STUDIES IN THE GENUS PHYTOPHTHORA DE BARY

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ELIZABETH IRENE LOVE MORGAN, B.Sc.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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General Introduction

The order Peronosporales includes many important plant pathogens. The most notable of these belong to the genera Phytophthora, Pythium and Plasmopara. Each year in both temperate and tropical zones species of these genera cause considerable damage to crops. Despite their obvious economic importance very little is known of the biology of these fungi. Mechanisms controlling variation are unknown. Factors controlling sexuality and its inheritance have yet to be determined. The place of meiosis in the life cycle and the ploidy of the somatic nucleus are still a matter of controversy.

Cytological methods have been used to investigate the stage at which meiosis occurs as a means of determining the ploidy of the somatic nucleus. This will be haploid if meiosis occurs in the oospore, or will be diploid if meiosis occurs in the developing gametangia prior to fertilisation.

Evidence supporting both haplontic and diplontic types of life cycle has been presented. Meiotic figures in the germinating oospores have been reported in Sclerospora graminicola (McDonough, 1937), Plasmopara viticola (Bosc, 1947) and Albugo evolvuli (Thirumalachar et al, 1949). Sansome working with Pythium debaryanum, Sclerospora sorghi, Phytophthora erythroseptica, P. cactorum and Achlya sp. (1961, 1963, 1965),

considered that reduction division occurs in the antheridium and oogonium. However figures similar to those of Sansome have been demonstrated in the hyphal tips of Phytophthora infestans (Marks, 1965).

Some indication of the ploidy of the nucleus of certain organisms, e.g. Neurospora crassa (Norman, 1954) has been obtained by extrapolation of the killing curves of X-rays and ultra violet irradiation. No work with X-rays has been reported with species of Phytophthora. Buddenhagen (1958) calculated that the effect of ultra violet on the zoospores of P. cactorum indicated the existence of 2-3 targets or genomes per zoospore. However similar investigations by Clarke (1964) on P. infestans and Shaw (1965) on P. cactorum indicated over 100 targets. This casts doubts upon the reliability of this method of estimating the ploidy of the nucleus.

The most satisfactory method of determining ploidy and the stage at which meiosis occurs is the analysis of the segregation of nuclear markers after sexual reproduction. Progress in the Peronosporales in this field has been hindered by

(1) The persistent dormancy of the oospore,

(2) The difficulty in obtaining suitable nuclear markers.

However, recently within the genus Phytophthora, the oospores of several homothallic and heterothallic species have been germinated. Also an analysis of the segregation of markers such as mating type, pigment production, and colony morphology has been attempted (Galindo and Zentmyer, 1967; Satour and Butler, 1968). These studies have shown that parental and recombinant types exist among the oospore progeny of a mating but each oospore only produces one phenotypic class in its zoospore progeny. These

results can be explained if meiosis occurs in the gametangia or if meiosis occurs in the oospore and only one product survives. Thus further genetical studies are required to demonstrate which hypothesis is correct.

The aim of the work described in this thesis was to make a genetical study of a suitable species of Phytophthora, and thus to determine the ploidy of the somatic nucleus.

As it is of advantage to use a heterothallic species to ensure that the products of sexual reproduction are crossed and not selfed, Phytophthora palmivora (Butl.) Butl. was chosen as one of the test organisms. The first section of the thesis deals with attempts to germinate the oospores of this species.

Phytophthora cactorum (Leb. and Cohn) Schroet. was chosen as the other test organism for two reasons: (1) 67% germination of the oospores of this species has been obtained (Shaw, 1965), (2) Resistance to streptomycin has been found in this species (Shaw, 1965; Shaw and Elliott, 196

Segregation in the selfed progeny of a homothallic species will occur if the markers used are dominant. For this reason drug resistance was studied as this type of mutation usually shows dominance.

Section II of the thesis is concerned with the search for resistance to other drugs in P. cactorum.

SECTION I - OOSPORE DORMANCY AND GERMINATION IN PHYTOPHTHORA PALMIVORA (BUTL.) BUTL.

Oospore Dormancy and Germination in Phytophthora palmivora

Introduction

Dormancy is any rest period or reversible interruption of the phenotypic development of the organism (Sussman, 1965). For convenience this interruption is divided into two somewhat overlapping categories depending on the factors imposing it. Constitutive dormancy is caused by some internal factor whereas exogenous dormancy is imposed by an adverse environment.

The occurrence of a dormant period which permits species to survive unsuitable environmental conditions is a general phenomenon. It is so widespread, being found in all divisions of the plant kingdom, and has such ecological significance that very many mechanisms of dormancy have evolved. These range from purely mechanical barriers to complex biochemical systems not yet fully understood.

The ability of a spore to germinate depends on its stage of development. Both development of the wall and questions of nuclear fusion and cytoplasmic organisation must be considered. The failure of certain zygospores to germinate may be related to cytoplasmic immaturity for here the resting stage precedes nuclear fusion. As soon as nuclear fusion has occurred the spore germinates (Allen, 1965). In other cases germination does not occur when the spore is placed in conditions favourable to growth even though the development of the wall and all nuclear and cytoplasmic events have apparently been completed. This situation may be called true dormancy, but to distinguish it from immaturity requires a knowledge of development too often lacking. The truly dormant spore must evidently

go through some further internal changes, as yet unknown, before it can germinate; these changes constitute "after-ripening". In practice it is usually considered that after-ripening, but not maturation can be shortened by activation treatments (Sussman, 1965).

The time required for after ripening processes to occur naturally in fungal spores varies greatly. At the two extremes in the Phycomycetes are the oospores of Pythium angustatum which Sparrow (1931) was able to germinate immediately after formation, and the oospores of Peronospora destructor which McKay (1935) found to require four years before germination occurred. Other times are found within these extremes depending on the species. Zeigler's (1948) intensive studies on the oospores of many members of the Saprolegniaceae showed that the after-ripening period varied from 30 hours to 3 months. De Bary (1860) found that the oospores of Aphanomyces stellatus germinated after three months. Blackwell (1943) found that the oospores of Phytophthora cactorum required nine months.

Methods of shortening the after-ripening period

In any fungal spore studies, whether genetical or physiological, it is of advantage to be able to germinate the spores in quantity simultaneously. Thus many techniques of shortening the after-ripening period have been developed. These methods fall into four main categories:

1) Temperature
   a) high temperature
   b) low temperature
2) Chemical
3) Biological
4) Light

1. a) High Temperature Treatments

The observation that the spores of certain organisms appeared to germinate better after they had passed through the intestines of animals led to the suggestion that it might be the body heat of the animal which acted as the stimulant.

Welsford (1907) tested this possibility and concluded that incubation at 38°C in cow dung broke the dormancy of the ascospores of Ascobolus furfuraceus.

Dodge (1912) was the first to use temperatures above body temperatures, when he achieved germination of A. furfuraceus ascospores and other related species with a heat treatment of 60 - 70°C for 15 - 30 minutes. It has now been found that the ability to be activated by heat appears to be a general property among the coprophilous ascomycetes (Sussman, 1965).

Apart from ascomycetes only a few other fungi have been found to respond to a heat treatment. The spores of Ustilago striiformis (Krietlow, 1943), Phragmidium mucronatum (Cochrane, 1945) and the spores of the phycomycete Phycomyces blakesleanus (Sommer and Halbsguth, 1957) are reported to be activated by heat.

In all cases of heat stimulation the spores must be returned to room temperature before germination will occur. Lowry and Sussman (1958) working with Neurospora tetrasperma found that the heat resistance was a property of the dormant spore wall and once the spore was activated it became heat-sensitive - thus the necessity of returning the spores to room temperature.

1. b) Low Temperature Treatments

Many workers have found that an "overwintering" period is of importance in the after-ripening process of some fungal spores. Methods of imposing this artificially and thus breaking dormancy have been found to be successful with certain species.

The temperatures which have been found to be effective range from freezing point, e.g. the teliospores of Puccinia glumarum (Raeder and Bever, 1931), to 10°C, e.g. the ascospores of Taphrina coryli (Martin, 1924). The times for which the spores must be held at low temperatures vary from being as short as three hours with the aeciospores of Puccinia graminis (Eriksson and Henning, 1896) to six months with the spores of Physoderma dulichii (Johns, 1958).

There are several reports of low temperature treatments shortening the dormant period of oospores. Sparrow (1931) obtained germination of the oospores of Pythium adhaerens by storing them outside from February until April. Blackwell (1943) in controlled temperature experiments with Phytophthora cactorum obtained maximum germination by exposing oospores two to nine months old to temperatures just above freezing for one week.

As with high temperature treatments all spores have to be returned to room temperature before germination will take place.

2. Chemical Treatments

Compounds ranging from organic solvents, e.g. alcohols and acetone, esters, organic acids and other metabolites to inorganic materials, e.g. salts and acids have been found to stimulate the germination of some fungal spores (Sussman, 1965).

Acids were shown to be effective in promoting germination of the teliospores of Puccinia graminis (Sibilia, 1930) but the effect was one of acidity rather than of a particular acid. Providing that the pH of the medium = 2.0 both inorganic and organic acids were equally effective in breaking dormancy. Allen (1955) showed that coumarin and dinitrophenol enhanced the germination of the urediospores of Puccinia graminis tritici by reversing the effects of a volatile self inhibitor. Sussman (1953) found that furfural and many of its derivatives could be used instead of a heat shock to break the dormancy of the ascospores of Neurospora tetrasperma.

3. Biological Stimulation

There are several reports in the literature of the spores of plant pathogenic fungi being stimulated to germinate in the presence of host roots or host plant exudates. Hooker et al (1945) found that mustard oils produced in low concentrations by the roots of cabbage plants stimulated the germination of the resting spores of Plasmodiophora brassicae. Scharen (1960) reported that he obtained maximum germination of the oospores of Aphanomyces euteiches when they were in the vicinity of pea roots. Also Ghafoor (1964) found that the percentage germination of the oospores of Aphanomyces raphani was increased by a factor of fifteen when the spores were adjacent to radish roots.

Animal stimulation is also known. The ascospores of Onygena corvina require egestion by owls before germination will occur (de Bary, 1887) and germinating spores of Russula and Lactarius species have been found in toads (Voglino, 1895).

Gregg (1957) obtained germination of the oospores of P. erythroseptica after they had passed through the gut of the land snail Helix aspera, and Shaw (1965, 1967) obtained 67% germination of the oospores of P. cactorum which had been fed to water snails Planorbarius corneus.

An interesting example of self-stimulation was found in P. graminis tritici (French, 1961). Terpenes and related volatile compounds, in crude extracts, from urediospores stimulated urediospore germination.

4. Light Treatment

Light has been regarded as an inhibitor of germination for a long time (see Gottlieb, 1950 for review). Recently, however, increasing attention has been paid to its role in activating the dormant spores of some species. Many spores including smut spores, urediospores and oospores have been found to respond to a light treatment.

The intensity of the light required for activation varies. Some spores, e.g. the resistant sporangia of Physoderma maydis require as little as 0.1 - 0.2 foot candles for activation (Herbert and Kelman, 1958). The spores of Tilletia contraversa and other smut species have been found to require 50 - 150 foot candles (Gassner and Neiman, 1954) and the oospores of Phytophthora cactorum give maximum germination with a light intensity of 600 - 700 foot candles (Berg and Gallegly, 1966).

The quality of the light is also important. Blue light has been found the most effective with P. cactorum (Berg and Gallegly, 1966) and Oidium monilioides (Sempio and Castori, 1950). Light of longer wavelengths, 550 - 750 mμ was found to inhibit O. monilioides.

Alternating light and dark has been found to activate the oospores of Phytophthora drechsleri (Galindo and Zentmyer, 1967a) and Peronospora

tabacina (Lucas and Person, 1954).

Materials and Methods

The isolates used in the present study were obtained from the Commonwealth Mycological Institute, Kew, Phytophthora palmivora serial numbers IMI.63552 and IMI.79233, P. cactorum serial number IMI.21168 They have been cultured on oatmeal agar for several years and subcultured at regular intervals. Abundant oospores are produced by these isolates under suitable conditions.

A semi-synthetic medium has been used successfully for oospore production (Shaw, 1965). This medium is composed of a minimal synthetic medium (Elliott et al, 1964) supplemented with an aqueous extract of garden peas and a light petroleum ether extract of oats. The oat extract supplies sterols which have been proved necessary for oospore production (Hendrix, 1964, 1965; Elliott, Hendrie, Knights and Parker, 1964; Leal, Friend and Holliday, 1964; Harnish, Berg and Lilly, 1964).

Composition of the Minimal Medium

Sucrose	10 gm
L-asparagine	1.0 gm
Magnesium sulphate	0.25 gm
Potassium dihydrogen phosphate	0.5 gm
Thiamin hydrochloride	1.0 mg
Trace element solution	1 ml
Deionised water	1000 ml

Composition of the trace element solution

$Na_2B_4O_7 \cdot 10H_2O$	88 mg	$Na_2MoO_4 \cdot 2H_2O$	50 mg
$CuSO_4 \cdot 5H_2O$	393 mg	$ZnSO_4 \cdot 7H_2O$	4430 mg
$Fe_2(SO_4)_3 \cdot 5H_2O$	910 mg	EDTA	5 gm
$MnCl_2 \cdot 4H_2O$	72 mg	Water	1000 ml

Aqueous extract of garden peas

150 gm of frozen garden peas were brought to the boil in 1000 ml of deionised water. The peas were filtered off and the clear liquid was added to an equal volume of twice concentrated minimal medium.

The minimal medium was prepared at four times the concentration stated above and is dispensed into 1 litre capacity polythene bottles. Aqueous pea extract is also stored in polythene bottles. These bottles were kept until required at -15°C. This freezing procedure cuts out the necessity of autoclaving the media before storage.

Petroleum extract of oats

100gm of crushed oats were refluxed with light petroleum ether for one hour. This process yields on evaporation of the solvent approximately 4 gm of a yellow brown oil. This is added to the standard medium (minimal medium + aqueous pea extract) at a concentration of 1% w/v. The medium is solidified with 10 gm/l Oxoid No. 3 agar and autoclaved at 15 lbs/sq.in. for 15 minutes.

Plates of solid standard medium + 1% oat extract were inoculated with both isolates of *P. palmivora* or with a single isolate of *P. cactorum*. The inoculum was a 4 mm disc cut from the edge of an actively growing colony on solid standard medium. The plates are incubated in the dark at 24°C as neither of the species form oospores in the light. Initially

oospores are formed in the region between the two isolates of P. palmivora and then throughout both colonies.

As light has recently been found to be an important factor in the germination of many Phytophthora species including P. cactorum (Shaw, 1965), P. heveae and P. erythroseptica (Leal and Gomez-Miranda, 1965), P. erythroseptica, P. heveae, P. megasperma var. sojae, P. palmivora and P. infestans (Berg and Gallegly, 1966), all germination experiments were incubated in the light and in the dark. The light source consisted of two 11 inch 8 watt warm white fluorescent tubes. Depending on the position of the plates in the incubator this gave a light intensity of 8 - 13 foot candles.

Techniques which had proved successful in germinating the oospores of Phytophthora species were tried with the dormant oospores of P. palmivora.

Attempts to Break the Dormancy of the Oospores of Phytophthora palmivora

The Effect of Biological Stimulation on Oospore Dormancy

Although the oospores of several species of Phytophthora have been germinated (Blackwell, 1943; Gregg, 1957; Savage and Gallegly, 1960; Erwin, 1966; and others) the final germination observed in spores harvested from the parent cultures has been only a few percent. Shaw (1967) however obtained 67% germination of 21-day old oospores of Phytophthora cactorum after they had passed through the intestinal tract of water snails. The involvement of the snail has been further investigated (see Appendix).

It was decided that this method should be tried first using the oospores of P. palmivora.

Attempt to germinate the oospores of P. palmivora using water snails

28-day old cultures of P. palmivora on solid standard medium + 1% oat extract were placed in needle boxes containing 200 ml of deionised water. Three snails per culture were added. After 48 hours the faeces, consisting mainly of oospores, were withdrawn in a Pasteur pipette and homogenised with a little water in a tissue grinder. They were then centrifuged, the water discarded, and an equal volume of mercuric chloride (60 µg/ml) was added to surface sterilise the spores (This was twice the concentration used by Shaw.). After five minutes treatment, the mercuric chloride was centrifuged off and the "snailed" oospores were washed in four changes of sterile deionised water before being plated on non-nutrient agar (10 gm/litre). Oospores from control plates which had not been fed to snails, ("non-snailed"), were treated in the same way. The plates were incubated in light and dark at 24°C and examined at intervals for germination. No germination was recorded after 14 days.

As oospore age has been found to be a factor influencing germination of P. cactorum, this technique was repeated using oospores of P. palmivora of different ages ranging from 16 days to 13 weeks. No germination was recorded.

Discussion

Very little work has been done on the action of the snails on oospores. Shaw (1965) tried a cytase preparation with the oospores of P. cactorum but this was not successful.

Gregg (1957) using Helix aspera observed that the oogonial and

antheridial wall appeared unaltered after the oospores of P. erythroseptica had passed through the gut. This is in contrast to the situation in P. cactorum where most of the oospores are free after digestion. With P. palmivora the oogonial wall of most of the spores is still present and this may be a reason why no germination was obtained.

Another possible reason for the negative germination results obtained may be due to the fact that the wrong species of snail was used. Turner (1964) has proved that the giant African snail Achatina fulica is an agent of transmission in nature of foot-rot disease of Pepper, caused by P. palmivora. Although his experiments were conducted using only one isolate, and thus the effect of the snails on oospores was not studied, it may be that this species of snail might break the dormancy of these oospores.

The Effect of Chemical Treatments on Oospore Dormancy

As mentioned in the introduction some fungal spores are stimulated to germinate by certain chemicals. This has also been observed with several phycomycetes. McKay (1939) found that 0.01% - 0.02% potassium permanganate solution induced the oospores of Peronospora schleideniana to germinate; Uppal (1924) obtained germination of the oospores of P. infestans using a 1% glucose solution; Savage and Gallegly (1960) found 2-4 dichlorophenoxy acetic acid, coumarin, caffeine and gallic acid successful with P. infestans oospores.

Germination has also been induced using unknown complex chemicals. Some of these are produced by the particular host plant; Trow (1901) found that cabbage water stimulated the oospores of Pythium ultimum to

germinate; Scharen et al (1960) using plant debris, horse dung infusion or pea sand leachate broke the dormancy of the oospores of Aphanomyces euteiches and obtained between 2% and 40% germination. Horse dung infusion has proved successful also with the oospores of Phytophthora infestans. Barton (1957) found that the oospores of Pythium mamillatum could be germinated in the presence of radish roots and Smoot et al (1958) obtained 3 - 4% germination of the oospores of P. infestans using potato soil leachate.

Attempts to germinate the oospores of P. palmivora using supplemented media

(a) Media supplemented with known chemicals

13-week old "snailed" and "non-snailed" oospores of P. palmivora were plated on the following media:

(1) 4 times normal strength minimal medium

(2) 2 times normal strength minimal medium

(3) 1 times normal strength minimal medium

(4) standard medium

(5) non-nutrient agar

Three replicate plates of each medium were incubated in the light and dark at 24°C. No germination was observed after seven days.

(b) Media supplemented with complex unknown chemicals

(1) Aqueous pea extract

(2) Coconut milk

21-day old "snailed" and "non-snailed" oospores were plated on the following media:

(1) 100, 75, 50, 25, 10, 5, 1, 0% aqueous pea extract diluted with deionised water and solidified with 1% agar.

(2) 10, 3, 1, 0.3, 0.1, 0% coconut milk prepared as above.

Three replicate plates of each concentration of each of the two treatments were incubated in light and dark at 24°C. No germination was observed on the aqueous pea extract plates after one week, or on the coconut milk plates after three weeks.

It has been found with several species of Phytophthora that the constituents of the medium on which the oospores are produced may influence their subsequent germination.

Leal and Gomez-Miranda (1965) obtained 90% germination of the oospores of Phytophthora heveae when the nitrogen source in the medium was valine and none when it was citrulline. Their investigations showed that the carbohydrate source also could influence germination, fructose and lactose leading to abnormal oospores, starch and mannitol giving a high percentage of germination.

Berg and Gallegly (1966) working with several species of Phytophthora including P. palmivora and P. cactorum found that they obtained maximum germination when their medium was supplemented with practical grade methyl linoleate.

These investigations by Leal and Gomez-Miranda and by Berg and Gallegly were carried out on oospores germinating in situ, i.e. on the parent cultures. As this is not convenient for genetical work it was decided to use these media but to harvest and replate the oospores on other media.

The effect of changing the nitrogen source from Asparagine to Valine

The basal medium used was the minimal medium detailed on page (10) with no nitrogen source. To this was added:

(1) DL-Valine 1g/l

(2) DL-Valine 2g/l

(3) L-Asparagine 1g/l

Aqueous pea extract and 1% oat extract were added as usual. The media were solidified with 1% agar.

8-week old "snailed" and "non-snailed" oospores from each of these media were plated on non-nutrient agar and incubated in light and dark at 24°C. No germination was observed after seven days.

Oospore production was not as abundant on valine as on asparagine medium. This may relate to a requirement for L-amino acids.

The effect of medium on oospore and chlamydospore formation

In previous experiments where oospores were plated on media other than non-nutrient agar chlamydospore germination and growth tended to obscure the oospores after a few days incubation. Preliminary attempts to separate these spores by filtering through cotton wool or centrifugation in a sucrose density gradient solution were not successful.

In view of the possibility that different media might increase the proportion of oospores : chlamydospores formed the following media were tested:

(1) standard medium + 1% oat extract

(2) standard medium + 1% oat extract + 5 µg/ml $Ca(NO_3)_2$

(3) standard medium + 1% oat extract at pH 7.0

(4) maize meal agar (15 gm/litre)

The media were scored by withdrawing two 4 mm diameter cylinders, equidistant from the two inocula, from each replicate plate. The hyphal mat was sliced off and squashed as evenly as possible in water under a coverslip. Three low power microscope fields from each sample were scored for oospore and chlamydospore numbers. None of these media altered the oospore : chlamydospore ratio.

The effect of the carbohydrate source of the medium on the numbers of oospores and chlamydospores formed was then investigated.

The basal medium used was the minimal medium on page (10) with no sucrose.

It was supplemented as follows:

(1) Sucrose 10.0 gm/l.

(2) Glucose 10.0 gm/l.

(3) Starch 10.0 gm/l.

Aqueous pea extract was added to these three media as usual and the following concentrations of oat extract - 1, 0.5, 0.1, 0%.

Oatmeal medium (15 gm/l.) also supplemented with 1, 0.5, 0.1, 0% oat extract was included also.

Three plates of each treatment were inoculated and incubated at 24°C in dark. The plates were scored as before after one week.

Results

Table 1. $\frac{\text{Oospore}}{\text{Chlamydospore}}$ production on different media

Conc. of oat extract carbohydrate	1%	0.5%	0.1%	0%
Sucrose	1.5 : 1	2.1 : 1	1.4 : 1	0.7 : 1
Glucose	0.2 : 1	0.4 : 1	0.2 : 1	no oospores formed
Starch		1.6 : 1	1.4 : 1	no oospores formed
Oatmeal	0.7 : 1	4.4 : 1	21 : 1	4.5 : 1

From the results it is seen that oatmeal + 0.1% oat extract gives the highest oospore : chlamydospore ratio. This medium was used in the following experiments.

The effect of methyl linoleate in the growth medium and as an adjunct in the plating medium

Oospores of P. palmivora were produced on (1) Oatmeal + 0.1% oat extract, and (2) Oatmeal + 0.1% oat extract + 200 µg/ml methyl linoleate.

After eight weeks "snailed" and "non-snailed" oospores from these cultures were plated on non-nutrient agar and non-nutrient agar supplemented with 10, 50, and 200 µg/ml methyl linoleate.

Three replicate plates of each treatment were incubated in light and dark at 24°C.

No germination was observed on any of the plates in the light.

Less than 1% germination was observed on non-nutrient agar + 50 µg/ml methyl linoleate and non-nutrient agar + 200 µg/ml methyl linoleate. These oospores had been "snailed" and came from both types of growth media.

Attempts to induce germination using Gibberellic Acid in the growth medium and in the plating medium

The media used to produce oospores were - (1) Oatmeal + 0.1% oat extract, (2) Oatmeal + 0.1% oat extract supplemented with 10 µg/ml, (3) 50 µg/ml, and (4) 200 µg/ml gibberellic acid.

The gibberellic acid was dissolved in alcohol and added to the media before autoclaving.

After four weeks "snailed" and "non-snailed" oospores from these plates were plated on

(1) non-nutrient agar

(2) non-nutrient agar + 10 µg/ml Gibberellic Acid

(3) non-nutrient agar + 50 µg/ml Gibberellic Acid

(4) non-nutrient agar + 200 µg/ml Gibberellic Acid

Two plates of each treatment were incubated in the light and dark at 24°C.

No germination was observed on any of the plates in the dark after three days.

A few germinated oospores distributed apparently randomly were recorded on the plates incubated in the light.

Information in Plant Protection Data Sheets quoted by Briggs (1963) states that gibberellic acid is broken down during autoclaving. In view

of this it was decided to retest unautoclaved gibberellic acid for activity in breaking the dormancy of P. palmivora oospores.

Oatmeal media had not been found very successful in the production of large numbers of oospores and standard medium + 1% oat extract was used.

Attempt to induce germination of the oospores of P. palmivora and P. cactorum using unautoclaved gibberellic acid in the growth medium

The media used were standard medium + 1% oat extract supplemented with 0 and 200 µg/ml gibberellic acid. The gibberellic acid was dissolved in sterile bicarbonate solution, 27.1 mg $KHCO_3$ in 10 ml sterile water to which 111.6 mg gibberellic acid was added, and 4 ml of this was added to the medium before it was poured, thus giving a final concentration of 200 µg/ml. P. cactorum was included in this experiment.

6-week old "snailed" and "non-snailed" oospores of both P. palmivora and P. cactorum were plated on non-nutrient agar and incubated in light and dark at 24°C.

No germination was recorded on any of the P. palmivora plates. Gibberellic acid had no effect on the oospores of P. cactorum either, the only germination being recorded was in the snailed, light treatment, and a few percent in the snailed dark treatment.

Discussion

So few chemicals were tried that no general conclusions regarding the negative results obtained can be made.

It is interesting to note Hockings (1967) results with the zygospores of Phycomyces blakesleeanus and gibberellic acid. He also obtained a very few germinating spores scattered over his treatments. It would

appear that although gibberellic acid is very effective in breaking seed dormancy it has no effect on fungal spore dormancy.

The Effect of Heat Shock Treatments on Oospore Dormancy

As discussed in the Introduction the zygospores of Phycomyces blakesleanus can be stimulated to germinate when given a heat shock of 50°C for three minutes (Sommer and Halbsguth, 1957).

It was decided to investigate the effect of a heat treatment on the oospores of P. palmivora. Only "non-snailed" oospores were used in these experiments.

3-week old oospores of P. palmivora were ground in deionised water until a homogenous thick creamy paste was obtained. 0.2 ml of this suspension was added to test tubes containing 4 ml of liquid minimal medium. These test tubes were in water baths at the required temperatures. The temperatures tested were 60°C, 50°C, 40°C, 30°C and room temperature. Samples were withdrawn from each temperature treatment at time intervals of 10, 20, 40 and 80 minutes from the start of incubation.

Replicate samples were plated on non-nutrient agar and incubated at 24°C in light and dark.

After seven days no germination was observed.

A second experiment was set up in which three-week old oospores were incubated in different aqueous environments during a heat shock treatment of 40°C for 20 minutes.

The incubation media used were:

(1) liquid standard medium

(2) Ringers salt solution

(3) 2% sucrose solution

(4) deionised water

Samples were plated as before on non-nutrient agar. No germination was observed after one week.

Discussion

Several reasons may be suggested to account for the failure of heat shock treatment to break the dormancy of the oospores of *Phytophthora palmivora*.

With *Neurospora* it has been found that the heat treatment triggers off some enzyme system which enables the activated spore to respire trehalose as well as lipid which is the respirable substrate in the dormant spore. It may be that no such system exists in the oospores of *P. palmivora*.

The wall of dormant ascospores has been found to be heat-resistant although this property is lost as soon as the spores are activated (Lowry and Sussman, 1958). Although oospores have a thick wall this may not confer any heat resistance on the spore and exposure to high temperatures may result in the death of the spore. This could be investigated by treating the spores after heat shock with some vital dye which depends on reaction with an enzyme system, e.g. a dye of the tetrazolium series.

It may simply be that the temperatures and times used were not suitable for *P. palmivora* oospores.

The Effect of Light on Oospore Dormancy

Leal and Gomez-Miranda (1965) obtained germination *in situ* of the oospores of *P. heveae*, *P. cactorum* and *P. erythroseptica* by exposing them to light after a period of incubation in the dark.

More recently Berg and Gallegly (1966) reported germination *in situ* of several *Phytophthora* species including *P. cactorum* and *P. palmivora* under strong fluorescent lights.

As all germination experiments using harvested oospores of *P. palmivora* had given negative results it was decided to try and germinate these oospores *in situ*.

Media very similar to that used by Berg and Gallegly (1966) was used in the first experiment:

(1) standard medium + 1% oat extract

(2) glucose-asparagine medium + methyl linoleate 2.5 ml/l

(3) glucose-asparagine medium + β-sitosterol 10 mg/l

(4) glucose-asparagine medium + methyl linoleate + β-sitosterol

Six plates of each medium were inoculated with *P. cactorum* and *P. palmivora*. These plates were incubated in the dark at 24°C for 12 days. After this period it was found that sufficient quantities of oospores had been formed only on standard medium + 1% oat extract and on the other media oospores were very scarce if present at all. This is probably due to the very low sterol content of these media. Berg and Gallegly used practical grade methyl linoleate which is only 75% pure and possibly has sterol contaminants, whereas the methyl linoleate used in the above media was 99% pure.

Different media were then tried -

(1) standard medium + 1% oat extract

(2) standard medium + β-sitosterol 30 mg/l + methyl linoleate 2.5 ml/l

(3) sucrose-asparagine medium + β-sitosterol + methyl linoleate

Again six plates of each type of medium were inoculated with P. cactorum and P. palmivora. These plates were then incubated in the dark at 24°C for eleven days. On subsequent examination abundant oospore formation was recorded on all the media.

Three replicate plates of each treatment were then placed under a bank of fluorescent tubes. The incident light intensity was between 450 - 650 foot candles. Control plates were kept in the dark. The plates were examined at intervals for germination in situ. After 28 days exposure to light no germination had been observed.

Discussion

It was surprising that no germination of either P. palmivora or P. cactorum was observed using Berg and Gallegly's technique.

The most probable reason for this is the impure methyl linoleate which they used possibly contains some active chemical.

General Discussion

In the discussion at the end of each experimental section suggestions are made as to why these particular treatments which have been found successful with other Phytophthora species did not work with the oospores of P. palmivora.

It may in fact be that some of these treatments were successful in

activating the oospores but the germination conditions given after or during the treatments were not those required by P. palmivora, e.g. temperature of 24°C may be too high or low, perhaps these spores have a requirement for liquid water, or the light source may not have been adequate.

Many other factors may have been responsible for the negative results, e.g. age of the oospores, immaturity of the oospores, or the requirement of the oospores for some specific substance produced by their host plant.

Further work on the dormancy of these spores might include investigations along any of these lines. Also it would be interesting to investigate the permeability of the dormant oospore wall.

SECTION II - THE SEARCH FOR DRUG RESISTANCE IN PHYTOPHTHORA CACTORUM (LEB. AND COHN) SCHROET

The Search for Drug Resistance in Phytophthora cactorum

Introduction

Since the beginning of the century resistance to antimicrobial drugs has attracted much interest and several reviews on the different aspects of this subject have been published (Abraham, 1953; Bryson and Szybalski, 1955; Moyed, 1964; and others). Most of the research has been carried out in the medical field owing to the importance of refractory viruses and bacteria in the treatment of infectious diseases. However in recent years a little more attention has been paid to drug resistant mutants in fungi as these have been found to be useful tools in many biochemical and genetical studies.

Comparative studies on drug resistance in different organisms have demonstrated two, and a possible third, mode of origin: (1) Genetic change; (2) Non-genetic adaptation; and (3) Composite changes, a combination of (1) and (2), requiring a genetic change before the cell is physiologically competent to adapt phenotypically.

For many years the role of the drug in the appearance of resistant strains was disputed but techniques such as the Luria-Delbrück fluctuation analysis (Luria and Delbrück, 1943) and the Newcombe respreading technique (Newcombe, 1949) can distinguish between drug-selected and drug-induced mutants.

(1) Resistance due to Genetic Change

This type of resistant organism arises through a change in its chromosomal material and can be detected in both bacteria and fungi

by characteristic progeny segregation patterns.

Studies of the survival curves of bacterial populations exposed to a range of concentrations of drug demonstrated two classes of resistance within this group. Demerec (1948) categorized these as the Streptomycin and Penicillin Types. These categories are now known to represent changes in one gene and more than one gene respectively and are called one-step and multi-step resistance. It was thought that each drug usually showed one resistance pattern, i.e. either one-step or multi-step. However a detailed study of mutants of Escherichia coli resistant to proflavine showed that in this organism both mechanisms of drug resistance are present (Thornley and Yudkin, 1959). Also Wilkie and Lee (1965) obtained both one-step and multi-step resistance to actidione in Saccharomyces cerevisiae. Warr and Roper (1965) tried to obtain multi-step resistance to malachite green and teoquil in A. nidulans but had no success.

In recent studies on drug resistance in Salmonella typhimurium Anderson and Lewis (1965 a & b) found that resistance to several drugs may be transferred via the episomes during conjugation of certain strains of S. typhimurium and E. coli.

At first canavine resistance in Neurospora crassa was believed to be under polygenic control (Horowitz and Srb, 1948) but Teas and Horowitz (1948) showed that it is due to a one gene effect.

Apart from multi-step resistance to actidione in yeast the few fungal drug resistant mutants which have been analysed genetically have been shown to be one-step mutants. These include sulphonilamide resistance and dependence in Neurospora crassa (Emerson and Cushing, 1946), actidione

and acriflavine resistance in N. crassa (Howe and Terry, 1962), acriflavine resistance in A. nidulans (Roper and Kafer, 1957) and others.

Rate of Mutation to Drug Resistance

It is difficult to estimate the rate of mutation to drug resistance accurately unless it is known definitely how many genes are involved. Also, the stage of the life cycle treated (Sinai and Yudkin, 1959) and the composition of the medium (Rosanoff and Sevag, 1951) have been shown to alter the numbers of mutants obtained.

Roper and Kafer (1957) found that mutation in A. nidulans to acriflavine resistance occurred with a frequency of between 5×10^{-6} - 1×10^{-8}/conidium. Howe and Terry tried to obtain spontaneous mutation to acriflavine resistance with N. crassa but had no success. After U.V. treatment they found the mutation rate was between 1 and 6×10^{-6} per viable conidium. They also found that the frequency tended to decrease as the concentration of selective agent increased.

(2) Resistance due to Non-Genetic Adaptation

This type of resistance in many cases is induced by the presence of the drug in the environment and cannot be correlated with a change in the nuclear material. The ability of a cell to adapt phenotypically is controlled by its genotype (Jinks, 1959), but differing abilities to adapt within the same clone are under cytoplasmic control (Jinks, 1959).

The stability of induced resistance is variable. In cases of enzyme induction, where a newly formed enzyme system inactivates the drug, the resistance may be only of a temporary nature. A similar situation is found with β-galactosidase in E. coli which is only produced when lactose

is present (Cohn and Monod, 1953). In contrast penicillinase continues to be produced by Bacillus cereus in the absence of penicillin in the medium (Pollock, 1953).

Where resistance is correlated with loss or change of a cytoplasmic particle it is usually of a more permanent nature, e.g. streptomycin resistance in Chlamydomonas (Sager and Tsubo, 1962).

Physical factors in the medium may allow sensitive cells to continue to grow in a toxic environment. Sinai and Yudkin (1959) found that the acid produced by B. lactis aerogenes in a glucose salts medium allowed the bacteria to tolerate the gradual addition of proflavine.

(3) Resistance due to Composite Changes

This type of resistance requires that mutation must occur before the cell has the physiological capacity to adapt phenotypically. Lam and Sevag (1955) have proposed such a mode of origin for streptomycin resistance in micrococci.

Resistance to Toxic Agents in the Oomycetes

Prior to Shaw's work with P. cactorum (Shaw, 1965; Shaw and Elliott, 1968) no study of drug resistance in this group of fungi had been made. Spontaneous and ultra-violet induced mutation to several chemicals including streptomycin, actidione and sulphanilamide were investigated (Shaw, 1965). A streptomycin resistant and also a streptomycin dependent mutant were obtained. No segregation was obtained in the sexual or asexual progeny of either strain and thus it could not be determined whether the mutation was nuclear or cytoplasmic in origin. The rate of reverse mutation from dependence to non-dependence was estimated

tentatively to be between 2×10^{-5} and 4×10^{-5} per spore.

In any genetical study it is of advantage to be able to sample single nuclei. This is usually done by using uninucleate asexual spores. In Phytophthora species sporangia can be induced to release asexual uninucleate zoospores which germinate immediately to form daughter colonies. A small percentage of binucleate spores is produced also but under conditions favouring maximum zoospore release in P. cactorum the percentage of these is small (Buddenhagen, 1958).

Materials and Methods

Media

The basal medium in all experiments was standard or minimal medium prepared as described on page 10.

Pea Meal Agar

300 gms Frozen Garden Peas (blended)

1000 ml deionised water

80 gms Oxoid No. 3 Agar

The medium is autoclaved at 15 lb/sq.in. for 20 minutes and stored in the dark until used. Pea meal medium supports vigorous mycelial growth and is a suitable source of actively growing mycelium for use in the production of the asexual spores.

Ringer's Salt Solution

NaCl	2.15 gm	$CaCl_2$	0.12 gm
KCl	0.075 gm	Deionised water	1000 ml
$Na_2S_2O_3.5H_2O$	0.5 gm	pH = 6.6	

This solution is prepared at 10x strength and stored in a refrigerator until required.

Preparation of Drugs, Mutagens and Nitrogenous Bases

Sterile stock solutions were prepared in one ounce bottles. Drugs and nitrogenous bases were added to autoclaved media which had been allowed to cool.

Acridine yellow Acriflavine Actidione 5-Bromodeoxyuridine Crystal Violet Cytosine Malachite Green Methionine Thymine*	dissolved in sterile water *required gentle heating
5-Bromouracil Guanine	dissolved in dilute HCl. Appropriate amounts were added to control treatments.
Adenine 0.05M	solution supplied by Genetics Department, University of Glasgow.
Aminopterin	dissolved in $\frac{N}{100}$ NaOH
5-Amino acridine Proflavine Sulphanilamide	added by weight

ethylmethane sulphonate	Stock solutions were prepared by dispersing appropriate amounts of ethylmethane sulphonate in sterile deionised water on a shaker for 30 minutes immediately before use.

Production of Sporangia

This was a modification of the method used by Shaw (1965). Six discs, 8 mm diameter, of thick pea meal agar were placed on the lids of sterile petri dishes. These were then inoculated with a 4 mm disc of the required strain. A few ml of sterile deionised water were pipetted into the bottom of the petri dishes to prevent the media drying out. The lids were then carefully replaced, and the plates were incubated at 24°C in darkness for three days. After this time the pea meal discs were transferred to petri dishes containing non-nutrient agar. They were then incubated in darkness for a further three days. After this period microscopic examination of the plates revealed that abundant quantities of sporangia had been formed.

Liberation of Zoospores

To obtain zoospore suspensions the plates of non-nutrient agar + the pea meal discs were transferred to 15°C and flooded with 5 ml of sterile deionised water at 15°C. After 35 minutes 2.5 ml of 4% sucrose solution at 15°C were added. 10 minutes later another 2.5 ml of 4% sucrose solution at 15°C were added, thus making the final sucrose concentration 2% - the concentration found to give optimal zoospore survival (Shaw, 1965). The plates were then removed to room temperature and the sucrose solution, now containing zoospores was carefully withdrawn by pipette and stored in sterile 1 oz bottles.

Shaw (1965) found that by adding 0.1 ml of a 10x Ringer's salt solution the viability of the suspension was improved and the suspension could be stored for several hours before being used. Zoospore dilutions are made into "sucrose-salt" solution.

The main advantages of this method of sporangial production are that zoospores can be obtained after six days instead of eleven and there is less vegetative growth prior to sporulation.

The Search for Resistance to Actidione and Sulphanilamide

Introduction

Gene mutation giving resistance to actidione or sulphanilamide has been found in several species of fungi. Hsu (1963) obtained seven dominant mutants resistant to actidione in Neurospora crassa. These were shown to be due to mutation at one of two loci. Dominant and semi-dominant actidione resistant mutations have been obtained in Aspergillus nidulans (Warr, 1963; Warr and Roper, 1965). In an extensive examination of actidione resistance in Saccharomyces cerevisiae Wilkie and Lee (1965) obtained six mutant strains. In diploids, one of these showed full dominance, three were semi-dominant and two were recessive. They also obtained mutation at two modifier loci which did not confer any resistance to actidione on their own but in combination with the actidione mutants could increase the level of resistance five-fold. Emerson and Cushing (1946) obtained a dominant sulphanilamide mutant in Neurospora crassa.

Shaw (1965) attempted to select spontaneous and ultra-violet induced resistance to actidione or sulphanilamide in P. cactorum. He obtained several "resistant" growths in his selection flasks but on subsequent sub-culture and testing this increased tolerance was shown to be only of a temporary nature.

An attempt to select actidione and sulphanilamide resistance was made for two reasons.

(1) The majority of actidione resistant mutants obtained in fungi are dominant and semi-dominant. The only reported sulphanilamide mutant was also dominant.

(2) Shaw obtained resistance of a temporary nature in P. cactorum quite readily to these drugs. If resistance of a temporary nature is obtained it might be possible to get a similar resistance conferred by gene mutation.

Attempts to select spontaneous resistance to actidione or sulphanilamide

Actidione

Fifteen glaxo bottles, 2 litre capacity, were used in this selection experiment. Seven contained liquid minimal medium and the remainder liquid standard medium. To fourteen of them 1 ml of a 5 mg/ml stock solution was added to give a final concentration of 10 µg/ml - the selective dose used by Shaw (1965). Each was inoculated with 6×10^4 zoospores. After several days vigorous growth was observed in the control flasks only. No resistant growths were obtained.

Sulphanilamide

A similar experiment was set up using 3440 µg/ml of sulphanilamide instead of actidione. No resistant growths were obtained after three weeks incubation.

Attempts to induce mutation to actidione and sulphanilamide resistance using chemical mutagens

Ultra-violet irradiation, a mutagenic treatment which has been found successful in producing drug resistant mutants of Aspergillus nidulans (Howe and Terry, 1962; Hsu, 1963) did not induce drug resistance in

P. cactorum (Shaw, 1965). In view of this attempts were made to induce resistance to actidione or sulphanilamide using chemical mutagens.

Chemical mutagens may be classified according to their supposed mode of action on D.N.A. Two groups differing in this respect are (1) Alkylating agents, (2) Base analogues. One compound from each of these groups was used in attempts to induce drug resistance in P. cactorum.

1) Alkylating Agents

Mutagens in this category, which includes ethylating agents and the mustard compounds, react directly with the amino groups of D.N.A. Ethylmethane sulphonate, for example, is thought to react with the 7-nitrogen atom of guanine and the resulting compound 7-ethyl guanine tends to be removed from the D.N.A. chain by hydrolysis (Bautz and Freese, 1960). The removal of this base can result in the more or less random incorporation of any of the four bases opposite the gap at the following replication. The new base will incorporate its own partner at the next replication and thus a base pair may have been changed.

Ethylmethane sulphonate has been found to be mutagenic in phage (Loveless, 1959; Krieg, 1963), bacteria (Eisenstark, 1964), Drosophila (Fahmy and Fahmy, 1958), barley (Konzak et al, 1965) and in several fungal genera including Saccharomyces (Costello et al, 1963; Lindegren et al, 1965), Puccinia (Baker and Teo, 1966), Neurospora (Auerbach, 1960) and Aspergillus (Alderson and Clark, 1966).

Clarke (1964) tried to obtain biochemical mutants of P. infestans using ethylmethane sulphonate as mutagen but had no success.

P. cactorum was treated with ethylmethane sulphonate in two ways:

(1) Zoospores were shaken with ethylmethane sulphonate

(2) Ethylmethane sulphonate was included in the growth medium.

1. Attempt to obtain mutation to actidione or sulphanilamide by treating zoospores with ethylmethane sulphonate

This method has been found successful in inducing mutation in several species; e.g. Saccharomyces cerevisiae (Costello and Bevan, 1964), Puccinia graminis avenae (Baker and Teo, 1966), Aspergillus nidulans (Alderson and Clark, 1966). The concentrations which have been found successful range from 0.5×10^{-2} to 0.2 M.

To find a suitable treatment for P. cactorum samples of zoospores were shaken with different concentrations of ethylmethane sulphonate. 1 ml samples were withdrawn after five and ten minutes, and diluted in sucrose-salt solution to give approximately 50 survivors per plate. Three replicate plates were scored for survival.

Results

Table 2. The effect of different concentrations of ethylmethane sulphonate on the viability of zoospores

Conc. EMS µg/ml	% Survivors	
	After 5 min Treatment	After 10 min Treatment
0	100	100
250	82.6	66.6
500	83.0	44.7
1000	55.3	30.3
2000	44.0	25.7
4000	0	0
8000	0	0

A treatment of 2000 µg/ml for 10 minutes which causes a 75% kill was thought to be suitable.

Attempt to obtain resistance to actidione or sulphanilamide using treated zoospores

Flasks containing selective doses of actidione and sulphanilamide were inoculated with zoospores which had been shaken with 2000 µg/ml ethylmethane sulphonate for 10 minutes. Control flasks (lacking drugs) which were inoculated with non-treated and treated zoospores showed growth after

three days incubation. No growth was observed in any of the drugged flasks after two weeks.

2. Attempts to induce mutation to actidione or sulphanilamide by incorporation of ethylmethane sulphonate in (a) the growth medium

(b) the sporangial production medium.

(a) Auerbach (1960) obtained mutation to LiCl resistance in N. crassa by incorporating chloroethyl methane sulphonate in the medium. This method of inducing mutation was attempted with P. cactorum.

Plates of solid standard medium containing 0, 1, 10, 100, 1000 and 10,000 µg/ml ethylmethane sulphonate were inoculated with approximately 30 zoospores per plate. Six replicate plates of each concentration were incubated for three days at 24°C. After this time all plates, apart from the 10,000 µg/ml treatment, had approximately 20 colonies. No growth had occurred on the 10,000 µg/ml treatment. Five plates of each treatment were overlaid with standard medium + actidione to give a final concentration of 10 µg/ml actidione after diffusion. The remaining plate of each treatment was overlaid with non-drugged standard medium. After ten days incubation at 24°C only the plates overlaid with non-drugged medium showed growth on the surface. The appearance of the colonies arising from the ethylmethane sulphonate treatments was identical to the control colonies.

(b) Pea meal medium containing 0 µg/ml and 2000 µg/ml ethylmethane sulphonate was used to produce sporangia. Eight flasks of 1 litre capacity containing 500 ml medium with 10 µg/ml actidione, and eight containing 3440 µg/ml sulphanilamide were prepared. Four of each drug were inoculated with approximately 1.4×10^5 zoospores obtained from the control medium or

the ethylmethane sulphonate medium. Two control flasks, containing non-drugged media, were inoculated with zoospores from either media.

After five days incubation vigorous growth was observed only in the control flasks. No resistant growths were obtained in any of the drugged flasks after three weeks incubation.

2) Base Analogues

These compounds, as their name suggests, are structurally very similar to the naturally occurring nitrogenous bases found in D.N.A. Two of these compounds, 5-Bromouracil and 2-Ammopurine, which are analogues of thymine and adenine respectively have been found to be mutagenic in phage (Freese, 1959; Litman and Pardee, 1959 and others), bacteria (Rudner, 1960), fungi (Ishikawa, 1962; Brockman and de Serres, 1963), higher plants and mammalian cells (Kihlman, 1966). It is believed that these chemicals exert their mutagenic effect by causing wrong base pairing to occur. For example, there is evidence to suggest that bromouracil can assume a tautomeric form quite readily and in this form it exhibits the pairing behaviour of cytosine, and thus either enters the D.N.A. chain opposite guanine or replicates incorrectly bringing in guanine instead of adenine. Since their mutagenic action therefore depends on D.N.A. replication base analogues have to be supplied to actively growing cells.

An interesting feature of the action of base analogues is their apparent site specificity. This phenomenon is well known in phage (Benzer, 1961) and has been demonstrated in Neurospora crassa by Ishikawa who obtained fifteen independently isolated mutants at the same site of the ad-8 locus following bromodeoxyuridine treatment.

Bromouracil and bromodeoxyuridine have been found to be incorporated more easily than 2-ammopurine (Kihlman, 1966) and were chosen for this investigation with P. cactorum.

Before 5-bromouracil or 5-bromodeoxyuridine can be incorporated by cells the organism in question must be rendered thymine deficient. The most convenient way of obtaining this situation is to use a thymine requiring strain of the organism. However, if this is not available thymine synthesis may be inhibited by the use of - e.g. aminopterin (Freese, 1959), sulphanilamide (Litman and Pardee, 1959), or (less commonly) fluorodeoxyuridine (Ishikawa, 1962). Aminopterin is an analogue of folic acid which is required for thymine synthesis. Sulphanilamide inhibits the synthesis of folic acid.

Attempt to induce actidione or sulphanilamide resistance using 5-bromouracil as mutagen and aminopterin as thymine synthesis inhibitor

One litre capacity flasks containing 500 ml of liquid standard medium were supplemented as shown below:

Treatment	Conc. BU µg/ml	Conc. Amin µg/ml	Conc ACT µg/ml
1	-	-	-
2	100	100	-
3	500	500	-
4	100	100	10
5	500	500	10
6	-	-	10

The experiment was carried out in duplicate. Each flask was inoculated with approximately 32,000 zoospores. As bromouracil mutation

is of the delayed effect type and nuclear division must occur before it is incorporated, the actidione was added to treatments 4, 5 and 6 24 hours after inoculation. By this time several nuclear divisions should have occurred if P. cactorum follows a pattern similar to that found in P. infestans by Milne (1967).

After seven days incubation one flask of treatment 5 contained a growth. This was isolated onto solid standard medium + 10 µg/ml actidione, and after five days appeared to be growing better than a control isolate on this medium. It was sub-cultured onto non-drugged standard medium and then back again onto standard medium + 10 µg/ml actidione. It made no growth. Its response was identical to wild type.

This experiment was repeated using 3440 µg/ml sulphanilamide instead of actidione. No growths appeared in any of the treatments.

Aminopterin by itself, at the concentration used, inhibited growth. If it was inhibiting only thymine synthesis, treatments 2 and 3 should have supported growth. The absence of growth suggested that the addition of 5-bromouracil alone did not annul the inhibition caused by aminopterin. As folic acid is an intermediate on several biosynthetic pathways there was a strong possibility that the lack of some important end product was the limiting factor. Before mutagenic work with bromouracil was continued an attempt was made to find a medium which, while not supporting growth, would do so on the addition of either thymine or 5-bromouracil; in such a medium thymine deficiency would be the sole limiting factor.

First it was supposed that cytosine synthesis was affected. Cytosine as well as thymine, 5-bromouracil and 5-bromodeoxyuridine were used as

shown below to supplement solid standard medium + aminopterin. Three replicate plates of each treatment were inoculated with approximately 60 zoospores. The effect of two concentrations, 100 µg/ml and 500 µg/ml, of the supplements was investigated.

	Supplements				
Treatment	Aminopterin	Cytosine	Thymine	Bromouracil	Bromodeoxyuridine
1	-	-	-	-	-
2	+				
3	+		+		
4	+	+			
5	+			+	
6	+				+
7	+	+	+		
8	+	+		+	
9	+	+			+

Macroscopic growth was observed only in treatment 1 after three days incubation. Microscopic examination of the other plates showed that the zoospores had produced a germ tube but had made no further growth.

A similar experiment using thymine, methionine, adenine and guanine singly and in combination was investigated. Only one concentration, 100 µg/ml, of each supplement was investigated.

Results

Table 3. Total number of colonies arising from a zoospore plating on supplemented media

Supplements in media	No. of colonies	
	Big	Small
Control	132	-
A	-	-
A + Ad	74	20
A + M	-	-
A + M + Ad	7	19
A + T	-	-
A + T + Ad	77	32
A + G	1	1
A + G + Ad	44	5
A + T + M	-	-
A + T + M + Ad	6	35
A + M + G	-	-
A + M + G + Ad	21	20
A + T + G	-	-
A + T + G + Ad	61	20
A + T + G + M	-	-
A + T + G + M + Ad	12	32

ABBREVIATIONS

A = Aminopterin

Ad = Adenine

G = Guanine

M = Methionine

T = Thymine

It is interesting that all the media containing adenine showed growth. The presence of methionine in any of these media partially annulled this effect. The growth observed however was not as good as control and two types of colony appeared which were classified as 'Big' and 'Small', 'Big' being a sparse colony about 1.5 cm diameter and 'Small' being about 2 mm diameter after four days incubation. The addition of thymine in the media did not appear to give increased growth.

From the above experiments it was concluded that creating thymine deficiency in P. cactorum is more difficult than has been encountered in phage, bacteria and Neurospora.

Thus under these experimental conditions neither ethylmethane sulphonate nor base analogues induced mutation to actidione or sulphanilamide resistance in P. cactorum.

The Search for Resistance to Acridines

Mutants resistant to acriflavine have been found in Aspergillus and Neurospora. Roper and Kafer (1957) obtained three resistant mutants in Aspergillus nidulans resulting from mutation in one gene. Two of these were semi-dominant and one was recessive. In subsequent work with these Ball and Roper (1966) showed that one of the semi-dominant strains was cross-resistant to acridine yellow and proflavine and that the recessive mutant also showed resistance to acridine yellow. Howe and Terry (1962) obtained 31 acriflavine-resistant mutants of Neurospora crassa after ultra violet irradiation.

An acriflavine-resistant mutant of Phytophthora infestans has been recently obtained (K. M. Swiezynski, personal communication to Dr. C. G. Elliott). Whether it has resulted from nuclear or cytoplasmic change is not known.

Shaw (1965) attempted to select an acriflavine-resistant mutant of P. cactorum with no success.

Four acridines were used in this work with Phytophthora cactorum.

Acriflavine obtained from British Drug Houses

Proflavine " " " " "

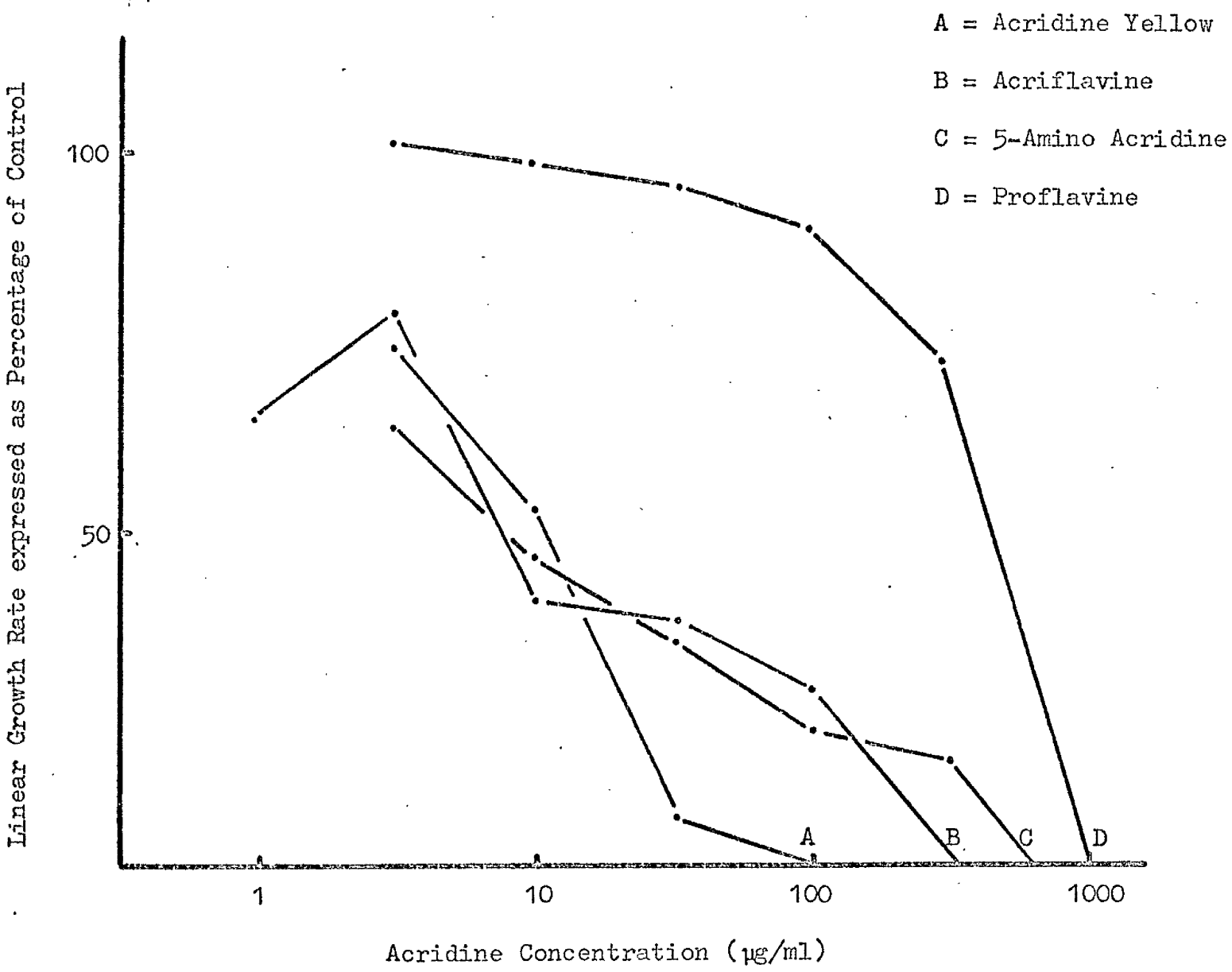
Acridine Yellow " " E. Gurr

5-Amino Acridine" " British Drug Houses

Determination of the Minimal Inhibitory Concentrations of the Acridines

The minimal inhibitory concentrations of the four acridines were

Figure 1. Dosage Response of Phytophthora cactorum to Acridines



determined in two ways.

(1) The inhibition of colony growth from a disc inoculum of hyphae.

(2) The inhibition of colony establishment from zoospores.

(1) Inhibition of Colony Growth

Plates of solid minimal medium, containing a range of concentrations of the acridines were inoculated with a 4 mm disc taken from the edge of an actively growing colony on solid standard medium. Six replicate plates of each concentration were incubated at 24°C. At least four measurements of two diameters of each colony were taken at two to three day intervals. From these measurements the rate of linear growth in mm per 24 hours was calculated for each acridine concentration. As there is variation between controls in different experiments the rate of growth on drugged medium is expressed as a percentage of the control. The results are shown in Figure 1.

(2) Inhibition of Colony Establishment from Zoospores

Six replicate plates of each drug concentration were spread with 0.2 ml of a suspension of 2×10^2 spores/ml. The plates were incubated at 24°C for 96 hours. The acridine concentrations which prevented the establishment of a macroscopic colony in this time are shown in Table 4.

Table 4. Minimal Inhibitory Level of Acridine Preventing Colony Formation from Zoospores

Acridine	Minimal Conc. ug/ml
Acriflavine	10
Acridine Yellow	32
5-Amino Acridine	562
Proflavine	1000

A problem in obtaining mutants in P. cactorum is screening large enough numbers of zoospores. As a suppressive effect of a sensitive population on resistant spores present in it was found by Shaw (1965) it is necessary to use large volumes of medium. Glaxo bottles of 2 litre capacity each containing 500 mls of the required medium were found to be satisfactory. However, owing to the large size of the bottles and the numbers used in each selection experiment, incubation in an incubator was impossible. The bottles were incubated in a dark cupboard, the temperature of which varied between 20-22°C.

Spontaneous Mutation to Acridine Resistance

(1) Proflavine

In this selection experiment fourteen glaxo bottles containing 500 mls of liquid minimal medium were used. To thirteen of these 500 mg of proflavine powder was added to give 1000 µg/ml. They were then left for 24 hours to allow the proflavine to dissolve. Each bottle

was then inoculated with approximately 80,000 zoospores. Thus over 1,040,000 spores were screened against proflavine. After three weeks there were no growths in any of the drugged bottles.

(2) Acridine Yellow

Eleven glaxo bottles, ten of which contained 40 µg/ml acridine yellow were used. Each bottle was inoculated with 50,000 zoospores. After 11 days incubation there were many growths in each of the drugged bottles. Over 70 of these growths were isolated onto solid minimal medium + 40 µg/ml acridine yellow. They made no growth on this medium thus their tolerance was indistinguishable from wild type.

So many growths arising in all the flasks raised the question of the stability of this medium after 10 days incubation. To investigate this plates of medium containing 40 µg/ml acridine yellow were prepared up to 10 days before inoculation with P. cactorum. No growth was made on any of the plates. Thus this medium had not lost its potency.

(3) Acriflavine

A very large scale selection experiment was set up. To 45 glaxo bottles 5 mg acriflavine was added giving a final concentration of 10 µg/ml. One bottle without drug was used as control.

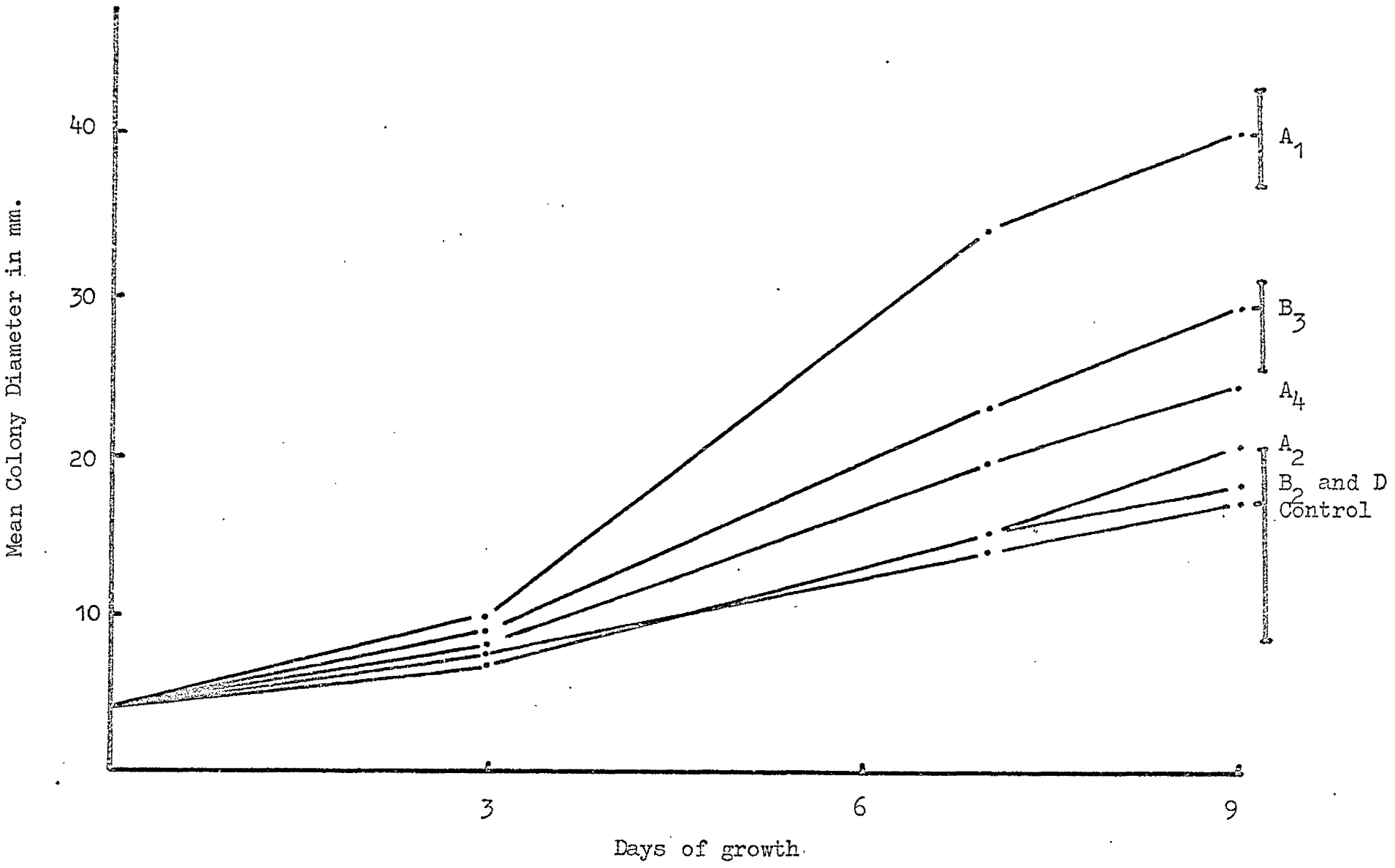
To 19 of the bottles approximately 105,000 zoospores were added.

To 20 of the bottles 10,500 zoospores were added.

To six of the bottles 1,050 zoospores were added. In all over 2×10^6 zoospores were screened against acriflavine.

After eight days incubation growths were present in seven of the

Figure 2. Comparison of Growth of Isolates and Wild Type on Medium containing 10 µg/ml Acriflavine



bottles (not apparently correlated with the density of the zoospore suspension). From these bottles a total of 54 growths were isolated and transferred to solid medium supplemented with 10 µg/ml acriflavine. Six control growths were also transferred for comparison.

After seven days six of the growths called A_1, A_2, A_4, B_2, B_3 and D appeared to be making better growth on drugged medium than wild type; they were then subcultured onto solid drug-free standard medium prior to testing. The response of the other isolates was indistinguishable from wild type.

Response of Isolates on Acriflavine Media

The growth of the six isolates and wild type on solid minimal medium + 10 µg/ml acriflavine was studied.

Plates of drugged medium were inoculated with 4 mm discs, taken from actively growing cultures on solid standard medium. Six replicate plates were used for each isolate and 24 control plates. Colony diameters were measured as before. The results are shown in Figure 2. From these results A_1 and B_3 were chosen for further investigation.

Zoospore Response of Isolates A_1 and B_3

The zoospore response on drugged medium of the two isolates A_1 and B_3 was compared with wild type. Four replicate plates of solid minimal medium + drug were used. Zoospore suspensions of the three strains were prepared as usual. Both isolates gave very sparse zoospore suspensions and these were used undiluted. The concentration of the control suspension

was adjusted to give approximately 50 zoospores per plate. The results are shown in Table 5.

Table 5. Number of Colonies Arising from Platings of Zoospores of Isolates A_1, B_3 and Wild Type on Different Concentrations of Acriflavine

Isolate	Concentration of Acriflavine µg/ml						
	0	1	3.2	10	32	56.2	100
Wild Type	196	3	-	-	-	-	-
A_1	93	5	-	-	-	-	-
B_3	43	27	28	50	49	32	27

Cross Resistance

A feature of acriflavine resistance appears to be an ability to withstand toxic concentrations of other drugs.

Roper and Kafer (1957) found that the three acriflavine-resistant mutants which they obtained from Aspergillus nidulans showed more resistance to malachite green and crystal violet than the wild type strain.

Ball and Roper (1966) in further investigations on these mutants showed cross resistance to acridine yellow and in one strain to proflavine. A recessive mutant resistant to malachite green was shown to be resistant recessively to acriflavine. It showed dominance, however, in its resistance to acridine yellow.

In view of these findings isolate B_3 was investigated for resistance to other drugs.

Three replicate plates of solid minimal medium supplemented with the appropriate concentrations of each of the drugs were inoculated with 0.2 ml of a 3×10^2 zoospores/ml suspension. The results are shown in Table 6.

Table 6. Zoospore Response of Isolate B_3 on Different Drugs

Drug	Conc. µg/ml	Wild Type			Isolate B_3		
		Replicates			Replicates		
		1	2	3	1	2	3
---	-	60 cols/plate			c. 60 cols/plate		
Acriflavine	100	1	-	-	19	8	11
Acriflavine	200	-	-	-	12	11	8
Malachite Green	0.3	-	-	-	-	-	-
Crystal Violet	1	Small stunted growths			Small stunted growths		
Acridine Yellow	40	-	-	-	-	-	-
Amino Acridine	562	-	-	-	-	-	-
Proflavine	1000	-	-	-	-	-	-
Actidione	10	-	-	-	-	-	-
Sulphanilamide	3440	-	-	-	-	-	-

It is seen from the results that no cross resistance was found.

Discussion

Increased tolerance of P. cactorum to acriflavine

Without further evidence very little can be said about the nature of the change conferring increased tolerance to acriflavine in P. cactorum.

The absence of cross resistance in this strain to other drugs may indicate that the change is not nuclear, as in all other cases of gene-determined acriflavine-resistance in fungi cross resistance to malachite green has been observed. However resistance to malachite green is usually recessive and thus if P. cactorum is diploid this would not be expressed.

Heterokaryon Formation in Phytophthora cactorum

Introduction

Heterokaryosis is the co-existence of genetically dissimilar nuclei in the same somatic hypha. It can arise by mutation in one nucleus in the mycelium or by hyphal fusion and subsequent nuclear migration.

A detailed investigation of the factors controlling heterokaryon formation in Neurospora crassa revealed that it is under complex genetical (Garnjobst, 1953, 1955; Holloway, 1955). The two hyphae must be of the same mating type and be similar at two other loci each with two alleles.

Recent studies in the homothallic species Aspergillus nidulans have indicated a comparable situation (Grindle, 1963; Jinks and Grindle, 1963; Jinks, Caten, Simchen and Croft, 1966). Heterokaryon compatible strains must be similar at a minimum of five loci and are practically indistinguishable phenotypically. If such mechanisms are operating in other species it may explain the failure to obtain heterokaryons in Venturia inaequalis and Cladosporium fulvum (Fincham and Day, 1965).

Heterokaryosis is of widespread occurrence in the Ascomycetes and the Basidiomycetes both under natural and laboratory conditions. In the Phycomycetes, however, there are only two reports of synthesised heterokaryons, morphological in Mucor rammanianus (Evans, unpublished) and nutritional in Rhizopus javanicus (Minami and Ikedo, 1962). Couch believes that it may be a possible explanation of the mycelia of mixed reaction in Dictyuchus (Raper, 1960).

Heterokaryosis is a useful genetical tool and has been used for detecting complementarity (Beadle and Coonradt, 1944) and for determining

dominance relationships of mutant alleles (Roper and Kafer, 1957). Also nuclear and cytoplasmic changes can be distinguished by the so-called "heterokaryon test", which involves combining the two parents in a heterokaryon (Jinks, 1954). When the heterokaryon is resolved into its component homokaryons segregation of the original differences under test with the parental nuclear markers indicates that these differences were under nuclear control. If, however, the homokaryons are alike, or if the differences recombine with the nuclear markers, it may be concluded that the original differences were under cytoplasmic control. It was hoped that this test might help to elucidate the nature of the changes conferring acriflavine and streptomycin resistance in P. cactorum.

The streptomycin resistant strain used, designated Sr was obtained by Shaw. It is resistant to 1000 µg/ml streptomycin.

Inspection of the literature indicates that heterokaryons have been synthesised in several ways. Plates of a suitable medium, which will not permit the growth of either strain are inoculated with (a) a dense mixed conidial suspension; (b) a dense suspension of hyphal fragments; or (c) hyphal tips of both strains. Single hyphal tips of any areas of vigorous growth arising on the selective medium are subcultured and presence of the different kinds of nuclei tested for in the uninucleate asexual or sexual progeny.

As the acriflavine resistant strain B_3 did not produce sufficient quantities of zoospores hyphal disc inocula and hyphal suspensions were used.

Attempt to Obtain a Heterokaryon using Hyphal Disc Inocula on Selective Media

Plates of solid minimal medium and solid standard medium supplemented with 100 µg/ml acriflavine and 100 µg/ml streptomycin were inoculated in one of two ways:

(1) A 4 mm diameter disc of medium was removed from the petri dish and replaced with a similar disc taken from the edge of an actively growing colony of isolate Sr growing on solid standard medium. A disc of isolate B_3 was then placed on top so that the hyphal surfaces were in contact.

(2) 4 mm disc inocula of each isolate were laid a few mm apart on the surface of the selective medium. Each isolate was inoculated separately onto medium containing either 100 µg/ml acriflavine or 100 µg/ml streptomycin as controls. Six replicate plates were incubated at 24°C. After three weeks no vigorous sectors were observed on any of the plates. Microscopic examination at intervals during the three weeks revealed no evidence of hyphal fusion.

Attempt to Obtain a Heterokaryon by Growing Both Inocula on Non-Drugged Medium then Overlaying with Drugged Medium

Plates of solid minimal medium and solid standard medium were inoculated with isolates B_3 and Sr as in the previous experiment. Six replicate plates were incubated at 24°C for seven days. By this time considerable growth had occurred, colony diameters being 62 mm approximately on solid standard medium and 57 mm approximately on solid minimal medium.

These plates were then overlaid with either solid minimal medium

or solid standard medium containing 150 µg/ml of each acriflavine and streptomycin, giving a minimum final concentration of 75 µg/ml in the upper layer. This medium, when poured, was just above the solidifying point. Control plates were overlaid with medium containing neither drug.

After three weeks incubation at 24°C there was a dense mycelial mat on the surface of the control plates. There were no surface growths on any of the drugged plates.

Attempts to Obtain a Heterokaryon using a Mixed Hyphal Fragment Suspension

Strains B_3 and Sr were grown separately in liquid minimal medium for seven days. They were then blended together in a Wareing blender for half a minute, to give a dense mycelial suspension. This hyphal suspension was treated in two ways:

(1) 0.5 ml of the suspension was spread on plates of drug-free solid minimal medium and solid standard medium and incubated for four days at 24°C. These plates were then overlaid with either solid minimal medium or solid standard medium containing 150 µg/ml acriflavine or streptomycin. Control plates were overlaid with drug-free medium. After three weeks incubation at 24°C growth had only occurred on the surface of the control plates.

(2) Plates of solid standard medium and solid minimal medium supplemented with 100 µg/ml acriflavine and streptomycin were inoculated with 0.5 ml of the hyphal suspension. After seven days incubation at 24°C one plate

had a vigorously growing sector. This growth was subcultured onto drugged solid standard medium and continued to show resistance to both drugs.

Unfortunately at this point work had to be terminated before the drug response of the zoospore and oospore progeny of this "heterokaryon" could be investigated.

Conclusion

From these preliminary studies it appears that it may be possible to induce heterokaryon formation in Phytophthora cactorum. If this is also true in other species of Phytophthora, it may prove a very useful genetical tool in this genus, especially in those species where oospore germination has not yet been obtained.

Appendix - The effect of passage through the snail on oospore germination in P. cactorum

Five-week old oospores of P. cactorum were subjected to the following treatments:

(1) "Snailed" - oospore cultures were placed in needle boxes containing deionised water and snails

(2) Soaked - oospore cultures were placed in needle boxes containing deionised water

(3) Control - no water or snails

After 48 hours oospores from all treatments were homogenised, surface sterilised, washed and plated on non-nutrient agar as usual. Three replicate plates were incubated in light and dark for four days. The results are shown in Table 7.

Table 7. The effect of "snailing" and soaking treatments on the dormancy of the oospores of P. cactorum

Treatment	% Germination	
	Light	Dark
"Snailed"	36.9	4.7
Soaked	0	<1
Control	<1	0

The results shown in Table 7 indicate that passage through the snail is required for germination of the oospores of P. cactorum. The effect of this treatment can be detected only after exposure to light, but light alone is ineffective. Light-enhanced germination of snailed oospores of P. cactorum has previously been demonstrated by Shaw (1965, 1967) but it could not be determined whether the important feature of this treatment was passage through the snails as control treatments became overgrown due to chlamydospore germination and hyphal fragment growth. These latter effects were reduced by plating the oospores thinly after they had been thoroughly homogenised. Apart from the obvious effects of snail gut enzymes it was possible that the loss of dormancy might be due to an increase in permeability resulting from a mechanical rasping of the oospore wall against the radula of the snail. Oospores were shaken with various grades of carborundum powder and the resulting increase in permeability assayed by increase in uptake of various dyes including Nile Blue Sulphate. Although a slight increase in uptake was observed no germination occurred following carborundum treatment.

Table 8. The effect of "snailing" and soaking on the uptake of Nile Blue Sulphate by oospores of P. cactorum

Treatment	% Stained
"Snailed"	16.6
Soaked	50
Control	37

In Table 8 it is seen that increase in permeability was greatest in oospores which had been soaked and lowest after passage through the snail. It is thus evident that increase in permeability, as measured here, is not correlated with an increase in the ability to germinate.

Time did not permit any further investigations.

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