THE UNIVERSITY OF HULL

Physiology of reproduction in *Phytophthora.*

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by

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CONTENTS

GENERAL INTRODUCTION

The genus Phytophthora. a large genus of some forty species of facultative parasites, has been one of the most intensely studied of phytopathogenic genera, and yet remains one in which many of the major problems are still unsolved. The group is of considerable economic importance, and is responsible for diseases in a wide range of main crop host plants throughout tropical and temperate regions. The economic significance of the genus has resulted in a long and persistent search by pathologists and mycologists for answers to several important problems, some of which are outlined below.

The genus Phytophthora was first monographed by Tucker (1931), and recently reviewed by Hickman (1958). Taxonomically the genus is complex, owing to extreme variability both in the field and in culture. Earlier taxonomic efforts were made by Tucker, and by Leonian (1934), each author publishing a key to the species. A recent taxonomic revision was undertaken by Waterhouse (1963), who has divided the species into six groups on the basis of sporangial, oogonial and antheridial characters, "but the grouping is not necessarily intended to imply that this is a 'natural' classification." Waterhouse modestly proposes that "This key is put forward very much as a first effort, to be tested and moulded by use." It is typical of the difficulties involved in working with Phytophthora isolates that, even with this recent and comprehensive undertaking at our disposal, isolates are apt to possess characters intermediate between two or more

species, and sometimes cannot with certainty be assigned to either. New techniques, such as the serological technique recently used for separating P. citricola and P. cactorum (Burrell et al.1965), or knowledge of chromosome numbers, will be required if Phytophthora isolates are to be identified with more certainty.

The variability of Phytophthora isolates, whether of pathogenic variation in the field, or cultural variation on the agar plate, constitutes another complex major problem of the genus, and has been the subject of a recent review (Erwin et al.1963). Pathogenic variation, and the occurrence of physiologic races in the field, has been demonstrated in several species, and is well known in P. infestans. Some species have narrow host ranges (P. infestans on Solanaceae, P. primulae on polyanthus and primula), whilst others such as P. palmivora and P. cactorum have very wide host ranges. Phytophthora isolates show considerable variation in the agar plate, and sectoring is frequent; single zoospore isolates from one original isolate may often show significant morphological and growth rate variation. Single isolates show morphological variation under different cultural conditions, and the variation between isolates of the same species even when isolated from the same host plant, can be large enough to cast doubt upon the use of morphological criteria for species identification. The mechanisms of variation in Phytophthora are at present unknown, and attention has been paid to the possible occurrence of heterokaryosis and mitotic recombination within the thallus. The karyotype of the thallus, whether haploid or diploid, remains uncertain, and workers have been unable to obtain biochemical mutants or a reasonable percentage of oospore

germination for examining possible recombinant progeny. Some cytological evidence (Sansome,1961) suggests that the greater part of the life cycle is diploid, with meiosis occurring in the oogonia and antheridia.

The mechanism of sexual reproduction in Phytophthora comprises another important problem, and has been outlined by Hawker (1957), and briefly reviewed by Erwin et al (1963). Just over half the species described in Waterhouse's key are true homothallic species, producing sex organs and oospores readily in single isolate culture. Other species appear to be 'heterothallic', with oospores formed only when two strains of opposite mating type are brought together (e.g. P. palmivora), whilst some species exhibit a mixture of homothallism and heterothallism (e.g. P. nicotianae v. parasitica). Two schools of thought have emerged with regard to the nature of sexuality in the 'heterothallic' or partially 'heterothallic' species; the first supports the concept of strict morphological heterothallism between the two isolates of each mating type, with one isolate behaving as a male, and the other as a female. The second supports a concept of physiological heterothallism, involving chemical stimulation of sexuality between bisexual strains of each mating type; hybridisation might still occur between the two isolates of each mating type under these conditions. Both inter-specific and intra-specific matings have been discerned, so that the possibility exists of inter-specific hybridisation within the genus. The controversy over the nature of sexuality remains unresolved, and is further aggravated by a low percentage germination in oospores produced on crossing isolates of each

mating group; this has resulted in a lack of knowledge with regard to recombinant progeny and the occurrence of meiosis within the oospore.

The problem of sexuality in 'heterothallic' and partially 'heterothallic' Phytophthora species can be extended to the field, where in some species no observations of sex organs or oospores have been made in the host in spite of their widespread occurrence as crop disease organisms. The absence of evidence demonstrating the occurrence of sexual reproduction in the field in these species, throws doubt upon the role played by oospores in survival of the pathogen, and in pathogenic variation of the fungus. Alternative means of survival might include secondary host plants, a competitive saprophytic existence in the soil, or production of resistant soil-borne chlamydospores, which are formed mostly by the 'heterothallic' species. In homothallic species, oospores are often formed in the host and are quite likely to play a major role in survival, although not enough confirmatory evidence has been obtained. The problem of survival is discussed by Hickman (1958).

Work on the physiology of the genus has been limited, by and large, to the more important pathogenic species, and remains a field of almost unlimited scope; this is partly due to the wide variation between species, and between isolates of the same species, in the agar plate. For many years, the sexual stage of Phytophthora could be obtained only on complex vegetable media, limiting the scope for investigation into the nutritional aspects of reproduction, but even research into the effects of temperature and light upon growth and reproduction using vegetable media has been scant.

The field of reproductive physiology in Phytophthora has been re-opened by the recent discovery that sterols incorporated into certain synthetic, defined media, stimulate sexual reproduction in several species. The result has been that the role played by sterols in growth and reproduction of Phytophthora has become a new, yet refreshing problem of the genus. With the aid of this new knowledge, better standardisation of working conditions can be obtained, and many basic problems such as those outlined above can be more conveniently investigated.

The problem :- "Physiology of reproduction in Phytophthora."

The research described in this thesis does not involve a large number of species, nor a particularly large number of isolates, but is restricted to certain species found in Group II of Waterhouse's key, and characterised by having strongly papillate, usually deciduous sporangia, not proliferating internally, antheridia all amphigynous, and oospores often not formed in single strain culture. (For terminology in Phytophthora see Blackwell,1949). The work has been centred around a group of isolates of a fungus which caused an epiphytotic of Black Pepper, Piper nigrum, in Sarawak during the years 1953-6. The disease outbreak was thoroughly investigated for the Colonial Pool of Plant Pathologists by P. Holliday and W.P. Mowat (Holliday and Mowat,1963), though the causal organism was first named Phytophthora palmivora var. piperis by Muller (Muller,1936) during his earlier investigations of this disease in Indonesia. Holliday and Mowat, with confirmatory advice from G.M. Waterhouse, also placed the

isolates in the P. palmivora group.

Additional isolates of P. palmivora, from other host plants and geographical regions, together with isolates of P. arecae, P. nicotianae v. parasitica, and P. heveae, were also used in this work. The greater part of the work, however, is concerned with P. palmivora, a tropical species with a wide host range, and of considerable economic importance, as illustrated by the following quotation from Hickman (1958) :-

"P. palmivora parasitises plants of fifty-one genera in twenty nine families of flowering plants. Common host plants are cocoa, rubber, various palms, cotton, citrus, pawpaw, pineapple, pepper, breadfruit, jak, mango, guana, cinchona, and a number of orchids."

Phytophthora palmivora is a 'heterothallic' species, and whilst the work has largely concerned this species, an isolate of the related homothallic species, P. heveae, from Hevea brasiliensis in Malaya, has also been used throughout much of the work, providing a taxonomic 'shadow' to the results obtained with P. palmivora.

All the major problems of the genus, described earlier, apply to P. palmivora, and some are magnified by its wide host range and geographical distribution. In keeping with its economic importance, a large amount of phytopathological and mycological work has been published on this species, but many unsolved problems remain.

Taxonomically, P. palmivora is closely related to several other species in Waterhouse's Group II, including P. arecae, P. meadii, P. nicotianae v. nicotianae and P. nicotianae v. parasitica. All five species are

'heterothallic', with isolates falling into two mating groups, although P. meadii, P. nicotianae v. nicotianae, and P. nicotianae v. parasitica, are also partially homothallic, as described by Waterhouse (1963) for P. nicotianae v. nicotianae :- "oogonia formed promptly by some isolates in single culture, but those that form them sparsely or not at all after some weeks produce them abundantly in dual cultures with an 'opposite' strain." The divisions between these five species are not very distinct, and many isolates which are considered as intermediate forms occur. Oogonia are produced readily in matings between P. palmivora, P. arecae, and P. meadii, provided that both mating types are present, and are also formed between P. nicotianae v. parasitica and P. palmivora. Leonian (1925) and Thomas (1948) considered P. palmivora and P. nicotianae v. parasitica to be varieties of one and the same species, and saw no adequate grounds for maintaining them as separate species. Tucker (1931), believed P. palmivora and P. nicotianae v. parasitica to be valid species, but merged P. palmivora, P. arecae, and P. meadii into one species under P. palmivora. Whilst the author believes that there is probably a good case for merging several of these species, the specific nomenclature laid down in Waterhouse's 1963 key may be used when referring to isolates involved in the present work. Thus, P. palmivora, P. arecae, and P. meadii will be called as such, P. parasitica may be referred to as P. nicotianae v. parasitica, and P. parasitica v. nicotianae may be referred to as P. nicotianae v. nicotianae. All five species will be referred to as the 'palmivora' group.

Very little work has been done on the mechanisms of pathogenic and

cultural variation in P. palmivora (Gadd,1924; Orellana,1959, Tucker, 1931; Turner, 1960; 1961, etc.), but the work does indicate the existence of physiologic races within the species. Barely anything is known of the means of survival of the fungus in the field in the absence of the host plant, and at the time of writing the author has found no published account of the occurrence of oospores in the host other than that by Ramakrishnan and Seethalakshmi (1956), who found oospores in the pericarp of diseased areca fruits.

Earlier research into the physiology and nutrition of growth and reproduction of P. palmivora was limited by the reliance upon complex vegetable media for obtaining the sexual stage. Reports of the effects of light and temperature upon growth and reproduction have been few, and usually associated with work of a different nature; the greater part of physiological investigation has been concerned with either zoospore production or the mating reaction.

The research described in this thesis consists of an investigation into the physiology and nutrition of reproduction in P. palmivora and P. heveae, and an investigation of the mating reaction in the 'palmivora' group. An attempt to obtain sexual reproduction of P. palmivora in the host plant is also described. No attempt has been made to germinate oospores, to investigate pathogenic and cultural variation mechanisms, or to examine the validity of the taxonomic criteria within the 'palmivora' group.

The thesis is divided into three sections, the first of which deals

with the effects of temperature and light upon growth and reproduction of four isolates, three of P. palmivora and one of P. heveae, in agar culture, and the production of oospores of P. palmivora in the host. The physiological knowledge obtained for these four isolates was used in the second section, in which the nutrition of reproduction is investigated. Special attention was paid to the role of sterols in growth and reproduction of P. palmivora and P. heveae, and a search was made for a defined minimal medium suitable for obtaining sexual reproduction in the 'palmivora' group. In these first two sections, a reasonably detailed knowledge is obtained of the basic effect of a cultural environment upon reproduction in the four Phytophthora isolates. In the third section the mating reaction in the 'palmivora' group is examined; the more general work in this section incorporates a larger number of isolates, including isolates of P. arecae, P. nicotianae v. parasitica, P. nicotianae v. nicotianae, and other P. palmivora isolates; work of a more specialised nature involves the four isolates used in the physiological and nutritional investigations. Each section is preceded by an introduction, and concludes with a discussion; a general discussion is provided at the end of the thesis.

SECTION I

The effects of temperature and light upon sexual and asexual reproduction in *Phytophthora palmivora* and *Phytophthora heveae*, and the production of oospores in the host plant by *P. palmivora*.

Chapter 1.

INTRODUCTION

Although a considerable amount of morphological and physiological data has been accumulated for the genus Phytophthora. it is equally true that a certain amount of confusion and conflict has partly arisen from it. Phytophthora isolates are such plastic organisms that the definition of a strain, or of a species, or an analysis of the behaviour of an isolate under different cultural conditions, may become a problem within a problem. In an attempt to offset this variability, a tendency has arisen for some workers to use large numbers of isolates, whether of the same species or of different species, in cultural work. Sometimes this may lead to a lack of sufficient detail in results. The very fact that two isolates of the same species may behave differently in culture suggests that each isolate should be treated as an 'individual in its own right', and that an experimental emphasis on detailed results from a few isolates would often be better than an emphasis on accumulated results from many. For this reason the number of isolates used in this section has been restricted to four.

A lack of standardisation of cultural conditions, combined with isolate variability, has to a certain extent increased the difficulty of interpreting results obtained by different authors. For example, oatmeal

agar has been preferred by many workers for use with P. palmivora. but their methods of preparation, incubation temperatures, and quantities of medium per plate have been far from standard; french bean agar, corn meal agar, carrot agar, lima bean agar and potato agar have been widely used with P. palmivora and other Phytophthora species. Defined synthetic media previously came low on the list of media in use, but with the recent discovery that sterols stimulate oospore production, their perfection will greatly assist in standardising techniques in future work.

A considerable difference exists between methods used by authors to estimate spore frequency in agar plate cultures, especially where relative frequency symbols, such as +, ++, etc. are involved. Some authors have used more accurate quantitative assessment methods involving counting techniques. Spore distribution in Phytophthora crosses is complicated by the presence of two cultures, and for this reason attention was devoted in Materials and Methods (Ch.2) to the development of a standard spore frequency assessment technique.

The mechanism of sexuality in P. palmivora remains an outstanding problem; in order to provide a basis for a detailed examination of the mating reaction, a study of the environmental factors, other than nutrition, affecting sexual reproduction were required, since little previous work of this nature exists. An examination of these factors was undertaken, and described in the present section, in which an attempt was made to rationalise the approach to reproductive physiology of P. palmivora using three isolates. A fourth isolate, of P. heveae. was included in these

studies, to contrast the reactions to the environment of related heterothallic and homothallic organisms.

Some attention was paid to the value of different media under varied temperature regimes, and carrot agar was found to be preferable to oatmeal agar; carrot agar was therefore used in further work investigating the effects of temperature and light upon sexual and asexual reproduction. Knowledge of the physiology of the four isolates obtained in these experiments provided a useful basis for further experimentation on the nutrition of reproduction and the mechanisms of sexuality described in the two later sections. The final experiments in this section are partly an extension of the agar culture work, and at the same time an investigation into the problem of oospore production by P. palmivora in the field. Work with two P. palmivora isolates and their original host plant (Piper nigrum), demonstrated that the production of oospores under controlled laboratory conditions can be obtained in host tissue.

Chapter 2.

MATERIALS AND METHODS.

The following isolates were used in this section :-

No.	Species	Origin	Host	No.of donor
P4 :	Phytophthora palmivora (Butl.) Butl.	Sarawak	Piper nigrum	*P72
P18 :	" " " "	"	" "	*P81
P24 :	" " " "	Jamaica	Theabroma cacao	IMI 75548
P28 :	Phytophthora heveae Thompson	Malaya	Hevea brasiliensis	IMI 36528

* P72 isolated by P. Holliday, and P81 by J.G. Turner.

These isolates will sometimes be referred to as P4, P18, P24 and P28 in the text. Isolates P4 and P18, chosen because the Piper nigrum fungus was selected for special study, produced abundant oospores when mated together in preliminary mating experiments, and belong to different mating groups (see also Section III). Isolate P24 was chosen because it produced abundant sporangia in single isolate culture, and because of its different host plant and geographical origins. Isolate P24 produces oospores when mated with P18, but not with P4. Isolate P28 is a homothallic fungus, producing abundant oospores in single isolate culture. Within the context of this and the following section, the terms homothallic and heterothallic

will be defined as follows :-

Homothallic isolate : a single hyphal tip culture of an isolate which produces oogonia in single culture within 10 days of incubation at 20-25°C.

Heterothallic isolate : a single hyphal tip culture of an isolate which produces no oogonia in single culture after incubation for 21 days at 20-25°C, but which produces oogonia within 10 days in mixed culture with another self-sterile, but sexually compatible isolate of opposite strain or mating type.

Isolates P4, P18 and P24 are heterothallic, and P28 homothallic, within the context of these definitions. Further discussion of these terms will be found in Section III.

Four culture media, oatmeal agar, carrot agar, cornmeal agar, and a synthetic medium (Medium A) were used in this section, and details of their preparation and contents are given in the Appendix. All media were sterilised at 10 lb. for 10 minutes. The slight sediment in the carrot medium was allowed to settle before pouring. The recipe for the synthetic sterol medium (Medium A + S) was kindly supplied by Dr. Antonio Leal, who was at the time investigating the value of synthetic media for use with Phytophthora, Phythium, and Saprolegnia. The carrot, cornmeal, and synthetic media have the advantage of being clear, whilst the oatmeal medium, widely used by Phytophthora workers, is opaque. These media will sometimes be referred to as CA (carrot agar), CMA (cornmeal agar), OMA (oatmeal agar), and Med A + S (synthetic sterol medium) in the text.

Large (13 cm. diameter) petri dishes were used for measurement of radial growth rates. Standard (9 cm. diameter) petri dishes were used for stock cultures and most other work. Small (5 cm. diameter) petri dishes, which were more economical with medium, were used for crosses, after preliminary experiments had shown that the use of small dishes did not adversely affect oospore production. 30, 15, and 5 ml. of medium were used in the large, standard, and small dishes respectively.

The cultures used in this work were stored (over long periods) under sterile liquid paraffin, on OMA slopes in 10 ml. screw cap bottles. Two types of oil culture were maintained for each isolate :-

1) A mass isolate culture, as the donor culture.

2) A single hyphal tip subculture from a water agar plate.

Only the hyphal tip subcultures were used in experiments. Stock cultures for experimental purposes were usually maintained on carrot agar, but also on Med A + S, oatmeal agar or water agar medium at 25°C for specific purposes; inocula were taken from the edges of growing colonies with a No. 1 cork borer. The inoculum was usually placed in the centre of the dish, except in crosses, where agar plugs of each isolate were placed at opposing edges of the dish (see Fig. 2.1.). All plates were replicated, and incubated in the dark unless stated otherwise.

Special Techniques

1. Spore frequency assessment.

For comparative investigation of environmentally controlled quantitative effects upon spore production, a standard method of spore

frequency assessment is required. Klebs (1899) estimated the frequency of water mould oogonia in liquid cultures using the relative numbers I, II and III, and Pieters (1915) used counting methods to further this technique. Leonian (1925) working with Phytophthora isolates in agar culture, used the symbols 0, I, II, III, IV and V to indicate relative quantities of aerial mycelium, sporangia and oogonia.

Many recent workers have used quantitative methods of spore frequency assessment with Phytophthora, but these techniques are often dependent upon direct observation of the agar plate with the low or high power objective of a microscope, and therefore dependent upon light transmitted through the medium. These methods have some drawbacks, since light transmission may be impeded in an opaque medium such as oatmeal agar, or in a culture where surface aerial mycelium is dense; in addition many Phytophthora species form spores within the medium, so that a direct observation method is best suited to conditions of shallow depth in a clear medium. Opaque media, including oatmeal agar, were required for much of the work described in this thesis, and as a means to overcome some of these problems the following slide technique was developed :-

Slide technique. Small pieces of culture required for microscopic examination are cut from the agar plate using the tip of a sharp scalpel, and transferred to a microscope slide with a needle. Ideally these pieces are somewhat smaller than the coverslip to be used; care has to be taken not to drag away any aerial mycelium from the piece(s) during removal. A drop of lactophenol (with stain if required) is added, and the slide

gently warmed over a bunsen until the agar begins to melt. The specimen piece(s) shrinks in depth as the melting agar and lactophenol runs off, and is absorbed with blotting paper. A further drop of lactophenol is added, followed by a coverslip, and the slide gently reheated. The weight of the coverslip on the specimen(s) squeezes out much of the remaining liquid agar, but leaves the mycelium intact. If necessary gentle pressure may be applied to the coverslip from above using blotting paper; the excess liquid is then absorbed from around the edges of the coverslip.

By this method a good semi-permanent preparation, cleared in lactophenol, can be obtained; the original depth of the agar has been greatly reduced, and the depth of field to be examined with a microscope is almost the same as that for a normal slide. With careful handling, the width of a piece of medium selected for examination remains unchanged, so that the method is suitable for accurate spore counting. Morphological detail in Phytophthora oogonia and sporangia is preserved, and observation is easy; such preparations are also useful in photomicrography, biometry, and various quantitative techniques, especially where the spores were originally embedded in the medium. More permanent preparations can be made by sealing the coverslip with nail varnish in the traditional way.

The above slide technique was used in the development of two spore frequency assessment methods, in conjunction with high and low power counts in the field of a microscope. The first method will be referred to as the 'Quick Estimation Method' (Q.E.M.) in the text; the second is

a development of the Q.E.M. and will be referred to as the 'Numerical Estimation Method' (N.E.M.).

a) The Quick Estimation Method (Q.E.M.): (see Fig. 1).

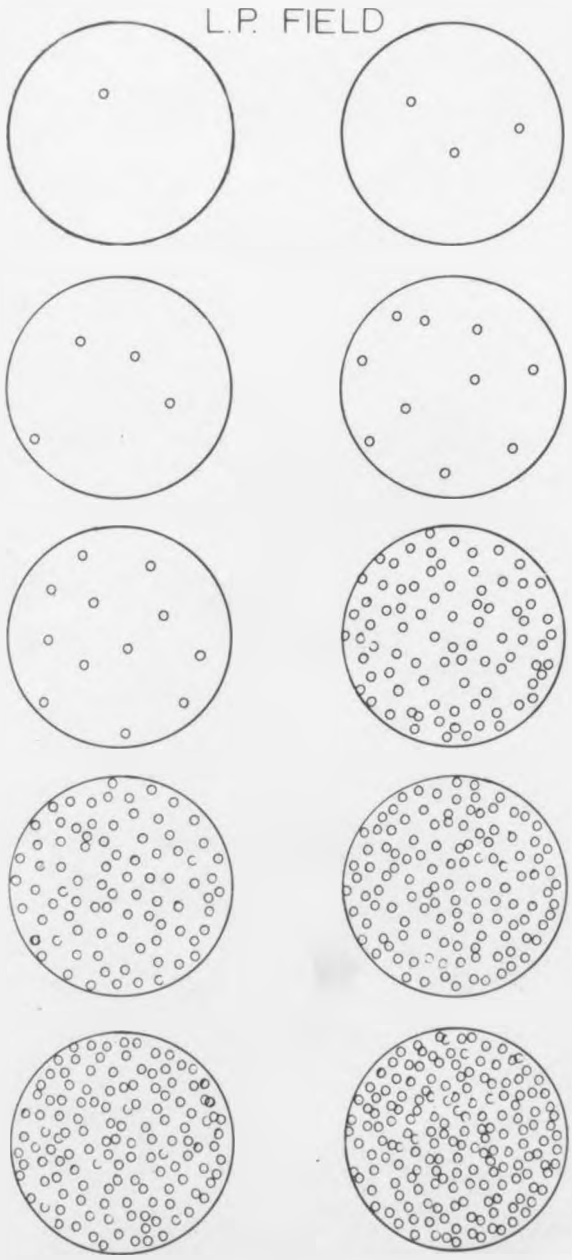
This method, used for applying relative spore frequency symbols (+, ++ etc.), involves the use of small samples from the culture under examination, and slides prepared by the above technique. The method relies on a personal assessment of evenness of distribution of spores between samples, combined with a rough spore frequency count. Culture sampling positions, different for single isolate and cross cultures, are shown in Fig. 2.1.

In single isolate cultures, nine samples, each 5 mm. square, are removed from the positions shown in Fig. 2.1. The samples lie on circles of 10, 20 and 30 mm. radius from the centre of the inoculum in a standard petri dish. The slide prepared samples are first examined with the low power (x 100) field of the microscope to assess evenness of spore distribution, and one spore count is made from a random low or high power (x 400) field, for each of the nine samples. The total spore count, divided by nine, gives a rough spore frequency for the culture. The process is repeated for the replicate plates to give a mean rough spore frequency, with which, taking evenness of spore distribution into account, a relative frequency symbol is applied. This method is used for both sporangia and oogonia.

In cross cultures, oogonia are usually concentrated along the line of conjunction of the two isolates, on both sides of the 'repulsion zone',

SPORE FREQUENCY ESTIMATION.

L.P=low power(×100) Frequency=average spore frequency
E.D=even spore distribution U.D.=uneven spore distribution



RARE

+

L.P. Frequency 1 or <1 (U.D.)

OCCASIONAL

++

L.P. Frequency 1-10 (E.D.)

FREQUENT

+++

L.P. Frequency 11-80 (E.D.)

FREQUENT/ABUNDANT

+++/++++

L.P. Frequency 80-120 (E.D.)

ABUNDANT

++++

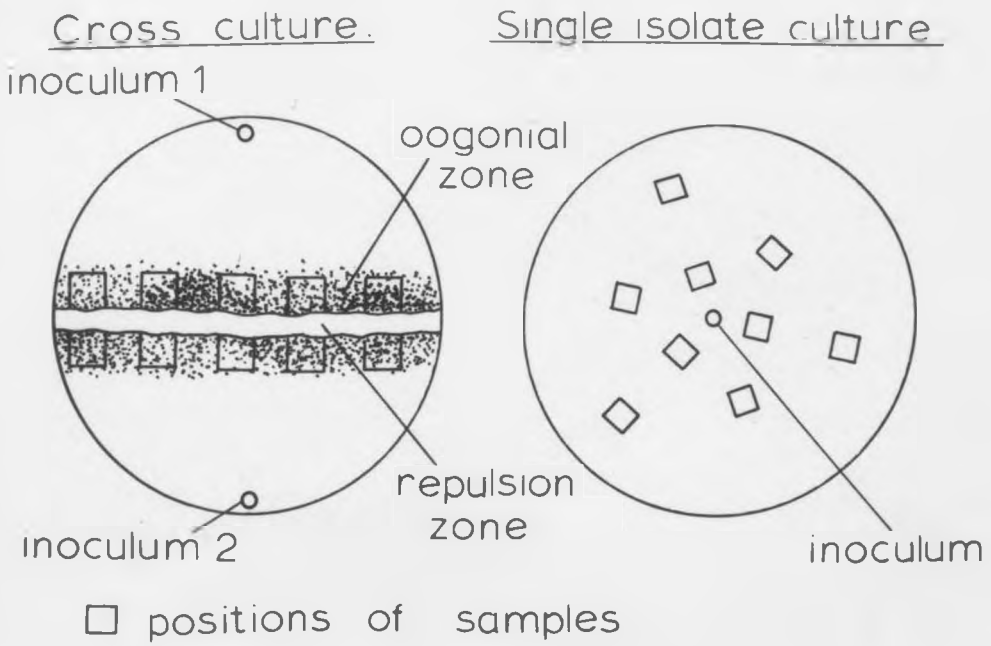
L.P. Frequency 120+

and are often absent or scarce in the rest of the plate. For this reason, a different sampling technique is used (see Fig. 2.1.). Ten samples, each 5 mm. square, are removed from the positions shown, a slide prepared, and the samples examined under low power for spore distribution assessment. Oogonia in crosses tend to be formed in patches, and distribution is very variable; some samples may contain no oogonia at all. It was therefore found necessary to select the five samples with the least oogonia and ignore them for counting purposes. Although this could be considered as introducing a bias into the counting technique, it was found to be efficient in overcoming variations in spore distribution, and the method was standard to all cross plates and replicates. From each of the remaining five samples, two random low or high power field counts are made, and the total oogonial count, divided by ten, gives a rough spore frequency. The process is repeated for the replicate plates to give a mean rough spore frequency, with which, taking spore distribution into account, a relative frequency symbol is applied according to the system below. This system was used for both single isolate and cross cultures.

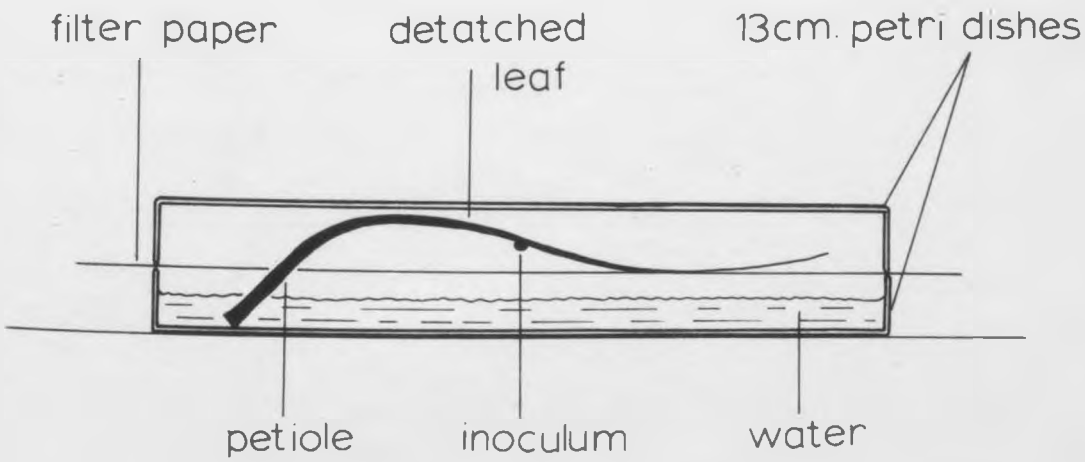
System for Quick Estimation Method. (see Fig.1.).

Symbol	Spore distribution and rough spore frequency.
a) None - ;	
b) Rare + ;	Under L.P. total no. of spores observed in all 9 or 10 samples on slide few, perhaps only 1, sometimes up to 10. Spore distribution uneven, rarely more than 3 per L.P. field; L.P. frequency 1 or < 1, often 0.

1. SAMPLING FOR SPORE FREQUENCY ESTIMATION



2. METHOD OF CULTURING DETATCHED LEAVES



c) Occasional ++ ; Under L.P. about 3-10 spores is usual per field. Spores should be evenly distributed in samples; L.P. frequency 1-10; never 0.

d) Frequent +++ ; Under L.P. about 11-80 spores is usual per field. Spores fairly evenly distributed; L.P. frequency 11-80; H.P. frequency 2.5-20.

e) Abundant ++++ ; Under L.P. spores very dense, sometimes approaching 50% of field or more; L.P. frequency 120+ ; H.P. frequency 30+ .

f) Intermediate symbols.

+/++ ; Under L.P. spores rare, but sometimes clustered 1-5, usually scarce or absent from the remaining fields. Uneven spore distribution. All samples must be taken into account.

++/+++ ; Under L.P. spores patchy; L.P. frequency 8-14, but spores unevenly distributed.

+++/++++ ; Distribution of spores often even under L.P. L.P. frequency 80-120; H.P. frequency 20-30.

L.P. = Low power (x 100); H.P. = High power (x 400).

The intermediate symbols +/++, and ++/+++, are used in cases where spore distribution is very uneven and the frequency symbol uncertain. A further symbol, +++++, was introduced at a later date to cover exceptional circumstances.

b) The Numerical Estimation Method (N.E.M.).

In this method, which is a development of the Quick Estimation Method, culture samples and slides are prepared as for the Q.E.M., but a larger number of random microscope field counts are made. A total mean spore frequency is calculated and used for graphic representation of

results.

When dealing with sample slides from single isolate cultures, five random high power (x 400 and sometimes H.P. x 2.25) field counts are made from each of the nine samples per plate, and the mean H.P. spore frequency for the plate obtained; the mean H.P. spore frequency for the replicate plates is also found, and the total mean for the replicates is calculated. Three replicate plates were used in Section I, but owing to the difficulties involved in making large numbers of counts, and the non-significant variation between replicates, two replicate plates were used in Section II. An analysis of variance in a single isolate culture series is given in Table 1.

When slide samples from cross cultures were used (this applies only to Section I, Chapter 5), nine random H.P. field counts were made from each of the five samples to be counted, and a mean spore frequency for the plate obtained. This was repeated for the replicate plates, and the total mean H.P. spore frequency calculated. An analysis of variance for a cross culture series is given in Table 2; the use of only five out of ten samples per slide greatly reduced the variation between replicates in cross culture plates.

In both the single isolate and cross culture series, variation between series means was highly significant, and variation between replicate means was not significant.

The H.P. spore frequencies obtained by this method were used for plotting relative spore frequency as a function of the environment (see

TABLE 1.

Analysis of variance in a single isolate culture series :-

data taken from Section I, Ch.5, are mean oogonial frequencies of P28 at a range of temperatures (15-30°C), with three replicate plates at each temperature. (See Fig. 5 & 6.).

DATA : Mean spore frequency (N.E.M.)

Series	15°C	21°C	22.5°C	25°C	27.5°C	30°C
Replicate no.						
1	38.0	53.6	56.1	60.2	37.0	17.6
2	43.5	41.5	47.9	52.5	31.4	20.6
3	39.7	49.9	45.1	50.5	37.4	15.5
Total mean	40.4	48.3	49.7	54.4	35.3	17.9

ANALYSIS OF VARIANCE.

	Sum of squares	d f	Mean square	F ratio
Between series	2627.6	5	525.5	F = 29.5
Between replicates	68.1	2	34.05	F = 1.9
Within	178.1	10	17.8	
Total	2873.8	17		

$F_{.99}(5,10) = 5.64$; $F_{.99}(2,10) = 7.56$.

TABLE 2.

Analysis of variance in a cross culture series :-

data taken from Section I, Ch.5, are mean oogonial frequencies of P18 x 4 at a range of temperatures (15-30°C), with three replicate plates at each temperature. (See Fig. 3.).

DATA : Mean spore frequency (N.E.M.)

Series	15°C	17.5°C	20°C	22.5°C	25°C	27.5°C	30°C
Replicate no.							
1	26.7	39.2	30.7	15.9	14.5	5.4	3.5
2	21.3	40.1	32.8	17.0	14.2	6.0	1.6
3	24.7	38.5	30.8	16.6	14.8	3.7	0.8
Total mean	24.2	39.3	31.4	16.5	14.5	5.0	1.9

ANALYSIS OF VARIANCE

	Sum of squares	df	Mean square	F ratio
Between series	3313.25	6	552.2	F = 277.5
Between replicates	2.57	2	1.28	F = .065
Within	23.92	12	1.99	
Total	3339.74	20		

$F_{.99}$ (6,12) = 4.82; $F_{.99}$ (2,12) = 6.93.

Figs. 3, 4 and 5). Owing to the different counting technique used in each case, no direct comparison can be made between spore frequencies of single isolate and cross culture plates under the same environmental conditions. Interesting comparisons can be made, however, between the graphic optima obtained for different cultures and isolates, though the spore frequencies themselves are not significant, and do not constitute a total spore count.

2. Estimation of aerial mycelium in petri dish cultures.

The relative symbols +, ++ and +++ were used when assessing aerial mycelium, according to the following scheme :-

-	None
+	Sparse
+/++	Sparse to downy, often confined to region of inoculum.
++	Moderate
++/+++	Moderate to dense; patchy, or intermediate.
+++	Dense

3. Light Experiments.

For light experiments, a large wooden inoculation box, painted white inside, with a hinged glass front, was placed in a window so as to receive normal daylight and darkness. A temperature of 22.5 or 25°C ± 1°C was maintained by means of a 60 watt electric lamp enclosed in a tin box, and by adjusting the hinged front of the light box to vary the degree of ventilation. The light box was used for both agar plate and leaf inoculation experiments, with culture plates placed singly on the floor of the box.

4. The use of living plant material.

In October 1964, twelve nodal cuttings were struck from a mature vine of Piper nigrum L. maintained at the University Botanic Garden, and the eight surviving cuttings were used as experimental material in October 1965; in experiments whole potted plants and excised leaves were used. Inoculations of detached young leaves with Phytophthora palmivora were carried out in the following manner :-

A leaf was cut from the main stem at the base of the petiole, surface sterilised with 95% alcohol, air dried, and a horizontal incision made on the abaxial mid-rib with a sterile scalpel. A drop of sterile water was added to the incision, and aerial mycelium from an oatmeal agar culture of the respective Phytophthora isolate placed beneath the flap of plant tissue with an inoculating needle. Inoculated leaves were incubated over a 13 cm. petri dish of water by supporting the leaf with a large filter paper (see Fig. 2.2.). When crosses were made, one isolate was inoculated at the proximal and the other at the distal end of the leaf mid-rib. When leaves on intact plants were inoculated a polythene bag was placed over the plant and pot.

Examination of plant material.

A segment 6 x 2 cm. long was cut from the middle of a leaf; this was then cut into three smaller segments each 2 cm. square, and the segments macerated with KOH and HCl using the method described by Priestly and Scott (1938). After maceration, the abaxial and adaxial sides of each segment were gently teased apart, and soaked overnight in lactophenol. The half

segments were placed on a slide with the epidermis downwards, and a drop of cotton blue in lactophenol added, followed by a coverslip. Good, semi-permanent preparations were obtained.

Chapter 3. The effects of temperature and medium upon radial growth and growth rate of Phytophthora.

Knowledge of the optimum temperature for growth of a fungus under defined conditions is aprerequisite of any further physiological studies. Holliday and Mowat (1963) showed the optimum temperature for radial growth of a P. palmivora isolate from Piper nigrum to vary according to the medium used. This effect was further investigated in the following experiment, in which three media widely used in Phytophthora investigations, carrot, oatmeal and corn meal agars, were tested. Isolates P18, P24 and P28 were used, and the cultures incubated at 25°C in the dark.

Two large (13 cm.) plates of each medium were inoculated with water agar inocula of each isolate, and colony radii (4 radii) measured at 24 hour intervals from the time of inoculation. The mean radial growth rate, in mm./10 hr. was calculated for the second to fifth days of growth. The results, given in Table 3, are not expressed as mean colony diameters because some isolates commenced growth earlier on one medium than on another.

TABLE 3.

Mean radial growth rates from 2nd-5th days on 3 media at 25°C.

(mm./10 hr.)

Isolate no.	Carrot agar	Oatmeal agar	Cornmeal agar
P18	3.8	3.0	1.5
P24	4.3	3.3	2.9
P28	3.9	3.3	3.3

The result confirmed the observation of Holliday and Mowat that radial growth rate varies with the medium. Each isolate grew fastest on carrot agar; since other workers (Spence,1961; Ramakrishnan and Seethalakshmi,1956) have shown carrot agar to be very favourable for good growth and sporulation of P. palmivora, and since this medium has the added advantage of being transparent, carrot agar was selected for use in further physiological studies.

A second experiment was conducted to determine the optimum temperatures for radial growth of isolates P4, P18, P24 and P28 on carrot agar. Two large carrot agar dishes of each isolate were incubated at 15.0, 21.0, 22.5, 25.0, 27.5, 30.0 and 32.5°C in the dark. Radial growth measurements (4 radii) were taken at 24 hour intervals from the time of inoculation, and the mean colony radius for each isolate on the sixth day plotted against temperature. The results are shown in Figs. 3, 4 and 5. The radial growth optima for P18 and P24 lay close to 30°C, for P4 27.5°C, and for P28 25°C. The rate of radial growth of P4 at 25°C during the 2nd - 5th days from the time of inoculation was 3.5 mm./10 hr., somewhat slower than that recorded for the other P. palmivora Piper nigrum isolate, P18 (see Table 3). The optimum temperature for radial growth of these two isolates also differed.

Chapter 4. The effects of temperature and medium upon oogonium formation.

Before the effect of temperature upon reproduction of the four isolates on carrot agar was examined in more detail, the following experiment, using three different media, was performed. Incubators set at 15.0, 17.5, 20.0, 22.5, 25.0, 27.5, 30.0 and 32.5°C were used, in conjunction with the

following isolate combinations : P18 x P4, P18 x P24, and P28; P4, P18, and P24 (controls). Small petri dishes, and carrot, oatmeal and Med.A + S agars were used. The above isolate combinations, with three replicates on each medium, were incubated at the stated range of temperatures in the dark for 21 days, and examined for oogonia and sporangia using the Quick Estimation Method. The results are given in Tables 4 and 5.

TABLE 4.

The effects of temperature and medium upon oogonium formation by P18 x 4 and P18 x 24.

Medium	P18 x 4			P18 x 24		
	CA	OMA	Med.A + S	CA	OMA	Med. A + S
Temperature °C						
15.0	+++/++++	++++	-	+++	+++	-
17.5	++++	++++	+++	++++	+++	++/+++
20.0	++++	++++	++/+++	++++	+++/++++	++
22.5	+++	++++	++	++++	+++	*/++
25.0	+++	+++	++	+++	+++	+
27.5	+++	+++	+	+++	+	+
30.0	++/+++	+	-	-	-	-
32.5	-	-	-	-	-	-

TABLE 5.

The effects of temperature and medium upon aerial mycelium, oogonium and sporangium formation by P28.

Medium	CA			OMA			Med.A+S		
Temperature °C	A. myc.	Sporang.	Oog.	A.myc.	Sporang.	Oog.	A.myc.	Sporang.	Oog.
15.0	-	-	++++	-	-	+++/++++	-	++	+++
17.5	-	-	++++	-	-	++++	+	++	++++
20.0	-	-	++++	-	-	++++	+	++	++++
22.5	-	-	++++	-	-	++++	+	++	++++
25.0	-	-	++++	-	-	++++	+	++	++++
27.5	+/++	++	++++	+	++	++++	+	++	++++
30.0	++	+++	+++	+	+++	++/+++	++	++/+++	+++
32.5	+++	++++	-	+	+++	-	++	++/+++	-

A.myc. = Aerial mycelium; Sporang. = Sporangia; Oog. = Oogonia.

Oogonium formation in the P. palmivora crosses reached a maximum frequency around 17.5 - 20°C on all three media, though on the simpler Med. A + S medium oogonium formation was poor, and the temperature range for sexual reproduction more limited. Oogonia were not formed above 30°C on any medium, and the results suggested an optimum temperature for sexual reproduction of P. palmivora close to 20°C. No oogonia were found in the single isolate control plates, and aerial mycelium and sporangium production was fairly uniform in all the series. In contrast oogonia in the P28 P. heveae plates were abundant on all three media between 17.5 and 27.5°C, with no indication of an optimum temperature for sexual reproduction. Further, sporangia, accompanied by aerial mycelium development, were formed only at the highest temperatures (27.5 - 32.5°C) on the two richer media, and were associated with a marked decline in oogonium formation.

These results suggested that temperature was an important factor in determining the frequency of oogonia or sporangia, and showed that these effects were modified to a lesser extent by the type of medium used. Carrot agar was found to be a good medium for sexual reproduction, and in the next experiment a more detailed investigation was made using this medium alone.

Chapter 5. The effects of temperature upon oogonium formation.

The above experiment was repeated using carrot agar only, and with spore frequencies assessed by means of the Numerical Estimation Method. Carrot agar was chosen in preference to oatmeal because it is more suited to the counting technique, being a clearer medium on which the isolates

develop less aerial mycelium, and because it is also a better medium for radial growth (see Ch.3).

Small (5 cm.) petri dishes were used for the P18 x 4 and P18 x 24 crosses, and for the single isolate controls; 13 cm. petri dishes were used for the P28 cultures. Three replicates were used for each series, and the mean high power spore frequency obtained from each (see Tables 1 and 2). The results are given in Table 6, and graphically in Figs. 3, 4 and 5. One replicate culture of P28 at 30°C developed 'fluffy' and 'non fluffy' (normal) sectors, in which separate spore frequency counts were made and are given in Table 6. Sporangial frequency counts were also made in all the P28 plates; these results will be given in the next chapter which deals with sporangium formation.

TABLE 6.

Mean high power oogonial frequencies of P18 x 4, P18 x 24, and P28, at a range of temperatures (see Figs. 3, 4 and 5).

Temperature °C :	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5
P18 x 4	24.2	39.3	31.4	16.5	14.5	5.0	1.9	-
P18 x 24	5.8	38.0	62.2	38.6	10.3	9.8	-	-
P28	40.4	N.D.	48.3	49.7	54.4	35.3	17.9	-

Sectored Plate 30°C, 'fluffy' : 13.5

" " " 'non fluffy' : 17.1

N.D. = no data

It is clear from the results that P. palmivora has a definite temperature optimum for oogonium formation close to 20°C, with a sharp decline in oogonium formation above and below this temperature. This confirms the results obtained in the previous experiment. The results of the two crosses varied slightly : the 18 x 4 cross produced oogonia over a wider temperature range (15 - 30°C) than the 18 x 24 cross (15 - 27.5°C), and in the latter cross, the frequency of oogonium formation dropped more sharply above 20°C. The optimum temperature for sexual reproduction in P. palmivora appears to be very distinct from that for radial growth.

In the P28 plates, the optimum temperature for oogonium formation, close to 25°C, coincided with that for radial growth. Only a gradual rise in oogonial frequency was observed between 15 and 25°C, which explains why an optimum temperature for sexual reproduction was not suspected in the previous experiment. Above 25°C, a sharp fall in oogonial frequency was observed (accompanied by an increase in sporangial frequency), and at 32.5°C no oogonia were formed (see Fig. 6). The fall in oogonial frequency also coincided with a fall in radial growth rate of the isolate (see Fig. 5), and an increase in aerial mycelium production (see Fig. 7). The plate of P28 at 30°C which sectored is shown in Fig. 7.2 ; the 'fluffy' sectors were abnormal in developing an unusually dense aerial mycelium, whilst the 'non-fluffy' sectors developed little. The 'fluffy' sectors had a lower oogonial frequency (and a higher sporangial frequency) than the 'non-fluffy' sectors or the other plates at 30°C. It is not known how the 'fluffy' sectors arose, but this problem will be examined further in Chapter 7.

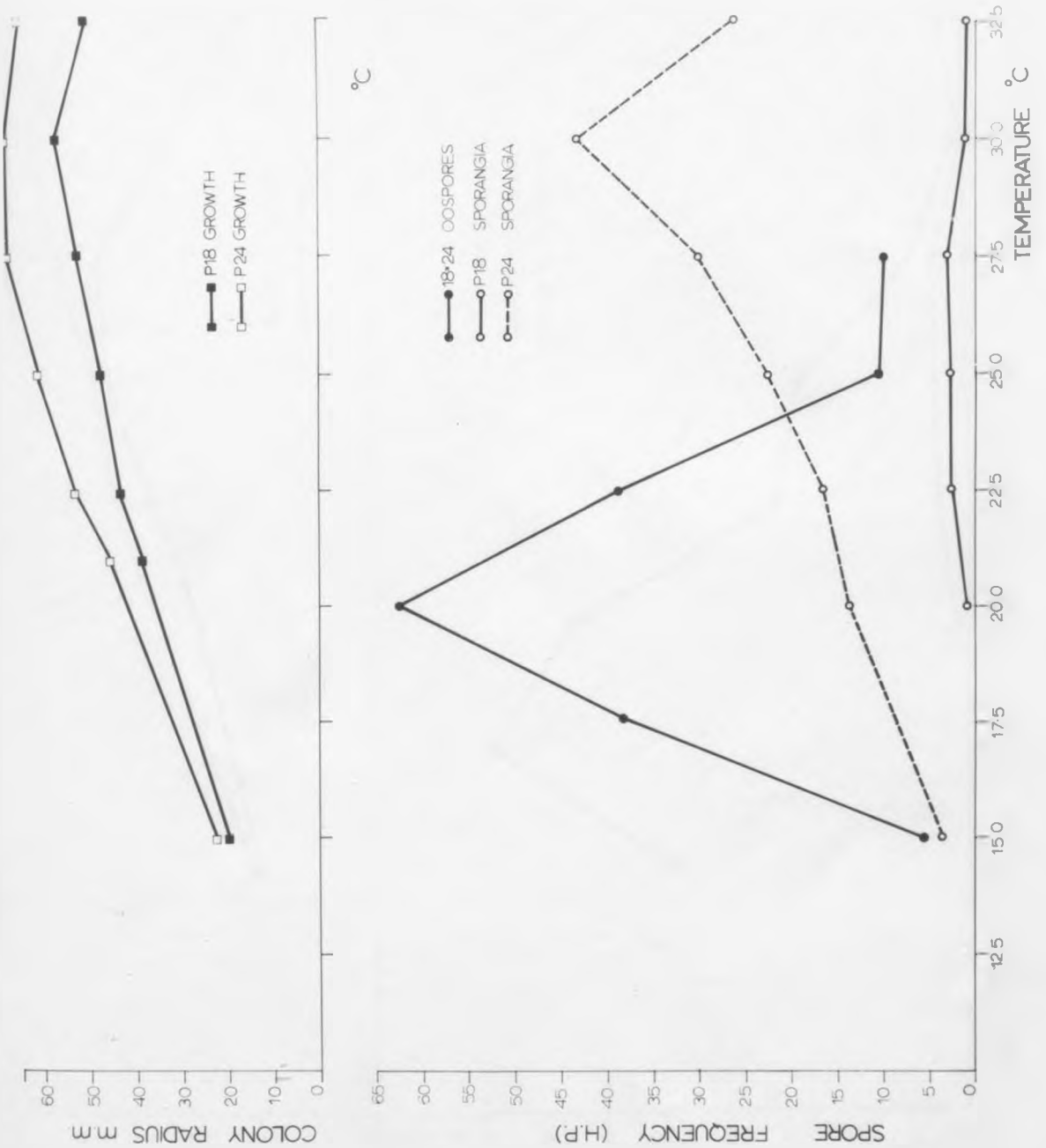


Fig.3 The effects of temperature upon radial growth and sporangium formation of isolates P24 and P18, and oogonium formation by P18 x24.

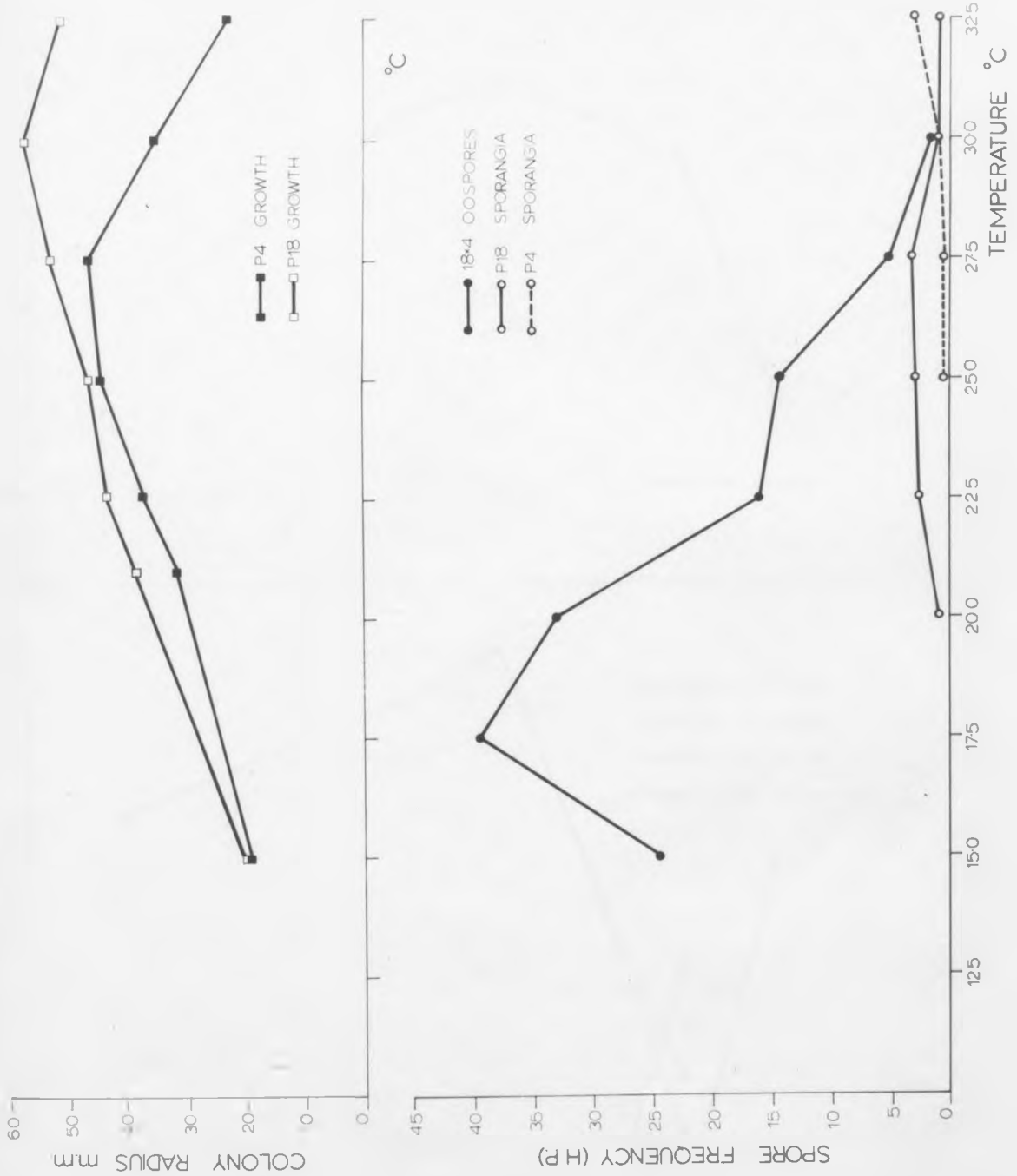
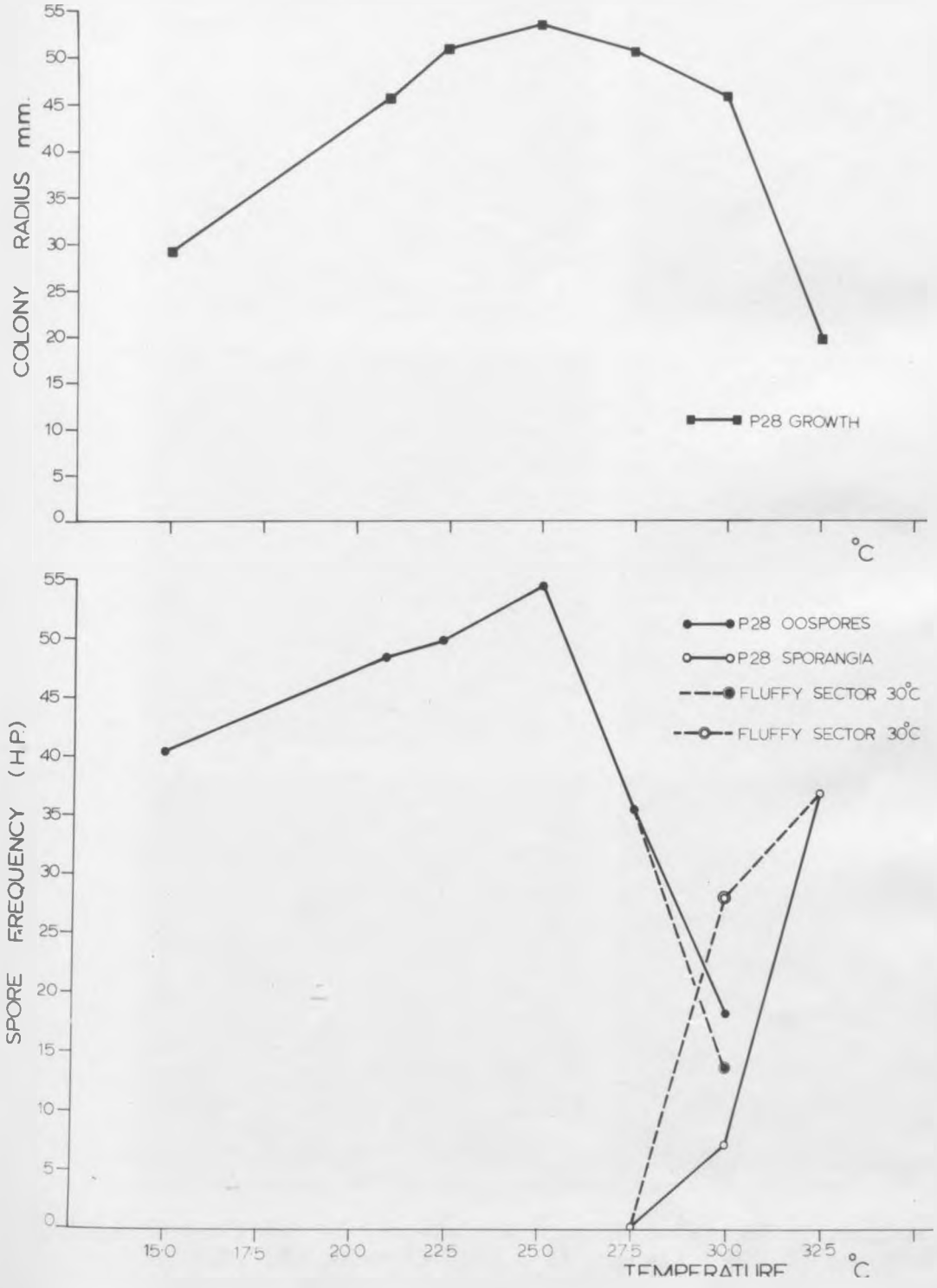


Fig.4. The effects of temperature upon radial growth and sporangium formation of isolates P18 and P24, and oogonium formation by P18 x 4.

Fig.5. The effects of temperature upon radial growth, sporangium formation, and oogonium formation of isolate P28 (P. heveae).



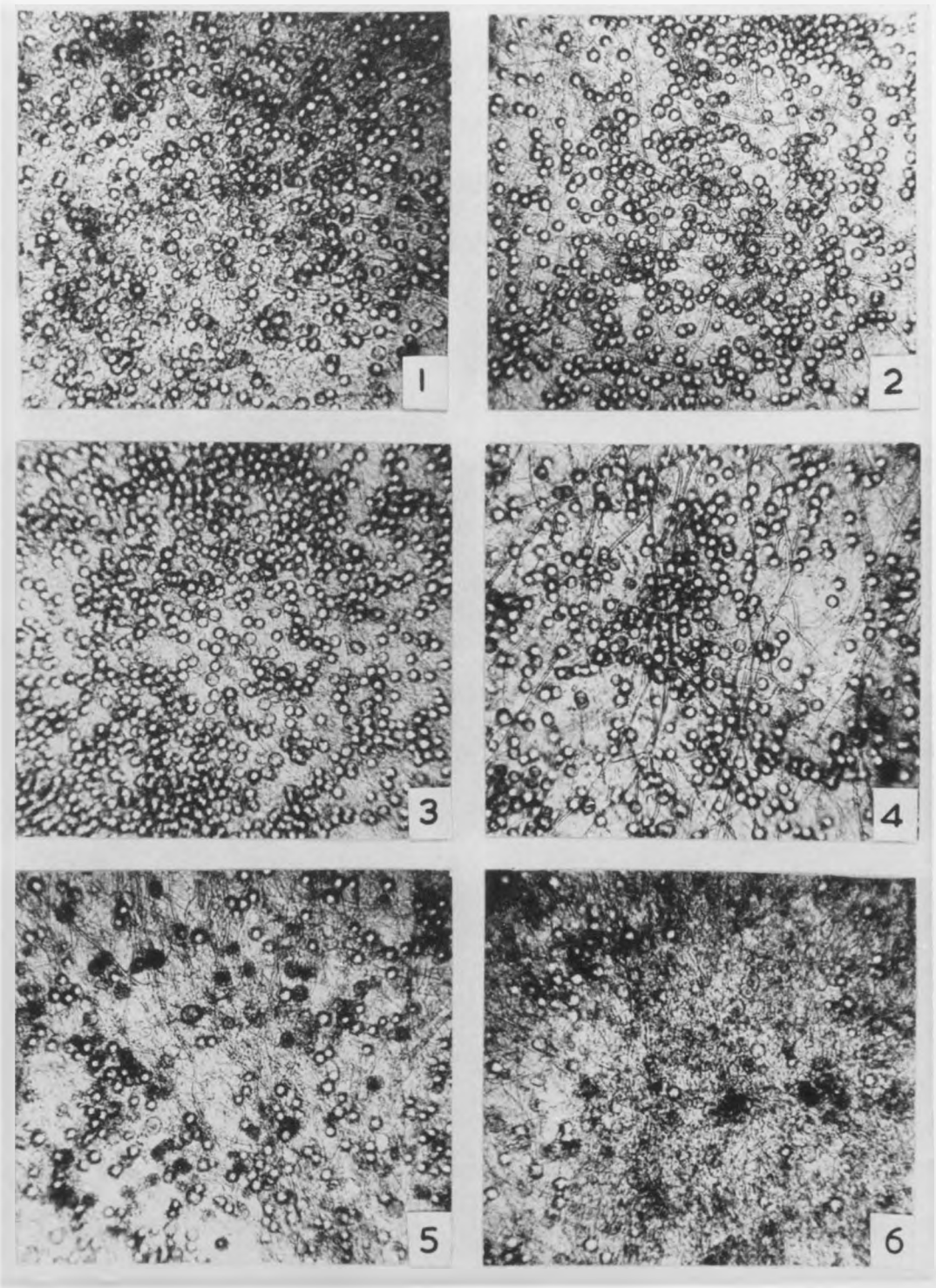


Fig.6. The effects of temperature upon oogonium and sporangium formation by P28, low power (x 100) : 1) oogonia 15°C ; 2) oogonia 20°C ; 3)oogonia 25°C ; 4) oogonia 27.5°C ; 5) oogonia and sporangia 30°C, 'non fluffy' sector; 6) oogonia and sporangia 30°C, 'fluffy' sector.

The experiments demonstrated conclusively that temperature optima for sexual reproduction could be obtained for both the heterothallic P. palmivora and the homothallic P. heveae; in the next experiment the effect of temperature upon asexual reproduction in these organisms is examined.

Chapter 6. The effects of temperature upon sporangium formation.

Some isolates of Phytophthora form sporangia readily on the agar plate, whereas others may require the presence of free water before they will do so. For this reason, two lines of approach were used in the investigation of sporangium formation by the four isolates, the first involving the use of a solid medium as in the previous experiment, and the second incorporating the use of free water.

a) The formation of sporangia on carrot agar plates.

Standard (9 cm.) carrot agar plates were inoculated with isolates P4, P18 and P24 in single isolate culture, and incubated (3 replicates) at 15.0, 20.0, 22.5, 25.0, 27.5, 30.0 and 32.5°C in the dark for 21 days. The plates were assessed for sporangial frequency by the Numerical Estimation Method, and the results, plotted against temperature, are shown in Figs. 3, 4 and 5, and in Table 7. The results shown for P28 were obtained in the previous experiment; sporangial frequencies in the 'non-fluffy' (normal) plates and 'fluffy' sectors at 30°C are given separately.

The two Piper nigrum isolates of P. palmivora, P4 and P18, differed significantly from isolate P24 (from Cacao) in their ability to produce sporangia on solid medium. Isolates P4 and P18 produced very few sporangia

TABLE 7.

Mean high power sporangial frequencies of isolates P4, P18, P24 and P28 at a range of temperatures (see Figs.3,4 & 5).

Temperature °C	15.0	20.0	22.5	25.0	27.5	30.0	32.5
P4	-	-	-	1	1	1.0	2.9
P18	-	0.7	2.5	2.7	3.0	1.0	0.9
P24	3.7	13.6	16.5	22.5	30.0	43.2	26.3
P28	-	-	-	-	0.3	N.Fl. 6.9 Fl. 27.9	36.6
A.myc.	-	-	-	-	+/++	N.Fl. ++ Fl. +++	+++

A.myc. = aerial mycelium; N.Fl. = 'non fluffy' plates;

Fl. = 'fluffy' sectors.

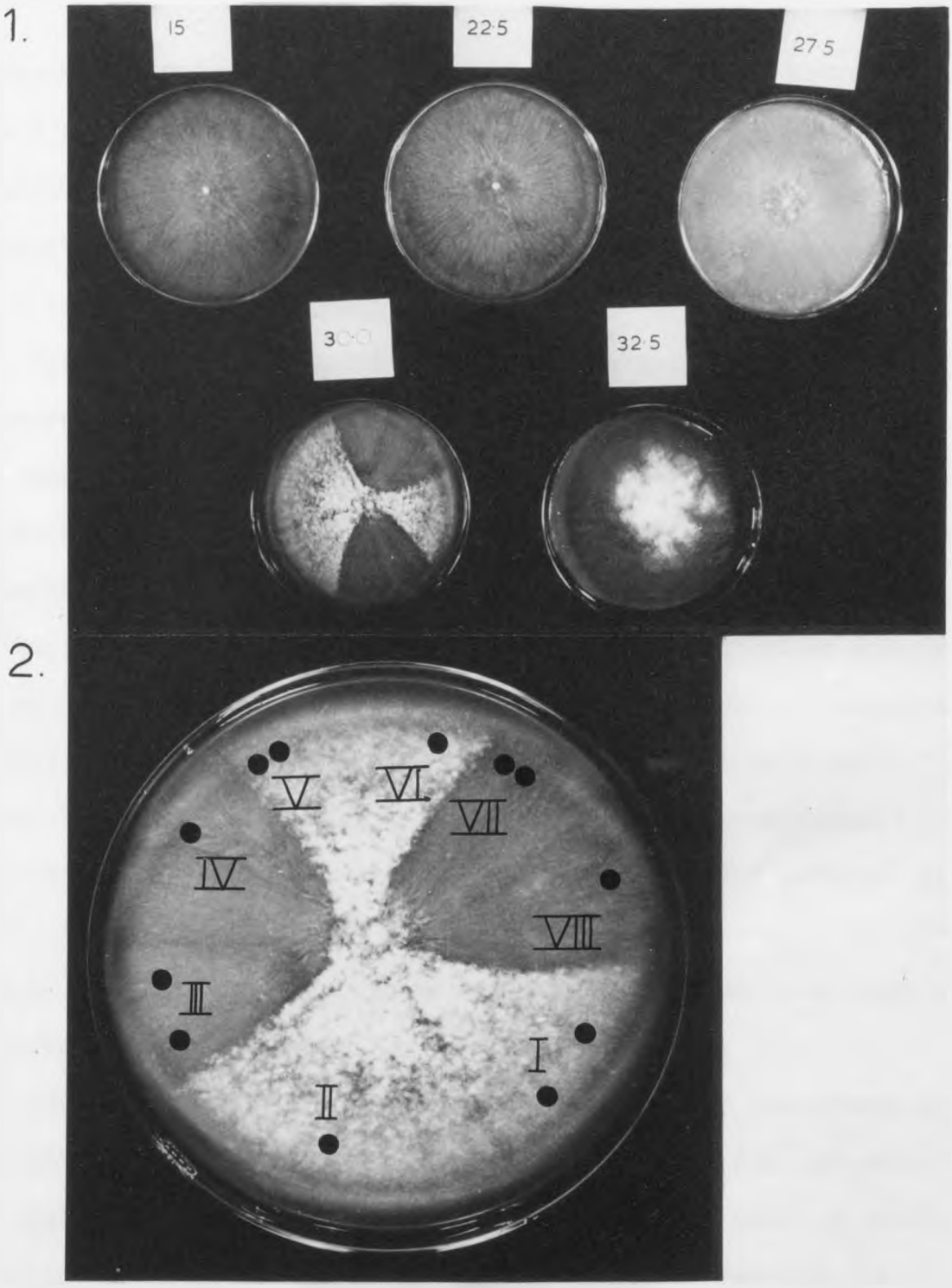


Fig.7. 1. Aerial mycelium in P28 plates at different temperatures (°C).

2. Sectored plate at 30°C showing 'fluffy' and 'non fluffy' sectors, and subsectors I - VIII (see Ch.7).

at any temperature, whereas P24 produced large numbers of sporangia between 20 and 32.5°C, with a marked optimum temperature close to 30°C coinciding with the optimum temperature for radial growth. There was no significant change with temperature of aerial mycelium formation by any of these three P. palmivora isolates.

The P. heveae isolate P28 only produced sporangia at 27.5°C - 32.5°C, in company with a simultaneous increase in aerial mycelium production (see Figs. 5, 6 & 7). This increase in aerial mycelium and sporangia was concurrent with a decrease in oogonial frequency and radial growth rate, suggesting that a fine temperature sensitive balance exists between vegetative reproduction, vegetative growth, sexual reproduction and growth rate in this homothallic organism. The difference between sporangial and oogonial frequencies in the 'fluffy' and 'non fluffy' forms at 30°C accentuates this relationship, with a considerably higher sporangial frequency, and a lower oogonial frequency, in the 'fluffy' sectors. At 32.5°C, many sporangia, but no oogonia were formed, in contrast to 25°C, where no sporangia but many oogonia were formed. Between these temperatures, intermediate stages occurred.

This experiment demonstrates a temperature optimum for sporangium production in a single isolate culture of P. palmivora. P24, and also distinguishes it physiologically from P4 and P18 on the basis of relative sporangium production in agar culture. The optimum temperature for sporangium formation in P. palmivora P24 is close to the growth rate optimum, and distinct from the optimum for sexual reproduction. In P. heveae a

relationship between sexual and asexual reproduction was observed.

b) The formation of sporangia in solid/liquid cultures.

Isolates P4, P18 and P28 formed few sporangia on carrot agar, suggesting that these isolates might have a free water requirement for sporangium formation on solid media in the dark. This possibility was investigated in the following experiment. Agar plugs, each 1 cm. in diameter, were taken from the edges of growing colonies of isolates P4, P18, P24 and P28 on carrot agar at 25°C; the plugs were transferred to 5 cm. petri dishes and a little unsterile Petri solution added to each dish until liquid was level with the top of the agar plug. Cultures of this type (3 replicates) for each of the four isolates were incubated at the range of temperatures shown in Table 8, and examined for sporangia after 5 days. A modification of the Quick Estimation Method was used in which counts were made of sporangia in the mycelium around the perimeters of the agar plugs.

Isolate P24 formed abundant sporangia, as in the solid cultures. The presence of free water also induced isolate P18, but not P4, to produce abundant sporangia between 22.5 and 30°C,. All three P. palmivora isolates produced considerable vegetative mycelium in the liquid, with the most between 22.5 and 27.5°C. Isolate P28, however, produced neither mycelium nor sporangia in these liquid cultures.

TABLE 8.

Sporangium formation in solid/liquid cultures at a range of temperatures.

Temperature °C	15.0	20.0	22.5	25.0	27.5	30.0
P4	-	-	-	++	++	-
P18	++	++	+++/++++	+++/++++	+++/++++	+++/++++
P24	+++	+++	+++/++++	+++/++++	+++/++++	+++/++++
P28	-	-	-	-	-	-

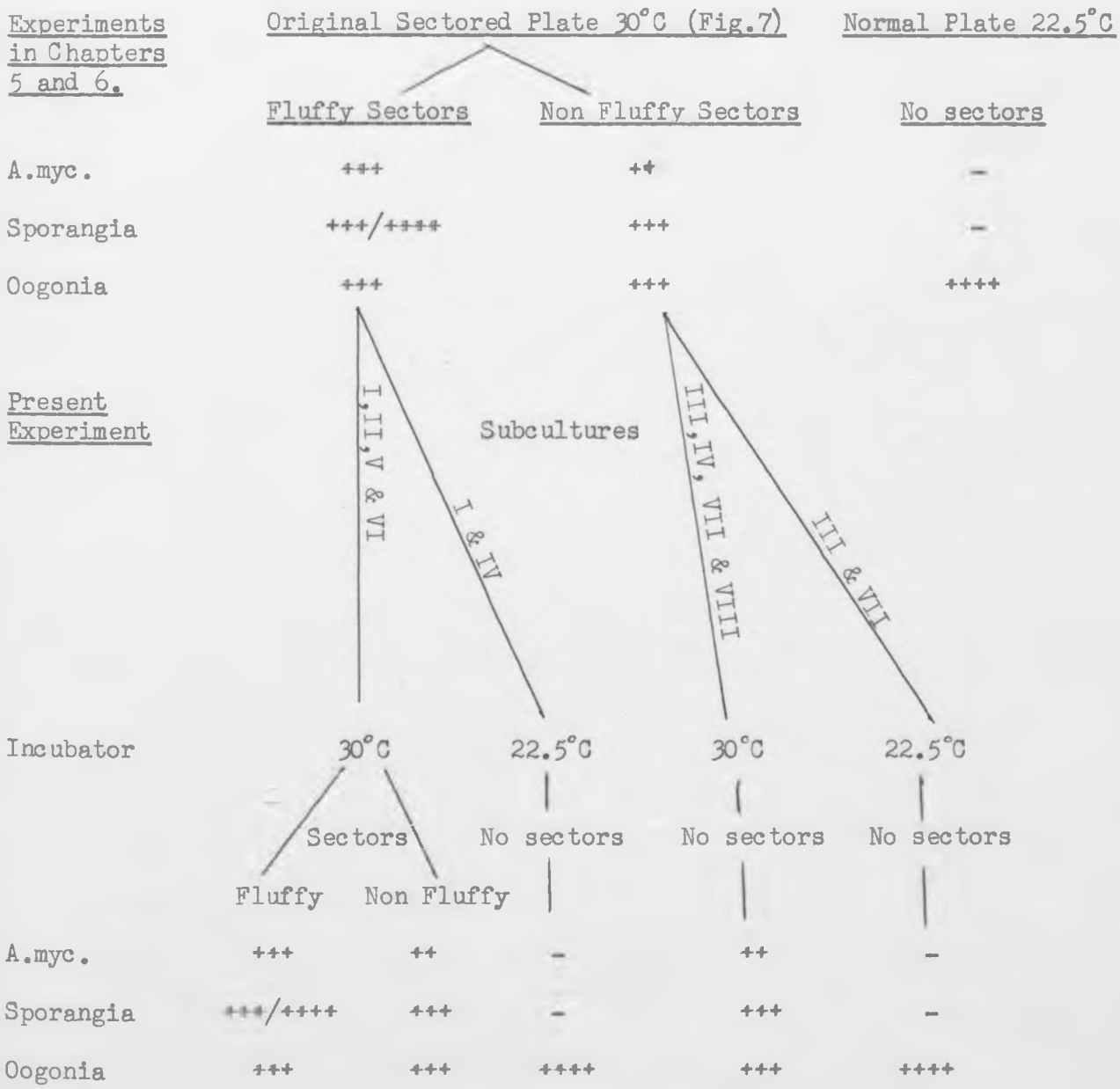
Chapter 7. The effects of temperature upon the stability of subcultures from 'fluffy' and 'non fluffy' sectors of P. heveae.

In a replicate plate of P. heveae P28 on carrot agar at 30°C (described in Chapter 5), two types of sector, a new 'fluffy' form and the normal 'non fluffy' form, were observed (see Fig. 7.2.). An unusually high sporangial frequency, abundant aerial mycelium, and a lower oogonial frequency were recorded from the 'fluffy' sectors in comparison with the 'non fluffy' sectors and the other two normal ('non fluffy') replicate plates at 30°C. Experiments described in Chapters 5 and 6 demonstrated an effect of temperature upon sporulation and vegetative growth in P. heveae, including induction of aerial mycelium at high temperatures (27.5 - 32.5°C). In the following experiment, the temperature stability of the new 'fluffy' form of the fungus was examined in comparison with the normal 'non fluffy' form.

The sectored culture of P28 was divided for convenience into eight subsectors (Fig. 7.2.), subsectors I, II, V and VI being 'fluffy', and III, IV, VII and VIII 'non fluffy' or normal. A No.1 cork borer inoculum plug was removed from each of the eight subsectors (the positions are shown in Fig. 7.2.), and each plug transferred to a standard carrot agar plate and incubated at 30°C. A further inoculum plug was taken from each of subsectors I, III, V and VI, transferred to a carrot agar plate, and incubated at 22.5°C. The plates were examined after 14 days of incubation; spore frequencies, assessed by the Quick Estimation Method, are shown in Table 9, and the appearance of the plates is shown in Fig. 8.

TABLE 9.

The temperature stability of 'fluffy' and 'non fluffy' subsectors and their sporangial and oogonial frequencies (Q.E.M.).



A.myc. = Aerial mycelium.

Fig.8. The effects of temperature upon subcultures from 'fluffy' and 'non fluffy' sub sectors of P. heveae P.28.

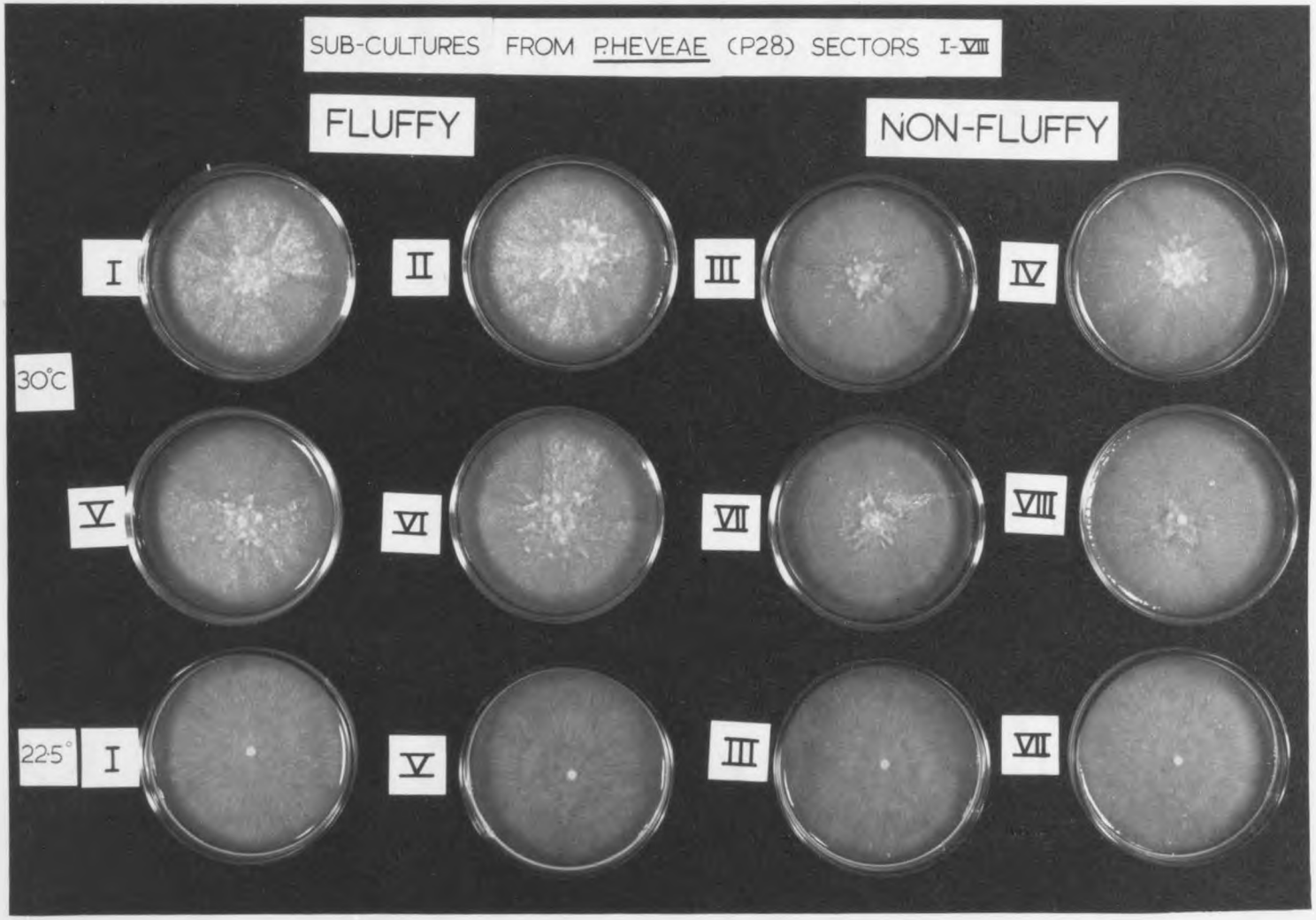


Fig. 8.

The 'fluffy' form of P28 was transferrable by subculture at 30°C, but was unstable, all four 'fluffy' subcultures developing one or more sectors of the normal 'non fluffy' (30°C) type. The 'fluffy' forms subcultured at 30°C were similar to those in the parent culture with regard to oogonial and sporangial frequencies and aerial mycelium development. At 22.5°C however, subcultures from 'fluffy' subsectors in the parent plate gave no indication of fluffiness or of sectoring, and were normal in sporulation and appearance.

Subcultures from the 'non fluffy' subsectors to 30°C were stable, and normal in sporulation and vegetative growth for P28 at this temperature; similarly, subcultures to 22.5°C from the 'non fluffy' subsectors behaved normally.

The experiment demonstrated that the 'fluffy' form of P. heveae, which arose as a sector, was a temperature sensitive form, expressed at high temperatures (30°C), but not at low temperatures (22.5°C). It was also unstable and frequently reverted back to the normal type (by sectoring) when subcultured to 30°C. This result is further evidence for the fine balance between vegetative growth, sexual reproduction, asexual reproduction, and growth rate observed in earlier experiments with P. heveae. (Chapters 5 and 6).

It was later observed that exposure of the 30°C 'fluffy' subsector subcultures to daylight at room temperature (20°C) led to an even greater development of fluffy aerial mycelium in the 'fluffy' sectors of these plates, whilst the remaining cultures, which were also exposed to the

light, showed little change. This indicated that light, in addition to temperature, could induce aerial mycelium changes in P. heveae. The problem of light and Phytophthora sporulation is examined in the next Chapter.

Chapter 8. The effects of light upon sporulation.

Effects of light upon sexual and asexual reproduction of Phytophthora species and isolates have been demonstrated by several authors (Aragaki and Hine, 1963; Merz, 1964; Leal, 1965; Harnish, 1965 etc.), but no detailed attention has been paid to Phytophthora palmivora. In the following experiment, the effects of daylight upon the growth and reproduction of isolates P4, P18, P24 and P28 were investigated, using single isolate cultures on standard (9 cm.) carrot agar plates, and cross cultures on small (5 cm.) carrot agar plates.

Two identical sets of plates (three replicates) were used. One set was placed in the light box, and exposed to normal daylight and darkness; the second set was placed in an incubator in permanent darkness. Each set was incubated at 22.5°C, a temperature close to the optima for oogonium formation in both P. palmivora and P. heveae (see Ch.5). The plates were examined by the Quick Estimation Method after 21 days incubation. The results are given in Tables 10 and 11, and in Fig. 9.

Exposure to light reduced the amount of aerial mycelium produced by the P. palmivora isolates in single culture; light exposed plates had a frosted appearance, and dark plates a fluffy appearance. Sporangium formation was greatly stimulated by light : isolate P4 produced many

TABLE 10.

The effects of light upon sporulation of single isolate cultures at 22.5°C: rough H.P. spore frequencies in parentheses.

Isolate		Light		Dark	
P4	A.myc.	++		++/+++	
	Sporangia	+++/++++	(24)	-	
P18	A.myc.	++		+++	
	Sporangia	++++	(38)	+++	(3.3)
P24	A.myc.	++		++/+++	
	Sporangia	++++	(100 +)	+++/++++	(21)
P28	A.myc.	+/++		-	
	Sporangia	+++/++++		-	
	Oogonia	++/+++		++++	

A.myc. = Aerial mycelium

TABLE 11.

The effect of light upon sporulation of cross cultures at 22.5°C:

rough H.P. spore frequencies in parentheses.

Cross		Light		Dark	
P18 x 4	A.myc.	++/+++		++/+++	
	Sporang. P4 side	++++	(72)	++	
	P18 side	++++	(87)	++	
	Oogonia	++/+++	(3)	+++	(17.5)
P18 x 24	A.myc.	++/+++		++/+++	
	Sporang. P18 side	++++	(44)	+++	(3)
	P24 side	++++	(100 +)	+++	(14)
	Oogonia	-		++++	

Sporang. = Sporangia; A.myc. = Aerial mycelium.

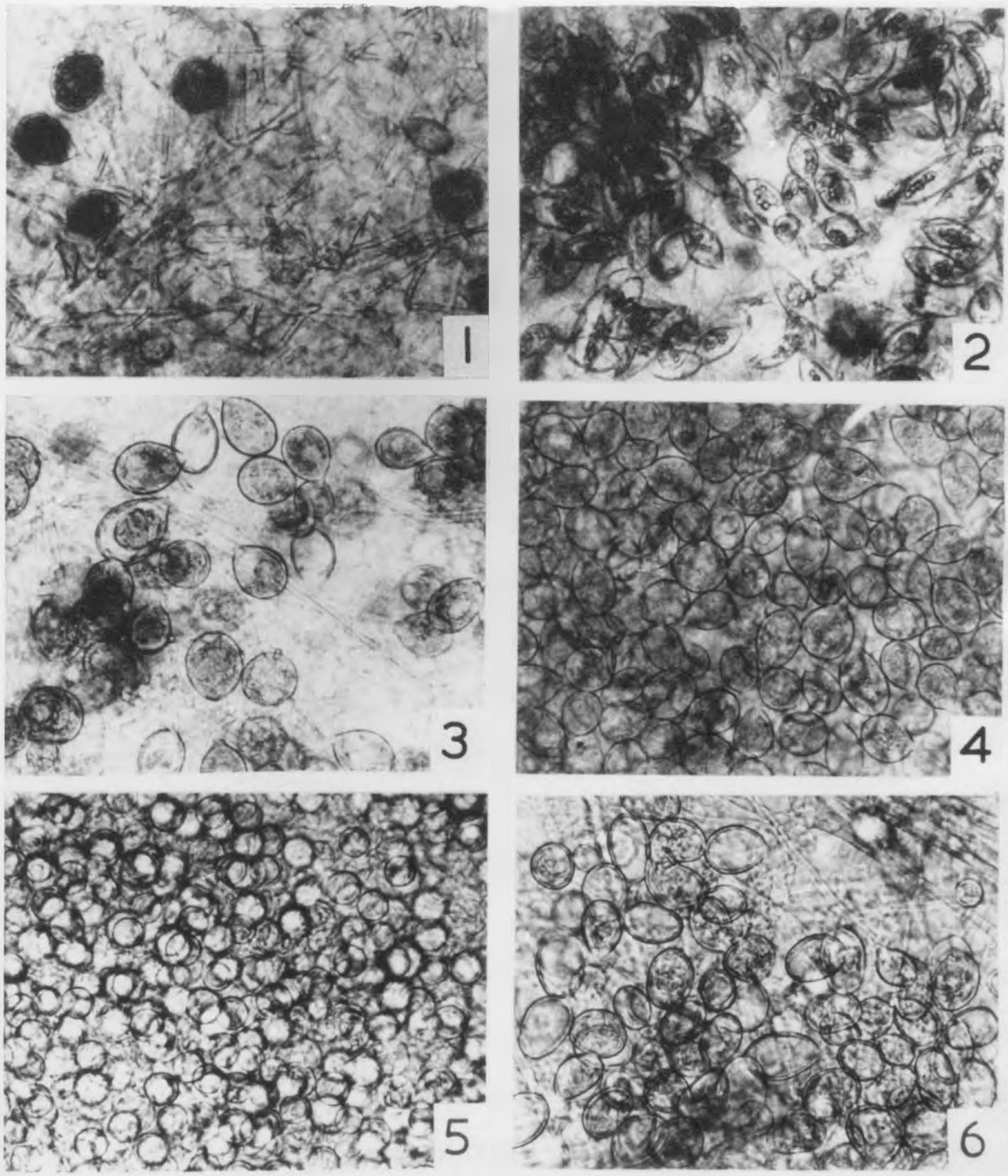


Fig.9. The effects of light upon sporulation of single isolate cultures of Phytophthora (L.P. = x 100 x 2.25); 1) P18, rounded sporangia in dark; 2) P18, elongated sporangia in light; 3) P24, sporangia in dark; 4) P24, abundant sporangia in light; 5) P28, oogonia in dark; 6) P28, clusters of sporangia, and occasional oogonia in light.

elongated sporangia with prominant papillae in the light, and none in the dark; isolate P18 produced sporangia similar to those of P4 on the agar surface in the light, but in the dark produced only a few submerged, rounded, weakly papillate sporangia; the frequency of P24 sporangia also increased significantly in the light. (see Fig. 9).

In the P. palmivora crosses, exposure to light both inhibited oogonium formation and stimulated sporangium formation. An interesting effect was observed in the P18 x 4 cross plates, where sporangia in the light plates were more than twice as frequent as in the single isolate cultures (see Tables 10 and 11). This apparent stimulation of sporangium formation in dual culture which did not occur in the P18 x 24 crosses or in the dark plates, may have been due to the use of small petri dishes.

Light induced aerial mycelium formation and sporangium formation in P. heveae (P28), the light plates having a frosted appearance with sporangia formed in scattered clusters on the surface of the medium (see Fig. 9). Light also inhibited oogonium formation by P. heveae.

Light is clearly active in stimulating sporangium formation and inhibiting oogonium formation by the isolates used in this experiment. In one isolate (P18), light also induced morphological changes in sporangium structure. The inhibition of oogonium formation by light was very marked, and the few oogonia formed in light plates were found at the bottom of the medium, where the inhibitory effects of light may have been reduced by absorbtion. The two species were distinguishable from one another on the basis of aerial mycelium formation, retarded in light with P. palmivora,

and induced in light with P. heveae. Isolate P24 was distinguishable from isolates P4 and P18 by its prolific sporangium formation in both light and dark.

The effects of light on these four isolates at 22.5°C were rather similar to the effects induced by a high temperature (30°C) in the dark, (see Chs. 5 and 6), though more marked. In P. heveae, a very similar effect was produced, with a simultaneous increase in aerial mycelium and sporangium formation coupled with a decrease in oogonium formation. No significant difference was observed, however, between growth rates of these four isolates in dark and light at 22.5°C, though such differences were observed with temperature in Chapter 3.

Despite the fact that a ringed appearance, due to alternating daylight and darkness, was obtained in the light cultures, the possibility that the effects of light were upon the medium rather than the fungus could not be ruled out. This suggested the following experiment. Five standard carrot agar plates were exposed to light for 21 days in the light box, and a further five plates simultaneously incubated in darkness. All ten plates were then inoculated with P18, and incubated in the dark at 22.5°C for 21 days. On examination, the plates were all fluffy in appearance and sporangia were few, rounded, and embedded in the medium. These characters were typical of P18 incubated in the dark at 22.5°C (see Table 10), confirming that the effect of light was primarily upon the fungus.

Chapter 9. The pathogenicity of isolates P4 and P 18 to Piper nigrum.

The value of detailed *in vitro* experiments upon sexual reproduction in *Phytophthora palmivora* would be considerably lessened if oospore formation did not take place in the field. The following chapters amount to a brief investigation of this problem, but before such an investigation could be undertaken, sustained pathogenicity of the isolates to be used had to be confirmed, because *Phytophthora* isolates often lose their pathogenicity in prolonged artificial culture.

In this and the following experiments, *P. palmivora* isolates P4 and P18 were used in conjunction with young cuttings of their original host plant, *Piper nigrum*. One of the cuttings is shown in Fig. 10.1. For the initial pathogenicity test, two mature young leaves of *Piper nigrum* were inoculated with isolate P4, and two with P18; the four leaves were incubated at 27.5°C (close to the optimum temperature for radial growth of the isolates) in the dark. In the P18 inoculated leaves, signs of lesion development were observed after 24 hours; all four leaves had developed lesions characteristic of the *Piper nigrum* foot rot disease (described by Holliday and Mowat, 1963) after 90 hours incubation (see Fig.10.2.). The lesions were circular and dark brown, with fimbriate edges, and developed rapidly along the midrib, leaf veins, and petiole, so that 'green islands' were eventually formed (see Fig.10.3.). Lesion development with P18 was considerably faster than with P4, and compared with the faster growth rate of P18 on artificial media (see Ch.3.)

After five days of lesion development, the two fungi were re-isolated

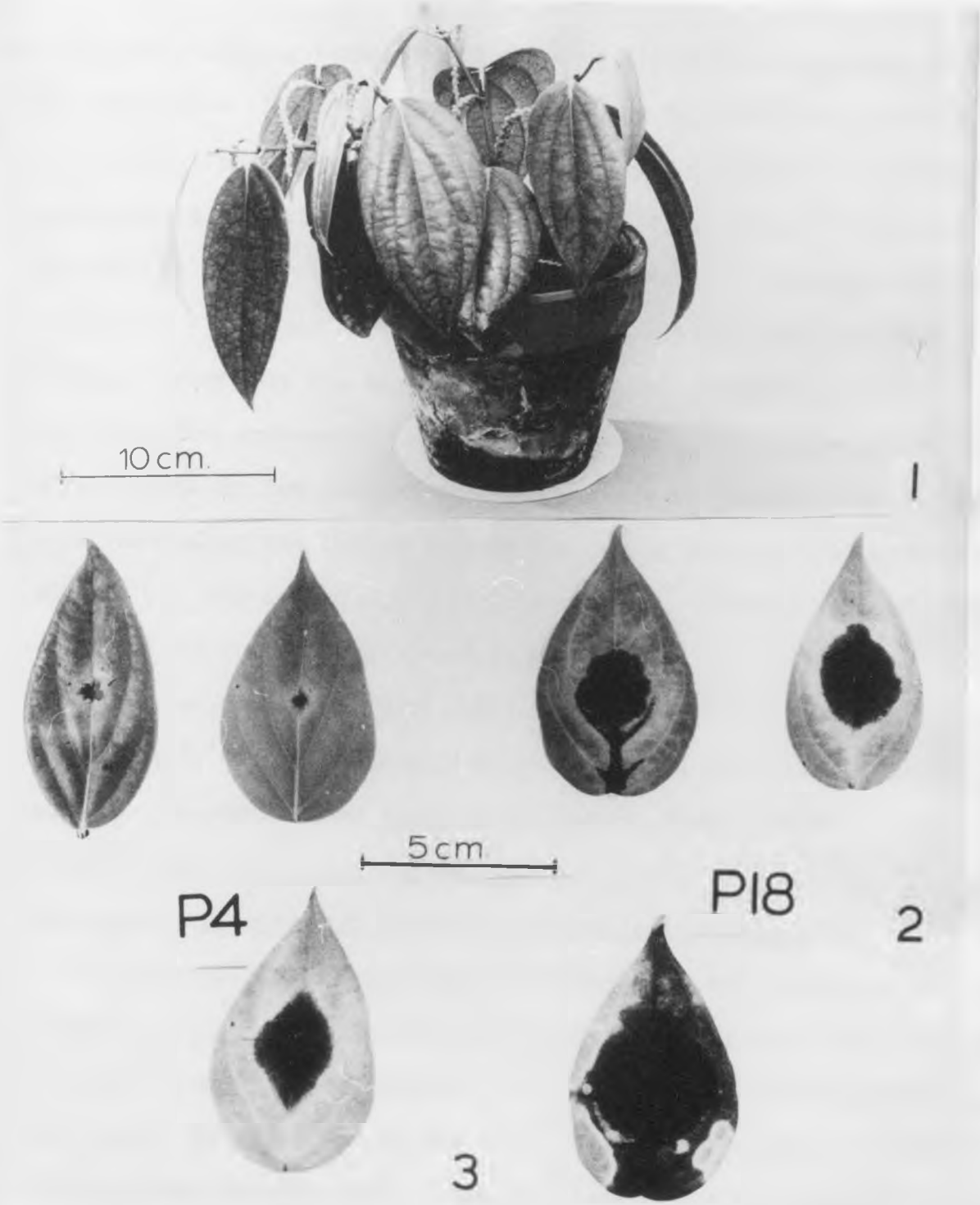


Fig.10. The pathogenicity of Phytophthora palmivora isolates P4 and P18 to Piper nigrum; 1) twelve month old cutting of Piper nigrum ; 2) leaf lesions after 90 hours incubation at 27.5°C; 3) leaf lesions after 145 hours incubation at 27.5°C.

using the following technique. A piece of leaf 1 cm. square was cut from the outer edge of the lesion in both the P4 inoculated leaves, and each piece transferred to a standard petri dish of Hollomon's selective Phytophthora isolation medium (Hollomon,1965); the same procedure was adopted for the two P18 inoculated leaves. Two further plates were dual inoculated with a piece from a P4 leaf and a P18 leaf, so that two potential mating crosses were set up. All six plates were incubated at 20°C. Fluffy white mycelial colonies developed around all the leaf pieces, and in the cross plates the two colonies met on the sixth day. After 10 days, oogonia were found along the line of conjunction of the two isolates in the cross plates, but none in the single isolate plates, confirming the re-isolation of P4 and P18 from the inoculated leaves.

This experiment confirmed that P4 and P18 had retained their pathogenicity to Piper nigrum, despite at least 4 years in artificial culture. No oogonia were found in the leaves, though the high temperature (27.5°C) might have been limiting.

Chapter 10. Oogonium and oospore formation in detached leaves.

The possibility that oogonia are formed when multiple leaf infection occurs in the field was investigated in the following experiment, in which a range of temperatures were used. Ten detached leaves were inoculated with P4,and incubated for one day at 27.5°C to allow earlier development of this slower growing fungus. The same leaves were then inoculated with P18 (at the opposite end of the leaf to P4), incubated a further day at 27.5°C, and four of the five dishes, each containing two leaves,

transferred to 15.0, 20.0, 25.0 and 30.0°C. Another two pairs of leaves were inoculated singly with P4 and P18 for control purposes and incubated at 20°C; shortage of plant material made it impossible to use further controls.

At 30°C, the P4 and P18 lesions had merged by the fourth day from the time of inoculation of P18, and the leaves completely blackened by the fifth. Lesion development was slower at lower temperatures; at 27.5°C and 25°C the leaves were fully blackened by the 10th day, and at 20°C by the 12th day. At 15°C, the lesions did not merge until the 18th day, and had only blackened completely after 24 days incubation. The rates of lesion development at different temperatures were comparable with growth rates in the agar plate, with a high optimum temperature in both instances.

The leaves were examined for oogonia, using cotton blue stained preparations, after 10 days (27.5°C and 30°C), 12 days (25 and 20°C), and 24 days (15°C) incubation. Mycelium was abundant in all the leaf sections examined. Oogonia were found in the dual inoculated leaves at 15.0, 20.0, 25.0 and 27.5°C, but none at 30°C, nor in the singly inoculated leaves at 20°C. The oogonia were patchily distributed, and of variable frequency (mostly 'occasional' or 'frequent'); around 50% contained oospores. They were usually present in the leaf segment in which the two isolate lesions had merged, and were typically situated amongst the vascular tissue of the midrib (often between the xylem elements), along the epidermal cells adjacent to the midrib, and in the intercellular spaces of the mesophyll adjacent to the midrib (see Fig. 11.).

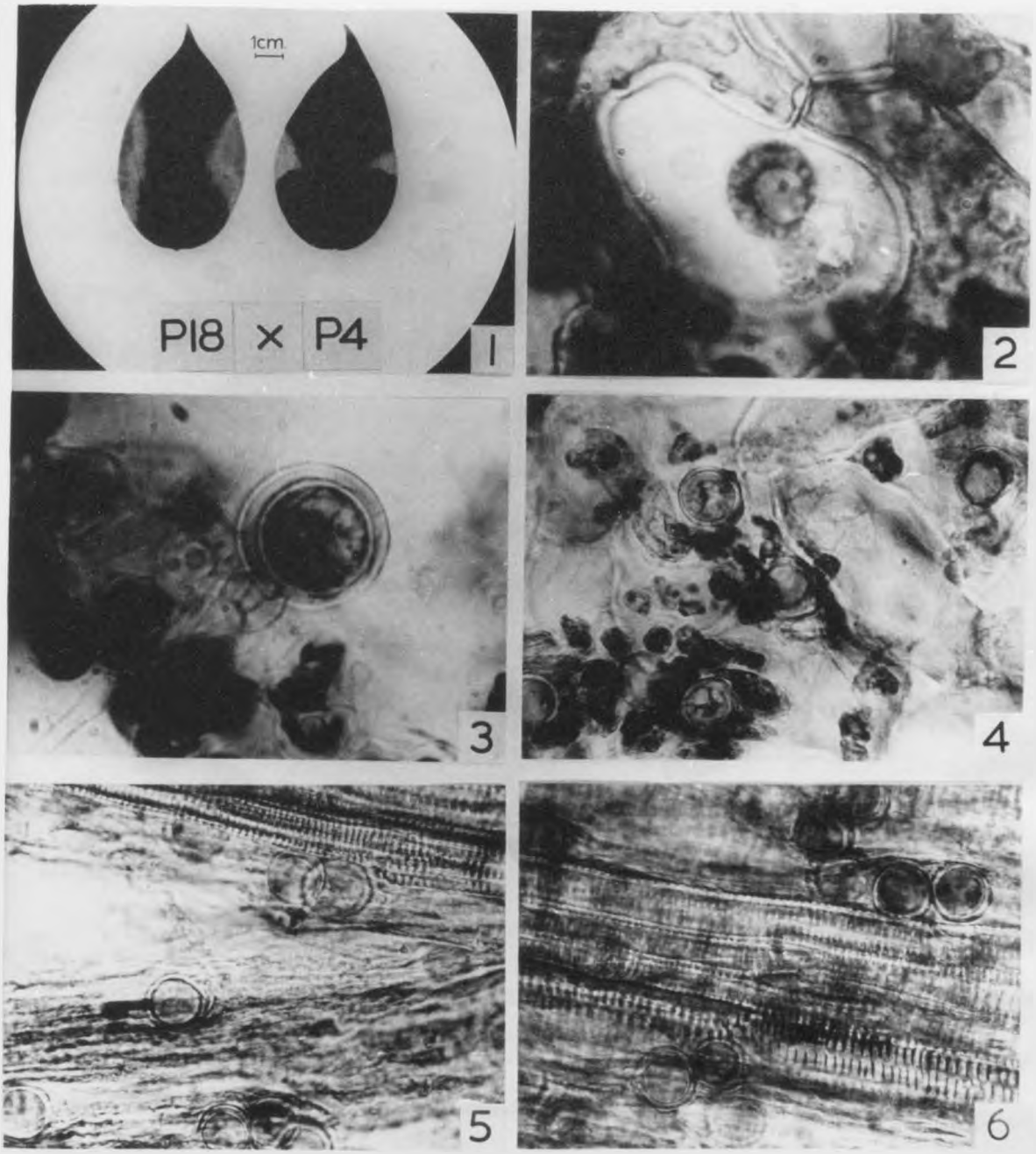


Fig.11. Oospore formation in the host plant by isolates of P. palmivora: 1) dual inoculated leaves after 18 days incubation at 15°C; 2) developing oogonium in mesophyll (x 400); 3) oogonium and oospore in mesophyll (x 400); 4) oogonia and oospores in mesophyll (x 100); 5) and 6) oogonia and oospores amongst vascular tissue (x 100).

This experiment demonstrated that oogonium and oospore formation could take place in host tissue under laboratory conditions in the presence of two complementary P. palmivora isolates. As in the agar plate, a high temperature (30°C) inhibited oogonium formation; single isolate infections were self-sterile at the optimum temperature for oogonium formation on carrot agar (20°C).

Chapter 11. An investigation of oogonium formation in non-detached leaves.

The following experiment was set up in an attempt to obtain oogonium formation in the intact host plant rather than in detached leaves. Four leaves on each of two potted cuttings were dual inoculated with P4 x P18, and the plants covered with a polythene bag; four leaves on each of two further plants were inoculated separately with P4 and P18 as controls. These four plants, together with a fifth non-inoculated control plant, were left in a glasshouse under normal daylight conditions at 21°C (max. 23°C, min. 15°C).

In the dual inoculated plants, the petioles of the 8 leaves, close to one or the other of the two inocula, blackened rapidly as the nearest lesion developed; by the sixth day, all eight dual inoculated leaves had abscissed, although the two lesions on each leaf had not yet merged. Singly inoculated leaves on the two control plants, in which the lesions were developing from the middle of the leaf, abscissed after eight days, although in this case the petioles were not always diseased. All the abscissed leaves fell to the soil surface of the pot, where lesion

development continued normally. No leaf abscission took place in the non-inoculated control plant.

The leaves were examined for oogonia 22 days after inoculation, by which time the dual inoculated leaves had completely blackened. In the 24 leaf segments taken from the 8 dual inoculated leaves, oogonia were only found in four segments, each from a separate leaf. The oogonia, which were widely scattered and rare or occasional in frequency, lacked oospores and contained yellow, oily contents (after leaf maceration). This result contrasted with that obtained in the previous experiment when oogonia and oospores were plentiful (Chapter 10).

In the singly inoculated control leaves, no oogonia were found, but mycelium was abundant. In one such leaf (P18), the nearest lesion edge was still 35 mm. from the point of abscission of the healthy petiole, and in a subsequent search no mycelium was found in the petiole. This suggests that leaf abscission can be induced before spread of the lesion causes petiolar necrosis. Such premature abscission of diseased Piper nigrum leaves was also observed in the field by Holliday and Mowat (1963). No lesions developed in the non-inoculated control plant, and no abscission occurred.

No further signs of disease development were observed in the four inoculated plants after a 5 week observation period, indicating that abscission of infected leaves may be a factor in disease escape. However, despite apparently normal lesion development, good oogonium formation was not obtained in the dual inoculated leaves, suggesting that some factor

other than temperature was limiting sexual reproduction. With the abscission of the leaves concerned, the experimental conditions, including temperature and humidity, were very similar to those used with the artificially detached leaves in the previous experiment, the most exceptional factor being light.

Light was found inhibitory to oogonium formation by P18 x 4 in agar cultures; in the next experiment, the effect of light upon oogonium formation in detached leaves was examined.

Chapter 12. The effects of light upon oogonium formation in detached leaves.

In this investigation, four mature young leaves were dual inoculated with P18 x P4; one petri dish containing two leaves was put in the light box at 25°C, and the other incubated in permanent darkness at the same temperature. All four leaves, which were almost completely blackened after 8 days incubation, were examined for oogonia and oospores after 12 days. In those leaves exposed to the light, no oogonia were found in any of the six segments examined; in the dark incubated leaves, oogonia with oospores were 'occasional' to 'frequent' in two out of three segments examined for each leaf.

This experiment shows that light inhibits oogonium formation by P. palmivora in dual inoculated leaves at a temperature normally favourable to oogonium formation in the dark; this result probably explains the absence of substantial oogonium formation in the previous experiment (Ch.11.), and compares with the light effects obtained in agar cultures.

The rate of lesion development with temperature, and the temperature maximum for oogonium formation in detached leaves (27.5°C) also compare with effects observed in the agar plate, suggesting that *in vitro* environmental effects may in some circumstances be validly related to natural conditions.

Chapter 13.

DISCUSSION.

There has been little standardisation of technique in Phytophthora work; this applies particularly to P. palmivora, where authors who have previously produced papers involving experiments upon the physiology of reproduction in P. palmivora and related species have worked with a wide variety of isolates, media and incubation temperatures; some examples are given in Table 12.

It is clear from the results obtained with the four isolates used in this section that the incubation temperature and type of medium used are of great significance when a particular spore form is required. An initial comparison of radial growth rates and sporulation of the four isolates on several media resulted in the selection of carrot agar for further physiological studies (see Tables 3, 4 and 5, and Table 13). This medium has also been used with favourable results by a number of other authors (Johann, 1928; Ventakaryan, 1932; Ramakrishnan and Seethalakshmi, 1956; Spence, 1961; Royle, 1963 etc.). Although as a vegetable medium, carrot agar is in itself not standard since "it is well nigh impossible to standardise such media owing to the variable composition of suitable vegetable material" (Gadd, 1927); it was a considerably better medium for sexual reproduction of P. palmivora than the only synthetic medium, Med. A+S, available at the time, and also better than oatmeal agar. It is interesting to note that sexual reproduction and growth rate of P. heveae

TABLE 12.

Media and temperatures used by selected authors in Phytophthora investigations.

Author	Date	Media	Temperature(s)
Rosenbaum	1917	CMA, OMA, cooked carrots, potato cylinders;	18-20°C
Gadd	1924	FBA, MMA;	22-26°C
Lester-Smith	1927	BA, MMA, OMA; ice chest &	26°C
Ashby	1928,9	CMA, FBA, MMA, OMA;	20-25°C
Leonian and Geer	1929	Nucleinic acid (defined) medium;	25°C
Narasimhan	1930	OMA;	not given
Ventakaryan	1932	CA;	" "
Marudarajan	1941	CMA, FBA, OMA;	20°C
Thomas et al	1947	OMA;	26°C
Ramakrishnan and Seethalakshmi	1956	CA, FBA, MMA, OMA, PDA;	26-29°C
Apple	1959	OMA;	20°C
Koyeas	1959	OMA;	25°C
Orellana	1959	Potato agar, Tomato agar;	lab.temp.(27°C)
Turner	1962	OMA;	60°F
Haasis and Nelson	1963	Hemp seed decoction;	25°C
Harnish	1965	Lima bean.	wide range

BA = bean agar; CA = carrot agar; CMA = cornmeal agar; FBA = french bean agar; MMA = maizemeal agar; OMA = oatmeal agar; PDA = potato dextrose agar.

was less affected than P. palmivora by the type of medium used (see Tables 3 and 13).

TABLE 13.

Oogonium formation on three media over a range of temperatures (see Ch.4).

Temperature °C	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	Isolates
Medium									
CA	———	———	———	———	———	———	———		P18x4
	———	———	———	———	———	———			P18x24
	———	———	———	———	———	———	———		P28
OMA	———	———	———	———	———	———			P18x4
	———	———	———	———	———				P18x24
	———	———	———	———	———	———	———		P28
Med.A+S			———	———	———				P18x4
			———	———					P18x24
	———	———	———	———	———	———	———		P28

——— oogonium formation (+/++ to ++++)

With isolates P4, P18 and P24, the optimum temperature for growth of P. palmivora on carrot agar was found to lie between 27.5 and 30°C, confirming the results of many previous authors (e.g. Tucker, 1931); the optimum for P. heveae, close to 25°C, was the same as that given in Waterhouse's key (Waterhouse, 1963).

Earlier workers frequently expressed a belief that low temperatures

stimulated sexuality in P. palmivora, but no detailed confirmatory work was performed. Ashby (1929) wrote "at tropical temperatures the development of oospores had been comparatively slow", and later stated sexual organs were "formed freely within a week on maizemeal agar at moderate temperatures" (20-25°C). Lester-Smith (1927), working at room temperature (26°C) and with an ice chest, also thought sexual reproduction of P. faberi (= P. palmivora) possibly characterised by a low temperature. Kheshwalla (1935) paired cultures of P. palmivora at 22°C, since no oogonia were formed at 28°C. Thomas et al (1947) found no oospores in paired cultures during March, April and May (lab. temperature 28-31°C), but obtained them when the lab. temperature was below 26°C. Marudarajan (1941) studying isolates of P. palmivora, P. arecae and P. meadii, stated "inoculated plates and tubes were incubated at 20°C, as there was no oospore formation in the laboratory temperature of 27-28°C." The results obtained with P18 x 4 and P18 x 24 confirm that the optimum temperature for oogonium formation in P. palmivora lies close to 20°C, and suggest that this temperature should be used in work on sexual reproduction of this species. Orellana (1929) failed to obtain oogonia in paired cultures of P. palmivora on OMA at lab. temperature (around 27°C) in Costa Rica and Ceylon, and Johnson and Valleau (1954) failed to do so with P. parasitica v. nicotianae on oatmeal agar at 30°C. From the examples of earlier workers, and the results obtained in this section (see Table 13 and Figs. 3, 4 and 5), it is probable that the incubation temperatures used by Orellana, and Johnson and Valleau were too high for oogonium

formation. No previous work has been found describing the effects of temperature upon oogonium formation by P. heveae.

The optimum temperature for sporangium formation of P. palmivora P24 on carrot agar, close to 30°C, agreed with the results obtained by Reinking (1923) of 27-30°C, and Tucker (1926) of 27°C; P4 and P18 however, required free water for reasonable sporangium formation in the dark (as also observed by Holliday and Mowat (1963) for the Piper nigrum fungus). This fundamental physiological difference between isolate P24 and isolates P4 and 18 is similar to that described by Ashby (1929 a), who divided Phytophthora isolates into two groups, typical and atypical, on the basis of abundant or sparse sporangium formation; P24 appears to be a 'typical' isolate and P4 and P18 'atypical'. Of workers who have used liquid cultures, Husain and Ahmed (1961) found the optimum temperature for sporangia of isolates of P. parasitica v. piperis from Piper betle to be 27-30°C, whereas Wills (1954) and Gooding and Lucas (1959) found the optimum for P. parasitica v. nicotianae to be 20-25°C; Aragaki and Hine (1963) found the optimum for sporangia of P. parasitica on solid medium to be 25°C. The results obtained by these authors, and the results obtained with P4 and P18 (Chapter 6) suggest that the Piper betle fungus is more similar to the Piper nigrum (P. palmivora) fungus than to P. parasitica or P. parasitica v. nicotianae.

With P. heveae, for which no previous work has been found, sporangia were only formed on carrot agar at very high temperatures (27.5°C - 32.5°C), and were not induced by water either at low or high temperatures.

Some authors have recently examined the effects of light upon the sporulation of Phytophthora isolates (Gooding and Lucas, 1959; Aragaki and Hine, 1963; Merz, 1964; Harnish, 1965; Leal, 1965 etc.). Their results, involving a variety of species and isolates, have in general shown that light inhibits oogonium formation, and that sporangium formation is either stimulated by light or that light is no better than darkness, depending on the isolate; little work has been devoted to P. palmivora in this respect. Work with P. palmivora isolates P4, P18, and P24 has confirmed that the general trend also applies to this organism, but whereas P24 produced abundant sporangia in the dark and very abundant sporangia in light, isolates P4 and P18 produced few sporangia in the dark and abundant sporangia in light; sporangia of P4 and P18 formed in light were also morphologically distinct from those formed in darkness (see Fig. 9.). This result accentuated the physiological distinction between isolate P24 and isolates P4 and P18. The results obtained with P. heveae P28 confirmed those of Leal (1965), who observed stimulation of sporangium formation and inhibition of oogonium formation in the light with the same isolate.

The mechanism by which sporangium formation is light stimulated in some Phytophthora isolates has yet to be investigated, though Aragaki and Hine (1963) found that for P. parasitica radiation was effective within the limits 300 - 600 mμ. Effects of light upon sporulation in other fungi are well known, and are reviewed by Hawker (1957) and Cochrane (1958). Hawker considers that light may act by supplying energy leading to a

photochemical reaction, which takes part in a chain of metabolic reactions leading to spore formation, and Cochrane postulates an enzymatic basis for light stimulation.

A 'balance' between asexual and sexual reproduction, in the form of distinctly different temperature optima for sporangia and oogonia, was observed with both P. palmivora and P. heveae, and a similar reciprocal effect of this nature was observed in light and dark. This effect, shown in Table 14, is reminiscent of the hypothesis of Lester-Smith (1927) with regard to the sporulation of P. faberi : "The view is held that a normal strain of P. faberi is capable of both asexual and sexual reproduction, that the former is induced by a high rate of metabolism characterised by a high water content and a certain ratio of food materials; the latter by a low rate of metabolism characterised by a low water content, a different ratio of food materials and possibly a low temperature. The rate of metabolism will be greatly influenced by the presence of a competing organism, especially when the two growths approximate more closely to each other." Although this observation was hypothetical, the formation of sporangia, and reduction in oogonium formation by P. palmivora at high temperatures (25°C +) or in light could be due either to direct inhibition of oogonia under these conditions, or perhaps to indirect inhibition as a result of a greater asexual reproductive capacity and a change in growth rate. The same problem applies to P. heveae. Effects of temperature upon two spore types have also been observed in other fungi (e.g. Barnett and Lilly, 1950), and the possible role

of temperature in retarding mycelial growth has been considered important in spore formation (Cochrane, 1958).

TABLE 14.

Oogonium and sporangium formation on carrot agar over a range of temperatures (see Figs. 3, 4 and 5).

Temperature °C	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5
P18x4 oogonia	———	———	———	———	- - -	- - -	- - -	- - -
P18,P4 sporangia				- - -	- - -	- - -	- - -	- - -
P18x24 oogonia	- - -	———	———	———	———	- - -	- - -	- - -
P24 sporangia	- - -	- - -	- - -	- - -	- - -	———	———	———
P28 oogonia	———	———	———	———	———	———	- - -	- - -
sporangia							- - -	———

- - - - - - under +++/++++ ——— +++/++++ and over

Interactions between temperature, medium, light etc., and their effect upon fungus metabolism, growth rate and sporulation are obviously complex and require detailed biochemical investigation outside the scope of the

present work. The objective, to determine the simple temperature and light requirements for sporulation of the four isolates, was however successfully achieved.

If the in vitro effects of temperature and light upon isolates P4, P18, P24 and P28 apply equally well in the field (though there is little supporting evidence for this) it is of interest to note the mean monthly temperatures, given in Table 15, to which these isolates are normally subjected. The Singapore temperatures are close to the optimum for growth and oogonium formation of P. heveae, and the Port of Spain temperatures would be good for sporangium formation and poorer for oogonium formation of P. palmivora P24, (other conditions permitting). Sandakan (N. Borneo) temperatures would be good for growth and sporangium formation by P4 and P18 in the presence of water, but inhibitory to oogonium formation.

TABLE 15.

Mean monthly temperatures from sites near the geographical origins of isolates P4, P18, P24 and P28: (data from The Oxford Atlas, Oxford University Press; °F converted to °C).

	J	F	M	A	M	J	J	A	S	O	N	D
P24 Port of Spain	24	24	24.5	25.6	26.2	25.6	25.6	25.6	25.6	25.6	25.0	24.5
P18,P4 Sandakan	26.6	26.6	27.2	27.8	28.3	27.8	27.8	27.8	27.8	27.2	27.2	26.6
P28 Singapore	25.6	25.6	26.2	26.6	27.2	26.6	26.6	26.6	26.2	26.6	26.2	25.6

The requirement for water for sporangium formation by the Piper nigrum fungus should be readily satisfied since according to Holliday and Mowat (1963) "The wettest months are October to March with a range of means of 12.7 to 26.1 inches. The remaining 6 months have a range of 7.5 to 10.1 inches." Further, disease spread on Piper nigrum plantations is largely by zoospore emission from sporangia and "disease outbreaks are highest during the wetter months, which have frequent overcast humid days. At such times mean maxima may fall to about 26°C and mean minima to 21°C. Minimum temperatures may fall to 18°C." The absence of prolonged cool periods in the tropics may be a factor limiting frequent sexual reproduction of P. palmivora. but the last statement suggests some chance of seasonal low temperature stimulation of oogonium formation.

The possibility of oogonium formation in the field has long been in doubt, and so in consequence has the role of the oospore as a means of pathogenic variation, or as a resting stage. In his classic paper on P. faberi in 1924, Gadd described the fungus as "one of the most destructive known", but in 1927 cited "the presence of oospores in pure cultures of P. faberi and P. palmivora has not been recorded in nature or in any culture medium." The same held true in 1947, when Thomas et al wrote "the part played by non-crop hosts in the survival of the pathogen, the formation of sexual bodies when the host becomes infected by two sexual strains, and possible production of new strains by sexual reproduction, cannot be overruled." In 1956, Ramakrishnan and Seethalakshmi reported discovering oospores in the pericarps of diseased

areca fruits, but no other report has been found of oospore formation in the host by P. palmivora.

Thus the discovery (see Chapter 10) that oogonia and oospores of P. palmivora can be formed in host material suggests that under the right conditions oospores may play a part in field variation and survival. The fact that plant pathologists have not (or only once) found oospores may be due to a combination of factors : oogonium formation might require

1) the presence of both mating types in the same locality;
2) multiple infection of the host by both mating types;
3) optimum environmental conditions (temperature, light, nutrition etc.).

Isolates P4 and P18 both came from the Serian district of Sarawak, suggesting that condition (1) above will be satisfied. Further, Holliday and Mowat (1963) observed multiple leaf infections of Piper nigrum : "sometimes an infected leaf has more than one lesion and several, scattered circular areas are the result of multiple infection. Before all the leaf becomes necrotic the leaf abscisses." Thus the second condition could be satisfied. Leaf abscission was also observed when cuttings of Piper nigrum were inoculated in the present experiments (see Chapter 11).

Climatic conditions in Sarawak, Borneo,and other tropical regions, (already discussed), may be unsuitable for oogonium formation owing to high mean temperatures; oogonia might be formed only during the cooler periods. Light inhibited oogonium formation in detached leaves (Chapter 12) and may be a further limiting factor, since sexual reproduction may

be restricted to the more woody tissues such as stems and roots not penetrated by light. If this were the case, the minute spores could easily escape detection by field workers. Circumstantial evidence does suggest therefore that the chances of oogonium formation in any one crop plant (whether of Piper nigrum or of another host), and of their observation by pathologists, may be small.

In his review, Hickman (1958) commented that the pathogen (Phytophthora) must ultimately be released into the soil, but that the importance of mycelium and oospores in survival was not clear. Working with the Piper nigrum fungus, Holliday and Mowat (1963) found saprophytic survival of mycelium in the soil to be short lived; if this is the case, the need for a resting stage might well be met by oospore formation, or by survival on non-crop hosts. Holliday and Mowat (1963) successfully inoculated several other Piper species with the Piper nigrum fungus, including Piper sarmentosum which was laterfound to be indigenous to Sarawak, and which "may indicate reservoirs on wild hosts." This observation is more significant now that oospore formation in host tissue has been obtained; it is possible that a dynamic inoculum source of different physiologic races and mating types exists on other Piper species in the rain forests,and that a resting inoculum of oospores resides in the soil.

Finally, it is encouraging to note that comparisons may be drawn between results of in vitro and in vivo experiments concerning the same organisms. Observations upon environmental factors affecting the growth

and sporulation of facultative parasites on the agar plate are difficult to justify in terms of natural behaviour. It is therefore valuable knowledge that, when both mating types are used, oogonia are formed in the host as on agar, and that in both cases they are formed over a similar temperature range and inhibited by light.

SECTION II.

The nutrition of reproduction in *Phytophthora palmivora* and *Phytophthora heveae*.

Chapter 1.

INTRODUCTION

The lack of a suitable synthetic medium in which sexual reproduction of Phytophthora would take place was for many years a hindrance to the advance of research in this field. This era was terminated in 1964 with the almost simultaneous publication by several independent authors of papers concerning the effect of sterols in stimulating reproduction in the genus (Haskins, Tulloch and Micetich, 1964; Hendrix, 1964; Elliott, Hendrie, Knights and Parker, 1964; Leal, Friend and Holliday, 1964; Harnish, Berg and Lilly, 1964). Earlier contributions to our knowledge of Phytophthora nutrition included the discovery of thiamin as an essential growth factor by Leonian and Lilly (1938) and Robbins (1938), and work upon the nitrogen, carbon and mineral requirements for growth (Volkonsky, 1934; Robbins, 1938; Mehrotra, 1951; Wills, 1954; Lopatecki and Newton, 1956; Sakai, 1961; Christie, 1958; 1961; Dimitman and Zentmyer, 1960; Erwin and Katzenelson, 1961; Cameron and Milbraith, 1965; Leal, 1965; Roncadori, 1965 etc.). Many species of Phytophthora were brought into artificial culture, and many synthetic growth media tested, but none was found in which sexual reproduction would take place, although Leonian and Lilly (1937) and Zentmyer (1952) obtained from peas and avocado roots respectively simplified organic extracts active in stimulating sexual reproduction in Phytophthora. Until 1964, all media used for the study

of sexuality in Phytophthora contained extracts of vegetable or animal origin; such media, being largely undefined, complex and variable, impeded the definition of the broader nutritional requirements of sexual reproduction, and obstructed the search for minute quantities of substances possibly produced by isolates in a mating reaction.

The discovery that sterols stimulated sexual reproduction in Phytophthora opened the way for the establishment of defined simple media for work upon the mechanisms of reproduction in the genus. In this section, a brief investigation into some of the nutritional requirements of reproduction of P. palmivora and P. heveae is described. The initial intention of this work was to obtain a simple medium suitable for use during later investigations of sexual mechanisms; as a basis, a serine/glucose/salts/sterol medium (Med. A + S) was used, recommended by Dr. Antonio Leal as being suitable for oogonium formation by P. heveae. However during the course of these experiments a number of interesting effects were observed, especially the effects of β-sitosterol upon asexual and sexual reproduction. For this reason, and because of current interest in the effects of sterols upon Phytophthora isolates, special attention was devoted to the role of β-sitosterol in the growth and sporulation of P. palmivora and P. heveae.

Chapter 2.

MATERIALS AND METHODS.

(additional to those described in Section I)

Isolates P4, P18, P24 and P28 were used in this section. Medium A was used as a basal medium for the nutrition studies; the composition was as follows :-

Medium A + Sterol (Basal)	
DL-serine	2.0 g.
Glucose	5.0 g.
KH_2PO_4	0.5 g.
$MgSO_4.7H_2O$	0.5 g.
β-sitosterol	10 mg.
Thiamine	10 mg.
Davis agar	15 g.
Trace element solution	1 ml.
Distilled water to	1 litre

Trace element solution.	
$FeSO_4.7H_2O$	0.05 g.
$ZnSO_4.7H_2O$	0.05 g.
$CuSO_4.7H_2O$	0.002 g.
$MnCl_2.4H_2O$	0.002 g.
$NaMoO_4.2H_2O$	0.002 g.
Distilled water to	100 ml.

Medium A + Sterol = Med. A + S

Alternative nutrient sources or concentrations were substituted when required. Glucose was autoclaved separately from other constituents.

The initial pH of any medium was adjusted to pH 5.5 using N HCl and N NaOH unless it already lay between pH 5 and 6, or was an indicator medium. In the first experiment, pH was not adjusted, but measured after autoclaving and after growth using B.D.H. Universal Indicator. All media were autoclaved 10 lb./10 minutes. β-sitosterol was added to the powdered agar in ether solution (100 mg./ml.) and the ether evaporated; this ensured dispersal of the sterol throughout the medium. 'Analar' grade chemicals were used wherever possible and Davis agar was used throughout. Yeast extract, peptone, malt extract and vitamin-free casamino acids obtained from Difco Laboratories were used at a concentration of 2 g./l. Proprietary brands of castor oil, and corn, peanut and sunflower seed cooking oils were used; olive and linseed oils, linoleic, oleic, palmitic and stearic acids, and amino acids were obtained from British Drug Houses Ltd., and β-sitosterol from Kodak Ltd. Vegetable oils and fatty acids were used at a concentration of 3 ml. or 3 g./l.

15 ml. of medium was used in 9 cm. dishes, and 5 ml. of medium in 5 cm. dishes. When the medium contained oils or fatty acids, the dishes were cooled to 2°C, and the medium to 48°C, and shaken before pouring; this ensured rapid solidification of the medium and maintained a fine suspension of well dispersed oil droplets. A lipase detecting medium used was a modification of that described by Starr (1941); the preparations of this and V-8 juice agars are given in the Appendix. Colour reaction tests for sterols obtained from "Biochemistry of Steroids" (Heffmann and Mosettig, 1960), are also given in the Appendix.

Stock cultures for experiments were maintained on Med. A + S, Med. A - S (no sterol) or water agar at 25°C in the dark, and No.1 cork borer inocula taken from the edges of growing colonies. All cultures were incubated in the dark unless otherwise stated. Incubation temperatures used were those closest to the observed optimum for the particular spore type required (see Section I). Two replicate plates of each culture series were used, and spore frequencies assessed by either the Quick Estimation or Numerical Estimation Method. Spore dimensions were the mean measurements of 50 spores taken from random high power microscope fields using slides prepared for spore frequency assessment; spore content percentages were obtained similarly, but 100 spores counted.

Chapter 3. The value of alternative carbon and nitrogen sources in oogonium formation.

In Section I (Chapter 4) Med. A + S was found considerably less adequate for sexual reproduction of P. palmivora than either carrot or oatmeal agars, but was equally good for sexual reproduction of P. heveae. In this experiment a wide variety of complex and simple carbon and nitrogen sources were substituted into Med. A + S in various combinations for the purpose of finding a simple effective medium for sexual reproduction of P. palmivora, and for determining the broader nutrient requirements of sexual reproduction in both species. A comparison was made between three much used vegetable media,(carrot, oatmeal and V-8 juice agars), and the range of synthetic media mentioned above, given in Table 16.

Of six 5 cm. petri dishes of each of the 21 media concerned, two were inoculated with P18 x 4, two with P18 x 24 and two with P28 using Med. A + S inocula. The P. palmivora plates were incubated for 21 days at 20°C, and the P. heveae plates for 21 days at 25°C, and examined for oogonia using the Quick Estimation Method. The results are given in Table 16; the results for P18 x 24, not given in the table, were similar to those for P18 x 4, but the oogonial frequencies were generally lower, and no oogonia at all were formed on media 3, 8 or 15. Sporangia of P24 were abundant in all the P18 x 24 plates,however, and may explain the lower oogonial productivity of this cross when compared with the P18 x 4 plates,in which sporangia were only occasional.

TABLE 16.

The effects of various carbon and nitrogen sources upon oogonium formation by P. palmivora and P. heveae.

Medium	P18 x 4 Oogonia	P18 x 4 Original pH	P18 x 4 Final pH	P28
1) Aspar. + glucose	-	5	7	ND
2) Medium A	+++	5.5	6.5	++++
3) Aspar.+ ser., + glucose	++	5	8	ND
4) Peptone + glucose	+++	5	6.5	++++
5) Yeast extract + glucose	+++/++++	5	7	++++
6) Casam. + glucose	+++/++++	5	6.5	++++
7) Malt extract + glucose	+++	5	5	+++
8) $NaNO_3$ + glucose	+++	5	8.5	++++
9) Casam. + corn oil	++++	5.5	5	+++++
10) Casam. + peanut oil	++++	5.5	5	+++++
11) Casam. + olive oil	+++++	5	6	+++++
12) Casam. + castor oil	++++	6	5.5	ND
13) Casam. + linseed oil	+++++	5.5	5	ND
14) Casam. + palmitic acid	-	4.5	4.5	ND
15) Casam. + oleic acid	-	5	5	ND
16) $NaNO_3$ + corn oil	+++++	6	8.5	+++++
17) $NaNO_3$ + peanut oil	+++++	6	8.5	+++++
18) $NaNO_3$ + olive oil	+	6	8	+++++
19) Carrot agar	++++	6	8.5	++++
20) Oatmeal agar	++++	5.5	6	++++
21) V-8 Juice agar	+++	6	8.5	++++

Table 16 (cont.)

All nitrogen sources at 2g./l.

ND = no data; Aspar. = L-b-asparagine; ser. = DL-serine;

Casam. = casamino acids.

With a glucose carbon source, P. palmivora produced more oogonia on the complex nitrogen sources than on the simpler ones; the best nitrogen source for both P18 x 4 and P18 x 24 was casamino acids, which represented a considerable improvement upon results obtained with Medium A (serine/glucose medium) in Section I. No oogonia were formed with L-b-asparagine (2g./l.), and the addition of L-b-asparagine to 1g. DL-serine (medium 3) appeared to inhibit the formation of oogonia in the presence of serine. $NaNO_3$ was a reasonable nitrogen source for oogonium formation by P18 x 4, but not for P18 x 24, where P24 produced little growth on the medium. As an alternative carbon source to glucose, the vegetable oils stimulated substantial oogonium formation by both of the P. palmivora crosses, with either casamino acids or $NaNO_3$ as a nitrogen source, but single fatty acids were poor in the combinations tested (media 14 and 15).

For the homothallic P. heveae, all the media tested proved good for oogonium formation, but the vegetable oil media were exceptionally so. In both P. palmivora and P. heveae, oogonium formation on the oil media was greater than on the three vegetable media (media 19, 20 and 21).

With P. heveae, oogonia were piled in very dense dumps on the agar surface of the oil media producing a deep yellow colour. With P. palmivora, similar clumps of oogonia caused a yellow colouration on certain oil media (media 11, 13, 16 and 17). It was evident that complex oils provided a better medium than glucose for the formation of oogonia. Glucose has previously been shown to be equal to, or better than, most sugar carbon sources for the growth of Phytophthora isolates (Volkonsky, 1934; Wills, 1954; Erwin and Katzenelson, 1961; Chee, 1965 etc). However, little attention has been paid to the value of lipid carbon sources, with the exception of Hendrix and Apple (1964), who found growth of P. parasitica v. nicotianae better on vegetable oil or fat carbon sources than on glucose, and demonstrated the production of lipases by this organism, and Klemmer and Lenney (1965), who observed lipid stimulation of sexual reproduction in Phytophthora and Pythium.

The effect of oils upon sexual reproduction observed in the present experiment may have been due to a direct preference for a lipid carbon source, a faster rate of uptake of lipids, or perhaps to a synergistic effect due to the presence and probable absorbtion of sterols or other substances, finely dispersed in the complex oils. Possible causes of stimulation of growth by lipids were discussed by Hendrix and Apple (1964). The possibility that sterols were present in the oils acting as a source of reproductive stimulation, and also of lipase production by the four isolates, was investigated in the next experiments.

It can be seen (Table 16) that the original pH of a medium (after

autoclaving in this case) bore no relationship to the pH after growth, which was apparently governed by the nutrients present. The pH's of nitrate, glucose and vegetable media rose during growth of the fungus, whilst those of the casamino acid/oil media remained relatively unchanged or fell. The final pH's appeared to have little effect on the formation of oogonia over the range pH 5 - 8.5, since abundant oogonia were formed by P18 x 4 at both pH 5 and pH 8.5. In future work, however, the initial pH was adjusted to 5.5 where initially it was not between pH 5 and 6.

It is preferable in the search for a simple effective medium for oogonium formation by P. palmivora, to discard complex nitrogen and carbon sources, such as casamino acids and vegetable oils, owing to their indefinite composition. Of the alternative simple nitrogen sources tested, asparagine was poor, serine only moderate, and $NaNO_3$ ineffective for P18 x 24. However, all the nitrogen sources were used at a concentration of 2g./l. : since the actual nitrogen concentration used would vary with the source, comparisons between nitrogen sources on this basis were not completely valid.

In an attempt to produce a defined medium it is better to test ranges of concentrations of the nutrients concerned, especially with nitrogen and carbon sources. In further experiments, some effects of amino acid and glucose concentrations upon sporulation were investigated by substitution into the basal medium (Med. A + S).

Chapter 4. The production of lipases by P. palmivora and P. heveae.

In the previous experiment, lipids, in the form of complex vegetable oils, were found stimulatory to oogonium formation in P. palmivora and P. heveae, suggesting that these fungi might produce exogenous lipases for rapid absorbtion of the hydrolytic products of the oils, glycerol and fatty acids. In the following experiment the four isolates were tested for lipase production using a Spirit Blue medium, modified from the formula of Starr (1941); this method was used by Hendrix and Apple (1964) for the detection of lipases in P. parasitica v. nicotianae.

Castor and sunflower seed oils were substituted into the medium as an alternative to Starr's cotton seed oil (see Appendix). On setting, the media were pale grey-blue/green in colour. Standard (9 cm.) dishes of each medium were inoculated with isolates P4, P18, P24 and P28, and control plates with sterile agar plugs; the cultures were incubated at 25°C. On both castor oil and sunflower seed oil media, the fungal colonies produced a densely fluffy aerial mycelium, and in each case a deep blue colouration was developed beneath and just beyond the limit of growth, as a result of the pH change with extracellular hydrolysis of the oils to component fatty acids. This demonstrated the ability of all four isolates to produce lipases (see Fig. 12.).

Chapter 5. The presence of sterols in complex vegetable oils.

The possibility that lipid stimulation of oogonium formation might be due, in part at least, to the presence of well dispersed sterols

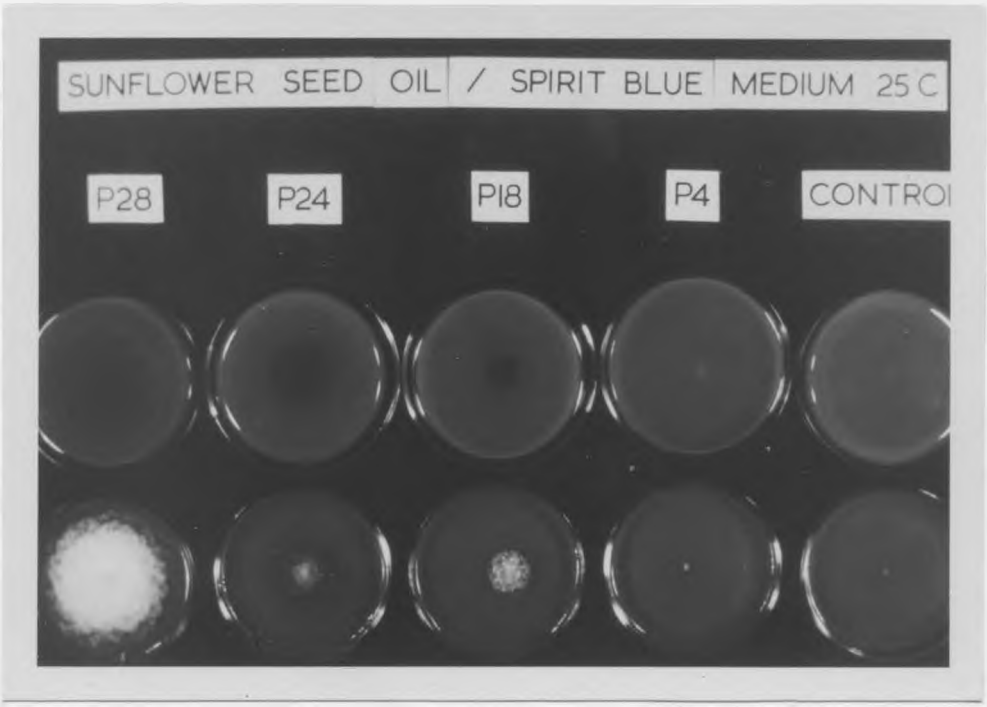


Fig.12. The production of lipases by P. palmivora and P. heveae.

Top row : plates reversed, showing dark blue colouration beneath colonies.

Bottom row : plates upright.

in the oils, was examined by testing oils and fatty acids for sterols using colour reactions. The results of these tests are given in Table 17.

TABLE 17.

Colour tests for sterols on a variety of lipid sources.

Test / Source	Liebermann-Burchard	Salkowski	Tschugaeff	Sterols present
β-sitosterol	+	+	+	+
Castor oil	+	+	+	+
Corn oil	+	+	+	+
Linseed oil	+	+	+	+
Olive oil	+	+	+	+
Peanut oil	+	+	+	+
Sunflower seed oil	+	+	+	+
Linoleic acid	+	+	+	+
Oleic acid	+	+	+	+
Palmitic acid	-	-	-	-
Stearic acid	-	-	-	-

+ = test positive; - = test negative.

Clearly sterols are present not only in the vegetable oils, but

also in some of the purer fatty acids. It is therefore possible that the stimulatory effect of vegetable oils upon oogonium formation (observed in Chapter 3) may be due in part to the ease of absorbtion of sterols from the oils as well as to the nutrient value of the fatty acids and glycerol.

Chapter 6. The effects of serine and asparagine concentrations upon growth and sporulation.

In the first nutrition experiment (Chapter 3), DL-serine proved to be a better source of nitrogen for oogonium formation than L-β-asparagine at 2 g./l., despite the fact that asparagine is "often one of the best sources of nitrogen for fungi and actinomycetes" (Cochrane, 1958). However, Cochrane also stated that the concentration of the amino acid and the incubation period are important; therefore an experiment was designed to investigate the effects of the concentrations of these two amino acids upon growth and sporulation of P4, P18, P24 and P28, in single isolate cultures. Special attention was paid to sporangium formation by P. palmivora isolate P24, and oogonium formation by P. heveae isolate P28, since these two isolates usually produced these spore types in abundance; (oogonium formation by P. palmivora crosses was also investigated, but spore frequencies were too low to be significant). The following media, adjusted initially to pH 5.5, were used in this experiment:-

Medium no.	1	2	3	*(7)	4	*(8)	*(9)	5	6
DL-serine or L-β-asparagine g./l.	0.1	0.5	1.0	1.5	2.0	3.0	4.0	5.0	5.0
Glucose g./l.	5	5	5	5	5	5	5	5	0
Medium A + S basal	+	+	+	+	+	+	+	+	+

* Medium 7, asparagine only; 8 and 9, serine only.

Two standard (9 cm.) dishes of each of media 1-6 were inoculated with the four isolates using water agar inocula, and incubated at 27.5°C (P. palmivora) and 25°C (P. heveae) for 21 days, the cultures photographed (see Figs. 13, 14, 15 and 16) and examined for pH, growth and sporulation. Three further media (7, 8 and 9) were prepared later and inoculated similarly, but with P24 and P28 only.

The effect of amino acid concentration upon aerial mycelium formation is shown in Table 18, and also in Figs. 13 - 16. With P. palmivora, an increase in sub-agar and aerial mycelium formation occurred with increase in concentration of serine or asparagine in the medium. The best growth was obtained at 1 - 2 g./l. serine, and 0.5 - 1 g./l. asparagine. At 5g./l., asparagine appeared to be toxic, causing irregular hyphal development and limited growth. Although P. heveae (P28) produced very little aerial mycelium, sub-surface mycelial density was greatly influenced by amino acid concentration, and the effect resembled

TABLE 18.

The effect of serine and asparagine concentrations upon aerial mycelium formation (for growth see Figs.13 - 16).

Isolate	P4		P18		P24		P28	
Medium no.	S.	A.	S.	A.	S.	A.	S.	A.
1	+	++	+	++	+	++	-	-
2	++	++/+++	++	++/+++	++	+++	-	-
3	++	++/+++	+++	++/+++	+++	+++	+/++	-
(7)					ND	++	ND	-
4	++	+/++	+++	++	+++	++	+/++	-
(8)					++/+++	ND	+/++	ND
(9)					++/+++	ND	+/++	ND
5	+	+/++	++/+++	+	++	-	+/++	-
6	-	+	+/++	+	+	-	-	-

ND = no data; S. = serine; A. = asparagine.

Medium	Concentration of Amino acid.
1	0.1 g./l.
2	0.5 g./l.
3	1.0 g./l.
4	2.0 g./l.
5	5.0 g./l.
6	5.0 g./l.
-	no glucose.

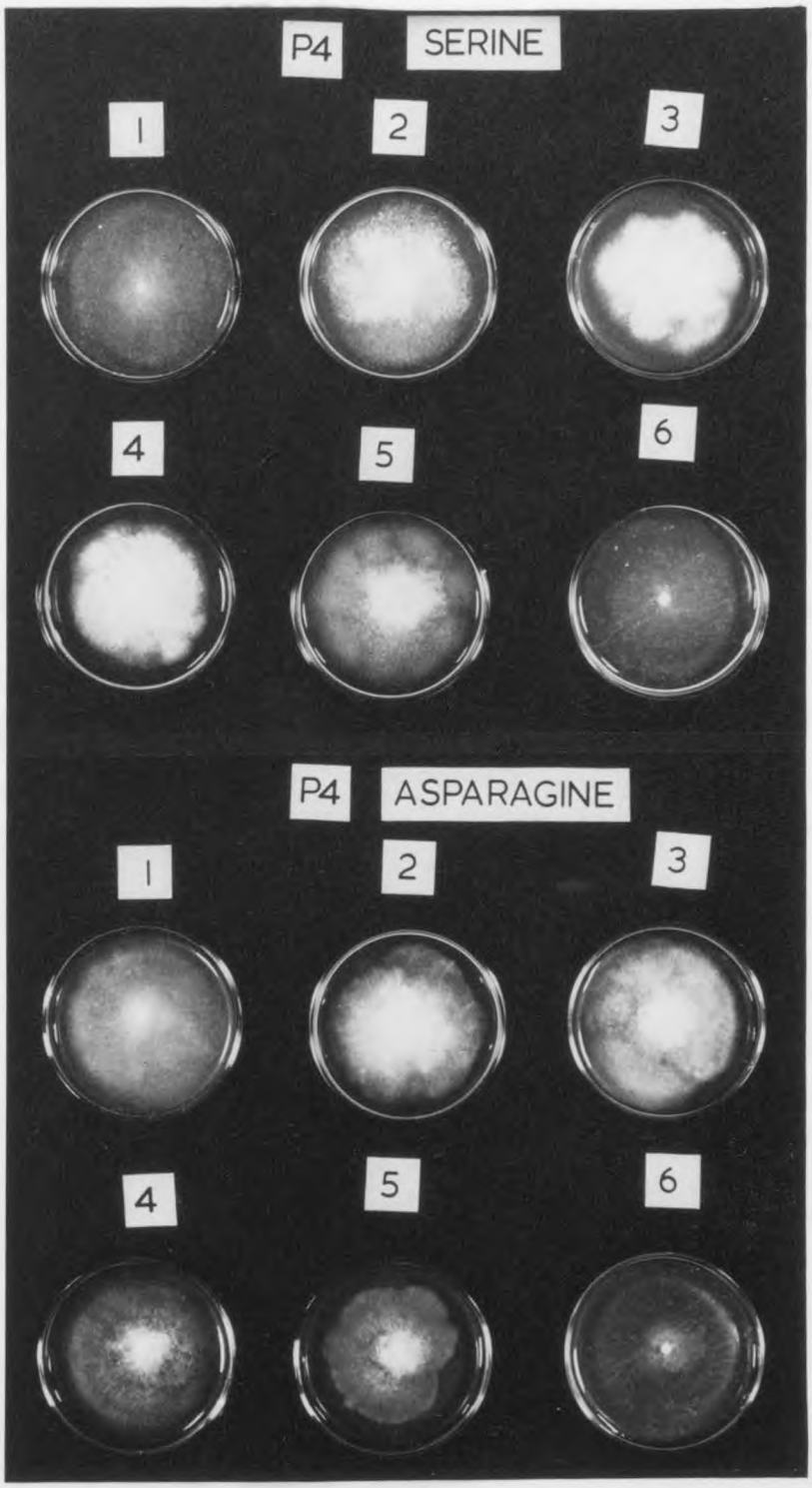


Fig.13. The effects of serine and asparagine concentrations upon growth of P. palmivora isolate P4.

Medium	Concentration of Amino acid
1	0.1 g./l.
2	0.5 g./l.
3	1.0 g./l.
4	2.0 g./l.
5	5.0 g./l.
6	5.0 g./l.
	- no glucose.

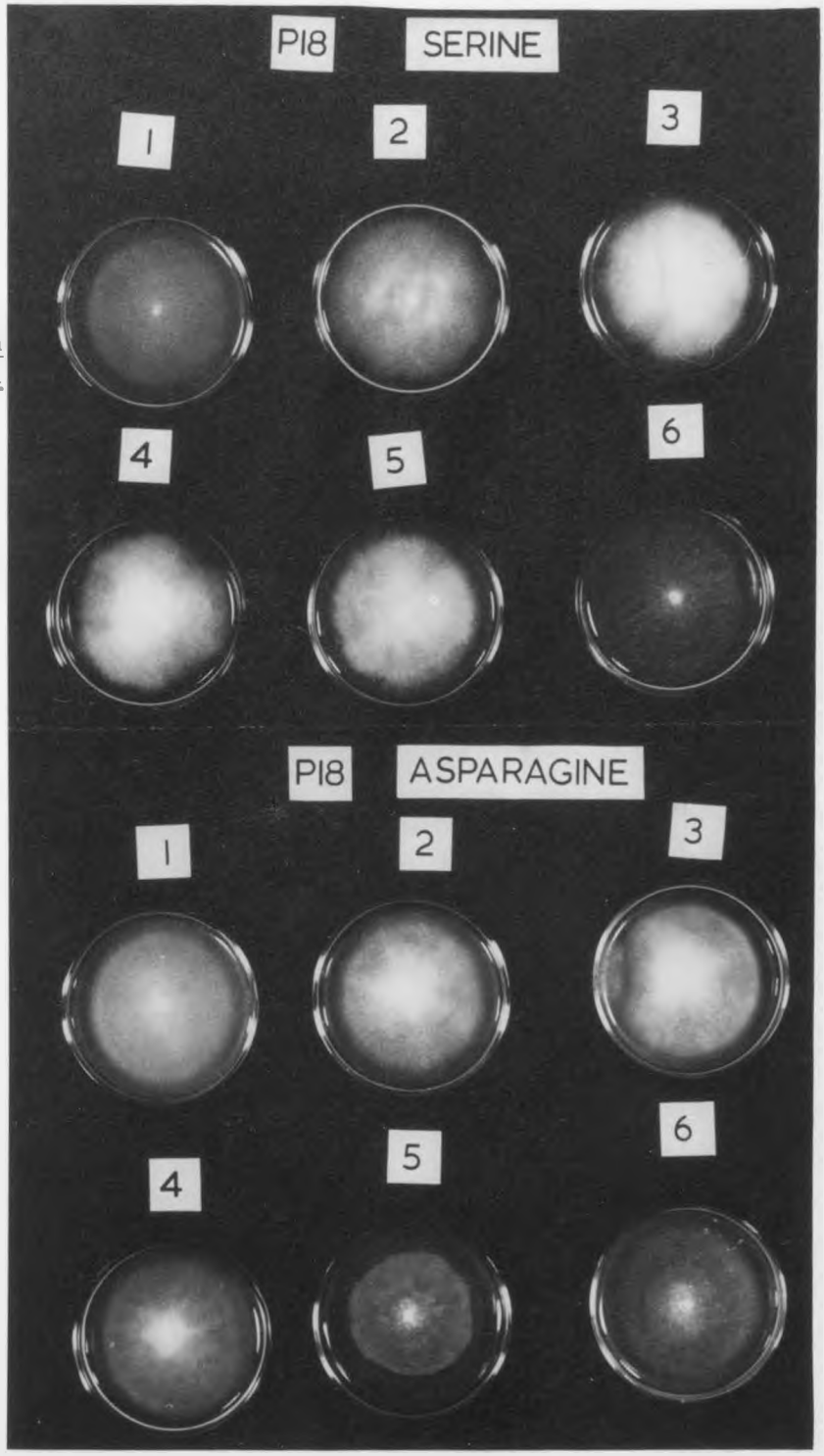


Fig.14. The effects of serine and asparagine concentrations upon growth of P. palmivora isolate P18.

Medium	Concentration of Amino acid.
1	0.1 g./l.
2	0.5 g./l.
3	1.0 g./l.
4	2.0 g./l.
5	5.0 g./l.
6	5.0 g./l.

- no glucose.

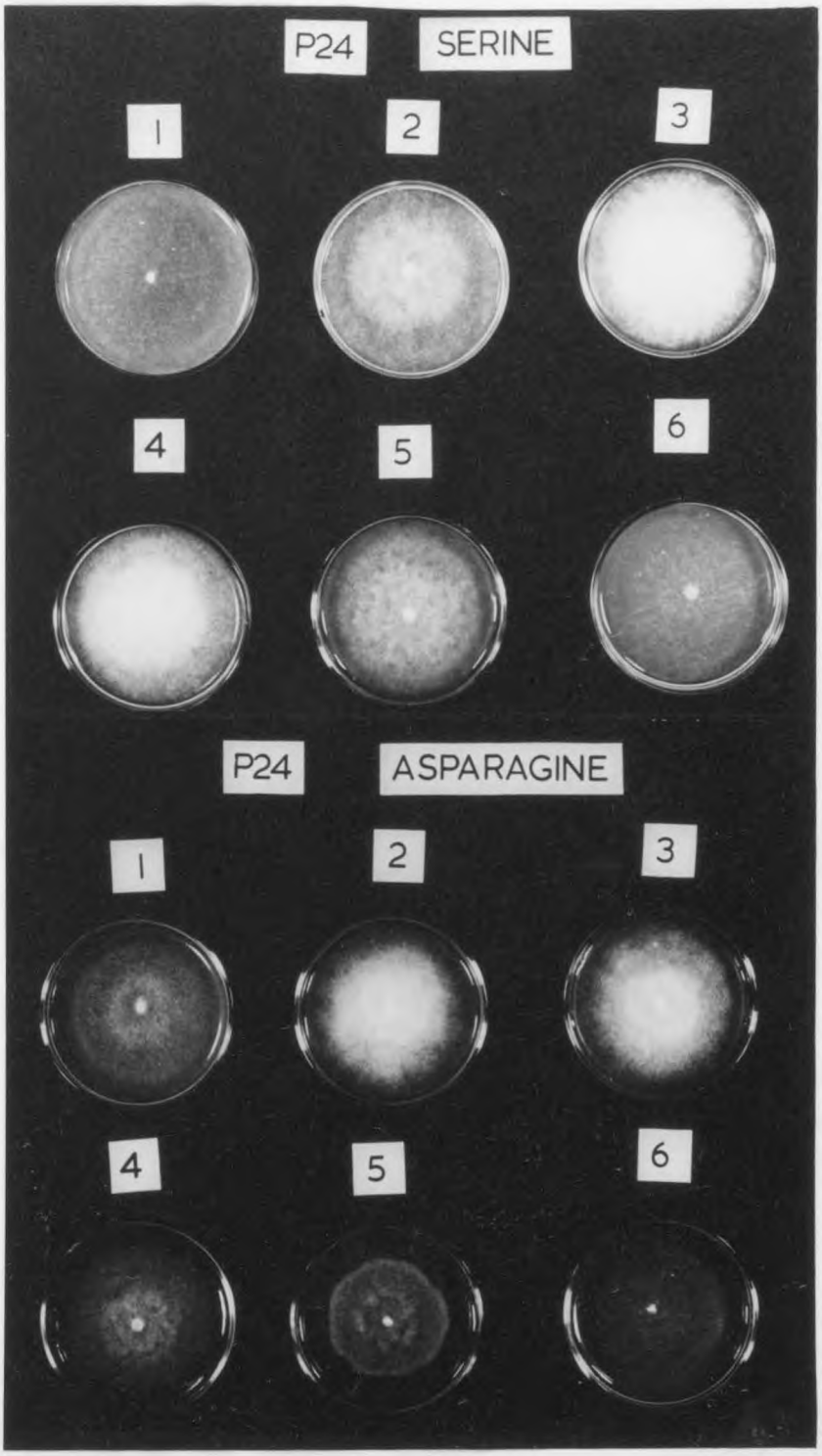


Fig. 15. The effects of serine and asparagine concentrations upon growth of P. palmivora isolate P24.

Medium	Concentration of Amino acid.
1	0.1 g./l.
2	0.5 g./l.
3	1.0 g./l.
4	2.0 g./l.
5	5.0 g./l.
6	5.0 g./l.

- no glucose.

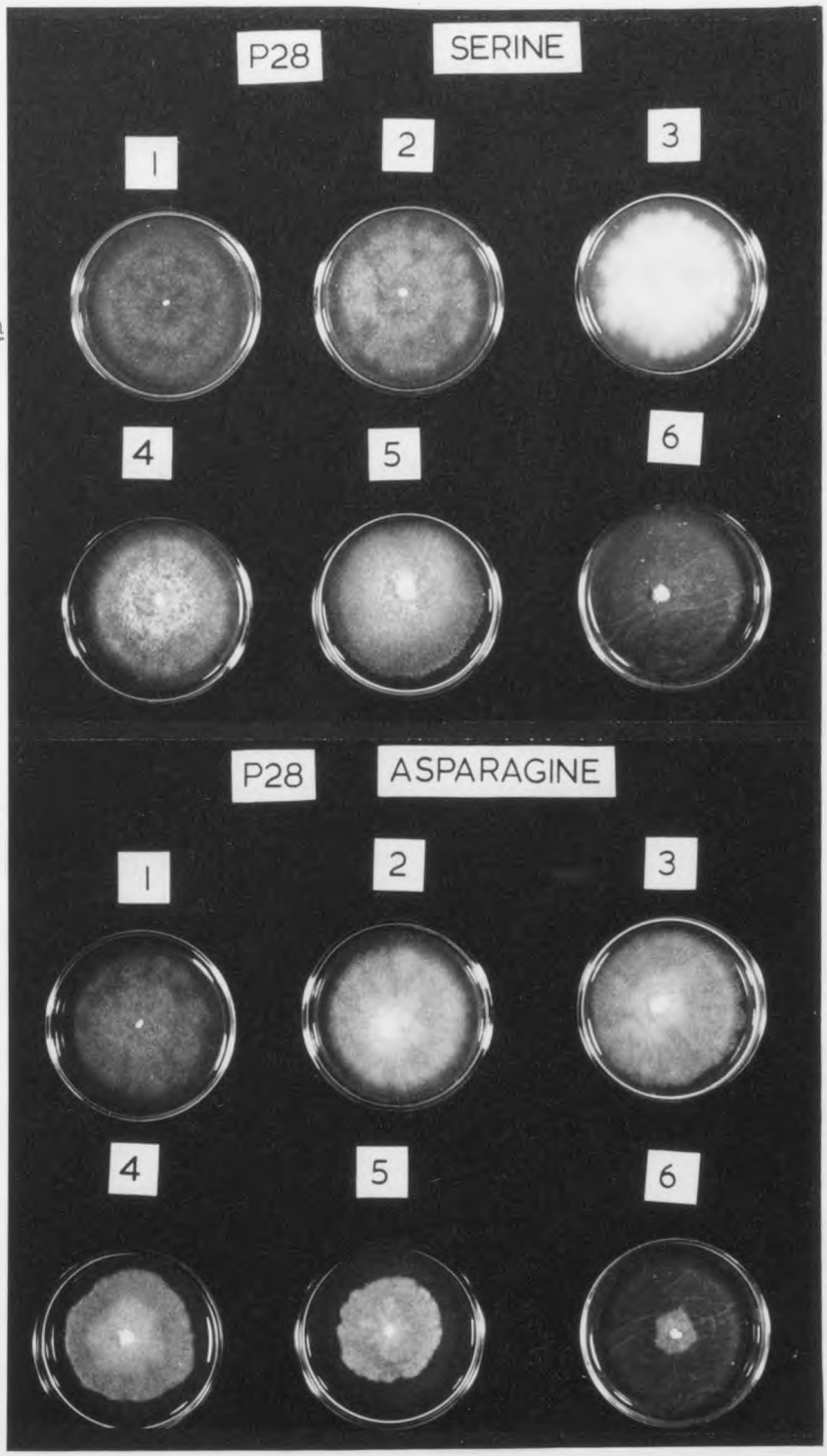


Fig.16. The effects of serine and asparagine concentrations upon growth of P. heveae isolate P28.

that observed with aerial mycelium formation of P. palmivora (see Fig. 16.). Thus good growth was obtained with these four isolates at a nitrogen concentration of 0.13 - 0.26 g./l. for DL-serine, and 0.11 - 0.21 g./l. for L-β-asparagine, suggesting that nitrogen concentration, as well as the amino acid used, was exerting a significant effect on growth. At 5 g./l. serine or asparagine, in the absence of glucose (medium 6), slight growth was obtained with all four isolates which presumably used the amino acid as a carbon source.

Spore frequencies in the plates, assessed by the Q.E.M. are given in Table 19. A more detailed analysis of sporangium and oogonium formation by P24 and P28 was performed using the N.E.M.; these results are given in Table 20 and Fig. 17. Sporangium formation by P4 and P18 was poor on all the media, and gave little indication of the effects of amino acid concentration, although for all four isolates sporulation at a high concentration of asparagine (5 g./l., medium 5) was nil. Interesting concentration effects were obtained with P24 and P28 however, and are shown in Fig. 17 expressed as a function of total nitrogen concentration.

Sporangium formation by P24 increased almost lineally with increase in nitrogen (amino acid) concentration, and was greater per gram asparagine nitrogen than per gram total serine nitrogen. The optimum concentration of serine was 2 - 3 g./l. (0.26 - 0.4 g./l. total nitrogen), and of asparagine close to 1.0 g./l. (0.21 g./l. nitrogen); at these optimum concentrations, the sporangial frequencies were comparable, but

TABLE 19.

The effect of serine and asparagine concentrations upon sporulation.

Isolate	P4		P18		P24		P28	
Medium no.	S.	A.	S.	A.	S.	A.	S.	A.
1. Sporang.	+	+/++	++	++	++	+++	++	++
Oogonia							++++	++++
2. Sporang.	-	++	-	++	+++	+++/++++	++	++
Oogonia							++++	++++
3. Sporang.	++	++	++	++	+++	++++	++	-
Oogonia							++++	++++
(7) Sporang.					ND	+++	ND	-
Oogonia								++++
4. Sporang.	++	++	++	-	+++/++++	+++	++	-
Oogonia							++++	+++ *
(8) Sporang.					+++/++++	ND	++	ND
Oogonia							++++	
(9) Sporang					+++	ND	++	ND
Oogonia							++++	
5. Sporang.	++	-	++	-	+++	-	- *	-
Oogonia							++++	-
6. Sporang.	++/+++	-	++	-	+++	-	++	-
Oogonia							+++	-

ND = no data; * = no oospores present.

Sporang. = sporangia; S. = serine; A. = asparagine.

above the asparagine optimum the frequency fell sharply, whereas a more gentle decline in sporangial frequency occurred above the serine optimum. These results were comparable with the effects of serine and asparagine concentrations upon growth and aerial mycelium formation, by P24 (Fig. 15 and Table 18).

The effects of amino acid concentration upon oogonium formation by P28 were similar; at the optimum concentrations of asparagine (0.21 - 0.31 g./l. nitrogen) and serine (around 0.26 - 0.4 g./l. total nitrogen) the oogonial frequency was considerably greater with asparagine. At 5.0 g./l. serine and 2.0 g./l. asparagine no oospores were formed, and above 2g./l. asparagine no oogonia were formed.

In terms of nitrogen concentration, the effects of the two amino acids upon growth were similar, and with both, low concentrations were more favourable for both growth and sporulation. However, it is not known whether the D form of DL-serine was utilised; if not, the effective concentration of serine nitrogen would be half that observed, and optimum nitrogen concentrations for spore formation on serine and asparagine would be closer; spore frequency results obtained for P24 sporangia per gram serine and asparagine nitrogen would then be very similar, whereas those for P28 oogonia would remain quantitatively distinct (see Fig.17). Nevertheless, asparagine was undoubtedly a better nitrogen source than serine, especially for oogonium formation by P. heveae, confirming the observation of Cochrane (1958), who discusses possible reasons for its effectiveness; this is not explainable simply in terms of carbon/nitrogen

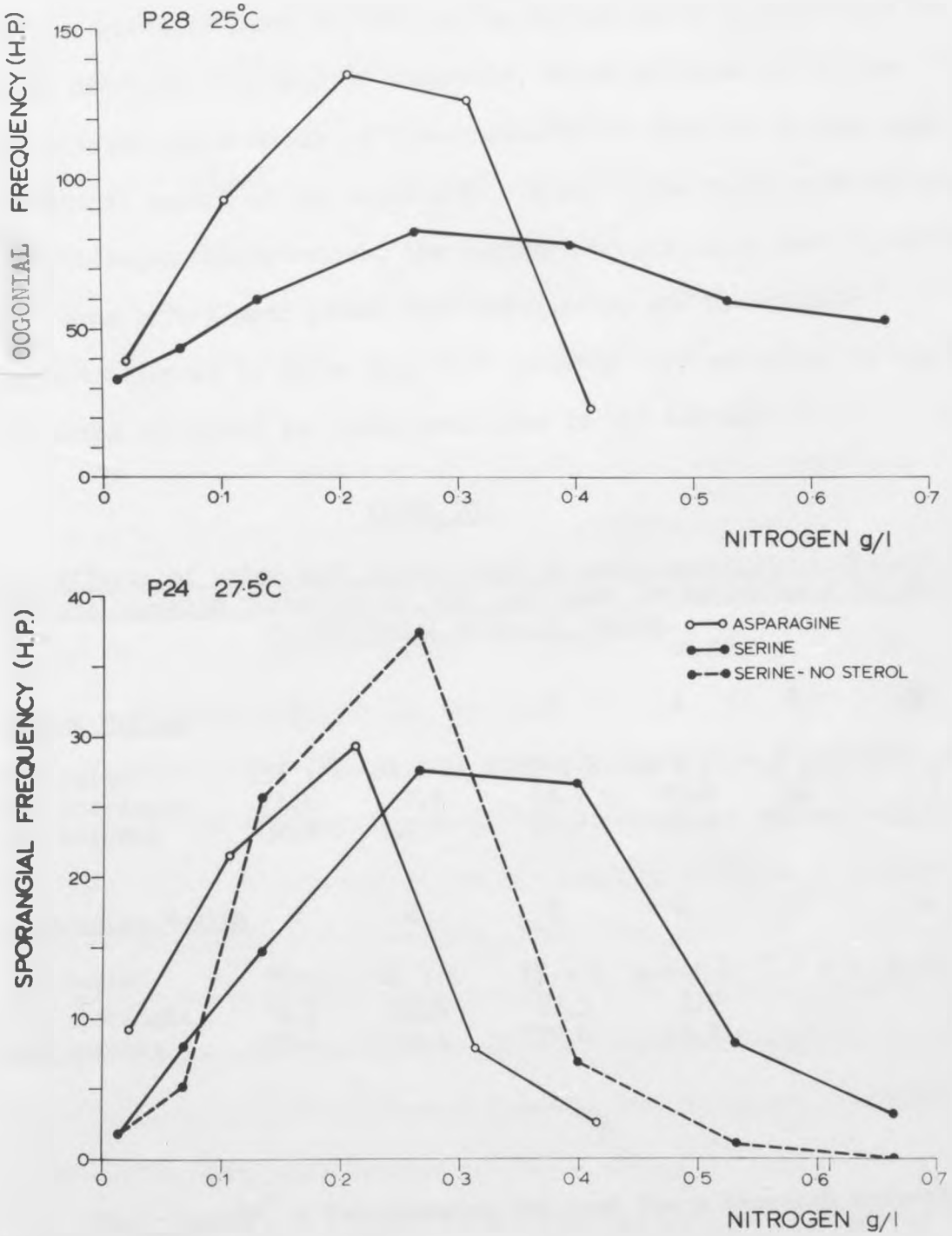


Fig.17. The effects of serine and asparagine concentrations, expressed as total nitrogen, upon sporangium formation by P. palmivora isolate P24 and oogonium formation by P. heveae isolate P28.

ratios, given in Table 20, but may be the result of a preference for amide nitrogen, a source of aspartate, extra nitrogen and carbon assimilated per molecule, a faster absorbtion rate, or to some such structural aspect of the amino acid. Since spore frequencies increased with nitrogen concentration, the carbon/nitrogen ratio must conceivably have some effect upon growth and sporulation, and the optimum concentration of an amino acid will probably vary according to the level of carbon (glucose) and other nutrients in the medium.

TABLE 20.

The effects of amino acid concentrations upon sporangium formation by P24 and oogonium formation by P28, and upon the approximate total carbon/total nitrogen ratios.

Serine Medium	1	2	3	4	5	6
C/N ratio	153 : 1	31 : 1	17.5 : 1	10 : 1	5.6 : 1	2.6 : 1
P24 sporangia	1.8	7.9	14.7	27.6	3.1	3.3
P28 oogonia	32.9	40.3	59.9	82.4	50.3	19.5

Asparagine Medium	1	2	3	4	5	6
C/N ratio	96: 1	21 : 1	11 : 1	6.6 : 1	3.6 : 1	1.75 : 1
P24 sporangia	9.1	21.6	29.3	2.5	-	-
P28 oogonia	37.1	93.1	125.6	23.3	-	-

This experiment demonstrates the need for a thorough investigation of concentration effects for any nitrogen source used, and suggests that data obtained when several nitrogen sources are used at the same

concentration per litre, or even at the same weight of nitrogen per litre using a constant carbon source, cannot be justly compared. For this reason, only generalisations can be made from the results obtained in the first experiment (Chapter 3). Concentrations of asparagine in excess of 1.5 g./l., with 5 g./l. glucose, are likely to be too high for growth and sporulation of Phytophthora isolates under the conditions of this experiment; thus the ineffectiveness of asparagine in the first experiment (Ch.3) may be explained in terms of the high concentration used. In further experiments on varied nutrient concentrations, serine was used as a nitrogen source at 2 g./l.; asparagine was not used because of the narrowness of its useful range of concentrations in Medium A.

Chapter 7. The effects of glucose concentrations upon growth and sporulation of P24 and P28.

Cochrane (1958) states that glucose "is utilised for growth by virtually all cultivatable fungi," and glucose has indeed been amongst the best simple carbon sources for Phytophthora isolates (Volkonsky, 1934; Wills, 1954; Erwin and Katzenelson, 1961; Chee, 1965 etc.). Although with a good nitrogen source, glucose at 5 g./l. was adequate for growth of the isolates under study, a brief experiment was performed to observe the effects of greater and lesser glucose concentrations substituted into Medium A + S, using isolates P24 and P28 only.

Serine was used in the medium at 2g./l. (to give 0.68 g./l. initial carbon), and four media prepared at 1, 5, 10 and 20 g./l. glucose to give total concentrations of 1.08, 2.68, 4.68 and 8.68 g. carbon/l.; the

initial pH of all media was 5.5. Two plates of each medium were inoculated with P. palmivora.P24.using water agar inocula, and incubated at 27.5°C (for sporangia); four plates of each medium were inoculated with P. heveae.P28. two incubated at 25°C (for oogonia) and two at 30°C (for sporangia). The plates were examined for aerial mycelium and spore formation after 21 days incubation (N.E.M. for P24, and P28 at 25°C; Q.E.M. for P28 at 30°C); the results are given in Tables 21 and 22.

TABLE 21.

The effects of glucose concentrations upon sporulation and growth of P24.

Glucose g./l.	0*	1	5	10	20
Total carbon g./l.	1.7	1.08	2.68	4.68	8.68
Aerial mycelium	+	+/++	+++	+++/++++	++++
Sporangia	+++	+++	+++/++++	+++	+++
Sporang. frequency	3.3	18.1	22.2	16.3	5.6

* Result of previous experiment, serine 5 g./l.

For both isolates, an increase in aerial mycelium formation was associated with increased glucose concentration; this may have been a carbon/nitrogen ratio effect. At 10 and 20 g./l. glucose, the dense aerial mycelium obstructed assessment of spore frequencies. P24 sporangium formation was best at 5 g./l. glucose, and the excess of vegetative growth at higher concentrations probably reduced sporangium

formation. With P. heveae, P28, at both 25 and 30°C, the increase in aerial mycelium was associated with a simultaneous increase in sporangium formation and decrease in oogonium formation; this 'balance' between vegetative reproduction and growth, and sexual reproduction, was first observed with temperature and light effects upon P28, on carrot agar, in Section I. Similarly, oogonia of P28 lacked oospores at 30°C (as in Section I.).

TABLE 22.

The effect of glucose concentrations upon sporulation and growth of P28.

Glucose g/l.	*0	1	5	10	20
Total carbon g./l.	1.7	1.08	2.68	4.68	8.68
1) 25°C					
Aerial mycelium	-	+	++	+++	+++
Oogonia	+++	++++	++++	+++/++++	+++/++++
Oogonial frequency	19.5	58.3	78.3	ND	ND
Sporangia	++	-	++	+++	+++
2) 30°C					
Aerial mycelium	ND	-	+/++	+++	++++
Oogonia	ND	++++	++++	++	++
% oospores		<1%	<1%	<1%	<1%
Sporangia	ND	++/+++	+++	+++/++++	+++

* Result of previous experiment, serine 5 g./l.;
ND = no data.

5 g./l. was the best glucose concentration for sporulation of both isolates, and will be used in further experiments with Medium A. It must be pointed out, however, that serine at 2 g./l. provided an additional carbon source (0.68 g./l.), and that in the previous experiment serine alone (at 5 g./l., i.e. 0.67 g./l.nitrogen and 1.7 g./l. carbon) provided enough carbon for limited growth and sporulation of P24 and P28. This factor must be taken into account in assessing the effects of the glucose concentrations.

Chapter 8. The effects of KH_2PO_4 concentrations upon growth and sporulation of P24 and P28.

Less attention has been paid, in fungal physiology, to the nutrition of the inorganic elements K, Mg, P and S than to carbon and nitrogen nutrition. Cochrane (1958) discusses fungal requirements for these elements, and reports the requirements for potassium and phosphorus, as KH_2PO_4, to be 0.001 - 0.004 M and 0.004 M respectively, and for magnesium and sulphur, as $MgSO_4$, to be 0.001 M and 0.0001 - 0.0006 M. In previous experiments, these two compounds have been provided at 0.5 g./l. i.e. 0.037 M (KH_2PO_4) and 0.002 M ($MgSO_4.7H_2O$). The following experiment investigated the combined effect of two of these elements, potassium and phosphorus, by substitution of a range of KH_2PO_4 concentrations into Medium A + S (2 g./l. serine, 5 g./l. glucose).

Five media, each containing 0.1, 0.5, 1.0, 2.0 and 3.0 g./l. KH_2PO_4 respectively, were prepared, and plates of each medium inoculated with P24 and P28 and incubated as in the last experiment. The initial

pH's of all media ranged from 5 - 5.5; results are given in Tables 23 and 24, and Figs.20 and 21.

TABLE 23.

The effects of KH_2PO_4 concentrations upon growth and spore formation of P24.

KH_2PO_4 g./l.	0.1	0.5	1.0	2.0	3.0
Aerial mycelium	+++	+++	+++	+++	+++
Sporangia	+++/++++	+++/++++	+++/++++	+++/++++	+++/++++
Rough frequency*	25.1	26.1	23.9	26.3	22.8
% contents	53.7	62.3	80.3	85.3	83

* Q.E.M., 18 counts per slide.

KH_2PO_4 concentration had little effect upon growth or sporangial frequency of P. palmivora.P24. However, sporangia increased significantly in size over 0.1 g./l. to 0.3 g./l., an effect not observed with changes in amino acid or glucose concentrations. Further, a large proportion of sporangia at 0.1 g./l. KH_2PO_4, had disorganised, or missing protoplasmic contents, whilst at high KH_2PO_4 concentrations (1.0 - 3.0 g./l.), a large proportion of the sporangia had normal, brown, granular, dense, protoplasmic contents, after 21 days incubation (see Fig.20).

Aerial mycelium formation and spore frequencies were similarly relatively unaffected by increased KH_2PO_4 concentration in the P. heveae

P28 plates. The percentage of oogonia containing oospores, however, was poor at 0.1 g./l., and good at 0.5 - 3.0 g./l. Oospores are not normally formed at 30°C, and this was the case at all KH_2PO_4 concentrations, but sporangia increased significantly in size with increased KH_2PO_4 concentration (see Fig. 21).

TABLE 24.

The effects of KH_2PO_4 concentrations upon growth and spore formation of P28.

KH_2PO_4 g./l.	0.1	0.5	1.0	2.0	3.0
1) 25°C					
Aerial mycelium	++/+++	++	++	++	++
Oogonia	++++	++++	++++	++++	++++
*Rough frequency	78.5	82.3	67.9	70.2	63.0
% oospores	48	83	85	85	90
Sporangia(Q.E.M.)	-	++	++	++	++
2) 30°C					
Aerial mycelium	+/++	+/++	+/++	+/++	+/++
Oogonia (Q.E.M.)	++++	++++	++++	++++	++++
% oospores	<1	<1	<1	<1	<1
Sporangia(Q.E.M.)	+++/++++	+++	+++	+++	+++

* Q.E.M., 18 counts per slide.

The effect of KH_2PO_4 concentration upon spore size and internal organisation (or percentage contents, if oospores are considered as the

oogonial contents in a manner similar to the sporangial contents) may be due to an effect of phosphorus concentration in governing glucose utilisation (Cochrane, 1958); similarly, potassium has been associated with sugar utilisation and carbohydrate metabolism (Cochrane, 1958). Experiments have indicated so far that carbon and nitrogen concentrations have a significant effect upon quantity of vegetative and reproductive growth, whereas inorganic nutrients are likely to affect spore quality, possibly by affecting carbon and nitrogen metabolism.

Since only qualitative improvements in sporulation were observed above 0.5 g./l. KH_2PO_4 , this concentration will be used in further experiments involving changes in concentration of Medium A + S constituents; in order to keep the medium standard the concentration of 0.5 g./l. $Mg.SO_4.7H_2O$ will also be maintained.

The effects of β-sitosterol upon growth and reproduction of Phytophthora palmivora and Phytophthora heveae.

In the following experiments a brief investigation was made into the qualitative and quantitative effects of β-sitosterol upon growth and reproduction of isolates P4, P18, P24 and P28. β-sitosterol, a naturally occurring plant sterol, was shown by Leal et al (1964) to be present in oats and peas, and to be satisfactory in the 'chemical' form for inducing oospore formation by P. heveae on synthetic media; however, little attention has been paid to the effects of sterols upon P. palmivora.

Chapter 9. An investigation of the effects of Medium A, with and without β-sitosterol, upon sexual reproduction of P. palmivora and P. heveae.

Other workers have shown that sterols, added to synthetic media, stimulate oogonium and oospore formation by Phytophthora isolates, where these would not otherwise have been produced. This particular effect was investigated in the following experiment, in which two media, Medium A + S (β-sitosterol 10 mg./l.) and Medium A - S (no sterol), were used.

Two small (5 cm.) petri dishes of Med. A + S were inoculated with P18 x 4 using Med. A + S inocula, and incubated at 20°C. After 3 days growth, two plates of Med. A - S were inoculated with P18 x 4, using agar plugs from one of these first two plates (series A plate 1). This process was repeated at three day intervals to give a series of seven replicated subcultures, the first series (series A) cultured on Med. A + S, and the remaining six (B to G) on Med. A - S, each series subcultured in steps from series A_1 :-

$$\begin{array}{ccccccccccccc} A_1 & \rightarrow & B_1 & \rightarrow & C_1 & \rightarrow & D_1 & \rightarrow & E_1 & \rightarrow & F_1 & \rightarrow & G_1 \\ & \searrow & & \searrow & & \searrow & & \searrow & & \searrow & & \searrow & \\ A_2 & & B_2 & & C_2 & & D_2 & & E_2 & & F_2 & & G_2 \end{array} .$$

An identical procedure was undertaken for P. heveae, P28, in the form of a homothallic 'cross', P28 x 28, using standard (9 cm.) petri dishes and incubating at 25°C (see Fig.18.). The cultures were examined for growth and sporulation 3, 5, 7 and 9 weeks after the inoculation of series A; the results are shown in Tables 25 and 26.

TABLE 25

The effects of Medium A with and without β-sitosterol upon sexual reproduction by P. palmivora cross P18 x 4 (20°C)

Series		Incubation period (weeks)		
		3	5	7 (to 9)
A.	Oogonia	++/+++	++/+++	+++
	% oospores	>50%	>50%	>50%
	Sporangia	++	++	++
B.	Oogonia	-	-	++
	% oospores	-	-	<1%
	Sporangia	++	++	++
C.	Oogonia	ND	-	++/+++
	% oospores		-	<1%
	Sporangia		++	++
D.	Oogonia	ND	-	++
	% oospores		-	<1%
	Sporangia		+/++	+/++
E to G.	Oogonia	ND	ND	++
	% oospores			<1%
	Sporangia			+/++

Q.E.M. used.

ND = no data.

TABLE 26

The effects of Medium A with and without β-sitosterol upon sexual reproduction of P. heveae P28(25°C).

Series		Incubation period (weeks)		
		3	5	7 (to 9)
A.	Oogonia (Q.E.M.)	++++	++++	++++
	frequency (N.E.M.)	81.9	ND	83.5
	% oospores	>50%	>50%	>50%
B.	Oogonia	-	+++	++++
	frequency	-	6.3	ND
	% oospores	-	<1%	<1%
C.	Oogonia	-	+++	++++
	frequency	-	9.7	79.2
	% oospores	-	<1%	<1%
D.	Oogonia	ND	+++	++++
	frequency		ND	ND
	% oospores		<1%	<1%
E.	Oogonia	ND	ND	++++
	frequency			ND
	% oospores			<1%
F.	Oogonia	ND	ND	++++
	frequency (N.E.M.)			87.8
	% oospores			<1%
G.	Oogonia	ND	ND	++++
	frequency			ND
	% oospores			<1%

ND = no data.

All the P. palmivora series were similar in appearance, with aerial mycelium ++ on the P4 side and +++ on the P18 side. In the plates of series B to G, however, hyphae were irregular, and oogonia only developed after 5 weeks incubation. These oogonia, which lacked oospores, were found amongst the aerial mycelium and not on the surface of the medium as in series A.

More detailed results were obtained from the P. heveae plates owing to the greater abundance of spores. P28 x 28 cultures were similar in appearance throughout series A to G (see Fig.18.), with aerial mycelium +/++; hyphal branching and growth was irregular in series B to G, and sporangia (++) were only formed in series A. In the absence of β-sitosterol, no oogonia were formed after 3 weeks incubation, but oogonia were frequent after 5 weeks and abundant after 7 weeks incubation; no oospores were formed in these oogonia(except in the repulsion zone between the two colonies). In contrast, abundant oogonia and oospores were formed within 3 weeks in the presence of the sterol (see Fig.19.).

Thus on medium containing β-sitosterol, both P. palmivora and P. heveae produced sex organs and oospores promptly, but in its absence, hyphal growth was irregular and sexual reproduction abnormal. There was no indication of any 'carry over' effect of sterol from series A to series B, since series B was similar to the other non-sterol series. The P28 oogonial frequency in series A after 3 weeks incubation was 81.9, and in series C and F after 7 weeks incubation were 79.2 and 87.8 respectively; these frequencies are comparable with the frequency of 82.4 obtained after

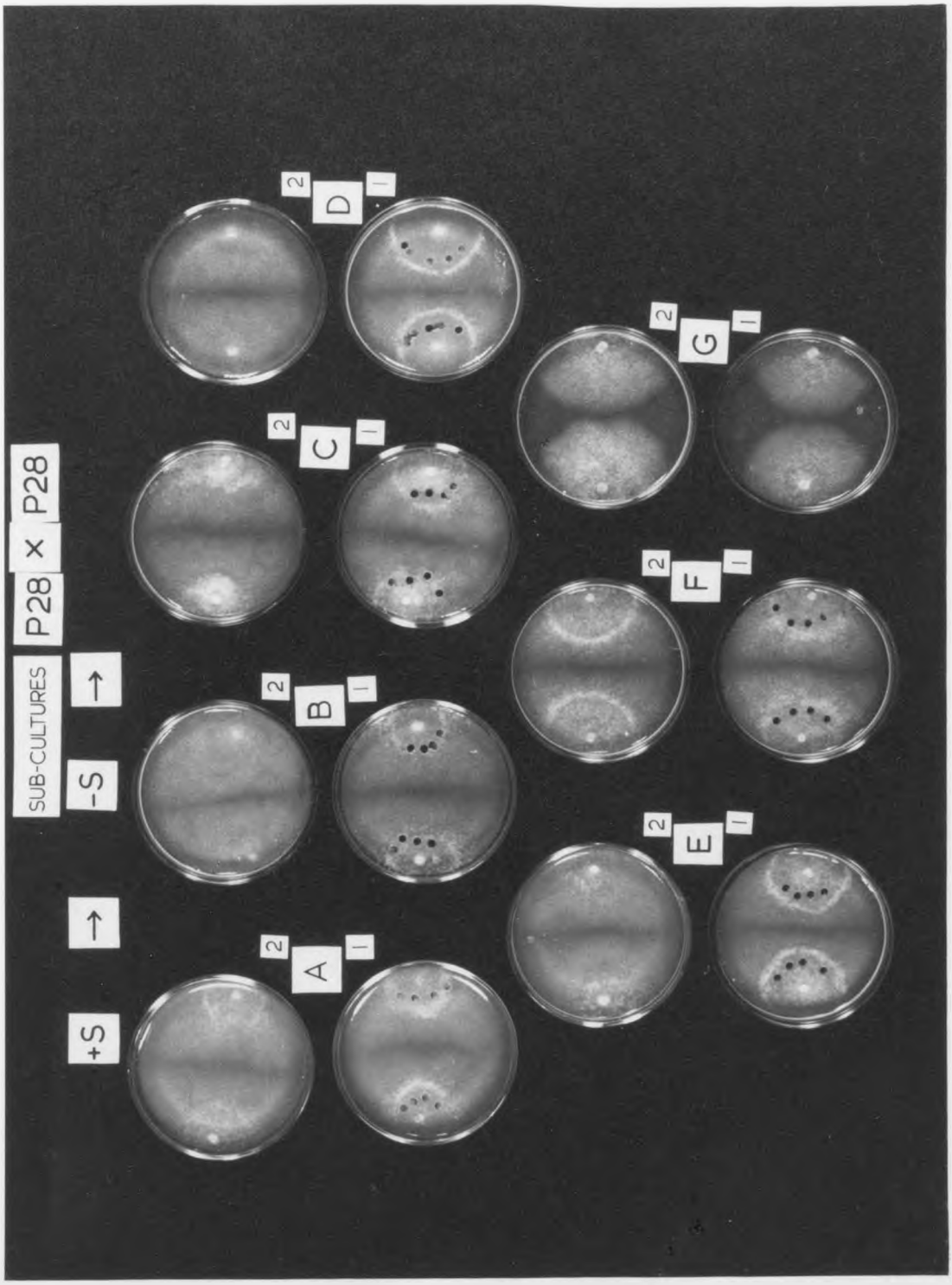


Fig.18. The effects of Medium A with and without β -sitosterol upon the growth of P. heveae (P28 x P28); series A, sterol 10 mg./l.; series B to G, no sterol. (White rings are aerial mycelium, produced as a result of brief exposure to light when subculturing).

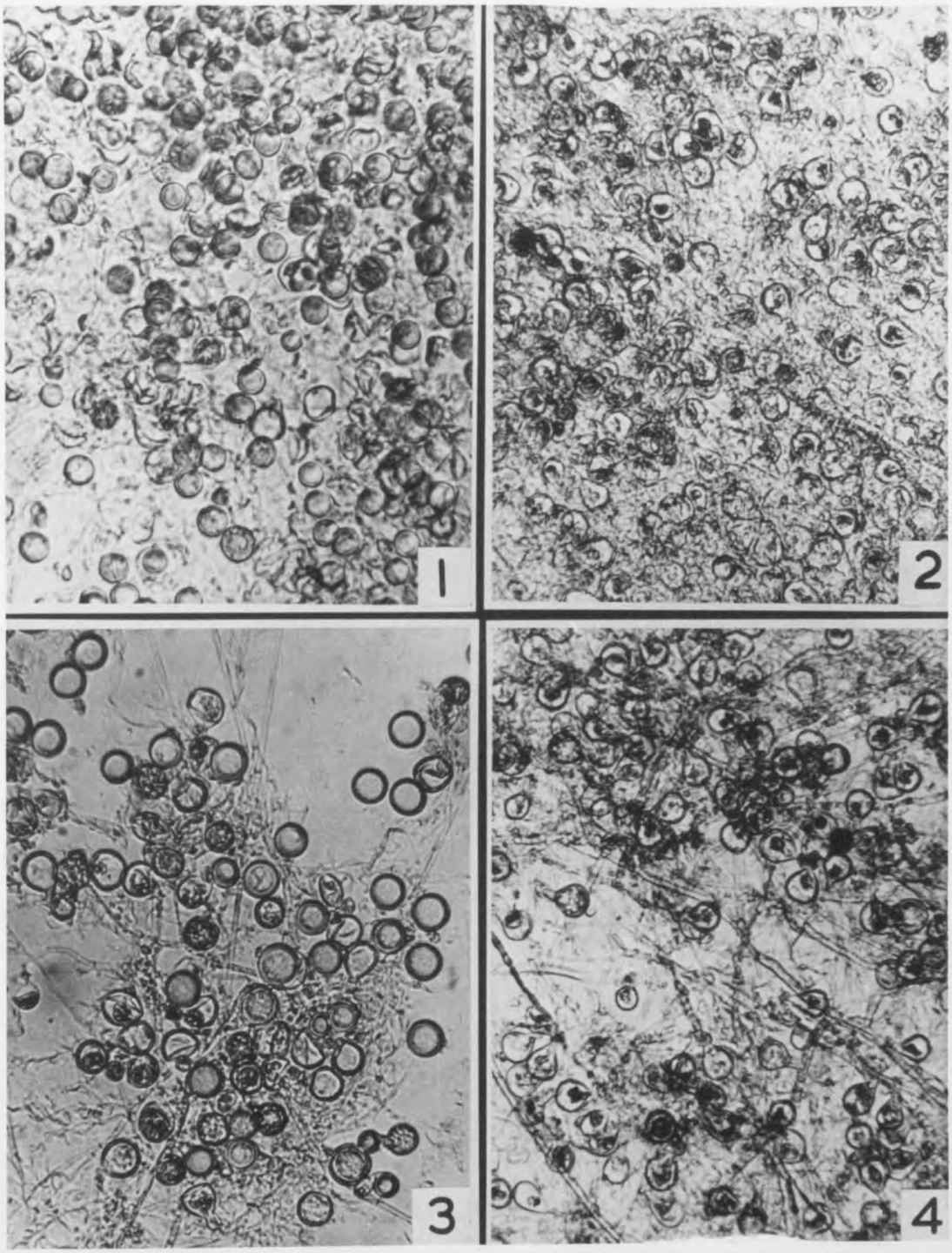


Fig.19. The effects of medium A with and without β-sitosterol upon sexual reproduction of P. heveae (P28 x P28); (L.P. = x 100 x 2.25); 1) series A, oogonia and oospores; 2) series C, oogonia lacking oospores; 3) series F, oogonia and oospores - repulsion zone between P28 colonies; 4) series F, oogonia lacking oospores - from middle of a colony.

3 weeks at 2 g./l. serine, 10 mg./l. β-sitosterol in Chapter 6, and suggest that oogonium formation, though delayed, remains influenced by nutrient concentration in the absence of the sterol.

Chapter 10. The effects of β-sitosterol concentrations upon oogonium formation by P. palmivora and P. heveae.

In the following experiment, in which various concentrations of β-sitosterol were substituted into Medium A, casamino acids were used (at 3 concentrations) in addition to serine (2 g./l.), since this was the best nitrogen source for sexual reproduction of P. palmivora (Chapter 6); the concentration of KH_2PO_4 was also varied, and the following 12 media prepared :-

Medium no.	1	2	3	4	5	6	7	8	9	10	11	12
Medium A (glucose 5 g./l.)	+	+	+	+	+	+	+	+	+	+	+	+
Casamino acids g./l.	1.5	2	2.5	1.5	2	2.5	2	2	———			
Serine 2 g./l.	———								+	+	+	+
KH_2PO_4 g./l.	0.5	0.5	0.5	0.5	0.5	0.5	2	2	0.5	0.5	0.5	0.5
β-sitosterol mg./l.	10	10	10	50	50	50	10	50	10	25	50	100

Two small (5 cm.) petri dishes of each medium were inoculated with P18 x 4 using Med. A + S inocula, and incubated at 20°C, and two standard (9 cm.) petri dishes of each medium were inoculated with P28 and incubated at 25°C. The cultures were examined for growth and sporulation after 21 days using the Quick Estimation Method; the results are shown in Table 27.

TABLE 27

The effects of β-sitosterol concentrations upon sexual reproduction of P. palmivora and P. heveae.

Medium no.	P18 x 4 Oogonia	P28 Aerial mycelium	P28 Oogonia	
1	+++	-	++++	
2	+++/++++	-	++++	(1)
3	+++	+	++++	(2)
4	+++	-	++++	
5	+++/++++	-	++++	
6	+++	-	++++	
7	+++/++++	-	++++	
8	+++/++++	-	++++	
9	++/+++	++	++++	
10	++/+++	+/++	++++	
11	++/+++	+/++	++++	
12	++/+++	+	++++	

Oogonium formation by P. palmivora was apparently unaffected by either β-sitosterol or KH_2PO_4 concentrations. At 0.5 g./l. glucose, the best concentration of casamino acids in Medium A was 2.0 g./l.; this was considerably better for sexual reproduction of P18 x 4 than 2.0 g./l. serine, irrespective of the sterol concentration. Sporangia were occasional (++) on all 12 media.

Oogonium formation by P. heveae varied little on the 12 different

media, and both sources of nitrogen were adequate. On the media containing serine, a slight increase in the density of oogonia, which resulted in a yellow/brown colouration around the inoculum, was observed at 50 and 100 mg./l., β-sitosterol suggesting that sterol concentration affected oogonial frequency.

From these and earlier results, the best simple medium for inducing the sexual reproduction of P. palmivora. consisted of casamino acids substituted into Medium A at a concentration of 2 g./l. The variation of β-sitosterol concentration, which had little effect upon the formation of oogonia by P. palmivora. did have some effect upon oogonium formation by P. heveae. This effect was examined in more detail in the next experiment.

Chapter 11. The effects of β-sitosterol concentrations upon growth and sporulation of P. heveae at 25 and 30°C.

Seven media were prepared by substituting β-sitosterol into Medium A at concentrations of 0, 1, 5, 10, 25, 50 and 100 mg./l. Four standard plates of each medium were inoculated with P28 using Med. A - S inocula, two incubated at 25°C (for oogonium formation), and two at 30°C (for sporangium formation). The plates were examined for sporulation and growth after 21 days, using both the Quick and Numerical Estimation Methods. The results are given in Tables 28 and 29, and Fig. 20.

TABLE 28

The effects of β-sitosterol concentrations upon sporulation and growth of P28 at 25°C.

β-sitosterol mg./l.	0	1	5	10	25	50	100
Aerial mycelium	++	++	++	++	+/++	+/++	+
Oogonia(N.E.M.)	-	++	+++	++++	++++	++++	++++
Oogonial frequency	-	0.7	14.9	81.9	103.4	126.3	132.9
Oospores(frequency)	-	0.2	9.9	65.6	66.5	61.0	58.5
Calculated % oospores	-	28	67	80	64	48	44
Sporangia (Q.E.M.)	-	++	++	++	++	+++	+++

The oogonial frequencies recorded after 21 days at 25°C rose with increase in sterol concentration from 0 to 50 mg./l. However, this apparent effect of sterol concentration controlling oogonial frequency was shown, in a previous experiment (Chapter 9), to be a temporary effect resulting from a "lag" in oogonium formation in the absence of sterol: after a longer incubation period (7 weeks), oogonia in plates lacking sterol reached a frequency close to that at 10 mg./l. β-sitosterol after 3 - 7 weeks incubation. This apparent limit to oogonial frequency at both 0 and 10 mg./l. β-sitosterol after 7 weeks incubation (a frequency of around 80, see Table 26) was exceeded in the present experiment after only 21 days incubation at higher β-sitosterol concentrations, suggesting that the sterol may effect some control over the initial rate of oogonium formation, and that this control appears after a short incubation period to be an effect on oogonial frequency.

Oospore frequencies recorded after 21 days rose up to 10 mg./l. β-sitosterol, but remained fairly constant above this concentration, possibly because other nutrients (nitrogen, carbon etc.) became limiting. Less aerial mycelium was formed at higher sterol concentrations, and sporangia were more frequent at 50 and 100 mg./l. than at 1 - 25 mg./l.; none were formed on 0 mg./l. In contrast, sporangia were frequent on 0 mg./l. at 30°C, but were exceptionally small (see Fig . 20). Sporangial frequencies were slightly higher at 25 - 100 mg./l. than at 0 - 10 mg./l., and sporangial size increased significantly with increase in sterol concentration up to 25 mg./l. Oogonial frequencies at 30°C were less affected, and the temperature induced absence of oospores at 30°C was unaffected, by alterations in sterol concentration.

TABLE 29.

The effects of β-sitosterol concentrations upon sporulation and growth of P28 at 30°C.

β-sitosterol mg./l.	0	1	5	10	25	50	100
Aerial mycelium	++	++	+/++	+/++	+/++	+/++	+
Oogonia (Q.E.M.)	+++	+++	+++	++++	++++	++++	++++
% oospores	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Sporangia (Q.E.M.)	+++	+++	+++	+++	+++/++++	++/++++	+++/++++

This experiment confirmed the observation (in the previous experiment) that sterol concentration might control oogonial frequency of

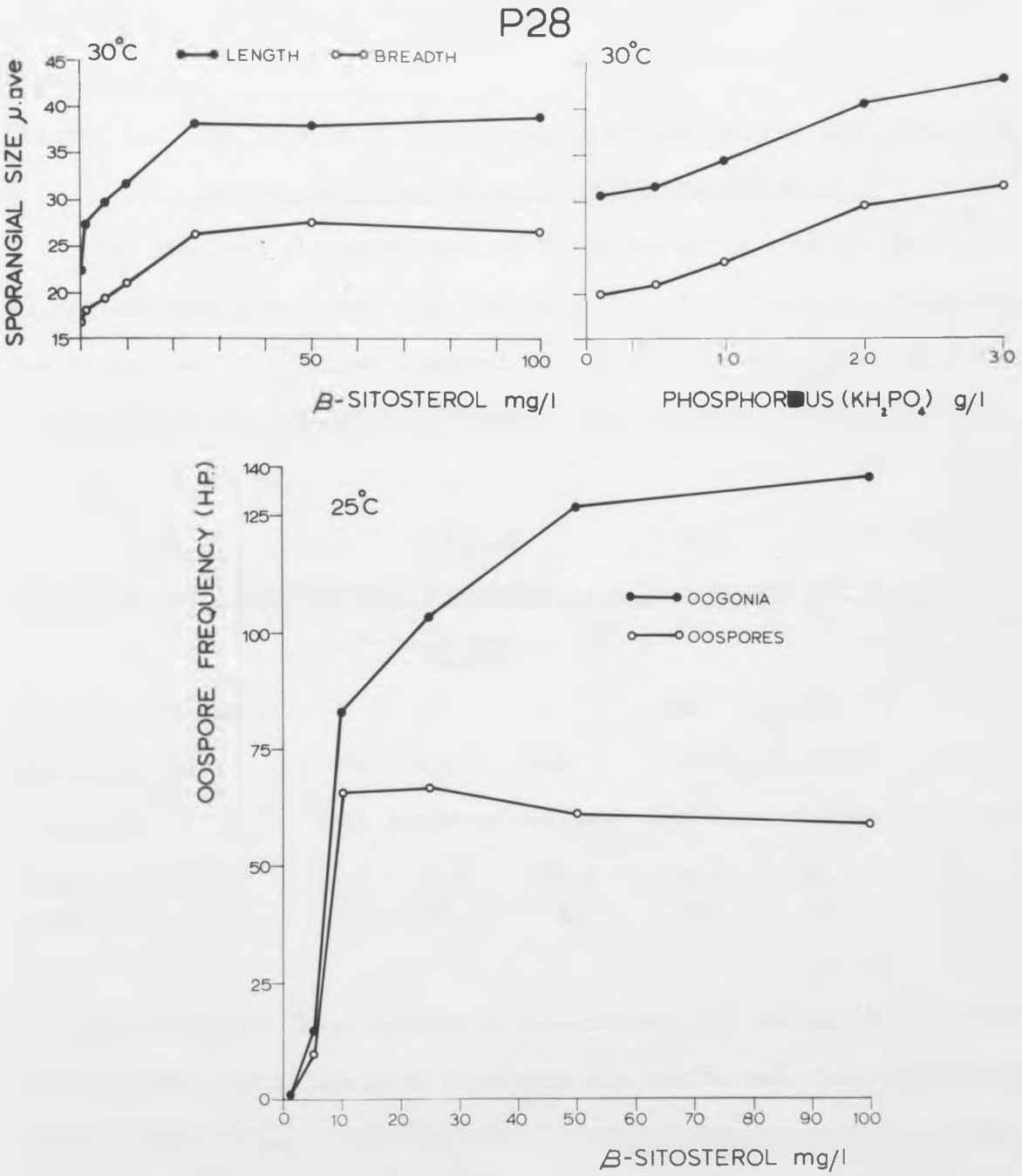


Fig.20. The effects of β-sitosterol and KH_2PO_4 concentrations upon oogonium and oospore formation (25°C), and sporangial size (30°C), of P. heveae isolate P28.

P. heveae at 25°C; this effect was not repeated at 30°C, however, possibly as a direct result of the many more sporangia formed at this temperature.

Chapter 12. The effects of β-sitosterol concentrations upon growth and sporangium formation of P. palmivora, P24, at 27.5°C.

Two standard plates of each of the seven media used in the last experiment were inoculated with P24 using Med. A - S inocula, incubated for 21 days at 27.5°C, and examined for growth and sporangium formation using the Numerical Estimation Method. The results are given in Table 30 and Figs. 21 and 22.

TABLE 30.

The effects of β-sitosterol concentrations upon growth and spore formation of P24.

β-sitosterol mg./l.	0	1	5	10	25	50	100
Aerial mycelium	+++	+++	+++	+++	+++	+++	+++
Sporangia	++++	+++/++++	+++/++++	+++/++++	+++/++++	+++/++++	+++/++++
Frequency(N.E.M.)	38.2	25.9	27.0	26.1	26.2	25.0	23.3
% contents	25	26	45	62	85	98	96

No effect of β-sitosterol concentration upon radial growth rate was observed, and aerial mycelium formation was unaffected; but as previously observed for P18 and P28 (in Chapter 9), hyphal growth was irregular on 0 mg./l. The mean sporangial size rose as the β-sitosterol concentration was increased, especially between 0 and 1 mg./l. where the sharp size increase

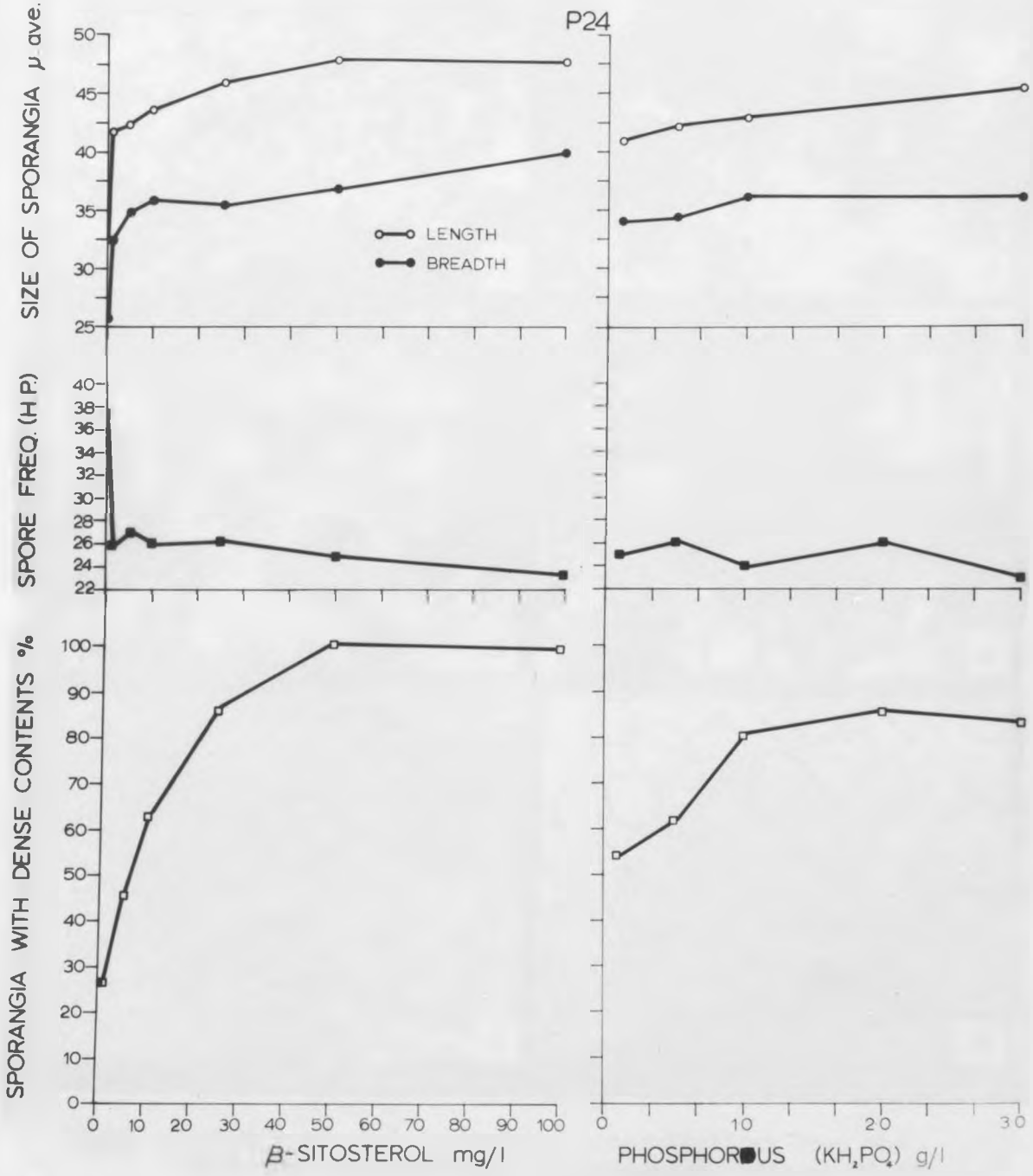


Fig.21. The effects of β-sitosterol and KH_2PO_4 concentrations upon sporangium formation and sporangial size of P. palmivora isolate P24.

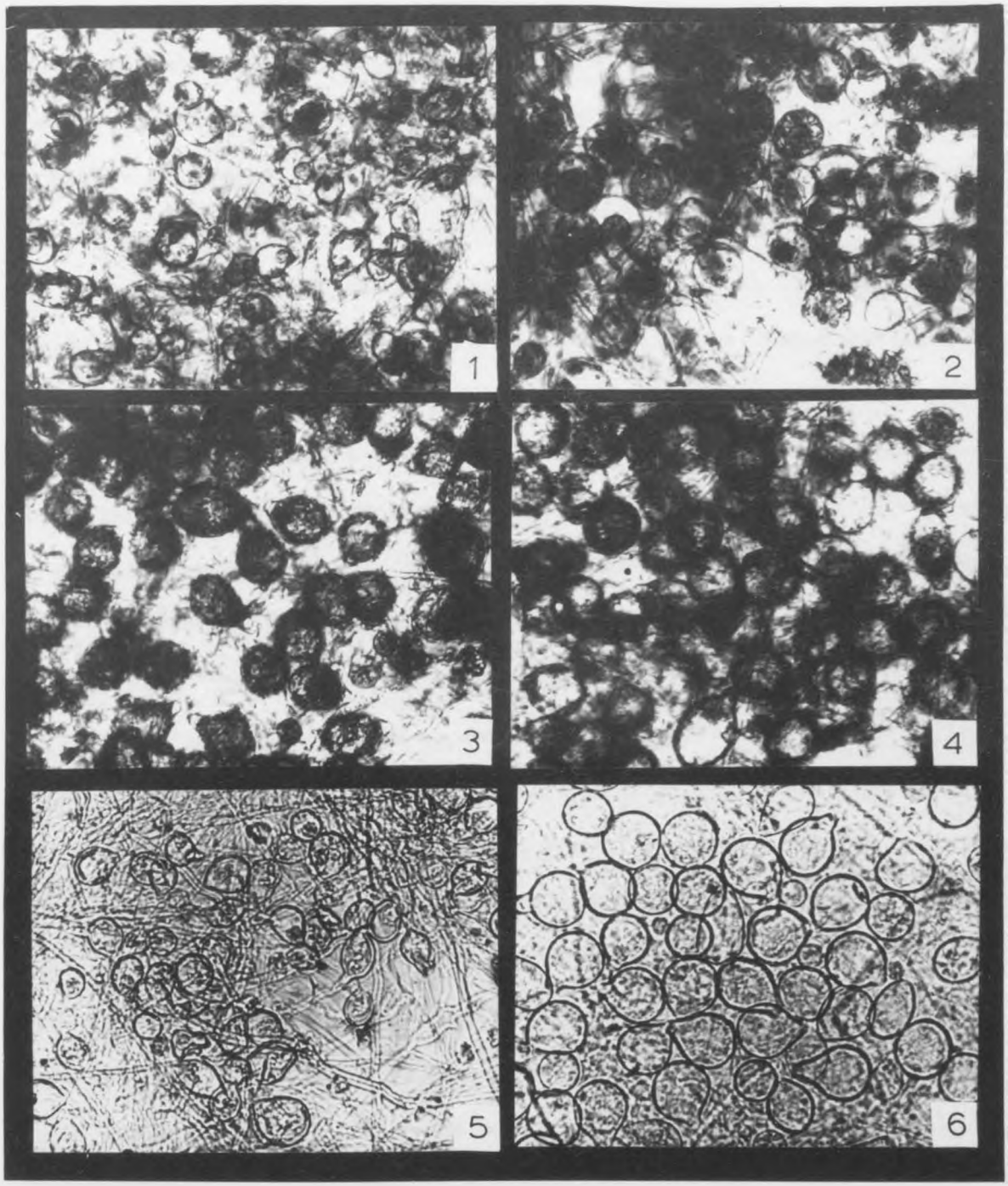


Fig.22. The effects of β-sitosterol concentrations upon the formation of sporangia by P. palmivora. P24, at 27.5°C (low power = x 100 x 2.25); 1), 2), 3) and 4) 0, 1, 25 and 50 mg./l. β-sitosterol to show size and contents of sporangia; 5) 0 mg./l. to show size of sporangia; 6) 100 mg/l. β-sitosterol to show size of sporangia.

was accompanied by a simultaneous fall in sporangial frequency. Thus the loss of 'sporangial volume' resulting from the formation of very small sporangia at 0 mg./l. was partially compensated by a higher sporangial frequency.

The concentration of β-sitosterol also affected the internal organisation of the sporangia in a manner similar to that observed with different KH_2PO_4 concentrations (Chapter 8). A certain proportion of internally disorganised sporangia were observed at each concentration: characteristically, the protoplasts of such sporangia had either shrunk to a small residual mass (remaining in the centre or near the papillum), or disintegrated into small peripheral clumps; other sporangia were empty of contents. The papillae of such sporangia were often lost, or were degenerate and saw-edged in appearance. The percentage of sporangia retaining normal contents (after 21 days) increased over 0 - 50 mg./l. and was clearly related to the β-sitosterol concentration. The density of the protoplasm, estimated by the relative depth of brownness of the sporangia, also increased as sterol concentration increased. Thus sporangia with apparently less dense protoplasm at lower sterol concentrations degenerated in culture more rapidly than those at higher concentrations.

Sterol concentrations were not active in controlling oogonial frequencies of P. palmivora, P18 x 4, in a previous experiment (Chapter 10), and from the results of the present experiment appear to exert a more significant affect upon internal control of the fungus. The affect of sterols upon P24 sporangial organisation poses the problem, investigated in the next Chapter, of their affect upon direct germination of sporangia

(by zoospores) via the papillum.

Chapter 13. The effects of β-sitosterol concentrations upon sporangium germination in solid/liquid cultures of P. palmivora,P24.

The effects of β-sitosterol upon P24 sporangium size and internal organisation observed in the last experiment suggested that,in the absence of sterol, zoospore release in liquid cultures might be inhibited as a result of poor protoplasmic differentiation or abnormal papillum structure. This possibility was investigated in the following experiment.

Five media were prepared by substituting β-sitosterol into Medium A at concentrations of 0, 10, 25, 50 and 100 mg./l., and 2 standard plates of each medium inoculated with P24 using Med. A - S inocula and incubated at 27.5°C. Two 1 cm. diameter agar plugs were removed from the edge of each growing colony after two days, and individually transferred to a small (5 cm.) petri dish. Each dish was irrigated with sterile distilled water level with the surface of the inoculum disc, and incubated at 27.5°C for 7 days to allow development of mycelium and zoosporangia. The cultures were then examined for growth and sporangium formation using the Quick Estimation Method, cooled for 10 minutes at 10°C to induce zoospore formation and emergence, and examined for zoospores after 1 hour at room temperature. The results, given in Table 31 and Fig. 23, showed that zoospore release was inhibited in the absence of β-sitosterol.

At 0 mg./l. zoosporangia,though abundant,were extremely small, produced no zoospores and often contained disorganised contents which were occasionally extruded as an undifferentiated mass; the free protoplasts

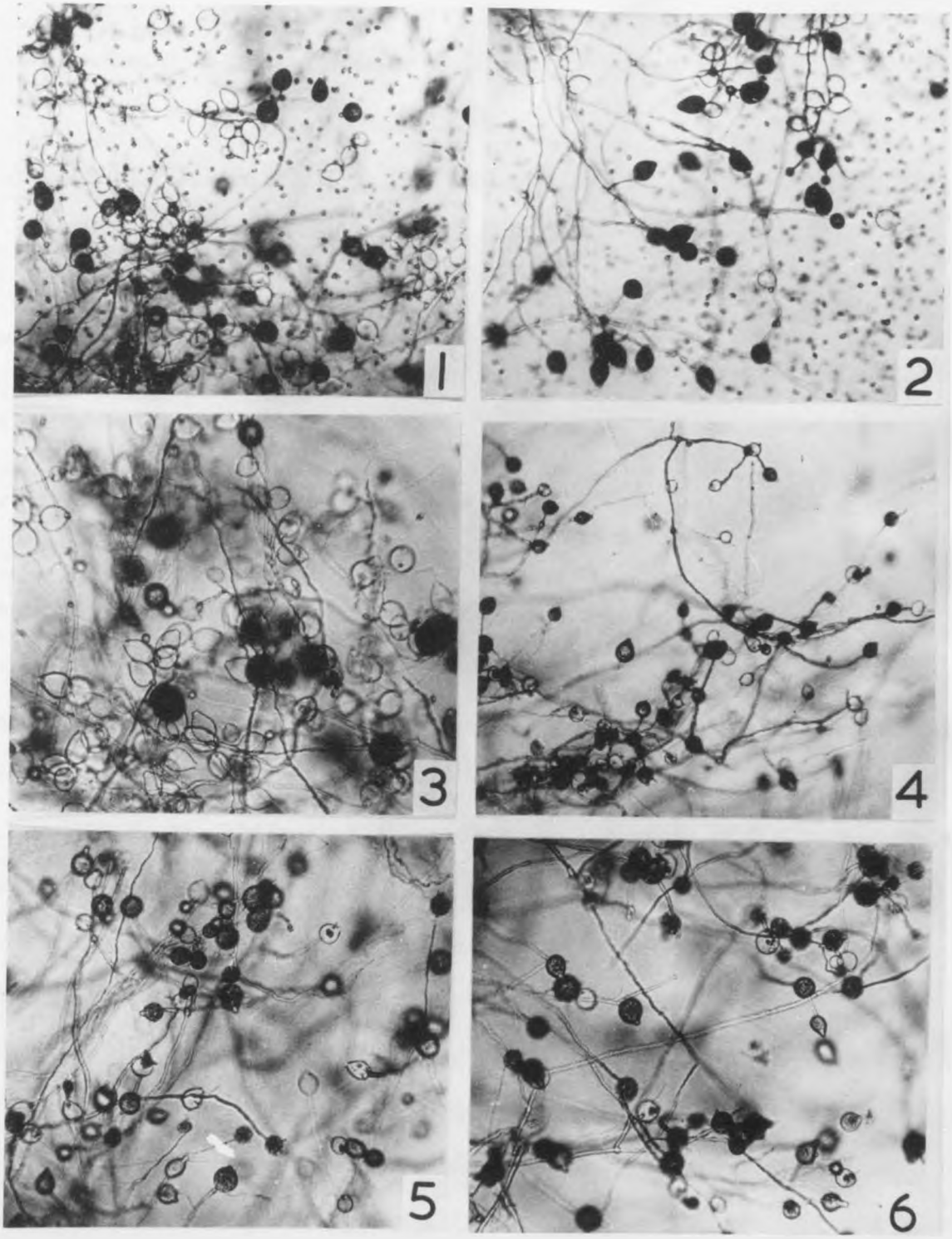


Fig.23. The effects of β-sitosterol concentrations upon zoospore formation by P. palmivora isolate P24; 1) and 2) 10 mg./l., sporangia large, zoospores formed (x 100); 3) 50 mg./l., sporangia large, zoospores formed (x 400); 4) 0 mg./l., sporangia small, no zoospores (x 100); 5) and 6) 0 mg./l., sporangia small, contents abnormal, no zoospores (x 400).

either adhered to the ostiole or sank to the bottom of the dish.

TABLE 31.

The effects of β-sitosterol upon zoospore release by P. palmivora P24.

β-sitosterol mg./l.	0	10	25	50	100
Zoosporangia	++++	++++	++++	++++	++++
Size of zoosporangia	small	large	large	large	large
Zoospores released	-	+++/++++	++++	++++	++++

In contrast, zoosporangia at 10 mg./l. were considerably larger and zoospore release was normal; those at 25 - 100 mg./l. were still larger and discharged a slightly greater number of zoospores (as estimated from their density after 120 minutes).

Thus in the absence of β-sitosterol, sporangia of P24 were unable to differentiate to form zoospores, but occasionally discharged their contents, suggesting that sterols are required for normal protoplasmic organisation within the sporangium.

Chapter 14. The combined effects of light and β-sitosterol upon growth and sporulation of P. palmivora and P. heveae.

Earlier experiments, described in Section I, demonstrated that both temperature and light exerted quantitative and qualitative controls upon reproduction of P. palmivora and P. heveae. and in the present section it has been shown that nutrients and growth substances, such as serine,

KH_2PO_4, and β-sitosterol, can exert similar controls. The sterol was particularly interesting because of its activity in very small concentrations, and for this reason the interplay of this chemical reproductive stimulant with a physical stimulant, light, was investigated in the following experiment.

Two media, Med. A + S and Med. A - S, were used, and four standard plates of each medium inoculated singly with P4, P18, P24 and P28 using Med. A - S inocula. Two plates were incubated at 22.5°C in the light, and two at 22.5°C in the dark. After 21 days the cultures were examined using the Quick Estimation Method. The results are shown in Tables 32 and 33, and Fig. 24.

TABLE 32.

The quantitative effects of light, darkness and β-sitosterol upon sporulation of P. palmivora and P. heveae.

Isolate / Conditions	P4	P18	P24	P28
- S LIGHT				
Sporangia	+++	+++/++++	+++++	+++
Oogonia				-
+ S LIGHT				
Sporangia	+++	+++/++++	+++++	+++/++++
Oogonia				-
- S DARK				
Sporangia	-	++	++++	-
Oogonia				- (* +++ at 4 weeks)
+ S DARK				
Sporangia	-	++	+++/++++	-
Oogonia				++++

* no oospores

TABLE 33.

The qualitative effects of light, darkness and β-sitosterol upon sporulation of P. palmivora and P. heveae.

Isolate	Conditions		Effects
P4	- S	L	Sporangia small, rounded, poorly papillate; protoplasmic contents thin, disorganised, or lacking.
P4	+ S	L	Sporangia large, elongated, with prominent papillae; protoplasmic contents dense and granular.
P4	- S	D	No sporangia.
P4	+ S	D	
P18	- S	L	Sporangia small, rounded, poorly papillate; protoplasmic contents thin, disorganised, or lacking.
P18	+ S	L	Sporangia large, elongated, with prominent papillae; protoplasmic contents dense and granular.
P18	- S	D	Sporangia small, rounded; protoplasmic contents thin, disorganised, or lacking.
P18	+ S	D	Sporangia large, rounded, poorly papillate; protoplasmic contents dense and granular.
P24	- S	L	Sporangia small; protoplasmic contents thin, disorganised,or lacking.
P24	+ S	L	Sporangia large; protoplasmic contents dense and granular.
P24	- S	D	Sporangia small to very small; protoplasmic contents thin, disorganised, or lacking.
P24	+ S	D	Sporangia large; protoplasmic contents dense and granular.
P28	- S	L	Sporangia small, papillate; protoplasmic contents mostly disorganised or lacking.
P28	+ S	L	Sporangia large, with prominent papillae; protoplasmic contents dense and granular.
P28	- S	D	Oogonia lacking oospores.
P28	+ S	D	Oogonia with oospores.

L = light.

D = dark.

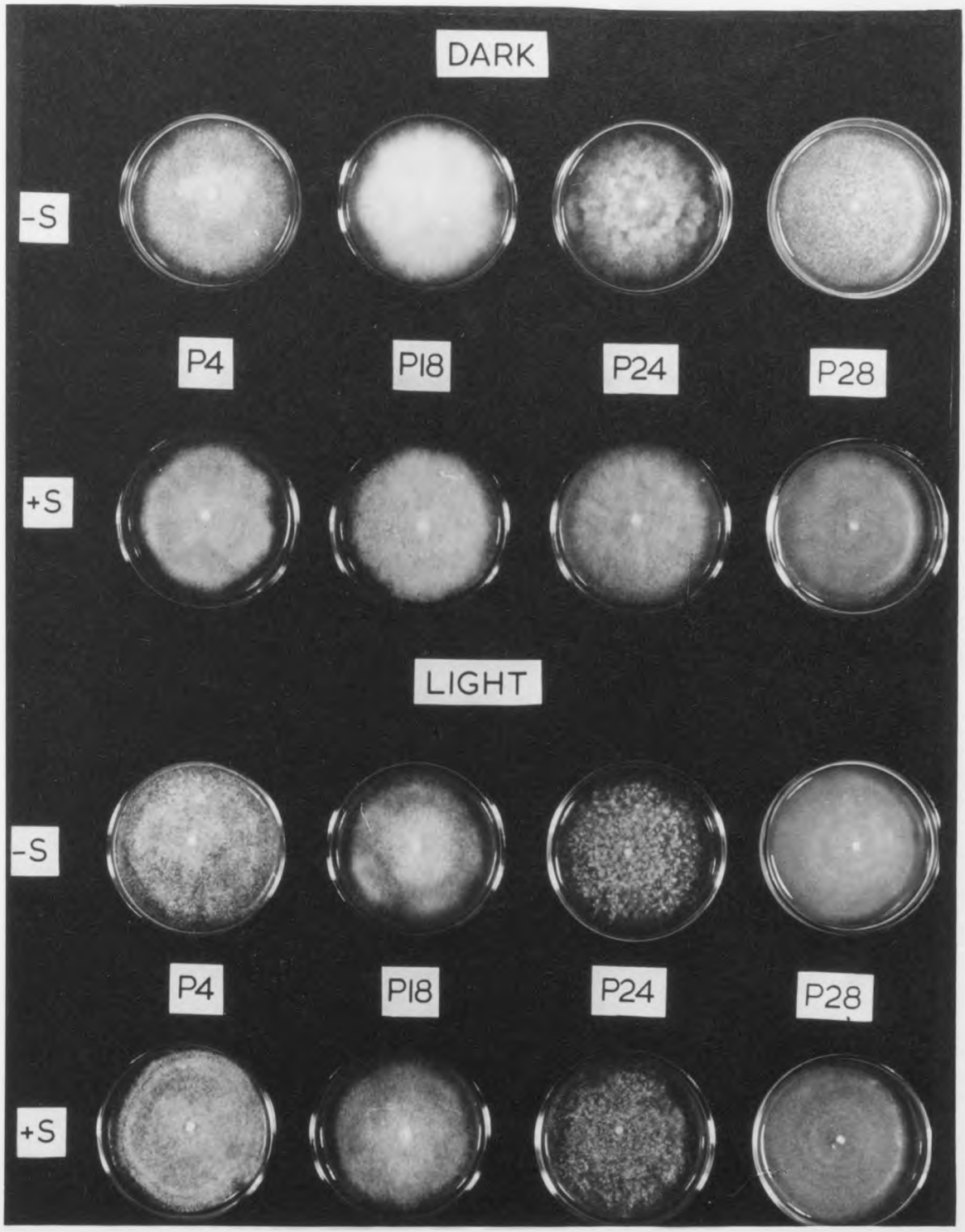


Fig.24. The effects of light, darkness and β-sitosterol upon growth of isolates P4, P18, P24 and P28; - S, no sterol; + S, 10 mg./l. β-sitosterol.

It is evident from Fig. 24 that the fluffy aerial mycelium formed by P4, P18 and P24 in darkness, and the frosty appearance induced by light, were not greatly affected by the presence or absence of β-sitosterol. Further, higher sporangial frequencies, the inhibition of P28 oogonium formation, and the formation of elongated strongly papillate sporangia by P4, P18 and P28, all resulting from exposure to light (and observed in previous experiments), were relatively unaffected by the presence or absence of sterol.

Similarly, certain effects resulting from the presence or absence of β-sitosterol were not altered by exposure to light or darkness. These factors included irregular hyphal growth and the formation of small internally disorganised sporangia in the absence of sterol, and the formation of larger sporangia in the presence of sterol.

The experiment demonstrated little interaction between the two environmental factors, light and β-sitosterol, and indeed the effects of the two factors, one physical and the other chemical, remained independent, suggesting that their mechanisms of action are distinct.

Chapter 15. The effects of serine concentrations upon growth and sporulation of P. palmivora, P24, and P heveae, P28, in the absence of sterol.

In Chapter 6, a range of serine concentrations substituted into Medium A (β-sitosterol 10 mg./l.) demonstrated quantitative effects of nitrogen concentration upon oogonial frequencies of P28 and sporangial frequencies of P24. Further experiments using 2g./l. serine have

demonstrated quantitative and qualitative effects of β-sitosterol upon reproduction of these two isolates (see Chapters 9, 11, 12 and 13), and in the last experiment the interaction of light and sterol effects were investigated. In the following experiment, the effects of a range of serine concentrations upon P24 and P28 were re-examined in the absence of sterol. Seven media were prepared by substituting DL-serine into Med. A - S at 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 g./l. Two standard plates of each medium were inoculated with P24 using Med. A - S inocula and incubated for 21 days at 27.5°C, and two with P28 incubated at 25°C for the same period. The plates were examined using the Quick and Numerical Estimation Methods, and the results are given in Tables 34 and 35, and Fig. 17.

TABLE 34.

The effects of serine concentrations upon growth and spore formation of P28 at 25°C in the absence of sterol (c.f. Table 18 and Fig. 16).

Serine g./l.	0.1	0.5	1.0	2.0	3.0	4.0	5.0
Aerial mycelium	+	+	++	++	++/+++	++/+++	++/+++
Sporangia	-	-	-	-	-	-	-
Oogonia(N.E.M.)	+++	-	-	-	++++	+++/++++	+++/++++
% oospores	± 20%	-	-	-	<1%	<1%	<1%

From Table 34 it is clear that the 'lag [illegible]' in P28 oogonium formation on Med. A - S, previously observed at 2 g./l. serine in Chapter 9, and here also apparent at 0.5 and 1.0 g./l., was absent at

high serine concentrations (3 - 5 g./l.) where oogonia were formed promptly. The usual absence of oospores on Med. A - S was unaffected by serine concentration (except at 0.1 g./l.). Since no oogonia were formed at 0.5 - 2.0 g./l. after 21 days, no optimum serine concentration was obtained, but oogonia were abundant at 3.0 g./l. The effects of serine concentrations upon mycelial growth were similar to those when sterol was present, though slightly more aerial mycelium was formed at higher serine concentrations. No sporangia were formed at any serine concentration however.

TABLE 35.

The effects of serine concentrations upon growth and sporulation of P24 at 27.5°C in the absence of sterol (see Figs.15 and 17).

Serine g./l.	0.1	0.5	1.0	2.0	3.0	4.0	5.0
Aerial mycelium	+/++	++/+++	+++	+++	+++	++/+++	++
Sporangia	+++	+++	+++/++++	++++	+++	+/++	+

In contrast to the large sporangia usually formed at 10 mg./l. β-sitosterol (see Chapters 6 and 12), the sporangia of P24 were very small and their protoplasts thin and disorganised at all serine concentrations. Further, sporangial frequencies on these Med. A - S plates were significantly higher than those previously obtained on Med. A + S (Chapter 6). Nevertheless, the basic effect of increase in sporangial frequency with increase in serine concentration, with an optimum serine concentration close to 2 g./l. was similar to that observed

on Med. A + S in Chapter 6 (see Fig. 17). Thus generally higher sporangial frequencies on Med. A - S were partially offset by a reduction in sporangial size. Sporangial size and contents were unaffected by serine concentration. Mycelial growth in this experiment was similar to that on Med. A + S (see Fig. 15).

The experiment demonstrated that the effects of nitrogen (serine) concentrations upon spore frequencies of P24 and P28 were not dissimilar in the absence of sterol, and that the effects of sterols upon spore structure were relatively unaffected by nitrogen concentration.

Chapter 16.

DISCUSSION

Until recently, the sexual stage of Phytophthora was unobtainable on simple media, and as a result most of the earlier nutritional work was devoted to aspects of vegetative growth and reproduction. The technical approach was very varied. Often, large numbers of carbon and nitrogen sources were substituted into various basal media, frequently at fixed or equivalent concentrations. Many species and sometimes large numbers of isolates were used (see Volkonsky, 1934; Mehrotra, 1951; Wills, 1954; Lapatecki and Newton, 1956; Christie, 1958, 1961; Sakai, 1961; Erwin and Katzenelson, 1961; Cameron and Milbraith, 1965; Roncadori, 1965; etc.). The present work has indicated that serious limitations are incurred if ranges of nutrient concentrations are not tested. The experiments have therefore been more quantitative in approach than those of most other workers. Only a few nutrient sources were examined in any detail, and particular attention was paid to the nutrition of sexual reproduction and the sterol problem. Any comparison of the present results with those of other authors (other than those associated with the sterol problem) would therefore be of limited value owing to the separate intent. For this reason problems relating to carbon and nitrogen nutrition (already examined in the text)

will not be discussed in detail.

As a simple medium for obtaining oospores of Phytophthora palmivora, a casamino acids/glucose/sterol medium is preferable to a serine medium because of the consistently better results obtained. Although the vitamin-free casamino acids medium is not completely defined, owing to the indefinite amino-acid composition of hydrolysed casein, it has proved adequate for obtaining oogonia from all compatible crosses tested, and has subsequently been used during work upon the mechanism of sexuality in this organism (see Section III). Any further refinements of this medium would involve the determination of specific amino acid requirements for reproduction, possibly for each isolate. Auxanographic techniques (not described) were attempted but were unsuccessful. Difficulties inherent in this problem are apparent from the differing responses to serine and asparagine by three P. palmivora isolates (Chapter 6). However, the assortment of amino-acids supplied by hydrolysed casein is probably no less likely to supply the requirements of a particular isolate than a pre-determined mixture. The homothallic P. heveae was noticeably less demanding in its nutrient requirements for sexual reproduction than P. palmivora, probably because in the latter, two isolates and a complex mating reaction are involved.

Little attention has been paid by other workers to the effects of nutrient concentrations upon Phytophthora. Experiments investigating the concentration effects of five nutrients (asparagine, serine, glucose,

KH_2PO_4, and β-sitosterol) upon growth and reproduction of the four isolates, demonstrated that carbon and nitrogen sources exerted significant effects upon quantitative aspects of development, such as mycelial growth and spore frequency; KH_2PO_4 and β-sitosterol affected more qualitative aspects, such as spore size and contents. These points are summarised in Table 36.

TABLE 36.

The relative effects of some nutrients upon growth and reproduction of P. palmivora, P24, and P. heveae, P28.

Nutrient	Nitrogen (serine)	Carbon (glucose)	KH_2PO_4	β-sitosterol
Aerial mycelium	+	+	-	+
Sporangial frequency	+	+	-	+
Sporangial size	-	-	+	+
Sporangial contents	-	-	+	+
Zoospore release	ND	ND	ND	+
Oogonial frequency	+	+	-	+
% oospores	-	-	+	+

ND = no data; + marked effect; - no marked effect.

Since carbon and nitrogen are major structural components of cells, quantitative effects of concentrations are perhaps to be expected. With both these elements, high concentrations suppressed sporulation,

and with glucose this was clearly related to excessive vegetative growth, resulting perhaps in exhaustion of other nutrients, and accumulation of staling substances,as suggested by Hawker (1957). The two nitrogen sources, asparagine and serine,not only exerted clear optimum concentration effects, but differed markedly from each other in their effects upon sporulation,both per gram nitrogen per litre and at their optima. Clearly a wide range of concentrations must always be used when testing nutrient sources.

Little is known about the fungal nutrition of the major mineral nutrients magnesium, potassium, phosphorus and sulphur. In the trial experiment involving KH_2PO_4 concentrations, sporangial size and contents and oospore contents were affected. Phosphorus is known to play a major role in primary metabolism, especially in glucose utilisation (Cochrane, 1958). It is therefore possible that these effects are secondary effects relating to phosphorus availability. However, more work would be required to distinguish between the individual effects of both phosphorus and potassium.

Much current interest has figured in the effects of sterols upon Phytophthora isolates. It is probable that the especially good sexual reproduction obtained on vegetable oil media (see Chapter 3) was partly due to the high sterol content of the oils. Similar effects were observed by Haskins et al (1964), Hendrix and Apple (1964) and Klemmer and Lenney (1965). It is also possible that a further non-sterol

growth promoting factor is present in complex oils. Sakai (1961) working with P. infestans detected a lipid growth promoting factor additional to cholesterol in cow liver, and Klemmer and Lenney (1965) have reported a non-sterol substance from oils stimulating sexual reproduction in other Phytophthora species. The chemical and physical factors influencing growth of P. parasitica v. nicotianae on vegetable oils have been investigated and discussed by Hendrix et al (1966). It would be of interest to know whether lipids are used as a major carbon source in nature. If so, production of exogenous lipases by P. heveae and P. palmivora. and also by P. parasitica v. nicotianae (Hendrix and Apple, 1964) may be of pathogenic significance. The general role of lipids in growth, reproduction, and metabolism, and the possible existence of a further growth promoting factor, are problems requiring further investigation.

Despite the investigations of many other workers (including Sakai, 1961; Haskins et al. 1964; Harnish et al. 1964; Hendrix, 1964; Elliott et al. 1964; Leal et al. 1964; Klemmer and Lenney, 1965; Hendrix, 1965; Chee and Turner, 1965; Hendrix et al. 1966; Hendrix,1966; Elliott et al. 1966) into the relationships between Phytophthora and sterols and the recording of many interesting phenomena, the true function of these substances in development remains obscure. The more striking effects of β-sitosterol upon P. palmivora and P. heveae. already described, may be briefly summarised as follows :-

1) The activity of β-sitosterol is detectable at low concentrations (1 mg./l.).

2) Hyphal growth is irregular in the absence, and normal in the presence of β-sitosterol.

3) Sporangia tend to be unusually small, though more frequent, in the absence of β-sitosterol; β-sitosterol concentrations affect sporangial size and frequency.

4) The protoplasmic contents of sporangia are affected by β-sitosterol concentration.

5) Zoospores are not produced (by P. palmivora P24) in the absence of β-sitosterol.

6) Oogonium formation and frequency (P. heveae P28) may be affected by β-sitosterol concentration.

7) Oospores are not formed in the absence of β-sitosterol.

In some cases effects similar to these have been observed by other workers, usually with different Phytophthora species, but in general little other work has been published dealing specifically with concentration effects of sterols. Leal (1965) demonstrated that the concentration of active substances in peas and oats affected the rate of formation of fusion organs by P. heveae and P.cactorum. Similarly, Klemmer and Lenney (1965) reported an effect of sterol (wheat germ oil fraction) concentrations upon oogonium formation in Pythium graminicola. Hendrix (1965) reported effects of cholesterol upon asexual reproduction of several Phytophthora species that are similar to those described above. Most isolates he tested produced sporangia abundantly in the

presence of sterol. On non-sterol media the sporangia were either absent, or if present they were abnormal or small, pale and more numerous. P. parasitica sporangia produced from non-sterol media did not release zoospores. Further, sexual reproduction was usually stimulated by cholesterol, and abortive oogonia were sometimes produced on sterol-free media.

In addition, sterols have been noted to stimulate mycelial growth (e.g. Sakai, 1961) and affect mycelial dry weights (e.g. Elliott et al. 1966). The activity of sterols in stimulating oogonium and oospore formation has been related to the structural configuration of the sterol molecule (Haskins et al. 1964; Elliott et al. 1966). It has also been shown that exogenous supplies of sterols are required by Phytophthora isolates because of a probable complete block in sterol biosynthesis (Elliott et al. 1964; Hendrix, 1966).

These diverse effects suggest that sterols cannot be considered only as reproductive stimulants, but rather as metabolic regulators exerting secondary effects upon many aspects of Phytophthora development. It is interesting to note that the distinct 'lag' in the formation of oogonia by P. heveae on a non-sterol medium (Medium A - S) resembles that described by Fries (1965) for mycelial growth in the absence of a typical dispensable growth factor. Indeed, the activity of sterols in very small concentrations, continued through irregular growth in their absence, and the complete block in sterol biosynthesis, do indicate that sterols may fulfil a role as some form of non-essential growth factor.

Sterol concentration effects upon mycelial growth rate, oogonium and oospore formation, and the size and internal organisation of sporangia, suggest a form of general metabolic control. It may be significant that effects of KH_2PO_4 concentrations and thus of phosphorus concentrations upon sporangia in some ways resemble those of β-sitosterol concentrations (see Table 36), since phosphorus is of importance as an energy source in many key metabolic pathways. Activity in very small concentrations might even be interpreted as suggestive of hormonal (and thus regulatory) control. Indeed, steroid hormones are widespread in animals. In this respect, and in view of the non-essential role of sterols in Phytophthora. the statement by Selye (1947) that "hormones do not bring about any essentially new metabolic activity but merely regulate the course of phenomena which can progress to some extent even in their absence" may be of some significance. Recent work suggests that animal steroids act either by affecting membrane permeability or by activating an enzyme (Villee and Engel, 1961). Sterols have also been implicated in phospholipid-sterol complexes in cell membranes (Engström and Finean, 1958). If sterols fulfilled such roles in Phytophthora. indirect effects upon hyphal structure, mycelial growth, spore size and organisation, and zoospore formation might be explained in terms of direct effects upon cell membrane and cell wall structure or certain enzyme systems, and related effects upon nutrient uptake and general metabolism. However, in view of the limited evidence accumulated

so far, theories pertaining to the function and mode of action of sterols, both in Phytophthora and in other organisms (see review by Buetow and Levedahl, 1964), are necessarily circumspect. A fresh approach to the problem is needed, and Hendrix (1966) has suggested two aspects requiring further research: firstly, the nature of the metabolic blocks in sterol biosynthesis and secondly, the function of sterols in metabolism.

In Section I, two physical environmental factors, temperature and light, were shown to exert both qualitative and quantitative control upon the sporulation of the four isolates tested. Investigation of the chemical (nutritional) environment has again demonstrated both qualitative and quantitative effects. In this case the physical environment was kept reasonably constant. Results have also shown, however, within one experiment, that specific effects of physical and chemical environmental factors, such as light and dark, sterol and no sterol, in reciprocal combinations (see Tables 32 and 33), can be distinguished. Further, the probable effects of individual chemical components of the environment can be resolved, as say in the effects of serine concentrations upon sporangium formation, both in the presence and absence of β-sitosterol (see Fig. 17). Nevertheless, despite measurable effects attributable to specific environmental components, whether physical or chemical (see Table 36), the overall balance of the components and their interaction with the genotype of the isolate

must be of considerable, if undetermined significance, influencing the internal environment of the fungus and, ultimately, the phenotype.

SECTION III

The mechanism of sexual reproduction in the 'palmivora' group.

Chapter 1.

INTRODUCTION

In addition to taxonomic divisions, based on morphological characteristics, Phytophthora species can be broadly divided into two groups on the basis of their ability to form oospores in single isolate culture. One group is homothallic and produces sex organs and oospores promptly and abundantly (see definitions on page 15). Of these species, some have paragynous antheridia (e.g. P. cactorum), some amphigynous antheridia (e.g. P. heveae), and some have both amphi- and paragynous antheridia (e.g. P. fragariae; Waterhouse,1963). In the second group oospores are not, or only rarely, produced in a single isolate culture, but are usually formed promptly in paired cultures comprising two isolates of opposite mating or compatibility type. Only two major mating types appear to exist in nature, so that nearly all isolates, regardless of species, can probably be assigned to one or other mating type. When two isolates of opposite mating type meet in paired culture sex organs usually form in the region of hyphal intermixing within a few days. The antheridia are always amphigynous. Paired cultures between isolates of the same mating type are sterile. This second group is conventionally referred to as the heterothallic group. Many important phytopathogenic species such as P. infestans, P. palmivora and P. nicotianae v. parasitica are representatives of

this group.

For over fifty years the nature of the sexual mechanism in the heterothallic group has been a controversial problem. This problem, reviewed by Tucker (1931), Hawker (1957) and Erwin et al (1963) and also the subject of the present investigation, had early beginnings. Clinton (1908) suspected the absence of oospores in single cultures of P. infestans to be due to the existence of heterothallism within this species, and considered that separate antheridial and oogonial strains existed. He was unable to obtain oospores by pairing P. infestans isolates, but later reported the formation of P. infestans oospores in mixed cultures with P. phaseoli (Clinton, 1910). Ashby (1922) reported the formation of oospores in mixed cultures of P. faberi (syn.P. palmivora) and in mixed cultures of P. faberi and P. parasitica. Later Gadd (1924, 1927) declared P. palmivora heterothallic, and reported that his isolates fell into two mating groups which he termed the 'cacao' and 'rubber' groups. These two terms have been used for P. palmivora by many subsequent authors (e.g. Turner, P.D., 1960, 1961), but isolates from cacao and rubber are by no means restricted to either mating group.

Subsequent investigations demonstrated situations similar to that reported in P. palmivora for many other species, including P. arecae (Uppal and Desai, 1929; Narasimhan, 1930), P. capsici (Kreutzer, 1940), P. cinnamomi (Galindo and Zentmyer, 1964; Haasis et al, 1964), P. drechsleri (Barrett, 1948), P. infestans (Smoot et al, 1957;

Galindo and Gallegly, 1960), P. meadii (Peries and Dantanaraydna, 1965), P. nicotianae v. nicotianae (Johnson and Valleau, 1954; Apple, 1959), and P. nicotianae v. parasitica (Koyeas, 1953). Other major investigations involving P. palmivora have been those of Lester-Smith (1927), Ashby (1929a), Leonian (1931), Thomas et al (1947), Thomas and Ramakrishnan (1948) and Turner, P.D. (1960, 1961). The two mating types have been variously designated 'cacao' and 'rubber', I and II, A_1 and A_2, male and female and so on. In addition to intra-specific oospore formation, many instances of oospore formation between paired compatible isolates of different species have also been reported (e.g. Clinton, 1910; Ashby, 1922, 1928, 1929b, 1929c; Lester-Smith, 1927; Narasimhan, 1930; Ventakaryan, 1932; Thomas et al. 1947; Barrett, 1948; Thomas and Ramakrishnan, 1948; Cohen, 1950; Waterhouse, 1950; Stamps, 1953; Apple, 1959; Savage and Clayton, 1962; Haasis and Nelson, 1963; Peries and Dantanarayana, 1965). Oospores formed by a mating reaction between isolates of different species may or may not be true hybrids. Work by Barrett (1948), Cohen (1950), Stamps (1953) and Savage and Clayton (1962) suggests that hybrid reproductive organs are formed, but true hybrid status cannot be established until more oospores are germinated and their progeny typed.

With regard to the nature of the sexual mechanism in the heterothallic species, two schools of thought emerged following the investigations of earlier workers. The first supported the concept of morphological heterothallism, which supposes that each isolate is individually self-

sterile and unisexual, producing either male or female sex organs exclusively. Other workers believed each isolate to have bisexual potentialities, and thus capable of forming both antheridia and oogonia, but normally self-sterile. In a mixed culture involving two compatible isolates, sexual stimulation, possibly chemical, takes place, and the bisexual nature of each isolate is expressed. This is possibly in terms of intrinsic and relative strengths of maleness and femaleness within each isolate (Galindo and Gallegly, 1960). Under these circumstances, oospores might be formed either as hybrids, or as selfs or both, within any one cross. These two concepts may be referred to as morphological and physiological mechanisms of heterothallism within the definitions of Whitehouse (1949).

Evidence in support of the first concept would require proof that in all cases of compatible mating one isolate provided only the antheridia and the other the oogonia. At the same time it would be necessary to rule out any possibility of bisexual behaviour. This can be achieved by searching for sex organs in single isolate cultures under a variety of cultural conditions, and by carefully tracing the organs of antheridial and oogonial hyphae in paired cultures. Few authors have provided convincing evidence of this nature, probably because the techniques involved are laborious owing to the difficulty in following individual hyphae through the dense mycelium on either side of the repulsion zone (see Fig. 2). In order to reduce the hyphae involved in the sexual reaction, Narasimhan (1930) placed strips of mica perforated

with tiny holes between the advancing mycelia of compatible isolates, and demonstrated antheridial and oogonial incepts to be produced exclusively on the separate thalli, which he termed male and female respectively. Leonian (1931) traced oogonial and antheridial hyphae by the use of a transparent agar, and divided isolates into male and female strains. Barrett (1948) claimed to have established maleness and femaleness in P. drechsleri and several other species, and Cohen (1950) demonstrated the formation of hybrid oospores between an isolate of P. palmivora and one of P. cinnamomi by tracing the antheridia and oogonia to their respective thalli on the coverslip of a Van Tiegham cell.

However, some evidence obtained by hyphal tracing has indicated that the isolates involved are bisexual. Stamps (1953) using a glass slide culture technique demonstrated that in fertile mixed cultures of P. cinnamomi and P. cryptogea, the antheridia were provided by P. cinnamomi and the oogonia by P. cryptogea . Later, typical fusion organs of P. cinnamomi, morphologically distinguishable from the hybrids developed in the same plates. More recently Galindo and Gallegly (1960), using a combination of the glass slide and perforated aluminium foil techniques, showed that in fertile P. infestans pairings they examined, the isolates behaved bisexually. Similar observations were made with an isolate of P. capsici and one of P. parasitica by Savage and Clayton (1962).

Direct evidence for an intrinsically bisexual nature in some normally self-sterile isolates suggests that chemical stimulation of

reproduction might take place between compatible isolates in paired cultures. This concept was first proposed by Lester-Smith (1927) who on obtaining typical oospores of both P. palmivora and P. parasitica in a paired culture concluded that the production of oospores in mixed cultures was "due to the influence of one vegetation on the other, acting through its effect on the medium or on certain constituents in the medium". His theory was supported by Ashby (1929a) who attributed the above phenomenon to "setting in of conditions under which sexual organs can develop in the other vegetation, possibly by removing inhibiting substances". Nevertheless, little direct evidence of chemical stimulation between isolates has been forthcoming. One method of approaching this problem is to use culture filtrates. This technique was first tried by Galloway (1936), who induced the formation of oospores in single isolates of P. meadii and P.colocasiae by the use of culture filtrates from paired cultures. More recently Koyeas (1953) obtained a few oospores when one P. parasitica isolate was inoculated into the sterile culture filtrate of another. Another technique involves the separation of compatible isolates by a semi-permeable membrane. This method was also used by Koyeas (1953), who obtained oospores of P. parasitica in liquid cultures by mating them on either side of a porous clay barrier. Other evidence for chemical stimulation was provided by Mukerjee and Roy (1962). These authors obtained oospores from a self-sterile isolate of P. parasitica v. sabdariffae by growing it in mixed culture with a bacterium, probably a species of Xanthomonas.

In spite of these highly suggestive results, this field has remained relatively open and promising for further development.

Attention should also be drawn to the many authors who have used biometrical methods to analyse the sexual reaction, by obtaining from inter and intra-specific pairings the mean oogonium and/or oospore dimensions. The results have often been interpreted as indicating either hybridism or selfing (Gadd, 1924; Lester-Smith, 1927; Ashby, 1929b, 1929c; Narasimhan, 1930; Thomas et al. 1947; Thomas and Ramakrishnan, 1948; Waterhouse, 1950; Koyeas, 1953; Stamps, 1953; Apple, 1959; etc.). Such indirect evidence is difficult to interpret unless it is accompanied by the tracing of antheridial and oogonial hyphae to their respective thalli (Narasimhan, 1930; Stamps, 1953) or by very distinct and characteristic morphological differences between the fusion organs and oospores of different species (Ashby, 1929b, 1929c; Waterhouse, 1950; Stamps, 1953). Nevertheless the work of Koyeas (1953) is of particular interest. Koyeas used oogonial measurements to show that some of his P. parasitica isolates behaved as females in one pairing and males in another. In other pairings he obtained two peaks for oogonial size suggesting that in some cases both isolates were contributing oogonia. This indirect evidence for bisexual behaviour of single isolates was supported by the observation that of 200 isolates tested, 18% produced oogonia in single isolate culture after an incubation period of 2 - 6 months.

Apple (1959) recorded that 30 out of over 200 isolates of

P. parasitica v. nicotianae produced oospores in single cultures, but in most other heterothallic species single isolate oospore formation is probably a rare event and the ability quickly lost in culture. Nevertheless, references to single isolate oospore formation can be found for most species, e.g. P. arecae. Coleman (1910); P. capsici. Kreutzer (1940); P. cinnamomi. Ashby (1929c), Zentmyer (1952), Royle and Hickman (1964); P. colocasiae. Thomas and Ramakrishnan (1948); P. cryptogea. Ashby (1929c); P. infestans. Tucker (1931); P. meadii. McRae (1918); P. palmivora. Tucker (1927), Ramakrishnan and Seethalaleshmi (1956); etc.

This review suggests that whilst the division of heterothallic Phytophthora isolates into two compatibility groups is almost universally accepted, much further evidence is required before the true nature of the sexual mechanism can be elucidated. Much of the evidence accumulated so far is of an indirect and circumstantial type, largely owing to the well known cultural difficulties involved in the handling of this organism. Some of these have already been mentioned elsewhere in this thesis. Not least amongst the difficulties, and of significance to the present topic, must be the low percentage germination so far obtained with Phytophthora oospores (Hickman, 1958; Erwin et al. 1963), and the lack of convenient genetic markers. These factors have so far prevented a formal genetic analysis, which would otherwise be a valuable tool for the investigation of sex in this fungus. The difficulty encountered in inducing biochemical mutants or auxotrophs (Erwin et al. 1963) together with the available

cytological evidence (Sansome, 1961, 1962, 1963) suggests that this may be a diploid organism. If this is so, the elucidation of the sexual mechanism will be of considerable genetic and phylogenetic interest.

The following section comprises an investigation into the mechanism of sexual reproduction in the heterothallic species P. palmivora and the 'palmivora' group. Although the work was centred around the Black Pepper isolates of P. palmivora from Sarawak, P. palmivora isolates from other host plants and geographical origins, together with isolates of P. arecae, P. nicotianae v. parasitica and P. heveae were included. Five lines of approach were used.

1. The isolates' behaviour in single isolate culture was recorded. Stock cultures on plates or in bottles were retained and cultured for an extended period (up to 12 weeks) rather than discarded. It was hoped that by this means the ability of an isolate to produce sex organs in single culture would be detected.

2. Paired cultures were used to determine the mating or compatibility types of the isolates. The relative strengths of the mating reactions, as assessed by the frequency of oospores in pairings, were also investigated.

3. Using sterile culture filtrates, an attempt was made to demonstrate chemical stimulation of sexual reproduction between compatible isolates. Semi-permeable membranes were also used to separate compatible isolates in liquid cultures.

4. Cellophane membranes were incorporated into agar cultures to separate compatible isolates. It was hoped that chemical stimulation of sexual reproduction might be demonstrated. The interaction between an isolate of the homothallic and morphologically distinct P. heveae and single P. palmivora isolates was also examined by this method.

5. The effects of two chance contaminants upon single isolate oospore formation were examined. One organism was a bacterium and the other an unrelated species of fungus.

Large numbers of isolates were required for the investigation of single isolate oospore formation and the establishment of mating types (1 & 2). In the work of a more specialised nature (3, 4 and 5) the isolates used earlier for the physiological and nutritional investigations were employed. No attempts were made to obtain biochemical markers by induced mutation, or to germinate the oospores. The work provides evidence in support of the bi-sexual nature of the organism, and suggests some interesting possibilities for further research.

Chapter 2.

MATERIALS AND METHODS

(Additional to those described in Sections I and II).

The following isolates were used in this Section.

1.

	No.	Species	Origin	Host	No. of Donor
*	P28	P. heveae	Malaya	Hevea sp.	CMI 36528

2. P. palmivora, isolated from Piper nigrum in Sarawak.

	No.	District of Origin	Isolated by	No. of Donor
	P1	Miri	P. Holliday	Phy. 38
	P3	Sarikei	"	" 59
*	P4	Serian	"	" 72
	P15	Tarat	J. Turner	" 76
	P16	Serian	"	" 79
	P17	Serian	"	" 80
*	P18	Serian	"	" 81
	P19	Kamping Tarat	"	" 82
	P53	Batu Kawa	"	" 102
	P54	Matang	"	" 305
	P55	Kuchung	"	" 209
	P56	Lundu	"	" 91
	P57	Niah	"	" 206
	P58	Sarikei	"	" 86

3. P. palmivora. isolated from other hosts and geographical regions.

	No.	Origin	Host	No. of Donor
	P10	unknown	Anona squamosa	CMI 46333
	P14	Puerto Rico	Piper nigrum	unknown
	P23	unknown	Citrus reticulata	CMI 32268
*	P24	Jamaica	Theobroma cacao	CMI 75548
	P37	U.S.A.	Tobacco	N.Carolina State Coll., 162
	P41	Trinidad	Theobroma cacao	CMI 74798
	P42	St. Lucia	Theobroma cacao	CMI 79570
	P43	Ceylon	Hevea brasiliensis	CMI 80028
	P44	Ceylon	Hevea brasiliensis	CMI 80030
	P45	Ceylon	Hevea brasiliensis	CMI 80031
	P46	Ceylon	Hevea brasiliensis	CMI 80032
	P47	Ceylon	Hevea brasiliensis	CMI 80033
	P48	Ceylon	Hevea brasiliensis	CMI 80034
	P49	Panama	Theobroma cacao	CMI 79234
	P50	Dominica	Theobroma cacao	CMI 79571
	P51	Jamaica	Theobroma cacao	CMI 79572
	P52	Ceylon	Hevea brasiliensis	CMI 80029
	P59	Ceylon	Hevea brasiliensis	CMI 80027

4. Isolates of species related to P. palmivora ('palmivora' group).

No.	Species	Origin	Host	No. of Donor
P25	P. nicotianae v. parasitica	Mauritius	Nigella damascena	CMI 75548
P26	P. arecae	India	Areca catechu	CMI 62656
P27	P. arecae	India	Areca catechu	CMI 62655
P35	unknown	Sarawak	Citrus sp.	Dept.Agric. F 170
P38	P. nicotianae v.parasitica	U.S.A.	English boxwood	N. Carolina State Coll.,25

5. Other fungi.

Trichoderma viride Pers. ex Fr. Isolated at Department of Botany, University of Hull by C.M. Brasier. Subsequently kindly confirmed as typical T. viride by M.A. Rifai, Department of Botany, University of Sheffield.

Gliocladium virens Miller, Giddens et Foster. Two isolates of this species, one gliotoxin and one viridin producing, were kindly supplied by Dr. J. Webster, Department of Botany, University of Sheffield. These isolates were used for physiological studies in Sections I and II.

Oatmeal agar (OMA), widely used by workers investigating mating types in large numbers of Phytophthora isolates (see Table 12), was retained for the same purposes in this section. Carrot agar (CA) was used in the more specific investigations owing to its advantages for

microscopic work outlined in Section I, and for liquid cultures an extract of carrots was prepared. A synthetic medium (Casam. + S), shown in Section II to be suitable for obtaining oospores of P. palmivora, was also used in some experiments. Details of the preparation of these media are given in the Appendix.

Stock cultures for experimental purposes were hyphal tip sub-cultures maintained on standard OMA plates at 25°C. Inocula were agar plugs taken from the edges of growing colonies with a No.1 cork borer. Small (5 cm.) petri dishes were used for pairings, the two inocula being placed 0.5 cm. from the edge on opposing sides of the dish. Standard (9 cm.) dishes were used in other experiments. All cultures were incubated in the dark, and the incubation temperatures are given in the text.

Spore frequencies were assessed by the Quick Estimation Method. In pairings, oogonial frequencies for each isolate were assessed separately from the samples on each side of the repulsion zone (see Fig. 2).

Special Techniques

1. Cellophane membrane technique (see Fig. 25).

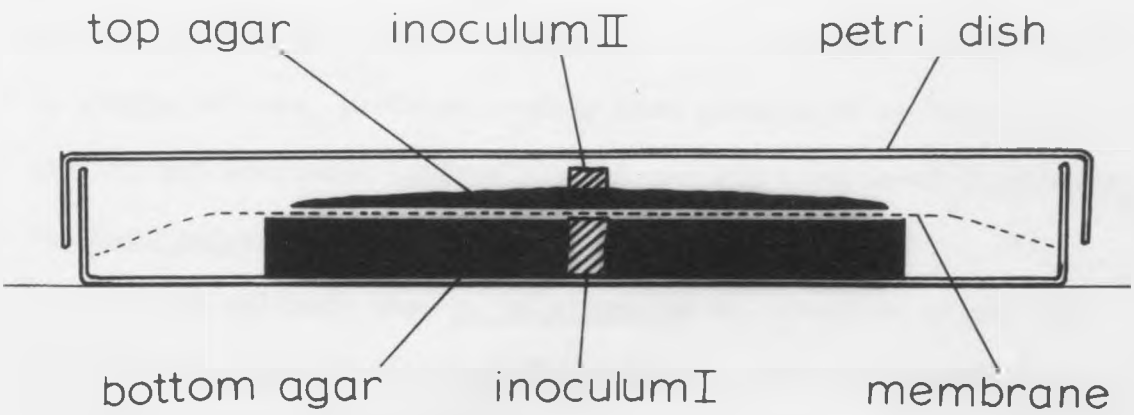
The following method was employed to separate Phytophthora isolates in agar culture by a cellophane membrane. Discs 8.5 cm. in diameter of non-lacquered cellophane were boiled for one hour in distilled water, washed, and sterilised by autoclaving under distilled water. From standard (9 cm.) agar plates of the required medium, an outer ring of

agar was cut away with a 6 cm. diameter metal pastry cutter and removed with forceps, leaving an agar disc in the plate. If this 'bottom agar' was to be inoculated, an agar plug was removed and replaced with an inoculum plug of the same size. A cellophane membrane was drained of excess surface water and placed over the agar disc. Any air bubbles trapped between the two surfaces were removed by drawing a spatula firmly across the cellophane. In this way the membrane perimeter extended beyond the edge of the agar disc such that the fungus inoculated beneath the membrane was unable to reach the upper surface easily. A second thin 'top agar' was poured on the membrane overlying the bottom agar, and inoculated if required. Sterile instruments were employed throughout.

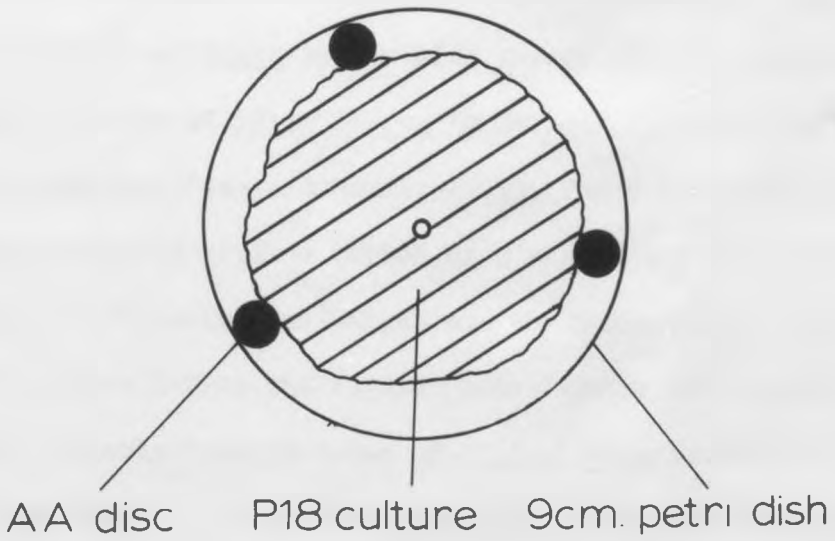
2. Use of antibiotic assay discs (see Fig. 25,2).

In the final experiment (Chapter 16), antibiotic assay discs were used to assay liquid culture filtrates of Trichoderma viride. The absorbent paper discs, 1 cm. in diameter, were sterilised over propylene oxide in an enclosed dish. When required, assay discs were assayed on a flat sterile surface and 0.1 ml. of filtrate pipetted onto each disc. Several extra drops were added to ensure saturation. Three discs were then pressed firmly into position on the agar surface of each culture plate with the aid of forceps. This arrangement is shown in Fig. 25, 2.

1. AGAR/CELLOPHANE MEMBRANE TECHNIQUE.



2. USE OF ANTIBIOTIC ASSAY DISCS.



Chapter 3. The formation of sex organs in single isolate cultures.

The question as to whether Phytophthora palmivora forms sex organs in single isolate culture prompted Waterhouse (1963) to insert in a description of this species,"oogonia never (? or very rarely) formed in single culture, produced readily when grown with an 'opposite' strain, and sometimes (sparsely) with certain strains of nicotianae and var. parasitica."

It is unlikely that P. palmivora is an exception within the 'palmivora' group in not producing oogonia, even occasionally, in single isolate culture. Indeed reports exist by Tucker (1927) and Thomas and Ramakrishnan (1948) of oospore formation by single strains of P. palmivora on OMA, and by Ramakrishnan and Seethalakshmi (1956) who described oospore formation by single isolates on the pericarps of diseased fruits of Areca spp. Also Turner (1962), working with the foot rot pathogen of Piper nigrum in Sarawak (later identified as P. palmivora (Holliday and Mowat, 1963)), found a majority of 31 cultures producing oospores after a period of 2 - 4 months in pure culture on OMA.

In the following investigations the formation of sex organs by single isolates during the regular subculturing of stock cultures was examined. Twenty four isolates of Phytophthora were used. The isolates were hyphal tip cultures stored under oil, and derived from the donor's mass isolate culture. For some investigations (Table 37, IV-VII) OMA stock culture plates were not discarded but retained and incubated for a period of 4 weeks. Other investigations comprising OMA slopes in

10 ml. bottles (investigations I-III) were incubated for a longer period (5 - 12 weeks). All cultures were incubated at 25°C in the dark. The total number of successive subcultures for each isolate following upon the original subculture from the oil stock is given in Table 37.

Twelve isolates produced sex organs and oospores within the first three subcultures from the oil stock, five producing them in considerable numbers. With the exception of isolates P3, P4 and P15 however, there was a distinct decline in the capacity to produce sex organs on further subculturing.

The behaviour of isolates P3, P4 and P15 may be explained from the following observations. Isolate P4, mislaid after investigation III, regained the ability to produce sex organs in investigation IV when freshly subcultured from the oil stock culture. Thereafter it followed the general pattern and quickly lost the ability to form sex organs. Similarly, isolate P3 became heavily contaminated with a bacterium during investigation IV and was again subcultured from the oil stock culture for investigation V.

Isolate P15 developed an unusual pinkish brown colour in investigation V producing even more sex organs than in previous investigations. A piece of this culture was transferred to 10 ml. of sterile nutrient broth and incubated at 37°C for forty eight hours. Similar contamination tests were made with investigation V cultures of P16, P17 and P18. The broth cultures became cloudy, showing the

TABLE 37.

The formation of sex organs in single isolate cultures at 25°C.

(Mean oogonial diameters, in parentheses, are from measurements of 100 oogonia).

INVESTIGATION NO.	I	II	III	IV	V	VI	VII
INCUBATION PERIOD (WEEKS)	10	5	12	4	4	4	4
ISOLATE NO.							
P1		+++ (30.04μ)	-	-	-		-
P3		+++ (30.1μ)	+++/++++ (29.2μ)		*+		-
P4		++++	-	++/+++ (29.1μ)	-		-
P10	-		-	-	-		-
P14		+	-	-	-		
P15		++/+++	+	++/+++ (28.3μ)	++++	-	-
P16		-	-	-	-	-	-
P17		++/+++ (27.8μ)	-	-	-	-	+
P18	+++ (29.6μ)		-	-	-	-	-
P19		-	+	-	-		-
P23	-		-	-	+		-
P24	-		-	-	-		-
P25			-	-	-		-
P26	-		-	-	-		-
P27	-		-	-	-		-
P35			++/+++	-	-		-
P37			-	-	-		-
P38			+++	-	-		-
P53			+	+	-		-
P54			+	-	-		-
P55			-	-	-		-
P56			-	-	-		-
P57			-	-	-		-
P58			++/+++	+	++		+
No. of subcultures (since oil stock culture)							
P10,18,23,24,26,27.	3	-	6	8	9	-	13
P1,3,4,14-17,19.	-	2	4	6	7	8	11
P35,37,38,53-58,25.	-	-	2	4	5	-	9

*P4 reinoculated from oil.
¥P3 " " "

presence of bacteria in the cultures of isolates P15 - P18. Further, a drop of P15 broth culture developed bacterial colonies at 37°C when streaked across a nutrient agar plate. The cultures of isolates P15 - P18 were freed from bacterial contamination by subculturing to water agar plates incorporating a Van Tieghem ring, and transferring de-contaminated inocula to standard OMA plates for investigation VI. Sex organs were not found in cultures of investigation VI. This result suggested that the prolonged ability of isolates P3 and P15 to form sex organs was due to stimulation in the presence of a bacterial contaminant. The nutrient broth culture, P15, was retained for a further experiment (Chapter 10).

The remaining twenty cultures were freed from possible bacterial contamination following investigations V and VI. Broth tests were not made. In a final investigation involving all twenty four sterile cultures (VII) the formation of sex organs was sparse, but still evident. (Bacterial contamination probably arose in these cultures from the breath of the author during subculturing; a breathing mask was used during later inoculation work). These investigations demonstrated the ability of twelve out of nineteen hyphal tip subcultures of P. palmivora to form sex organs in single isolate culture. The formation of sex organs by ten of fourteen isolates from Piper nigrum in Sarawak confirms the observations of Turner, J.G. (1962) with regard to the foot rot pathogens. Mean oogonial measurements (Table 37) were consistent with those obtained by Turner. One unidentified Phytophthora and one isolate of P. nicotianae

v. parasitica also formed sex organs.

Evidently P. palmivora is not strictly self-sterile, but the conditions under which the fungus forms sex organs in single isolate culture are limited. Sterile cultures fresh from stock may do so readily, but this ability is lost with continual subculturing unless perhaps some special stimulus is supplied. Bacterial contaminants appeared to provide such a stimulus, possibly chemical. The induction of oospore formation in self-sterile single isolate cultures by a bacterial contaminant was also observed (with P. parasitica v.subdariffae) by Mukerjee and Roy (1962). This aspect will be further examined later in the section.

Single isolates capable of forming both oogonia and antheridia are bisexual. Morphological heterothallism cannot therefore be considered as the sole sexual mechanism operating between paired compatible isolates. Neither are these fungi homothallic within the definition given on p.15, since they are normally self-sterile. An explanation for the presence of two mating or compatibility types must therefore be attempted within the terms of physiological heterothallism. The mating reaction between paired isolates of P. palmivora is investigated in the following chapters.

Chapter 4. Mating groups amongst isolates of Phytophthora palmivora from Piper nigrum in Sarawak.

P. palmivora isolates were first divided into two mating groups by Gadd (1924), who termed the groups 'cacao' and 'rubber' groups. The

heterothallic behaviour of this species has since been confirmed by many other workers. Holliday and Mowat (1963) however were unable to demonstrate the presence of mating groups within isolates of the foot rot pathogen of Piper nigrum in Sarawak, or to find fusion organs in the host, nor, unlike Turner (1962) and the author (Chapter 3), did they find sex organs of the organism in single isolate cultures. Nevertheless, they did obtain fusion organs "in a mixture of a black pepper isolate (No.71) and one of the palmivora group from Theobroma cacao (North Borneo)," and "between a black pepper isolate (No.72)* and one of the palmivora group from Citrus reticulata (Sarawak)." This indicated that mating types existed among these isolates.

In a fresh attempt to establish the presence of mating groups within the organism infecting Piper nigrum the fourteen isolates examined in Chapter 3 (from various geographical locations in Sarawak) were paired in all possible combinations, in small petri dishes of OMA, and incubated at 20°C for twenty one days.

The experiment showed the presence of two mating groups within the isolates from Piper nigrum. Eleven of the isolates fell into one group, termed for convenience group A, and three into the other group, B. (Fig. 26). Sex organs and oospores were formed in all the A x B group pairings, but not as a rule within A x A or B x B group pairings. Estimates of oogonial frequency were made for each isolate in a compatible pairing. These are given in Fig. 28, Chapter 5. In compatible pairings,

* No.72 = P4.

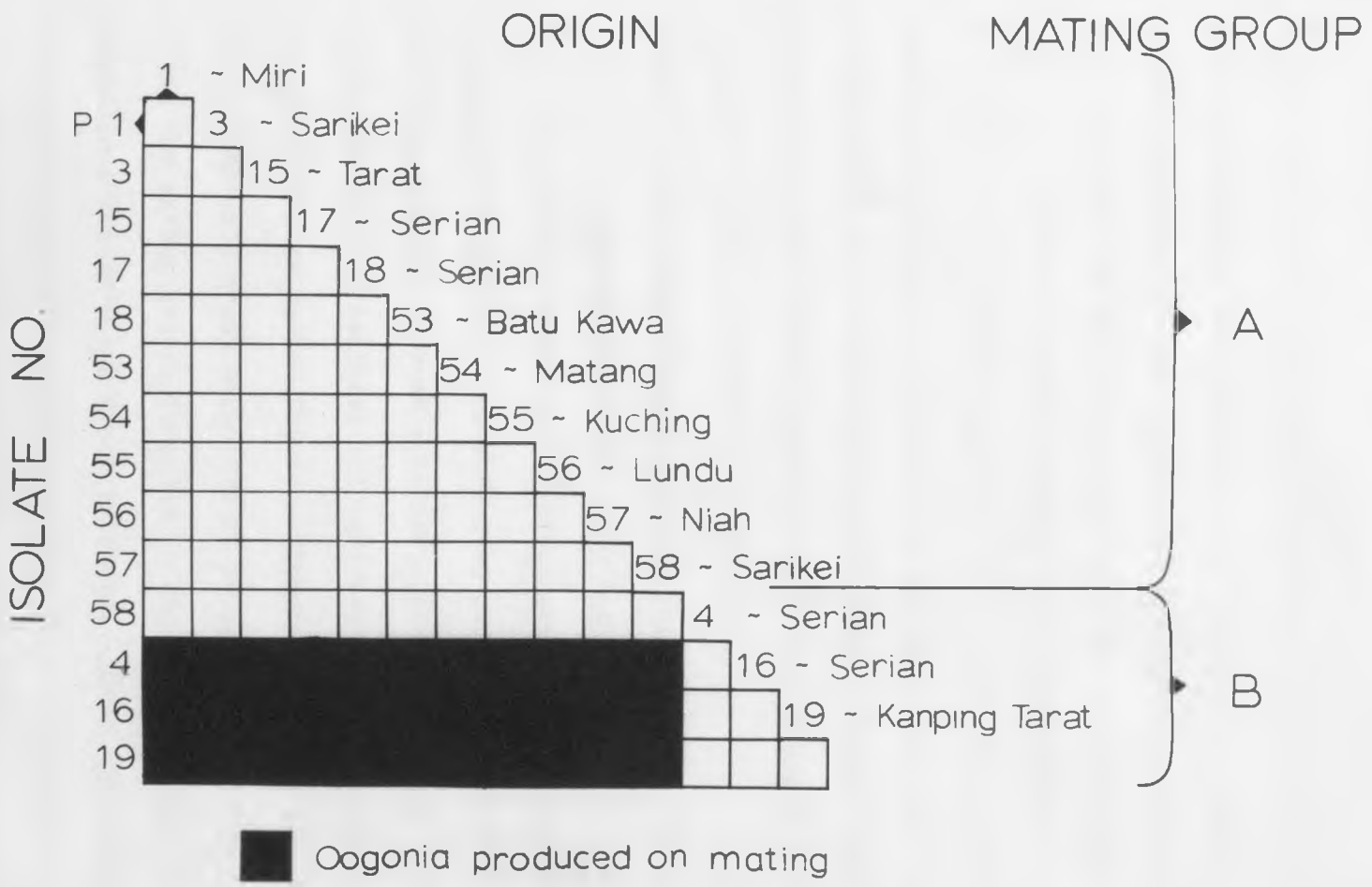


Fig.26. The mating or compatibility group, and district of origin, of isolates of P. palmivora from Piper nigrum in Sarawak.

oogonia were usually frequent or abundant, but in A x A or B x B pairings they were rare, and probably produced in 'single isolate culture' fashion and not as a result of a mating reaction.

The division of the isolates into two mating groups is shown in Fig. 28. It is of interest to note that both 'A' and 'B' mating groups are to be found in the Serian district, since this enhances the possibility of oospore formation in the field (pp. 64 - 66). The preponderance of one mating group (A) among isolates received from Sarawak may have reduced Holliday and Mowat's chances of finding oogonia, whether in paired cultures or in the field.

Chapter 5. The relationships between isolates from Piper nigrum and isolates from other host plants.

In further pairings the mating reactions between the isolates from P. nigrum (Sarawak) and the other ten isolates referred to in Chapter 3 were investigated, in such a manner that all possible paired combinations of the twenty four isolates had been tested. The techniques described in Chapter 4 were used. The remaining ten isolates fell into either the A group or B group with respect to mating behaviour. The composition of the two mating groups is shown in Fig. 27. Oogonia were very rarely formed in A x A or B x B group pairings. These were probably produced in the manner of single isolates (Chapter 3).

The oogonial frequency within each individual culture of an A x B group pairing was assessed by the Quick Estimation Method. The relative symbol +, ++, +++ or ++++ was assigned to each isolate in

the pairing concerned; +++/++++ was included with +++. In Fig. 27 the frequencies of occurrence of oogonia on each side of a pairing are shown by cross hatching. These data represent the means of the rough frequencies from two replicate plates. The results of the previous experiment (Chapter 4) are included in Fig. 27.

Some further observations were made from Fig. 27.

1. Host plant interactions of isolates.

In Fig. 27 all A x B group pairings except those between isolates from Piper nigrum are between isolates derived from entirely different host plants. It was noticed that pairings between A x B group isolates from Piper nigrum, including P14, appeared generally to show higher oogonial frequencies than A x B group pairings between isolates from Piper nigrum and isolates from different host plants. This was investigated in the following way.

The relative frequency symbols used in Fig. 27 were each assigned an arithmetic value, given in the key. (The spore frequency estimation scale appears to be a geometric and not an arithmetic progression. However, an analysis along such lines would have been extremely complex). These arithmetic values were used as variables, X_A or X_B being the value for each isolate of a pairing according to its mating group.

Fig. 27. The division of twenty four palmivora group isolates into two mating groups, A and B, and the frequency of oogonia within each compatible pairing. The oogonial frequency score in the culture of the A isolate (X_A) or B isolate (X_B) in a pairing is given separately. Isolate P10 was lost, and is not included in the calculations.

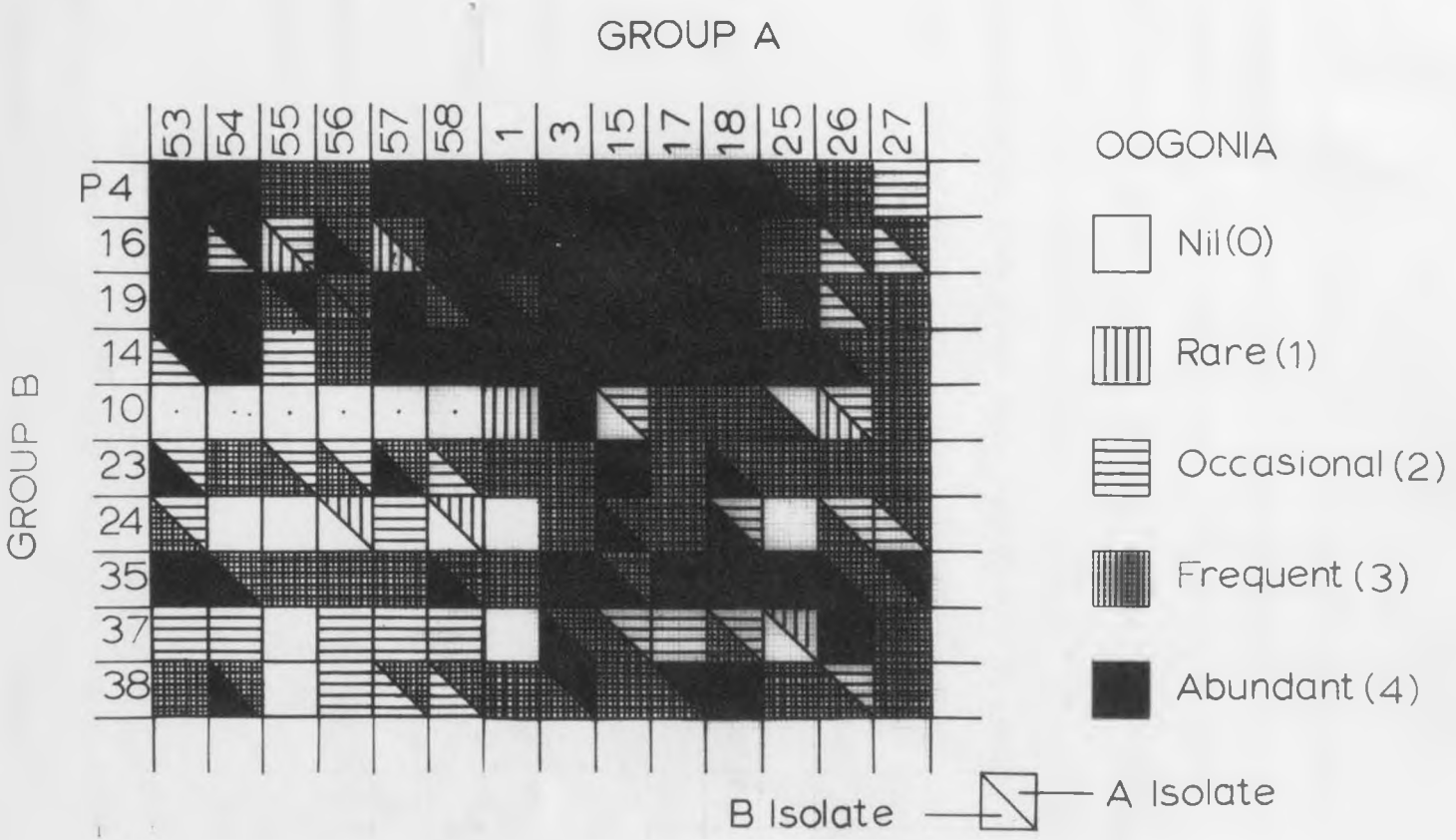


Fig. 27

An analysis of variance was performed on two sets, *Piper nigrum* A group x *Piper nigrum* B group isolates, and *Piper nigrum* A group x other host plant B group isolates, using the X_A's of the A isolates only. The data are given in Tables 38 and 39. The results confirmed that oogonial frequencies were generally higher in the cultures of the *Piper nigrum* A group isolates when these were paired with B group isolates from the same host plant.

TABLE 38.

Oogonial frequency in the cultures of *Piper nigrum* A group isolates.

Sets	Number of pairings	ΣX_A
Piper nigrum A isolates paired with *Piper nigrum* B isolates	44	164
Piper nigrum A isolates paired with other host plant B isolates	55	136
All *Piper nigrum* A isolates x all B isolates	99	300

TABLE 39.

Analysis of Variance

Item	df	SS	MS	vr	P
Between sets	1	38.47	38.47	37.7	< .01
Within sets	97	100.44	1.035	-	
Total	98	138.91	-	-	

2. The consistent mating behaviour of the isolates.

It was noticed from Fig. 27 that the oogonial frequency scored on each side of a pairing was often different, (in 41 of 126 pairings i.e. 32%). Further, some isolates appeared to score consistently low frequencies in all their possible pairings, some moderate, and others mostly high frequencies. This is shown in Fig. 28, where the frequency scores of six selected isolates, P 3 and P4 (high), P23 and P27 (moderate), P24 and P55 (low) are shown as histograms. This suggested that oogonial frequencies may reflect intrinsic mating strengths or potentials in the isolates. Since the data from the replicate plates used in compiling Fig.27 were no longer available, it was not considered possible to undertake a statistical analysis. However, a qualitative mating strength index (I) was calculated for each isolate from the isolate's pairing frequency scores (X_A's or X_B's) using the following

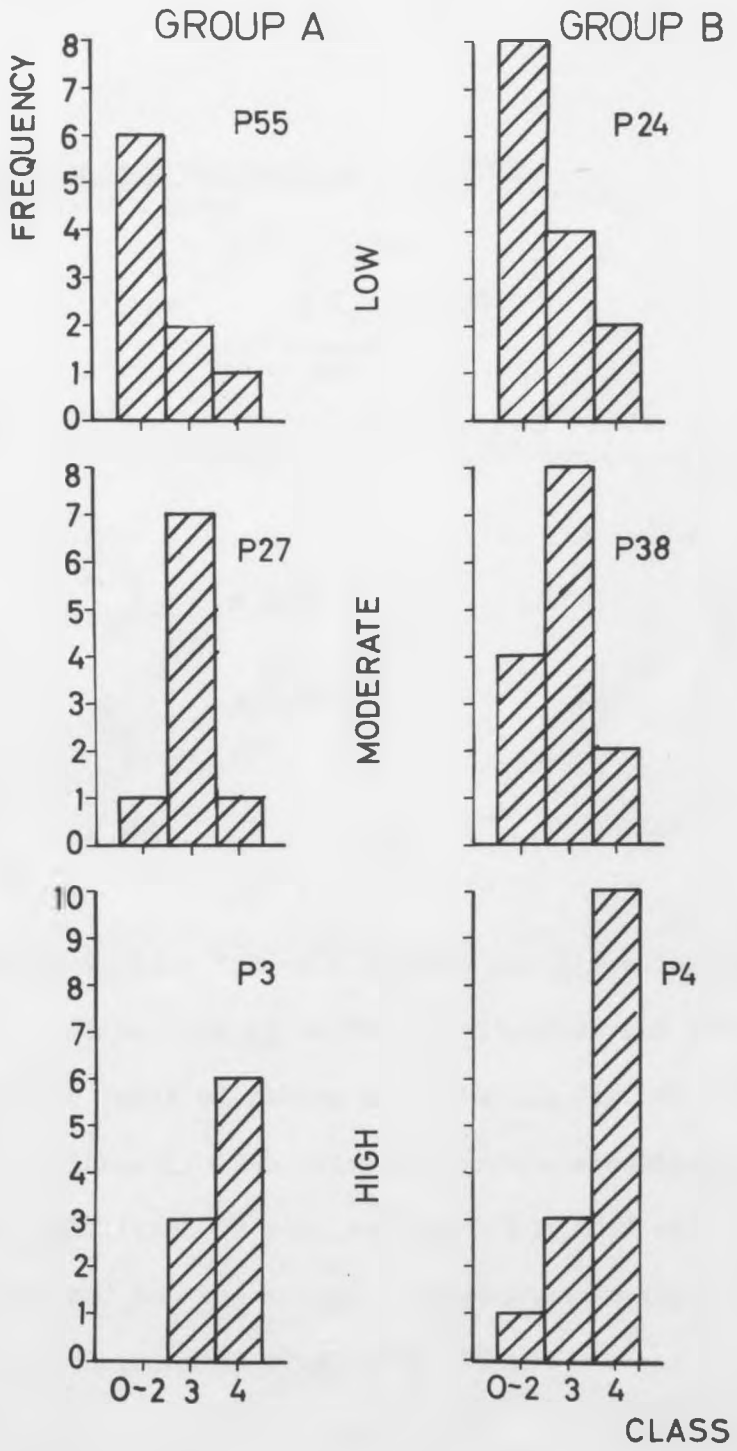


Fig.28. The oogonial frequency scores of six selected isolates (see data in Fig. 27). The oogonial frequency classes nil (0), and rare (1), are included in occasional (2).

*

formula :

$$I = \frac{\text{total of frequency scores for isolate}}{\text{maximum possible score}} \times 100\%$$

$$= \frac{\sum_{1}^{n} X_A}{4n} \times 100\% \quad \text{or} \quad \frac{\sum_{1}^{n} X_B}{4n} \times 100\%$$

e.g. for P23 (group B)

$$I_{P23} = \frac{\sum_{1}^{14} X_B}{56} \times 100\%$$

$$= \frac{45}{56} \times 100\%$$

$$I_{P23} = 80\%$$

The mating strength indices for each isolate are given in Tables 40 and 41. A range of indices from 43 to 92 was obtained, and the isolates arranged along an 'axis of mating strength' in Fig. 29. The isolates were divided arbitrarily into three sub-groups according to mating strength index. The first or weak sub-group included all isolates with an index between 40 and 62, the second or moderate sub-group 63-82, and the third or strong sub-group 83 plus, (Fig. 29).

* n = number of pairings

TABLE 40.

1. Mating strength indices for isolates in mating group A.

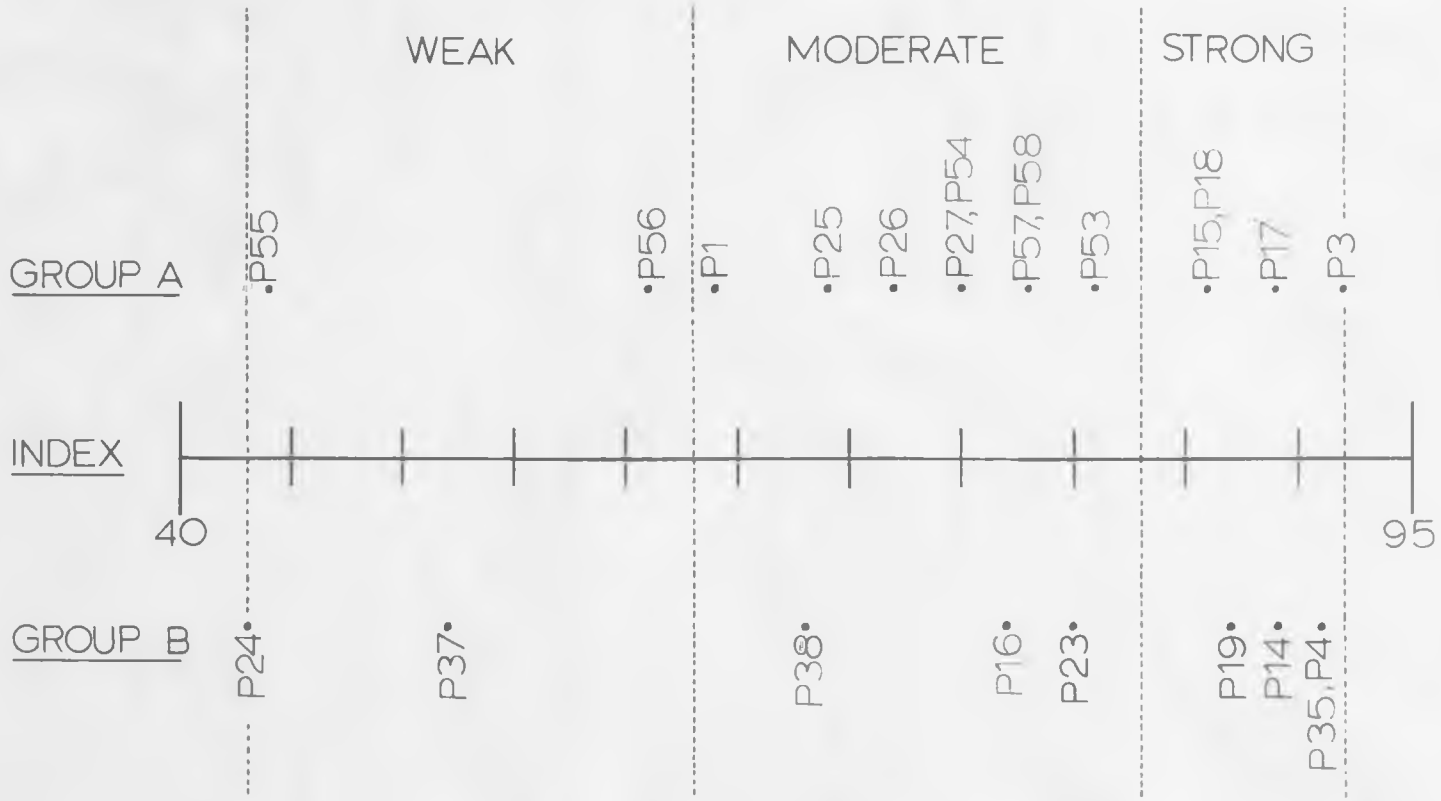
Mating group.	Isolate Number.													
A.	P1	3	15	17	18	25	26	27	53	54	55	56	57	58
$\sum_{1}^{n} X_A$	23	33	31	32	31	25	26	27	29	27	16	22	28	28
4n	36	36	36	36	36	36	36	36	36	36	36	36	36	36
I	64	92	86	89	86	69	72	75	81	75	44	61	78	78

TABLE 41

2. Mating strength indices for isolates in mating group B.

B.	P4	16	19	14	23	24	35	37	38
$\sum_{1}^{n} X_B$	51	43	49	50	45	24	51	29	38
4n	56	56	56	56	56	56	56	56	56
I	91	77	87	89	80	43	91	52	68

Fig. 29. The arrangement of isolates of both mating groups in order of mating strength indices, and their qualitative division into weak, moderate and strong sub-groups.



It was thought that the mating strength index might indicate the numbers of sex organs formed by an isolate within its own culture i.e. by selfing, in response to a stimulus from the other compatible isolate. Thus strong isolates would form more selfs (antheridia and oogonia formed in the manner of single isolates (Chapter 3)) than moderate or weak isolates. On this basis a relationship between mating strength and bisexual ability might be expected, with strong isolates having a greater tendency towards the formation of sex organs in single isolate cultures. Table 42 indicates that such a relationship probably does exist. These data are based upon results given in Table 37, Chapter 3.

TABLE 42.

The relationship between mating strength (I) and formation of sex organs in single isolate cultures.

Sub-group	Weak	Moderate	Strong
No. of isolates in sub-group	4	11	8
No. of isolates forming sex organs in single culture (%)	0	6(55%)	8 (100%)
Mean of mating indices of isolates in sub-group.	50	74.3	88.3

Mating strength may therefore be related to the degree of bisexual behaviour of an isolate. It may also reflect the numbers of oogonia

formed by an isolate on hybrid sex organ formation with a compatible isolate (the latter supplying the antheridia). It could be suggested that this is in turn related to the vigour of an isolate e.g. physiological vigour in terms of nutrition, more specifically that concerned with the formation and development of oogonia and oospores. Further, the wide variation in mating strengths, irrespective of mating group, suggests that these two factors may be independent of each other. The compatibility reaction may merely indicate sexual differentiation by overcoming the self-sterility of the isolates. These possibilities will be discussed in Chapter 18.

Chapter 6. The relationship between the 'cacao' and 'rubber' mating groups of Gadd (1924) and the Sarawak A and B mating groups.

Gadd (1924) divided P. palmivora isolates into two mating groups, 'cacao' and 'rubber' on the basis of host plant origins and morphological characteristics. Turner (1961) examined over 200 isolates of P. palmivora from cacao and rubber from 22 countries, and found sexually complementory strains of the pathogen on both hosts. In general, however, oospores were readily produced in mixed cultures of isolates from rubber and cacao. Turner did not find both mating groups (I and II) on the same host within the same country. Satchuthananthavale (1963) however, found both mating groups from rubber isolates in Ceylon, and in the

present work two mating groups have been found in isolates from Black Pepper in Sarawak. In order to discover the relationships between isolates from cacao and rubber, and the Sarawak A and B groups, the following investigation was carried out.

Thirteen isolates, eight from rubber in Ceylon and five from cacao in the Caribbean area, were obtained from the Commonwealth Mycological Institute. The thirteen isolates were paired in all possible combinations, using the techniques described in Chapter 4. Although the isolates formed somewhat abnormal oogonia and antheridia, they fell into two distinct mating groups (Table 43). No oogonial frequency estimates were made.

TABLE 43.

The relationships between cacao and rubber isolates.

First mating group - 'cacao'.		Second mating group - 'rubber'.	
Isolate No.	Host	Isolate No.	Host
P41	Cacao	P45	Rubber
P42	Cacao	P46	Rubber
P43	Rubber	P47	Rubber
P44	Rubber	P48	Rubber
P49	Cacao	P52	Rubber
P50	Cacao	P59	Rubber
P51	Cacao		

Since all five cacao isolates fell into the first mating group, and six of the rubber isolates fell into the second, these two groups can probably be equated with the 'cacao' and 'rubber' groups of Gadd. The presence of the two rubber isolates in the 'cacao' group, confirms the observation by Satchuthananthavale (1963) that both mating groups occur on rubber in Ceylon. Oogonia were not formed in single isolate stock cultures of P41 - P59, nor in pairings between 'cacao' x 'cacao' or 'rubber' x 'rubber' group isolates.

The above thirteen isolates were paired with seven selected isolates representing the Sarawak A and B mating groups. These isolates, including several species from various hosts and geographical locations, were P18, P25 and P26 (A group) and P14, P16, P24 and P28 (B group). Sex organs formed only in pairings between isolates from group A and isolates in the rubber group, and between isolates from group B and isolates in the cacao group. The results demonstrated that the Sarawak A group and the 'cacao' group, and the Sarawak B group and the 'rubber' group, were the same groups. Isolate P24 from cacao in Trinidad was thus equated with the 'rubber' group, confirming the observation of Turner (1961) that both mating groups occur on cacao.

These investigations have confirmed that in the palmivora group of species two mating or compatibility groups are found, irrespective of species, host plant or geographical origins of the isolates (Table 44). Both mating types can occur on the same host plant within a defined geographical location.

TABLE 44.

Mating group, host plant and origin of 37 'palmivora' group isolates.

Sarawak A group ('cacao' group).

Species	Host plant	Origin	Number of isolates.
P. palmivora	Piper nigrum	Sarawak	3
"	Cacao	Caribbean	5
"	Rubber	Ceylon	2
"	Piper nigrum	Caribbean	1
P. arecae	Areca sp.	India	2
P. nicotianae var. parasitica	Nigella sp.	Mauritius	1
			14

Sarawak B group ('rubber' group).

Species	Host plant	Origin	Number of isolates.
P. palmivora	Piper nigrum	Sarawak	11
"	Cacao	Caribbean	1
"	Rubber	Ceylon	6
"	Anona sp.	Unknown	1
"	Boxwood	U.S.A.	1
"	Citrus	Caribbean	1
Unknown	Citrus	Sarawak	1
P. nicotianae var. parasitica	Tobacco	U.S.A.	1
			23

Chapter 7. Attempts to demonstrate chemical stimulation of sexual reproduction by use of liquid culture filtrates.

Evidence obtained so far by other workers and in the present investigations has favoured the theory that heterothallic isolates of Phytophthora are weakly self-fertile, forming sex organs when stimulated chemically by the presence of an isolate of opposite mating group. Many of the isolates in the present work, shown to be bisexual under certain conditions (Chapter 3) were considerably stimulated by the presence of an isolate of complementary mating type (Chapters 4 and 5). If a chemical stimulant(s) is involved in the interaction of A and B group isolates, such a substance might be transferred from one culture and under the right conditions, stimulate sexual reproduction in a liquid culture of a complementary isolate. This was successfully demonstrated by Galloway (1936) and Koyeas (1953).

Brief outlines of several methods used by the author in similar, though unsuccessful, attempts are given below. Cultures were grown in 100 ml. conical flasks containing 20 ml. of carrot extract, and incubated at 20°C. P. palmivora isolates from the strong mating sub-group, P3, P4, P15, P16, P17, P18 and P19, and P. heveae isolate P28, were used. Both cold culture filtrates, prepared by using bacteriological sintered glass filters, and autoclaved filtrates, prepared by using filter papers, were tested.

Method 1.

Culture filtrates of an A group isolate were added to liquid

cultures of a B group isolate (in various stages of growth) and vice versa.

Method 2.

Culture filtrates of an A x B group pairing were added to liquid cultures of both A and B group isolates. Oogonia formed only in the original paired cultures.

Method 3.

Culture filtrates of A x B group pairings, and of single A or B group cultures, were inoculated with A or B group isolates. Oogonia formed only in the original paired cultures. However, on re-inoculation of an A x B pairing filtrate with both of the original isolates, growth occurred,but oogonia failed to form. Evidently the culture filtrates were now either inhibitory to the original isolates or too nutritionally impoverished to support the formation of oogonia.

Method 4.

Culture filtrates of P. heveae (P28) used as in methods 1 - 3 above, failed to stimulate sexual reproduction in P. palmivora A or B group isolates. P. heveae, however, formed oogonia abundantly when re-inoculated into its own culture filtrate.

Method 5.

A and B group isolates were separated in liquid cultures by porous clay vessels. Various combinations of A x B paired cultures and single isolate cultures were tried.

Method 6.

A perspex liquid culture vessel was constructed which consisted

of three chambers, each separated by a window, containing a bacteriological membrane filter (this apparatus was similar to that used by Marx and Haasis (1965)). A and B group isolates were placed in separate chambers. Oogonia were not formed, and the cellulose acetate membranes were eventually penetrated by the Phytophthora isolates.

That no formation of oogonia was observed may have been due simply to the non-existence of the postulated stimulatory substance(s). It is equally possible however that the conditions within were inadequate for the expression of any stimuli. Whilst environmental conditions were adequate for formation of oogonia in the A x B pairings, the culture filtrates of these pairings were self-inhibitory on re-inoculation. This may have been due to an accumulation of staling substances, to a critical reduction in nutrient status or to the filtering out of essential or stimulatory substances (such as sterols). Further, the inactivity of culture filtrates when added to growing cultures may in addition have been due to the extreme dilution or lability of any stimulants.

Owing to the difficulties inherent in perfecting the liquid culture technique, an attempt was made to develop a technique by which isolates could be separated by a semi-permeable membrane in a solid medium. These experiments, which were more successful, are described in the following Chapters.

Chapter 8. Initial membrane experiment.

Consideration was given to the possibility of separating an A and B group isolate in agar culture by a semi-permeable membrane, in the

hope that this would facilitate the passage of any substances stimulating sexual activity, but at the same time provide a physical barrier between the hyphae. In this way complementary isolates might be brought close enough together to allow substances formed by one isolate to diffuse readily into the mycelium of the other, providing a situation similar to that in a normal pairing. A technique devised for the incorporation of cellophane and similar membranes is described in Chapter 2 (p.138 and Fig. 25, 1). In the initial experiment cellophane discs .001 in. thick were used, with a carrot agar medium. The following combinations of isolates were used, with 3 replicates of each plate, and incubated at 20°C for fourteen days :

	I. Bottom agar inoculum	II. Top agar inoculum
Control plates	P3	-
	P3 x 4	-
	P4	-
	P18	-
	P24	-
	P28	-
Experimental plates	P3	P4
	P3 x 4	P4
	P28	P4
	P28	P18
	P28	P24

Unfortunately the cellophane membranes in all the control plates were penetrated from below by the various isolates within eight days of inoculation. After fourteen days, considerable mycelial growth was present on the top agar in each case. Thus, in the experimental plates,

hyphal contact probably took place between the isolates on each side of the membrane, thus casting doubt on the value of the results which are given in Table 45. Oogonial frequencies were estimated by the Quick Estimation Method, using samples taken above and below the membranes.

TABLE 45.

Formation of oogonia above and below the membranes in the experimental plates.

	Below (I)		Above (II)	
	Inoculum	Oogonia	Inoculum	Oogonia
	P3	+++	P4	+++
	P3 x P4	++++	P4	+++/++++
	P28	++++	P18	+++
	P28	++++	P4	+++
	P28	++++	P24	-
Control	P28	++++	-	++++

No valid conclusions could be drawn from the pairings of the P. palmivora A x B groups. However, the pairings involving the homothallic P. heveae P28 were particularly interesting.

The sex organs formed below and above the membranes in the P28 x P4 and P28 x P18 pairings were morphologically distinct, and closely resembled those of the type descriptions by Waterhouse (1963) for P. heveae and P. palmivora. Measurements of oogonia, antheridia and

oospores from above and below the membranes are given in Table 46, together with line drawings of the sex organs in Fig. 32.

TABLE 46.

Mean oogonial, antheridial and oospore sizes from the membrane cultures of P28 x P4 and P28 x P18, 100 spores in each case.

	Control I P28	I P28 x	II P4	I P28 x	II P18
Oogonial diameter μ Range	23.0 (19.5-24.3)	22.6 (17.8-29.5)	36.6 (31.8-37.5)	22.7 (20.3-24.2)	32.8 (26.8-37.9)
Oospore diameter μ Range	ND	ND	31.1 (26.0-32.0)	ND	ND
% oogonia with oospores	ND	85	86	80	27
Antheridial length μ Range	10.3 (8.7-10.8)	10.1 (7.2-10.6)	10.9 (7.7-17.4)	ND	ND
Antheridial width μ Range	8.7 (6.7-9.8)	8.7 (7.4-9.2)	13.6 (11.4-14.7)	ND	ND

ND = No data

Waterhouse (1963) gave the following spore measurements for P. heveae and P. palmivora. P. heveae: oogonia 25 x 28 μ, antheridia 10.5 x 9 μ; P. palmivora: oogonia 30 μ and antheridia 15 x 14 μ. Turner (1962) gave the following measurements for single isolates of the organism causing foot rot of Piper nigrum. P. palmivora: oogonia 26 - 45 μ (35 μ) and antheridia 11 - 15 x 11 - 23 μ (mean 13 x 15 μ). These measurements for P. heveae and P. palmivora agree closely with those given in Table 46 for isolate P28, and isolates P4 and P18

respectively. The sex organs formed in the mycelium of P28, P4 and P18 also correspond morphologically with the type descriptions (Fig. 32). Typically, P. heveae forms a spherical oogonium conically tapered towards the stalk, a spherical or ellipsoidal antheridium and a thick walled oospore loose in the oogonium, (Waterhouse, 1963). P. palmivora, however, forms a larger, non-tapered oogonium, spherical or oval antheridia, with the oospore nearly filling the oogonium.

The sex organs formed below the membranes in the P28 x 4 or P28 x P18 pairings were thus typical for P. heveae, whereas those formed above the membranes were sex organs of the P. palmivora type. The two types were confined to opposing sides of the membrane. No hybrid sex organs were detected either morphologically or biometrically. Thus close contact between a culture of the homothallic P. heveae and a single heterothallic P. palmivora isolate resulted in sexual stimulation of the latter, which responded by single isolate oospore formation. Isolates of both mating groups, A (P18) and B (P4), responded. That the response was probably due to the passage of a chemical stimulus through the cellophane membrane was suggested by the absence of P. heveae type or hybrid sex organs above the membranes. This indicated that there had been little if any physical contact between the isolates. The type of mechanism, whether by direct chemical stimulation, for example by sex hormone, nutrient or growth substance formation, or by indirect stimulation such as the removal of fertility inhibitors, remains unclear.

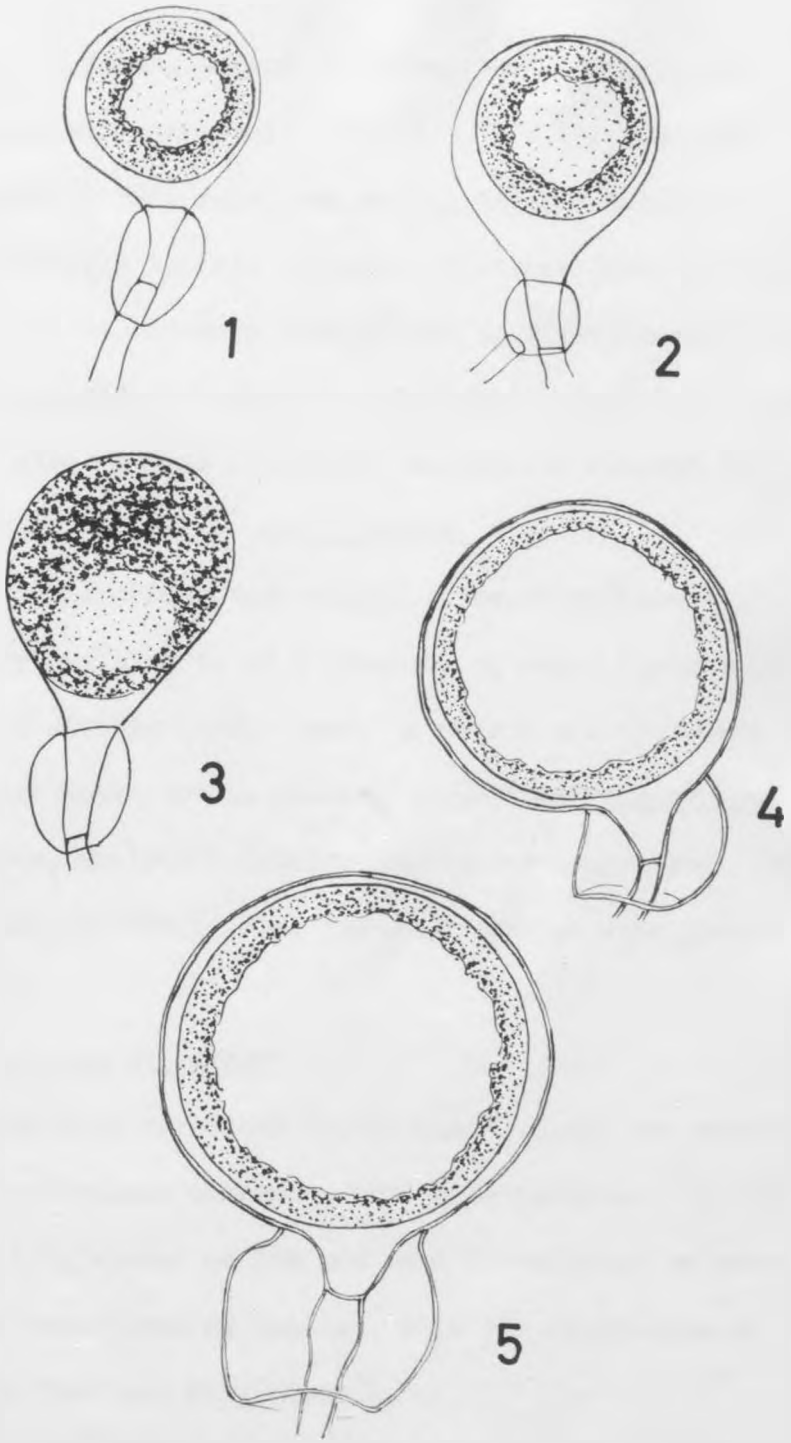


Fig.32. Morphology of oogonia, antheridia, and oospores from P28 x 18 membrane cultures. Not to scale.
Below membrane: 1) and 2) P. heveae type sex organs containing oospores; 3) P. heveae type lacking oospore.
Above membrane: 4) and 5) P. palmivora type sex organs containing oospores.

Both P4 and P18 are strong mating isolates, each found to form sex organs in single isolate culture in Chapter 3. In contrast, P24, which was not stimulated by P28, is a weak mating isolate which did not form sex organs in single isolate culture. This confirms the view that there is probably a relationship between mating strength and selfing ability, as suggested in Chapter 5. Isolates with the strongest mating potential, and also selfing potential, apparently respond more readily to stimulation, in this case by P. heveae.

These results do not confirm that sexual stimulation between compatible P. palmivora isolates is of a chemical nature. However, they strongly suggest that this is the case. A search was therefore made for a membrane that could not be penetrated by the Phytophthora isolates. Several types, including thicker cellophane membranes, dialysing membranes, and bacteriological filter membranes were tested without success.

Chapter 9. Further membrane experiment.

The membrane experiment described in the last Chapter was repeated with the same type of cellophane membrane, but using isolates P28 and P18 only. A casamino acid/sterol medium was used in addition to carrot agar. The plates were inoculated as follows, with six replicates of each, and incubated for fourteen days at 20°C.

The membranes were penetrated by P18 and P28 in the control plates. The non-inoculated control remained sterile. The results in the experimental plates, which developed good growth on both sides

of the membrane, are given in Table 47. As in the previous experiment, typical P. heveae sex organs formed below the membranes and P. palmivora sex organs above. These can be seen in Figs. 33 and 34.

	I Bottom Agar	II Top Agar	Medium
Control plates	-	P18	CA
	P28	-	CA
	-	-	CA
Experimental plates	P28	P18	CA
	P28	P18	Casam. + S

TABLE 47.

Formation of oogonia above and below the membranes.

		Below (I)			Above (II)		
Control plates	Medium	Inoculum	Oogonia	Type	Inoculum	Oogonia	Type
Six replicates	CA	P28	++++	H	-	++++	H
"	CA	-	-	-	P18	-	-
"	CA	-	-	-	-	-	-
Experimental plates							
Plate i	CA	P28	++++	H	P18	++/+++	P
ii	CA	P28	++++	H	P18	++	P
iii	CA	P28	++++	H	P18	++	P
iv	CA	P28	++++	H	P18	+/++	P
v	CA	P28	++++	H	P18	+/++	P
vi	CA	P28	++++	H	P18	++	P
Plate i	Casam + S	P28	++++	H	P18	++	P
ii	Casam + S	P28	++++	H	P18	++/+++	P
iii	Casam + S	P28	++++	H	P18	+/++	P
iv	Casam + S	P28	++++	H	P18	+/++	P
v	Casam + S	P28	++++	H	P18	+	P
vi	Casam + S	P28	++++	H	P18	-	P

H = P.heveae type sex organs. P = P. palmivora type sex organs.

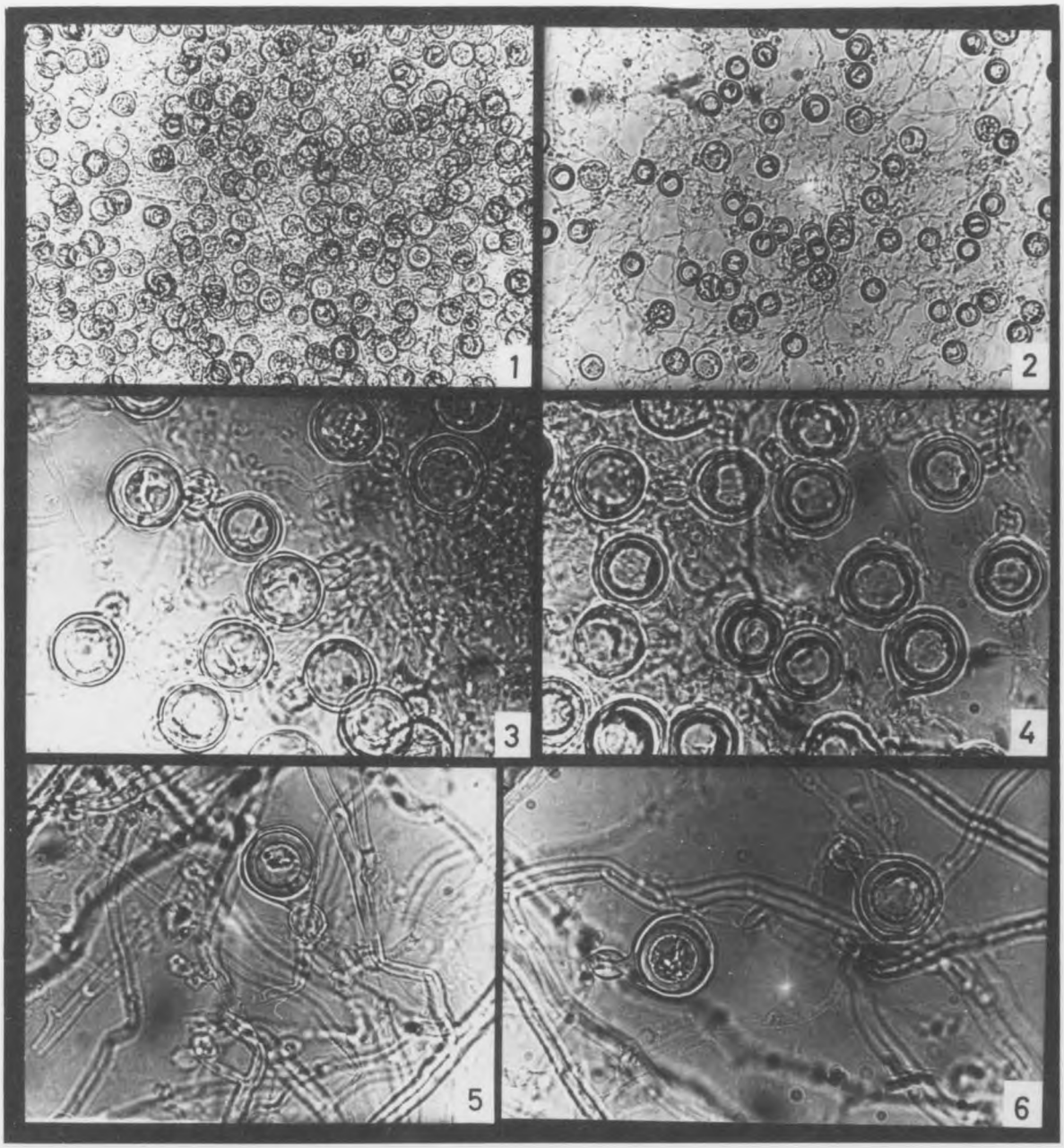


Fig.33. Oogonia of the P. heveae type below membrane; 1) and 2) low power; 3), 4), 5) and 6) high power to show details of morphology.

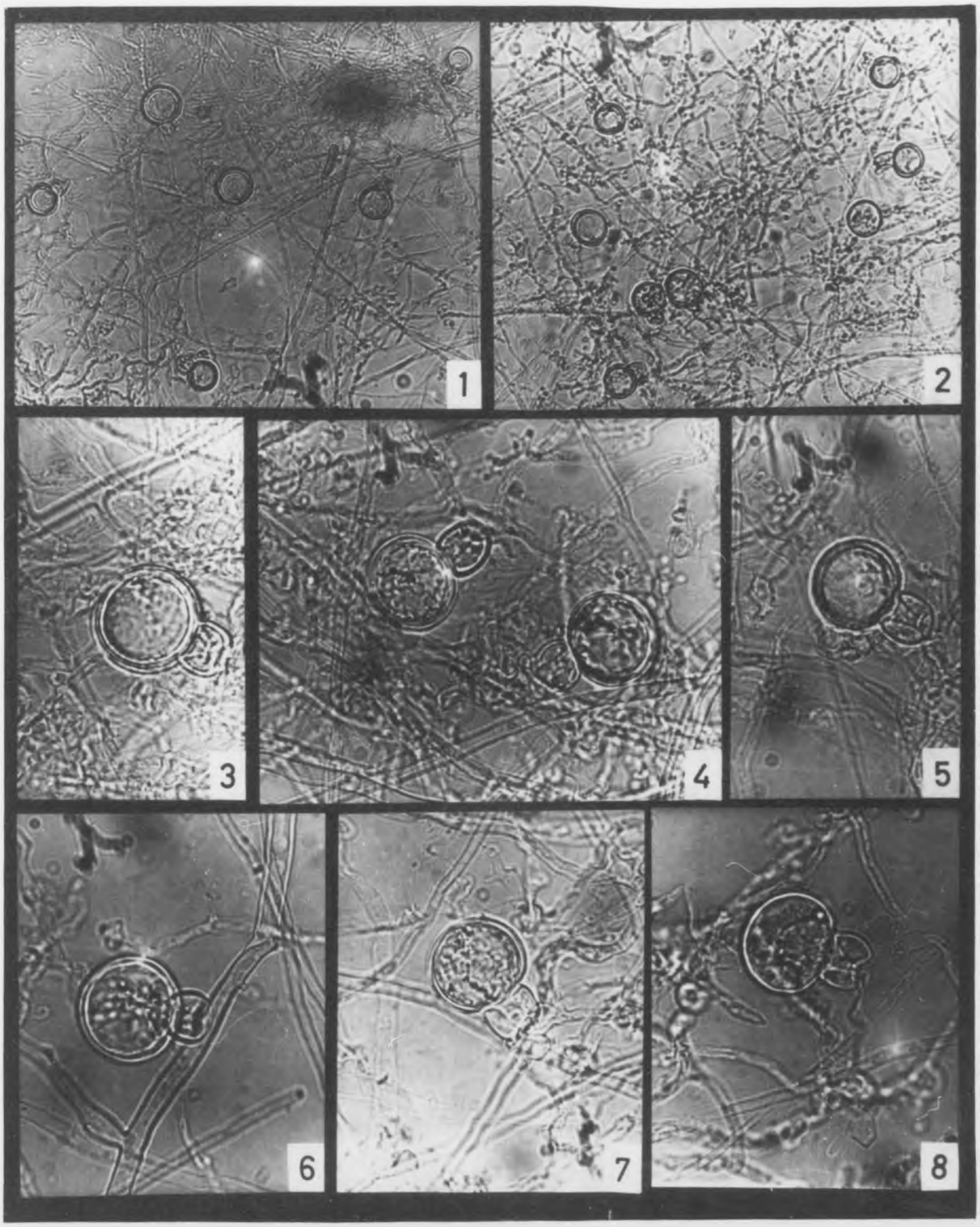


Fig. 34. Oogonia of the P. palmivora (Pl8) type above membrane; 1) and 2) oogonia under low power; 3), 4), 5), 6), 7), and 8) high power to show details of morphology.

Similar results were obtained on both carrot agar and the synthetic medium originally tested in Section II. This experiment confirmed the stimulation of sexual reproduction of P. palmivora in single isolate culture by P. heveae. and also indicated that the effect was not due to modification by P. heveae of substances specific to the carrot medium.

Chapter 10. The effect of a bacterium upon the mating reaction in P. palmivora.

During the investigations into the formation of oogonia by single isolates (Chapter 3) two isolates which formed large numbers of oogonia, P3 and P15, were found to be contaminated with bacteria. Isolate P3, consequently lost, was again subcultured from oil stock culture. Isolate P15, which developed a significant pinkish brown aerial mycelium during investigations IV and V, was shown to be contaminated by transferring portions of the culture to nutrient broth bottles.

The broth cultures were later found to contain a suspension of a gram positive rod shaped bacterium, which formed cream colonies on nutrient agar plates. Similar colonies, of a creamy pink colour, were also found at the edges of the P15 OMA stock culture during investigation V. Since it was suspected that the bacterium was stimulating sexual reproduction in P15, the following investigation was carried out.

Six cultures, taken from brownish patches in the P15 investigation V culture in which oogonia were formed abundantly, were transferred to plates of water agar incorporating glass Van Tiegham rings. Hyphae

of P15 growing beneath the rings were free from bacteria. Six inocula, one from each plate, were transferred to a single OMA plate, and incubated for one week at 25°C. The resulting culture of P15 was shown to be free from bacteria when small samples were incubated in nutrient broth. Oogonia did not develop in subsequent subcultures from the new P15 stock culture (investigations VI and VII). An attempt was therefore made to induce sexual reproduction in P15 by re-introducing the contaminating bacterium.

Fresh nutrient broth cultures of the bacterium were prepared and transferred to a sterile hand spray. A control spray containing sterile nutrient broth was also prepared. Six ten day old OMA cultures of P15 were lightly sprayed with the bacterial spray, and six others with the control spray. All twelve plates were incubated for one month at 25°C and then examined for oogonia.

The control plates developed a fluffy white aerial mycelium (+++) but no oogonia were found. The six plates sprayed with bacteria developed a pinkish brown aerial mycelium (++) similar to that in the initial contaminated culture, and bacterial colonies were found at the edges of the plates, but again oogonia were not present. Subcultures taken from these plates, and yet further subcultures, developed similarly, but still no oogonia were found.

Since oogonia were not formed after the removal of the bacterium it is probable that the bacterium was in some way chemically stimulating the formation of oogonia by P15. Re-introduction of the bacterium

failed to repeat this stimulation. It is possible that conditions during the course of the experiments were not adequate for the fungus/bacterium interaction to repeat itself. Alternatively, the selfing potential of P15 may have declined with the further subculturing, or the bacterium may not have been the sole cause of the original stimulus.

Chapter 11. The effect of Trichoderma viride upon the formation of oogonia by P. palmivora, P18.

I. Initial observations.

During an experiment investigating the effects of temperature upon sporangium formation (Section I), a culture of P18 (P. palmivora) on carrot agar at 22.5°C developed by chance a fungal contaminant. On examination of the plate, the golden brown oogonia and antheridia (++) were found, in addition to sporangia. Oogonia were not found in the replicate non-contaminated plates. The contaminant, isolated into pure culture, was identified as Trichoderma viride Pers. ex Fr.

The unexpected development of oogonia in a much subcultured single isolate culture of P. palmivora. appeared to be due to the presence of the contaminant, which was sporulating freely throughout the plate. In an attempt to confirm this two carrot agar plates were freshly inoculated with P18 and were simultaneously inoculated at the edge with conidia of T. viride. These were incubated with two P18 control plates for 14 days at 22.5°C. Oogonia were found only in the plates inoculated with T. viride. However the oogonia were very scarce (+),

possibly because the Trichoderma had completely surrounded and even killed the developing P18 colonies.

Chapter 12.

II. A preliminary experiment.

In the previous experiment normal growth and development of the isolate of Phytophthora was probably prevented by the rapid growth of the Trichoderma. Therefore the radial growth rate of T. viride on carrot agar was determined at six temperatures (15.0, 20.0, 22.5, 25.0, 27.5 and 30.0°C) by taking four radial growth measurements at twenty four hour intervals from four replicate plates. The optimum temperature for radial growth of T. viride lay between 22.5 and 25°C. At 22.5°C, the mean radial growth increment of T. viride per hour was 0.73 mm., nearly twice the rate of 0.375 mm./hr. recorded for P18 on carrot agar at 22.5°C in Section I. Evidently P18 must be inoculated well before the Trichoderma if it is not to be overgrown by the latter.

In the next experiment the P18 plates were not inoculated with T. viride until the colonies had almost reached the edges of the plates. Six cultures of P18 were inoculated at the edge with T. viride conidia when the Phytophthora colonies were approximately 2 cm. from the perimeter, and six others when the colonies were fully developed. A further six were retained as controls. All eighteen plates were incubated for fourteen days at 22.5°C.

Oogonia were frequent (+++) in all twelve plates inoculated with T. viride. No oogonia were found in the control plates. In the T. viride

plates, the thick, septate hyphae of Trichoderma could be traced from the inoculum throughout the P18 colony. Dark green conidiophore tufts were formed mostly on the side of the culture opposite to the T. viride inoculum. Oogonia of P18 were either clustered or were formed in lines along one hypha (Fig. 35). Measurements of 40 sex organs and oospores, and of the percentage of oogonia containing oospores (Table 48) were obtained using slide samples taken at random from six plates.

TABLE 48.

Mean measurements of the oogonia and oospores of P18.

	Mean	Range
Oogonial diameter μ	32.4	26.6 - 39.5
Oospore diameter μ	28.6	23.8 - 31.7
% of oogonia with oospores	29%	

These figures are in agreement with those given by Waterhouse (1963) and Turner (1962) for P. palmivora and the fungal pathogen of Piper nigrum (P. palmivora) respectively (Chapter 8). They are also comparable with those previously obtained for P18 both in single isolate culture (Table 37) and in membrane culture with P28 (Table 46).

This experiment confirmed the ability of Trichoderma viride to stimulate sexual reproduction in a single isolate culture of P. palmivora, and, further, that the time of inoculation of T. viride in relation to the growth of the Phytophthora colony was critical. Once again the intrinsically bisexual nature of a single isolate of P. palmivora, which formed morphologically normal sex organs and oospores, was evident.

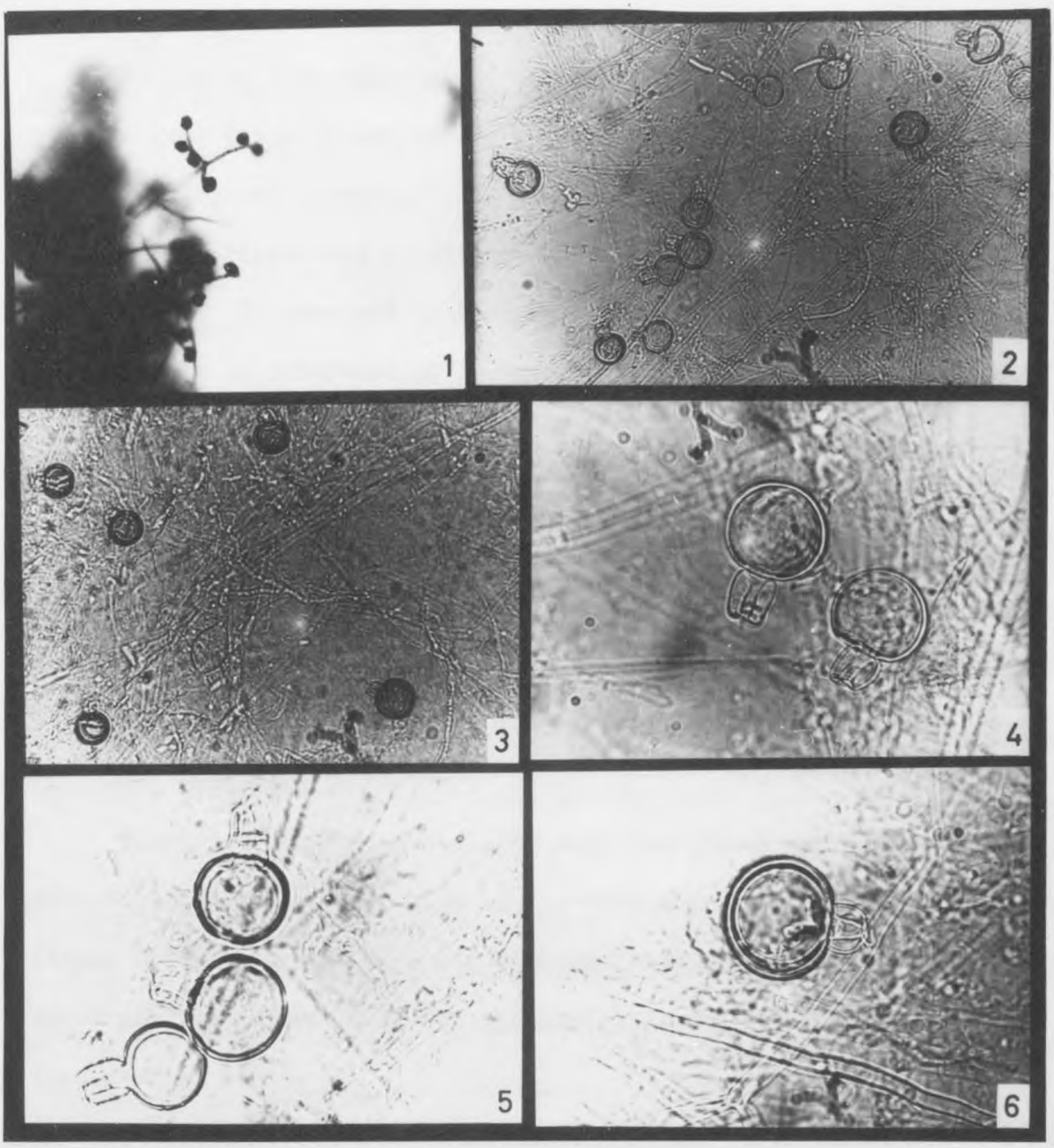


Fig.35. Stimulation of sexual reproduction in P. palmivora P18 by Trichoderma viride ; 1) conidia of Trichoderma viride, high power; 2) and 3) oogonia of P. palmivora under low power; 4), 5) and 6) oogonia of P. palmivora under high power showing morphology of sex organs and oospores.

Following this experiment, oil stock cultures of the T. viride isolate were prepared and retained for further experimentation when the physiological investigations in Sections I and II had been completed. The next experiment was conducted twelve months after the preliminary experiments. It remained to be seen whether after subculturing for stock plates at intervals of 3 weeks, P18 was still responsive to T. viride.

Chapter 13.

III. The critical time for the inoculation with T. viride.

The actual time for the inoculation of T. viride onto a growing culture of P18, and the ability of the P18 to form oogonia in response to T. viride after 12 months of subculturing, were investigated in the following experiment.

Thirty six carrot agar plates were inoculated with P18. Four were simultaneously inoculated at the very edge with conidia of T. viride (taken from a sporulating stock culture) and incubated at 22.5°C. To encourage faster growth of the Phytophthora, the remaining plates were incubated at 25°C.

After 24 hours the mean culture radii (4 radii) of a further 4 plates were measured. These plates were then inoculated with Trichoderma and incubated at 22.5°C. This was repeated (with four plates) at daily intervals for six days thus leaving only eight plates at 25°C. In these eight plates each colony of P18 was fully grown. Of these, four were inoculated with T. viride and the remaining four retained as controls.

These plates were then incubated at 22.5°C. Thus, 32 cultures of P18 with mean culture radii ranging from 0 to 45 mm. were inoculated with T. viride. The cultures were examined for oogonia two weeks after the date of inoculation with T. viride. These results are given in Table 49. The area covered by the P18 and T. viride colonies respectively, is shown in Fig. 36.

Clearly a minimum growth period was required by cultures of P18 before inoculation with T. viride if oogonia (and sporangia) were to be formed. The greatest sexual stimulus was achieved when the mean radius of the P18 colony lay between 27 - 39 mm. at the time of inoculation with T. viride. Cultures inoculated earlier were either less responsive (15 - 22 mm.) or were surrounded and probably killed by the other fungus (0 - 11 cm.). Growth of P18 was arrested where the two colonies met, whilst the Trichoderma, from slide examinations, continued to grow through the Phytophthora colony. In the case of the last four plates to be inoculated, this was characterised by a steady collapse of the aerial mycelium of P18 radially from the T. viride inoculum, (Fig. 36, 9). Trichoderma viride produced spores in all the inoculated plates. Oogonia were not formed in the control plates.

In the 27 - 39 mm. cultures, oogonia were scattered throughout the P18 mycelium, but in the 15 - 22 mm. cultures they were formed only along the line of conjunction of the two colonies. The oogonia, mostly golden brown in colour, with around 30% containing thin walled highly refractive oospores, appeared normal for P. palmivora.

TABLE 49.

The presence of oogonia in P18/T. viride cultures (Fig.36).

	Mean radius of P18 culture at time of inoculation with Trichoderma viride.		Date inoculated with T. viride.	Date examined (1965)	P18 sporing. present	P18 oogonial frequency
*	0	mm.	22/10	5/11	-	-
	0	"	22/10	5/11	-	-
	0	"	22/10	5/11	-	-
	0	"	22/10	5/11	-	-
	3.75	"	23/10	6/11	-	-
	3.75	"	23/10	6/11	-	-
*	4.25	"	23/10	6/11	-	+
	4.5	"	23/10	6/11	-	-
	7.5	"	24/10	7/11	+	-
	7.75	"	24/10	7/11	+	-
*	9.75	"	24/10	7/11	+	-
	11.25	"	24/10	7/11	+	-
*	15.25	"	26/10	9/11	+	+
	15.5	"	26/10	9/11	+	+
	16.0	"	25/10	8/11	+	+
	16.5	"	25/10	8/11	+	+
	18.25	"	25/10	8/11	+	-
	19.75	"	25/10	8/11	+	++
*	20.75	"	26/10	9/11	+	-
	22.5	"	26/10	9/11	+	++
	22.75	"	28/10	11/11	+	-
	27.0	"	27/10	10/11	+	+++
	28.25	"	27/10	10/11	+	+++
*	30.75	"	27/10	10/11	+	+++
	32.75	"	27/10	10/11	+	+++
*	37.5	"	28/10	11/11	+	+++
	38.5	"	28/10	11/11	+	+++
	39.25	"	28/10	11/11	+	+++
*	Full Plate		29/10	12/11	+	++
	Full Plate		29/10	12/11	+	++
	Full Plate		29/10	12/11	+	+
	Full Plate		29/10	12/11	+	+++
*	Full Plate		Control	12/11	+	-
	Full Plate		Control	12/11	+	-
	Full Plate		Control	12/11	+	-
	Full Plate		Control	12/11	+	-

* Appearance of plate is shown in Fig. 36.

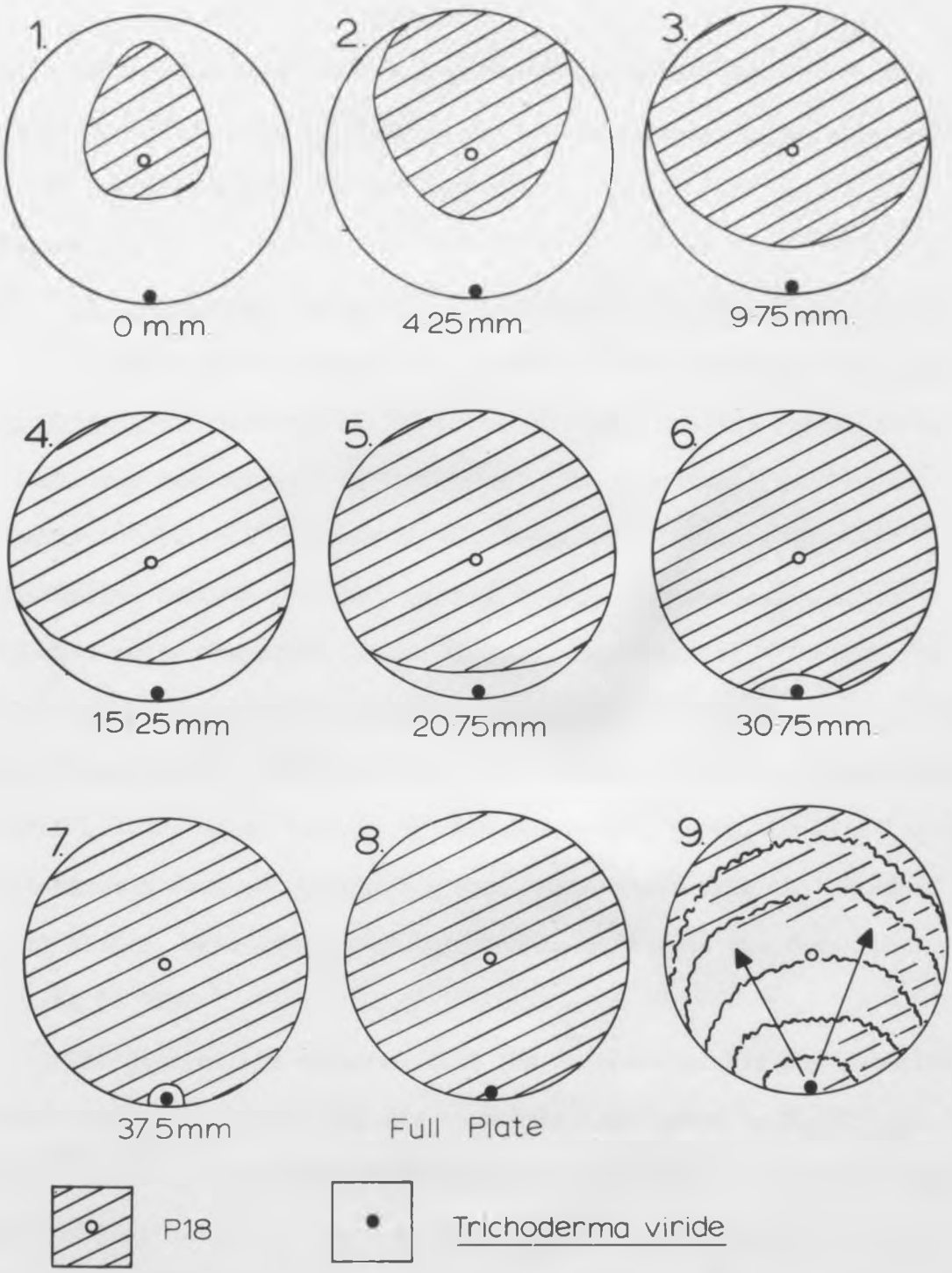


Fig.36. Appearance of P18 plates inoculated with T. viride at daily intervals. Figure is mean radius of P18 colony at time of inoculation with T. viride. See text and Table 49 for details.

This experiment confirmed that P18 was still responsive to sexual stimulation by T. viride, and that a minimum vegetative growth period was a requisite of the response.

Chapter 14.

IV. The interaction between P18 and strains of Gliocladium virens.

Hitherto some critical environmental factors influencing the Trichoderma/P18 interaction have been defined, but the mechanism of stimulation has not been investigated. The two fungi involved are clearly so distantly related taxonomically that no inter-hyphal association between the two, such as hyphal anastomoses, could be expected to be the cause of the stimulatory mechanism. Further the mere presence of Trichoderma as a physical barrier is equally unlikely to be responsible. Other physical barriers, such as the presence in a paired culture of an isolate of the same mating group, or of other chance contaminants such as Penicillium spp., or contact with the edges of petri dishes, have never been observed to stimulate the formation of oogonia in P18.

The possibility remained that the response of P18 was associated with fungistatic inhibition due to substances formed by T. viride. A recent paper by Lomas (1964) developed on taxonomic grounds the view that production of the fungistatic substances gliotoxin and viridin, long attributed to Trichoderma viride was in fact restricted to a closely related species, Gliocladium virens. They were unable to detect gliotoxin or viridin in culture filtrates of true T. viride (see also Webster and

Brian and Hemming, 1945; Brian et al. 1946; Webster, 1964; Webster, Rifai and El Abyad, 1964).

An experiment similar to that in the last Chapter was performed with gliotoxin and viridin producing isolates of Gliocladium virens. Eighteen carrot agar plates of P18 at different stages of growth from a 25°C incubator were inoculated at the edge with conidia from either of the two isolates, incubated at 22.5°C, and examined for oogonia after fourteen days. The results are given in Table 50. The interaction between the P18 and cultures of G. virens was very similar to that observed with T. viride in the last chapter (Fig.36). The growth of the P18 cultures was arrested a little earlier, possibly due to the fungistatic activity of gliotoxin or viridin. Nevertheless the Gliocladium isolates failed to stimulate sexual reproduction in P18. Thus neither the physical contact between P18 and G. virens isolates, nor the production of the fungistatic substances, induced a sexual response from the Phytophthora.

Earlier experiments involving P. heveae P28 and P18 suggested that the stimulus to single isolate oogonium formation was of a chemical nature. It was now thought that the T. viride/P18 interaction may be similar. This possibility was investigated in the following Chapters.

Chapter 15.

V. Possible modes of action.

One of the following mechanisms may be operative if the stimulus is of a chemical nature.

TABLE 50.

The presence of oogonia in P18/Gliocladium virens cultures.

Mean radius of P18 culture at time of inoculation with G. virens.	P18 sporangia present	P18 oogonial frequency
1. Viridin isolate.		
12.75 mm.	-	-
21.0	+	-
25.25	+	-
34.25	+	-
35.5	+	-
36.5	+	-
37.0	+	-
Full plate	+	+ (2 only)
Full plate	+	-
2. Gliotoxin isolate.		
12.0 mm.	-	-
19.5	+	-
25.25	+	-
33.5	+	-
33.75	+	-
35.75	+	-
36.5	+	-
Full plate	+	-
Full plate	+	-
3. Controls.		
Full plate, four replicates	+	-

(i) Secretion by T. viride of a substance (s) active as a specific Phytophthora sex hormone.

(ii) Secretion by T. viride of a nutrient (s) required for sexual

reproduction, not present in the growth medium.

(iii) Modification by T. viride of a substance (s) already present in the growth medium such that it became active as a sexual stimulant through mechanisms (i) or (ii) above.

(iv) Removal or modification by T. viride of a substance (s) formed by P18 which normally inhibits sexual reproduction in the latter.

These mechanisms, in particular (i), (ii) and (iii), might also be performed by an isolate from the complementary mating group.

The following three experiments were performed to demonstrate the broad nature of the mechanism of stimulation. So far, the stimulus has been investigated using only isolate P18. It was thought that the Trichoderma stimulus could be either of a comprehensive nature, inducing a response from both mating groups, as with P. heveae, or specific to one mating group. A brief experiment was conducted using the B group isolate P4, a strong mating isolate which like P18 responded to P. heveae. (Chapter 8).

Six well developed carrot agar cultures of P4 were inoculated at their edges with conidia of T. viride, incubated for fourteen days at 22.5°C and examined for oogonia (Table 51). Oogonia did not develop on either the inoculated or control plates. This result suggested that (unless cultural conditions were unsuitable) the Trichoderma stimulus was specific to the A mating group.

TABLE 51.

The interaction between P4 and T. viride.

Mean radius of P4 colony at time of inoculation with T. viride.	P4 sporangia present	P4 oogonial frequency
33.5 mm.	-	-
35.0	-	-
35.0	-	-
36.5	-	-
37.0	-	-
Full plate	-	-
Controls full plate (4 replicates)	-	-

A second experiment entailing the use of the casamino acids/ sterol medium, investigated the possibility that T. viride was modifying one of the many substances already present in the complex carrot medium. Six Casam. + S plates inoculated with P18 and incubated at 25°C were later inoculated with conidia of T. viride. transferred to 22.5°C and examined for oogonia after fourteen days. The results are shown in Table 52.

TABLE 52.

Mean radius of P18 colony at the time of inoculation with T. viride.	P18 sporangia present	P18 oogonial frequency
27.0 mm.	+	+++
30.25	+	+++
31.5	+	+++
31.5	+	+++
31.75	+	+++
Full plate	+	+++
Controls Full plate (4 replicates)	+	-

T. viride supplied a stimulus equally well on the simpler medium. This was a strong indication that the mechanism of action upon P18 was more direct than through the modification of substances present in the carrot agar. With a direct stimulus (mechanisms (i), (ii) and (iv)), a substance might be secreted directly into the medium by T. viride. If the substance were labile, highly volatile, or formed in very small quantities, close contact between the hyphae of P18 and living hyphae of T. viride might be essential for the expression of the stimulus. However, if it were formed in large quantities, a substance might be detectable in the absence of living T. viride mycelium by the use of liquid culture filtrates, and if thermostable in autoclaved agar cultures of T. viride.

In a third experiment, autoclaved carrot agar cultures of T. viride were re-inoculated with P18. Eight carrot agar plates were inoculated centrally with T. viride. with agar plug inocula free from spores, and transferred to an incubator at 25°C with eight non-inoculated control plates. The cultures reached the edges of the plates after three days. On the fourth day, four inoculated and four control plates were autoclaved at 10 lb./10 minutes (110°C), cooled, and each plate inoculated with P18 and transferred to 25°C. This was repeated with the remaining eight plates on the seventh day. The cultures were examined for growth and sporulation of P18 twenty days after re-inoculation. The results are given in Table 53.

TABLE 53.

Sporulation and growth of P18 on autoclaved cultures of T. viride.

T. viride plates	Incubation (days)	P18 aerial mycelium	P18 sporangia present	P18 oogonial frequency
a	4	++	+	++
b	4	++	+	+/++
c	4	++	+	++
d	4	++	+	+++
e	7	-	+	-
f	7	+	+	-
g	7	+	+	+ (1 only)
h	7	-	+	-
Controls				
4 replicates	4	++	+	-
4 replicates	7	++	+	-

Normal growth of P18, with the formation of oogonia occurred on the plates incubated with Trichoderma for four days. However, on T. viride plates incubated for seven days, growth of P18 was extremely poor, and oogonia were not formed. This result indicated that a thermostable stimulatory substance was produced by T. viride within a few days of growth. Since the presence of living mycelium of T. viride was not essential to the stimulus, it was thought the substance was probably a normal product of Trichoderma metabolism secreted into the medium. Removal of an inhibitor by T. viride (mechanism (iv)) would be less probable under these circumstances unless a chemical interaction were involved. The poor growth of the Phytophthora and the absence of oogonia on seven day cultures of T. viride may have been due to the accumulation

of metabolic products (staling substances) or fungistatic substances. Such substances may have been important in the fine balance observed between growth of both organisms and the formation of oogonia (Chapter 13).

Various other methods, such as the pouring of a fresh top agar over an autoclaved culture of T. viride and then inoculating with P18, gave similar results. The evidence suggested that a direct stimulation mechanism along the lines of (i) or (ii) was probably involved. The testing of culture filtrates of T. viride is described in the next Chapter.

Chapter 16.

VI. Use of liquid culture filtrates.

Demonstration of direct secretion of a sex stimulating substance by T. viride required a simple defined medium which could be freed from mycelium and spores after growth of the Trichoderma. The liquid culture medium chosen was that used by Weindling and Emerson, (1936); Weindling, (1937, 1941) for the isolation of toxic substances from culture filtrates of Trichoderma and Gliocladium, later used by Brian and Hemming (1945) and Brian et al (1946) for the extraction of gliotoxin and viridin. The formula and preparation of the medium is given in the Appendix.

Phytophthora isolates are difficult to handle in liquid cultures. Therefore drops of Trichoderma liquid culture filtrates were added to agar plate cultures of P18 by means of antibiotic assay discs in the following experiment.

Four Roux bottles, each containing 200 ml. of Weindling's medium, were inoculated with conidia of T. viride and incubated at 25°C, together with a fifth non-inoculated control bottle. The inoculated bottles were shaken after 24 hours to disperse the developing mycelium. On the sixth day the well developed cultures were shaken vigourously and filtered twice through a No.1 filter paper. The filtrate was freed from debris, and sterilised by re-filtering through oxoid membranes. The resulting filtrate was clear and pale yellow in colour.

Earlier, three sets of eight carrot agar plates had been inoculated with P18 at intervals of 24 hours, and incubated at 25°C. The mean colony radii of the twenty four plates now ranged from 19.5 - 35 mm. Samples of the culture filtrate were added to sixteen of these cultures by the use of antibiotic assay discs (p.139 and Fig.25, 2). Three discs saturated with the culture filtrate were added to each plate of P18. Discs saturated with sterile Weindling's medium from the control bottle were added to the remaining eight plates. All twenty four plates were incubated for 21 days at 22.5°C. The remaining T. viride filtrate was stored in a refrigerator at 5°C. The results are given in Table 54.

The assay discs in all twenty four plates remained moist. Normal growth of P18 (aerial mycelium ++) occurred in all the plates, and there were no signs of growth inhibition around the discs. The experiment successfully demonstrated that a substance(s) stimulating the sexual reproduction of an isolate of P. palmivora, P18, was present in a culture filtrate of Trichoderma viride. Oogonia were clustered around the

filtrate assay discs, mostly within a few millimetres, but were scarce beneath, and further away from the discs. Stimulation of sexual reproduction did not occur with Weindling's medium in the control plates.

TABLE 54.

Oogonia of P18 formed in response to culture filtrates of T. viride.

Mean radius of P18 colonies on addition of assay discs.	P18 oogonial frequency around assay discs	P18 oogonial frequency in rest of culture
T. viride filtrate.		
19.5 mm.	++/+++	++
19.5	-	-
20.0	++	-
20.0	+	-
20.5	++/+++	-
20.75	+	+
20.75	++/+++	-
27.75	++/+++	+/++
28.25	++	+
28.5	++	+
28.75	++	+
28.75	++	-
29.0	+/++	+/++
30.0	++/+++	+
30.75	+++	-
35.0	+++	++
Weindling's medium.		
Eight plates. (19.5 - 33.75 mm.)	-	-

The localisation of the stimulus at the edges of the discs, suggests that the substance did not diffuse very readily into the agar

medium, and may therefore have been of low solubility in water. This result also suggests that the substance is not formed by modification of a constituent of the carrot agar. However, the substance could be a modified component of Weindling's medium common to Casam. + S (Chapter 15). It is far more probable that a synthetic metabolite of T. viride is involved in the stimulatory mechanism.

Chapter 17.

VII. Final observations.

The chemical nature of the active substance(s) in the Trichoderma viride filtrate is not yet known and must await further analysis. In concluding the work to date, however, it is of interest to record that Trichoderma species are already noted to produce several substances of biological interest.

Weindling (1932) found that antagonism occurred between Trichoderma lignorum and Phytophthora isolates, and later investigated toxic substances in the culture filtrates of Trichoderma and Gliocladium (Weindling and Emerson, 1936; Weindling, 1937, 1941). Kaster (1938) investigated the antagonism of Trichoderma to Phytophthora, and attributed the ability of Trichoderma to replace Phytophthora in culture, to a faster growth rate in the former. There was no evidence for chemical action at a distance. Kaster also found culture filtrates of Trichoderma inhibitory to Phytophthora, and observed that T. koningi was more effective in this respect than T. lignorum. The substances involved may have been similar to those inhibiting growth of P18 on

autoclaved cultures of T. viride (Chapter 15). However, no observation has been made of the stimulation of sexual reproduction in Phytophthora by Trichoderma. Nevertheless, Thomas (1940) may at least have suspected such a possibility. Describing an investigation of sexuality in P. arecae he writes "addition of Trichoderma lignorum filtrate did not facilitate oospore formation."

Peltier and Borchers (1947) found Trichoderma capable of producing riboflavin. Buston and Kahn (1956) observed stimulation of perithecium formation in Chaetomium globosum by T. viride, and showed that the stimulus was associated with formation of organic phosphates, (especially phosphoglyceric acid) by Trichoderma, and other micro-organisms, in fermented medium. The synthesis of palmitic, stearic, oleic, linoleic and linolenic acids by T. viride was demonstrated by Crombie and Ballance (1959) Lipids were shown to stimulate the sexual reproduction of P. palmivora and P. heveae in Section II.

Trichoderma viride has also been found to modify steroids. Wix et al (1956) observed production of 11α-hydroxyprogesterone from progesterone by cultures of T. viride, and Dulaney and McAleer (1958) 17α hydroxylation of steroids by the same fungus.

Sterols are already implicated in the sexual reproduction of isolates of Phytophthora (Section II). A crude ether extract was therefore made from the remainder of the T. viride liquid culture filtrate, and tested for sterols. A sample, 250 ml., of the pale yellow filtrate was extracted with ether five times in a separating

funnel, using 100 ml. of the solvent on each occasion. The colourless extract was washed with .1 litre of distilled water and dried over anhydrous Na_2SO_4. This was finally evaporated to 'dryness' in a 100 ml. Buchner flask, under reduced pressure, using a waterbath at 60°C. A slight residue, of an oily viscous nature, remained. The residue was re-dissolved in 6 ml. of chloroform, and tested in 2 ml. portions for the presence of sterols, using the colour tests in Table 55, (described in the Appendix).

TABLE 55.

Tests for sterols in the ether extract of T. viride culture filtrate.

Test	Result	Sterol present
Liebermann Burchard	Turned red	+
Salkowski	Turned red	+
Tschugaeff	Deep purple above/ green below.	+

+ = test positive

The tests suggested that sterols were present in the T. viride culture filtrate, as a result of direct secretion into the medium by Trichoderma. It is therefore possible that, among other substances, a specific sterol produced by T. viride is a chemical agent active in inducing sexual differentiation in the P. palmivora isolate, P18.

Chapter 18

DISCUSSION

In this work isolates of Phytophthora palmivora taken from Piper nigrum in Sarawak, together with other P. palmivora isolates and isolates of different species within the 'palmivora' group, fell into two distinct mating groups in a manner typical of heterothallic species of Phytophthora (p.125 et seq). The two groups were probably analagous to the classical 'cacao' and 'rubber' groups, as described by Gadd (1924). The presence of both mating groups, A and B, among isolates from Piper nigrum in Sarawak, together with the earlier observation (p.49) that oospores can be formed in host material infected with both A and B group isolates, suggests that oospores participate in survival and variation in nature (see also p.64).

The isolates were usually self-sterile in single culture, but formed oospores when paired with compatible isolates. In an initial test however, nearly 60% of isolates from both mating groups formed sex organs in single culture. This ability was sporadic, and declined with continued sub-culturing, though sex organs were still formed by single isolates in the presence of a stimulus provided by the homothallic P. heveae, by Trichoderma viride, and probably by certain bacteria, even after much subculturing. Thus, many of the isolates, though self-sterile, were found to be bisexual. This had already been demonstrated for several other species (Koyeas, 1953; Stamps, 1953; Galindo and Gallegly, 1960;

Savage and Clayton, 1962). Indeed, in view of the bisexual nature of their isolates of P. infestans, Galindo and Gallegly considered it inappropriate to refer to mating groups, but rather to term them compatibility groups. This suggestion will be adopted hereafter for the A and B groups.

The compatibility mechanism was found to involve a simple division of the isolates into two compatibility groups in the absence of any additional groups, intergrades, or neutrals - yet both isolates in one compatible A x B group pairing could be bisexual. It was therefore thought that the expression of sexuality was probably not closely associated with the compatibility reaction between two isolates. Indeed, it has already been suggested (Galindo and Gallegly, 1960) that sexual characteristics and compatibility group characteristics of such isolates are expressed independently: the mechanism of reproduction will be discussed from this angle.

What is the nature of the compatibility mechanism? No direct evidence was obtained for the compatibility reaction between A and B group isolates. However, the response of single isolates of P. palmivora to stimuli provided by P. heveae and Trichoderma viride demonstrated indirectly that this is in all probability a mechanism involving chemical stimulation. Phytophthora heveae stimulated sexual reproduction in single isolates of both compatibility groups. T. viride stimulated an isolate of the A group but not a B group isolate. The number of substances involved is uncertain and their chemical nature unknown, but it is possible that

the number of substances is only one or two.

For example, with two substances it could be argued that one substance is normally produced by the A group isolate to induce sexual differentiation in the B group isolate and vice versa for the other substance. Therefore P. heveae might produce both substances, but T. viride only one, that normally produced by B group isolates. The evidence is as yet too unsubstantial to accommodate a theory. However, it is thought that the compatibility group reaction involves a chemical secretory and possibly a hormonal mechanism which operates to overcome the self-sterility of the isolates. Sex hormones are already known from related aquatic Phycomycetes (Bishop, 1940; Raper, 1952; Machlis, 1966).

The sporadic formation of sex organs by single isolates on emergence from stock cultures is difficult to explain from the standpoint of a chemical compatibility mechanism. The accumulation of a substance(s) stimulating sexual reproduction, due to the activity or supression of certain metabolic pathways during vegetative dormancy may account for this uncertain but temporary phenomenon.

Since many isolates were bisexual, the compatibility reaction appeared to be independent of the sexual nature of the isolates. It also appeared to be independent of the strength of the mating reaction in terms of oogonial frequency. This varied greatly according to the isolates concerned in a pairing, and appeared to be correlated with intrinsic mating strengths or potentials (indices) calculated for the individual isolates (Chapter 5).

With regard to the cause of the wide variation in mating strengths observed, it is thought that the mating strength of an isolate (I) may be related to its physiological vigour, more specifically to its nutritional ability and capacity to form oogonia and oospores in single isolate fashion.

This 'theory' was suggested by the observations (i) that the mating strengths of compatible isolates in a pairing gave a predictable result in terms of the mean oogonial frequency within the pairing, and (ii) that isolates of the strong sub-group showed a greater tendency to form sex organs in single isolate culture than either moderate or weak isolates. Similar evidence comes from the work of Apple (1959) who found highly compatible isolates of P. parasitica v. nicotianae consistently able to produce oogonia in single isolate culture, whereas weakly compatible isolates did not do so. Strong or highly compatible isolates may be better equipped nutritionally than moderate or weak isolates to support the formation of oogonia and oospores when a stimulus is supplied. The expression of such vigour may have a multi-genic basis, and the genetic loci postulated may be those particularly associated with the nutrition and control of oogonium formation.

Koyeas (1953) also found, with P. parasitica, that the proportion of oogonia produced by each strain in a given mating was constant. He additionally proposed that all his isolates were bisexual, but that sexuality was relative, and obtained evidence with liquid cultures for the existence of sex hormones. However, he did not infer from his own

work that compatibility and sexual strength were distinctly expressed entities, the concept developed by Galindo and Gallegly (1960) and supported in this thesis.

Galindo and Gallegly, using hyphal tracing techniques, established that their isolates of P. infestans were bisexual, and that they exhibited a range of male and female characteristics. Some isolates behaved predominantly as males, some as females, and others equally as both. They concluded that femaleness was the expression of an ability to form oogonia in relation to the nutritional strength of the isolate concerned:

"preliminary data suggest reaction of an isolate may change with an alteration of its vigour of growth. In pairing of compatible isolates of equal sexual strength, hyphae of the A_1 (compatibility) type that had been starved produced only antheridia when paired with an unstarved isolate of the A_2 compatibility type, whereas the unstarved A_1 type produced only oogonia when the A_2 type was starved."

The conclusions reached in the present work with oogonial frequency measurements, and by Galindo and Gallegly with hyphal tracings, are similar. Indeed, it may be theoretically possible to align the two pieces of work by equating P. infestans predominantly male isolates with weak sub-group isolates in P. palmivora, and predominantly female isolates with strong sub-group isolates.

Thus, weak isolates, as already suggested, may be physiologically deficient, and unable to form oogonia in single isolate culture even if a suitable stimulus is supplied. In weak A x weak B pairings very few

oogonia, if any, are formed, although a normal compatibility reaction may occur. When paired with moderate or strong isolates, weak isolates behave as male, providing mostly antheridia. In contrast, strong isolates are physiologically equipped to form oogonia in single isolate culture if a stimulus to sexual differentiation is supplied. In pairings they form sex organs abundantly, and are equivalent to Galindo and Gallegly's ♀ or ♀♂ isolates, behaving as ♀ , ♀♂, or both, according to the vigour of the other isolate. Moderate isolates are less vigourous, more equivalent to a balanced ♀♂ , in general providing fewer oogonia than strong isolates, and able in some instances to form sex organs in single isolate culture.

Thus,if both isolates in a pairing are strong, both behave bisexually, but if one isolate is stronger (more vigourous) it provides more oogonia than the other, and hence it appears more ♀ , the other more ♂ . In this way the oogonial frequencies observed on each side of a pairing, and the mating strength indices calculated from them, may be directly associated with intrinsic sexual balance and potential, and have resulted from (i) an expression of the individual vigour or mating strength (I) of each isolate, and (ii) the additional interaction of the two mating strengths, by hybrid sex organ formation. The extra strong mating reaction between isolates from the same host plant (Chapter 5) may be equated with the above concept if it is assumed that when isolates are physiologically similar (owing to adaptation to living on a certain host and thus having a similar genetic background) the potential doubling of nutrient ability in the pairing of two strong isolates is

reinforced.

Significantly, both the present work and that of Galindo and Gallegly (1960) resulted in the conclusion that the compatibility reaction is probably independent of sexual expression, and that mating strength of an isolate may be an expression of the ability to form oogonia related to its physiological vigour. The compatibility reaction itself may be a chemical stimulation mechanism which serves to initiate sexual differentiation by overcoming the self-sterility of potentially bisexual isolates e.g. by induction or derepression. However, this general theory does not exclude the possibility that mating strength is in some way related to the quantity and quality of substances produced in the compatibility reaction. This theory may be verified by more experimental work, to which it is a guide.

Further work along the lines indicated in this Section should assist in the elucidation of the compatibility mechanism. One immediate advance would be the chemical identification of the sex stimulating substance produced by T. viride. In view of the uncertain nature of the evidence, the possibility that this substance is a steroid is purely speculative (Chapter 17). Another approach could be to adopt the antibiotic-assay disc technique used with T. viride to detect the activity of any stimulatory substances present in liquid culture filtrates of P. heveae or P. palmivora upon single isolates of P. palmivora in agar cultures. A search could also be made for semi-permeable membranes that are not penetrated by Phytophthora. With regard to the expression of sexuality and mating strength, a combination of hyphal tracing and starvation

techniques, together with oogonial frequency assessments, should give a clearer picture of the sexual mechanism.

The ultimate tool in the elucidation of the reproductive mechanism could well be a genetic analysis (based on oospore germination) of the segregation of loci concerned with expression of compatibility and sexuality. This approach will have no place, however, until suitable techniques have been developed for the germination and culturing of isolated oospores. Recent success with germination of P. heveae oospores (Leal, 1965) indicates that this problem could be overcome.

Other aquatic Phycomycetes (Couch, 1926; Bishop, 1940; Raper, 1940) and Peronospora parasitica (De Bruyn, 1937) exhibit a pattern of sexuality resembling that in Phytophthora. and recent cytological evidence suggests that both the Saprolegniales and the Peronosporales may be diploid in the vegetative state (Sansome, 1961; Sansome and Harris, 1962; Sansome, 1963). Diploidy, with meiosis taking place at or near gametangium formation rather than oospore formation, together with complex sexual mechanisms, may be characteristic of this group of fungi as a whole. Indeed, recent genetic evidence involving the germination and typing of oospores in Dictyuchus monosporus and Achlya bisexualis is consistent with the concept of diploidy (Mullins and Raper, 1965; Barksdale, 1966).

On the basis of a diploid life cycle, complex genetic models could be devised to explain the wide ranges from near unisexual non self-fertile to homothallic isolates found in certain species of this group of fungi. Raper (1966) has already suggested that in biflagellate

fungi "the inheritance of mating competence involves a more complex genetic basis than alternate alleles at a single locus." This observation certainly appears to apply to sexual expression in Phytophthora, where a wide range of mating potentials is to be found. The loci involved could be those governing nutritional, structural and hormonal aspects of the formation of antheridia and oogonia. However, in Phytophthora a distinct compatibility mechanism may be superimposed upon the pattern of relative sexuality as found in Achyla, Dictyuchus and Sapromyces. Genetic analysis will demonstrate whether or not the postulated loci for compatibility and sexuality segregate independently. This seems possible in view of the wide range of sexual strengths found in both compatibility groups.

Finally, it is of phylogenetic interest that P. heveae, a homothallic fungus, is able to stimulate sexual reproduction by single isolates of both compatibility groups in a related heterothallic species, since it is believed by some authors that many modern homothallic fungi are derived from heterothallic species (Whitehouse, 1949; Raper, 1954, 1959; Burnett, 1956; Olive, 1958). The close relationship between the two species suggested by the compatibility reaction indicates that the reproductive mechanism in the homothallic fungus may be essentially the same as that which occurs when A and B group isolates meet e.g. P. heveae may combine within one thallus all genetic loci controlling the compatibility reaction, whereas in heterothallic isolates the loci are functionally divided between the two compatibility groups. P. heveae may be a

representative of an ancestral type of fungus from which the two compatibility groups have been derived, or a homothallic organism synthesised from the two compatibility groups, perhaps by an aberrant genetic mechanism at syngamy.

CONCLUDING DISCUSSION

This work has inevitably created as many problems as it has solved. Some problems were brought to light during the course of accessory investigations into environmental effects upon reproduction. These would be well worth investigation for their own sake. Among these problems could be included (a) the mechanism behind light inhibition of oogonium formation, (b) the genetic mechanism behind the unstable 'fluffy' sectors of Phytophthora heveae. (c) the balance between sexual and asexual reproduction, and (d) the role of sterols in development.

Other problems, such as the chemical nature of the stimulatory substance(s) produced by T. viride are central to the problem of the mechanism of sexual reproduction in Phytophthora itself, and more in line with the objectives of this work. Looking back to the General Introduction, many other important problems of the genus remain untouched. Clearly a great deal still has to be learnt within the confines of this very small field. However, it is hoped that the approach to the problem, in first examining some of the fundamental environmental aspects of reproduction, using the experience gained to investigate the sexual mechanism, will have assisted in standardisation of techniques and provided some basis

for further work.

By the use of the same four isolates in all three sections it was hoped to introduce a degree of continuity to the experimental work. However, in face of the complex interactions observed

(1) Between the genotype of each isolate and its physical and chemical environment,

(2) between the genotypes of the different organisms, culminating in

(3) the overall interaction,

the division of the work into three sections appears, as it indeed is, distinctly artificial. For example, the sexual reproduction mechanism, itself complex, may involve (i) chemical expression of compatibility and, (ii) an expression of sexuality related to the variable physiological capabilities of the isolates themselves. The mechanism was divided on the basis of the evidence into (i) compatibility and (ii) sexual mechanisms (Section III). In Section I, physical factors of the environment, temperature and light, were found to control the frequency of oogonia, and to affect the balance between vegetative and sexual reproduction.

In respect of this evidence from these two sections, does the effect of temperature upon sexual reproduction in P. palmivora entail an effect upon the compatibility reaction, upon the balance of sexual expression within the isolates or upon general metabolism? Again, in Section II, small variations in nutrient balance such as the absence of sterol in the medium, clearly disrupted the normal course of the formation

of oogonia and oospores. Does this observation in addition hold implications for the compatibility mechanism, or for the mechanism of sex organ formation, as examined in Section III?

Experimental answers to developmental problems such as these would clearly link across the three sectional classifications of physiology, nutrition, and mechanism of sexual reproduction in Phytophthora.

APPENDIX

Notes on preparation of media and colour reaction tests.

1. Oatmeal agar (OMA).

100 g. rolled oats were soaked overnight in 700 ml. distilled water, warmed one hour at 60 - 70°C in a water bath, filtered through muslin, and the extract made up to 1000 ml. with distilled water. Difco agar was used at 15 g./l.

2. Carrot agar (CA).

200 g. washed carrots were ground in a Waring Blendor with 500 ml. distilled water, filtered through two layers of muslin, and the extract made up to 1000 ml. with distilled water. Difco agar was used at 15 g./l.

3. Cornmeal agar (CMA)

Oxoid Cornmeal agar was used.

4. Synthetic serine medium, Med. A + S.

The composition of this medium is given on p.71.

5. V-8 juice agar.

Campbells V-8 Juice	200 ml.
Agar	15 g.
Distilled water to	1000 ml.

6. Lipase detecting medium; modified from Starr (1941).

Yeast extract	5 g.
Tryptone	10 g.
Sunflower seed or Castor oil	10 ml.
0.3% alcoholic Spirit Blue	50 ml.

6. (continued)

Agar	15 g.
Distilled water to	1000 ml.

7. Medium suitable for sexual reproduction of Phytophthora palmivora.

As Medium A + S above (4), but substituting 2g./l. casamino acids in place of serine, and 50 mg./l. β-sitosterol.

8. Petri solution.

$Ca(NO_3)_2$	0.4 g.
$MgSO_4.7H_2O$	0.15 g.
KH_2PO_4	0.15 g.
KCl	0.06 g.
Distilled water	1000 ml.

9. Carrot extract.

Prepared as for carrot agar (2) but with 100 g./l. carrots.

10. Weindling's Medium.

Glucose	25.0 g.
Ammonium tartrate	2.0 g.
KH_2PO_4	2.0 g.
$MgSO_4.7H_2O$	1.0 g.
$FeSO_4$	0.01 g.
Distilled water to	1000 ml.

pH adjusted to pH 3.5 with phosphoric acid.

11. Colour reaction tests for sterols. (from Heftmann and Mosettig,1960)

Liebermann Burchard.

Dissolve in 2 ml. chloroform. To 1 ml. add 5 drops acetic anhydride, plus 1 drop concentrated sulphuric acid, and shake. Colour change from red to green denotes presence of sterols.

Salkowski.

Dissolve in 2 ml. chloroform. To 1 ml. add 1 ml. concentrated sulphuric acid. Mix carefully and note colours in both layers. Sterol layer turns red.

Tschugaeff.

Heat test substance in chloroform or glacial acetic acid with zinc chloride and acetyl chloride. Presence of sterol denoted by purple colour on addition of ferric chloride dissolved in a mixture of glacial acetic acid and concentrated sulphuric acid.

ABSTRACT

The homothallic species, P. heveae, formed oospores mainly at the optimum temperature for radial growth (25°C) and sporangia above 27.5°C, whereas the heterothallic P. palmivora formed oospores at 20°C (optimum) and sporangia between 27 - 30°C, the optimum for radial growth. Daylight induced abundant formation of sporangia, but supressed oogonium formation in both species, at 22.5°C.

When alternative concentrations of nutrients were substituted into a basal medium, serine (nitrogen) and glucose (carbon) concentrations affected the quantity of mycelial growth and the frequency of both sporangia and oogonia. KH_2PO_4 concentrations affected the size and contents of sporangia and the percentage of oogonia which contained oospores. β-sitosterol, which affected mainly qualitative aspects of growth and reproduction, was thought to act as a non-essential growth factor. In its absence, oogonium formation was delayed, oospores were not formed, and sporangia were small, their contents disorganised, and no zoospores were released.

Leaves of Piper nigrum inoculated with compatible isolates of P. palmivora were found to contain oogonia and oospores, suggesting that sexual reproduction may take place in the field.

Although nearly 60% of the isolates of P. palmivora were found to be bisexual from the examination of single isolate cultures, they fell into two distinct compatibility groups, A and B, on the basis of oogonium formation in paired cultures. Single isolates of both compatibility groups were stimulated to produce sex organs (without

hybridisation) by an isolate of P. heveae. Liquid culture filtrates of Trichoderma viride stimulated the formation of sex organs and oospores in agar cultures of an A group isolate only. These results were interpreted as indirect evidence for the existence of a chemical compatibility mechanism between bisexual, but normally self sterile, A and B group isolates.

Strong, moderate, and weak mating isolates were defined, within both compatibility groups, on the basis of the frequency of oogonia in their pairings. All strong isolates, and some moderate isolates, but none of the weak isolates, formed sex organs in single isolate culture. It was suggested that mating or sexual strength was independent of compatibility, but related to the nutritional vigour of an isolate.

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