SOME ASPECTS OF THE LIFE ~~CYCLES~~ HISTORIES OF CERTAIN SMUT FUNGI

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Abstract

Accounts of the sporidial stages of members of the genus Entyloma are very confusing, and the role of the sporidia in the life cycles of these fungi is uncertain. A sporidial stage described by Stempell (1935) for E.calendulae has been stated by Derx (1948) and Olive (1952) to represent a fungus, Itersonilia perplexans, independant of the life cycle of the Entyloma, and present on the lesions as a contaminant. The accounts of Derx (1930) and Nyland (1950) of the widespread occurrence of Tilletiopsis spp. on the leaves of "healthy" plants suggests that members of this genus may likewise have been occasionally assigned to Entyloma.

Exploratory work on nine species of Entyloma revealed that "sporidia" approximating to Derx's and Olive's descriptions of Itersonilia perplexans are prevalent on lesions of Entyloma, but are also widespread on lesions of other fungal pathogens and occasionally present on "healthy" leaves. Fungi approximating to Nyland's descriptions of Tilletiopsis washingtonensis and T.minor are present on most leaves, whether or not attacked by Entyloma.

A detailed investigation of E.calendulae and E.dahliae showed that needle-shaped and allantoid sporidia are formed when the chlamydospore germinates, and are discharged at the surface of the lesions of the smut. Inoculation experiments proved that the disease is reproduced by the needle-shaped

sporidia; all other inoculation experiments had negative results. The allantoid sporidia superficially resemble the ballistospores of Tilletiopsis washingtonensis, but the two kinds of spore can be distinguished when they germinate. No connection was established between the life cycle of Entyloma and I.perplexans or T.washingtonensis, and it is highly probable that the latter fungi are present on the lesions as contaminants.

Contents

I HISTORICAL

Introduction

Among the members of the Ustilaginales, the "white" smuts are those which form lesions on the leaves of their hosts. The production of conidia at the surface of the host has been described for several of these fungi. Woronin (1882) first described such a "stage" from Tuburcinia trientalis, and "conidia" or "foliar sporidia" have been reported from species of Thecaphora, Doassansia, and Entyloma. (Ainsworth and Sampson, 1950, pp. 21-24).

In 1948, Derx threw doubt on a description given by Stempell (1935) of a "conidial stage" of Entyloma calendulae, and he maintained that the fungus described by Stempell was present on the surface of the smut lesion as a contaminant unconnected with the life cycle of the Entyloma. The fungus, he claimed, should be included in the Sporobolomycetaceae.

Since the "conidial stage" of the smuts has been given little attention, and its connection with the life cycle still remains in doubt, it seemed desirable to investigate this stage (or these stages) in as many species of the genus Entyloma as were readily available. The literature on the foliar sporidia of Entyloma is summarised in the following pages, followed by a short survey of the literature on the members of the Sporobolomycetaceae.

Throughout the literature, there is much confusion in the terminology used, especially in the use of the terms

"chlamydospore" and "sporidium". In the Ustilaginales, the thick-walled spore in which nuclear fusion takes place has for long been called a "chlamydospore", although it is not a chlamydospore in the normally accepted sense of the word, since it forms part of the sexual reproductive phase of the smut. The term has, however, been so widely applied to this phase in smut fungi that it must be accepted and is used here.

The term "sporidium" was formerly applied to a "basidiospore of the Uredinales and Ustilaginales" (Ainsworth and Bisby, 1945), but some authorities used the term "conidium" in this sense. "Sporidium" has also been used for a vegetative conidial stage, so that the two terms have become practically synonymous. Ainsworth and Sampson ("The British Smut Fungi", 1950, p.20) state "We have decided ... to use the term "sporidium" for all types of exogenous, thin-walled spores, whether abstricted from the promycelium directly, from subsequent growth in culture, or from mycelium in the host ... Segments of the germ tube which simulate sporidia but which remain attached are referred to as promycelial branches. They have been called sporidia by some writers". This terminology is accepted in the latest edition of the "Dictionary of the Fungi" (Ainsworth and Bisby, 1954), and is used here.

Derx (1930) proposed the name "ballistospore" for spores discharged by the drop excretion mechanism. He used it primarily for spores produced by members of

the Sporobolomycetaceae, but also applied it to basidiospores. It is thus a term which can be legitimately used for those smut sporidia which are discharged by the drop excretion mechanism, and it has been occasionally used in this way in the following pages.

The classification of the genus Entyloma is much confused, and in general two schemes are followed by systematists. The one is based on the morphology of the fungus, the other on that of the host, and recognises as a separate species each smut that parasitises a different host plant, even where there is no morphological difference between the fungi. The latter leads to the accumulation of a vast number of species, especially in those smuts which parasitise groups like the Compositae (Ciferri, 1938). The first scheme was followed by Savile (1947), who divided the Entylomas on North American Composites into two species, E.calendulae and E.polysporum, on the criteria of the size of the chlamydospores and the density with which they are crowded in the lesion. Ainsworth and Sampson (1950) included as forms of E.calendulae all those forms which attack members of the Compositae. Thus, E.bellidis Kreiger becomes E.calendulae (Oudem.) de Bary f. bellidis (Kreiger) Ainsworth and Sampson. A similar change had been proposed by Viégas (1944) for E.dahliae Sydow which becomes E.calendulae (Oudem.) de Bary f. dahliae (Sydow) Viégas.

While the nomenclature used by Ainsworth and Sampson

is accepted in principle, it has been decided here not to refer to the Entylomas attacking members of the Compositae as forms of E.calendulae. This decision was reached because the present work is not concerned with the complex taxonomy of the genus, and if the system used by Ainsworth and Sampson had been followed, it would have entailed the degradation of E.tanaceti Sydow. This did not fall within the scope of this work, and should clearly not be attempted without comparison of the particular smut in all its aspects with other forms of the species E.calendulae (in sensu Ainsworth and Sampson). In all other respects the system of nomenclature followed is that of Ainsworth and Sampson.

A. Entyloma

The one constant feature in the life cycles of all smut fungi is the formation of the uninucleate chlamydospore from an original binucleate hyphal segment, and the chlamydospore forms the logical starting point for a description of the life cycle of a smut. The mode of germination of the chlamydospore also determines to which sub-order the smut is assigned, and was the stage first described in the early literature.

The type of the genus Entyloma, on Ranunculus repens, was first assigned by Unger (1833), to Protomyces, a member of the Exoascales forming resting spores in the leaves of its hosts. De Bary (1874) obtained the germination of these resting spores after placing them for twenty four hours in water in a warm room, and thus demonstrated that the fungus belonged to the Ustilaginales, and he renamed Protomyces microsporus Unger as Entyloma ungerianum. This was later corrected to E.microsporum (Ung.) by Schroeter (1874).

Chlamydospore germination:- De Bary's account of Chlamydospore germination in E.microsporum is typical, with some minor variations, of the descriptions for the genus. He showed how a promycelium emerged from the spore, and after growing for some distance, produced an apical crown of four to eight branches into which the protoplasm of the spore passed via the promycelium which became septate as it emptied of

contents. The promycelial branches, (de Bary's term was "conidia"), then fused in pairs apically or at their bases, the contents of one passing over into its conjugate pair, the branches still remaining attached to the promycelium. After fusion, the branches occasionally grew out forming either a mycelium or long narrow sporidia ("secondary conidia"), which fell off and germinated to give a mycelium. Germination of chlamydospores of Entyloma calendulae was substantially similar. There was, of course, no account of nuclear behaviour in de Bary's description, but Kaiser (1936), working with E.calendulae, gave a very similar account to the above, and showed that four nuclei were present in the young promycelium, (presumably the result of meiosis on germination), and that the promycelial branches each contained one nucleus before they conjugated. The nuclei of both parent branches passed into the narrow sporidium which was thus binucleate. Unpaired branches occasionally grew out to form a mycelium, or uninucleate, "needle-shaped" sporidia. Paravicini (1917) claimed that the promycelial branches only fused after abstriction in E.calendulae, but this does not agree with the results of other workers. Nyland (1950) described how, in E. compositarum on malt agar, the fusion of the promycelial branches was followed by the formation of a mycelial colony. Sporidia, discharged by the drop excretion mechanism, were formed by this mycelium after a week to ten days; later chlamydospores indistinguishable from those produced

on the host occurred. Kaiser (1936), also obtained a mycelial colony in culture from germinating chlamydospores of E.arnoseridis on which "needle-shaped" sporidia, 30 to 40 μ long were formed.

Accounts of germination in other species follow variations of this story, very often going no further than the stage where promycelial branches were produced, owing to the difficulty of maintaining pure cultures.

In several cases the sporidia were described as septate: E.fuscum, E.matricariae (Ainsworth and Sampson, 1950); E.scirpicola (Thirumalachar and Dickson, 1949).

Winter (1880) described the occasional germination of the chlamydospores while still in the leaves of the host plant in E.microsporum, but it is only in certain species that germination occurs as soon as the spores are fully formed in the leaf, others, like E.ficariae, need long periods of maturation. Naturally it is largely the members of the first group for which the course of germination has been described, and for many fungi classed as species of Entyloma, the germination is stated to be "unknown".

The fate of germinated chlamydospores of Entyloma species in nature, and the entry into the host of mycelium or sporidia of chlamydospore origin has not so far been described, although mass inoculations of chlamydospores by de Bary resulted in the production of lesions in nine days for E.calendulae, and in eleven to fourteen days ~~in~~ for E.microsporum.

Recently, many smut fungi have been recorded which form chlamydospores, and thus complete their life cycles, in culture. Nyland reported chlamydospore formation in cultures of E.compositarum, originating from single chlamydospores obtained from the host, but their germination was not described. Stempell's claim (1935), to have grown normal chlamydospores of E.calendulae in culture will be referred to later.

Foliar sporidia:- In 1874 Winter, describing Entyloma ungerianum f.ficariae on Ranunculus ficariae (E.ficariae), suggested that the fungus described by Berkeley in 1837 as Cylindrosporium ficariae might be the conidial stage of the Entyloma. The following year Berkeley and Broome reported chlamydospores in the type specimen of Cylindrosporium ficariae. Winter also suggested that the Fusidium ranunculi described by Bonorden in 1871 was connected with the Entyloma, and in 1880 he noted the production of conidia on the mycelium before chlamydospore production in some species of Entyloma. He divided the genus into two groups, one including those species that produced conidia or sporidia (from germinating chlamydospores) "on the living host," and the other including those without conidial formation and in which sporidial production had not been observed on the living host. This method of classification was followed by Plowright (1889), and Clinton (1904). De Bary (1884), in

the "Comparative Morphology of Fungi, Mycetozoa and Bacteria" (English edition of 1887), cited Schroeter's evidence that in E. ranunculi, (E. ficariae) and E. serotinum, "the mycelium in the leaves sends a large number of short branches, often closely crowded together, into the air, partly through the stoma, partly through the lateral walls of the epidermis, and from the extremities of these branches single spores (or perhaps several successively) are abjointed. The spores are narrowly fusiform in shape, like the secondary sporidia of the promycelium, and it may be assumed, though it has not been observed, that their mode of germination is the same. They are formed on the mycelium before the resting spores." De Bary considered these, like those described by Woronin (1882) in Tuburcinia trientalis, to be equivalent morphologically to secondary sporidia, which had been interpolated in the life cycle between successive series of "carpospores" (resting spores), in the course of development.

Marshall Ward (1887) described the production and germination of these aerial spores in E. ficariae, and showed them to be "true conidia", as they were developed independently of the resting spores, and his infection experiments appeared to establish their connection with the Entyloma. Marshall Ward distinguished a slight difference in shape amongst these aerial spores, and described as "normal conidia"

the club-shaped or long oval bodies, slightly curved and more pointed at the attachment end, while in wet weather and in water he recognised longer, more curved and relatively thinner ones. Hanna (1938), examined nine species, in seven of which he found both of these kinds, and in two the sickle-shaped ones only. The following is a table of his results:

Species	Host	Filiform spores	Sickle-shaped
E.menispermi	Menispermum canadense	present	present
E.australi	Physalis pruniosa	"	"
E.linariae	Linaria vulgaris	"	"
E.melioti	Meliotis indica	"	"
E.ranunculae	Ranunculus maconnii	"	"
E.nymphaeae	Nymphaea advena	absent	"
E.lobeliae	Lobelia inflata	"	"
E.compositarum	Ambrosia trifida	"	absent
E.polysporum	Gaillardia sp.	"	"

Hanna pointed out that these results did not agree with Clinton (1906) who classified the Entylomas on the presence or absence of filiform sporidia but only mentioned one instance of sickle-shaped sporidia. Hanna expressed doubt on the value of these aerial spores as specific diagnostic characters since, in the published descriptions of most species, no clear distinction had been made between the two types, and like Marshall Ward he thought the presence of either type might vary with the age and condition of the host. He also showed that in cultures made from single sickle-shaped spores of E.menispermi, E.lobeliae, and E.linariae, some variation existed between cultures of a single species. In

E.nymphaeae Hanna found two distinct cultures of smaller and larger sporidia, the former making a potato-dextrose agar medium turn dark brown, while the larger never behaved in this way. Hanna showed that the sickle-shaped spores were always discharged by the drop excretion method first described by Buller (1933) for the allantoid sporidia of Tilletia caries, while the filiform spores were not violently discharged, but were easily detachable.

Ciferri (1928) separated E.matricariae Trail and E.traillii Massee on the size of the aerial sporidia.

Pethybridge (1928), and Green (1932), both working on E.dahliae, described "needle like bodies" on the leaf surface, some of which were produced from the promycelia of germinating chlamydospores, and were assumed to be all sporidia of like origin. Green described them as "needle like, some slightly curved, with no cross walls, one end generally more pointed than the other, showing where the sporidium was released from the hypha on which it was produced." Germination of the aerial spores was not observed.

The number of nuclei in the foliar sporidia has only rarely been described. Stempell (1935) isolated crescent-shaped spores from E.ficariae and E.calendulae which were being actively discharged at the surface of their host leaves, and he showed them to be uninucleate when shed. Hanna (1938) described as "for the most part uninucleate" his sickle-shaped

spores of E.menispermi, E.linariae, and E.lobeliae.

Stempell also reported an entirely different type of spore from the above, which he found in the deposit of spores obtained by suspending a leaf of Calendula officinalis infected with E.calendulae over the surface of an agar medium. These he described as "half-moon-shaped"; they were bi-nucleate, and germinated to give a wide hypha which formed clamp connections at regular intervals, quite distinct from the fine mycelium formed by the crescent-shaped spores in culture. Kaiser (1936) working with E.fergussoni (E.serotinum) on Symphytum officinale found two different kinds of sporidium from the upper and lower surfaces of young lesions, although he could not determine whether they were produced before chlamydospores were formed in the host. From the lower surface he found ellipsoidal sporidia 15-20 x 5-7μ, and from the upper surface thread-like sporidia 30-40 x 1.5-2μ, the latter in greater abundance. The thread-like sporidia were very similar to the sporidia formed in cultures of germinated chlamydospores of E.arnoseridis ~~(see p.)~~. Both types of sporidium from Symphytum had two nuclei, which divided to give three or four, the newly formed pairs of nuclei remaining close together. Neither type of sporidium formed clamp mycelium on germination.

Origin of the Foliar sporidia

For all the different types of aerial spores described above

there are, as has already been mentioned, two possible origins within the life cycle of the smut:-

(a) germinating chlamydospores, producing their sporidia at the leaf surface, and

(b) the parasitic mycelium, abstricting "conidia" at the surface of the host.

It is of course possible that conidium formation may take place even in species where the chlamydospores have germinated *in situ*, and in these cases aerial spores of either origin are difficult to tell apart.

When interpreting the descriptions in the literature, another difficulty is incurred when the branches formed at the tip of the promycelium are termed "sporidia" on the same basis as the crown of filiform sporidia which form at the distal end of the promycelium in *Tilletia*. Although these structures perform the same function in both genera, in *Entyloma* they seldom drop off the end of the promycelium, and it has seemed less confusing to call them "promycelial branches" in this present account. (see p. 6)

The connection of the sporidial stages with the parasitic phase.

The efforts which have been made to establish the connection between the many types of sporidium described for *Entyloma* species and the diseases associated with these smuts can be divided into three groups. First, direct observation

of the production of sporidia by the parasitic mycelium; secondly, reinfection of the host with the smut by inoculation with sporidia, and thirdly, inducement of the smut to complete its life cycle in culture. The first two groups are considered below, and the last will be discussed later. (p. 27)

The actual attachment of the sporidium to the mycelium of the Entyloma in the host has not always been considered by the authorities. Many, (Stempell, Hanna) collected sporidia discharged by the drop-excretion mechanism by suspending pieces of leaf infected with Entyloma over a surface, and examining the deposit of sporidia, and did not trace the original connection of the sporidia with the parasitic mycelium. In this Marshall Ward provided an exception when dealing with the "conidial" type of sporidium found in E.ficariae. He described how the mycelium in older lesions entered the sub-stomatal space and projected through the orifices of the stomata, abstricting sporidia at the ends of the hyphae. He also showed some hyphae forcing their way between adjacent epidermal cells.[1]

Schroeter (1877) sectioned leaves of Papaver infected with E.fuscum and showed how the promycelia of the germinating chlamydospores passed through the stomatal opening and formed long, spindle-shaped sporidia at the surface of the host.

Pethybridge (1928) described the germination in situ

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[1] And also see p. 13 for Schroeter's description of the "conidial type".

of the chlamydospores of E.dahliae. He traced the promycelium through a stoma to the surface of the leaf where it developed a crown of branches, and later large numbers of sporidia. Other authors made no reference to the connection of the sporidia with the mycelium in the host, and Stempell in particular did not trace the origin of his half-moon-shaped sporidia.

The second way of establishing the identity of the sporidia as part of the life cycle of the Entyloma, by reinfecting with the sporidia, has not so far been satisfactorily demonstrated. Marshall Ward was the first to attempt to reinfect the host, and he made a mass sowing by means of a camel-hair brush of sporidia of Entyloma ficariae, obtaining characteristic lesions and a new crop of sporidia three to four weeks later. Kaiser (1936) induced infection with a suspension of sporidia of E.fergussoni in water, resulting in chlamydospore formation in the host.

In most other smuts uni-nucleate sporidia conjugate before infection takes place. Marshall Ward showed a figure of what appeared to be two sporidia which had conjugated, and stated that "this is a very common event when they lie close together." He also said that whether or not the sporidia conjugated first, entry to the host was effected in exactly the same way, through a stoma. Stempell (1935) claimed to have found pairs of sporidia which had already conjugated on

the surface of leaves infected with E.calendulae, although he never actually observed the conjugation, and in E.ficariae "all attempts to obtain conjugation between ... the sickle-shaped conidia ... were unsuccessful."

Infection experiments with monospore and paired sporidial cultures have not been described.

The parasitic mycelium in smuts has generally been assumed to be dicaryotic, a state which begins with the conjugation of sporidia or promycelial branches, or hyphae, (after the short haploid phase of the life cycle, when the smut is independent of the host) and which ends with the fusion of two nuclei in the young chlamydospore. These two nuclei are generally accepted as being the direct descendants of those nuclei which first became associated at conjugation of the sporidia. This is one of the many assumptions generally held to be true of all smut fungi, although supported by little evidence, as Miss Sampson pointed out in her Presidential Address to the British Mycological Association (1939). Since the whole of the parasitic phase of the life cycle occurs between these two events it may be supposed that some form of conjugate division of the nuclei such as is found in the Higher Basidiomycetes takes place. Very little is known of the parasitic mycelium of smuts, as attention has been focussed mainly on the beginning or end of the parasitic phase, the entry to the host, or the formation of the

chlamydospores. Examination of the parasitic mycelium has been followed with difficulty owing to its fineness, and its twisting course through the host. The small size of the nuclei makes it difficult to count them.

Churchward (1940), working with Tilletia caries, found that association of nuclei was not permanent and occurred three times in the life cycle of this smut; the first two taking place before entry into the host. The first association occurred after the fusion in pairs of sporidia which form in a crown at the tip of the promycelium. These nuclei later separated and passed into narrow hyphae which gave rise to uninucleate secondary sporidia. The second association occurred after the fusion of hyphae derived from the secondary sporidia and resulted in the production of a wider mycelium. The nuclei of this mycelium divided conjugately for a time, but after penetration of the host the coenocytic mycelium contained many nuclei which were no longer in pairs. The third association could be observed immediately prior to chlamydospore formation, but at what stage this association was set up was not observed, as the mycelium was only examined in its earlier and latest stages.

An examination of parasitic smut mycelium for clamp connections was made by Seyfert (1927). He worked mainly with Ustilago Vuijckii on Luzula species, and although many of his figures are quite convincing, those few in which the

nuclei are stained do not resemble typical clamps very closely. When compared with Buller's figures (1931) of clamp mycelium in Coprinus lagopus it can be seen that none of Seyfert's figures of stained material depicts clearly the fusion between the tip of the clamp branch and the main hypha. In fact Seyfert's drawings of young mycelium are very similar to Harder's "pseudoschnallen" (1927). Harder studied the relation between nucleus and protoplasm in Schizophyllum commune by micro-operations removing one nucleus of a dikaryon. He found that structures superficially resembling clamp connections were formed in secondarily haploid mycelium for some time after the removal of the other nucleus. In these "pseudoschnallen", there was never an open passage between the tip of the clamp branch and the mycelium behind the original cross-wall. Seyfert also examined the parasitic mycelium of many other smut genera and species for clamp connections and his figures for Entyloma calendulae show what appear to be normal clamps in material from a young chlamydospore sorus teased apart. Seyfert found clamp connections in parasitic mycelium of E. ficariae (E. ranunculae), and E. chrysosplenii.

Production of mycelial sporidia:- If the assumption, supported by Seyfert's observations, is correct, and the parasitic mycelium in some species of Entyloma is dicaryotic at least in its later stages, the problem remains of how so large a

range of foliar sporidia is formed on this mycelium. In those species where the chlamydospores germinate *in situ* they provide a possible source of sporidia, but where they do not, the sporidia must be abstricted directly off the mycelium. In those sporidia, (usually of the cylindrical or allantoid type) which have been shown to have one nucleus, this involves the fusion or separation of the two nuclei of the dikaryon. Diploid nuclei are unusual in smuts except in the mature chlamydospore, and vegetative segregation of nuclei should result in two genetically different strains of sporidia. Where mycelial sporidia are stated to be binucleate they would presumably contain one dikaryon and would constitute a means of dispersal of the dikaryotic phase. Stempell indicated the parallel between his half-moon-shaped sporidia (which were binucleate and produced mycelium with clamp connections in culture) and the uredospores of rusts, which also perpetuate the dikaryophase. Stempell himself remarked on the strange existence within the life cycle of one smut, of an unstable dikaryotic parasitic mycelium producing uninucleate sporidia, and so stable a dikaryotic saprophytic mycelium as results from the germination of the half-moon-shaped sporidia. Kaiser (1936) also pointed out that two kinds of binucleate sporidium had been reported for *Entyloma Candulae*, Stempell's half-moon-shaped kind, and his own needle-shaped kind which were formed when the chlamydospores of the *Entyloma* germinated. Kaiser thought that the two accounts

were not necessarily contradictory, since he was dealing with sporidia of chlamydospore origin, and Stempell with "conidia" produced by the mycelium. Kaiser found clamps in the parasitic mycelium of E. calendulae, but considered them "rudimentary." Derx (1948) was extremely sceptical of the existence of two types of dikaryotic mycelium in one smut. It has already been stated that very little is known of the parasitic mycelium in smuts, and the only evidence that it is dikaryotic in Entyloma is Seyfert's observations of clamps in three species, recorded above. The fusions between allantoid sporidia prior to penetration seen by Marshall Ward and Stempell for two of the same species (E. ficariae and E. calendulae respectively) afford some support for the supposition that the parasitic mycelium is dikaryotic.

In certain strains of Ustilago maydis (Stakman et al., 1929), monosporidial ("solo-pathogenic") lines were shown to infect the host. Should a similar occurrence take place in Entyloma, it might account for the production of uninucleate sporidia which are rarely seen to fuse, but not for the binucleate ones.

Growth of smuts in culture. It has long been known that the promycelium in smuts is capable of living for a limited time outside the host as a saprophyte, and uninucleate sporidia produced by this mycelium have been successfully grown in culture, especially in the Ustilaginaceae. The dikaryotic

mycelium, which is formed by the fusion of promycelial branches or of sporidia, was described in the early literature as unstable in culture (see Sampson, (1939); Christensen and Rodenhiser, (1940)). Dickinson (1927) reported that in Ustilago hordei the dikaryotic mycelium reverted to the haploid state in culture. In 1941, however, Thren, working on Ustilago nuda, showed that the dikaryotic mycelium in culture underwent a stable course of nuclear division followed by septum formation resulting in a row of hyphal segments, the middle one with two nuclei, and those at each side with one nucleus each. The two isolated monokaryons fused with each other, thus maintaining the dikaryophase. This course of events was essentially like the formation of clamp connections, although the typical appearance of the usual type of clamp was not evident.

Typical clamp connections were recorded in saprophytic mycelium. These in only two other instances, were on the germination of the half-moon-shaped sporidia described for Entyloma calendulae by Stempell, and for E. dahliae by Sampson (Ainsworth and Sampson, 1950). Derx (1949), however, maintained that these "sporidia" did not belong to the life cycle of the Entyloma.

The half-moon-shaped sporidia collected by Stempell in the deposit of sporidia shot off a lesion of E. calendulae, germinated on agar to give a strong mycelium, three to

four times as wide as that formed by the allantoid sporidia obtained from the same Entyloma material, and it grew much faster. At regular intervals on the mycelium typical clamp connections were formed, and Stempell followed their formation and the behaviour of the conjugate nuclei, and showed that the process was exactly like that found in the Hymenomycetes. The mycelium grew embedded in the medium, but a system of aerial hyphae formed in young cultures, and at the tips of these hyphae half-moon-shaped sporidia arose singly, and were discharged by the drop-excretion mechanism. When the cultures were three to four weeks old, Stempell described how "typical chlamydospores" formed on the tips of hyphae and agreed in shape and size with the chlamydospores produced in the host plant. At the base of each chlamydospore was a clamp connection. The only difference which Stempell recorded between the cultural chlamydospores and those found on the host was that the former were binucleate throughout, and he stated that "one had great difficulty in finding spores with only one nucleus, that is, with fused nuclei," and he thought that some cultural condition must have hindered the fusion of nuclei.

These cultural chlamydospores germinated soon after their formation to give a normal promycelium and sporidia, but only if they were taken from younger cultures. In older cultures the germination was irregular. Sometimes a germ

tube resembling the beginnings of a promycelium produced a secondary spore at the tip, in other cases two germ tubes arose from the same chlamydospore, and occasionally a mycelium with clamp connections was formed by the germinating spore. Stempell did not examine cytologically any of the cultural chlamydospores showing "normal germination", and his figures of this germination are not very convincing, but he claimed to have observed the entire life cycle of Entyloma calendulae in culture.

Chlamydospores have often been reported in cultures of other smut fungi, but they have seldom been shown to germinate, and their nuclear condition has not always been determined. Sartoris (1924) described cultural chlamydospores of Ustilago heuffleri which germinated in the normal manner, and Leach, Lowther and Ryan (1946), working with U.striiformis, reinoculated the host by means of chlamydospores obtained in culture. Nyland (1950) obtained chlamydospores of Entyloma compositarum in culture which germinated in the same way as those formed on the host.

Stempell also followed the germination and further growth on agar of his uninucleate crescent-shaped sporidia from lesions of both E.ficariae and E.calendulae. He described how they multiplied for a short time by budding, but later grew out to form a sparsely branched hypha without cross walls. The latter were only formed in older cultures, where they cut off ageing portions of mycelium. The nuclei

in young mycelium lay widely separated from one another, and in older segments of mycelium only one nucleus was present. Stempell claimed that the crescent shaped sporidia and cultures originating from them were "without doubt haploid."

After a few days in culture, sickle-shaped sporidia were produced on short unbranched hyphae similar to those found on the host plant, together making a powdery mass at the surface of the colonies from whence they were actively shot off.

About five weeks later the colony became yellowish-brown in colour due to the production of chlamydospores resembling those found in the host plant in size and appearance, but this time they had one nucleus which Stempell presumed to be haploid. They germinated by a hypha and eventually formed mycelial colonies which were rather denser than those formed by the sporidia, and in which chlamydospores were eventually formed either in chains or solitarily on the tips of the hyphae.

Stempell therefore described two types of mycelial colony derived from foliar sporidia of E.calendulae, that originating in uninucleate allantoid sporidia, and that formed by the binucleate half-moon-shaped sporidia. He also described two sorts of chlamydospore formed by these mycelia, one haploid type and one diploid type, but otherwise closely

resembling each other and the chlamydospores found in the host. Stempell did not reinfect the host with any of the sporidia he isolated from it or with chlamydospores formed in culture.

B. Sporobolomycetaceae

Introduction:- The organisms classified together in the Sporobolomycetaceae are a heterogenous collection of forms all of which alike produce unicellular hyaline spores on sterigmata, and these are violently discharged by the drop excretion mechanism. These characteristic spores were given the name of "ballistospores" by Derx (1930), in reference to their manner of dispersal.

The first of the organisms in this group to be seen was a pink, yeast-like fungus isolated by Fischer and Brebeck (1894) from a Japanese source and placed provisionally by them in Blastoderma. Kluyver and van Niel, (1924-5) examined this isolate and showed that unicellular spores were being discharged from the surface of colonies on wort agar by the drop-excretion mechanism typical of the discharge of basidiospores. They placed Blastoderma salmonicolor Fischer and Brebeck in a new genus Sporobolomyces as S.salmonicolor (Fischer and Brebeck) Kluyver and van Niel, and at the same time described two other new species, S.roseus and S.tenuis.

In 1930 Derx described the ballistospore flora obtained when leaves of many plants are suspended over the surface of an agar medium. This could be divided into three main groups by the appearance of the colonies formed when the ballistospores germinated. The first group was made up of species of Sporobolomyces, forming pink, yeast-like

colonies, together with two species of Bullera (Derx), which formed similar but colourless colonies. In the second group were mycelial colonies which were produced by the germination of colourless curved ballistospores, and these colonies formed similar ballistospores on the ends of short sterigmata developed by certain of their hyphae. These ballistospores resembled very closely Brefeld's pictures of the secondary sporidia of Tilletia tritici. They were so like the sporidia shown by Buller and Vanterpool (1925) to be developed on the saprophytic mycelium of Tilletia tritici and violently projected from it, that Derx wondered whether his fungi were not cultural forms of certain Tilletiaceae, and provisionally called them Tilletiopsis. He isolated five strains, distinguished by the dimensions of their ballistospores.

The third type of colony found in Derx's ballistospore impression consisted of mycelium forming clamp-connections, and he considered that the ballistospores which produced this type of mycelium probably belonged to one or more of the members of the true Basidiomycetes.

Nyland (1950), examining the impressions of leaves of twenty-eight different healthy plants, found the flora to consist of Sporobolomyces species in great abundance with large numbers of ballistospores belonging to Tilletiopsis, although these were not quite so common as those of Sporobolomyces species. He found that the collections of

Tilletiopsis fell into two well-defined groups neither of which fitted exactly into any of Derx's five groups, and which he named T.washingtonensis and T.minor. Derx (1930), had previously found species of Sporobolomyces and Bullera in great abundance on the leaves of a variety of plants. They were present in greatest number on old leaves and especially on damaged spots and places already invaded by parasitic fungi and greenfly, and he said there was no doubt that they live on the exudates provoked by the parasite.

In 1948 Derx described a fungus which he first isolated in 1925 from a pustule of Puccinia malvacearum on Althaea rosea. The large, laterally-compressed, sub-reniform ballistospores were found amongst an impression on agar of the basidiospores of the Puccinia, and were of about the same size, but unlike the rust basidiospores they germinated to give a true mycelium with many clamp connections. The mycelium grew partially submerged in the medium, but soon formed an aerial system of tapering hyphae which produced at their tips more ballistospores of the same shape as those originally collected. Derx drew attention to the close resemblance between his fungus and Stempell's (1935) Mycelium II, isolated from Entyloma calendulae, and considered the two to belong to an independent fungus which he described as constituting a new genus and species - Itersonilia perplexans. Derx stated that he isolated the fungus from

very diverse material including Althaea rosea, where it was present as a secondary infection, and he considered that it was as a secondary infection that Stempell had isolated the fungus from Entyloma calendulae.

In 1952 Olive described a fungus which he collected in a spore impression of a pustule of Kunkelia nitens on a dewberry leaf. The ballistospores formed by this fungus covered a wider range of measurement than those of Derx's Itersonilia perplexans, but he considered it to be the same species. Olive was of the opinion that the fungus had first been seen and illustrated by Stempell, stating that "it is fairly certain that Stempell's Entyloma material was contaminated with Itersonilia perplexans."

Nyland (1948) and (1949), described two very similar fungi, one, isolated from a dead leaf of Acer macrophyllum, he considered to be another species of Itersonilia, I.pyriformis, and for the other he erected a new genus, Sporidiobolus, with one species, S.Johnsonii.

The family, Sporobolomycetaceae, was proposed by Derx in 1930 to include the two genera, Sporobolomyces and Bullera. Both these genera budded like yeasts and produced ballistospores.

In 1948 Derx distinguished four genera in the family:-

Sporobolomyces Kluyver et van Niel (1924)

Bullera Derx (1930)

Tilletiopsis Derx (1948)

Itersonilia Derx (1948)

He amended his diagnosis of the family, since some species of Sporobolomyces had been shown to form pseudomycelium, and Tilletiopsis and Itersonilia true mycelium. Nyland's Sporidiobolus (1948) although in many ways resembling Itersonilia, has not been included in the family Sporobolomycetaceae by any authority.

Morphology of the members of the family Sporobolomycetaceae.

The following short descriptions, taken from Lodder and Kreger van Rij (1952) are of the four genera usually included in the family.

Sporobolomyces Kluyver et van Niel

Red or salmon-pink organisms with oval-elongate to hyphal cells. True mycelium and pseudomycelium may be present. Vegetative reproduction mainly by budding. Ballistospores are kidney-shaped or sickle-shaped and develop in an oblique position to the aerial sterigmata.

Bullera Derx

Colourless, palid, pale yellow, creamy-coloured to yellowish organisms with round to oval cells. No mycelium or pseudomycelium. Vegetative reproduction by budding. Ballistospores are symmetrical, round or

oval and develop in an oblique position to the aerial sterigmata.

Tilletiopsis Derx

No yeast cells.[1] Septate hyaline mycelium forming aerial sterigmata on which single smooth, hyaline, curvated, sickle-shaped ballistospores are formed.

Itersonilia Derx

No yeast cells. Septate hyaline mycelium forming clamp connections connected with swollen terminal cells which after further growth may become intercalary aerial sporophores, not ramified, at the apex thinner and changing gradually into sterigmata on which develop ballistospores singly and terminally. They are asymmetrical - but not sickle-shaped - rather thick, smooth, hyaline, at one side slightly dented.

Species of two of the above genera have been mentioned in connection with smut fungi; Tilletiopsis for the resemblance which the ballistospores bear to the secondary sporidia of Tilletia, and Itersonilia perplexans has, according to Derx, actually been assigned by Stempell (1935) to Entyloma, as a stage in the life cycle of Entyloma calendulae, therefore descriptions of these two genera and their species

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[1] A yeast-like budding stage has recently been reported for Tilletiopsis washingtonensis and T.minor by Nyland (1950).

will now be given in greater detail.

Tilletiopsis Derx. The generic name was suggested provisionally by Derx in 1930, who stated that he had isolated five species distinguished by the dimensions of their spores.

Strain 1	...	ballistospores	9μ	x	2μ
" 2	...	"	7μ	x	1.6μ
" 3	...	"	15μ	x	2μ
" 4	...	"	13μ	x	2.3μ
" 5	...	"	12μ	x	3μ

These ballistospores on germination gave a very fine, ramifying mycelium which formed a felty, coherent, cartilaginous mass, white or colourless to begin with, later more or less brown according to the species. This mycelium formed, on sterigmata, slender spores which were curved or sickle-shaped, and were projected at maturity. In 1948 Derx validated the genus with a Latin diagnosis giving his strain 4 as the type species.

Nyland (1950) described two fungi which he named Tilletiopsis washingtonensis and T.minor. The spore dimensions differ slightly from Derx's, although they fit into the general range,

T.washingtonensis	...	ballistospores	(18-19) x (2-2.6)μ (average 14 x 2.4)μ
T.minor	...	"	(5.8-14)x(1.5-2)μ (average 9 x 1.8)μ

Since Derx's Strain 4 was no longer obtainable in culture, Nyland proposed his larger strain, T.washingtonensis, as the

type species. The descriptions given in Nyland's paper are similar in essentials to those of Derx, but the latter (1948) did not mention the budding of the cells, which was profuse in both Nyland's species. The budded cells were somewhat longer than the ballistospores and more or less straight, rather than falcate. A further character described by Nyland was the formation of chlamydospores in old cultures of both T.washingtonensis and T.minor.

Nyland showed that the ballistospores, vegetatively budded cells, and chlamydospores were uninucleate. The last were very difficult to stain and he could not determine whether or not there was a binucleate stage in the young chlamydospore. The chlamydospores germinated to form either a mycelium or vegetative budding cells. In both species there was a mucilaginous form of colony in which budding was profuse, besides the mycelial type.

In 1952, Tubaki described sixteen strains of Tilletiopsis isolated in Japan, six of which he identified as T.minor, one he recognised as a new variety T.minor var. flava, two strains as belonging to a new species T.lilacina and five to a new species T.cremea. According to Tubaki, the variety differed from T.minor in the colour and the consistency of the colony, while the two new species differed from T.washingtonensis in the colour and consistency of their colonies and the number of chlamydospores formed.

Itersonilia Derx. The first published description of this fungus was that of Derx in 1948, although it is possible that it had previously been mistakenly described by Stempell in 1935 as a stage in the life cycle of Entyloma calendulae. Derx himself took this view, and was satisfied to refer to Stempell for a cytological description of his fungus. Derx gave a diagnosis of the new genus and species Itersonilia perplexans, together with some morphological and physiological details. A much fuller description was given however by Olive (1952), who also isolated the fungus from a rust pustule. The dimensions of the ballistospores given by Derx and Olive differ slightly, those of Olive covering a greater range in length than those of Derx. The two figures are given below:

Derx	8-10	x	14-15 μ
Olive	7.6-10.6	x	12.2-18 μ

Both authors confined their descriptions to the fungus in artificial culture, that of Olive being very detailed.

Olive showed that the spores (or ballistospores of Derx), were formed singly at the ends of long, tapering sterigmata which he called "sporophores." The latter arose from inflated "sporogenous cells," each with a clamp connection at its base. A sporogenous cell was formed at the end of a vegetative hypha, and germinated without a rest, its entire contents passing into the sporophore and

thence into the spore. Occasionally a second sporogenous cell formed at the tip of the sporophore, or a ballistospore arose directly from a vegetative hypha. The ballistospores were somewhat curved and apiculate, and germinated by repetition or by means of germ tubes.

Chlamydospore-like cells produced singly or in short chains were found in cultures several days old, above and below the surface of the agar. They became filled with oil droplets, and upon ageing developed walls slightly thicker than the walls of the hyphae. Olive stated that "their function has not been determined, but it is considered likely that under appropriate conditions they may germinate vegetatively."

In the mycelium, clamp connections with conjugate division were found. In ballistospore formation, the two nuclei from the sporogenous cell were shown to pass via the sporophore into the ballistospore, which was discharged in the binucleate condition. The chlamydospores produced by the dikaryotic mycelium were shown to be binucleate, and Olive concluded that they were vegetative chlamydospores. In older cultures many chlamydospores underwent nuclear degeneration and their walls often collapsed. They ranged in shape from globose to clavate and were often irregular.

If cultures grown on yeast-beef-extract agar were flooded with water, large numbers of uninucleate conidia

were formed, budded off near the ends of slender hyphae which were without clamp connections. The conidia were curved and tapered towards each end.

Single conidial cultures on agar media formed yeast-like colonies and little mycelium. Some ballistospores were formed at the surface of such colonies. If a portion of such a colony was subcultured on to Sabouraud agar, a mycelium was formed very like that typical for the fungus, but with incomplete clamp connections, the clamp branch failing to fuse with the hypha. Ballistospores formed by such a mycelium were morphologically similar to, and behaved like, those from typical cultures of the fungus, but were uninucleate, and the incomplete clamp mycelium was shown to consist of uninucleate cells.

Itersonilia pyriformis Nyland was described by Nyland (1949). It differed from I. perplexans in that the chlamydospores have no clamps associated with them, and in one or two minor differences. Nyland's "chlamydospores" include Olive's "sporogenous cells," and Olive (1952) suggested that Nyland's fungus might be a variant of I. perplexans.

Nyland's Sporidiobolus may be conveniently mentioned here. This fungus, described in 1949, with one species, S. Johnsonii Nyland, produced a well developed mycelium with clamp connections at almost every septum. The drawings of these clamps show them to be unlike the usual, regular type

such as is produced by true Basidiomycetes and Itersonilia. The mycelium formed uninucleate ballistospores, and yeast-like budding cells, also uninucleate. Thick-walled resting spores were formed, which germinated after six days or more either to give a mycelium or the yeast-like cells. The chlamydospores were uninucleate when young, but later became binucleate, presumably by division, and these two nuclei fused. This fusion is unlike anything so far reported in the Sporobolomycetaceae. On germination, the cells formed were uninucleate and Nyland assumed them to be diploid. Nyland could not state where in the life cycle of the fungus reduction division occurred. Because of the unique fusion of nuclei in the resting spore, Nyland could not put this fungus in the Sporobolomycetaceae, nor could he place it amongst the smuts as it is non-parasitic. He therefore considered it to be a Heterobasidiomycete of unknown affinities.

Distribution of the Sporobolomycetaceae. The isolations of Itersonilia perplexans from rust pustules by Derx and Olive have been followed by others from plants infected with fungi. Wilkinson (1952) isolated Itersonilia sp. from a parsnip canker, and claimed to have induced symptoms of the disease in parsnip by reinoculation with Itersonilia. Species of Tilletiopsis have been isolated from a very wide range of leaves (Derx, 1930; Nyland, 1950; Tubaki, 1952) but were not

particularly associated with fungal parasites. Gregory (1954) reported that a large proportion of the spora of the atmosphere in a barley crop consisted of spores of Tilletiopsis. Last (1955), in a study mainly concerned with Sporobolomyces sp., found that for the plants tested, the number of contaminated leaves and the area contaminated with T.minor increased with age, but none of the dead leaves was contaminated. He found some evidence that Sporobolomyces and Tilletiopsis compete on barley leaves.

Sporobolomyces has been reported from Great Britain by several writers, either as S.roseus or simply as a record of the genus. Tilletiopsis sp. was recorded from Great Britain by Gregory (1954) and T.minor by Last (1955), but neither T.washingtonensis nor Itersonilia has been recorded from this country.

Relationships of the Sporobolomycetaceae. All the fungi now classified in the Sporobolomycetaceae are placed there because of the manner of discharge of their ballistospores, and because they have not so far been found a place in any other group. Many may be imperfect stages of other fungi which are already classified elsewhere, and a search for such connections still continues. Until such connections have been found, there is the problem of where to place the family as it stands.

The systematic position of the Sporobolomycetaceae

has been fully discussed by Lodder and Kreger van Rij (1952), who gave a review of the literature on the subject.

The original Sporobolomycete was placed amongst the yeasts by Fischer and Brebeck (1894) who used the genus Blastoderma for all pellicle forming yeasts except those showing "endogenic cell formation." When Kluyver and van Niel (1924) showed the manner in which the spores were discharged, this fungus was transferred to the new genus Sporobolomyces, and eventually Blastoderma became a nomen nudum, but the now increased number of members of the Sporobolomycetaceae are, even today, usually classified among the non-ascosporogenous yeasts.

From the first there has been much discussion on whether the sporobolomycetes can be considered to be Basidiomycetes on the sole evidence of the manner in which the ballistospores are discharged. Originally only Sporobolomyces and Bullera were involved, but with the discovery of Itersonilia and Sporidiobolus another character of this group, that of clamp connections, was added.

As soon as Kluyver and van Niel (1924) discovered the manner of spore discharge in Sporobolomyces they suggested that the fungus might belong to the Basidiomycetes, a view which was also held by Derx (1930). Buller (1933) went further, and said that since spore discharge by drop excretion was only found in Basidiomycetes, Sporobolomyces

was a Basidiomycete. He also maintained that all spores discharged by the drop-excretion mechanism were basidiospores. This view was based solely on morphological considerations and did not take into account the cytological history of the spores. All normally produced basidiospores are uninucleate as a result of meiosis, in the basidium, of a diploid nucleus formed by the fusion of the two nuclei originally present in each young basidium (or teleutospore of rusts, chlamydospore of smuts). No such preliminary fusion occurs in the formation of the ballistospores of members of the Sporobolomycetaceae, and furthermore some of the secondary sporidia (allantoid sporidia formed after the fusion of the needle-shaped sporidia) of Tilletia tritici, and the ballistospores of Itersonilia, are binucleate. For this reason Lohwag (1926), and Stempell (1935) considered that ballistospores formed otherwise than as a result of meiosis in a basidium are conidia and not basidiospores.

If the Sporobolomycetaceae are accepted as Basidiomycetes the problem remains of where to classify them within that group. The various members of the family are obviously not all closely related to each other, and there is a strong possibility that some of them are imperfect stages in the life cycles of some Basidiomycetes already recognised by their perfect stages. Until such biological relationships can be traced, the position of the

Sporobolomycetaceae within the group remains very much a matter of choice. The various alternatives which have at one time or another been suggested are surveyed fully in the book by Lodder and Kreger van Rij.

Kluyver and van Niel put Sporobolomyces in the "Hemibasidii," and those who follow their lead think them nearer the Smuts ~~rather~~ than the Rusts. Lunder (1940) even gave a complicated derivation of Sporobolomyces from Ustilago scabiosae by a reduction of the chlamydospore and promycelium, and a decrease in the number of spores, and he gave intermediate examples taken from the smuts. Stempell had already suggested that Tilletiopsis might belong to Tilletia and be a free-living form of sporidium, which its ballistospores so closely resemble. Nyland (1950) furthermore stated "in fact there is sufficient morphological similarity between Tilletiopsis and Entyloma to entertain the thought that they may be identical. However, the fact that Tilletiopsis was isolated from the surfaces of apparently healthy leaves of twenty-eight unrelated hosts ... is considered good circumstantial evidence that these fungi are not species of Entyloma." The fact that Tilletiopsis species are not parasitic prevented Nyland from classifying Tilletiopsis amongst the smuts. Nyland (1949) also claimed that his genus Sporidiobolus, which also produces ballistospores, could not be classified within the Sporobolomycetaceae as it stood,

as fusion of nuclei occurred in the cultural "chlamydospores" and "constitutes a sexual phase of the order of that occurring in the smuts." Gäumann (1949), thought that Sporobolomyces might be a vegetative ("sprouting") stage of certain smut fungi. Finally, the Sporobolomycetaceae have been considered by some, eg. Martin (1940), as imperfect stages of certain Tremellales, some of which have a conidial phase.

Uninucleate conidia were described by Maxwell (1954) from several genera of the Thelephoraceae of the Agaricales. They were produced both by dikaryotic clamp mycelium and "simple-septate haploid" mycelium. The conidia were grown in artificial culture and all germinated to give a mycelium without clamp connections. These conidia could be induced to fuse with each other, and were shown to contain nuclei of two reaction types identical with those of the two nuclei in the dikaryon, and also with those of the nuclei of the parent basidiospores. Mostly, they were produced in groups from swollen, simple conidiophores, or from lobed ones. In Corticium roseo-pallens, however, they were produced on sterigmata arising directly from cells of the vegetative mycelium. (Maxwell quoted this last evidence from Lyman (1907), and Nobles (1937).) It is possible that Tilletiopsis may represent some such conidial phase. Bulat (1953) described the isolation of ballistospores from cultures of Dacrymyces Ellisii, which formed cultures with

all the characteristics of Tilletiopsis, and which he believed to be members of this genus. Bulat did not state, however, whether this fungus was obtained from monospore cultures of the Dacrymyces, and it is possible that he was dealing with a contaminant present on its surface.

Leaving the Basidiomycetes, there has been one instance of spore discharge by the drop excretion method reported from the Ascomycetes, by Wieben (1927), who collected aerial conidia very like the ballistospores of Sporobolomyces from cultures of Taphrina pruni made from germinating ascospores. Lodder and Kreger van Rij, who repeated the experiment, got a mixture of Taphrina and Sporobolomyces by Wieben's method of isolation, and therefore said that there is a strong suspicion that Weiben's cultures were contaminated with a species of Sporobolomyces.

Thus, members of the Sporobolomycetaceae may probably be produced as conidial forms, which are capable of living as independent saprophytes, from fungi which are already placed in the Basidiomycetes or elsewhere. Until such biological connections have been traced, it is difficult to decide where the group as it stands should be classified.

If they are not to be included in the Basidiomycetes, the Sporobolomycetaceae must rank as Fungi Imperfecti. Lodder and Kreger van Rij, following Clements and Shear (1931) and Martin (1940), considered that there is no suitable place

for the family within the existing orders of this group, unless it was alongside the anascosporogenous yeasts (Cryptococcales or Pseudosaccharomycetaceae) in the Moniliales. Lodder and Kreger van Rij, however, excluded Tilletiopsis and Itersonilia from the yeasts as they thought that they had no budding stage. Recently, however, Nyland (1950) described a budding stage in Tilletiopsis washingtonensis and T.minor and also in Sporidiobolus. If, however, the Sporobolomycetaceae are considered as a family of equal rank with the Cryptococcaceae, the question of the absence of budding is not of great importance to their classification.

II METHODS

Sources of Material

For purposes of exploration, an attempt was made to isolate sporidia from the lesions of as many different species of Entyloma as possible for ~~purposes of~~ comparison. Of the fourteen species recorded in the literature for Great Britain (see Appendix 2), material of three species only, E. ficariae, E. calendulae, and E. dahliae was readily available in this country. When, however, in 1950, an opportunity arose for study in France, the range of available material was greatly enlarged, as a high proportion of the European species have been recorded from France, and the commoner ones are more readily obtainable.

Altogether ten species were collected and examined from various French localities, and these collections are summarised in Table 1. The record of E. tanaceti is new for France, having been described from Germany by Sydow (1936).

Later, E. calendulae and E. dahliae were studied in greater detail and collected from as many different localities as possible both in England and France. In England, E. dahliae was more easily obtained than E. calendulae.

The sources of material of the three species found in England are listed below, those places marked "main source" being where regular collections were made throughout the period of study and which are the sources of the material described in the following pages.

Sources of British material

Entyloma ficariae	Lacey Green, Bucks Egham hill, Surrey	(Main source)
Entyloma calendulae	Lacey Green, Bucks South Mundham, Sussex Datchet, Bucks	) both subsequently) grown at (main source)
Entyloma dahliae	Wisley, Surrey Englefield Green, Surrey Lacey Green, Bucks Datchet, Bucks Wraysbury, Bucks Bradfield, Berks Bournemouth, Hants	(main source)

Table 1 Species of Entyloma collected in France

Species	Host	Locality	Date
Entyloma ficariae (Berk.) Fisch. v. Waldh.	Ranunculus ficariae L	Versailles	March, 1951
E. calendulae (Oudem.) de Bary	Calendula arvensis L	Near Paris	May, 1951
	C. officinalis (cult.)	St. Germains	May, 1951
		Versailles	July, 1951
E. dahliae Sydow	Dahlia spp. (cult.)	Versailles	June, 1951
E. tanaceti Sydow	Tanacetum vulgare L	Versailles	November, 1950
E. bellidis Kreiger	Bellis perennis L	Versailles	March, 1951
		Rambouillet	April, 1951
E. achilleae Magn.	Achillea millefolium L	Versailles	October, 1950
E. serotinum Magn.	Symphytum officinale L	Versailles	October, 1950
E. fergussoni (B. and Br.) Plowr.	Myosotis arvensis (L) Hill	Near Paris	June, 1951
		Rambouillet	April, 1951
	M. stricta	S.E. France	May, 1951
E. eryngii (Corda) de Bary	Eryngium campestre L	Near Mantes	July, 1951

Collection of Material

Wherever possible, living material was used, especially for collecting foliar sporidia, and these were grown in pure culture. Fixed and stained material was used for cytological investigations. The living leaves, infected with *Enyloma* species, were collected in sterile tins or sterile polythene bags, and set up as soon as possible for the collection of sporidia. The way in which this was done depended on the manner of discharge of the sporidium. *Ballistosporic sporidia* from lesions of *Entyloma* species were collected by making use of the forcible manner in which they are discharged. Whole leaves, where these are small as in *Ranunculus ficaria*, or pieces of large leaves like those of *Dahlia*, were washed well in running tap water, then in sterile glass distilled water, dried slightly with filter paper and fixed to the lids of Petri dishes with strips of moistened cellophane. The ballistospore deposit was either collected on a suitable medium in the lower half of the dish, or on slides treated with Mayer's slide fixative for later cytological investigation, and these slides were placed in the bottom of the dish on a piece of filter paper damped with water to maintain a moist atmosphere. When it was necessary to examine the entire ballistospore flora of a leaf surface, ie. including members of the *Sporobolomycetaceae*, the leaf was not washed before sticking it to the lid of the

dish, and even after repeated washings it was very difficult to remove all sporobolomycetes, as they adhere very closely to the leaf, especially around lesions of fungal parasites. Disinfecting the leaf surface by other means than washing was liable to kill the parasitic mycelium of the Entyloma. Other sporidia not forcibly discharged were collected by scraping the surface of the leaf gently with a sterile glass rod. The adhering sporidia were removed by dipping the rod in a small drop of sterile distilled water which was then examined microscopically or transferred to an agar medium. Ballistospores of members of the Sporobolomycetaceae were collected from leaf surfaces in the same way as ballistosporic sporidia isolated from lesions of Entyloma.

Culture Methods

A solution of 2% malt extract solidified with 2½% powdered agar was used as the standard medium for growing stages of the smut in culture, and for culturing members of the Sporobolomycetaceae, but in most cases the fungi grew equally well on Sabouraud agar, potato extract agar, and potato dextrose agar.

Monospore cultures were made by picking up individual spores from the surface of an agar medium. If these were ballistospores, a thin scattering was obtained on the surface of the medium by slowly rotating the lid of a Petri dish on which was fixed the material which was actively discharging

ballistospores. If the spores required in monospore culture were of a type which is not actively discharged, they were spread over the surface of the medium in dilute aqueous solution. The agar surface was then examined microscopically for isolated sporidia which were picked up by hand on the end of a dry, finely drawn-out glass rod, and transferred to a marked area on a fresh agar surface. A binocular dissecting microscope (~~x~~) was used for this operation.

Germination of chlamydospores: The preparation of clean chlamydospore material from mature lesions is made the more difficult because the spores have to be teased out of a leaf which is already heavily invaded by secondary infections of Sporobolomycetes, bacteria, etc. The most effective means of obtaining clean cultures from chlamydospores was to cut mature lesions out of the infected leaf and wash them in continuous running tap water for ten minutes. The pieces of leaf were then ground in a mortar in .001% mercuric chloride solution, left to soak for ten minutes, then the mercuric chloride solution was decanted off after spinning the suspension of chlamydospores twenty times in a hand centrifuge. The spores were shaken in six changes of sterile glass distilled water, and the liquid decanted after each washing by centrifugation.

A suspension of individually separated chlamydospores could thus be obtained in a suitable concentration by gently

centrifuging, and set up in hanging drops on coverslips placed on glass rings cemented on to glass microscope slides with vaseline. A drop of sterile water was placed inside the ring, and the whole slide kept in a Petri dish at 24°C in the light. After a period of growth depending on the stage of development of the spores required, the coverslip was picked up in a pair of forceps and the drop lightly touched on to the surface of an agar medium in a Petri dish. The drop was spread by gently tilting the dish, and in a short while the film of water spread sufficiently to allow the growth of the promycelium and sporidia to be followed under the microscope. It was not found practical to germinate chlamydospores directly on the surface of agar, as certain contaminants which had not been eliminated by the previous washing always became established early; this was not the case when the chlamydospores were started off in water suspension.

For subsequent staining, hanging drops were transferred to slides spread with a thin layer of Mayer's fixative and allowed to dry out at room temperature.

Measurements

The thin walls and rapid germination of the sporidia of Entyloma and the ballistospores of the sporobolomycetes make it difficult to determine accurately the measurements of these spores. If collected on a dry surface they rapidly

become desiccated, but in water they may swell, and even a coverslip placed over a sporidium may distort its thin walls. Since any method introduces inaccuracies, the same method was used consistently in order that the measurements would be valid for purposes of comparison. The following procedure was followed:

Sporidia recently discharged (within the last hour), were collected on the surface of 2% malt agar, and a number "0" coverslip lowered gently into position over them. The film of liquid on the surface of the medium filled the spaces between the spores and kept the coverslip from pressing too hard on them. One hundred spores were measured in each sample.

Chlamydospores, with their more rigid walls, were measured in water, under a coverslip.

All drawings were made with the aid of a camera lucida, and their dimensions calibrated by means of a slide scale.

Cytological Technique

The material to be examined was either in the form of sporidia and chlamydospores and the mycelium derived from their germination, or consisted of parasitic mycelium within the tissues of the host. The former could be fixed as a smear for further treatment, but for examination of the latter, a method of clearing the host tissue or of sectioning was

needed. The methods used for preferential staining of the mycelium in the host will be described first.

Chloral hydrate method: McBride's method (1936) was used for following the course of the parasitic mycelium in the leaf without sectioning. Pieces of leaf tissue were put in a saturated solution of chloral hydrate (5 grams of chloral hydrate in 2 ccs. of water), and the air was removed from the tissues with a vacuum pump. After a week or more the leaf tissue was effectively cleared, and was placed in a mixture comprising 1 cc. of 2% acid fuchsin in 70% alcohol, 12 ccs. of chloral hydrate solution, and 8 ccs. of 95% alcohol for one to two days; the excess stain was then removed by returning the material to chloral hydrate solution. After passing through 80% and 93% alcohol to absolute alcohol, the material was mounted in "Euparal."

"Pectozyme" method: Another method used for following the parasitic hyphae, made use of a commercial preparation of pectinase to separate the cells of the host and display the mycelium, (Fraymouth and Hawker, 1952).

A 2% suspension of commercial "Pectozyme" in McIlvaine's phosphate/citrate buffer at pH 4 was shaken intermittently for one hour at 25°C, and then filtered, and the filtrate added to an equal volume of water tinged with aniline blue. Plant material preserved in formalin-alcohol was sectioned by hand, washed in water, and put in enzyme

extract over night. The sections were then mounted on a slide, a coverslip added, and gentle pressing on the coverslip was sufficient to separate the cells of the host, leaving the mycelium intact.

Sectioned material: Finally, fixed leaf material, embedded in paraffin wax, was sectioned with a microtome at 8 µ, and the sections mounted on slides for staining.

Examination of the nuclei: Throughout the investigation it was important to know the position and number of nuclei present in sporidia, germinating chlamydospores and mycelium. For this purpose a large number of methods for staining the nuclei were tried, of which only two were found to be satisfactory. These were a modification of Haidenhain's iron alum haematoxylin technique, and Olive's propiono carmine method (Olive, 1952).

The fluid used for fixing depended on the staining technique to be subsequently applied. Material to be stained in haematoxylin was fixed in Sansome's modification of Navaschin's fluid for twenty-four hours or more. The 0.5% aqueous solution of haematoxylin was ripened for six weeks before use, and 3% ferric chloride was used for mordaunting prior to staining, and for differentiating after staining. After differentiating, the material was dehydrated in increasing concentrations of alcohol, and mounted in "Euparal."

This method was used for chlamydospores and

promycelia as well as for sporidia and ballistospores and the mycelium obtained when they germinated.

Olive's method, using propiono carmine, was used on material prepared as slide smears, and previously fixed for half an hour in a modified Carnoy's fluid consisting of one part glacial acetic acid, two parts absolute alcohol, and three parts chloroform. The slides were then passed through decreasing concentrations of alcohol to water, and mordaunted in 4% ferric alum solution before staining in a 2% solution of carmine in 40% propionic acid. The progress of staining could be watched under the microscope, and when complete, the slides were washed, dehydrated, and mounted in "Euparal."

This method was not used for chlamydospores, where the penetration of the fixative was not sufficient through the thick walls.

III EXPERIMENTAL WORK

Introduction

The lack of knowledge of sporidial stages of the genus Entyloma made it seem desirable to survey the different kinds of sporidium found associated with as many species as possible within the genus. Although the number of species which were available was small in comparison with the total number in the genus, even these species represented too large a field for detailed investigation. For more intensive work, therefore, the smuts on Dahlia and Calendula were chosen, and in the following pages fuller accounts are given of these two species.

In order to determine how many of the different kinds of "sporidium" isolated from Entyloma calendulae and E.dahliae lesions actually represent stages in the life cycle of these smuts, the preliminary survey was followed by:

(1) An investigation of the proportion and order in which the various kinds of sporidium are produced,

(2) a study of the germination of the chlamydospores of the smuts,

(3) examination of the parasitic mycelium of the smuts,

(4) inoculation experiments using single and paired sporidial isolates, and

(5) an examination of the ballistospore flora of leaves not exhibiting the symptoms associated with an infection of Entyloma, and of leaves attacked by

other fungi, to determine the distribution of members of the Sporobolomycetaceae which might have been confused with stages in the life cycle of Entyloma.

The terminology used throughout the experimental part of this study varies somewhat from the accepted use. Where isolations are made from the surface of a lesion it is difficult to determine, without detailed investigation of each isolate, whether it is part of the life cycle of the smut or not. In the following pages the term "sporidium" is not used strictly in its accepted sense, since some of the "sporidia" isolated were later found to have no part in the life cycle of the Entyloma. All thin walled spores isolated from the lesions of Entyloma and from its chlamydospores, and all similar thin-walled spores formed by these isolates in culture are here called sporidia.

Preliminary Survey of Sporidia from Several Species of Entyloma.

Foliar sporidia were isolated from lesions of as many species of Entyloma as possible, and the isolates compared with one another. The isolations were made over an extended period, as the species examined were not all available at the same time, but wherever possible the sporidia were maintained in pure culture for later comparisons. The sporidia isolated were either discharged forcibly from the leaf (ballistospores), or became detached without force; the former were more numerous. Several different kinds of sporidium were distinguished, but commonly the shape of the sporidia was either allantoid, half-moon-shaped or needle-shaped.

In the earlier records the distinction between two types of allantoid sporidium, indistinguishable when discharged, but which germinate to give mycelial colonies of different appearance, was not appreciated, and separate records were not made of these two types. Later work on the lesions of E.calendulae, E.dahliae, and E.ficariae, revealed their presence, and the accounts of these three species include descriptions of both types of allantoid sporidium. It is possible that more than one type was present in other species from which allantoid sporidia were isolated, but at the time of isolation they were not recognised.

The sporidia found in association with the lesions

of the smuts investigated in the preliminary survey will now be described briefly.

Entyloma ficariae

From lesions of this species only allantoid sporidia were isolated. On germination two distinct types of sporidium were consistently recognised from amongst the allantoid sporidia.

Allantoid sporidia: (19-25) (average 22) x (2-4) μ; thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both surfaces of the leaf. Uninucleate.

Type (a): Germination takes place in water and on the surface of an agar medium at both ends to give a fine, mycelium (1-2) μ wide, rarely branching and with very occasional septa. A characteristic of this mycelium is the twisting course followed by the hyphae on the medium. This, together with the limited branching, gives a sparse mycelium which slowly extends over the surface of the medium. No blastospores are formed, but allantoid ballistospores are produced at the surface of the colonies at the ends of short side branches. (see fig. 1, p.77)

Type (b): Germination takes place in water and on the surface of an agar medium at both ends to give a fine, branched mycelium, (1-2) μ wide; the sporidium as it empties of its contents becomes septate, but the hyphae are rarely

so, although septa are more common in the mycelium from this type of sporidium than in that from the type (a) above. The twisting of mycelium so characteristic of type (a) sporidia does not occur here, and the mycelium grows more quickly over the surface of the medium. (see Fig. 1, p. 77)

Germination of the type (b) sporidium is often followed by the formation of many yeast-like blastospores. Allantoid ballistospores, like those isolated in the impression from the smut lesion and like those formed by mycelia from type (a) sporidia, are later produced at the surface of the colonies at the ends of short side branches. Chlamydospores (12-14) μ in diameter, hyaline, are formed occasionally in old cultures.

Since the two types of sporidium can only be told apart after they have germinated, it cannot be stated with absolute certainty that both types have a single nucleus in each sporidium. However a large number of ballistospore impressions were examined cytologically, some at least of which should have included representatives of both types of sporidium, and all these were uninucleate. Similar impressions tested by germination were usually found to consist of both types (a) and (b). The measurements of the allantoid sporidia isolated from lesions of E. ficariae, given above, represent the range of sporidium size found in an impression of a lesion, and thus cover both types of

sporidium. By measurements made later (see p.132), the range of size of ballistospores of type (b) sporidia was found to fall within the measurements given for both the types taken together.

Entyloma calendulae

Four different kinds of sporidium were isolated from lesions of this species, two allantoid types, (a) and (b), one half-moon-shaped kind, and one needle-shaped kind. The two allantoid types can only be distinguished from each other when they germinate, like the allantoid sporidia isolated from lesions of E.ficariae.

Allantoid sporidia: (11-24) (average 15.5) x (2-3.5) μ; thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both surfaces of the leaf. Uninucleate.

Type (a): Germination takes place in water and on the surface of an agar medium at both ends to give a fine mycelium (1-2) μ wide, rarely branching, and with very occasional septa. The mycelium follows a twisting course on the surface of the medium giving rise to a colony resembling that formed by the type (a) sporidia isolated from lesions of E.ficariae. No blastospores are formed, but allantoid ballistospores, like those discharged from the leaf, are produced at the surface of the colonies at the ends of short side branches. Occasionally, very long, narrow,

needle-shaped sporidia are formed from similar side branches, and project into the air above the colony. These sporidia, very few of which were measured, are about (20-40) x 2 μ in size, and are not forcibly discharged.

Type (b): The second type of allantoid sporidium found in leaf impressions of Entyloma calendulae is exactly like the type (b) found in association with E.ficariae, and germinates to give a fine, branched mycelium (1-2) μ wide, which produces a rather more quickly growing colony than that formed by type (a) sporidia. Blastospores are often formed on germination instead of hyphae. Allantoid ballistospores are later produced at the surface of the colonies at the ends of short side branches; needle-shaped spores, however, are never produced. Chlamydospores, (12-14) μ in diameter, are occasionally formed in older cultures.

Since this type of sporidium and the type (a) sporidium cannot be distinguished until after they have germinated, the number of nuclei and the range of sporidium measurement applies to both types taken together, as in the examination of E.ficariae.

Half-moon-shaped sporidia: (13-21) (average 16) x (7-15), (average 11) μ, thin walled, hyaline. Shape approximately that of a 1/6th segment of a sphere of which the corners are rounded. Forcibly discharged from both leaf surfaces. Binucleate.

The sporidia germinate in water and on the surface of an agar medium by one, rarely two, germ tubes which arise from the curved surface of the spore. These form a broad hypha, (3-5) μ wide, the spore rapidly emptying of contents which are soon also withdrawn from the base of the germ tube, which becomes septate. These septa are simple, but afterwards cross walls are only formed in association with clamp connections. These clamps arise at intervals, and a side branch often arises later from the clamp branch, or opposite it. In young cultures half-moon-shaped sporidia are formed at the tips of tapering side branches. These are similar to the sporidia isolated from the smut lesion, and are discharged by the drop excretion mechanism. In older cultures, chlamydospores, (10-15) (average 13) μ in diameter, are formed in the ageing parts of the mycelium.

When half-moon-shaped sporidia are allowed to germinate in contact with a hard surface such as glass, or the epidermis of a leaf, their mode of germination differs from that followed on agar. The germ tube does not at first form a mycelium, but swells up to give a lobed appressorium-like structure with dense contents. From this structure one or more hyphae arise, which grow out to form a typical clamp mycelium. (see fig. 3, p. 79)

Needle-shaped sporidia: (27-57) (average 37) x (1-2) μ; thin walled, hyaline, usually straight, but sometimes slightly

curved, tapering at the distal end. Detached from the parent hypha by simply falling off, and are not forcibly discharged. Binucleate.

Germination takes place in water and on the surface of agar at both ends to give hyphae (1-2) μ wide, which form a rarely septate, sparsely branched mycelium like that formed by the allantoid sporidia of type (a). Similarly also, the hyphae follow a twisting course on the surface of the medium. Needle-shaped sporidia are abstricted from the ends of short side branches, and allantoid ballistospores are formed on short sterigmata.

Entyloma dahliae

Four kinds of sporidium were isolated from lesions of this species, two allantoid types, (a) and (b), one half-moon-shaped kind, and one needle-shaped kind.

In this species, as in the two species of Entyloma already dealt with, the two types of allantoid sporidium could only be distinguished on germination.

Allantoid sporidia: (11-22) (average 15) x (2-3) μ; thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both leaf surfaces. Uninucleate.

Type (a): Germination occurs at both ends of the sporidium to give a fine, rarely septate, sparsely branched mycelium, (1-2) μ wide. The twisting course, characteristic of

mycelium from type (a) sporidia from lesions of E.ficariae and E.calendulae, occurs and leads to the same kind of sparse colony. Both allantoid ballistosporic sporidia, and needle-shaped sporidia which are not forcibly discharged, are produced by this mycelium.

Type (b): This type of allantoid sporidium resembles the type (b) sporidium found in association with E.ficariae and E.calendulae, and on germination forms either a fine branched mycelium, (1-2) μ wide, or a series of blastospores. The colony is dense and more quickly growing than that formed on germination of type (a) sporidia. Allantoid ballistosporic sporidia are formed at the ends of short side branches; needle-shaped sporidia however never occurred. Chlamydospores, (12-14) μ in diameter, are occasionally formed in older cultures.

Half-moon-shaped sporidia: (12-17) (average 14) x (7-14), (average 11) μ; thin walled, hyaline. Forcibly discharged from both surfaces of the leaf. Binucleate.

Germination in water and on the surface of an agar medium is by one, rarely two, germ tubes to form a hypha (3-5) μ wide which initiates a mycelium with clamp connections at intervals as in similar sporidia isolated from lesions of E.calendulae. Half-moon-shaped ballistospores, and later chlamydospores, (10-15) (average 13) x (10-15), (average 12.5) μ in diameter are formed in culture. This type of

half-moon-shaped sporidium looks and behaves exactly like that isolated from lesions of E.calendulae.

Needle-shaped sporidia: (25-67) (average 42) x (1-2) μ; thin walled, hyaline, usually straight, but sometimes slightly curved, tapering at the distal end. Detached from the parent hypha by simply falling off without drop excretion. Binucleate. Germination occurs at both ends to give a hypha (1-2) μ wide, which forms a rarely septate, sparsely branched, twisting mycelium from which more needle-shaped sporidia and also allantoid ballistosporic sporidia are formed as in E.calendulae.

The following records are included for comparison with the more detailed observations given above. They are, however, incomplete, as at the time they were made the possibility of there being two types of allantoid sporidium present was not explored. It is not, therefore, significant that in each case only one type of allantoid sporidium is recorded as no germination tests were made.

Entyloma tanaceti

Two kinds of sporidium were isolated from lesions of this smut, an allantoid kind and a half-moon-shaped kind.

Allantoid sporidia: (11-21) (average 16) x (2-4.5) (average 3.5) μ, thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both

surfaces of the leaf. Uninucleate.

Germination occurs in water and on the surface of an agar medium at both ends to give a fine, branched, rarely septate mycelium, (1-2) μ wide, the emptying sporidium becoming septate. Ballistospores like those discharged from the lesion are later produced at the surface of the colonies at the ends of short side branches.

Half-moon-shaped sporidia: (12-18) (average 15) x (7-13) (average 9.5) μ; thin walled, hyaline. Forcibly discharged from both leaf surfaces. Binucleate. Germinate in water and on the surface of an agar medium by one, rarely two, germ tubes to form a hypha, (3-5) μ wide, which later gives a mycelium with clamp connections at intervals like that formed by the sporidia isolated from lesions of E.calendulae and E.dahliae. Half-moon-shaped ballistosporic sporodia, and later, chlamydospores, (8.5-13.5) (average 11) x (8.5-14) (average 11) μ, are formed in culture.

Entyloma bellidis

One kind of sporidium was isolated from lesions of this species.

Allantoid sporidia: (12-18) x (1.5-3) μ; thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both surfaces of the leaf. Number of nuclei not determined.

Entyloma achilleae

One kind of sporidium was isolated from lesions of this species.

Allantoid sporidia: (11-19) x (1.5-3) μ; thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both surfaces of the leaf. Number of nuclei not determined.

Entyloma serotinum

Two kinds of sporidium were isolated from lesions of this smut, an allantoid kind, and a half-moon-shaped kind.

Allantoid sporidia: (11.5-25) (average 18) x (1.5-4.5) (average 3.5) μ; thin walled, hyaline, cylindrical and straight, rather narrower at the attachment end. Forcibly discharged from both surfaces of the leaf. Uninucleate. Germinate in water and on the surface of an agar medium at both ends to give a fine, branched, rarely septate mycelium (1-2) μ wide.

Half-moon-shaped sporidia: (11.5-17) (average 14) x (7.5-14) (average 10) μ; thin walled, hyaline. Forcibly discharged from both surfaces of the leaf. Binucleate.

Germinate by one, rarely two, germ tubes to form a hypha (3-5) μ wide which later gives a mycelium with clamp connections at intervals as in the mycelium formed by similar sporidia isolated from lesions E.calendulae, E.dahliae, and E.tanaceti.

Half-moon-shaped ballistospores, and later chlamydospores, (8-14) x (8-13) (average 11 x 11) μ, formed in culture.

Entyloma fergussoni

Two kinds of sporidium were isolated from the lesions of this species, one allantoid, and one half-moon-shaped.
Allantoid sporidia: (12-20) x (1.5-3.5) μ; thin walled, hyaline, cylindrical and straight, rather narrower at the attachment end. Forcibly discharged from both leaf surfaces. Number of nuclei not determined. Germinate at both ends to give a fine, branched, rarely septate mycelium (1-2) μ wide.
Half-moon-shaped sporidia: (12-18) x (7.5-12) μ; thin walled, hyaline. Forcibly discharged from both surfaces of the leaf. Number of nuclei not determined.

Germinate in water and on the surface of an agar medium by one, rarely two germ tubes, to form a hypha (3-5) μ wide which forms a mycelium with clamp connections.

Entyloma eryngii

One kind of sporidium was isolated from lesions of E. eryngii.
Allantoid sporidia: (12-24) x (2-3) μ; thin walled, hyaline, slightly curved and more pointed at the attachment end. Forcibly discharged from both surfaces of the leaf. Number of nuclei not determined.

Germinate in water and on the surface of an agar

medium at both ends to give a fine, branched, rarely septate mycelium, (1-2) μ wide, the sporidium becoming septate as it empties.

Ballistosporic sporidia like those isolated from the surface of the smut lesion, are later formed at the ends of short side branches and discharged at the surface of the colony by the drop excretion mechanism.

Because of the small amount of material available it was impossible to investigate cytologically sporidia isolated from E.bellidis, E.achilleae and E.fergussoni.

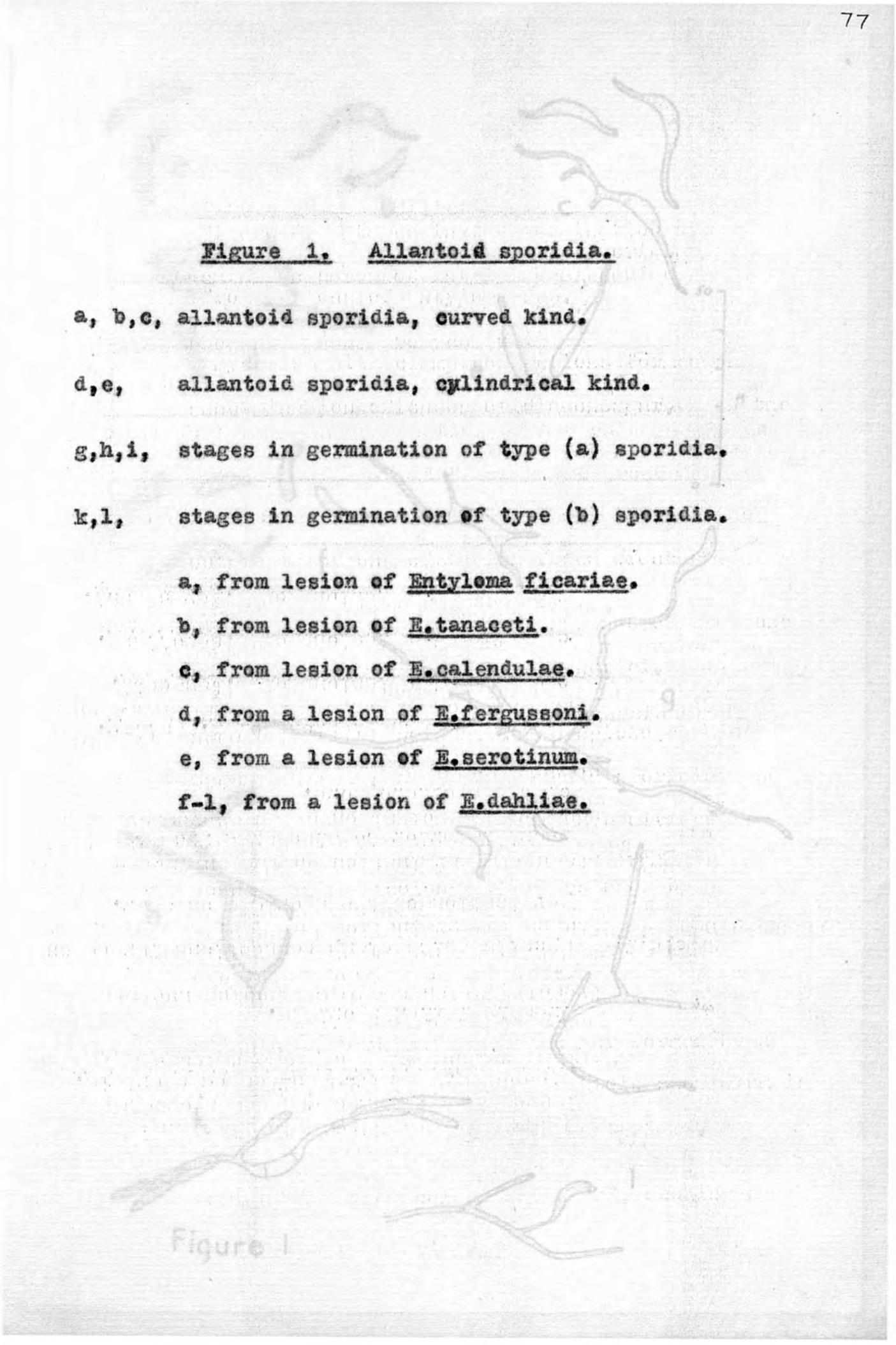


Figure 1. Allantoid sporidia.

a, b,c, allantoid sporidia, curved kind.

d,e, allantoid sporidia, cylindrical kind.

g,h,i, stages in germination of type (a) sporidia.

k,l, stages in germination of type (b) sporidia.

a, from lesion of Entyloma ficariae.

b, from lesion of E.tanaceti.

c, from lesion of E.calendulae.

d, from a lesion of E.fergussoni.

e, from a lesion of E.serotinum.

f-l, from a lesion of E.dahliae.

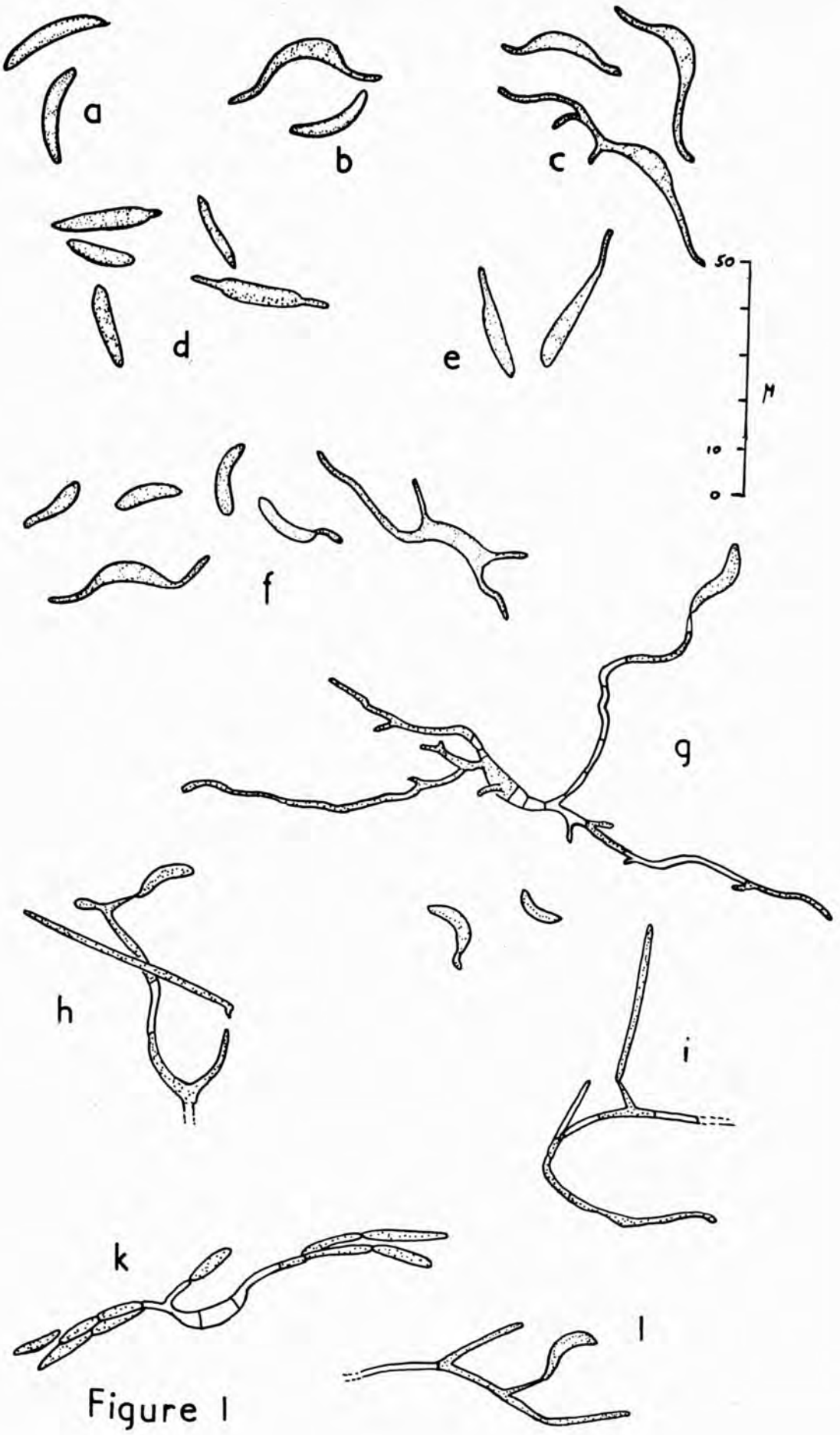


Figure 1

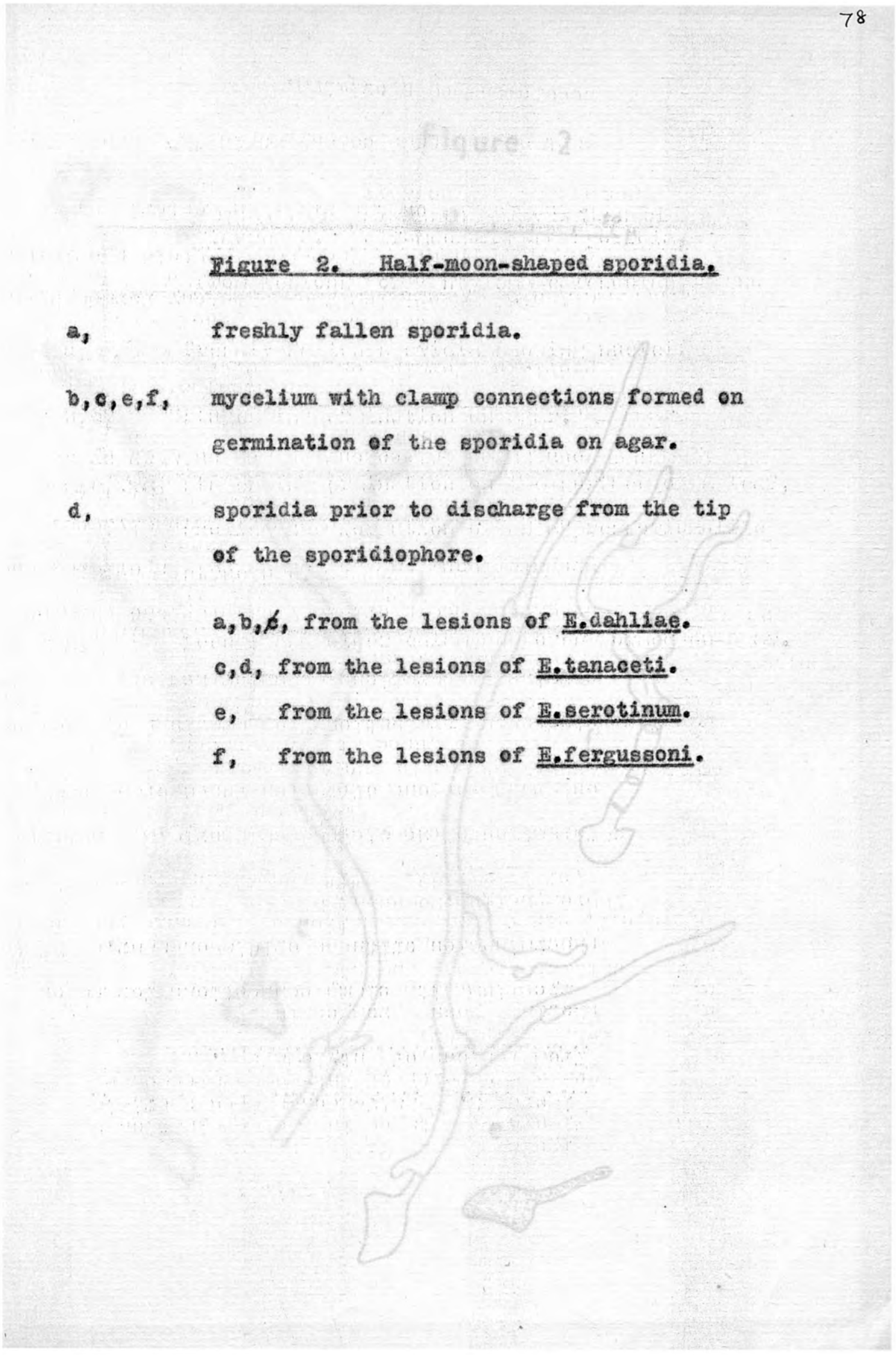


Figure 2. Half-moon-shaped sporidia.

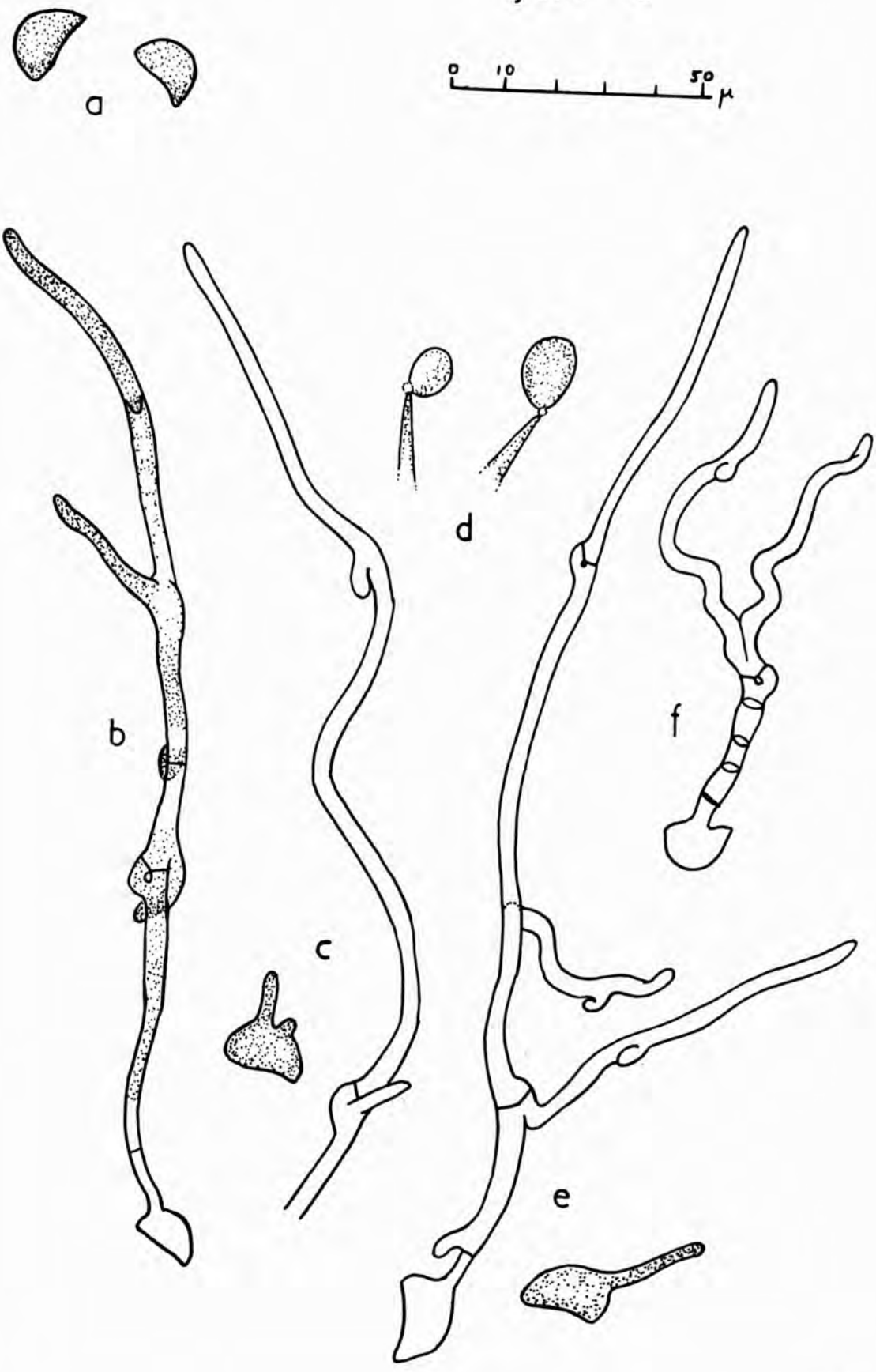
a, freshly fallen sporidia.

b, c, e, f, mycelium with clamp connections formed on germination of the sporidia on agar.

d, sporidia prior to discharge from the tip of the sporidiophore.

a, b, c, from the lesions of E.dahliae.
c, d, from the lesions of E.tanaceti.
e, from the lesions of E.serotinum.
f, from the lesions of E.fergussoni.

Figure 2



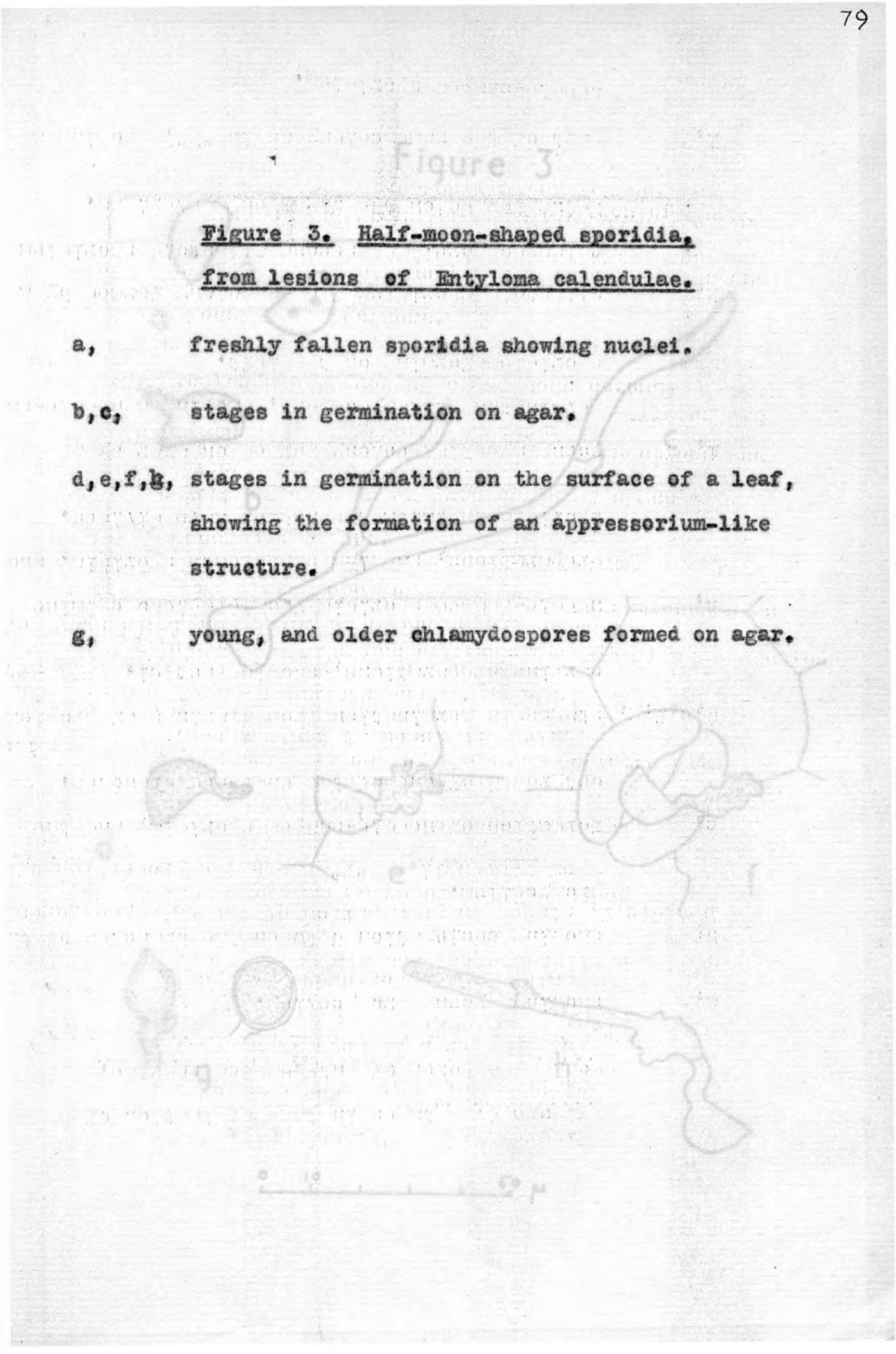


Figure 3. Half-moon-shaped sporidia, from lesions of Entyloma calendulae.

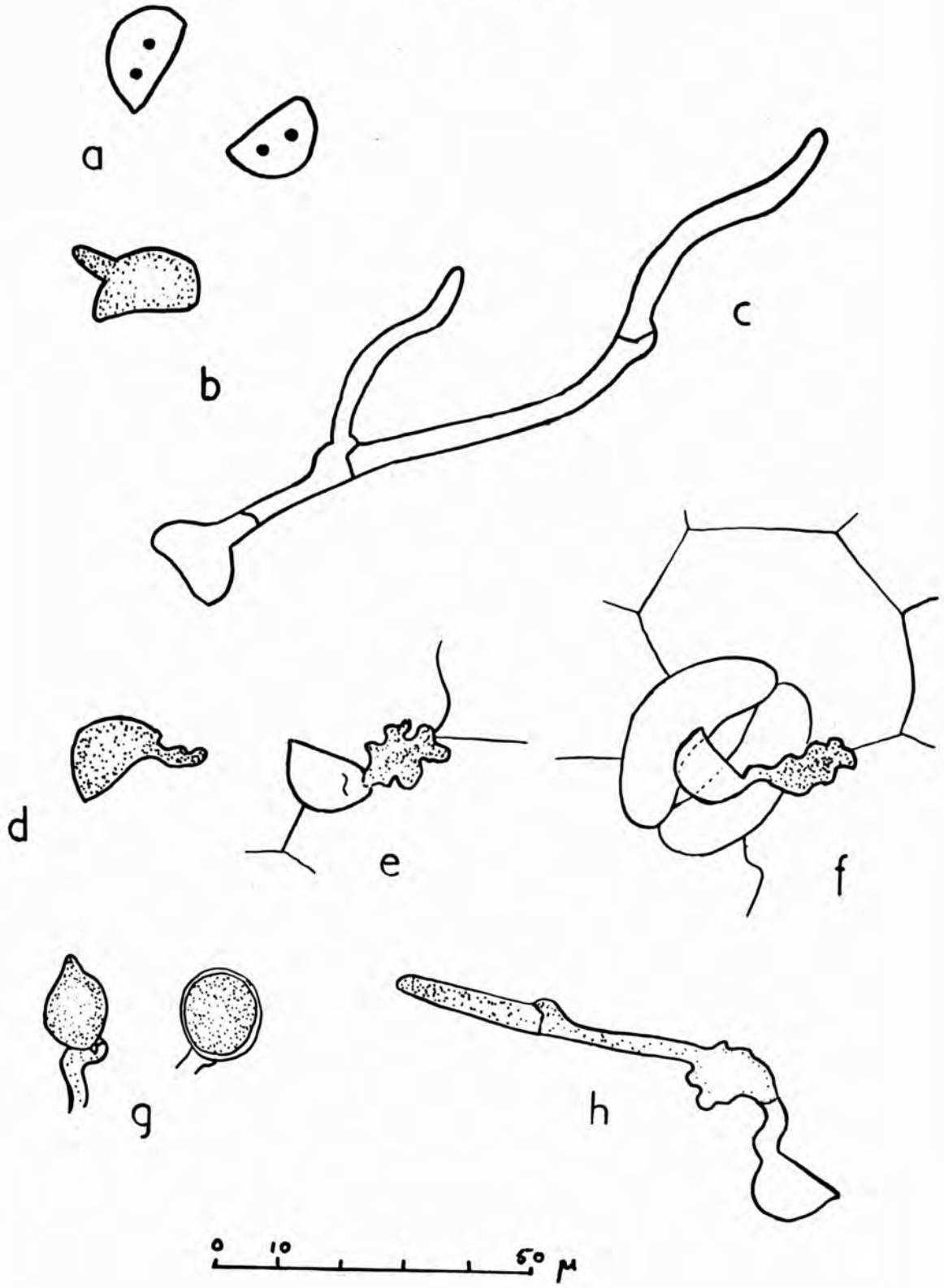
a, freshly fallen sporidia showing nuclei.

b, c, stages in germination on agar.

d, e, f, g, stages in germination on the surface of a leaf, showing the formation of an appressorium-like structure.

g, young, and older chlamydospores formed on agar.

Figure 3



Summary of the Preliminary Observations on Sporidia

It appears from the foregoing descriptions that all the sporidia isolated from the lesions of the species of smut examined can be placed in one of three groups according to their shape.

1. Allantoid sporidia, which are uninucleate where investigated, and germinate to give a narrow, rarely septate, branching mycelium, more or less twisting.

2. Half-moon-shaped sporidia, which are binucleate where investigated, and germinate to give a wide, branching, mycelium, with clamp connections at intervals, which produces chlamydospores.

Sporidia of both the above groups are discharged by the drop excretion mechanism.

3. Needle-shaped sporidia, which are also binucleate, and germinate to give a narrow, twisting mycelium, and are not forcibly discharged.

Some smut lesions, like those of E.eryngii, apparently produce sporidia from only one of these groups. Others, like E.calendulae, may produce sporidia from all three main groups, including two types of allantoid sporidium.

Within the allantoid group of sporidia distinctions can be made both on shape and behaviour. In the majority of cases, the allantoid sporidia are curved or crescent-shaped, but sporidia isolated from smut lesions on

boraginaceous hosts, (E.serotinum and E.fergussoni) are cylindrical and straight. The cylindrical kind was not studied in detail, but on germination produces a narrow mycelium as does the curved kind in other species. The germination of the allantoid sporidia was followed closely in material from E.calendulae, E.dahliae and E.ficariae, all species from which curved sporidia were isolated. In each case, two types of sporidium could be recognised by the kind of mycelium formed, that of type (b) showing a greater tendency to branch, and occasional formation of budded cells.

With some kinds of sporidium, the mycelium formed on germination produces two kinds of sporidium, one like, and one unlike the sporidium which gave rise to the mycelium. Thus, needle-shaped sporidia in E.calendulae form a mycelium which produces both needle-shaped and allantoid sporidia.

The number of nuclei present in the three main kinds of sporidium is clearly of great importance. Where cytological techniques were used, the allantoid types were consistently uninucleate, and the half-moon-shaped and needle-shaped kinds binucleate.

The kind of mycelium produced by the two binucleate sporidia is strikingly different, the half-moon-shaped kind forming clamp mycelium, such as is characteristic of Basidiomycetes in the dikaryophase, while the needle-shaped sporidia produce a mycelium without clamps, and which closely

resembles the mycelium formed when uninucleate allantoid sporidia of type (a), isolated from lesions of the same species, germinate, as in E.calendulae. In fact throughout the whole range of sporidia there appear to be three morphologically clear cut types of mycelium, that formed by allantoid sporidia of type (a) or by needle-shaped sporidia, that formed by allantoid sporidia of type (b), and that formed by half-moon-shaped sporidia.

From this preliminary survey, summarised in Table 2, the number of different kinds of sporidium isolated from eight different smuts, though large, can be grouped into a number of types each of which can be found in association with the lesions of several different species of smut. This may indicate a certain uniformity in the sporidia produced by the members of the genus Entyloma, but it is not certain how many of these types of sporidium represent stages in the life cycle of the Entyloma, and how many, if any, are stages of other fungi living on the surface of the lesions. The uniformity of sporidial types may merely be evidence that the same fungal contaminants are being isolated each time from different species of Entyloma.

Table 2

Kinds of Sporidium found on the Lesions of Entyloma spp.

Species	Sporidia found
Entyloma ficariae	Allantoid type (a) Allantoid type (b)
E. calendulae	Allantoid type (a) Allantoid type (b) Half-moon-shaped Needle-shaped
E. dahliae	Allantoid type (a) Allantoid type (b) Half-moon-shaped Needle-shaped
E. tanaceti	Allantoid Half-moon-shaped
E. bellidis	Allantoid
E. achilleae	Allantoid
E. serotinum	Allantoid (cylindrical) Half-moon-shaped
E. fergussoni	Allantoid (cylindrical) Half-moon-shaped
E. eryngii	Allantoid

III 1 Conditions and Order of Sporidium Production

During the preliminary survey and the more detailed investigations which followed, a large number of lesions of the following smuts were examined for the presence of sporidia: E.ficariae, E.calendulae, E.dahliae, E.tanaceti, E.serotinum. The first three species were studied throughout five seasons, the last two during one season, when several hundred lesions of the former, and nearly a hundred of the latter were examined.

The production of sporidia was sporadic in all species, probably due to their delicate nature, and climatic conditions. While many sporidia were produced after a period of dull weather or rain, few were formed following a period of hot weather and drought. The age of the lesion appeared to have some influence on the production of sporidia, and older lesions often produced a better crop of ballistospores than younger ones. Since, in most lesions, chlamydospores are formed very early, it was difficult to determine whether any sporidia were produced before the formation of chlamydospores. Allantoid sporidia of type (b) were, however, occasionally found on very young lesions of E.calendulae. The needle-shaped sporidia of E.calendulae and E.dahliae were found on all but very young lesions, and were not found in very dry conditions. If a lesion collected on a dry day was put in a refrigerator for a few hours, needle-shaped sporidia would sometimes develop. The fact that a large number of

the lesions examined yielded no sporidia at all was probably due to the influence of external conditions and the age of the lesion on sporidium production.

At the time of collection, notes were taken of whether the different kinds of ballistosporic sporidia fell simultaneously or in succession, and from the same or different parts of a given lesion. A selection of these recordings is given in Table 3, with the proportion and order of formation of allantoid sporidia and half-moon-shaped sporidia from lesions of species from which both types were isolated. In the table, no distinction is made between the two types of allantoid sporidium, since in E. calendulae and E. dahliae they cannot be told apart until after they have germinated, and in E. tanaceti and E. serotinum no distinction was made between the two shapes.

In general, allantoid sporidia fall before half-moon-shaped ones, which tend to be present on older lesions; lesions which are kept on the lid of a Petri dish will go on producing half-moon-shaped sporidia for several days. Occasionally, the order of production is reversed, and the half-moon-shaped sporidia are produced first. In the later work on E. calendulae and E. dahliae the sequence of production of the two types of allantoid sporidium was noted. The allantoid sporidia of type (b) were found to behave like the half-moon-shaped ones, and continued to be formed when the

leaf had been suspended over agar for some time, and after the type (a) sporidia had ceased to fall.

Sometimes an impression of a lesion is made up of more than one kind of sporidium, and these may fall simultaneously. When this happens, the different kinds of sporidium tend to occur in localised parts of the lesion, with little or no overlap. Some such impressions are analysed in Table 3, pp 89-90

If the entire leaf impression is considered, and not only the impression of the lesions, the allantoid sporidia of type (a) are localised under the lesions, irrespective of the species of smut, and the half-moon-shaped sporidia are nearly always beneath the lesion, (in two cases only were half-moon-shaped sporidia found on the uninfected parts of the leaves attacked by Entyloma species.) Sporidia of type (b) occur on the healthy part of the leaf as well as beneath the lesion. Species of Sporobolomyces, and very small, yeast-like, allantoid ballistospores probably belonging to Tilletiopsis species are also found on the healthy part of the leaf. Both these may also occur beneath a lesion, but when half-moon-shaped and allantoid sporidia of types (a) and (b) are being discharged from the surface of the lesion, the Sporobolomyces species and the very small ballistospores tend not to be found there.

The results of a more detailed survey of the

distribution of ballistospore-producing fungi on leaf surfaces are given on pp. 127 - 147.

Table 3.

Sequence and Position of the Fall of Allantoid and Half-moon-shaped Sporidia in Entyloma calendulae

Abbreviations: Allantoid sporidia ..... Al
Half-moon-shaped sporidia ..... H-m

The Petri dish lid was rotated after each recording.

Lesion	Time over agar	Sporidia fallen	Order	Position
1	19 hrs.	Al newly fallen H-m germinating	H-m first	Mixed
	plus 24 hrs.	Al many H-m few		
	Total 43 hrs.			
2	46 hrs.	Al germinating	Al first	Distinct patches
	plus 18 hrs.	Al germinating H-m newly fallen		
	plus 48 hrs.	Al only		
	Total 112 hrs.			
3	23 hrs.	Al newly fallen	Al first	Distinct patches
	plus 24 hrs.	Al germinating H-m newly fallen		
	plus 24 hrs.	Al newly fallen H-m germinating		
	plus 46 hrs.	Al germinating H-m germinating		
	plus 24 hrs.	H-m only, newly fallen.		
	plus 24 hrs.	H-m only, newly fallen.		
	Total 165 hrs.			

Table 3 (cont.)

Lesion	Time over agar	Sporidia fallen	Order	Position
4	48 hrs.	Al only	Al first	Some mixing
	plus 23 hrs.	Al germinating H-m newly fallen		
	plus 30 hrs.	Al newly fallen H-m germinating		
	plus 18 hrs.	H-m only, newly fallen		
	Total 119 hrs.			
5	24 hrs.	Al newly fallen H-m newly fallen	Both together	Distinct patches
	plus 24 hrs.	Al germinating H-m germinating		
	plus 30 hrs.	H-m only, some germinating		
	Total 78 hrs.			
6	20 hrs.	Al newly fallen H-m newly fallen (no further fall)	Both together	Some mixing
	Total 20 hrs.			

Table 3 (contd.)

Analysis of the sequence and position of sporidium-fall in E. calendulae.

Sequence

Allantoid sporidia fell first	3 lesions
Half-moon-shaped sporidia fell first	1 lesion
Both kinds fell simultaneously	2 lesions
Total	6 lesions

Position

Distinct patches	3 lesions
Distinct patches (some mixing)	2 lesions
Mixed	1 lesion
Total	6 lesions

III 2 Germination of the Chlamydospores of the Species of *Entyloma* Studied

In the literature the origin of the sporidia from germinating chlamydospores is often stressed. In the species of Entyloma examined in the preliminary survey, the chlamydospores formed in the host germinated in situ in three cases only, E.calendulae, E.dahliae, and E.eryngii, in the others after a period of rest. Sporidia found associated with the lesions of smuts in the latter group could not therefore have been formed directly by the germination of chlamydospores.

Of the three species of Entyloma mentioned above, material of E.eryngii was not easily obtainable, and for most of the work on chlamydospore germination E.calendulae and E.dahliae were used.

Sections cut through mature lesions reveal many chlamydospores in process of germination and the empty walls of those already germinated, and spores isolated from the lesion germinate in a few hours if set up in hanging drop cultures in sterile water. The early stages can be followed with ease in hanging drops, and the promycelia develop vigorously, but later stages are not so easily seen, as the spores tend to "clump" at the bottom of the drop, and the mycelium develops a starvation growth with the lack of nutrient substrate. If, however, a drop containing young, active promycelia is transferred to 2% malt agar in a Petri dish, and the germinating spores separated from one another

by spreading the drop across the plate, individual spores can be watched as they grow into colonies, and parts of the mycelium examined under the high power of the microscope.

It was not found practical to initiate germination directly on the surface of agar, as under these conditions the aerobic bacteria which remain after even the most careful washing of spores, over-grow the promycelia before the latter can develop far. In hanging drops, however, the promycelia are at an advantage and get ahead before they are transferred to agar.

The course of germination of the chlamydospores was thus followed in material grown in hanging drops and later transferred to agar medium (for details see p.95). In the early stages of the work examination of the nuclei was made in material fixed in Navaschin's fluid and stained with Haidenhain's haematoxylin. Although this method is good for establishing the position of the nuclei, the nucleus itself appears very large, and there is always a possibility that cytoplasm as well as the nucleus is being stained. When it is of importance to establish the number of nuclei, such as in a dicaryon, this method of staining is unreliable. Later, the method of staining adopted was that used by Olive (1952) for staining the nuclei of Itersonilia, using propiono carmine after mordaunting with ferric chloride. This method was found to be very satisfactory for staining the nuclei of the

germinating chlamydospores of Entyloma, while other methods which made use of lacmoid or carmine directly were useless. The carmine stains have so far not been applied extensively to smut nuclei, although Sampson, (Ainsworth and Sampson, 1950), recorded the successful application of lacmoid to the nuclei of the promycelium of Ustilago hordei.

Chlamydospore germination in Entyloma calendulae

In the very young chlamydospore of E.calendulae two nuclei were shown by staining with propiono carmine. In the mature chlamydospore a single very large body stains deeply with Haiderhain's haematoxylin, although it is difficult to say with certainty whether this is the nucleus or a storage product. (see fig.4a) Propiono carmine is not successful for mature chlamydospores.

Germination of the chlamydospore is first evident when the wall cracks and the promycelium emerges. In E.calendulae germination sometimes begins while the chlamydospore is still in the tissues of the host, and so in a hanging drop the promycelium may be seen to emerge at any time between one and twenty-four hours after the drop was set up. The promycelium widens almost at once, remaining constricted at its base where it leaves the chlamydospore. The elongation of the promycelium is extremely rapid at this stage, and it is very difficult to fix and stain a stage before the four, presumably haploid, nuclei are ranged

along its length, but occasionally a preparation shows a single nucleus at the base of a young promycelium. ~~(see fig.)~~ This nucleus is presumably the same one that is stainable with haematoxylin in the mature chlamydospore, and by analogy with other smuts, is assumed to be diploid. Likewise, it is assumed that the four nuclei found in the young promycelium arise by meiosis from the diploid nucleus. The four or more nuclei that result from the first divisions of the original single nucleus are arranged in a line near the base of the promycelium as the latter elongates. (see fig.4d) Meanwhile, some very small protruberances appear at the tip of the promycelium and rapidly elongate to form four or more, often up to eight, promycelial branches. Not until these branches are well formed do the nuclei move up the promycelium to take up a position near the bases of the branches into which they pass one by one at about the same time that the branches reach their maximum length. (see fig.4e) All the contents of the chlamydospore meanwhile pass into the promycelium and accumulate in its tip. Vacuolation occurs at the base, giving the appearance of many cross walls; in fact one, or occasionally two, cross walls are formed, cutting off the empty spore from the developing promycelium.

The length of the promycelium varies greatly with the conditions in the drop, reaching anything from 8 to 40 μ, the width being between 2 and 8 μ. The promycelial

branches are straight, measuring (10-20) x (2-3) μ, and in liquid medium they stand out at a wide angle in a crown at the tip of the promycelium. On solid medium they often diverge less from one another. Each branch has a single large nucleus, which takes up a position near the centre of the branch.

Conjugation of the branches in pairs occurs mainly at the tip, occasionally at the base, and is often brought about by means of a long conjugation tube, though sometimes by a shorter one. It is difficult to obtain stages in which the formation of this conjugation tube can be traced, but occasionally two unfused branches can be seen, each with a distal extension, and it is probable that fusion occurs between the tips of two such protruberances, and not from the fusion of a single prolongation of one branch with the tip of an unextended branch. Alternatively, the two extensions seen in these few cases may have been outgrowing branches destined never to fuse, as happens when an odd number of branches occurs in a crown. (see fig. 4 c)

From the distal part of the conjugation tube a hump appears into which the protoplasm with the two nuclei from the conjugated promycelial branches passes. This hump extends to form a slender, straight, or sometimes slightly curved, binucleate sporidium, pointed at its tip and measuring (27-57) x (1-2) μ. These needle-shaped sporidia are easily

detached, but in a hanging drop transferred to agar, often three or four such sporidia remain attached to a germinated chlamydospore, and can be picked off individually with a glass rod.

Unconjugated promycelial branches often grow out at the tip to form mycelium, but needle-shaped sporidia were never found connected with unfused promycelial branches, nor were uninucleate needle-shaped sporidia ever seen.[1] Detached sporidia often show three or four nuclei, presumably formed by the division of one or both of the two nuclei of the sporidium, and the two products of one division remain close to one another in the early stages of sporidium development. (see fig. 4 h)

Sporidia germinate easily on the surface of agar, usually at both ends, to give a narrow, sparsely branched mycelium (1.5-2) μ wide. This mycelium has few cross walls, and grows in a markedly "twisting" manner. The occasional side branches often grow out above the medium and develop into needle-shaped sporidia very like the parent sporidium, and which are easily detached but not forcibly discharged. Occasionally also, allantoid sporidia are formed on short side branches, and are forcibly discharged by the drop excretion method.

Although repeated attempts were made to stain the

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[1] Kaiser (1936) found occasional uninucleate needle-shaped sporidia formed by unconjugated promycelial branches.

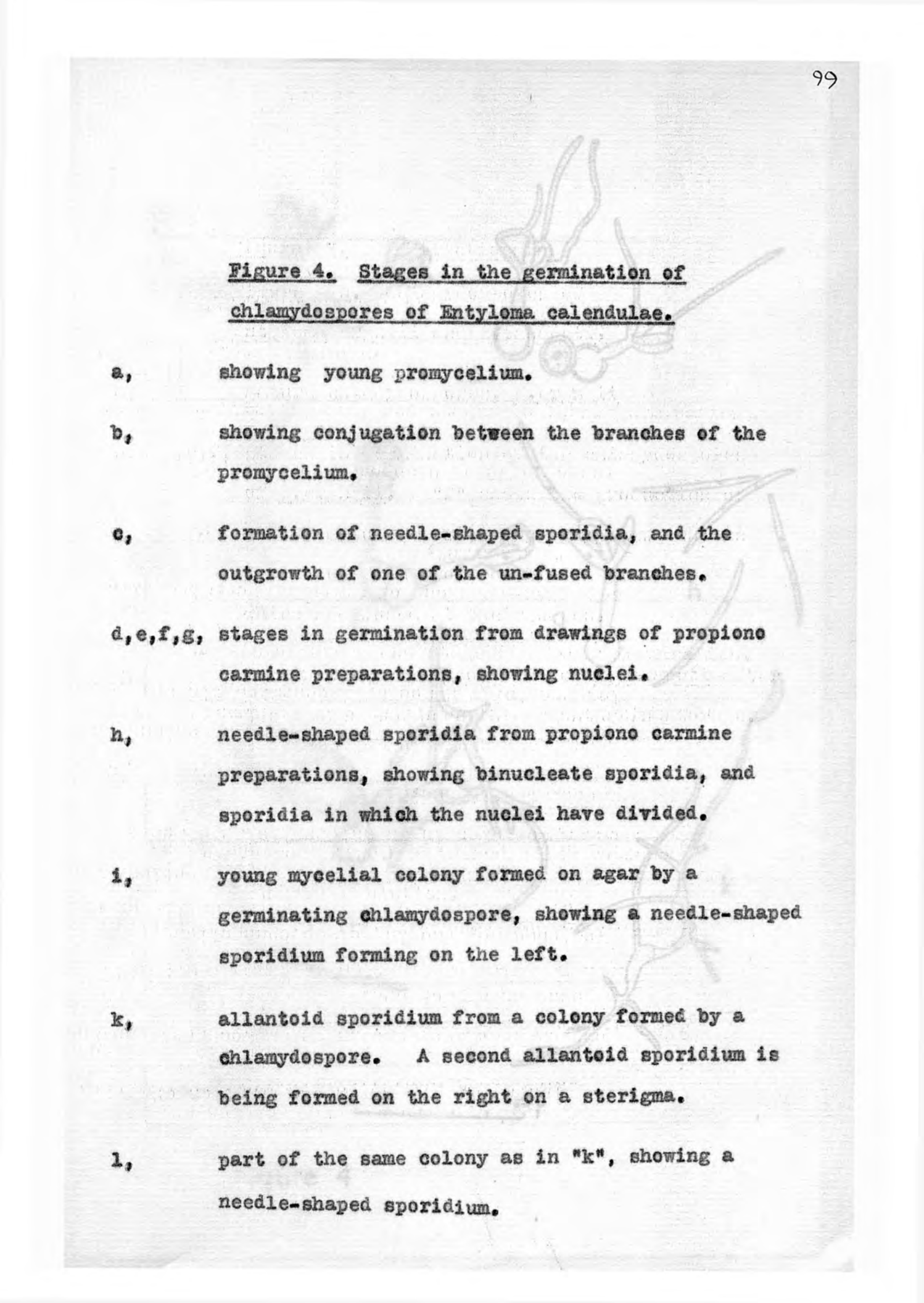


Figure 4. Stages in the germination of chlamydospores of Entyloma calendulae.

a, showing young promycelium.

b, showing conjugation between the branches of the promycelium.

c, formation of needle-shaped sporidia, and the outgrowth of one of the un-fused branches.

d,e,f,g, stages in germination from drawings of propiono carmine preparations, showing nuclei.

h, needle-shaped sporidia from propiono carmine preparations, showing binucleate sporidia, and sporidia in which the nuclei have divided.

i, young mycelial colony formed on agar by a germinating chlamydospore, showing a needle-shaped sporidium forming on the left.

k, allantoid sporidium from a colony formed by a chlamydospore. A second allantoid sporidium is being formed on the right on a sterigma.

l, part of the same colony as in "k", showing a needle-shaped sporidium.

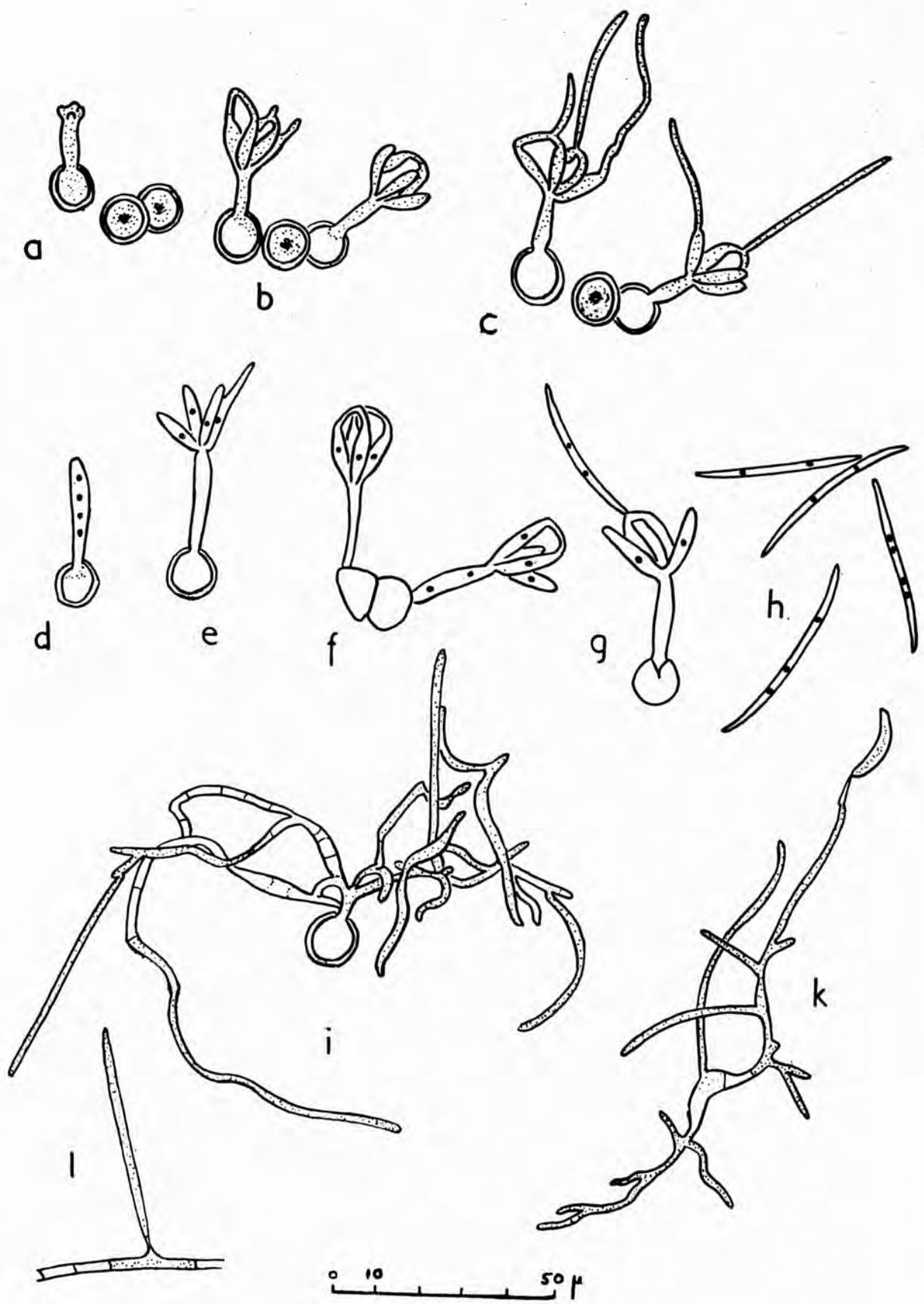


Figure 4

nuclei of both allantoid and needle-shaped sporidia produced in culture, no successful preparations were obtained.

Chlamydospore Germination in other Species

The germination of the chlamydospores, and the kinds of sporidium obtained, are the same in E.dahliae as in E.calendulae. In Entyloma eryngii also the chlamydospores germinate while still in the tissues of the host, which makes examination of the course of germination easy. This follows closely the E.calendulae story, except that no needle-shaped sporidia were found in the same conditions of hanging drop and agar culture. Instead, hyphae arise from the conjugated promycelial branches, from which allantoid sporidia are cut off directly. (see fig. 5f)

In all the other species examined, the chlamydospores do not germinate directly in the leaf, but require a period of rest. Lesions of certain of these smuts were cut from the leaf and stored in sachets of "Windowlite" buried about one inch below the surface of garden soil. At monthly intervals a lesion was removed and hanging drop cultures made of the chlamydospores. After several months storage the chlamydospores of E.ficariae, E.serotinum and E.tanaceti germinated in the manner typical of Entyloma species, forming a promycelium with four to eight distal branches. (see fig 5) It was very difficult to obtain such cultures free from contamination by bacteria, and for this reason the further development of

Figure 5. Chlamydospores of Entyloma eryngii and E.tanaceti.

a, mature chlamydospores, E.eryngii

b,c,d,e,f, stages in germination of the chlamydospores of E.eryngii. "f" shows the production of two allantoid sporidia by a hypha which has grown out from two promycelial branches which had fused at their bases.

g, Germinating chlamydospores of E.tanaceti after the spores had been three months in storage.

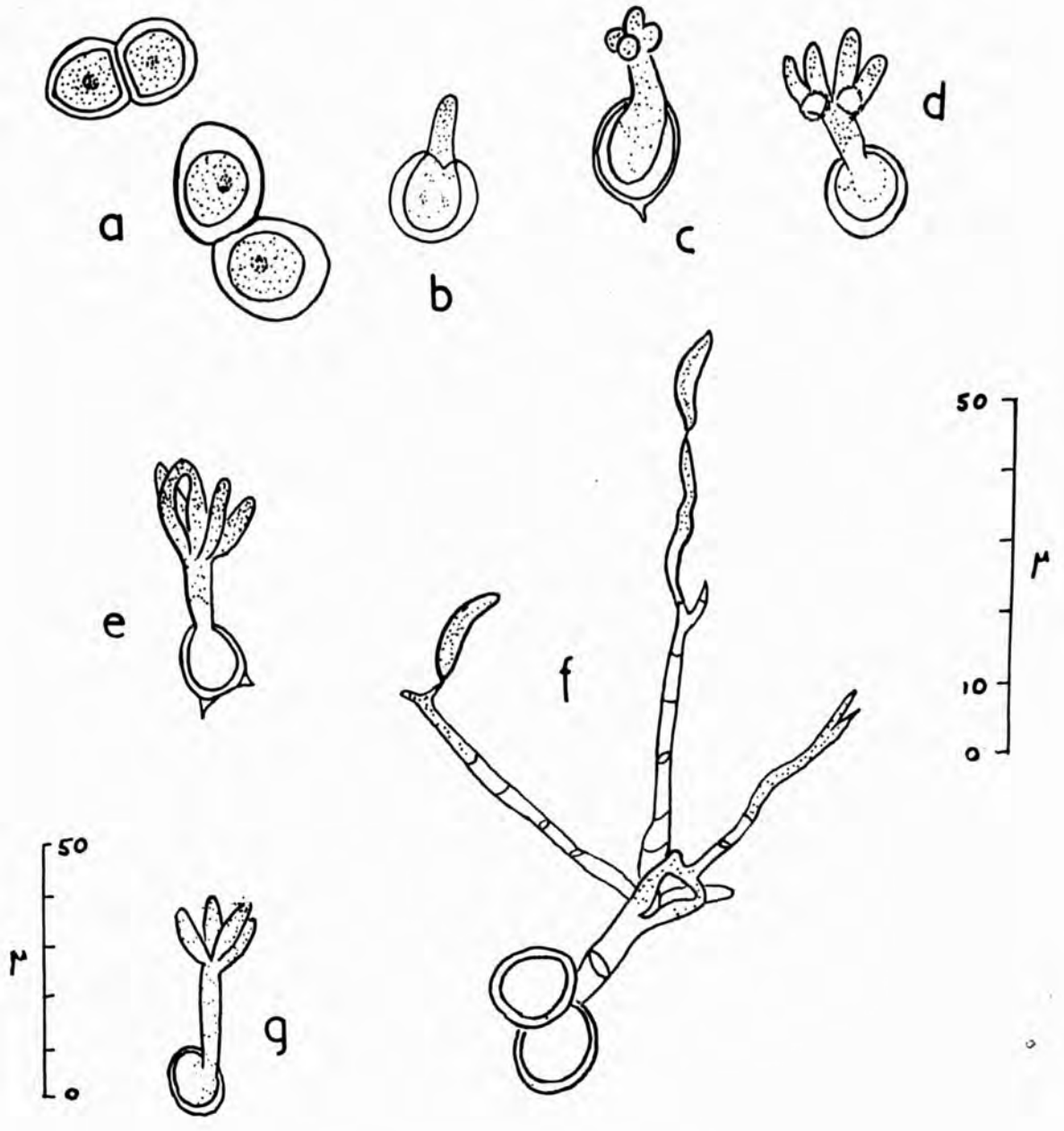


Figure 5

these spores was not followed. The period that elapsed before the first chlamydospores of the three species germinated was as follows:

E.ficariae germinated in January, after seven months storage
E.serotinum " " April " four " "
E.tanaceti " " March " three " "

Comparison between the sporidia formed by the chlamydospores and those isolated from lesions.

Four kinds of sporidium were constantly found associated with the lesions of several different species of Entyloma: two allantoid ones (types (a) and (b)), a half-moon-shaped kind, and a needle-shaped kind. In E.calendulae and E.dahliae, on which most of the work on chlamydospore germination was carried out, all four kinds of sporidium were isolated from the lesions. However, when the chlamydospores of these species germinate, only two kinds of sporidium are formed, needle-shaped and allantoid. The needle-shaped sporidia are morphologically like those isolated by scraping the surface of the lesions, and on germination they form the same kind of mycelium. This suggests that a large number of the foliar needle-shaped sporidia must be formed as a result of the germination of chlamydospores in the host. Such sporidia were not found on the lesions of E.eryngii, nor are they formed when chlamydospores of this species germinate under the experimental conditions.

In E.calendulae and E.dahliae, the mycelium formed by the needle-shaped sporidia follows a twisting course on agar, and slimes off more needle-shaped sporidia and also produces allantoid sporidia which are discharged by the drop excretion mechanism. Both kinds of sporidium reproduce the twisting mycelium on germination. Thus the second kind of sporidium, the allantoid kind, is formed by the needle-shaped sporidia, which in turn are formed by the germinating chlamydospores. The allantoid sporidia are apparently identical with the type (a) allantoid sporidia which are discharged from the surfaces of lesions of E.calendulae and E.dahliae, and which give a twisting mycelium on germination. This suggests that some of the allantoid sporidia of type (a) are formed as a result of the germination of chlamydospores in the host. In E.eryngii allantoid sporidia are formed directly from the fused branches of the promycelium of the germinating chlamydospore without the intermediate production of needle-shaped sporidia. It is probable that here too many of the allantoid sporidia isolated from the surface of the lesion originated from germinating chlamydospores.

It is stressed that in E.calendulae and E.dahliae, and E.eryngii, the germinated chlamydospores must give rise to some of the type (a) allantoid sporidia, but may only give rise to a certain proportion of these. In E.ficariae, and other species where the chlamydospores do not germinate

in situ, the foliar allantoid sporidia must have some other source such as the vegetative parasitic mycelium, or be unconnected with the life cycle of the smut. It is thus probable that, in those species in which the chlamydospores do germinate while still in the tissues of the host, (*E. calendulae*, *E. dahliae*, and *E. eryngii*), some of the foliar sporidia may arise independently of the germination of these chlamydospores. Similarly, in *E. calendulae* and *E. dahliae*, occasional needle-shaped sporidia are formed by the mycelium of germinated allantoid sporidia of type (a), and some of the foliar needle-shaped sporidia may come from allantoid sporidia and not directly from germinated chlamydospores.

Germinating chlamydospores never produce sporidia of type (b), nor half-moon-shaped sporidia, although both these kinds of sporidium are obtainable from the lesions of both *E. calendulae* and *E. dahliae*.

III 3. The Parasitic Mycelium of the Species of *Entyloma* Studied

In surface view the lesions of the species of Entyloma studied are level with the leaf surface, except in E.eryngii, where the epidermis of the host is raised in the region of infection giving a "pimply" effect to a diseased leaf. The size of the lesion varies with the species; in E.dahliae the disease spots are very large, up to $2\frac{1}{2}$ cms in diameter, while in E.tanaceti and E.eryngii they may measure only 1 mm. across. Thus large lesions tend to be found on species with the larger expanse of leaf surface. This is probably a reflection of the internal anatomy of the leaf, as the lesions are restricted where they impinge on a major vein, and this also results in the angular outline of those lesions which abut on large veins. Where the expansion of the lesion is not impeded by veins, the disease spot is circular in outline. The margin of the spot is paler than the rest of the leaf as the host tissue is discoloured, and forms a "halo" round the dark necrotic centre where chlamydospores are being produced by the older mycelium. In very old lesions the centre may drop out, giving a "shot-holed" effect.

If the surface of a leaf which is discharging sporidia is examined under the low power of the microscope, the "frosty" appearance of the surface is shown to be partly caused by tufts of hyphae protruding from the stomata and bearing sporidia. In older lesions a mat of mycelium is formed by the sporidia which have germinated on the leaf

surface, and secondary invaders growing on the surface of the lesion may contribute to this mycelial mat. The frosty effect of young lesions is increased where tufts of sporidiophores force the guard cells of the stomata apart and air is admitted to the intercellular spaces of the mesophyll.

The parasitic mycelium of E.calendulae and of E.dahliae, mounted fresh in water, measures 1.3 to 2.0 μ across. A picture of the extent of the parasitic mycelium in three dimensions was constructed by the use of the following three methods.

An entire small leaf, or part of a larger one, was cleared in chloral hydrate, and the mycelium stained in acid fuchsin. By this method the horizontal extent of the mycelium is revealed. The vertical system of hyphae is best seen in sections taken at right angles to the leaf surface, although the hyphae of this system follow a winding route and tend to be so crowded in the intercellular spaces that it is impossible to follow the course of individual hyphae. Finally, pieces of intact mycelium were extracted from the host tissues with the aid of "Pectozyme."

The limit of the lesion is best seen by viewing a cleared and stained piece of leaf, mounted with the lower surface uppermost on a slide, under an oil immersion lens. The hyphae radiate from the point of infection and run

parallel with the leaf surface, just below the lower epidermis, and spread outwards in remarkably straight lines, as they pass between the cells of the epidermis and mesophyll. The cross walls of clamp connections show up white against the purple contents of the hyphae at infrequent intervals.

The side branches of the horizontal hyphae, which are given off at an acute angle, often, but not always, arise in association with a clamp connection. Peg-like outgrowths, which might at first sight be taken to be haustoria, but which never penetrate the cell walls of the host, can actually be seen on focussing to be side branches which penetrate downwards between the cells of the lower epidermis, or upwards into the mesophyll, and which, although uniform in width, appear at one optical level to have swollen tips, due to their spiral growth at right angles to the parent hyphae, between the cells.

Young chlamydospores occur near the centre of the lesion, arising at the ends of short side branches, and in the extreme centre of even a young lesion a few mature chlamydospores can be found. In older lesions all intercellular spaces are filled with chlamydospores.

In vertical section, the intercellular spaces of the lower mesophyll are seen to be filled with a twisted mass of hyphae, and strands of twisted hyphae run up between the cells of the palisade. Hyphae fill the sub-stomatal chambers

of both leaf surfaces and from there pass out to the exterior. The mass of hyphae present in the intercellular spaces makes it very difficult to trace the origin of the sporidiophores. In the vertical leaf sections "chains" of chlamydospores are often evident between the cells of the palisade. This appearance is caused where many short side branches, each with a chlamydospore at its end, are given off from a strand of hyphae passing up the space between the cells.

Clamp connections occur both in the young, marginal mycelium, and in the older, central, part of the lesion, and are most clearly demonstrated in mycelium obtained in a state of isolation from the host by the action of "Pectozyme" on thick hand sections of the leaf. (see fig. 6) The pectinase dissolves the middle lamella, and the cells can easily be separated by mounting the section under a coverslip and tapping on it gently. While this description of the parasitic mycelium applies particularly to Entyloma calendulae, examples of clamp connections have also been found in E. dahliae, E. tanaceti, E. eryngii and E. ficariae. In no case, whatever the method of investigation, was a genuine case of a haustorium seen. The ease with which the mycelium can be separated from the host cells indicates the absence of haustoria.

Figure 6. Parasitic mycelium of Entyloma.

a,b, c,d, *Entyloma dahliae*, showing clamp connections and a young chlamydospore.

e,f,g, *E.calendulae*, showing clamp connections.

h, *E.ficariae*, mycelium with clamp connections and chlamydospores in an intercellular space of maccerated tissue.

i,k, *E.tanaceti*, mycelium with clamp connections in an intercellular space of maccerated tissue.

a-g from microtomed leaf sections,

h-k from sections of a leaf maccerated with "Pectozyme",

Sporidia formed by the parasitic mycelium

On p.106 it was stated that tufts of hyphae protrude from the stomata of a leaf infected with Entyloma and bear sporidia at the surface of the host. These sporidia are very easily knocked off in preparations, but young stages sometimes remain attached.

In E.calendulae and E.dahliae the sporidia observed were either of the allantoid or of the needle-shaped types, but it was very difficult to decide whether they were being formed directly by the parasitic mycelium, or indirectly as a result of chlamydospore germination. Sometimes a sporidiophore was followed to its origin from a germinating chlamydospore; usually, however, the mycelium was too tangled to be traceable. A half-moon-shaped sporidium was never found attached to Entyloma mycelium issuing from the host plant.

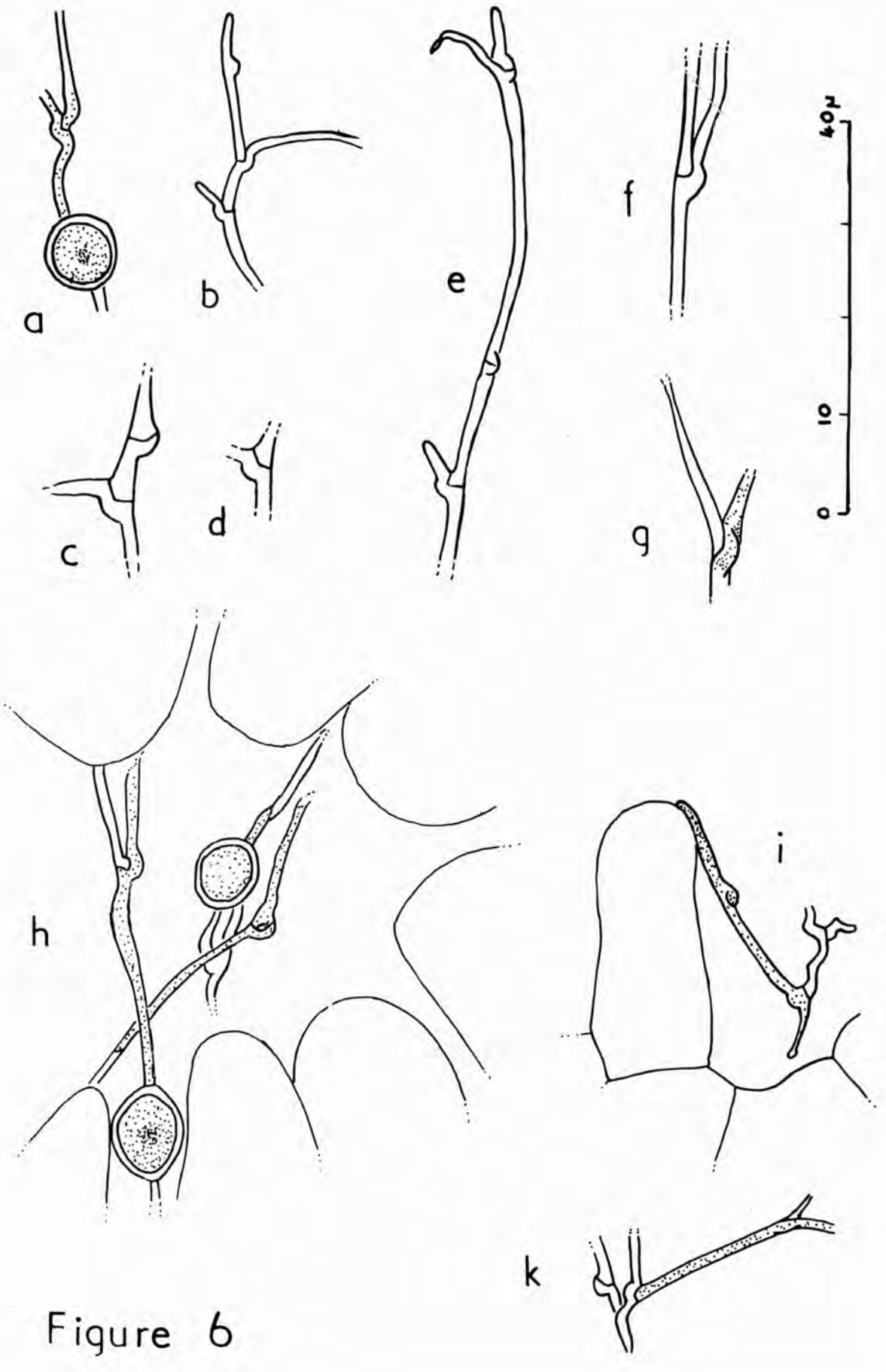


Figure 6

III 4. The Behaviour of the Sporidia in Relation to the Host

The observations already recorded have dealt with the isolation of different kinds of sporidium from lesions of Entyloma, and their behaviour in artificial culture. It remains to describe the behaviour of these sporidia in relation to the host.

Symptoms characteristic of an infection of Entyloma have been induced in a healthy plant by spraying an aqueous suspension of chlamydospores obtained from a lesion of the same species. This has been done using E.calendulae and E.dahliae. Within three weeks to one month of spraying, many of the typical lesions appear, with a pale coloured halo surrounding a region containing young chlamydospores which later can be isolated and shown to germinate in the manner typical of the species. Control plants sprayed with water only, do not develop these symptoms. It is probable that the greater part of this infection was brought about by the germinating chlamydospores, although the suspension may have also included some foliar sporidia, and it is possible that sporidia alone are capable of infecting the host in the absence of chlamydospores.

In order to determine how many of the sporidial types are capable of reproducing the symptoms of the disease, many experiments were made using sporidia of one type and of known origin. Mass inoculation of a single type of sporidium, and mono-spore and paired mono-spore cultures were made.

A. Attempts to obtain fusion between allantoid sporidia.

Both the half-moon-shaped and needle-shaped sporidia are binucleate, but the allantoid sporidia of types (a) and (b) are uninucleate. Some kind of fusion might be expected to occur between two uninucleate sporidia before invasion of the host could take place. Many attempts were made, therefore, to obtain fusion between allantoid sporidia under artificial conditions, and these will be described first.

In an impression of a leaf in which allantoid and half-moon-shaped ballistosporic sporidia occur, there is a tendency for the half-moon-shaped sporidia to appear on the agar after the allantoid sporidia, and in some impressions this appears to indicate that half-moon-shaped sporidia and clamp mycelium have arisen from allantoid sporidia on the agar. This is not so, however, because the individual sporidia can be isolated, as they fall, by frequent rotation of the Petri dish lid. Since, however, the allantoid sporidia are uninucleate and the half-moon-shaped binucleate, there is always the possibility that the latter may have arisen as a result of fusion between the former under conditions prevalent on the leaf, which are not reproduced on the agar medium. Some such fusion might also result in the production of some quite different kind of mycelium or sporidium.

Monospore isolations were therefore made of allantoid sporidia of both types (a) and (b) from lesions of Entyloma

ficariae, E.calendulae and E.dahliae. Those of the same type, and those of different types but isolated from lesions of the same species of Entyloma, were grown together and in pairs on the surface of an agar medium, and the zone where the two cultures met was examined microscopically at intervals for signs of fusions between hyphae.

Since it is possible that the sporidia lose any ability to fuse by a period in artificial culture, freshly fallen impressions of sporidia from a single lesion, and "mixed" impressions from several different lesions were examined. In the last case the impression often included sporidia of types (a) and (b).

The agar substrate was varied in an attempt to obtain conditions favourable to fusion. Three main variations of a 2½% agar medium were used: (1) a concoction of the leaves of the host, (2) malt agar and potato extract agar of varying dilutions of the nutrient constituent down to plain agar, and (3) potato agar of varying pH. The basic leaf decoction and potato extract was made by cutting up finely the leaves or peeled potatoes, and boiling 250 grams in water for half an hour. The cooled extract was decanted and made up to a litre with water. The dilutions of potato agar used for "fusion agars" were 50% normal strength, 25%, 10% and 2%, and of malt agar 2%, 1.5%, 1% and 0.5%, and plain agar. For variations of pH, a double strength potato

extract medium was diluted with an equal volume of McIlvaine's phosphate buffer to obtain a series of varying pH from pH 3, by 0.5 increases to pH 8.

Results: No positive results were obtained on any of the media with paired monospore cultures of any of the various origins. The only cases in which fusions were observed at all were those where ballistospores freshly fallen from a lesion were examined. In a very few of the large number of impressions examined, allantoid sporidia of type (a) isolated from lesions of E.ficariae and E.dahliae, showed fusions on very dilute media, 1% and 0.5% malt extract agar, and plain agar. These fusions could be detected by keeping an impression of sporidia under observation for a period of several days, when tips of branches were seen to approach each other and fuse. The fusions, however, occurred as often between branches from the same sporidium as from different sporidia, and the mycelium formed after a fusion was the same in appearance as the branches which took part in it ~~(fig.)~~. Since, at the dilutions of media concerned, the mycelium was exhibiting starvation growth, it seems probable that these fusions were unnatural occurrences induced by the lack of nutrient, although conditions of low nutrient level would be found on the surface of a leaf where fusion, if it takes place at all, might be expected to occur. Impressions of sporidia which had been allowed to fall on the epidermis of a healthy

leaf occasionally exhibited similar apparent "fusions". As the epidermis had to be stripped or the leaf cleared in order to observe these "fusions", there was no means of knowing whether they actually were fusions between mycelial branches coming originally from different sporidia, or had come from the same sporidium, as occasionally happens in artificial culture.

B. Inoculation experiments

The object of these experiments was to achieve controlled inoculation of the host with sporidia isolated from lesions of species of Entyloma, in order to determine whether the infection of Entyloma was reproducable by this means. The requirements for such experiments are:

1. clean, healthy plants of the host,
2. a suitable inoculum,
3. suitable conditions for inoculation.

These three requirements will now be treated in order.

The host: In order to avoid the possibility of the disease being systemic in perennating organs of the host, seedlings were always used for inoculation experiments. Of the species investigated, seedlings of Dahlia were the easiest to grow quickly, and were also less susceptible to attacks of mildew than were Calendula seedlings. The majority of inoculation experiments were made using sporidia isolated from E.dahliae, and in a few cases E.calendulae.

The strain of Dahlia used was Carter's "Coltness Gem", which was found to be easily infected by the method of mass spraying of chlamydospores described on p. 113. For Calendula seedlings, Carters' "Radio" was used.

Dahlia seeds were sown in boxes in soil which was not previously sterilized, but was taken from a border on which Dahlias had never within memory been grown. The boxes

were kept under glass, and after a fortnight the seedlings were pricked out into four inch pots and kept in a cool greenhouse. At the four to six leaf stage (after one month to six weeks), they were sufficiently large for use in inoculation experiments.

The inoculum: Four kinds of sporidium were used for inoculation experiments, allantoid types (a) and (b), half-moon-shaped, and needle-shaped sporidia. The needle-shaped sporidia were either scraped from the surface of the lesion or removed directly from germinating chlamydospores. All the other kinds of sporidium were taken from ballistospore impressions of lesions of Entyloma. None of the allantoid sporidia used had been isolated directly from germinating chlamydospores. Monospore cultures were made and maintained of all four kinds, and the following methods of inoculation were tried:

1. mass inoculation of the sporidia formed by an actively growing culture, recently isolated, on an agar medium,
2. single sporidia, taken either directly from the deposit of sporidia beneath a lesion, or directly from the germinating chlamydospore, were transferred straight to the leaf surface.

The methods used for applying the inoculum to the plant were modifications of what are probably the major

means of spread of the disease in nature - dispersal by air currents and by rain splash. Sporidia were either allowed to shoot on to the leaf, or they were applied in aqueous suspension.

In all the experiments, control plants were used in which the leaves were treated in exactly the same way as the inoculated ones, except that water only, or plain agar, was used instead of inoculum.

1. Mass Inoculation: Two methods were used. In the first, pieces of the culture were cut out and shaken in a small quantity of sterile water which was pipetted on to the surface of the leaf. In the second method, the surface mycelium and sporidia were removed from the colony on a dry needle and transferred to a small drop of liquid on the leaf surface. In the case of sporidia discharged by the drop excretion mechanism, agar colonies were suspended over the surface of the leaf, and the sporidia allowed to shoot directly on to the leaf surface. If paired cultures of monospore origin were required for mass inoculation, either the products of two cultures were mixed before being inoculated on to the host by one of the first two methods, or pieces of each culture in succession were suspended over the leaf so that ballistospores of each culture were shot on to the leaf in turn.

2. Direct Method: Single sporidia were picked up on the end

of a finely drawn out glass rod from a ballistospore impression of a lesion. They were placed either directly on the leaf surface, or in a small drop of water, within a previously marked area of the leaf. For paired spore inoculations, a second sporidium was placed on the same area close to the first. Needle-shaped sporidia, which are not forcibly discharged, were scraped off the surface of a lesion and suspended in a drop of water which was then spread over an agar surface, and the individual sporidia were then picked off the surface on the end of a glass rod. Alternatively, single needle-shaped sporidia were taken individually from the crown of sporidia surmounting the fused promycelial branches of a chlamydospore germinating on agar.

Conditions for inoculation: The conditions under which inoculation experiments are made are obviously very important. Negative results might be due to conditions unfavourable for inoculation, even though the sporidia were themselves capable of infecting the plant.

Many factors must affect the entry of a suitable inoculum in to a susceptible host (Brown, Brooks and Bawden, 1948). It was impossible to vary them all until it was known whether the sporidia were capable of infecting. An indication of the conditions most suitable for inoculation was deduced by observing the spread of the disease in nature. By observation of a border of infected Dahlia plants through

four successive seasons, the incidence of Entyloma dahliae was found to be higher in wet than dry seasons. Since suspensions of chlamydospores in water are capable of infecting the host (see p.113), it seems that a humid atmosphere is at least important for the early stages of infection.

In order to maintain a humid atmosphere, some plants were kept for the first two or three days after inoculation under bell jars in an atmosphere rendered humid by spraying with water. The bell jars were kept in the shade to prevent over heating. In other cases, the inoculated leaf was enclosed in a bag of polythene tubing 0.0015" thick, and the interior of the bag was sprayed with water before closing the mouth of the bag. In this way a humid atmosphere was maintained for periods of several weeks while allowing gaseous exchange to take place. Finally, small damp chambers were constructed to enclose the inoculated region of the leaf only. A glass ring was fixed on to the leaf with vaseline, and a coverslip similarly secured on top of the ring. Where sporidia were discharged from the surface of a culture on agar, the culture was either stuck on to the inner surface of the polythene bag, or on to the lower side of the coverslip of a damp chamber.

In both the polythene bag method and the damp chamber method, the very humid atmosphere encouraged moulds which grew over the surface of the leaf. For this reason,

the bell jar method was found most satisfactory. The bell jar was removed two or three days after inoculation, and thereafter the inoculated surface was kept covered to protect it from falling dust and spores, while allowing free circulation of air. Small shades of polythene were supported on sticks to shade the inoculated leaf while the lesion developed.

Results: A large number of inoculation experiments were made with the four different types of sporidium isolated from lesions of E. dahliae, using the various methods just described. These are summarised in Table 4 , p. 124. The results were as follows: (1) In no case did an inoculation of half-moon-shaped sporidia result in the infection of the plant with Entyloma. (2) Single or paired inoculations of allantoid sporidia of either the (a) or (b) types never resulted in infection. (3) The only cases where symptoms associated with an infection of Entyloma dahliae were induced in the host was when single needle-shaped sporidia were isolated directly from the ends of conjugated promycelial branches of a germinating chlamydospore of E. dahliae and placed on the upper surface of Dahlia leaves. Twelve such inoculations were made, and in three cases lesions typical of E. dahliae developed and were visible one month after inoculation. Chlamydospores were isolated from this lesion and were shown to germinate in the manner typical of Entyloma dahliae. (4) In none of the control leaves did symptoms of Entyloma develop.

Table 4.

Summary of the Inoculation Experiments on Dahlia.

The numbers in brackets refer to the number of experiments made.

Kind of Sporidium	Inoculation Method	Origin of Inoculum	Result
Allantoid Type (a)	1. Mass inoculation in water from a culture	Monospores(36)	Negative
		Paired spores (24)	"
	2. Mass inoculation by discharge from a culture.	Monospores(28)	Negative
		Paired spores (20)	"
Type (b)	1. Mass inoculation in water from a culture	Monospores(20)	Negative
		Paired spores (20)	"
	2. Mass inoculation by discharge from a culture	Monospores (6)	Negative
		Paired spores (6)	"
Types (a) or (b)	3. Single sporidia isolated from the impression of a lesion	Monospores(40)	Negative
		Paired spores (22)	"
Needle-shaped	1. Single sporidia from a scraping of a lesion	Monospores(24)	Negative
	2. Single sporidia from germinating chlamydospores.	Monospores(12)	3 Positive 9 Negative
Half-moon-shaped	1. Mass inoculation in water from a culture	Monospores(50)	Negative
	2. Mass inoculation by discharge from a culture.	Monospores(12)	Negative
	3. Single sporidia isolated from the impression of a lesion.	Monospores(36)	Negative
		Paired spores (10)	"

III 5. The Ballistospore Flora of Leaves not Infected with *Entyloma* Species

The Role of the lesion Sporidia in Relation to the Life Cycle of Entyloma Species

Of the four types of sporidium found associated with the lesions of various species of Entyloma, only two have proved to be actually part of the life cycle of any of these species. These are the needle-shaped sporidia, and the allantoid sporidia of type (a), which in E.calendulae and E.dahliae are both formed when the chlamydospores germinate. In E.dahliae the needle-shaped sporidia are capable of reinfecting the host.

The remaining two kinds of sporidium, the half-moon-shaped and the allantoid of type (b), have not been found when the chlamydospore germinates, nor have they been found to be connected with the vegetative parasitic mycelium. It is probable that they are not part of the life cycles of these species of Entyloma. ~~If~~ this ~~case~~ is so, they are of outside origin and only present on the surface of the lesions as contaminants. It seemed desirable to make a survey of both the ballistospore flora of the surfaces of leaves which were not infected with Entyloma, and of the ballistospore discharging fungi present on the surface of lesions of fungal parasites other than members of that genus. The results of that survey are recorded in the following pages.

Examination of the Ballistospore Flora of Leaves not Infected with Entyloma

A piece of meadow land with nearby trees in the grounds of the Royal Holloway College was selected, and the leaves of as many different species of plant as possible from this plot were tested for the presence of ballistospore-discharging fungi. The particular piece of ground was chosen as it was conveniently close to the laboratory, was not near any known source of species of Entyloma, and there was a good variety of different species of plant growing there.

Leaves were collected at fortnightly intervals over a period of eight months in a range of weather conditions, and were suspended over 2% malt agar for twenty-four hours before the leaf was removed, thus allowing for the possible effect of diurnal discharge of ballistospores should this occur.

The plates were kept at room temperature (about 22°C) for four to five days, when the colonies which had developed from the ballistospores discharged during the twenty-four hour period could be seen with the naked eye. Parts of these colonies were covered with pieces of coverslip and examined under the high power of the compound microscope before isolations were made from other parts of the colonies. Earlier stages in the development of these colonies could be examined under the ~~binocular~~ dissecting microscope at any time after the ballistospores had fallen on the plate. Isolations were

made on to slopes of 2% malt agar, and later monospore cultures were taken from these isolates.

The leaves examined were taken from plants in the tree, shrub, and ground layers, and the litter of the broad leaved trees was also sampled. The following is a list of the species used:

Agrimonia eupatoria	Primula vulgaris
Arrhenatherum elatius	Quercus robur
Castanea sativa	Ranunculus ficaria
Fagus sylvatica	Rubus sp. (Blackberry)
Fraxinus excelsior	Rumex acetosa
Glechoma hederacea	Stellaria holostea
Hedera helix	Taraxacum officinale
Larix decidua	Vicia cracca
Potentilla sterilis	Viola riviniana

Litter of the following:

Castanea sativa	Quercus robur
Fagus sylvatica	

Some species were tested consistently throughout the period (eg. Rubus sp., Glechoma hederacea, Arrhenatherum elatius, Potentilla sterilis, Stellaria holostea and Viola riviniana), but others for only part of the time, because the growing period was limited to certain seasons. Thus Ranunculus ficaria and Primula vulgaris were only available in the months of March to May, and the trees were bare in

winter months, although leaves of most of the shrub and ground layers could be found at all seasons. The number of dead leaves in the litter declined towards midsummer, as the dead leaves rotted, and from August on the dead leaves were those of the current season.

Isolations

The colonies which develop from ballistospores from this source fall into three groups. First, species of Sporobolomyces are easily distinguishable by the pink colonies they form and which make them at first sight the most striking part of a leaf impression, when they often shadow the veins of the leaf. They are of very frequent occurrence and were recorded from one hundred and fifty-two out of a total of two hundred and eleven leaves tested. Equally frequent, although less conspicuous, are the colourless, allantoid, slightly curved ballistospores, answering to Derx's description of Tilletiopsis, which form white to yellow-brown colonies in the spore deposit. These fungi were found in one hundred and forty-one of the two hundred and eleven leaves tested. Finally, from a small minority of the "healthy" leaves, (in fourteen of the leaves tested) half-moon-shaped ballistospores were isolated. These germinate to give a mycelium with clamp connections at intervals resembling in every way that originating by the germination of half-moon-shaped sporidia isolated from Entyloma species, and also

approximating to Derx's and Olive's descriptions of Itersonilia.

In addition to these three types of fungus, mycelial colonies occasionally developed where a spore had been shaken off the leaf on to the agar, and "true" yeasts appeared now and then. If any part of the leaf was accidently allowed to touch the agar surface, yeasts and mycelial fungi developed in profusion. Fungi resembling the needle-shaped sporidia which occur in lesions of Entyloma spp. were never isolated, either from "healthy" leaves or from the surface of lesions of fungi other than Entyloma. These sporidia are not forcibly discharged, and no exhaustive search was made for them on the surface of "healthy" leaves. Nevertheless, in a large number of scrapings of such leaves, no needle-shaped sporidia were found. Allantoid sporidia of type (a) (ballistospores), which also occur on Entyloma lesions, were not isolated when ballistospore impressions of "healthy" leaves were examined for sporobolomycetes. The germination of the allantoid ballistospores found in impressions of "healthy" leaves was always carefully followed, and no example of germination like that of the sporidia of type (a) was recorded.

Of the three kinds of ballistospore-discharging fungus, Sporobolomyces was not studied in any detail, and no attempt was made to differentiate between the species of this genus. The fungi resembling species of Tilletiopsis and

Itersonilia were isolated and grown on 2% malt agar in order to study their further growth and compare the isolates with one another.

Besides the isolations made from "healthy" leaves, Itersonilia was isolated from rust pustules on several occasions, and once from a smutted panicle of Arrhenatherum elatius. Ballistospores were obtained from amongst the teleutospores of the following three rusts, Puccinia malvacearum on Malva sylvestris, P.fragariastri on Potentilla sterilis, and P.violae on a garden pansy, but they were never found on any of the pustules of P.violacea on bramble which were examined. The panicle of Arrhenatherum elatius was completely smutted with Ustilago avenae, and this in turn was thickly covered with Itersonilia, so that a complete mirror-image in ballistospores of the inflorescence was obtained when the latter was suspended above an agar surface.

The allantoid ballistospores resembling those of Tilletiopsis vary greatly in size and in the appearance of the mycelium formed when they germinate, and it is possible that several different strains of species were present on the leaves tested. The isolates, however, consisted almost entirely of two distinct strains, one with larger ballisto-spores and forming an ivory-white "frosted" colony, the other with smaller ballistospores and a yellow-brown colony which later becomes chocolate-coloured. Occasionally,

isolates were made which did not agree with the descriptions of either of these strains, and it is possible that other strains were actually present in quantity on the leaf but they become over-grown by their faster-growing associates.

Descriptions of the two strains of Tilletiopsis and of the fungus apparently belonging to Itersonilia are given below.

Description of Tilletiopsis species with larger ballistospores.

The following description is based on a monospore culture (No.Y70), isolated from the surface of a leaf of Primula vulgaris on 24th August, 1955.

Habitat: Abundant throughout the year on the surfaces of living or dead leaves of a wide variety of species of Angiosperm.

Morphology: Ballistospores (12-20) µ x (2-3) µ, average 16 u x 2.5 µ, colourless, hyaline, allantoid, slightly curved, uninucleate, produced excentrically at the ends of short tapering sterigmata, and discharged by the drop excretion mechanism. Ballistospores germinate immediately after discharge at both ends to give a narrow mycelium (1.5-2.0) µ wide, rarely septate (ie. every 125-200 µ), or breaking up into blastospores (10-30) µ x (2-3) µ, hyaline, straight. Chlamydospores, (12-14) µ in diameter, spherical, hyaline, arise terminally or as intercalary

swellings, and are either solitary or in chains.

Colour of the colony on 2% malt agar, white when young, becoming ivory to deep cream with age. Young colonies have a "frosted" appearance, older ones a dull surface, but the consistency of the colony surface is always soft.

Blastospores are more frequently formed in young colonies, and the abundance in which they occur in some gives them a "slimy" appearance, while other young colonies grow without blastospore formation resulting in a more "open", branching mycelium. The former type of growth is more typical of colonies formed from ballistospores originating from newly sub-cultured colonies.

The occasional septa are usually accompanied in younger parts of the mycelium by the formation of a branch which arises just behind the septum, departing at an angle of 45° or less to the parent hypha. The length of mycelium just beyond a septum where a side branch has formed is often devoid of contents, thus the mycelium tends to consist of alternating living and dead regions. Septa are more frequent in the older parts of the mycelium, and are then less often accompanied by a side branch.

Formation of ballistospores commences shortly behind the actively growing margin of the colony. Ballistospores arise singly and excentrically at the tips of short, pointed side branches or sterigmata, and are forcibly discharged,

accompanied by a drop of liquid. They are carried to considerable distances and give rise to the scattered daughter colonies that appear beyond the margin of the main colony giving it a "ragged" appearance.

Another cause of the irregularity of the margin of the colony is the way in which branches at the edge tend to grow towards each other, even though they may arise at points quite far apart on the circumference of the colony. The hyphae converge, and then grow parallel and adpressed to one another, sometimes four or more aggregated to form a pointed extension beyond the edge of the more densely growing part of the colony. After growing parallel for some distance the hyphae may diverge again and approach and grow parallel to other hyphae further round the circumference of the colony. ~~(see fig.)~~

This aggregation of hyphae also occurs in the aerial system, which commences some distance behind the margin of the colony. This aggregation leads to the formation of "whisps" at the surface of the colony, and is partly responsible for the "frosty" surface consistency of young cultures. This appearance is increased by the production of ballistospores, many of which arise at the tips of hyphae near the ends of the "whisps".

Mature chlamydospores are found in the older, central parts of the colony, but hyphal swellings and early

stages in chlamydospore formation can be found quite near the margin of the colony.

Identification: The above description seems to agree with that of Nyland (1950) of Tilletiopsis washingtonensis, with minor differences such as the range of size of ballistospores, that given by Nyland being (8-19) μ x (2-2.6) μ, average 14 x 2.4 μ.

Description of Tilletiopsis species with small ballistospores.

The following description is based on a monospore culture (No.Y15), isolated from the surface of a dead leaf of oak, on 15th April, 1955.

Habitat: Abundant throughout the year on the surfaces of living or dead leaves of a variety of Angiosperm species.

Morphology: Ballistospores (5-10) μ x (1-2) μ, average 7 μ x 1.5 μ, hyaline, allantoid, slightly curved, uninucleate, produced excentrically at the ends of short, tapering sterigmata, and discharged by the drop excretion mechanism. Ballistospores germinate immediately after discharge at both ends to give further ballistospores; blastospores (8 - 10) μ x (1 - 2) μ, hyaline, straight; or a narrow mycelium, 1-1.5 μ wide, with occasional cross walls. Chlamydospores, (8 - 12) μ in diameter, hyaline, terminal or intercalary, formed singly or in chains.

Colour of the colony on 2% malt agar, light buff when young, becoming yellow-brown to chocolate with age, the

surface hardening in cartilaginous flakes.

Ballistospores are very frequent in young colonies, giving to the latter a "broken up " appearance, and assuring the fungus a quicker spread over the agar surface than occurs in T.washingtonensis, which forms mycelium or blastospores in the early stages of germination on malt agar.

The first hyphae of the young colonies tend to grow in a curve, and as the tips of the hyphae advance across the medium, the curve may be reversed, giving an undulating appearance to the mycelium.

There is often a side branch associated with the rare cross walls, and an empty region beyond the septum, which increases the "broken up" appearance of the mycelium. If the colony develops while covered with a coverslip, many side branches are formed; if no coverslip is placed over the developing colony, short, pointed sterigmata are formed instead of side branches.

With its abundant budding, and rarely septate mycelium, this fungus is not unlike a "true yeast". With slight variation in the range of ballistospore size, this strain of Tilletiopsis approximates to Nyland's description of T.minor. The measurements given by Nyland (1950) for the ballistospores of this species are: (5.8-14) µ x (1.5-2) µ, average 9 µ x 1.8 µ.

Figure 7. Tilletiopsis.

a-e, Tilletiopsis washingtonensis

f-k, T.minor.

a,b, ballistospores, and stages in their germination.

c, young colony producing blastospores.

d, ballistospore production from a hypha.

e, chlamydospores.

f,g, ballistospores, and stages in their germination.

h,i, mycelium, with ballistospore formation in "h".

k, chlamydospores.

b,g, from material stained with propiono carmine solution.

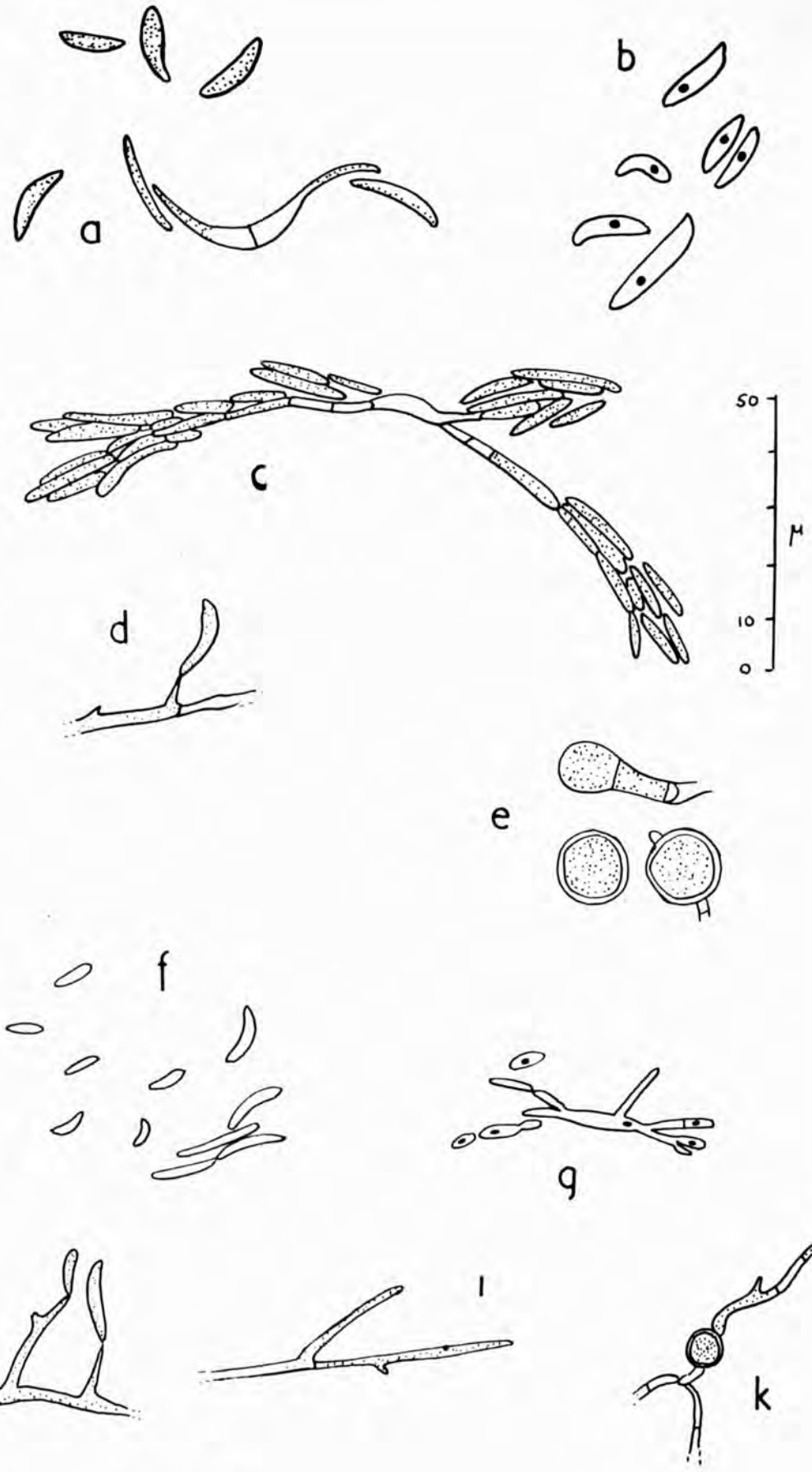


Figure 7

Description of Itersonilia species

The following description is based on a monospore culture, (No.I3), isolated from a leaf of Taraxacum officinale on 5th November, 1954.

Habitat: Found occasionally throughout the year on the surfaces of living and dead leaves of many Angiosperms, particularly those with hairy leaves. In summer less frequent on "healthy" leaves, and very frequent on the surface of lesions of fungal parasites.

Morphology: Ballistospores (12-21), (average 16) x (7-15) (average 11) µ, colourless, hyaline, half-moon-shaped (the shape of a 1/6th sector of a sphere), binucleate, produced excentrically at the ends of long, tapering sterigmata, and discharged by the drop excretion mechanism. Ballistospores germinate immediately after discharge by one or two germ tubes to give a hypha 3-5 µ wide. Mycelium branching, with typical clamp connections. Intercalary hyphal swellings common, terminal ones occasional, the latter sometimes grow out at the distal end to form mycelium. Chlamydospores, spherical to obovate, (10-15) µ in diameter, with a thick but hyaline wall and central oil globule, plentiful in older mycelium. Each chlamydospore with a clamp connection at its base. Chlamydospore contents often degenerate with time; germination of chlamydospores not observed.

Colour of the colony on 2% malt agar white to pale cream, the surface "frosted" at first, becoming shiny with age. Strong, unpleasant smell to colony on most media.

The method of germination of the ballistospores varies with the surface of the medium. The germ tubes, of which there are one or more, arise from the curved surface of the spore, rarely from the flat one. On an agar surface the hypha advances rapidly, the spore soon loses its contents, and simple cross walls cut off the empty regions at the base of the hypha. The first clamp connection is formed early, and thereafter cross walls are formed only in association with the formation of a clamp, except in very old mycelium, when simple cross walls are found. Occasionally, after growing for a short distance, the tip of the germ tube swells up to form another ballistospore. This is germination by repetition rather than by budding, as a short germ tube is always formed. When a ballistospore is allowed to germinate on a hard surface, such as a glass slide, or the surface of a leaf, a short germ tube forms which then swells at the tip to form a lobed appressorium-like structure with dense contents which stain deeply with cotton blue. (see fig.8,C) A hypha with typical clamp connections often arises from the surface of this lobed structure and forms mycelium, if sufficient nutrient is present.

The clamp connections formed are typical ones, with

fusion occurring between the tip of the clamp branch and the main hypha, just behind the cross wall (see figs. 8 & 9) The clamp connections are spaced irregularly along the length of the hypha. A side branch often, but not always, arises in association with a clamp, either from the main hypha behind the cross wall, or from the clamp branch itself, departing at an angle of 45° or less from the main hypha.

The surface of a young colony on 2% malt agar medium, if viewed under the low power of the microscope, is seen to be very irregular with a complex aerial system of "whisps", formed by the aggregation of hyphae. These "whisps" all tend to point in one direction at the same angle to the surface. Ballistospores are frequently formed on the hyphae which make up these "whisps".

If mycelium from an actively growing culture is transferred to liquid malt extract medium, spherical colonies of clamp mycelium develop in the medium. On examining these colonies, occasional cylindrical to allantoid cells can be seen to be formed as outgrowths or "buds" from the hyphae. These buds vary much in size, measuring (4 - 10) x (2 - 5) µ, and if stained in propiono carmine solution a single nucleus is evident. The further development of these cells has not been followed, but they appear to be similar to the "conidia" described for Itersonilia perplexans by Olive (1952).

Apart from slight differences in morphology

Figure 8. Itersonilia perplexans.

a,b,d, stages in germination on agar.

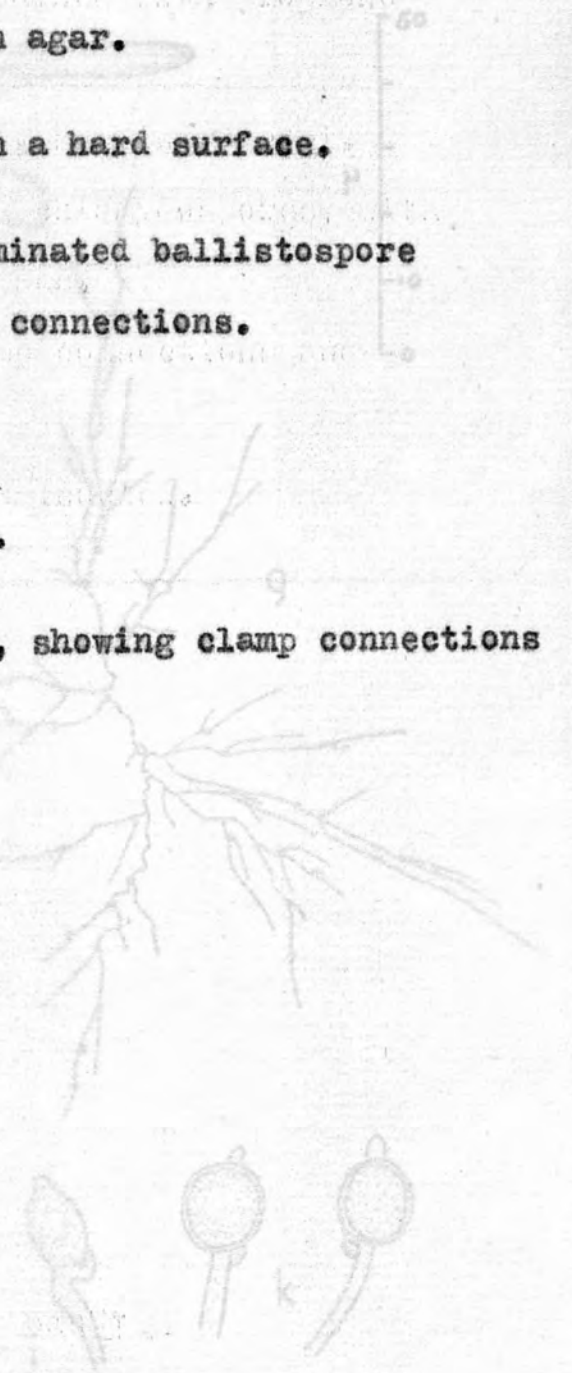
c,e, stages in germination on a hard surface.

g, young colony from a germinated ballistospore showing position of clamp connections.

f, clamp mycelium.

h, ballistospore formation.

i,k, chlamydospore formation, showing clamp connections at base of each.



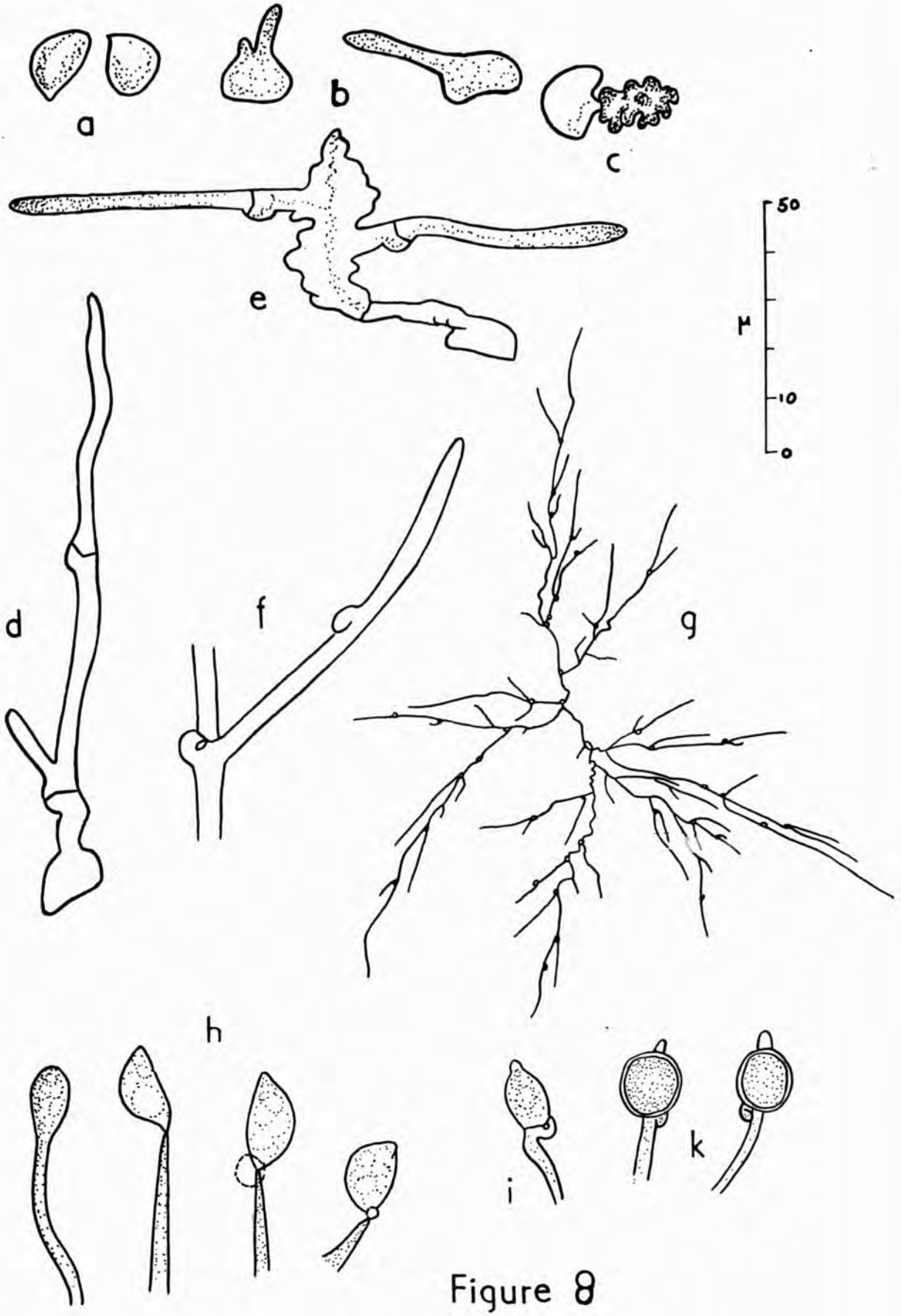


Figure 8

Figure 9. Itersonilia perplexans.

Drawings from preparations stained with propiono carmine solution.

a, freshly fallen ballistospores, showing nuclei.

b, c, "sporogenous" cells, or young chlamydospores, with clamp connections at their bases.

d, e, f, a single hypha showing clamp connections and paired nuclei.

g, "conidium" production, showing nuclei.

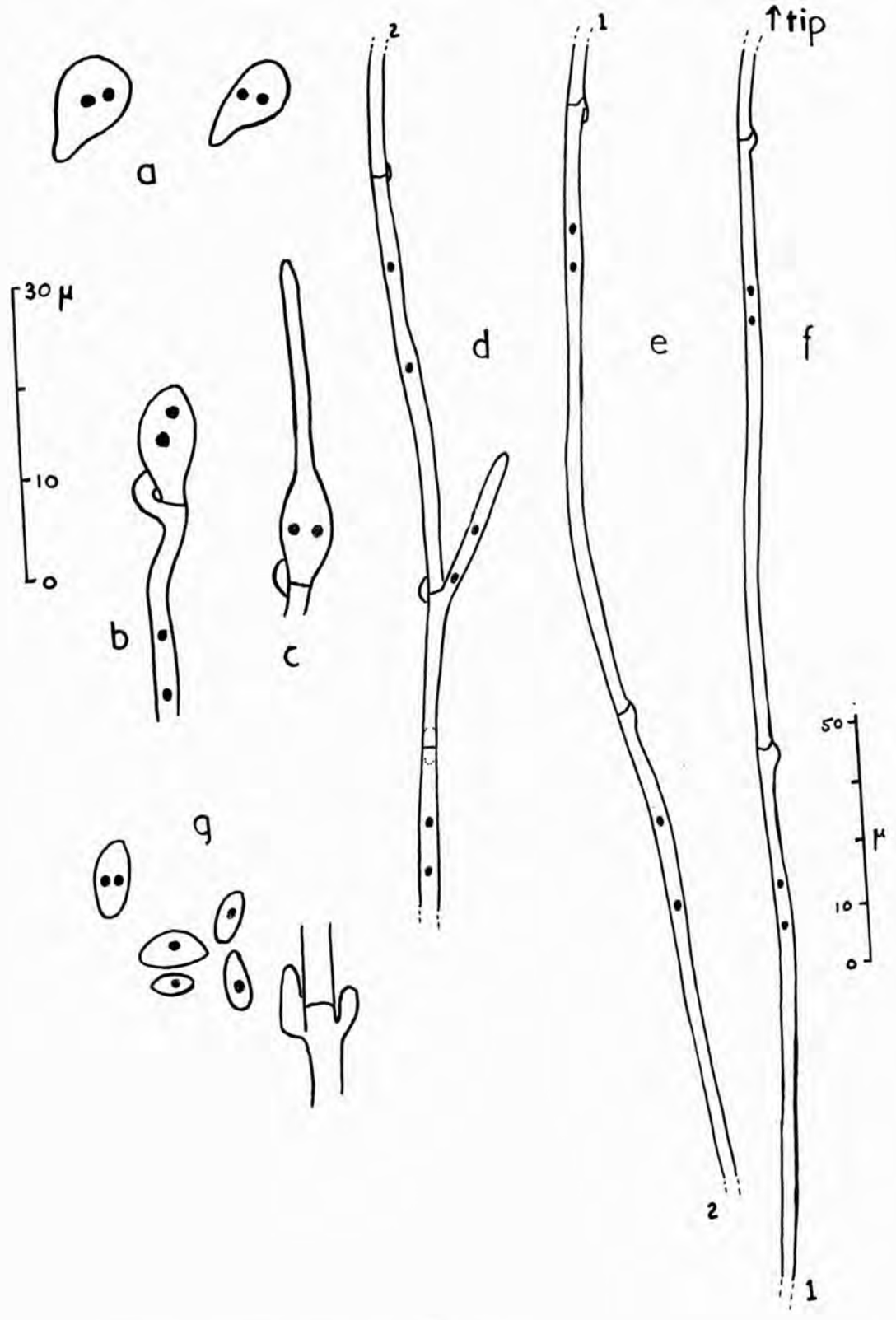


Figure 9

of the fungus described above with the Itersonilia perplexans described by Nyland (1950), and Olive (1952), the agreement seems to be such as to justify the identification of this fungus as I. perplexans.

There are differences in the measurements of ballistospores between Derx's and Olive's descriptions, Derx's covering a more restricted range. For purposes of comparison the two sets of measurements are repeated here:

Derx 14-15 μ x 8-10 μ

Olive 12.2-18 μ x 7.6-10.6 μ

The measurements of ballistospores of the present isolates, 12-21 μ x 7-15 μ, cover an even wider range, but as the walls of the ballistospores are thin, it is possible that differences in the conditions of measurement, such as the medium employed or the age of the ballistospores might account for this. Alternatively the differences may be due to the fact that different populations were measured in the three cases.

Olive described the formation of a swelling, the "sporogenous cell", from which grew out the "sporophore" which eventually produced the ballistospore at its tip. Although such structures were occasionally found in the material examined, they were by no means so constant in occurrence as Olive's description suggests, and the ballistospores were usually found to be produced at the ends

of very long, tapering hyphae which started as simple aerial branches of the mycelium. Ballistospore measurements are too large for Nyland's Itersonilia pyriformis, and the almost invariable formation of a clamp connection with each chlamydospore agrees with I.perplexans rather than I.pyriformis. There is, however, abundant development of aerial mycelium which Nyland claimed is more characteristic of I.pyriformis. Since Olive suggested that I.pyriformis is probably a variant of I.perplexans, it was decided to assign the fungus isolated to I.perplexans.

The seasonal distribution of the Sporobolomycetes. There are obviously many factors which may affect the incidence of sporobolomycetes on leaves, such as the age of the leaf, previous weather conditions, suitable conditions for the discharge of ballistospores, etc., and if the record for 1954 to 1955 of isolations from "healthy" leaves is examined in detail it is at once apparent that the records very considerably from one fortnight to another. The results are in no way quantitative, since the presence only of each of the two species of Tilletiopsis, of Itersonilia perplexans, and of the genus Sporobolomyces, was recorded for each leaf examined. Undoubtedly a similar series of observations made fortnightly throughout any other year would show wide fluctuations from the figures for 1954-55. When, however, the results of counts made over longer periods are considered,

there is a general trend towards an increase in total recordings throughout the summer, reaching a maximum between September and October, followed by a gradual decline to a minimum at the end of April. (see ~~Table~~ Figure 10, p.147)

Isolations of individual species follow this trend, except in the case of Itersonilia perplexans, which is rare on "healthy" leaves throughout the summer, but comparatively common in winter, although it is of course common during the summer months on lesions of primary parasites like Entyloma.

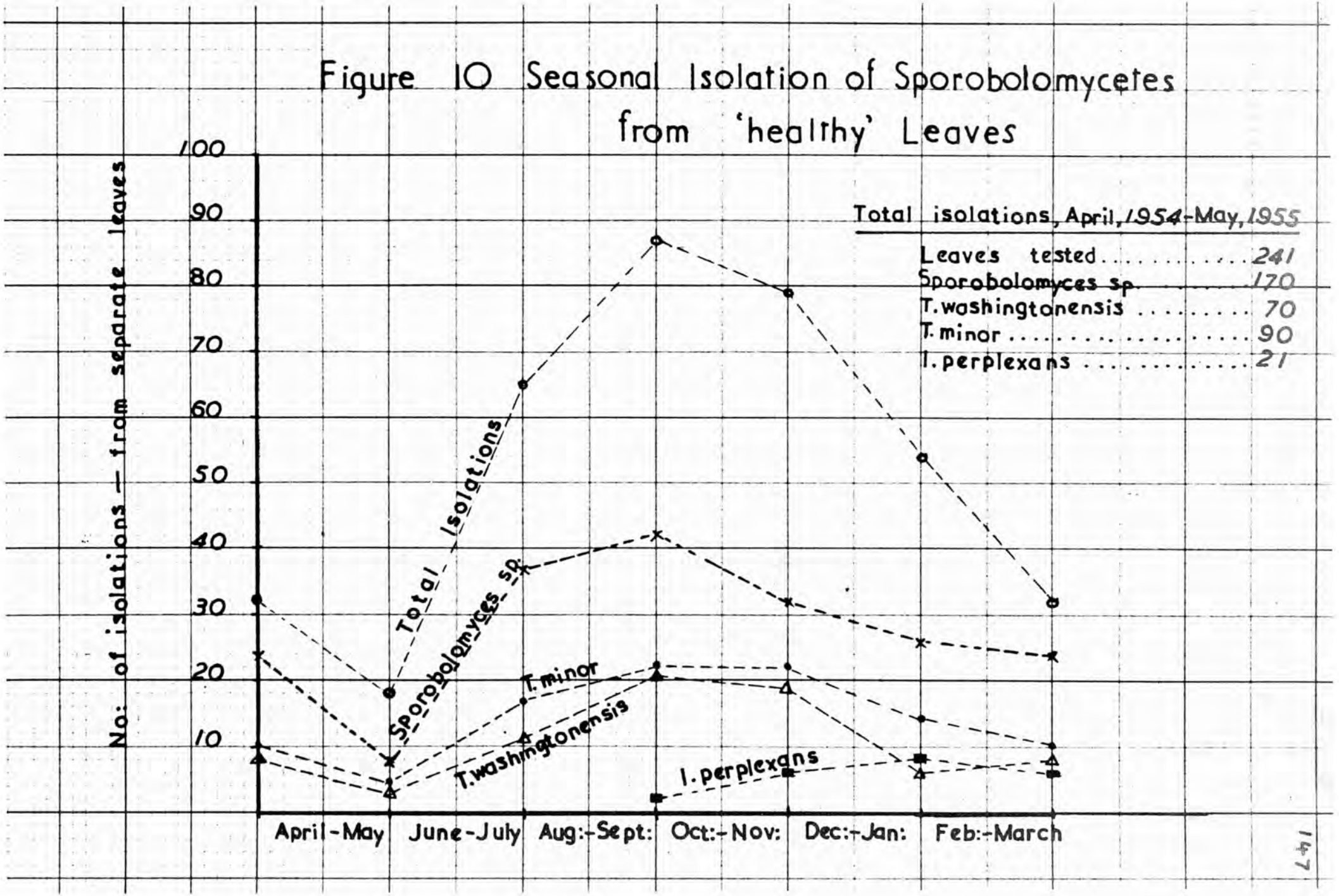
Although a sharp frost in autumn may cut down the number of sporobolomycetes isolated soon after to negligible numbers, if a period of warm weather follows, quite high numbers appear again within a short period, and it is obviously not merely the lower temperatures of winter which govern their decline, as the high isolation figures for December to January show.

The general seasonal trend is most probably the effect of available substrate. Individual leaves, which consistently bear numerically higher populations of sporobolomycetes are either diseased leaves, dead leaves, or, if living, hairy leaves. Diseased leaves increase in numbers throughout the summer and autumn and afford centres of dispersal of sporobolomycetes. The number of pustules of primary parasites decreases when the early frosts kill off their host plants, but at the same time of year there is a great deal of tree

litter from which some of the abundant sporobolomycetes can reinfect the remaining living leaves. By April and May much of this litter has decayed, the parasitic fungi are not yet widespread, and so there are no really dense centres of infection to recolonise healthy leaves after the occasional spring frosts until parasitic fungi become plentiful again.

Itersonilia perplexans is very rare on "healthy" leaves during the summer, although it is seldom absent from lesions of species of Entyloma, Puccinia malvacearum, etc. during the same period; in winter it becomes comparatively common on "healthy" leaves. This may be explained by the decline in numbers of fungal parasites, on which it grows preferentially, during the winter, which obliges the sporobolomycete to grow on the surfaces of uninfected living leaves and dead leaves.

Figure 10 Seasonal Isolation of Sporobolomycetes from 'healthy' Leaves



IV DISCUSSION

Comparison between the "sporidia" found on Entyloma lesions and the ballistospores of Sporobolomycetes found on "healthy" leaves

In the deposit of ballistosporic sporidia obtained by suspending leaves infected with Entyloma over an agar surface, ballistospores of Sporobolomyces sp. were often recorded as well as some very small hyaline allantoid ballistospores. Both kinds occurred as frequently beneath the clean part of the leaf as under the lesion. (see p. 87) The small ballistospores are identical with those of Tilletiopsis minor, isolated from "healthy" leaves, and described on pages 135 to 136. Ballistospores of this species of Tilletiopsis and of species of Sporobolomyces were present on nearly all leaves examined whether "healthy", infected by a fungus, or dead.

The allantoid sporidia of type (b), recorded from the deposit beneath lesions of Entyloma ficariae, E. dahliae, and E. calendulae, and also under "clean" parts of infected leaves, are identical with ballistospores of Tilletiopsis washingtonensis. This species of Tilletiopsis was present on the surfaces of most leaves living or dead, and whether or not previously infected with fungi.

T. washingtonensis and T. minor are shown in this investigation to have no direct connection with the life cycle of the Entyloma, insofar as they were not produced by the mycelium or chlamydospores of any of the species of Entyloma examined, and it is most likely that ballistospores

of T.washingtonensis are present on the surface of lesions as a secondary infection.

There is a close similarity between the ballistospores of Tilletiopsis washingtonensis (type (b) sporidia) and the allantoid sporidia (type (a) sporidia) of the Entyloma, and the two can only be told apart when they germinate. The "twisting" mycelium formed by the allantoid sporidia of the Entyloma is less dense than the mycelium formed by T.washingtonensis, forms needle-shaped sporidia but does not bud, and no chlamydospores have been recorded from such mycelium in this investigation. The mycelium of T.washingtonensis, on the other hand, buds frequently, especially when young, and forms chlamydospores, although the ability to form these spores varies with different isolates.

The half-moon-shaped sporidia, isolated from Entyloma lesions, look and behave exactly like ballistospores of Itersonilia perplexans isolated from lesions of other parasitic fungi and from "healthy" leaves. The measurements of ballistospores and of the chlamydospores formed in culture by the various isolates are repeated for purposes of comparison in Appendix 2. The fungi isolated from lesions of E.calendulae and E.dahliae were studied in more detail than the isolations from other species, and agree in every particular with isolations of I.perplexans from "healthy" leaves. The mycelium has the same kind of "whispy" growth,

and the same unpleasant smell, whatever the source of the isolate, and the ballistospores form the same appressorium-like structure when they germinate on a hard surface. Material from all sources produces uninucleate buds in liquid culture. It thus seems certain that the isolates of half-moon-shaped sporidia from Entyloma lesions belong to I.perplexans.

The sources of I.perplexans are almost all lesions of fungal diseases, with occasional occurrences on uninfected leaves, and it appears that the normal habitat of I.perplexans is the surface of lesions and pustules of fungal pathogens. It was not produced directly, either by the vegetative parasitic mycelium or by the germinating chlamydospores in this investigation, and although the possibility that the fungus is a stage in the life cycle of a smut fungus cannot be excluded, it seems most probable that here also the fungus is present as a secondary infection on the lesions of primary parasites such as the Entyloma. The way in which it is distributed on the lesions (see p. 87 and Table 3) is in agreement with this interpretation.

The remaining two kinds of sporidium isolated from Entyloma lesions, the allantoid type (a) and the needle-shaped, are formed when the chlamydospores of E.calendulae and E.dahliae germinate, and thus are part of the life cycle of the Entyloma. Neither of these kinds of sporidium

Table 5

Summary of the kinds of 'sporidium' found on lesions

& of ballistospores on 'healthy' leaves

Key:- Allantoid, type (a)
" " (b)
Half-moon-shaped kind
Needle-shaped "
Blastospores

Discharge by drop excretion ↑

" " abstriction →

Isolated from a lesion of *E. calendulae* or *E. dahliae*	Formed by the 'sporidia' in culture	Formed on germination of the chlamydospore of the *Entyloma*	Isolated from 'healthy' leaves
from a lesion of *E. ficariae*	formed in culture	formed by the chlamydospore	from healthy leaves
		not examined	
from a lesion of *E. eryngii*	formed in culture	formed by the chlamydospore	

occurred on the surface of "healthy" leaves. A summary of the kinds of sporidium isolated from the lesions of several species of Entyloma and the different kinds of sporidium formed by these in culture, together with a list of the Sporobolomycetes isolated from sources other than Entyloma is given in Table 5.

The Life Cycles of the Entyloma Species

It is difficult to obtain conclusive proof that I.perplexans and T.washingtonensis do not belong to the life cycle of Entyloma. The evidence presented here is mainly negative, and the widespread occurrence of both fungi in situations other than on Entyloma lesions may be a manifestation of the ability of particular stages of the Entyloma to live saprophytically. Neither fungus was produced in this investigation when the chlamydospores of the Entyloma germinate nor formed by the vegetative parasitic mycelium. Inoculation experiments were negative, but so were all inoculations using type (a) sporidia, as well as a large proportion of those using needle-shaped sporidia. A positive piece of evidence where I.perplexans is concerned, is the great difference in appearance between the clamp mycelium formed by I.perplexans in culture and the parasitic clamp mycelium of the Entyloma. The mycelium formed in culture by germinating chlamydospores of Entyloma does not form clamps, and is very unlike the saprophytic clamp mycelium of

I.perplexans. Taken in its entirety, the evidence seems sufficient to exclude I.perplexans and T.washingtonensis from the species of Entyloma examined.[1]

If Itersonilia forms no part of the life cycle of Entyloma, the latter is simpler, but is still complex. In two species of Entyloma (E.calendulae and E.dahliae) the two kinds of sporidium isolated from the lesions - needle-shaped and allantoid type (a) - are formed when the chlamydospores germinate. In E.ficariae, on the other hand, where type (a) sporidia also occur on the lesions, the chlamydospores do not germinate in situ, and it is only by analogy with E.calendulae that type (a) sporidia can be included in E.ficariae. The origin of type (a) sporidia in E.ficariae is still unknown, and the relationship of these sporidia to the life cycle remains to be traced. Further work also needs to be done on the relation of allantoid sporidia to the life cycle of the other species of Entyloma examined in the preliminary survey, and on the significance of the cylindrical and curved kinds of allantoid sporidium. Each species of Entyloma may have a different life cycle, as for example E.eryngii where needle-shaped sporidia are not formed when the chlamydospore germinates as they are in E.calendulae.

Although most work has been done on E.calendulae

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[1] When a ballistospore of I.perplexans germinates on a hard surface an appressorium-like structure is formed. The formation of an appressorium is an unusual property in smut fungi and has previously been mentioned only by Churchward (1940) for the dikaryotic infection hypha of Tilletia caries. Appressoria have not been described in the Sporobolomycetaceae which inhabit the surfaces of leaves.

and E. dahliae, there are still very many gaps in the life cycles of these species, especially concerning the association of nuclei in the mycelium. The, presumably haploid, nuclei from the promycelial branches associate in pairs in the needle-shaped sporidia. These needle-shaped sporidia are capable of infecting the host. They germinate in culture to give a twisting mycelium which forms more needle-shaped sporidia and allantoid sporidia, of which the number of nuclei was not ascertained. Allantoid sporidia isolated from the surface of lesions, however, are always uninucleate, and it appears that a dissociation of nuclei, such as was described by Churchward (1940) for Tilletia caries, may occur. If so, it is difficult to interpret the fact that fusions between allantoid sporidia were not obtained, unless the requirements for this fusion were not fulfilled in the conditions of the experiments. Fusion must occur at some point before chlamydospore formation, and it is most likely that it takes place at some time before or early in the parasitic phase, as clamp connections occur in young lesions. The nuclei were not observed in these clamps, but the connections themselves appeared normal and were not "pseudoschnallen". Clamp connections were not seen in Entyloma mycelium in culture, whether from mycelium from germinated chlamydospores, needle-shaped sporidia, or allantoid sporidia of type (a). Thus, if the association of nuclei begun in the needle-shaped

sporidia is permanent, there must be two kinds of dikaryotic mycelium, one saprophytic and without clamps, and one parasitic with clamps.

None of the species of Entyloma examined has been induced to complete its life cycle in culture - by forming chlamydospores - probably because the conditions for chlamydospore formation were not fulfilled. Infection of E. dahliae has, however, for the first time, been brought about in the host by inoculation with a single needle-shaped sporidium, with the formation of a lesion with viable chlamydospores.

The Bearing of this Investigation on past records.

The similarity between the ballistospores of Tilletiopsis washingtonensis and the allantoid sporidia of Entyloma may have caused confusion in the description of sporidial stages of Entyloma. The varied descriptions of sporidia given in the literature, even for one species of Entyloma, may in some instances have been due to the presence of Tilletiopsis on the lesions. Thus Hanna's description (see p. 15) of two kinds of "sickle-shaped conidium" in E. nymphaeae, one small and one large, can probably be explained by the presence of one or more strains of Tilletiopsis on the surface of the lesions. Hanna did not give measurements of his "sporidia", but the dimensions of his figures of the smaller strain agree with those of

T.minor, and he also found that this strain made potato dextrose agar turn dark brown. T.minor forms a brown colony, and a soluble brown pigment.

The figure of allantoid sporidia of E.dahliae given by Ainsworth and Sampson (1950, fig.1a, page 22) suggests the budding stage of germinated ballistospores of T.washingtonensis, and probably represents this fungus. Stempell (1935) described how "Mycelium I", formed by his "crescent-shaped conidia" of E.ficariae and E.calendulae, formed abundant chlamydospores in culture, and it is possible that he was dealing with a species of Tilletiopsis.

Although Derx (1948) and Olive (1952) each considered that the fungus described by Stempell as a stage in the life cycle of E.calendulae ("Mycelium II"), really represented an independent fungus, I.perplexans, neither author compared his isolate with material from a lesion of E.calendulae, but relied on Stempell's published description. The omission has been rectified in the present investigation, where isolates from E.calendulae and from other sources have been found to correspond. I.perplexans is, however, so frequent on the surface of lesions of species of Entyloma - especially those which appear during the latter part of the year - that it is strange that it has not been described more often from these sources. The only published records of a fungus approximating to I.perplexans in association with lesions

of Entyloma are those of Stempell (1935) (E.calendulae), Ainsworth and Sampson (1950) (E.dahliae), where fig.1,e-j, page 22 undoubtedly represents Itersonilia, and Brady (1953). In this last paper half-moon-shaped sporidia were reported from E.calendulae, E.dahliae, E.tanaceti, E.serotinum and E.fergussoni, and were illustrated for E.calendulae. This paper was a report of the preliminary stages of the present investigation, and the sporidia were those isolates of I.perplexans which are studied further here.

Summary

1 Lesions of the following species of Entyloma were examined for the presence of foliar sporidia:- Entyloma ficariae, E.calendulae, E.dahliae, E.tanaceti, E.bellidis, E.achilleae, E.serotinum, E.fergussoni, and E.eryngii.

2 Four distinct kinds of sporidium have been consistently isolated from the nine species:- two allantoid types, (a) and (b), and a half-moon-shaped kind, all discharged by the drop excretion mechanism, and a needle-shaped kind discharged without force. All four kinds were isolated from E.calendulae and E.dahliae. The allantoid types, where investigated cytologically, are uninucleate, and the half-moon-shaped and needle-shaped kinds are binucleate.

3 Allantoid sporidia of type (a), and needle-shaped sporidia are found only on leaves infected with Entyloma, and then always in association with the lesions. An account is given of chlamydospore germination in E.calendulae and E.dahliae. Needle-shaped and allantoid type (a) sporidia are formed. Allantoid sporidia alone are produced on germination of the chlamydospores of E.eryngii.

4 Sporidia have never been isolated from the vegetative parasitic mycelium. Clamp connections occur in old and in moderately young parasitic mycelium.

5 The allantoid sporidia of type (b), and the half-moon-shaped sporidia are shown to be ballistospores of Tilletiopsis washingtonensis and Itersonilia perplexans respectively.

These fungi are most probably present on the surface of the lesions as contaminants and not as part of the life cycle of the Entyloma. T.washingtonensis and T.minor occur on the surfaces of leaves of many different species of plant whether or not these are infected with other fungi. I.perplexans commonly occurs on the lesions of many rusts as well as on the lesions of six of the species of Entyloma and occasionally on the surfaces of "healthy" leaves. The records of T.washingtonensis and I.perplexans are new for Great Britain.

6 Inoculation of Dahlia with s ingle needle-shaped sporidia from chlamydospores of E.dahliae produced diseased spots with chlamydospores. All other inoculation experiments gave negative results.

7 Earlier accounts of sporidial stages of Entyloma are discussed in the light of these findings.

Acknowledgments

This work was carried out with the help of a Tutorial Research Studentship at the Royal Holloway College during the years 1949-51, and of an Exchange Scholarship from the Société Nationale de la Recherche Scientifique held at the Institut National Agronomique, Paris, in the year 1951-52. Thanks are extended to the authorities of these institutions for their generosity in providing facilities for research, and most grateful thanks to Dr. M.A.P. Madge of Royal Holloway College for her valued help and criticism, and also to Professor G. Viennot-Bourgin of the Institut National Agronomique, and to Miss K. Sampson for their encouragement throughout the course of study.

APPENDIX 1

Species of Entyloma recorded for Great Britain

Ainsworth and Sampson (1950) published the records of all Entyloma species so far recorded for Great Britain. They gave eleven species, including E. calendulae, which on their system of classification comprises four forms attacking members of the Compositae, which have been found in Britain.

A list of British species with their distribution, composed from data given by Ainsworth and Sampson, is given below:

Species	Host	Distribution
Entyloma achilleae Magn.	Achilleae millefolium	One record, 1909, Isle of Bute.
Entyloma calendulae (Oudem.) de Bary	Calendula officinalis and cultivated Calendula.	Cornwall, Kent, Norfolk, Suffolk
Entyloma calendulae (Oudem.) de Bary f. bellidis (Kreger) Ainsworth and Sampson.	Bellis perennis	One record, 1932, Aberdeenshire.
Entyloma calendulae (Oudem.) de Bary f. dahliae (Sydow) Viégas.	Cultivated Dahlia	Widespread.
Entyloma calendulae (Oudem.) de Bary f. hieracii Schroet.	Hieracium vulgatum and H. murorum	One record, 1889, nr. Aberdeen.
Entyloma chrysosplenii (B. & Br.) Schroet.	Chrysosplenium oppositifolium	Nr. Pitsligo, 1875 and Findhorn, 1878
Entyloma eryngii (Corda) de Bary	Eryngium maritimum	One record, 1908, Ayrshire.
E. fergussoni (B. & Br.) Plowr.	Myosotis arvensis M. caespitosa and M. scorpoides	Scotland, Norfolk, Suffolk, Essex, Yorks.

APPENDIX I (Cont.)

Species	Host	Distribution
E. ficariae (Berk.) Fisch. v. Waldh.	*Ranunculus ficariae* and *R. scleratus*	Widespread Norfolk, 1882
E. fuscum Schroet. (or *E. bicolor* Stromeyer)	*Papaver rhoeas*	Surrey, 1930
E. helosciadii Magn.	*Apium nodiflorum*	Co. Dublin and Tipperary, 1936 Wiltshire
Entyloma henningsianum Sydow	*Samolus valerandi*	One record 1907 Argyllshire.
Entyloma matricariae Rostr.	*Matricaria inodora*	Orkneys, Aberdeens, Argylls.
Entyloma microsporum (Ung.) Schroet.	*Ranunculus repens*, and *R. acris*	Scotland Yorks.

APPENDIX 2

Measurements of the Ballistospores and Chlamydospores of Different Isolates of *Itersonilia perplexans*

Source	Ballistospore dimensions	Diameter of Chlamydospores.
"Healthy" leaf of *Taraxacum officinale*	(12-21) x (7-15)μ average 16 μ, 11 μ	(10-15) μ, average 13 μ
Lesion of *Entyloma calendulae*	(13-21) x (7-15) μ average 16 μ, 11 μ	(10-15) μ, average 13 μ
Lesion of *Entyloma dahliae*	(12-17) x (7-14) μ average 14 μ, 11 μ	(10-15) μ, average 13 μ
Lesion of *Entyloma tanaceti*	(12-18) x (7-13) μ average 15 μ, 9.5 μ	(8.5-14) μ, average 11 μ
Lesion of *E.serotinum*	(11.5-17)x(7.5-14)μ average 14 μ, 10 μ	(8-14) μ, average 11 μ
Lesion of *Entyloma fergussoni*	(12-18) x (7.5-12)μ	Chlamydospores were not found

In the isolate from the lesion of *Entyloma fergussoni* less than one hundred ballistospores were measured, and average measurements were not calculated.

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