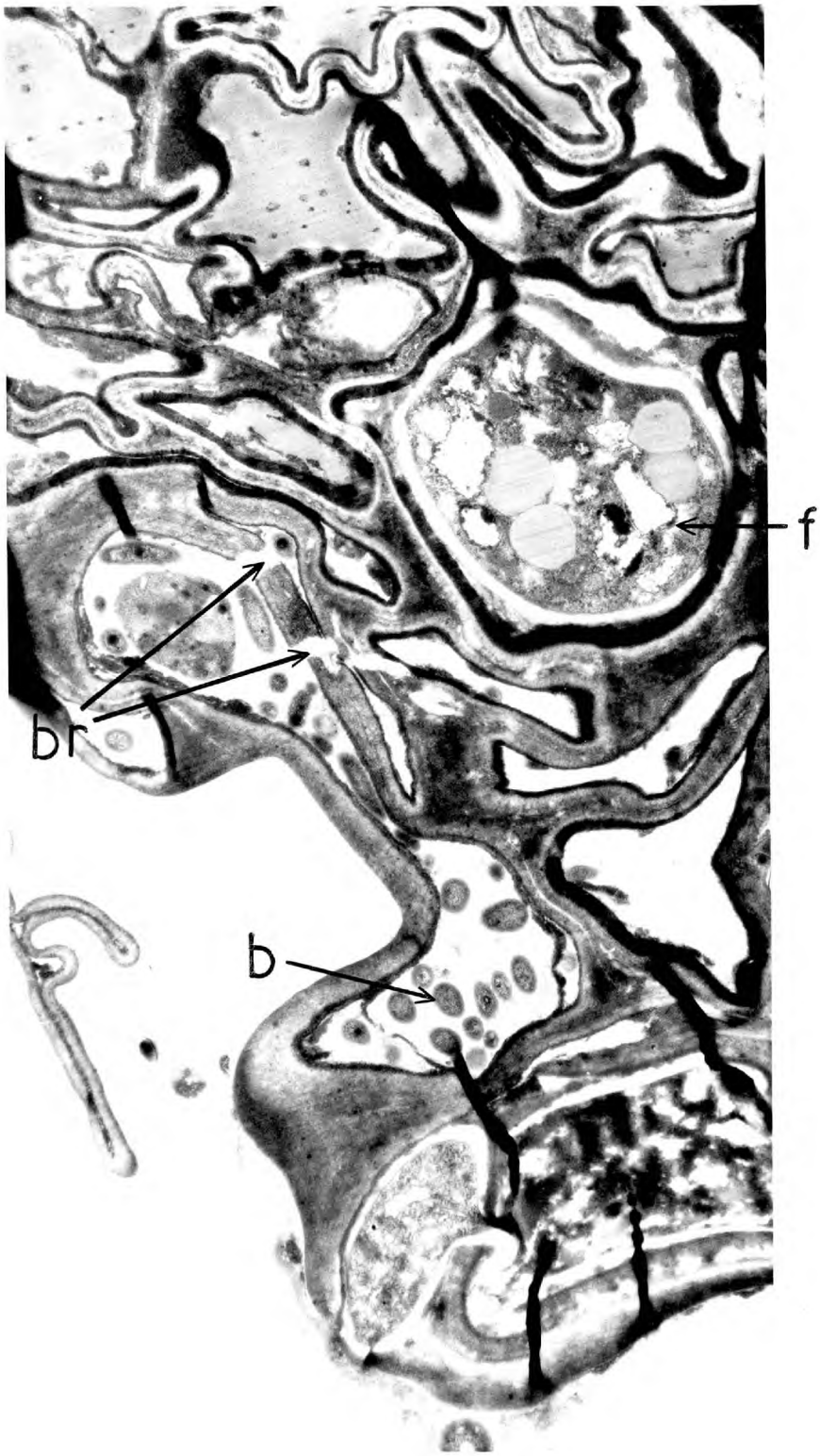
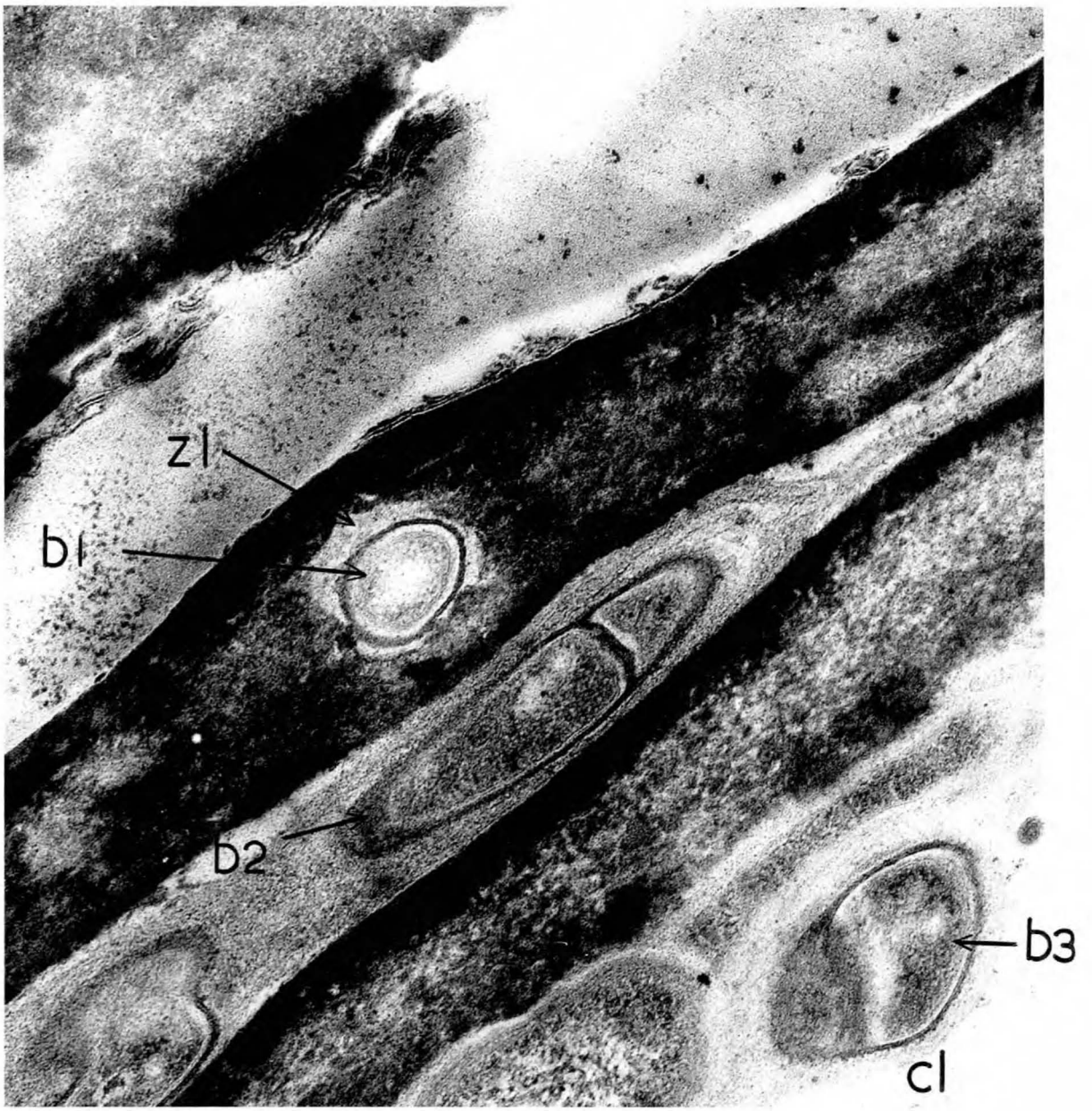


Fig. 45. Section through an overwintered cleistocarp showing a bacterium (b1) in the cell wall surrounded by a zone of lysis (z1), bacterium (b2) within the lumen of the wall cells and bacterium (b3) in the central lumen (c1) previously occupied by centrum cells and ascus. (x 67,000)

Fig. 45.



There was, however, little evidence of the means of entry of either bacteria or fungi into the cleistocarps. Fig. 47 may indicate direct penetration of a bacterium into the outer cell wall and some indication of fungal penetration is shown in Fig. 44. It is likely that if direct penetration is uncommon then entry of micro-organisms may take place via fractures in the wall (Fig. 41 & 44).

Bacteria and fungi were present also in the lumen of cells of the secondary mycelium (Figs. 48 & 49). Entry of the mycelium if not by direct means could have been achieved via the broken ends of hyphae. Damage to the mycelium, especially by soil insects feeding on both the cleistocarps and secondary mycelium was frequently observed in late autumn and early spring (p. 27).

Fig. 46. Section through an overwintered cleistocarp wall showing a fungal cell within the lumen of a wall cell. Note the presence of ellipsoidal bodies (eb). (x 29,000)

Fig. 47. Showing the possible entry of a bacterium directly through the wall of a cleistocarp. (x 78,500)

Fig. 46

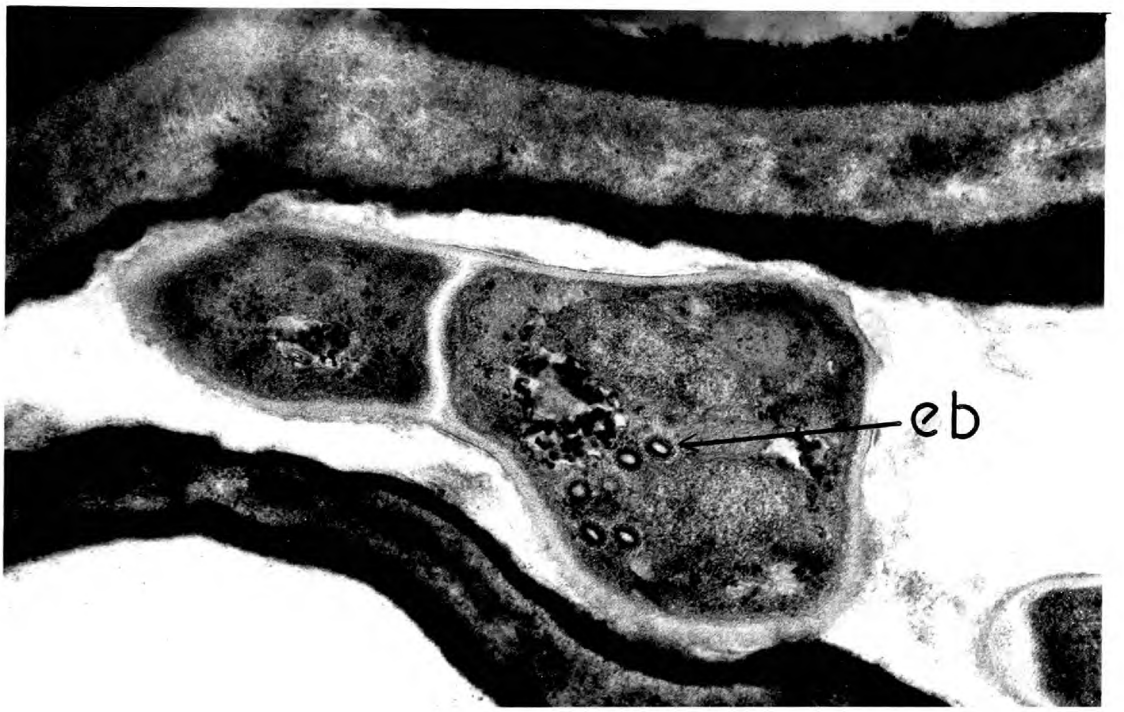


Fig. 47.

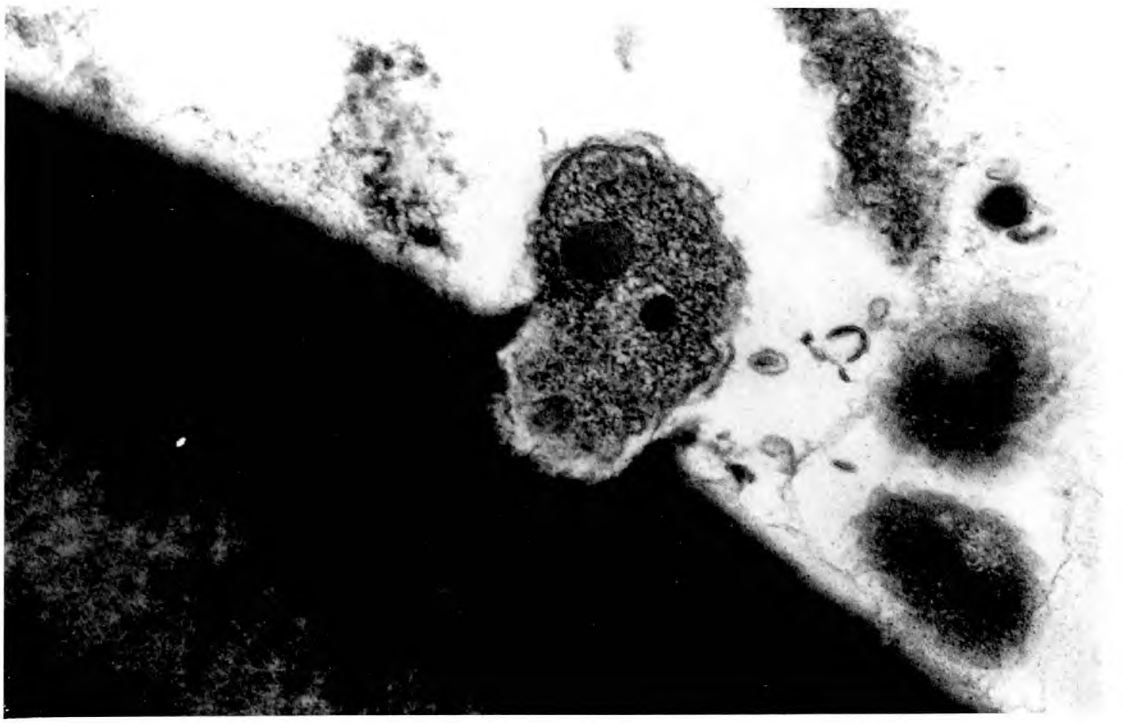


Fig.48 . Section through the secondary mycelium associated with overwintered cleistocarps. Note the presence of bacteria (b) within the lumen and wall of the mycelium. (x 29,800)

Fig.49 . Section through the secondary mycelium associated with overwintered cleistocarps showing the presence of a fungal cell within the lumen. Note the ellipsoidal bodies in the cytoplasm (eb) and the similarity between the wall of the mycelium and cleistocarp. (x 34,000)

Fig. 48.

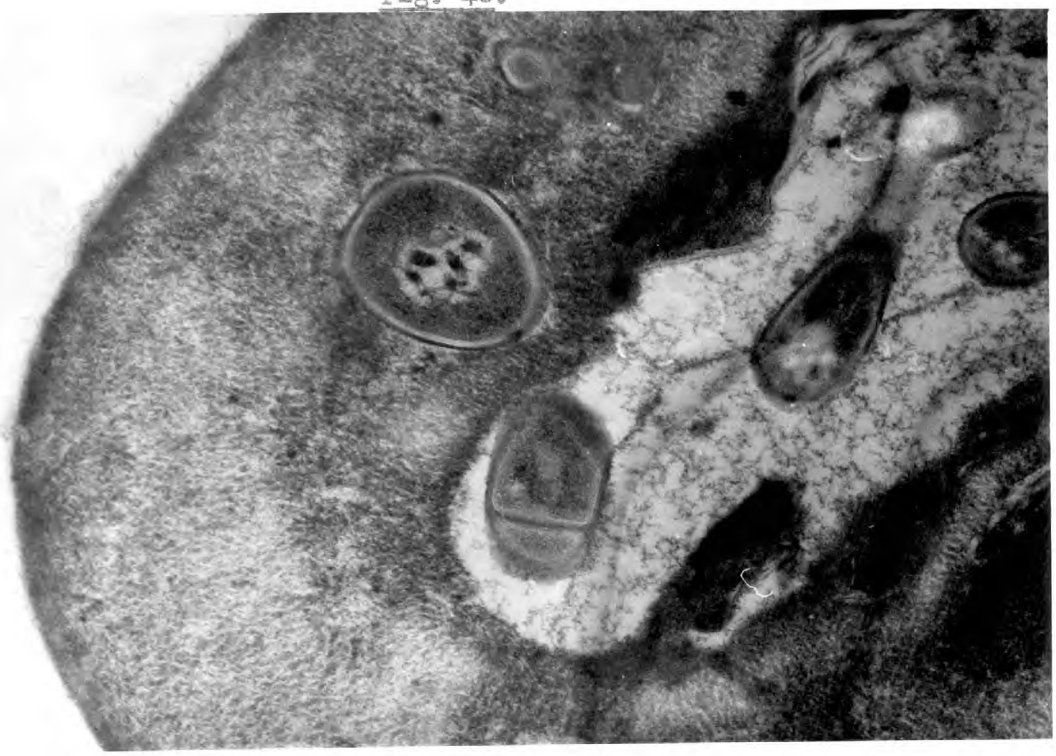
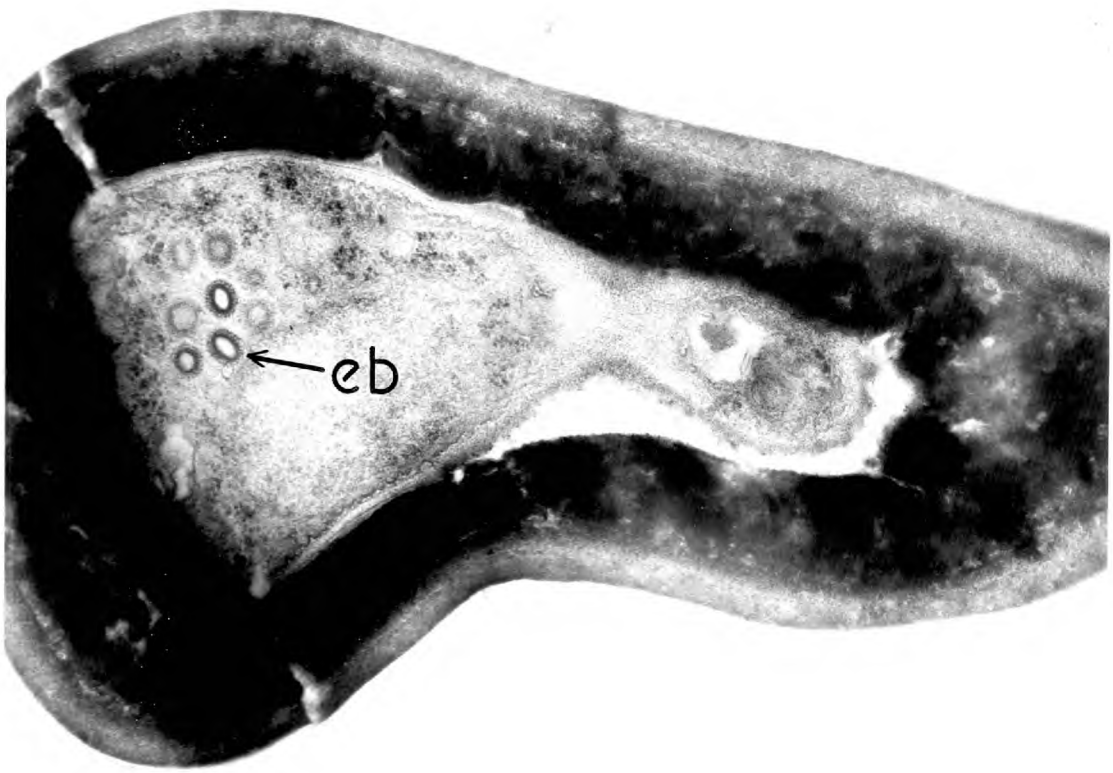


Fig. 49.



DISCUSSION

The present results substantiate those of Merriman and Wheeler (1968) and of Jordan (1966, 1967) that some cleistocarps of S. mors-uvae overwinter and discharge viable ascospores in the following spring. Additionally they indicate that ascospore discharge may occur over a longer period and earlier than previously supposed, depending on the time of cleistocarp formation and its relationship to leaf fall. For example, cleistocarps formed on leaves and placed on soil in early summer (July samples) discharged ascospores in late autumn; there was evidence of dehiscence in the field by 14 November (1969) and ascospores were trapped in dehiscence tests in the laboratory on 19 December. In contrast, discharge of ascospores from cleistocarps formed in late summer (September samples) was first noted on 10 February (1970), some three months later.

These results do not substantiate the speculation of Salmon (1914a) that only cleistocarps formed early in the season (in July and August) survive the winter. Indeed they indicate that cleistocarps formed later in the season (although relatively few in number) are potentially the most important, for not only do they discharge ascospores at a time when new leaves are formed by the black currant bush but also the number of ascospores discharged (relative to cleistocarps present) is greater than from any other cleistocarp collection (Table 37).

Although the time at which cleistocarps are formed may be important in relation to the successful perennation of S. mors-uvae, the low number of cleistocarps which discharge spores (<1% of the initial number

with differentiated ascospores) suggests that other factors influence survival substantially. Some of these are indicated in Fig.50..

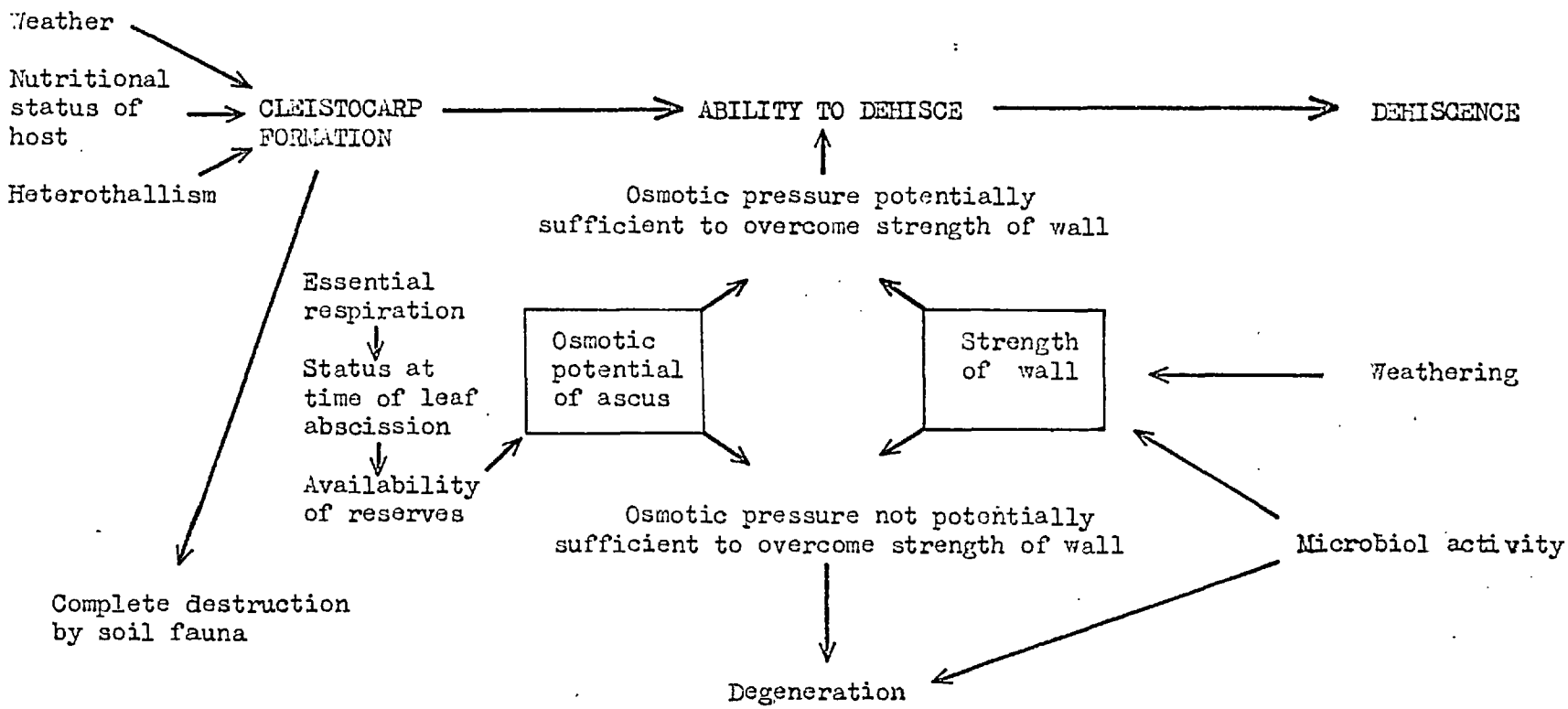
Table 37. Dehiscence of cleistocarps from time of black currant bud burst (1970).

Collection	No. ascospores/1000 cleistocarps
September	31.8
August-September	21.9
July-August	17.6
July	9.3
July-August-September	7.2
August	5.8

Cleistocarp formation on the black currant leaf is initially influenced by three factors: 1) the abundance of compatible (heterothalli strains, 2) the nutritional status of the host, 3) the weather. The present studies show that the fungus is heterothallic (Appendix I).

There are, so far, no critical studies on the relationships between the nutritive status of leaves and cleistocarp initiation, maturation and subsequent ability of cleistocarps to overwinter successfully. Generally, susceptibility to infection and mycelial development are positively related to plant vigour (Yarwood, 1957). For cleistocarp formation several factors have been implicated, for example, low night temperatures (Itoi et al, 1962), host senescence (Schnathorst, 1959; Prota, 1963), dry soil and low relative humidity (Laibach, 1930). That

Fig. 50. Summary of possible factors influencing cleistocarp survival.



the physiological condition of the leaf might be important is indicated in the experiments with leaf discs in controlled environments (Appendix I). Here, cleistocarp development was enhanced when discs were floated on sugar solutions though why this is so remains unknown.

The importance of the weather (especially rainfall and temperature) to cleistocarp development on the leaf and, subsequently, to cleistocarp survival is indicated in the overwintering experiments (p. 14). The August sample, although initially containing 30-40% cleistocarps with ascospores, survival poorly because many cleistocarps were immature when leaves were removed from the bushes on 1 September. This immaturity was perhaps a result of the cooler temperatures during August.

Many cleistocarps are destroyed entirely by soil insects. This was particularly marked in the July collections (p. 21). Other cleistocarps are lost from the leaves because insects remove or weaken the secondary mycelium around the cleistocarps which serves to anchor them to the leaf surface. In the winter months of 1968-69 over two-thirds of the cleistocarps from the leaf-discs were lost in this way and substantial losses from leaves also occurred in the winters of 1969-70 and 1970-71. The fauna most active in these respects are psocids, collembola, insect larvae, small slugs and worms. Many psocids were found on leaves placed on soil in the summer months and their ability to strip leaves of the mycelium and cleistocarps of S. mors-uvae was readily demonstrated in the laboratory. Ectopsoccis briggsi, the psocid commonly associated with the cleistocarps, has no diapause and as it survives the winter as adults or late instar nymphs (New, 1968) cleistocarps and secondary

mycelium may be removed from the leaves during the entire period the leaves are on the soil. Collembola, which are also known fungal feeders (see Butcher, Snider and Snider, 1971; South, 1961) were less common in the leaf litter during the winter months and were probably less important with regard to cleistocarp survival than psocids though, by feeding on secondary mycelium, they probably contributed to the loss of cleistocarps from leaves. The large faecal pellets of other animals (p. 23) also contained cleistocarps so these, too, obviously reduced the population of cleistocarps on the leaves.

Experiments reported here suggest that cleistocarps which are detached from leaves (either in faecal pellets or washed by rain from weakened secondary mycelium) are not important in perennation. In the two winters that cleistocarps were placed in the soil (1968-69 and 1970-71) degeneration occurred earlier than in cleistocarps remaining on the leaves and only in one instance were ascospores discharged. This occurred on 3 February 1971, approximately one month before bud-burst, in a sample remaining on the leaf in the soil. Similar results were reported by Rasulev (1965) for Uncinula necator except he did obtain dehiscence of cleistocarps overwintering in soil apart from host tissue. He suggested that micro-organisms were responsible for the degeneration of cleistocarps on leaves but provided no evidence to support this.

The survival and subsequent dehiscence of cleistocarps that escape destruction by the soil microfauna depend on many, interacting factors but they can be divided into two groups, 1) those which influence the

osmotic potential of the ascus and 2) those affecting the strength of the cleistocarp wall. In the simplest terms, a cleistocarp able to dehisce is one in which the osmotic potential of the ascus is sufficient to overcome the strength of the cleistocarp wall (Fig. 50.).

The present results suggest that after leaf-fall, differentiation within cleistocarps is arrested. This is in agreement with Kulikov (1953) and Merriman and Wheeler (1968). There was no evidence to support the observations of Jordan (1966,1967) that ascospores develop only in the spring. However, as cleistocarps do not dehisce until some considerable time after they are formed it must be assumed that either changes occur to facilitate dehiscence or dehiscence is inhibited - possibly by associated micro-organisms. The latter seems unlikely as cleistocarps which dehisce do so in the spring when microbial activity is greatest. Interest therefore centres on possible changes in the cleistocarp itself and in this respect changes in reserve food materials within the ascus are particularly relevant. Cleistocarps of S. morsuvae have abundant glycogen in the ascus at ascospore differentiation. Observations have shown that, once the wall of the cleistocarp is broken, ability of the ascus to swell and discharge ascospores is associated with a reduced glycogen content. Even samples taken from leaves on the bush contain some cleistocarps that can dehisce in this way and the ascospores have been shown to be viable and infective.

At leaf abscission, cleistocarps on leaves are in various stages of differentiation. Some are no more than unpigmented masses of cells with no distinct structure while others are darkly pigmented and have

fully differentiated walls and asci. Between these two extremes there are all stages of development. Thus, although 50% of the cleistocarps may contain ascospores initially many may not have reached the state of maturity (in terms of reserve materials) which will maintain them through the winter. Essential, respiratory processes may draw upon the reserve materials in many of these cleistocarps before natural dehiscence is possible, the result being that degeneration takes place by autolytic processes i.e. self-digestion by intra-cellular enzymes (Lloyd and Lockwood, 1966). Brian (1960) considers that autolysis brought about by exhaustion of an energy-yielding source may be a common explanation of lysis in fungi. However, many cleistocarps of S. mors-uvae still contain ascospores in the spring with no obvious signs of degeneration yet few discharge spores (p. 49). In these the turgor pressure of the ascus is presumably too low to break the wall and indeed a weakening of the cleistocarp wall may be an essential pre-requisite for natural dehiscence.

Weakening of the wall is possible either by chemical degradation by enzymes of micro-organisms or by the physical processes of weathering during the winter. Chemical analyses showed that about 35-40% of a mature cell-wall preparation of cleistocarps and secondary mycelium consisted of chitin and glucan (β-(1-3) and possibly β-(1-6) linked). This plus the ability of certain bacteria and actinomycetes which were associated with the overwintering cleistocarps to produce chitinases and glucanases (p. 142) suggested that weakening of the wall might take place by enzyme degradation. However, in incubation experiments with a bacterial chitinase and a fungal, β-(1-3) glucanase, hydrolysis of

mature walls was substantially less than that of immature, lightly pigmented, samples. This resistance of mature walls appears to be associated with their high melanin content (c. 25% by weight). The inhibition of lysis by melanin compounds has been reported with different fungi by several workers (Skujins, et al, 1965; Broomfield and Alexander, 1967; Potgieter and Alexander, 1966; Jones, Bacon, Farmer and Webley, 1968; Kuo and Alexander, 1967; Jones, 1970) and for at least one species, Aspergillus nidulans, a progressive increase has been shown to confer increasing resistance to lysis (Kuo and Alexander, 1967). Whether the resistance is a physical shielding of substrates or owing to a chemical complexing of the lytic enzymes is not known.

Much more work is required to demonstrate any such direct relationship between melanin and resistance to lysis in the mycelium and cleistocarps of a powdery mildew and, in this respect, S. mors-uvae is a particularly difficult subject because it is not possible to obtain samples which are uniform with regard to pigmentation or which are free of other micro-organisms.

Complete lysis of cell wall preparations of mature cleistocarps was obtained in liquid media at 25° inoculated with Streptomyces S_1 (p. 161) but it seems unlikely, from other evidence, that cell walls are degraded to anything like this extent during natural overwintering of cleistocarps. For example, although electron micrographs showed that there were many bacteria and fungi within the outer cell walls and, occassionally, there were zones of lysis around bacteria within

cell walls, there was no obvious, extensive degradation of the outer cell walls. Even after 8 months of weathering on soil there was little damage to cleistocarps that could be ascribed to enzyme hydrolysis.

Nevertheless, in some electron micrographs, bacteria were apparent in the central area of the cleistocarp and in these instances the ascus and centrum cells had completely disappeared. In some cleistocarps the walls of some cells appeared to have broken before they were fixed for electron microscopy, possibly as a result of weathering (p. 193). Small cracks such as these might allow the entry of micro-organisms and these could cause degeneration of the central region of the cleistocarp. At present, however, there is no direct evidence to substantiate this and cleistocarps would need to be sectioned from the time of iniation on the leaf and through the winter on the soil to test this hypothesis. As fungi were found in the outer wall cells when cleistocarps were still on the bush it is possible that entry at this early stage after cleistocarp formation can take place by direct penetration of hyphae through the walls. Similarly, the mechanism whereby Trichoderma viride and Coniothyrium minitans penetrates the pigmented rind wall of Sclerotinia sclerotiorum is still unknown (Jones and Watson, 1969).

In the above discussion degeneration has been considered in relation to starvation (i.e. depletion of energy-yielding substrates) and to lysis by micro-organisms. There remains the possibility that it is caused by antibiotics, themselves produced by other micro-organisms. The experiments with urea suggest that, despite the apparent barrier of

the outer cells of the cleistocarps, substances applied at the surface might adversely affect the ascus, for the results indicate that urea has a direct fungi-toxic effect, as it has on the pseudothecium of Venturia inaequalis (Ross and Burchill, 1968; Burchill, 1968). There is some circumstantial evidence to support an antibiotic hypothesis. Actinomycetes were found in association with cleistocarps and when cleistocarps placed on sterile soil at 10° were inoculated with one isolate (S_1) their asci degenerated rapidly. A similar, rapid degeneration occurred when isolated cleistocarps were placed in non-sterile soil. It is also of interest that Cephalosporium spp. were often found on cleistocarps in samples taken directly in the field for some species within this genus e.g. C. gramineum are known to produce antibiotics (Bruehl, Lai and Huisman, 1964). Other species are known hyper-parasites of fungi e.g. Kenneth (1964) has reported that C. acremonium directly invades the conidia, conidiophores and hyphae of Helminthosporium vagans (a fungus with pigmented cell walls) and others are capable of hydrolysing insoluble laminarin (Chester, Apinis and Turner, 1956). Obviously further work is required on these organisms and their effects on asci within cleistocarps.

However, evidence from overwintering studies of other powdery mildews indicate that effects of the microflora may not be important to successful perennation. For example, Cook and Wheeler (1967) found that c. 74% of the cleistocarps of Erysiphe cichoracearum dehisced naturally in the field (at Silwood Park) from November to March. The majority of cleistocarps of this powdery mildew reached maturity at

leaf abscission (in comparison to S. mors-uvae) and it is possible that reserve materials were adequate for survival. With such a high survival rate microflora would seem to play a small role in preventing successful perennation.

The release of ascospores is the final process in the overwintering of cleistocarps and for this moisture and temperature are the most important factors. Temperatures of 5° and over are adequate for ascospore discharge in S. mors-uvae. At 5° dehiscence continues over several days whilst at higher temperatures (e.g. 20°) it is complete in any sample within 12 h. Under these favourable conditions most dehiscence (c. 40-50%) occurred within the first 2 h, a result similar to those obtained by other workers with this and with different powdery mildews (Smith, 1968; Cook and Wheeler, 1967; Merriman, 1968).

Clearly this investigation has raised more problems concerning the perennation of cleistocarps than it has solved and this is especially so with regard to the role of micro-organisms that develop on the leaf litter. Structurally the cleistocarps, with its outer layers of thickened cells, darkly pigmented with melanin, appears well adapted to survive and invites comparison with other fungal resting-structures such as sclerotia. It is the more remarkable that relatively few cleistocarps do survive and function as perennating structures in the sense that they provide viable inoculum for the re-establishment of the fungus on its host.

SUMMARY

1. The number of cleistocarps of S. mors-uvae dehiscing in spring was less than 1% irrespective of the time of formation the previous summer. Dehiscence occurred over a longer period than previously recorded, in some collections beginning in November and December. However, those cleistocarps formed later in the season were considered to be most important as dehiscence coincided with leaf production in the black currant plantation.

2. Soil fauna, especially psocids and collembola, caused the removal of large numbers of cleistocarps and the secondary mycelium from leaves on the soil. This was particularly apparent in samples placed on the soil in the summer months.

3. Observations on the glycogen content of asci indicated that early depletion of this reserve material in cleistocarps wintering on the soil might explain their low survival rate. Comparisons between actual and the potential dehiscence suggested that the difference could be explained by the inability of the ascus to break the cleistocarp wall because of a reduced osmotic potential.

4. Cleistocarps placed in the soil either on or without the leaf failed to survive. It was considered unlikely that the large numbers of cleistocarps that are lost from the leaf surface (c. 2/3) are important in perennation of the fungus.

5. Cleistocarps borne on the stems of gooseberry degenerated by mid-November in the season of formation, substantiating earlier reports.

Those borne on leaves or stems placed on the soil survived and the ascospores infected gooseberry and black currant leaves.

6. Some cleistocarps collected at leaf abscission contained asci capable of swelling and discharging ascospores (once the wall was artificially split) which were viable. This together with the observation that cleistocarps survived well at -18° for 3 years may provide a means of maintaining cultures of S. mors-uvae over long periods of time.

7. Ascospore discharge was greatest at 20° and most spores, 40-50%, were released in the first 2 h after wetting either at 5° or 20°. At 5° discharge continued over several days whilst at 20° it was complete in 12 h.

8. Urea applied to leaves bearing cleistocarps after leaf abscission and in the spring stimulated microbial activity but failed to sustain the high populations. Urea was quickly lost from the leaves and even when degeneration resulted after its application it was considered to have been caused by a direct fungitoxic effect on the ascus wall. Leaves of black currant colonised by S. mors-uvae were observed to be extremely resistant to decomposition following urea treatment.

9. A Streptomyces sp. caused total degeneration when inoculated into soil tubes containing cleistocarps at 10°. Lysis of mycelium was not apparent although the isolate had the ability to produce chitinase and glucanase enzymes.

10. Mycelium and cleistocarps were taken from leaves bearing two different stages of S. mors-uvae development. One was lightly pigmented and immature, the other darkly pigmented and mature. Cell wall preparation were made and chemical analyses revealed that they contained hexosamine

(c. 10-13%) and hexan (c. 25-35%). The hexan component contained glucose, galactose and mannose with glucose the major constituent. Analysis of the hexosamine revealed that chitin (n-acetyl glucosamine) was the major component.

11. Analysis of the pigment of mature cleistocarps and secondary mycelium showed that melanin in a complex with glucosamine and protein was present (26% of cell wall weight). Melanin alone constitued c. 20%.

12. Organisms were not isolated capable of lysing a mature cell-wall agar, although lysis was obtained from a number of bacteria and actinomycetes (isolated from leaf washings) grown on an agar made from immature cell walls. Some of the lytic organisms were capable of producing chitinase and β-glucanase enzymes.

13. Enzyme hydrolysis with chitinase and glucanase confirmed the estimations made by direct chemical analysis of the chitin and glucan component of the cell walls. The glucan component was predominantly β-(1-3) linked although the presence of β-(1-6) linkages was possible.

14. Incubation experiments using chitinase and glucanase revealed that the mature cell wall preparations were more resistant to enzyme hydrolysis.

15. Penicillium javanicum and a Streptomyces sp. grown on cell wall preparations produced chitinase, β-(1-3) and β-(1-6) glucanase enzymes. Complete lysis of the walls occurred only with the Streptomyces sp. indicating that these three enzymes alone could cause reduction in wall thickness but not total hydrolysis of wall components.

16. Ultra-thin sections of mature and immature cleistocarps were made and viewed in the transmission E.M. Sections of naturally wintered cleistocarps showed the presence of micro-organisms in the wall cells and central region but degradation of the walls was slight. There was little evidence of direct penetration of micro-organisms but natural breaks, observed in the wall cells, may facilitate entry.

17. The results are discussed in relation to factors which might affect survival of cleistocarps from the time of initiation on the leaf until dehiscence the following spring.

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APPENDIX I

Studies on cleistocarp formation in S. mors-uvae.

Review of literature.

The formation of cleistocarps by powdery mildews has variously been attributed to the nutritive status of the host (Graf-Marin, 1934; Prota, 1963), the environmental conditions of the host plant and the mildew (Laibach, 1930; Jhooty and McKeen, 1962; Itoi, Nakayama and Kubomura, 1962) and the presence of two strains. Evidence that compatible mating strains (heterothallism) were necessary for cleistocarp formation in the powdery mildews was first obtained by Yarwood (1935) for Erysiphe cichoracearum on sunflower. Since then many other members of the Erysiphaceae have been found to be heterothallic, for instance E. cichoracearum on lettuce (Schnathorst, 1959) and on Zinnia elegans and Helianthus annus (Morrison, 1960), E. graminis on barley (Hiura and Tomada, 1959), E. palygoni on Lupinus sp., Heracheum sphondylium, Lathyrus pratensis and Pisum sativum, Microsphaera penicillata on Lathyrus ochroleucus and Uncinula necator on Pathenocissus sp. (Smith, 1970,1971). Within the genus Sphaerotheca, some strains of S. fuliginea have been shown to be homothallic (Homma, 1933) others heterothallic (Homma, 1937).

There have been few investigations into the initiation of cleistocarps of S. mors-uvae. Merriman (1968) produced cleistocarps on potted plants of black currant grown at 20° but not on plants at 10°. However, even at 20° the cleistocarps were not formed on all inoculated plants even though mildew grew well and sporulated abundantly. Griffee

(1967) investigated the possibility that cleistocarps of S. mors-uvae form in response to senescence of host tissue. Plants were heavily inoculated and subjected to several treatments leading to water stress but in none did cleistocarps develop.

Cleistocarp formation was investigated in S. mors-uvae not only to discover whether the fungus was homo- or heterothallic but also to determine whether cleistocarps could be produced readily on leaf tissue so that their survival could be investigated at different times of the year and under various environmental conditions.

Materials and methods.

i. Maintenance of conidial isolates.

Five conidial isolates, whose origin is given below were maintained on either whole leaves or leaf discs 2 cm^2 cut from the petiole end of the lamina. The discs or whole leaves were floated on distilled water in 9 cm Petri dishes and incubated at 10° in an illuminated cabinet with a 16 h photoperiod (c. 5000 lumens/m^2). Sub-cultures were made every 4-5 weeks.

Isolate no.	Conidia derived from:	Location	Date
1*	Ascospore inoculation (B.C.)	Silwood Park	1968
2	Bud infection (G.)	Silwood Park	1968
3	Bud infection (G.)	Silwood Park	1969
4	Bud infection (G.)	East Malling	1969
5	Leaf infection (B.C.)	Reading	1969

* isolate used by Merriman (1968)

B.C.- Black currant

G.- Gooseberry.

Leaves were taken from black currant plants maintained in a greenhouse under mercury vapour lights (c. 6500 lumens/m^2) for 16 h/day at 10-20°. The plants were grown from cuttings c. 20 cm in length taken from the plantation at Silwood Park, rooted in tap water and transferred after 6-8 weeks to 10 cm pots. Leaves were produced c. 8 weeks later and in all experiments the third leaf was used, i.e. the first fully expanded leaf of the plant. Leaves were also used, in the spring, from the

cultivars Amos Black and Wellington grown in the plantation. The leaf surface was always carefully inspected to ensure that it was uninfected.

ii. Inoculations of isolates onto leaf tissue.

Leaves bearing sporulating colonies were subjected to strong blasts of air from a teat pipette 48 h before use to remove old conidia and to standardise the age of inoculum. Fresh leaf material was inoculated on the abaxial surface by placing it at the base of a cardboard inoculating tower and allowing conidia, blown from an infected leaf held at the top, to settle on it (Griffee, 1967). This reduced clumping of conidia. The inoculated leaf material was floated on distilled water containing 20 ppm kinetin (6-furfurylaminopurine) (Merriman and Wheeler, 1968) either in 9 cm diameter Petri dishes or in polystyrene boxes (21.5 x 10.5 x 7.5cm). Streptomycin at 20 ppm was also included in some experiments to reduce bacterial contamination of the leaf discs. Cultures were incubated at 15° in a 16 h day (c. 4300 lumens/m^2) for 2-3 weeks. When crosses were made between isolates separate inoculating towers were used and the bench top and all instruments were sterilised with methyl alcohol after each inoculation.

iii. Production of single-chain conidial isolates.

Twenty single-chain inoculations of conidia from the five isolates were made using an eye-lash fixed to a glass rod (Smith, 1970). After 10-20 days one disc of each isolate was chosen and re-inoculated onto fresh leaf material.

Experimental.

1. Crosses of isolates inoculated on leaf tissue incubated on distilled water.

Conidia from the five cultures were inoculated onto leaf tissue in an attempt to produce cleistocarps as follows:

i. Sets of ten leaf discs were each inoculated with two of the five cultures so that crosses were made of all ten combinations.

ii. Similar inoculations were made onto pieces of leaf petiole. These were placed on moistened filter paper in the base of polystyrene boxes.

iii. Similar inoculations were made on the abaxial surface of leaf discs.

iv. Ten leaf discs were each inoculated with all five cultures. Control discs were inoculated with one isolate only.

No cleistocarps formed in any of these experiments although in crosses of isolates 2 x 5 and 2 x 3 in experiment i secondary mycelium was produced after 12 days. Spore development of this mycelium also occurred with isolates 2 and 5 alone. Experiment i was repeated with whole leaves but no cleistocarps formed although the mildew and leaf tissue remaind healthier and without contamination for a longer (c. 7 days) period than leaf discs.

2. Crosses of isolates inoculated on leaf tissue and incubated on sucrose solutions.

Five leaves were inoculated on the abaxial surface with conidia from all five cultures and incubated at 15° on the solutions indicated below. The numbers of cleistocarps formed after 21 days are given in Table 1.

Table 1. Cleistocarps developed on sucrose solutions.

Conc. sucrose (%)	Kinetin 10 ppm	Streptomycin	No. c'carps
a. 10	+	+	802
b. 1	+	+	72
c. 0.1	+	+	11
d. 10	-	+	1726
e. 1	-	+	5
f. 0.1	-	+	0
g. 0	+	+	0
h. 0	-	+	16
i. 0	+	-	0

Cleistocarps were seen after 10 days and a few turned dark brown and were counted before the leaves became completely contaminated. The large number of cleistocarps formed with 10% sucrose contrasted markedly with the few cleistocarps in the other treatments though, for the first time some cleistocarps were formed on leaf tissue incubated on distilled water. Kinetin did little to restrict contamination by delaying leaf senescence and it was not used in further experiments.

Concurrently with this experiment all five cultures were inoculated onto three potted plants and separately onto five more. All plants inoculated with the five cultures bore secondary mycelium after 10 days in which cleistocarps were developing; they were formed particularly on the new extension growth of the plants. None was found, however, on the plants inoculated with conidia from single isolates.

3. Conidial crosses to determine if S. mors-uvae is heterothallic.

The experiment above strongly indicated that not only were cleistocarps induced by changes occurring within the leaf caused by incubation on high sucrose solutions but that more than one conidial isolate was required before cleistocarps formed. Therefore, the isolates were crossed in pairs as in experiment 1i except that leaves were floated on a 10% sucrose solution.

Cleistocarps were formed only in crosses 3 x 4 and 2 x 5. In none of the others or in the single inoculations were they produced. This was strong evidence that S. mors-uvae was heterothallic on black currant leaves.

4. Production of cleistocarps on whole leaves on 10% sucrose, 5% glucose and water at 20° and 15°.

The effects of sugars on the leaf tissue and the total number of cleistocarps formed were observed in greater detail. Ninety black currant leaves were inoculated with S. mors-uvae using as a source of inoculum leaves from the plantation bearing conidia and cleistocarps. After inoculation these were divided into six lots of fifteen leaves. Three sets of fifteen leaves were incubated on either 10% sucrose, 5% glucose or distilled water at 15°. Comparable sets of leaves were incubated at 20°.

Cleistocarps were visible after 12 days on the leaves on sucrose and glucose at 15°; these appeared as silvery-white structures borne in a mycelium which was still sporulating. No cleistocarps were observed

on leaves on distilled water at 15° or on sucrose, glucose and distilled water at 20°. After 7days the leaves at 20° on the sugar solutions became pale-yellow and brittle. Contamination by species of Botrytis, Penicillium and Cladosporium was characteristic of incubations at this temperature and the mildew was quickly over-run by these fungi. Leaves at 15° also showed signs of contamination and the effect of being incubated on high concentrations of sugars and after 14 days these became pale yellow and contaminated. The control leaves, incubated on distilled water remained green c. 2 weeks after inoculation, although at this time the mildew began to senesce. This was in contrast to the mildew which developed on leaves on glucose and sucrose where, although the contamination was greater and the leaves appeared to have been effected by the sugar solutions, sporulation continued for a further 6-7 days.

Cleistocarps on sucrose were almost fully developed in size after 3 weeks from inoculation and a few turned from pale yellow to dark brown and contained asci but few contained ascospores. After 23 days contamination was too severe for further observations. The number of cleistocarps were counted and are given in Table 2 .

Analysis of the results showed that the differences between the 10% sucrose and 5% glucose were not significant ($P > 0.05$), whereas there were significant differences between the sucrose/glucose and water results ($P < 0.002$ and $P < 0.02$ respectively).

Table 2. Cleistocarps formed on whole leaves incubated on sucrose, glucose and water.

10% sucrose	5% glucose	Water
415*	16	0
42	120	2
389	10	6
255	19	0
402	85	0
75	487	3
83	14	21
17	0	10
31	56	26
764	92	0
1506	36	0
625	583	0
171	166	85
341	406	126
685	853	93
Totals 5801	2943	373

* Numbers relate to cleistocarps formed per leaf.

5. Production of cleistocarps on leaf discs on 10% sucrose, 5% glucose and water.

Leaf discs were used in place of whole leaves to reduce the variation in number of cleistocarps formed per unit area of leaf tissue. The experimental procedure was essentially the same as in 4 except that incubation of the three lots of fifteen leaf discs was at 15°. All discs were taken from leaves of similar ages and cut from the petiole region of the lamina.

As in the last experiment, the period of sporulation on the control leaves was considerably shorter than in the sugar treatments; complete senescence of the mycelium in the control occurring after 22 days whilst sporulation continued on the other discs until the end of the experiment, 3 weeks from inoculation. At this time leaf discs floated on sugar solutions were severely contaminated whereas those on water were not.

The development of cleistocarps was similar to the last experiment; they were observed on some discs after 10 days and on all those incubated on the sugar solutions after 12 days. Their subsequent development was hindered, however, by the contamination of the discs and few contained differentiated asci. The numbers of cleistocarps developed per disc was not more uniform within the treatments (Table 3) than on whole leaves.

The discs were uniformly inoculated and in all treatments the entire areas of the discs became colonised by mycelium at 96 h. But, on many discs there were areas where no cleistocarps developed. It was not known whether this was caused by unequal distribution of mating strains or

variations in the effect of the sugar solutions on leaf cells indirectly influencing cleistocarp development.

Table 3. Cleistocarps formed on leaf discs incubated on sucrose, glucose and water.

Replicate	10% sucrose	5% glucose	Water
	62	69	0
	196	210	0
1	209	157	0
	102	3	4
	173	28	2
Totals	742	667	6
	203	80	10
	65	18	0
2	97	272	5
	81	11	0
	165	38	0
Totals	611	419	15

6. Comparison of mycelial growth on sugar solution and water.

Leaf discs 2 cm^2 were inoculated with conidia as in 4 and floated on 10% sucrose, 5% glucose and distilled water. Twenty-five leaf discs were used for each treatment and inoculation was at 15°. Streptomycin (20 ppm) was included but not kinetin. Celloidin strips (Wenzil, 1939) were taken of the leaf surface of five discs from each treatment at

24, 48, 72 and 96 h after inoculation and assessments made of percentage germination, the number of germ tubes and their lengths and mycelial development.

From the results of the experiment several points are of interest:

(1) There was uniform inoculation of discs and mycelium was well established at 72 h.

(2) At 72 h initiation of conidiophores began on the discs on water. There were few formed on the leaves on sugar solutions.

(3) At 96 h the mycelium on leaf discs incubated on water had produced abundant conidiophores and many had a generative cell and 3-4 conidia. Discs on glucose bore mycelium with conidiophores but few had more than two conidia (including generative cell). In the sucrose treatment there were few conidiophores and none of the discs bore conidia.

(4) The number of conidia germinated after 24 h and 48 h is given in Table 4.

Table 4. Percentage germination at 24 and 48 h on sucrose, glucose and water.

Time (h)	Treatment	No. counted	No. germinated	%*
24	Sucrose	2633	2052	78.0
	Glucose	2453	1823	73.2
	Water	2588	1900	73.4
48	Sucrose	2448	1967	80.1
	Glucose	2377	1986	80.2
	Water	2573	2145	83.4

* Mean of 5 replicates of c. 500 conidia.

Analysis of the results showed that there was no difference in the percentage germination between the treatments at 24 and 48 h although there was a significant difference ($p < 0.001$) between germination at 48 h and that at 24 h. Table 4 shows that this difference was small and represented a mean increase after 24 h of 2-7%.

There was no difference at 24 h between the number of conidia with two germ tubes, nor any difference in the germ tube length of those conidia with one or two germ tubes incubated on the different solutions (Table 5).

Table 5 . Measurement of germ tubes of conidia at 24 h.

Time (h)	Treatment	Length of germ tubes µm *	
		1 germ tube	2 germ tubes
24	Sucrose	23.8	44.8
	Glucose	23.1	46.9
	Water	24.2	47.3

* Mean of 20 measurements taken on 5 leaf discs.

(5) The effects of incubation on sugar solutions became apparent at 48 h. Measurements of germ tubes from 48 h conidia (Table 6) showed that there were significant differences between sucrose/glucose and water treatments in the percentage of conidia with two germ tubes ($p < 0.05$) and three germ tubes ($p < 0.01$).

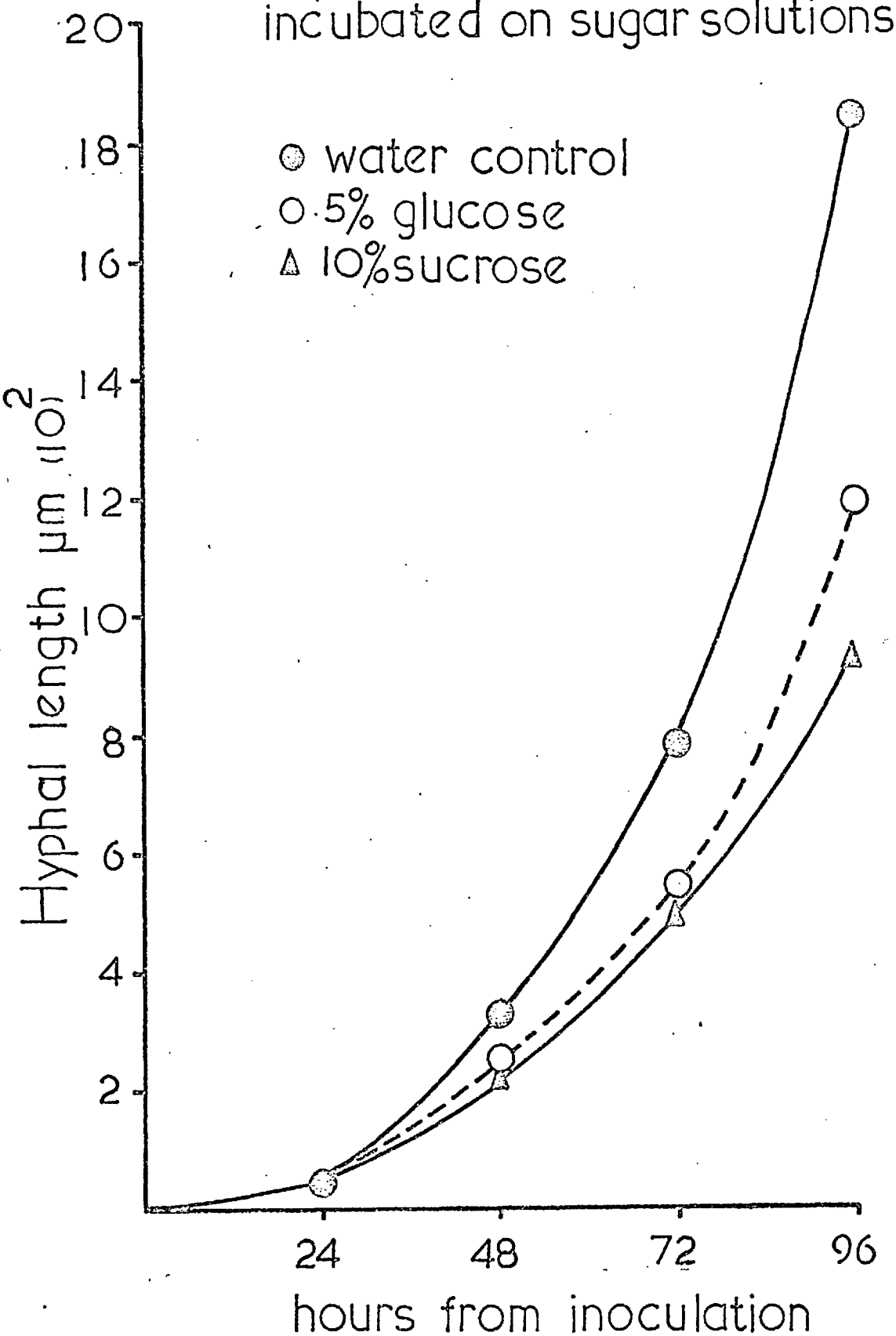
Table 6. Percentage conidia with two and three germ tubes.

Time (h)	Treatment	% conidia *	
		2 germ tubes	3 germ tubes
24	Sucrose	31.7	0
	Glucose	34.9	0
	Water	37.9	0
48	Sucrose	48.2	10.9
	Glucose	47.8	15.5
	Water	26.9	35.1

* Mean of c. 300 conidia observed on each of 5 leaf discs.

(6) The maximum development of 100 germ tubes at 48 h and 50 germ tubes (mycelium) at 72 and 96 h was measured. The increase in mycelial development in the three treatments is given in Fig. 1. At 96 h incubation, growth on discs floated on water was twice that on sucrose solutions. The measurements taken of the growth of hyphae on the three solutions were transformed to logarithms and regression coefficients calculated. Comparison of the coefficients revealed that only between the control and sucrose results was there a statistical difference ($p < 0.05$). This suggested that there were differences in the growth rates of hyphae on water and on the sugar solutions although in the early stages of growth variation within the treatments was relatively larger compared with that between treatments; only after 96 h incubation were the treatments substantially effecting growth rates.

Fig.1. Hyphal growth on leaves incubated on sugar solutions



Discussion.

Smith (1970) has suggested that heterothallic mildews might be expected to form cleistocarps frequently and relatively early in spring due to the presence of the different mating types within the one ascus, as found for instance in E. graminis tritici (Powers and Moseman, 1956). Failure of cleistocarps to overwinter would mean a delayed build-up of the appropriate mating types (perhaps derived from distant sources) and there would therefore be a tendency for late cleistocarp development. Observations on S. mors-uvae in a large planting of black currants at Southmoor, Oxfordshire substantiate the first view. Here in the two years 1969 and 1970 cleistocarps were produced in large numbers in June. However, in a small planting at A.D.A.S., Reading the bushes were heavily infected with S. mors-uvae in late June and secondary mycelium was observed on 2 July but not until the end of August were cleistocarps found on a few leaves of the bushes. Similarly, in the 3 years 1969, 1970 and 1971 cleistocarps on gooseberry bushes at Silwood Park were produced relatively late in the host growing season (late July - early August). This late development of cleistocarps is similar to their formation on Pisum sativum by E. polygoni (Smith and Wheeler, 1969), and probably reflects the presence of only a single strain or the incompatability of several strains of powdery mildew in the planting. With S. mors-uvae not only is the presence of compatable mating strains important but also the numbers of cleistocarps that survive the winter to produce inoculum the following year. In contrast to E. cichoracearum

on Arctium lappa (Cook and Wheeler, 1967) and E. polygoni on Heracleum sphondylium (Smith and Wheeler, 1969) very large numbers of cleistocarps degenerate in the spring and do not release ascospores. Probably no more than 1 or 2 every thousand formed release ascospores. Because of this low survival rate S. mors-uvae may exhibit both early and late cleistocarp formation depending upon the survival of cleistocarps in any particular plantation.

It is difficult to interpret the results of experiments in which cleistocarps developed on leaves and leaf discs incubated on sugar solutions. It was unlikely that the sugars were providing direct nutrient benefits to the mycelium as hyphal growth was considerably restricted during the early stages of development under these conditions (on leaf discs floated on sucrose and glucose). A similar effect was reported by Cole (1966) for potassium-deficient tobacco leaves inoculated with E. cichoracearum and incubated on 10% sucrose. Here soluble carbohydrate increased rapidly but hyphal growth was reduced. It was noted that although the leaves inoculated with S. mors-uvae were affected by the sugar solutions (becoming brittle and pale-yellow) mycelium continued to sporulate for c. 7 days longer than that on water. However, this longer survival was not implicated in cleistocarp initiation only in cleistocarp maturation. In all the experiments cleistocarps were first seen after only 10 days. At this time there were no noticeable differences between mycelial development and sporulation on the leaves on sugar solutions and distilled water.

One of the effects of incubation on sugars was to increase the OP of the cell sap and a 10% sucrose solution (0.3 M) caused c. 15-25% plasmolysis of the epidermal cells of black currant. Smith (1970) found a 50% increase in cleistocarps production by E. polygoni when pea leaf discs were incubated on 10% sucrose; this he attributed to increased carbohydrate of the leaf tissue available for fungal growth. He found that with incubation on Asterlaevis and Lathyrus ochroleucus, sucrose solutions reduced the number of cleistocarps formed although the OP was the same as in pea. He therefore considered that inhibition of mycelial growth on A. laevis and L. ochroleucus but not on pea could not be explained in terms of osmotic changes in the host. Weinhold and English (1964) studied the resistance of peach leaves to Sphaerotheca pannosa var. persicae when floated on high molar solutions of sucrose and found that susceptible leaves became resistant and the OP of cells increased. They could not however, attribute this change in resistance to an OP effect as detached leaves were found to be susceptible when the OP was above that found in resistant orchard leaves. If incubation on sugar solutions tends towards a restriction of growth of mildews then changes in host cells other than that of OP must be considered likely to promote development of cleistocarps. The very pale yellow of black currant leaves indicated that they were senescing c. 6 days after incubation. This would be accompanied by a reduction in assimilation as the rate of photosynthesis declines. During progressive yellowing there is a corresponding fall in protein-nitrogen (Michael, 1936) associated with the degradation of chlorophyll (Leopald, 1964) in the leaf tissue. In this

connection Weinhold and English (1964) reported that normally susceptible leaves of peach have higher amino-acid and lower sugar content than resistant leaves. Similar analysès for black currant tissue is required on both aging leaf tissue on the plant and the leaves incubated on sugar solutions. Only in this way can the observed effects on the leaves be linked to metabolic changes within the cell influencing cleistocarp formation.

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APPENDIX II

Media

For total counts of bacteria and yeasts: (Crosse et al. 1968).

	% (w/v)
Nutrient broth (Difco)	0.5
Yeast extract (Difco)	0.1
Glucose	0.1
Ionagar No. 2 (Oxoid)	1.5

pH 6.8-7.0.

For total fungi.

Czapek-Dox Yeast agar (C.M.I. Plant Pathologists Pocketbook).

	% (w/v)
Sodium Nitrate	0.3
Potassium dihydrogen phosphate	0.1
Magnesium sulphate	0.05
Potassium chloride	0.05
Ferrous sulphate	0.001
Sucrose	3.0
Agar (Davis)	1.5
Zinc sulphate	Trace
Copper sulphate	Trace

pH 4.0 adjusted with phosphoric acid.

Chitinoclastic organism (Veldkamp, 1955).

	% (w/v)
Chitin	0.2
Magnesium sulphate	0.1
di Potassium hydrogen phosphate	0.1
Bacto-agar (Difco)	1.5

pH 7.0

poured over c. 10 ml basal layer of 1% tap water agar.

For isolation of lytic organisms (Potgieter and Alexander, 1966).

Cell walls	0.15 % (w/v)
Magnesium sulphate	0.06
di Potassium hydrogen phosphate	0.07
Potassium di hydrogen phosphate	0.03
Calcium chloride	0.005
Potassium nitrate	0.40
Ferric chloride	Trace
Zinc sulphate	Trace
Soil	0.1

pH 7.0.

Cell wall inorganic salts media (Skujins et al, 1965).

Cell walls	0.001% (w/v)
di Potassium hydrogen phosphate	0.08
Potassium dihydrogen phosphate	0.02
Ammonium sulphate	0.05
Magnesium sulphate	0.02
Ferric chloride	Trace
Calcium chloride	Trace
Zinc sulphate	Trace

pH 7.2

Cell-wall agar.

	% (w/v)
Cell walls	0.15
di Potassium hydrogen phosphate	0.10
Magnesium sulphate	0.10
Bacto-Difco agar	1.5

pH 7.0

poured over a basal layer (c. 10 ml) of 1% tap water agar.

Glycerol-Asparagin agar (for actinomycetes).

	% (w/v)
Glycerol	1.0
Asparagine	0.1
di Potassium hydrogen phosphate	0.1
Agar (Davis)	2.0

pH 7.0

V8 Juice-agar.

	% (v/v)
'V8' vegetable juice	20.0
Agar (Davis)	2.0
Water	80.0

pH 6.0

For production of -(1-3) glucanase (Reese and Mandels, 1959).

	% (w/v)
Potassium dihydrogen phosphate	0.2
Ammonium sulphate	0.14
Urea	0.03
Magnesium sulphate	0.03
Calcium chloride	0.03
Yeast extract	0.01
Ferric chloride	Trace
Manganese sulphate	Trace
Cobaltous sulphate	Trace
Zinc sulphate	Trace

Table 1. Overwintering of cleistocarps in relation to time of formation; ascospore discharge at 15° and at [illegible] field temperatures.
a) Field temperatures.

Date	J				A				S				JA				AS				JAS			
	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72
5.1.70	1266	0	0	0	1945	0	0	0	1051	0	0	0	1502	0	0	0	1846	0	0	0	3980	0	0	0
13.1.70	2319	36	2	0	1621	0	0	0	1248	0	0	0	1896	12	0	0	2181	0	0	0	1957	8	0	0
20.1.70	2400	8	106	0	2016	0	0	0	1002	0	0	0	1355	0	62	8	2877	0	16	7	2003	0	27	0
3.2.70	2387	0	30	0	2539	12	0	0	842	0	0	0	2342	28	0	0	2445	8	0	0	1840	11	0	0
10.2.70	887	0	0	0	1609	0	0	0	562	17	13	0	2115	0	0	0	1588	3	4	0	4768	2	3	3
17.2.70	1188	0	0	0	1813	33	9	0	1429	6	6	10	3935	8	9	1	2748	1	0	5	1415	54	23	8
24.2.70	911	0	0	0	1951	0	0	0	651	0	0	0	1843	4	0	6	3412	0	0	8	930	0	0	0
3.3.70	1606	0	0	0	1940	0	2	4	771	0	0	0	1465	0	0	2	2801	0	6	9	1816	0	0	5
10.3.70	1089	0	0	0	1814	0	14	0	352	0	0	0	3862	14	20	0	2943	34	12	0	2323	11	6	0
17,3,70	1572	7	0	0	2482	14	4	0	1561	9	4	1	2672	94	9	0	814	10	0	0	3219	72	19	0
24.3.70	3411	94	13	7	2167	11	0	0	831	63	8	0	3064	79	7	0	2818	228	5	4	2225	10	0	0
31.3.70	1248	6	0	0	561	0	0	0	938	11	33	0	1115	1	0	2	2152	12	12	0	3039	0	11	0
7.4.70	3283	0	0	2	1173	2	0	0	1043	41	25	6	2488	21	24	8	1470	5	4	0	2408	0	0	0
14.4.70	1590	5	0	0	2087	6	0	0	479	7	0	0	1546	4	0	0	1245	16	0	0	1575	0	0	0
21.4.70	656	0	0	0	2402	28	0	0	829	8	0	0	305	8	0	0	926	0	0	0	1996	0	0	0
28.2.70	1980	0	0	0	1280	0	0	0	1154	0	0	0	957	0	0	0	2649	0	0	0	1370	0	0	0
Totals	27793	156	121	9	28120	106	27	4	14743	154	89	17	35208	273	131	27	34915	317	59	33	36864	168	89	16

Table 1 (continued). b) 15°.

Date	J				A				S				JA				AS				JAS			
	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72	No. c'carps	Hours 24	48	72
9.12.70	1002	6	0	0	N.R.	-	-	-	N.R.	-	-	-	N.R.	-	-	-	N.R.	-	-	-	N.R.	-	-	-
5.1.70	763	5	0	0	2003	0	0	0	1430	0	0	0	1511	0	0	0	2075	2	0	0	2635	12	0	0
3.1.70	1366	55	0	0	1746	0	0	0	916	0	0	0	2004	16	0	0	2514	0	0	0	2164	8	0	0
0.1.70	2220	0	38	0	1842	0	0	0	859	0	0	0	1580	18	0	0	3642	0	0	0	2291	7	0	0
3.2.70	2434	0	5	0	2801	12	0	0	916	0	0	0	2117	56	0	0	2528	12	0	0	1456	5	0	0
0.2.70	690	0	0	0	1749	0	0	0	633	9	0	0	1816	0	0	0	1481	0	0	0	3939	0	0	0
7.2.70	1140	0	0	0	1511	33	7	0	1665	54	0	2	3047	30	2	0	1472	33	0	0	1961	41	0	0
4.2.70	749	0	0	0	1781	2	0	0	616	0	0	0	1850	8	3	0	3224	12	22	0	759	1	0	1
3.3.70	1434	0	0	0	1694	0	0	0	745	0	9	1	1235	0	0	0	2567	0	12	0	1756	0	27	0
0.3.70	1187	5	0	0	2404	38	0	0	632	0	0	0	2867	19	0	0	2780	73	0	0	2507	13	0	0
7.3.70	1421	12	0	0	2506	21	0	0	1242	6	0	0	3681	34	1	0	544	6	0	0	3707	9	0	0
4.3.70	3755	48	0	0	1977	3	0	0	811	58	3	0	2643	127	0	0	2795	130	6	0	2164	9	0	0
1.3.70	1293	0	0	0	503	0	0	0	880	5	0	0	1504	0	0	0	2555	15	0	0	3056	3	0	0
7.4.70	3211	5	0	0	1253	0	0	0	1045	36	0	2	2186	23	0	0	1900	0	0	0	2227	0	0	0
4.4.70	1165	0	0	0	2169	23	0	0	412	0	6	0	1569	11	00	0	658	0	0	0	1765	4	0	0
1.4.70	649	0	0	0	3859	21	0	0	1001	5	0	0	2247	0	0	0	851	0	0	0	1745	0	0	0
8.4.70	2046	0	0	0	1068	0	0	0	1581	0	0	0	1027	0	0	0	2344	0	0	0	1356	0	0	0
Totals	25525	130	50	0	30866	153	7	0	15384	173	18	5	32884	342	6	0	33930	283	40	0	35488	140	27	1

N.R. No reading.

Table 2. Ascospore discharge at different temperatures.

a) 5°

Date	No. cleistocarps	No. spores	%	Angular transformation	No. swollen
	5639	340	6.03	14.2	18
27.2.71	4950	179	3.62	10.9	33
	4230	209	4.94	12.8	37
	6281	223	3.55	10.9	11
15.3.71	6256	299	4.78	12.7	11
	4872	97	1.99	8.1	12
	5553	66	1.19	6.3	4
9.4.71	7304	47	0.64	4.4	1
	6300	129	2.05	8.3	3
	51385	1789	3.48	9.84	130

b) 10°

Date	No. cleistocarps	No. spores	%	Angular transformation	No. swollen
	4638	275	5.92	14.1	0
27.2.71	3938	92	2.33	8.7	3
	4606	200	4.34	12.0	3
	4294	559	13.02	21.1	3
15.3.71	3262	179	5.49	13.6	7
	4265	200	4.69	12.5	3
	6504	481	7.40	15.8	1
9.4.71	6263	130	2.08	8.3	5
	5681	135	2.38	8.9	0
	43451	2151	4.95	12.78	25

Table 2. (Continued)

c) 15°

Date	No. cleistocarps	No. spores	%	Angular transformation	No. swollen
	5110	992	19.14	26.1	3
27.2.71	3681	897	24.37	29.6	2
	3946	680	17.23	24.5	2
	4329	540	12.47	20.7	0
15.3.71	5572	613	11.00	19.4	1
	4550	808	17.76	25.0	3
	6730	428	6.36	15.0	0
9.4.71	8210	214	2.61	9.3	1
	7474	772	10.33	18.7	0
	49602	5392	10.87	20.92	12

d) 20°

Date	No. cleistocarps	No. spores	%	Angular transformation	No. swollen
	4699	1530	32.56	34.8	2
27.2.71	4869	779	16.00	23.6	6
	5456	976	17.89	25.0	1
	5961	1161	19.48	26.2	
15.3.71	6447	1437	22.29		0
	4651	773	16.62		1
	6364	351	5.52	13.6	0
9.4.71	6667	369	5.53	13.6	0
	7210	511	7.09	15.5	0
	52324	7887	15.07	22.72	11

Table 2 . (Continued)

e) 25°

Date	No. cleistocarps	No. spores	%	Angular transformation	No. swollen
	6213	432	6.95	15.3	0
27.2.71	6983	363	5.20	13.2	0
	6163	274	4.45	12.3	1
	6202	268	4.32	12.0	0
15.3.71	4013	57	1.42	6.8	0
	5057	169	3.34	10.5	0
	8336	312	3.74	11.1	0
9.3.71	8941	584	6.53	14.8	1
	6754	244	3.61	10.9	0
	58662	2703	4.60	11.88	2

Table 3. Ascospore discharge per hour at 20° and 5°.

a) 20°

Hour	A	B	C	Total/h	% of total
1	256	68	103	427	40.4
2	51	33	57	141	13.3
3	18	26	10	54	5.1
4	45	13	25	83	7.8
5	15	24	24	63	6.0
6	39	19	14	72	6.8
7	29	0	22	51	4.8
8	14	19	15	48	4.6
9	16	21	11	48	4.6
10	0	8	8	16	1.5
11	19	16	0	35	3.3
12	16	4	0	20	1.9
	518	251	289	1058	

b) 5°

Hour	A	B	C	Total/h	% of total
1	61	51	31	143	40.9
2	5	4	19	28	8.0
3	11	4	15	30	8.6
4	17	2	2	21	6.0
5	13	5	7	25	7.1
6	5	8	5	18	5.1
7	6	0	5	11	3.1
8	6	0	10	16	4.6
9	10	11	10	31	8.9
10	13	5	4	22	6.3
11	4	1	0	5	1.4
12	0	0	0	0	0
	151	91	108	350	

Table 4 . Ascospore discharge at 20° and 5° over 5 days.

a) 20°

Time (h)	A	B	Total	%
2	200	191	391	43.7
4	61	57	118	56.9
6	31	58	89	66.8
12	105	43	148	83.4
24	50	26	76	91.8
36	7	6	13	93.3
48	12	12	24	96.0
60	12	12	24	98.7
72	1	0	1	98.8
96	0	8	8	99.7
120	3	0	3	100.0
144	0	0	0	100.0
Totals	482	413	895	

b) 5°

Time (h)	A	B	Total	%
2	10	18	36	18.4
4	0	4	4	20.4
6	0	8	8	24.5
12	8	10	18	33.7
24	12	18	30	49.0
36	14	1	15	56.6
48	6	1	7	60.2
60	0	19	19	69.9
72	8	3	11	75.5
96	3	31	34	92.9
120	0	3	3	94.4
144	3	8	11	100.0
Totals	64	124	196	

Table 5. Total bacteria and yeast autumn urea 1968-69. Treatment 1) 0.01% Spreadite.

Replicates	1/10		15/10		29/10		12/11		26/11		9/12		6/1		3/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	0.016	5.176	0.32	6.505	0.94	6.973	4.50	7.653	3.69	7.567	5.19	7.715	6.50	7.813	2.31	7.364
2	0.013	5.114	0.31	6.491	0.89	6.949	4.31	7.635	3.69	7.567	5.00	7.699	7.06	7.849	2.19	7.340
3	0.008	4.903	0.37	6.568	1.04	7.017	4.81	7.682	6.25	7.796	4.88	7.688	6.00	7.778	2.56	7408
4	0.013	5.114	0.22	6.342	0.96	6.982	3.69	7.567	6.69	7.825	3.31	7.520	6.31	7.800	1.88	7.274
5	0.013	5.114	0.22	6.342	1.18	7.072	4.56	7.659	4.63	7.666	5.25	7.720	4.13	7.616	3.44	7.537
6	0.014	5.146	N.R		1.08	7.033	3.75	7.514	6.19	7.792	4.31	7.635	5.50	7.740	2.75	7.439
7	0.013	5.114	N.R		1.12	7.049	3.19	7.504	N.R		5.13	7.710	6.38	7.805	N.R	
8	0.022	5.342	N.R		0.92	6.964	3.69	7.567	N.R		5.19	7.715	5.81	7.764	3.25	7.512
x	0.014	5.127	0.29	6.449	1.02	7.004	4.06	7.605	5.19	7.702	4.78	7.675	5.96	7.770	2.63	7.410

Replicates	17/2		3/3		17/3		31/3		14/4		12/5	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	1.13	7.053	5.25	7.720	2.06	7.314	0.46	6.663	0.58	6.763	0.44	6.644
2	0.56	6.748	4.00	7.620	1.44	7.158	0.60	6.778	0.29	6.462	0.50	6.699
3	1.31	7.117	5.38	7.731	1.00	7.000	0.46	6.663	0.60	6.778	0.44	6.644
4	1.38	7.140	4.69	7.671	1.69	7.228	0.58	6.763	0.65	6.813	0.46	6.663
5	1.50	7.176	3.63	7.560	1.69	7.228	N.R		0.59	6.771	0.51	6.708
6	N.R		3.94	7.596	2.25	7.352	N.R		0.64	6.806	0.30	6.477
7	1.31	7.117	4.50	7.653	1.69	7.228	N.R		0.59	6.771	0.25	6.398
8	1.81	7.258	6.19	7.792	1.94	7.288	N.R		0.41	6.613	0.25	6.398
x	1.13	7.087	4.70	7.667	1.72	7.232	0.53	6.716	0.54	6.722	0.39	6.578

Cols: Bacteria/cm^2 leaf tissue.
Log. Transformation applied.

Table 5 (continued). Treatment ii) 0.01% Spreadite + 5% urea.

Replicates	1/10		15/10		29/10		12/11		26/11		9/12		6/1		17/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			0.75	6.875	1.89	7.277	4.50	7.653	6.38	7.805	11.25	8.051	4.94	7.694	0.63	6.799
2			0.69	6.839	2.14	7.330	5.56	7.745	5.50	7.740	9.88	7.995	5.00	7.699	1.19	7.076
3			0.68	6.883	2.02	7.305	4.50	7.653	7.06	7.849	11.69	8.068	5.44	7.736	1.31	7.117
4			0.64	6.806	2.06	7.314	4.50	7.653	6.88	7.838	11.88	8.075	5.06	7.704	1.56	7.193
5			0.63	6.799	1.90	7.279	3.88	7.589	7.63	7.883	9.63	7.984	7.06	7.849	1.13	7.053
6			N.R		1.94	7.288	3.69	7.567	6.63	7.822	10.63	8.027	5.31	7.725	2.31	7.364
7			N.R		N.R		3.69	7.567	6.50	7.813	11.88	8.075	5.50	7.740	1.19	7.076
8			N.R		N.R		4.31	7.635	7.69	7.886	11.50	8.061	6.13	7.788	1.44	7.158
x̄			0.68	6.830	1.99	7.298	4.33	7.632	6.78	7.829	11.04	8.042	5.56	7.741	1.35	7.104

Replicates	31/2		3/3		17/3		31/3		14/4		12/5	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	2.31	7.364	1.75	7.243	1.38	7.140	1.26	7.100	0.64	6.806	0.48	6.681
2	1.75	7.243	1.00	7.000	1.31	7.117	1.20	7.079	0.68	6.833	0.45	6.653
3	2.31	7.364	1.31	7.117	1.63	7.212	1.16	7.066	0.59	6.771	0.46	6.663
4	2.13	7.328	1.19	7.076	1.19	7.076	1.15	7.061	0.68	6.833	0.39	6.591
5	1.69	7.228	1.50	7.076	1.69	7.228	N.R		0.60	6.778	0.45	6.653
6	1.75	7.243	1.44	7.158	1.50	7.176	N.R		0.58	6.763	0.39	6.591
7	2.38	7.337	N.R		1.38	7.140	N.R		0.69	6.839	0.54	6.732
8	2.13	7.328	0.94	6.973	1.19	7.076	N.R		0.64	6.806	0.35	6.544
x̄	2.06	7.309	1.30	7.106	1.41	7.145	1.19	7.076	0.64	6.803	0.44	6.638

N.R. No reading due to contamination.

Table 6 . Total fungi, autumn urea, 1968-69. Treatment i) 0.01% Spreadite.

Replicates	1/10		15/10		12/11		26/11		9/12		6/1		3/2		17/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	0.24	4.380	2.63	5.420	2.31	5.364	2.69	5.430	2.25	5.352	1.56	5.193	1.63	5.212	1.13	5.053
2	0.41	4.613	2.50	5.398	3.06	5.486	2.25	5.352	2.89	5.461	1.75	5.243	1.50	5.176	1.38	5.140
3	0.30	4.477	2.38	5.377	3.25	5.512	2.19	5.340	2.19	5.340	1.00	5.000	1.63	5.212	0.94	4.973
4	0.36	4.556	2.75	5.439	2.75	5.439	1.63	5.212	2.50	5.398	1.25	5.097	1.38	5.140	1.00	5.000
5	0.34	4.532	2.50	5.398	3.06	5.486	2.63	5.420	2.44	5.387	N.R.	N.R.	1.50	5.176	1.00	5.000
6	N.R		N.R		2.56	5.408	2.19	5.340	2.75	5.439	1.50	5.176	2.44	5.387	1.25	5.097
7	N.R		N.R		2.31	5.364	2.13	5.328	3.13	5.496	1.19	5.076	1.25	5.097	1.25	5.097
8	N.R		N.R		3.50	5.544	2.19	5.340	3.25	5.512	1.31	5.117	1.56	5.193	1.63	5.212
$\bar{x}$	0.33	4.511	2.55	5.406	2.85	5.450	2.24	5.345	2.68	5.423	1.36	5.128	1.62	5.199	1.19	5.071

Replicates	3/3		17/3		31/3		14/4		28/4		12/5	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	1.88	5.274	2.06	5.314	2.00	5.301	0.88	4.945	1.25	5.097	1.38	5.140
2	1.25	5.097	1.44	5.158	0.88	4.945	1.30	5.114	1.88	5.274	1.00	5.000
3	1.75	5.243	1.00	5.000	1.63	5.212	1.00	5.000	2.13	5.328	1.13	5.053
4	1.63	5.212	1.69	5.228	0.88	4.495	0.38	4.580	1.88	5.274	1.13	5.053
5	1.75	5.243	1.69	5.228	1.50	5.176	1.63	5.212	1.13	5.053	1.25	5.097
6	1.56	5.193	2.25	5.352	1.63	5.212	1.38	5.140	1.50	5.176	1.38	5.140
7	1.63	5.212	1.69	5.228	1.63	5.212	2.13	5.328	2.00	5.301	1.75	5.240
8	1.19	5.076	1.94	5.288	1.88	5.274	1.00	5.000	1.50	5.176	1.88	5.274
$\bar{x}$	1.58	5.193	1.72	5.224	1.50	5.103	1.21	5.039	1.65	5.209	1.36	5.124

Cols: Fungi/cm^2 leaf tissue ($x10^5$).
Log. transformation applied.

Table 6 (continued). Treatment ii) 0.01% Spreadite + 5% urea.

Replicates	1/10		15/10		12/11		26/11		9/12		6/1		3/2		17/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			2.06	5.314	4.44	5.647	3.56	5.551	4.13	5.616	1.44	5.158	1.38	5.140	0.94	4.973
2			2.75	5.439	3.94	5.596	3.00	5.477	3.13	5.496	1.50	5.176	0,75	4.875	1.06	5.025
3			3.19	5.504	4.25	5.628	3.19	5.504	3.81	5.581	1.31	5.117	1.06	5.025	0.81	4.909
4			2.25	5.352	4.25	5.628	3.63	5.560	6.13	5.788	1.13	5.053	0.81	4.909	1.25	5.097
5			2.31	5.364	4.31	5.635	2.88	5.459	3.81	5.581	1.25	5.097	1.19	5.076	1.13	5.053
6			N.R		5.13	5.710	3.88	5.589	4.44	5.647	1.50	5.176	N.R		1.06	5.025
7			N.R		4.44	5.647	3.50	5.544	4.44	5.647	1.13	5.053	1.25	5.097	0.69	4.839
8			N.R		4.25	5.628	4.19	5.622	3.00	5.477	1.25	5.097	0.88	4.945	1.13	5.053
x̄			2.51	5.394	4.37	5.639	3.48	5.538	3.98	5.604	1.31	5.115	1.04	5.009	1.00	4.996

Replicates	3/3		17/3		31/3		14/4		28/4		12/5	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	0.94	4.973	0.63	4.799	1.88	3.274	3.13	5.496	1.13	5.053	0.88	4.945
2	1.44	5.158	0.63	4.799	1.63	5.212	2.38	5.377	1.25	5.097	2.38	5.377
3	1.25	5.097	0.38	4.580	2,63	5.420	2.75	5.439	1.00	5.000	1.38	5.140
4	1.75	5.243	0.69	4.839	1.38	5.140	3.13	5.496	1.25	5.097	1.00	5.000
5	1.44	5.158	1.06	5.025	3.25	5.512	1.38	5.140	1.50	5.176	1.63	5.212
6	1.50	5.176	0.81	4.909	2.38	5.377	3.00	5.477	0.88	4.945	2.00	5.301
7	1.38	5.140	0.69	4.839	2.38	5.377	3.50	5.544	1.00	5.000	1.25	5.097
8	1.69	5.228	0.63	4.799	2.13	5.328	3.00	5.477	1.25	5.097	1.88	5.274
x̄	1.42	5.146	0.69	4.823	2.20	5.330	2.78	5.430	1.15	5.058	1.55	5.168

N.R. No reading due to contamination

Table 7. Total bacteria and yeast, spring application urea, 1969 Treatment i) 0.01% Spreadite.

Replicates	14/1		21/1		28/1		11/2		4/3		26/3		11/4		7/5	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	0.50	6.699	0.58	6.763	1.86	7.270	1.04	7.017	0.23	6.362	0.73	6.863	0.60	6.778	0.86	6.935
2	0.48	6.681	1.20	7.079	1.56	7.193	1.23	7.090	0.43	6.634	0.63	6.799	0.51	6.708	1.01	7.004
3	0.36	6.556	0.83	6.919	1.80	7.255	1.54	7.188	0.39	6.591	0.75	6.875	0.61	6.785	0.85	6.929
4	0.38	6.580	N.R.	N.R.	1.60	7.204	1.15	7.061	0.26	6.415	0.69	6.839	0.45	6.653	0.81	6.909
5	0.63	6.799	1.00	7.000	1.70	7.230	0.95	6.978	0.13	6.114	0.50	6.699	0.81	6.910	0.72	6.857
6	0.54	6.732	0.86	6.935	1.74	7.241	1.43	7.115	0.31	6.491	0.48	6.681	0.89	6.949	0.64	6.806
7	0.39	6.591	1.04	7.017	1.46	7.164	1.30	7.114	0.35	6.544	0.48	6.681	0.88	6.945	0.50	6.699
8	0.53	6.724	1.00	7.000	1.53	7.185	1.23	7.090	0.35	6.544	0.60	6.778	0.74	6.869	0.81	6.909
$\bar{x}$	0.48	6.672	0.93	6.959	1.66	7.217	1.23	7.086	0.31	6.461	0.62	6.776	0.69	6.824	0.78	6.881

Treatment ii) 0.01% Spreadite + 5% urea.

Replicates	14/1		21/1		28/1		11/2		4/3		26/3		11/4		7/5	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			1.26	7.100	2.68	7.428	6.25	7.796	3.25	7.512	6.00	7.778	3.00	7.477	6.88	6.945
2			1.23	7.090	3.53	7.548	5.88	7.769	2.63	7.420	5.00	7.699	2.25	7.352	1.44	7.158
3			1.51	7.179	3.18	7.502	7.63	7.883	4.00	7.602	4.50	7.653	4.00	7.602	1.85	7.267
4			1.53	7.185	2.86	7.456	7.88	7.897	4.00	7.602	5.00	7.699	3.13	7.496	1.48	7.170
5			1.74	7.241	4.19	7.622	9.38	7.972	2.25	7.352	5.63	7.751	3.38	7.529	1.25	7.097
6			1.25	7.097	3.03	7.481	7.00	7.845	1.88	7.274	5.38	7.731	3.00	7.477	1.10	7.041
7			1.73	7.239	3.98	7.600	7.38	7.868	1.75	7.243	5.13	7.710	3.00	7.477	1.14	7.057
8			1.93	7.286	2.36	7.373	8.00	7.903	2.63	7.420	5.25	7.720	2.50	7.398	1.50	7.176
$\bar{x}$			1.52	7.177	3.23	7.501	7.43	7.866	2.80	7.428	5.24	7.717	3.03	7.476	1.33	7.113

Cols: Bacteria/cm^2 leaf tissue($x10^7$).
Log. Transformation applied.

N.R. No reading due to contamination.

Table 8. Total bacteria and yeast, 1969-70. Treatment i) 0.05% Lissapol NX.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		18/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	0.17	6.230	3.20	7.505	1.80	7.255	0.70	6.845	2.36	7.373	1.92	7.283	0.74	6.896	0.72	6.857
2	0.15	6.176	6.20	7.792	1.94	7.288	0.64	6.806	2.24	7.350	1.48	7.170	0.60	6.778	0.36	6.556
3	0.14	6.146	4.60	7.663	1.70	7.230	0.56	6.748	2.84	7.453	1.22	7.086	0.74	6.869	0.66	6.820
4	0.13	6.114	6.20	7.792	1.76	7.246	0.70	6.845	2.54	7.405	1.28	7.107	1.74	7.241	0.62	6.792
5	0.08	5.903	5.90	7.771	2.52	7.401	1.30	7.114	N.R.	N.R.	1.88	7.274	1.06	7.025	0.58	6.763
6	0.08	5.903	6.20	7.792	3.08	7.489	1.52	7.182	1.90	7.279	2.08	7.318	0.68	6.883	0.56	6.748
7	0.08	5.903	6.60	7.820	2.80	7.447	1.24	7.093	2.36	7.373	2.36	7.373	0.92	6.964	0.76	6.881
8	0.09	5.973	6.00	7.778	3.12	7.494	1.32	7.121	2.36	7.373	2.02	7.305	0.92	6.964	0.54	6.732
x̄	1.15	6.043	5.61	7.739	2.34	7.356	0.99	6.969	2.57	7.372	1.78	7.239	0.92	6.942	0.60	6.768

Replicates	8/3		26/4	
	Cols	Log	Cols	Log
1	0.40	6.602	0.22	6.342
2	0.38	6.780	0.25	6.398
3	0.40	6.602	0.26	6.415
4	0.39	6.591	0.25	6.398
5	0.62	6.792	0.34	6.532
6	0.60	6.839	0.42	6.623
7	0.57	6.756	0.36	6.556
8	0.52	6.716	0.40	6.602
x̄	0.48	6.709	0.31	6.484

Cols: Organisms/cm^2 leaf tissue ($x10^7$).
Log. Transformation applied.

Table 8 (continued). Treatment ii) 0.05% Lissapol NX + 5% fructose.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/11		8/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			7.20	7.857	2.76	7.441	1.38	7.140	3.36	7.526	1.60	7.204	0.66	6.820	0.74	6.869
2			7.80	7.892	3.00	7.477	1.62	7.210	3.10	7.491	1.54	7.188	0.78	6.892	0.36	6.556
3			7.20	7.857	2.72	7.435	1.50	7.176	3.18	7.502	1.58	7.199	0.74	6.869	0.96	6.982
4			8.20	7.914	2.66	7.425	1.64	7.215	3.54	7.549	1.32	7.121	0.96	6.982	0.62	6.792
5			5.00	7.699	5.60	7.748	2.14	7.330	6.20	7.792	1.06	7.025	0.42	6.623	0.52	6.716
6			4.40	7.644	5.26	7.721	2.46	7.391	6.40	7.806	1.10	7.041	0.42	6.623	0.62	6.792
7			5.00	7.699	4.46	7.649	2.34	7.369	6.40	7.806	1.06	7.025	0.42	6.623	0.66	6.820
8			4.80	7.681	4.40	7.644	1.96	7.292	5.00	7.699	0.96	6.982	0.44	6.644	0.68	6.833
$\bar{x}$			6.20	7.780	3.85	7.567	1.88	7.265	4.64	7.646	1.27	7.098	0.60	6.759	0.64	6.795

Replicates	8/3		26/4	
	Cols	Log	Cols	Log
1	0.64	6.806	0.20	6.301
2	0.56	6.748	0.20	6.301
3	0.50	6.699	0.22	6.342
4	0.50	6.699	0.20	6.301
5	0.44	6.644	0.22	6.342
6	0.64	6.806	0.21	6.322
7	0.62	6.792	0.22	6.342
8	0.56	6.748	0.29	6.462
$\bar{x}$	0.55	6.742	0.22	6.339

Table 8 (continued). Treatment iii) 0.05% Lissapol 10x + 5% fructose + 5% urea.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			8.60	7.934	8.30	7.919	2.46	7.391	13.20	8.121	4.44	7.647	1.54	7.188	1.98	7.297
2			8.00	7.903	9.60	7.982	2.12	7.326	11.40	8.057	4.08	7.611	1.66	7.220	2.16	7.335
3			9.40	7.973	11.80	8.072	2.70	7.431	11.20	8.049	3.82	7.582	1.80	7.255	1.92	7.283
4			9.40	7.937	N.R.	N.R.	2.84	7.453	14.20	8.152	4.18	7.621	1.66	7.220	1.86	7.270
5			9.20	7.964	8.20	7.914	3.34	7.524	5.20	7.716	5.08	7.706	4.00	7.602	1.62	7.210
6			11.00	8.041	8.00	7.903	3.60	7.556	7.20	7.857	5.86	7.768	3.06	7.486	1.94	7.288
7			12.80	8.107	9.80	7.991	3.40	7.532	8.60	7.935	5.92	7.772	3.92	7.593	1.46	7.164
8			10.20	8.009	7.00	7.845	3.28	7.516	10.40	8.017	5.80	7.763	3.16	7.500	1.86	7.270
$\bar{x}$			9.82	7.983	8.95	7.946	2.96	7.466	10.17	7.988	4.89	7.683	2.60	7.383	1.85	7.264

Replicates	8/3		26/4	
	Cols	Log	Cols	Log
1	2.00	7.301	0.68	6.833
2	2.64	7.422	0.50	6.699
3	2.32	7.366	0.66	6.820
4	2.18	7.339	0.40	6.602
5	1.76	7.246	0.58	6.763
6	1.58	7.199	0.48	6.681
7	1.70	7.230	0.40	6.602
8	1.66	7.220	0.48	6.681
$\bar{x}$	1.98	7.290	0.52	6.710

N.R. No reading due to contamination.

Analysis of Variance

	s.s.	d.f.	m.s.	F ***
Treatments	8.97	2	4.49	24.94
Error	38.29	211	0.18	
Total	47.26	213		

Lissapol v. fructose

Difference of means = 0.05
S.E. for test of means = 0.0728

$$t = \frac{0.05}{0.0728} = 0.687 \text{ N.S.}$$

Fructose v. urea

Difference of means = 0.408
S.E. for test of means = 0.748

$$t = \frac{0.408}{0.0748} = 5.455^{***}$$

Table 9. Total fungi 1969-70. Treatment i) 0.05% Lissapol NX.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1	1.40	4.146	7.60	4.881	10.60	5.025	6.80	4.833	9.00	4.954	2.60	4.415	2.80	4.447	0.80	3.903
2	1.36	4.134	10.60	5.025	9.80	4.991	6.60	4.820	6.00	4.778	3.20	4.505	1.40	4.146	1.40	4.146
3	1.48	4.170	14.60	5.164	12.60	5.100	5.20	4.716	8.60	4.935	2.60	4.415	2.80	4.447	1.60	4.204
4	1.58	4.199	11.80	5.072	o0.40	5.017	5.40	4.732	9.80	4.991	4.40	4.644	2.40	4.380	1.60	4.204
5	0.80	3.903	6.60	4.820	6.40	4.806	3.40	4.532	5.80	4.763	6.40	4.806	3.00	4.477	2.80	4.447
6	0.78	3.892	7.40	4.869	6.40	4.806	2.20	4.342	6.40	4.806	6.60	4.820	1.80	4.255	3.00	4.477
7	0.76	3.881	6.20	4.792	6.80	4.833	3.20	4.505	5.60	4.748	9.00	4.954	2.80	4.447	3.00	4.477
8	1.00	4.000	6.60	4.820	6.60	4.820	2.20	4.342	7.80	4.892	N.R.	N.R.	3.40	4.532	2.80	4.447
$\bar{x}$	1.14	4.040	8.92	4.930	8.70	4.924	4.37	4.602	7.37	4.858	4.97	4.651	2.55	4.391	2.12	4.288

Replicates	8/3		26/4	
	Cols	Log	Cols	Log
1	3.00	4.477	2.80	4.447
2	2.40	4.380	1.40	4.146
3	2.00	4.301	3.00	4.477
4	2.20	4.342	2.20	4.342
5	2.60	4.415	4.20	4.623
6	3.00	4.477	2.00	4.301
7	3.80	4.580	2.80	4.447
8	3.00	4.477	1.80	4.255
$\bar{x}$	2.76	4.431	2.52	4.379

Cols: Fungi/cm^2 leaf tissue ($x10^4$).
Log. Transformation applied.

Table 9 (continued). Treatment ii) 0.05% Lissapol NX + 5% fructose.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			9.60	4.982	8.00	4.903	6.00	4.778	7.80	4.892	4.80	4.681	2.80	4.447	3.00	4.477
2			12.80	5.107	9.40	4.973	7.00	4.845	8.60	4.935	4.80	4.681	2.00	4.301	2.80	4.447
3			14.20	5.152	10.00	5.000	5.00	4.699	8.60	4.935	4.00	4.602	2.00	4.301	3.80	4.580
4			12.00	5.079	10.40	5.017	4.80	4.681	9.80	4.991	5.60	4.748	2.60	4.415	3.20	4.505
5			8.20	4.914	12.20	5.086	4.40	4.644	10.60	5.025	5.20	4.716	3.00	4.477	2.20	4.342
6			8.00	4.903	12.20	5.086	3.40	4.532	8.60	4.935	5.80	4.763	2.40	4.380	1.80	4.255
7			9.80	4.991	12.40	5.093	4.00	4.602	10.00	5.000	4.00	4.602	3.80	4.580	1.80	4.255
8			10.80	5.033	10.60	5.025	N.R.	N.R.	9.60	4.982	4.20	4.623	1.40	4.146	2.20	4.342
$\bar{x}$			10.67	5.020	10.65	5.022	4.95	4.683	9.20	4.961	4.80	4.677	2.50	4.480	2.60	4.400

Replicates	8/3		26/4	
	Cols	Log	Cols	Log
1	6.00	4.778	4.40	4.644
2	7.80	4.892	3.40	4.532
3	7.40	4.869	3.60	4.556
4	6.40	4.806	3.20	4.505
5	5.20	4.716	4.00	4.602
6	5.60	4.748	5.40	4.732
7	6.20	4.792	4.20	4.623
8	4.20	4.623	4.40	4.644
$\bar{x}$	6.10	4.778	4.07	4.604

Table 9 (continued). Treatment iii) 0.05% Lissapol NX + 5% fructose + 5% urea.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log	Cols	Log
1			5.40	4.732	8.60	4.935	5.60	4.748	11.80	5.072	3.60	4.556	1.40	4.146	2.60	4.415
2			5.20	4.716	11.00	5.041	4.80	4.681	14.20	5.152	2.60	4.415	1.40	4.146	3.00	4.477
3			5.00	4.699	10.60	5.025	5.40	4.732	13.60	5.134	4.40	4.644	2.00	4.301	1.60	4.204
4			4.60	4.663	9.60	4.982	4.60	4.663	10.60	5.025	3.80	4.580	2.00	4.301	3.80	4.580
5			15.60	5.193	13.40	5.127	6.20	4.792	7.00	4.845	3.20	4.505	3.60	4.556	2.40	4.380
6			13.60	5.188	N.R.	N.R.	5.40	4.732	6.60	4.820	3.00	4.477	3.40	4.532	3.20	4.505
7			11.20	5.049	9.60	4.982	6.00	4.778	7.80	4.892	3.00	4.477	3.20	4.505	1.60	4.204
8			10.60	5.025	13.20	5.121	5.00	4.699	8.60	4.935	3.40	4.532	3.40	4.532	1.60	4.204
$\bar{x}$			9.12	4.968	10.85	5.030	5.37	4.728	10.02	4.984	3.37	4.532	2.55	4.377	2.47	4.371

Replicates	8/3		26/4	
	Cols	Log	Cols	Log
1	8.00	4.903	3.20	4.505
2	8.20	4.914	4.00	4.602
3	7.00	4.845	4.60	4.663
4	6.80	4.833	3.80	4.580
5	6.20	4.792	3.60	4.556
6	6.00	4.778	2.80	4.477
7	5.80	4.763	2.00	4.301
8	6.20	4.792	2.40	4.380
$\bar{x}$	6.77	4.827	3.30	4.508

N.R. Contamination of plate.
1-4 & 5-8 Duplicate treatments.
Log. transformation applied.

Analysis of Variance

	s.s.	d.f.	m.s.	F. *
Treatment	0.54	2	0.27	3.86
Error	15.07	210		
Total	15.61	212		

Lissapol v. urea

Difference of means = 0.85
S.E. for test of means = 0.39

$$t = \frac{0.85}{0.39} = 2.18^{*}$$

Fructose v. urea

Difference of means = 0.35
S.E. for test of means = 0.421

$$t = \frac{0.35}{0.421} = 0.83 \text{ N.S.}$$

Table 10. Chitinoclastic organisms. I. Bacteria. Treatment i) 0.05% Lissapol NX.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	T	Cols	T	Cols	T	Cols		Cols	T	Cols	T	Cols	T	Cols	T
1	6.0	2.55	8.0	2.915	5.1	2.366	1.7	1.483	4.0	2.121	6.0	2.550	1.3	1.342	3.6	2.025
2	5.0	2.35	8.0	2.915	4.1	2.145	2.6	1.761	4.0	2.121	7.0	2.739	1.0	1.225	3.9	2.098
3	6.0	2.55	5.0	2.345	4.6	2.258	1.9	1.549	6.0	2.550	5.0	2.345	0.9	1.183	2.8	1.817
4	5.0	2.35	8.0	2.915	3.7	2.049	1.8	1.517	3.0	1.871	4.0	2.121	1.3	1.342	3.4	1.975
5	5.0	2.35	12.0	3.536	3.0	1.871	0.8	1.140	11.0	3.391	5.0	2.345	0.9	1.183	1.2	1.304
6	7.0	2.74	7.0	2.739	4.3	2.191	0.8	1.140	7.0	2.739	2.0	1.581	0.8	1.140	1.0	1.225
7	6.0	2.55	8.0	2.915	3.1	1.897	0.9	1.183	9.0	3.082	2.0	1.581	0.4	0.949	1.3	1.342
8	4.0	2.12	10.0	3.240	2.0	1.581	0.9	1.183	12.0	3.536	3.0	1.871	0.6	1.049	0.7	1.095
$\bar{x}$	5.5	2.44	8.25	2.94	3.74	2.04	1.43	1.37	7.0	2.68	4.25	2.14	0.90	1.18	2.23	1.61

Replicates	8/3		26/4	
	Cols	T	Cols	T
1	3.7	2.049	4.5	2.236
2	3.8	2.074	3.9	2.098
3	1.9	1.549	3.4	1.975
4	3.1	1.897	2.6	1.761
5	2.9	1.844	2.9	1.844
6	1.5	1.414	2.7	1.789
7	1.8	1.517	3.3	1.949
8	1.7	1.483	2.7	1.817
$\bar{x}$	2.55	1.73	3.25	1.93

Cols: Bacteria/cm^2 leaf tissue ($x10^5$).
$T = \sqrt{X + \frac{1}{2}}$ transformation.

Table 10(continued). Treatment ii) 0.05% Lissapol NX + 5% fructose.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T
1			17.0	4.183	6.0	2.550	1.0	1.225	17.0	4.183	4.0	2.121	5.0	2.345	2.0	1.581
2			15.0	3.937	8.0	2.915	1.2	1.304	16.0	4.062	3.0	1.871	5.0	2.345	1.5	1.414
3			11.0	3.391	8.0	2.915	1.0	1.225	10.0	3.240	2.0	1.581	1.0	1.225	2.0	1.581
4			14.0	3.808	10.0	3.240	1.5	1.414	27.0	5.244	0	0.707	3.0	1.871	1.6	1.449
5			20.0	4.528	7.0	2.789	2.1	1.612	26.0	5.148	4.0	2.121	4.0	2.121	1.0	1.225
6			28.0	5.339	4.0	2.121	1.8	1.517	19.0	4.416	2.0	1.581	2.0	1.581	1.5	1.414
7			15.0	3.937	6.0	2.550	1.5	1.414	18.0	4.416	3.0	1.871	3.0	1.871	1.4	1.378
8			26.0	5.148	4.0	2.121	2.0	1.581	18.0	4.301	1.0	1.225	1.0	1.225	0.7	1.075
$\bar{x}$			18.25	4.28	6.63	2.65	1.51	1.41	19.0	4.38	2.38	1.63	3.0	1.82	1.67	1.39

Replicates	8/3		26/4	
	Cols	T	Cols	T
1	2.4	1.763	3.1	1.897
2	2.6	1.761	1.9	1.549
3	1.8	1.517	2.4	1.703
4	2.6	1.761	2.8	1.817
5	1.9	1.549	2.0	1.581
6	1.4	1.378	1.4	1.378
7	1.4	1.378	1.7	1.483
8	1.9	1.349	1.0	1.225
$\bar{x}$	2.0	1.57	2.04	1.58

Table 10(continued). Treatment iii) 0.05% Lissapol NX + 5% fructose + 5% urea.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T
1			25.0	5.050	26.0	5.148	25.0	5.050	53.0	7.314	34.0	5.874	8.0	2.915	4.0	2.121
2			18.0	4.301	10.0	3.240	23.0	4.848	62.0	7.906	32.0	5.701	16.0	4.062	16.0	4.062
3			30.0	5.523	20.0	4.528	24.0	4.950	61.0	7.842	28.0	5.339	11.0	3.391	6.0	2.550
4			26.0	5.148	28.0	5.339	19.0	4.416	56.0	7.517	23.0	4.848	9.0	3.082	15.0	3.937
5			39.0	6.285	19.0	4.416	9.0	3.082	33.0	5.788	31.0	5.612	22.0	4.743	14.0	3.808
6			42.0	6.519	16.0	4.062	12.0	3.536	37.0	6.124	21.0	4.637	11.0	3.391	12.0	3.536
7			41.0	6.442	18.0	4.301	19.0	4.416	34.0	5.874	24.0	4.950	6.0	2.550	17.0	4.183
8			56.0	7.517	15.0	3.937	9.0	3.082	31.0	5.612	24.0	5.612	25.0	5.050	23.0	4.848
$\bar{x}$			34.63	5.84	19.0	4.37	17.50	4.17	45.90	6.75	27.13	5.32	13.5	3.65	15.29	3.63

Replicates	8/3		26/4	
	Cols	T	Cols	T
1	7.0	2.739	5.0	2.345
2	10.0	3.240	6.0	2.550
3	9.0	3.082	8.0	2.915
4	10.0	3.240	4.0	2.121
5	14.0	4.808	12.0	3.536
6	11.0	3.391	10.0	3.240
7	11.0	3.391	15.0	3.937
8	15.0	3.937	6.0	2.550
$\bar{x}$	10.88	3.35	8.25	2.90

Analysis of Variance

	d.f.	s.s.	m.s.	F.
Treatments	2	261.16	130.58	100.82 ***
Error	213	275.88	1.30	
Total	215	537.04		

Lissapol NX v. Lissapol + fructose

Difference of means = 0.35
S.E. for test of means = 0.1616

$$t = \frac{0.35}{0.1616} = 2.13^{*}$$

Lissapol + fructose v. urea.

Difference of mean = 2.14
S.E. for test of means = 0.219

$$t = \frac{2.14}{0.219} = 9.77^{***}$$

Table 11. Chitinoclastic organisms. II. Actinomycetes. Treatment i) 0.05% Lissapol NX.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T
1	9.0	3.082	1.0	1.225	0.1	0.775	1.8	1.483	1.7	1.483	4.0	2.121	4.0	2.121	5.0	2.345
2	8.0	2.915	0	0.707	0.1	0.775	1.9	1.549	1.8	1.517	2.0	1.581	2.0	1.581	1.0	1.225
3	8.0	2.915	1.0	1.225	0.1	0.775	1.5	1.414	1.9	1.549	1.0	1.225	3.0	1.871	2.0	1.581
4	10.0	3.240	1.0	1.225	0.1	0.775	1.5	1.414	2.5	1.732	3.0	1.871	5.0	2.345	2.0	1.581
5	7.0	2.739	2.0	1.581	0.1	0.775	2.0	1.581	1.0	1.225	0	0.707	3.0	1.871	3.0	1.871
6	8.0	2.915	1.0	1.225	0.2	0.837	2.1	1.612	0.9	1.183	2.0	1.581	2.0	1.581	7.0	2.739
7	10.0	3.240	1.0	1.225	0.2	0.837	1.4	1.378	2.5	1.732	3.0	1.871	2.0	1.581	4.0	2.121
8	11.0	3.391	1.0	1.225	0.1	0.775	1.1	1.265	1.2	1.304	1.0	1.225	4.0	2.121	1.0	1.225
$\bar{x}$	8.88	3.06	1.0	1.20	0.13	0.79	1.66	1.46	1.69	1.47	2.0	1.52	3.13	1.88	3.57	1.84

Replicates	8/3		26/4	
	Cols	T	Cols	T
1	6.0	2.550	1.0	1.225
2	7.0	2.739	1.6	1.449
3	13.0	3.674	1.0	1.225
4	5.0	2.345	0.9	1.183
5	1.6	1.449	3.3	1.949
6	1.8	1.517	3.2	1.924
7	2.6	1.761	2.8	1.817
8	1.6	1.449	12.5	3.606
$\bar{x}$	4.83	2.19	3.29	1.80

Cols: Actinomycetes/cm^2 leaf tissue ($x10^3$).
T. $\sqrt{X + \frac{1}{2}}$ transformation.

Table 11(continued). Treatment ii) 0.05% Lissapol NX + 5% fructose.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		18/1		8/2	
	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T
1			1.0	1.225	0.1	0.775	1.0	1.225	10.0	3.240	2.0	1.581	2.0	1.581	4.0	2.121
2			2.0	1.581	0.2	0.837	1.2	1.304	4.0	2.121	3.0	1.871	3.0	1.871	2.0	1.581
3			3.0	1.871	0.2	0.837	1.3	1.342	4.0	2.121	4.0	2.121	2.0	1.581	3.0	1.871
4			1.0	1.225	0.1	0.775	0.9	1.183	1.0	1.225	3.0	1.871	1.0	1.225	1.0	1.225
5			2.0	1.581	0.3	0.894	1.9	1.549	12.0	3.536	5.0	2.345	1.0	1.225	4.0	2.121
6			1.0	1.225	0.1	0.775	0.8	1.140	10.0	3.240	2.0	1.581	1.0	1.225	2.0	1.581
7			1.0	1.225	0.3	0.894	1.1	1.265	9.0	3.082	5.0	2.345	1.0	1.225	2.0	1.581
8			1.0	1.225	0	0.707	0.7	1.095	10.0	3.240	4.0	2.121	1.0	1.225	1.0	1.225
$\bar{x}$			1.5	1.39	0.16	0.81	1.11	1.26	7.50	2.73	3.5	1.98	1.5	1.39	2.38	1.66

Replicates	8/3		26/4	
	Cols	T	Cols	T
1	2.0	1.581	0.9	1.183
2	6.0	2.550	1.2	1.304
3	6.0	2.550	0.8	1.140
4	7.0	2.739	0.8	1.140
5	9.0	3.082	0.7	1.095
6	4.0	2.121	0.9	1.183
7	9.0	3.082	0.4	0.949
8	8.0	2.195	0.6	1.049
$\bar{x}$	6.38	2.58	0.78	1.13

Table 11(continued). Treatment iii) 0.05% Lissapol NX + 5% fructose + 5% urea.

Replicates	22/10		26/10		9/11		23/11		7/12		21/12		8/1		8/2	
	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T	Cols	T
1			6.0	2.550	0.2	0.837	3.0	1.871	23.0	4.848	6.0	2.550	2.0	1.581	2.0	1.581
2			6.0	2.550	0.3	0.894	5.0	2.345	12.0	3.536	6.0	2.550	4.0	2.121	6.0	2.550
3			2.0	1.581	0.2	0.837	8.0	2.915	14.0	3.808	6.0	2.550	1.0	1.225	10.0	3.240
4			3.0	1.871	0.3	0.894	7.0	2.739	5.0	2.345	5.0	2.345	5.0	2.345	5.0	2.345
5			2.0	1.581	0.2	0.837	2.0	1.581	3.0	1.871	6.0	2.550	3.0	1.871	11.0	3.391
6			4.0	2.121	0.2	0.837	4.0	2.121	5.0	2.345	7.0	2.739	3.0	1.871	3.0	1.871
7			1.0	1.225	0.1	0.775	2.0	1.581	3.0	1.871	3.0	1.871	8.0	2.915	3.0	1.871
8			3.0	1.871	0.4	0.949	2.0	1.581	3.0	1.871	15.0	3.937	3.0	1.871	4.0	2.121
$\bar{x}$			3.38	1.92	0.24	0.86	4.13	2.09	8.50	2.81	7.71	2.64	4.14	1.98	6.29	2.37

Replicates	8/3		26/4	
	Cols	T	Cols	T
1	6.0	2.550	5.0	2.345
2	8.0	2.915	6.0	2.550
3	9.0	3.082	4.0	2.121
4	7.0	2.739	7.0	2.739
5	17.0	4.183	4.0	2.121
6	17.0	4.183	5.0	2.345
7	15.0	3.937	4.0	2.121
8	15.0	3.937	3.0	1.871
$\bar{x}$	11.88	3.44	4.75	2.28

Analysis of Variance

	s.s.	d.f.	m.s.	F
Treatments	20.459	2	10.230	18.708 ***
Error	116.469	213	0.547	
Total	136.928	215		

Lissapol v fructose

Difference of means = 0.088
S.E. for test of means = 0.109

$$t = \frac{0.088}{0.109} = 0.807 \text{ N.S.}$$

Fructose v. urea

Difference of means = 0.560
S.E. for test fo means = 0.125

$$t = \frac{0.56}{0.125} = 4.510 \quad ***$$

Table 12. Ascospore degeneration after inoculation with two actinomycetes.

Date	Replicates	S_1	S_2	Control
29.11.70	1			36.25
	2			45.85
	3			39.92
	$\bar{x}$			40.70
9.12.70	1	35.54	50.00	36.99
	2	32.04	28.22	41.23
	3	31.28	39.79	32.15
	$\bar{x}$	33.44	38.60	37.49
29.12.71	1	0.55	41.15	56.51
	2	0	52.87	35.63
	3	0	43.45	32.43
	$\bar{x}$	0.26	45.16	44.39
18.1.71	1	0	16.52	48.26
	2	0	42.74	42.27
	3	0	51.00	39.66
	$\bar{x}$	0	38.05	43.80
7.2.71	1	0	17.65	34.52
	2	0	36.33	36.30
	3	0	2.350	32.66
	$\bar{x}$	0	25.70	34.60
27.2.71	1	0	18.49	29.35
	2	0	30.29	31.16
	3	0	8.94	29.44
	$\bar{x}$	0	22.12	29.96

S_2 V. control

Difference of means = 3.43
S.E. of test of means = 1.87

$$t = \frac{3.43}{1.87} = 1.83 \text{ N.Sign.}$$

S1 v. S2

Difference of means = 22.67
S.E. of test of means = 2.28

$$t = \frac{22.67}{2.23} = 9.94^{***}$$

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OVERWINTERING OF *SPHAEROTHECA MORS-UVAE* FROM GOOSEBERRY AS CLEISTOCARPS

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(With Plate 14 and 3 Text-figures)

It has been established that cleistocarps of *Sphaerotheca mors-uvae* (Schw.) Berk. formed on blackcurrant can overwinter naturally in England and give rise to viable and infective ascospores in spring (Jordan, 1966, 1967; Merriman & Wheeler, 1968), but there is doubt of the ability of those formed on gooseberry to do so. Salmon (1914) kept cleistocarps on gooseberry shoots in the laboratory through the winter and then observed discharge of ascospores when these were wetted, but he was unable to demonstrate this with cleistocarps overwintered naturally on gooseberry shoots; indeed, by spring, the ascospores had mostly degenerated. Similar results were obtained by Merriman & Wheeler (1968). The following reports the successful perennation of cleistocarps on gooseberry leaves.

About 250 leaves with cleistocarps of *S. mors-uvae* were collected from plants of the variety 'Careless' at Southmoor, Oxon., on 8 September 1969, and were overwintered on soil in terylene net bags at Silwood Park as described by Price (1970). The ascus and ascospore content of cleistocarps was assessed every 10–20 days by the methods of Merriman & Wheeler (1968). The ability of cleistocarps to dehisce was assessed by the following modification of the technique of Smith & Wheeler (1969): cleistocarps (*c.* 300–1000/sample) plus secondary mycelium were placed on damp blotting paper in the base of small, transparent polystyrene boxes and were inverted over a glass slide, cut to size and fixed in the lid with plasticine. The boxes were kept outside for 3 days and the glass slides then examined for discharged ascospores. The viability of discharged ascospores was assessed similarly by replacing the glass slides with disks cut from gooseberry and blackcurrant leaves. Here, after 3 days outside, the cleistocarps were removed and the boxes incubated at 20 °C until sporulating mildew colonies were observed on the leaf disks.

The general pattern of percentage cleistocarps with ascospores (Fig. 1) was similar to that observed by Merriman & Wheeler (1968) for cleistocarps on blackcurrant. The large fluctuations appeared to result from the substantial differences in the maturity of cleistocarps when the leaves were taken from the bushes in September; there was no evidence that ascospores matured within cleistocarps after this. Ascospores were discharged during March and April, and their viability established by infection of black-

currant and gooseberry leaf disks. Three points of interest arise from these observations: (i) The number of cleistocarps that dehisced was relatively small. Only 839 ascospores were trapped from 6676 cleistocarps; these are equivalent to the contents of some 105 cleistocarps, i.e. about 2 % of the total sample. (ii) Dehiscence began, and probably reached its peak, just before the marked decline in the percentage of cleistocarps with ascospores. (iii) This decline, as seen in samples taken in March and April, was caused mainly by an extensive degeneration of ascospores; very little was due to discharge of ascospores.

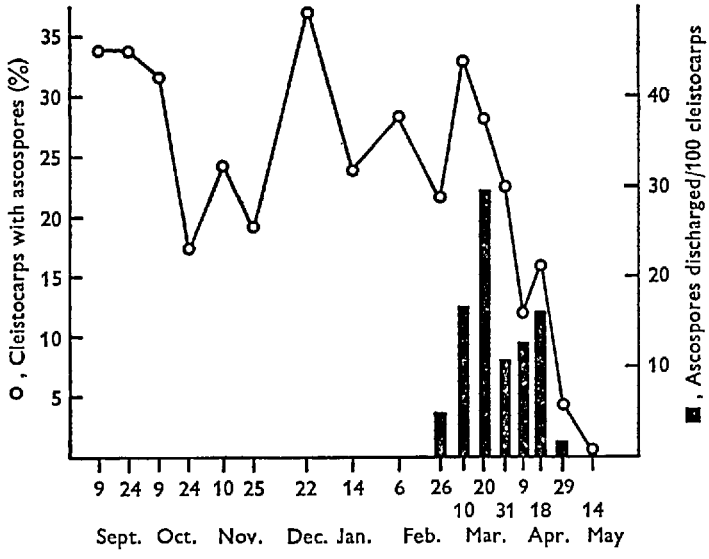

Fig. 1. Overwintering of cleistocarps of *Sphaerotheca mors-uvae* on soil.

The results on dehiscence are similar to those obtained by Merriman (1968) for cleistocarps from blackcurrant where they appear to be the sole means of survival of *S. mors-uvae*. Although perennation within dormant buds may be the most important method of survival for this fungus on gooseberry (Merriman & Wheeler, 1968), the results given here suggest that occasionally cleistocarps on leaves may be a further source of inoculum.

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