NATURAL AND INDUCED VARIABILITY IN *PHYTOPHTHORA CACTORUM* (L.&C.) SHROET. AND *PHYTOPHTHORA CINNAMOMI* RANDS

by

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INTRODUCTION

Members of the fungus genus Phytophthora occur throughout the world, and many are of great economic importance in causing plant diseases. The genus name was established in 1876 with P. infestans, the organism responsible for potato blight, as the sole species. Over 75 "species" and many varieties have been described since that time. Many of these species were proposed after examination of only a few individuals.

The paucity of characters suitable for use in identification, coupled with their variability and the practice of establishing very narrow species lines have combined to make identification of isolates impracticable. In spite of this, few reports on isolate or species variability have appeared.

Many reports of variability in Phytophthora are open to question since single zoospores were not used to establish genetically pure cultures, and genetic implications have seldom been appreciated. The rarity of oospore germination has precluded traditional genetic studies, and, in conjunction with the minute size of nuclei, has prevented obtaining a definite knowledge of the nuclear cycle. It is assumed that the oospore nucleus is the only diploid nucleus in the life cycle. Sexuality in the genus is not clearly

understood. Knowledge of the role of sexuality in the biology of the species is needed before an understanding of evolution and species limits can be obtained.

Mutation studies which have been so valuable in studying other fungi, have never been carried out on Phytophthora species. In fact almost no irradiation or mutation studies have been reported on any phycomycete.

Studies were undertaken to analyze for variability in natural isolates by the use of the single spore technique. The ease of mutability with ultraviolet light and X-rays, and the range of characteristics exhibited by induced mutants were investigated. Such analyses of variability and the production of clearly recognizable mutants will provide a groundwork for future studies in speciation and genetics of Phytophthora.

LITERATURE REVIEW

Taxonomy

The taxonomy of the genus Phytophthora is in a confused state. Tucker (71) in 1931 presented a taxonomic treatment in which species separation was based on sporulation and growth on different media, pathogenicity, temperature relations, and a few macroscopic and microscopic characters. Although this was a thorough treatment and is still the most useful taxonomic guide, it has not provided the average worker with the means to readily distinguish "species". Leonian (41) in 1934 presented a key to the species based mainly upon physiologic characters. The use of this key is also limited. Frezzi (23) in 1950 described the Phytophthora species in Argentina, and Waterhouse (72) in 1954 presented a key to the 16 species recorded in the British Isles. In 1956 she published a compilation of descriptions of all of the named species (73).

Variability and nuclear cycle

These and many other workers have recognized the great amount of variation which occurs within even one isolate. Leonian (41,43) especially has stressed the variability of all but physiologic characters. Nonetheless, few reports have appeared in which attempts were made to elucidate variability in the light of nuclear behavior or possible genetic mechanisms. This may partly be due to the fact that the

nuclear cycle has never been fully elucidated. Blackwell (9) reported that in P. cactorum the zoospores are uninucleate but germinating oospores and sporangia are multinucleate. In the developing antheridium and oogonium the numerous nuclei undergo one simultaneous division. Fusion of one antheridial and one oogonial nucleus occurs. The first two divisions of the fusion nucleus are conspicuous; prior to germination one to four or more divisions may occur. No mention was made of meiosis.

Nuclear behavior in P. macrospora has been described by Tasugi (66, p.37). He states, "Fusion of these two nuclei (in the oospore) takes place later during the maturation of the oospore. At the beginning of oospore germination the fusion nucleus becomes large in size and the chromosomes in the nucleus are easily observed. There are 24 chromosomes arranged in 12 pairs, scattered throughout the nucleus. This stage may be considered as (the) diakinetic phase in the course of the reducing division." This is the only reference to meiosis in Phytophthora which has been found.

The papillae have been considered to be functional in fertilizing the oogonia of P. himalayensis (49).

Meiosis in Phytophthora is assumed, mainly by analogy, to parallel that in the "related" genus Sclerospora. In S. graminicola meiosis occurs during the first two nuclear divisions of the zygote (47). A thorough study of nuclear behavior in species of Rhizopus, Mucor and other zygomycetes

has appeared (16). The first detailed study of meiosis in a phycomycete was presented in 1952 for Allomyces (76). An alternation of generations with a true diploid stage was found, as well as polyploidy in some species.

Zoospore fusion in P. infestans and in P. capsici has been reported (30,15). Cytological studies of this phenomenon were not successful.

Graham (28) stained nuclei in developing sporangia of P. infestans and concluded that a single nucleus enters a developing sporangium and that zoospores are uninucleate. He concluded that a single sporangium should be just as reliable as a single zoospore in establishing a genetically homokaryotic line. Leonian's observation (40,41) of dissociation in colonies of P. parasitica var. rhei derived from single sporangia would indicate that this does not apply to this species. The dissociation observed was the production of a morphologically visible colonial sector; later four other types were found among 100 colonies derived from single sporangia. Three of the dissociants were stable but one would alternate back and forth with the original type. The dissociants differed in pathogenicity, sporangial shape, and in other characteristics. It is significant that this dissociable strain would not produce oospores when paired with other strains of P. parasitica. Dissociation occurred in two other isolates which were unnamed. No attempt at a

rational explanation of the dissociation phenomenon was made.

Variation among different isolates of P. infestans regarding their pathogenicity to a series of differential host varieties has been well studied (6,7). Isolates are classified into "races", and "genes for pathogenicity" are differentiated on the basis of their pathogenic reactions to different potato varieties. Physiologic differences between two races of P. infestans have been reported (22). These differences concerned the ability to utilize asparagine, and the inhibitory action of NH_4NO_3.

So called "plasticity" in races of P. infestans also has been reported by several workers. The report by Mills (48) is one of the most thorough. He increased pathogenicity of potato isolates of P. infestans to tomato plants by repeated passage of the isolates through tomato foliage. Virulence increased gradually up to and including seven passages but not further. The changed isolates did not lose their pathogenicity to potato and differed from tomato isolates only in producing sporangia of smaller size on tomato plants. This work apparently was thorough and was repeated several times. The isolates which were changed were started from single zoospores, but mass transfers of zoospores were used with each passage. There seems to be nothing in this report which would contradict a hypothesis of selection of more successful mutants as an explanation of the phenomenon.

De Bruyn (17) found that race characteristics of P. infestans could be readily altered by passage through tomatoes or relatively resistant potato hybrids. The last passage determined the infective capacity of the parasite. She used cultures which originated from single zoospores. Although there was considerable variation among the experiments, she concluded that plasticity of the parasite makes race classification unreliable. She has informed me (personal communication) that she later questions some of this work.

Graham (29) presented a very rational discussion of variability in P. infestans, based upon research on Canadian races. He suggested that the mycelium is sometimes heterokaryotic for different races and that the host or medium exerts selective forces which select one or another nuclear type. He obtained 100 single zoospore cultures from a mycelial culture believed to be heterokaryotic, and tested them on a series of differentials. Ninety-six cultures gave identical pathogenic reactions, but four were avirulent. Single zoospore cultures of race "0" of P. infestans on artificial media for two years were not altered in host relationships. Zoospore-derived cultures of two other races also showed no change. He did not suggest that mutations would not occur, but rather that establishment of a pure line would enable studies of mutation rates and selective factors to be made.

Variation in pathogenicity among single zoospore isolates of P. parasitica var. nicotianae has been found (2). Fifteen isolates derived from zoospores taken from a four year old culture varied in reaction from non-pathogenic to pathogenicity greater than that of the parent culture. Slight variations in growth rates and colony morphology also occurred. Differences have been found in colony morphology (two types), growth rates, sexuality and pH optima among isolates of P. parasitica var. nicotianae obtained from different localities (1).

Tucker (71) recorded variability in different isolates of P. cactorum, and in other species, in such characters as pathogenicity and sizes of oogonia and sporangia. One of many instances mentioned by him concerned inoculations on green tomato fruits with P. cactorum; three isolates were virulently pathogenic, six were very weakly pathogenic and six were non-pathogenic.

Only one laboratory study of variation in P. cactorum has appeared (64). Single zoospores derived from a single zoospore colony produced colonies of differing morphology. As many as nine different types of colonies were produced, and changes from one colony type to another occurred readily when either single zoospore or mycelial transfers were made. Differences in pathogenicity to apple fruits and significant differences in spore sizes were found among some of the colony types. The author was unable to offer any explanation

for her observations, and concluded that proof of either gene mutation or cytoplasmic change would be equally impossible to provide. Her work seems to be thorough and she states that sufficient tests were run to show that variations in media or environment were not contributing factors.

Anomalous variability in Verticillium and Penicillium

Anomalous variability in Verticillium has recently been investigated (26). Single spore isolations from the parental strain produced four morphological types of colonies. Single spore cultures were made from the four colony types. From each colony type, the four morphological types were again obtained. Heterokaryosis and parasexuality were ruled out as explanations due to the uninucleate condition of the spores. Non-random mutation was considered as a possible explanation.

Unexplainable spontaneous variation has been studied in Wisconsin strains of Penicillium chrysogenum (65). In a large population of colonies derived from single spores, definite percentages of five colony types were produced. This behavior was called the "population pattern phenomenon". The types were arranged in order from the largest and most "normal" sporulating type to the smallest and most sterile type. The types were considered to

represent a "sliding downward series". When colonies were derived from spores of a given type, a population would be obtained which consisted predominantly of that type plus a few colonies of the types lower in the series. The tendency to sector increased progressively as the series was traversed, with the highest number of sectors appearing in the smallest and most sterile type. Sectors arising on colonies "low" in the series were usually of a more vigorous type higher in the series.

Heterothallism and oospore production

Oospore production in paired Phytophthora isolates and heterothallism were reported as early as 1924 (71). Many subsequent reports have appeared which have described interspecies and interstrain oospore production. Single zoospore cultures of isolates of the P. palmivora group produced oospores only when certain isolates were paired (51). It was reported that the strains could be distinguished as "male" or "female".

Leonian (42) paired various isolates of the palmivora-parasitica group and found 48 to be heterothallic and to always behave as "females" or "males"; about 40 isolates were either homothallic, neutral, or inconstant in their reaction. It was not stated if cultures originated from single spores.

Isolates of the palmivora group have been divided into two types based on oospore production when paired (67,68). Isolates long in culture gradually lost their reaction ability. An extract of the culture filtrate did not induce oospore formation in the complementary strain even when filter sterilized and used in amounts equal to the culture volume. Although the initials were not traced, on the basis of oospore production when paired the combination of eight species and varieties into one species was proposed.

The sexuality of P. parasitica was investigated in 1953 (39). Seventy-six strains from six hosts were examined in single and paired cultures and it was found that 18 percent produced oospores in two to six months as "single strain" cultures. When paired, oospores appeared in 15 days in some but not in other cultures. All isolates were bisexual. Sexuality was considered to be relative, resembling that in other oomycetes. Strains having the same sexual tendency were isolated almost constantly from the same host plant.

Oospores were formed when certain isolates of P. parasitica var. nicotianae were paired, but only when they were on agar containing fresh tobacco extract (37).

Barrett (4) has investigated oospore production in paired cultures and has published the only report of progeny testing for a character differing in the parents. He paired the two sexual strains of P. drechsleri and established 22 cultures from germinating oospores. The origin of these 22

cultures, whether from single zoospores or germ tubes, was not mentioned. None of these single cultures produced oospores, but when backcrossed to the parents, 14 produced oospores with the male parent, and six with the female parent only. He also described oospore production between P. infestans and P. drechsleri and germination of one such oospore. This work has apparently been neither continued nor confirmed.

Although these and other authors had inferred that oospores resulted from antheridia of one strain fertilizing the oogonia of another, no one had conclusively proved this point until the works of Cohen (14) and Stamps (63) appeared. Stamps paired P. cryptogea and P. cinnamomi in thin layers of agar on slides and was able to trace the initials and show that they arose on hyphae of the differing species. In these same cultures P. cinnamomi would later form a few non-hybrid oospores. All attempts to germinate the hybrid oospores failed.

Direct evidence of chemical stimulation of oospore formation was found when oospores were induced in non-paired cultures of P. meadii and P. colocasiae by addition to the medium of unheated filtrate from a paired culture (25). Oospore formation in these species does not normally occur except in paired cultures.

Although abundant oospore formation occurs when certain isolates are paired, this does not infer unisexuality or

complete heterothallism, for the component single cultures may rarely produce oospores on common media (63) or produce abundant oospores on the proper media. A substance in avocado feeder roots was found to stimulate the production of oospores in cultures of P. cinnamomi derived from single zoospores (77).

Abundant oospore production in paired cultures of isolates of P. infestans has recently been reported (24). Two compatible groups were demonstrated. Occasional oospore production in single cultures of P. infestans had been previously reported several times; oospore production in paired cultures of P. infestans and P. phaseoli was reported as early as 1910 (71).

Germination of oospores of P. cactorum has been reported (8). The oospores were aged three months, exposed to temperatures of 1° to 3° C. and soaked in water for two weeks.

Germination of oospores of P. infestans has recently been reported (27) and this may enable genetic tests to be made to solve, among other things, the question of heterothallism or sexuality, at least in that species.

Sexuality and heterothallism in other fungi

In many fungi, sexuality, the genetics of sexuality, and the effects of various sexual mechanisms on the biology of the organisms are well understood. Korf (38) and Whitehouse (74) have presented opposing ideas on heterothallism and sex

in the fungi. Lewis (45) has presented an excellent account of incompatibility systems in the fungi, and has discussed the possible relationships between compatibility systems and pathogenicity in fungi such as certain Uredinales and Ustilaginales which alternate between mono- and dikaryon stages differing in pathogenicity. Raper (56) has presented an excellent article on life cycles, sexuality and sexual mechanisms in the fungi. Burnett (11) has replaced the old terms hetero- and homothallism with the new terms heteromixis, homomixis, and amixis, and has divided heteromixis into three subdivisions. This new classification clarifies the relationships between mating systems and their significance in the biology and evolution of the fungi.

Radiation and mutation studies on fungi

Two general reviews of radiation effects on fungi are available (52,61). Radiation studies with fungi have been limited to a comparatively small number of species. Many of these studies were made before genetic effects were appreciated and they deal mainly with what was interpreted as damage or direct physiological effects upon growth rate. Mutation research has been widely used in industrial microbiology with the purpose of inducing mutants capable of higher production of a desired chemical (19).

Mutation studies have been most intensive on the ascomycete genera Neurospora (34), Aspergillus, Saccharomyces,

Venturia and *Ophiostoma* (52). A few other ascomycete genera have been investigated as have been *Ustilago* and *Coprinus* of the basidiomycetes, *Trichophyton* (52) and *Phoma lingam* (13) of the Fungi Imperfecti, and imperfect forms of *Penicillium* and *Aspergillus* (35,33).

In the phycomycetes, killing and growth stimulation effects of radiation have been reported for *Rhizopus* (18) and *Mucor* (50). The latter report is the only one which has been found in which induced mutation in a phycomycete is mentioned. In that study, conducted in 1925, two strains of *Mucor* were found which produced altered amounts of sporangia and zygotes.

An excellent review by Emerson (20) on biochemical genetics and mutation techniques employed in studies of fungi and bacteria has appeared. More detail on mutation techniques has been provided by Boone *et al.* (10) for *Venturia*, by Beadle and Tatum (5) for *Neurospora*, and by Hollaender *et al.* (33) for *Aspergillus terreus*, an imperfect species of *Aspergillus*.

Mutation techniques have been developed for use with the alga *Chlamydomonas moewusii* (44). Mutant production in motile unicells of *C. reinhardi*, a situation analogous to dealing with *Phytophthora* zoospores, has been reported (21).

In imperfect fungi the terms "mutant" and "phenotypic mutant" have been applied to apparently stable changed

colonies resulting from irradiation (33). Apparently stable changes in Aspergillus resulting from irradiation were accepted as genetic mutants by analogy with Neurospora work. Mutation-rate curves were obtained based upon grading colonies six to ten days after they had been transferred to Czapek's solution-agar slants. Sample size was 30 colonies from each exposure. A maximum mutation rate of 31 percent was obtained. When cultures were transferred to a more complete medium (PDA or malt extract agar) many more of them appeared normal and the mutation percentage was materially reduced. More than 80 percent of the mutants remained stable through ten mycelial subtransfers (57). In some cases cultures initially appearing as mutants developed into colonies indistinguishable from the normal type. A few others shifted back and forth between a mutant and a normal phenotype. No explanation for this was offered. Different batches of spores varied considerably in their susceptibility to killing by ultraviolet, and delayed colony development occurred from irradiated spores.

In Neurospora, when genetic tests were made, it was found that a high proportion (24 out of 60) of ultraviolet-induced mutants were multiple mutants (34). Gross morphological changes were correlated with microscopic differences in branching of the hyphae. A large proportion of the mutants produced small, dense growing colonies, and were termed "colonial".

The relationships of induced mutations to speciation in the fungi has been discussed (32). Some of the mutants produced by radiation closely resembled certain naturally occuring fungi which had been accepted as separate species. Other mutants were of a type which could be classified as new species if one did not know that they had originated from certain fungus cultures. It was calculated that there was sufficient ultraviolet present in radiation reaching the earth's surface from the sun to cause a significant increase in the mutation rates of some fungi. In actual experiments a six percent mutation rate in spores of Aspergillus terreus exposed to all wavelengths of sunlight was obtained. No detectable mutations in controls exposed only to wavelengths of sunlight over 4000 A were found. It was suggested that the ultraviolet in sunlight might be especially important in inducing mutations in plant pathogenic fungi.

Heterokaryosis and parasexuality

Parasexuality or mitotic recombination in the fungi was first reported in 1952 (59). The most thorough investigations of the phenomenon have been in the homothallic filamentous fungus Aspergillus nidulans (54,55). Buxton (12) has applied these ideas to Fusarium oxysporum and found heterokaryosis and parasexual recombination in that fungus. Heterokaryons were induced between marked mutants which differed in pathogenicity. Recombinants for the markers

and the pathogenic characters were produced. Some recombinants were pathogenic to pea varieties which neither of the parent strains could attack. These results open new possibilities for an understanding of variability in pathogenic fungi which have no sexual stages. The ideas of parasexuality are explained on the basis of heterokaryons forming diploids with subsequent recombination and segregation. Heterokaryon formation, which is basic to parasexuality, is a complicated phenomenon and has been investigated especially by Atwood and Pittenger (3) and by Pontecorvo (53).

METHODS AND MATERIALS

Media

Various natural media were used for different purposes. Potato dextrose agar (PDA) was used for a routine slant medium, and for growing P. cinnamomi in plates. Since P. cactorum does not sporulate readily on PDA, white bean agar (WBA) was employed for most work with this species. Later this medium was modified by the addition of glucose (WBGA). When bacterial contamination was probable, such as when plating irradiated zoospores, streptomycin nitrate (Phytomycin) was added to the media. A streptomycin concentration of 25 ppm. was found to be sufficient. Pea broth (PB) was used as a pre-sporulation medium. Difco potato dextrose agar (DPDA) was used for a zoospore plating medium since it was expected that deficient mutants would show up more readily on this less complete medium. Dung infusion was used in attempts to stimulate oospore germination, as reported by Gough (27).

Quantities given in the following media are in grams per liter unless otherwise indicated.

WBA: 50 g. dried Great Northern white beans, 20 g. agar. Beans were autoclaved ten minutes at 15 pounds pressure in 500 ml. distilled water. Agar was autoclaved similarly. Bean broth was strained through cheesecloth, combined with the agar and autoclaved 15 minutes.

WBGA: WBA plus 2.5 g. glucose.

PB: (A) 150 g. dried whole peas autoclaved five minutes, strained, autoclaved 15 minutes in one liter Erlenmeyer flasks. (B) 150 g. split peas, autoclaved three minutes, decanted or strained, autoclaved ten minutes in 500 ml. Erlenmeyer flasks.

Oat agar: 35 g. oatmeal were brought to boiling, steeped 30 minutes, strained. 10 g. dextrose and 20 g. agar added; autoclaved 15 minutes.

Corn meal agar: 50 g. yellow corn meal were brought to boiling, steeped 30 minutes. 20 g. agar added; autoclaved 15 minutes.

Dung infusion: Stock solution: 40 g. relatively dry horse dung stirred in 1 l. water, filtered and autoclaved 15 minutes. Used at dilutions of 1:10, 1:25, and 1:50.

B-Vitamin solution: (Prepared as a supplement to minimal media; used at a concentration of 6 ml./l. Figures are mg./6 ml.) Riboflavin 1.0, nicotinic acid 1.0, para-aminobenzoic acid 0.2, pyridoxine 0.5, thiamine hydrochloride 0.5, biotin 0.004, calcium pantothenate 2.0, folic acid 0.2.

Various synthetic media have been used for growth of Phytophthora (46), and as screening media in mutant studies with Neurospora, Aspergillus (20), and Venturia (10). Several minimal media (MM) were used in the present studies (Table 1). The minimal media were supplemented with B-vitamins (VIT) or amino acids (AA) for use in screening mutants for biochemical blocks. The vitamin solution containing eight B-vitamins was prepared and kept frozen in test tubes. A Difco vitamin-free casamino acid digest was used as the amino acid supplement at the rate of 3 g./l.

TABLE 1. SYNTHETIC MINIMAL MEDIA USED FOR SCREENING MUTANTS

Compound	Solution A	B	C	D
KH_2PO_4	100*	2	5	6
K_2HPO_4	-	-	-	4
$MgSO_4.7H_2O$	20	2	5	5
NH_4NO_3	15	1	5	5
$Ca(NO_3)_2$	-	5	2	0.1
KNO_3	-	4	5	-
minor elements	1ml.**	1	1	1
iron (chelate)	1ml.***	2	2	1
glucose	50g.	10g.	10g.	30g.
thiamine	1mg.	0.1mg.	0.2mg.	1mg.
final pH, adjusted with 0.1M NaOH and HCl	5	5.5	5	6.4

* milliliters of a 1 molar solution per liter.
** minor elements solution was adapted from that employed by Hoagland (31) for higher plants and consisted of (grams/liter) H_3BO_3 2.86, $MnSO_4H_2O$ 1.6, $ZnSO_4.7H_2O$ 0.22, $CuCl_2.2H_2O$ 0.05, H_2MoO_4 0.022.
*** chelated iron solution consisted of 1.2 g. sequestrene (12% iron) per 100 ml.

Solution A which contains very high amounts of salts and sugar was adapted from that employed by Robbins (58). Solution D was most satisfactory since the use of the two potassium salts produced a buffered solution of pH 6.4 and made pH adjustment unnecessary. Precipitation of the magnesium salt often occurred when the pH of the other

solutions was raised past about 5.5 with NaOH. In the early trials the glucose was sterilized separately and the thiamine was added aseptically without heat sterilization. Later it was found that satisfactory growth occurred without taking these precautions. For ease in preparation, a stock solution of thiamine was kept frozen in test tubes. The glucose was CP grade and the salts were reagent grade.

Zoospore induction

Two methods of inducing zoospore formation were used for P. cactorum. (1) Disks from colonies bearing sporangia on WBA or WBGA were placed in short 2 cm. vials and flooded with sterile distilled water. The vials were then immersed in a cold water bath (10° to 15° C.). (2) Colonies were grown in plates of pea broth, rinsed in several changes of sterile distilled water and left in sterile distilled water. If rinsing were done carefully the colonies would remain attached in the original petri dish in which they had developed. Sporangia, if not already formed, would form within 12 to 48 hours. The plates were then chilled at 10° or 15° C. to induce zoospore formation.

The ease with which zoospores could be induced varied with different isolates and different mutants. Some of these never produced zoospores under any conditions. In replicated plates, apparently handled identically, zoospores might be abundant in one plate and absent in another. Pea

broth B gave better results than pea broth A, and was used exclusively in the later studies.

The same procedures applied to P. cinnamomi did not result in zoospore formation. No technique was found whereby P. cinnamomi would form quantities of zoospores under sterile conditions.

Procedures used for mutation induction and screening

Ultraviolet: A 15 watt, 18 1/4 inch General Electric germicidal lamp was used as an ultraviolet light source. Catalog data rate the lamp with 90 percent of its ultraviolet output at 2537 angstrom units; 3 watts of output below 2800 A, 0.06 watts between 2800-3200 A, and 0.05 watts between 3200 and 3800 A. When checked on a spectrograph, the major line was at 2537 A, with other lines of much less intensity at approximately 2964, 3124, 3132 and 3646 A.

Ultraviolet irradiation was used only with P. cactorum. The irradiation and screening procedure found to be most satisfactory is shown in Figure 1. One milliliter of a zoospore suspension was pipetted into a vial two centimeters in diameter by 2.5 centimeters long, and irradiated. Lamp to specimen distances were 52 or 59 centimeters. A glass slide was used as a shutter. The irradiated zoospores were either poured directly into plates of WBGA or were serially diluted to the proper concentration in test tubes before plating. The zoospore concentration was checked at each

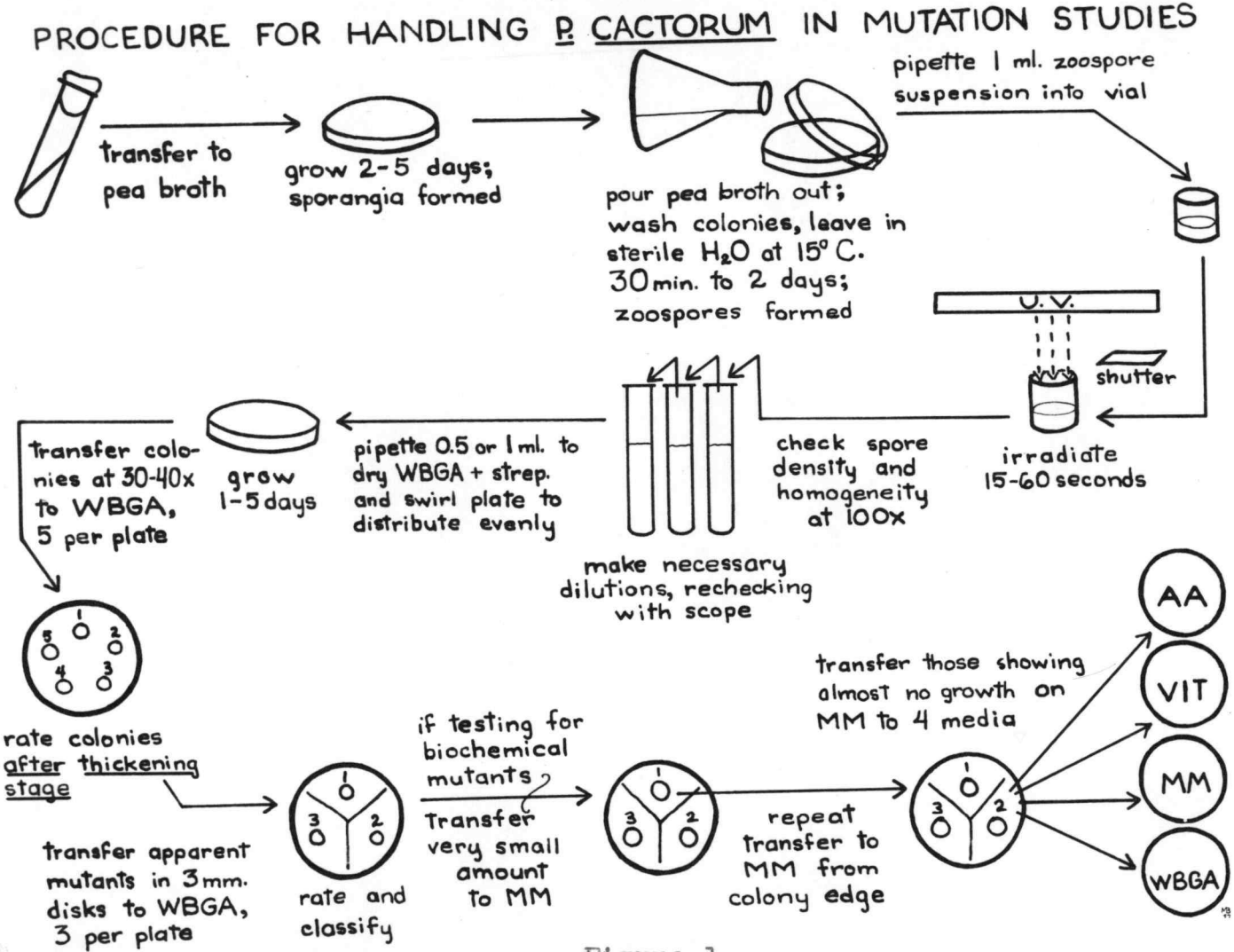

PROCEDURE FOR HANDLING P. CACTORUM IN MUTATION STUDIES
transfer to pea broth
grow 2-5 days; sporangia formed
pour pea broth out; wash colonies, leave in sterile H2O at 15° C. 30 min. to 2 days; zoospores formed
pipette 1 ml. zoospore suspension into vial
U.V.
shutter
irradiate 15-60 seconds
check spore density and homogeneity at 100x
make necessary dilutions, rechecking with scope
pipette 0.5 or 1 ml. to dry WBGA + strep. and swirl plate to distribute evenly
grow 1-5 days
transfer colonies at 30-40x to WBGA, 5 per plate
rate colonies after thickening stage
transfer apparent mutants in 3 mm. disks to WBGA, 3 per plate
rate and classify
if testing for biochemical mutants
Transfer very small amount to MM
repeat transfer to MM from colony edge
transfer those showing almost no growth on MM to 4 media
AA
VIT
MM
WBGA

Figure 1.

dilution by direct microscopic examination of one milliliter of suspension in a vial. A dilution that yielded well-separated colonies was obtained when two to four motile zoospores were visible in the field of the microscope at an enlargement of 100 diameters. One-half or one milliliter of suspension was pipetted to each plate. The plate was immediately swirled rapidly to spread the zoospores evenly over the agar surface. Colonies arising from irradiated zoospores were picked when they consisted of several branching hyphae but before they attained a diameter of one-half to one millimeter. A very small glass needle was used to pick and transfer colonies to WBGA. Five colonies were placed on each plate, and were rated after they had attained diameters of approximately 1.5 centimeters and were visibly thickened. The colonies rated as mutant on these plates were transferred (3mm. disks) to WBGA, three per plate. The colonies developing in these plates were given a final rating. Further transfers to appropriate media were made when screening for biochemical mutants.

X-ray: A standard mobile, oil-cooled X-ray therapy unit was used. The tube was a model Eureka TG3 oil-immersed tube with a 45 degree target range. Maximum input power was 150 KV but all dosages were applied at 115 or 130 KV and eight milliamperes. Calibration was done with a Victoreen r-meter, model 70.

Both P. cactorum and P. cinnamomi were irradiated with X-rays. Colonies of P. cinnamomi growing on PDA plates were irradiated. To obtain sufficiently high dosages on mycelial irradiations, working distance (from nozzle edge to agar surface) was reduced to four centimeters. A dosage rate of 200 r per minute was obtained at this distance with a setting of 130 KV and eight milliamperes. Petri plate covers were removed during the irradiation but plates were covered with a piece of sterile paper. Screening for mutants of X-ray-irradiated P. cinnamomi colonies was similar to the procedure outlined for P. cactorum except that hyphal tips were used and the medium was PDA. Zoospore suspensions from P. cactorum were irradiated in vials at a working distance of 2.5 centimeters and settings of 115 KV and eight milliamperes. A dosage rate of 225 r per minute was obtained at these settings. The screening procedure was the same as that used following ultraviolet irradiation.

Discussion of mutation and screening procedures

The handling and screening of colonies derived from irradiated zoospores of P. cactorum were patterned after methods employed with other fungi. However, such methods were not directly applicable to handling Phytophthora. In Phytophthora, color mutants could not be expected, so screening had to be based only on morphological, nutritional, or pathogenicity changes. Pathogenicity changes are not

amenable to large scale screening in P. cactorum. Screening for nutritional changes was complicated by the death of some of the very small colony fragments that were transferred. Screening for morphological changes was complicated by the growth-lag phenomenon. The growth-lag phenomenon refers to the characteristic of P. cactorum to grow to a diameter of one to two centimeters before thickening into a dense colony. Development of colonies arising from irradiated zoospores was often delayed up to one or two days even though no permanent change was produced. P. cactorum produced a spreading growth on agar media and the colonies soon grew together. This necessitated early picking and grading for mutants on plates one or two transfers removed from the zoospore colonies.

Best results were obtained when the plating medium was permitted to dry out slightly. The medium then absorbed the water more quickly, facilitating picking of the young colonies and reducing bacterial contamination. Also, table tops and equipment were swabbed with a 20 percent Clorox (5.25 percent sodium hypochlorite) solution and propylene glycol was atomized around the work area to reduce contamination.

Transferring colonies to tubes of a liquid medium was found to be unsatisfactory since grading in a liquid medium is limited and transferring to tubes requires too much time. In addition, the possibility of loss of isolates through

bacterial contamination is increased.

Micro-colonies developing from irradiated zoospores were checked at 100X to determine if they originated from single zoospores rather than from sporangia or multiple zoospores. Plates were first opened and sufficient sterile water was poured into them to barely cover the agar surface. This eliminated the refraction which sometimes made determination doubtful.

PHYTOPHTHORA CINNAMOMI

Morphological variability of isolates

Thirty-five different isolates of P. cinnamomi were obtained from diseased plants in Oregon and from other sources. These isolates were grown from mycelial transfers on PDA in replicated plates and their gross morphology was compared (Table 2.).

TABLE 2. ISOLATES OF P. CINNAMOMI, THEIR HOSTS AND MORPHOLOGICAL RATING

Host	No. of isolates	Morphology	Place of origin
Rhododendron	10(G4)*	x**	Oregon
Rhododendron	3(DAPD,L5a)	small	Oregon
Rhododendron	1(5A)	wide zonation	Oregon
Chamaecyparis	6(36A)	x	Oregon
Chamaecyparis	1	x	California
Chamaecyparis	2(39,32)	small	Netherlands
Avocado	2(LA1690)	x	California
Heather	1	x	Oregon
Heather	1	small	Oregon
Abies	1(Pc17)	small	Oregon
Taxus	1(Pc4)	small	Oregon
Taxus	1(Yew)	small	Netherlands
(Juniper) soil	1	x	Oregon
unknown	1(178)	small	California
Cinnamomum	1	small	Sumatra***
Cinchona	1	x	Peru
Pinus	1	x	Georgia

* Identifying symbol of the selected isolates shown in Figure 2.
** Large zonate growth type.
*** Type culture.

The range of variability which the 35 isolates exhibited is shown in Figure 2. Eleven isolates produced much smaller

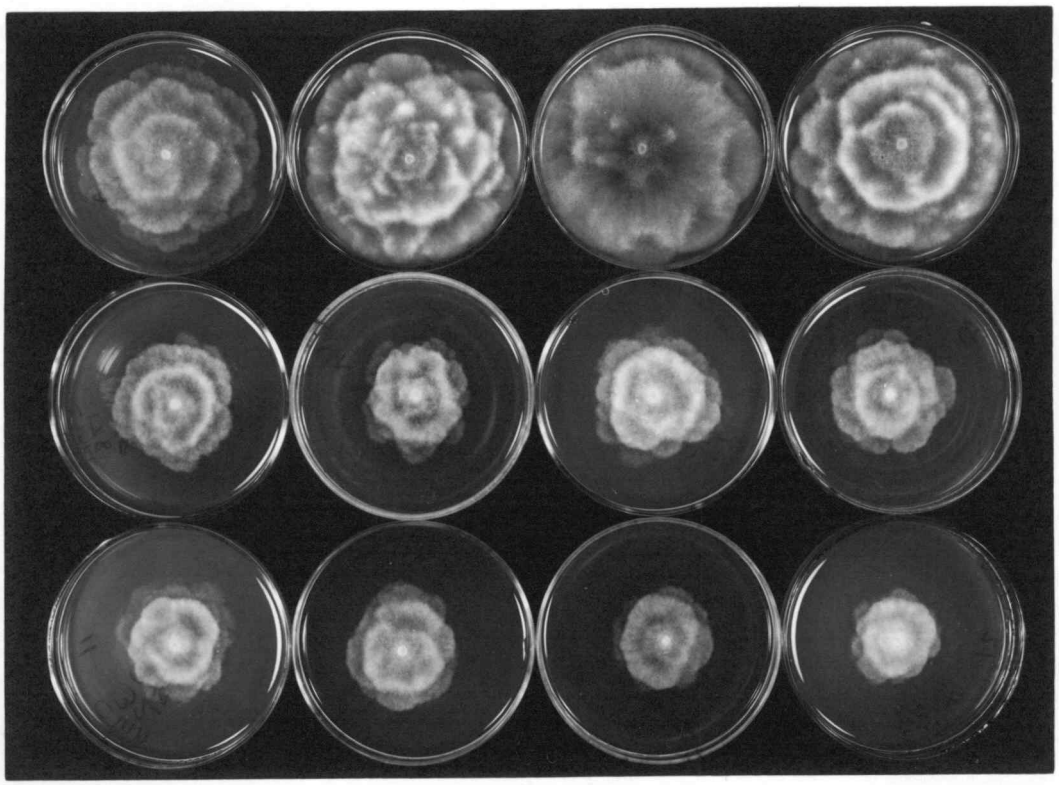


Fig. 2. P. cinnamomi isolates on PDA after 7 days at 20°C;
(upper, l. to r.) isolate G4, Pc7, 5A, LA1690;
(middle) 178, Pc17, DAPD, 39;
(lower) 32, L5a, Pc4, Yew.

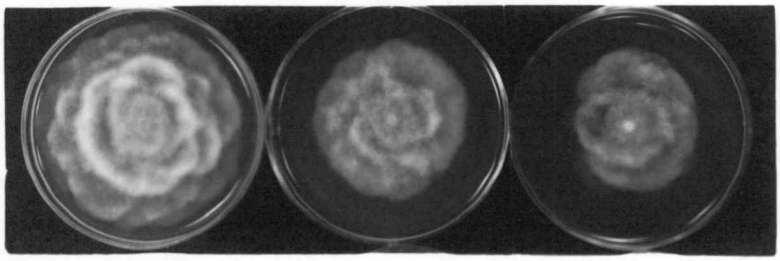


Fig. 3. Variation in colonies of P. cinnamomi derived from single hyphal tips, after 7 days at 20° C. on PDA.
(left) One of 58 typical colonies of isolate Pc7.
(middle) An atypical colony derived from a typical colony originating from a single hyphal tip.
(right) An atypical colony derived from a typical colony originating from a mass mycelial transfer.

colonies than the others and were rated as "small". The amount of zonation among the isolates also varied. P. cinnamomi is normally considered to produce vigorous, zoned colonies (Figure 2, isolates G4, Pc7, LA1690) and is identified on this basis coupled with the production of spherical swellings on the hyphae and absence of spores when grown on PDA. Variable amounts of spherical swellings were produced in all of these isolates.

Variability within one isolate

Variability within one isolate was tested in preparation for mutant studies to determine how much variability might be expected in non-irradiated colonies derived either from a single hyphal tip or from a mass transfer.

Zoospores were not obtained from P. cinnamomi by the pea-broth-water technique or by employing sterile soil leachates. A few were obtained in non-sterile soil leachate solutions but these were not used because of bacterial contamination. Therefore all work with P. cinnamomi was done with hyphal tips. The hyphal tips consisted of a tip with several branches, since poor survival was obtained when very short, unbranched tips were used. The nuclei in these branched tips might not have been identical.

Variability derived from a mass transfer was tested by taking 30 hyphal tips from a colony of an isolate (Pc7)

obtained from Rhododendron in Oregon and comparing the resulting colonies. Variability obtainable in colonies derived from a colony started from a single hyphal tip was also tested by transferring 30 hyphal tips from such a colony. The resulting colonies were retransferred to new PDA plates before comparisons were made. From each group one colony was obtained which was morphologically separable from the other 29 colonies (Figure 3).

The colony of reduced size and zonation that was obtained from a hyphal tip derived from a typical, large, hyphal-tip colony might indicate heterokaryosis within a hyphal tip. This condition could also be explained on the basis of a cytoplasmic change or a mutation.

Induction of mutants

Cultures of isolate Pc7 (wild type) were irradiated with X-rays at dosages from 400 to 4000 r. After six days of post-irradiation growth, colony size was smallest in the plates irradiated with 4000 r. From these plates 33 hyphal tip transfers were made. Twelve of these produced colonies which were graded initially as mutants. After several subtransfers only nine were sufficiently different from wild type to be classed as mutants. One of these, a very weak mutant, died after 12 days. Eight of the mutants are shown in Figure 4.

Three mutants produced sectors. The assumption was made

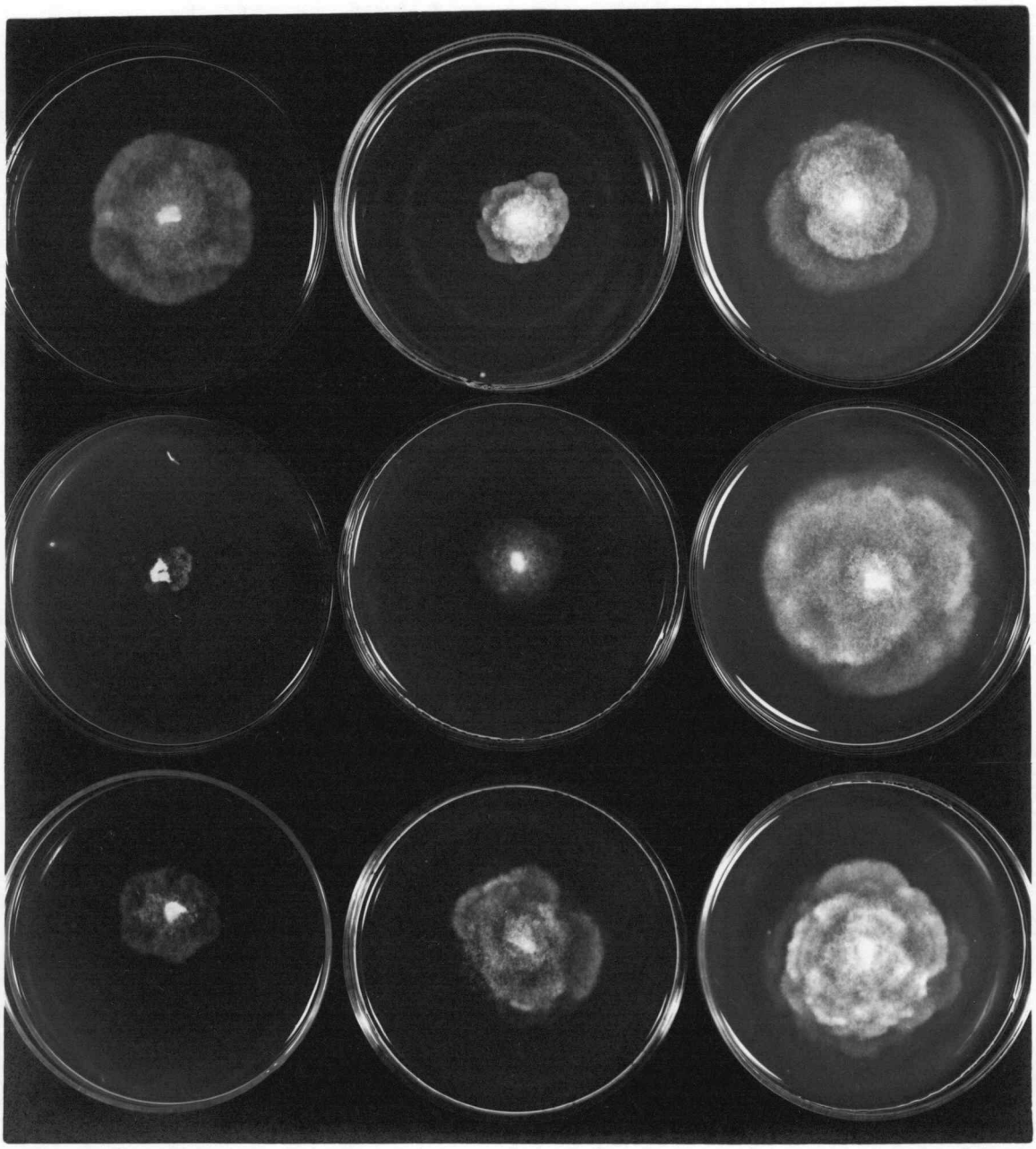


Fig. 4. X-ray induced mutants of P. cinnamomi, after 5 days at 20° C. on PDA;
(upper) 8a, 8b, 9;
(middle) 1, 3b, 4;
(lower) 3a, 7, wild type.

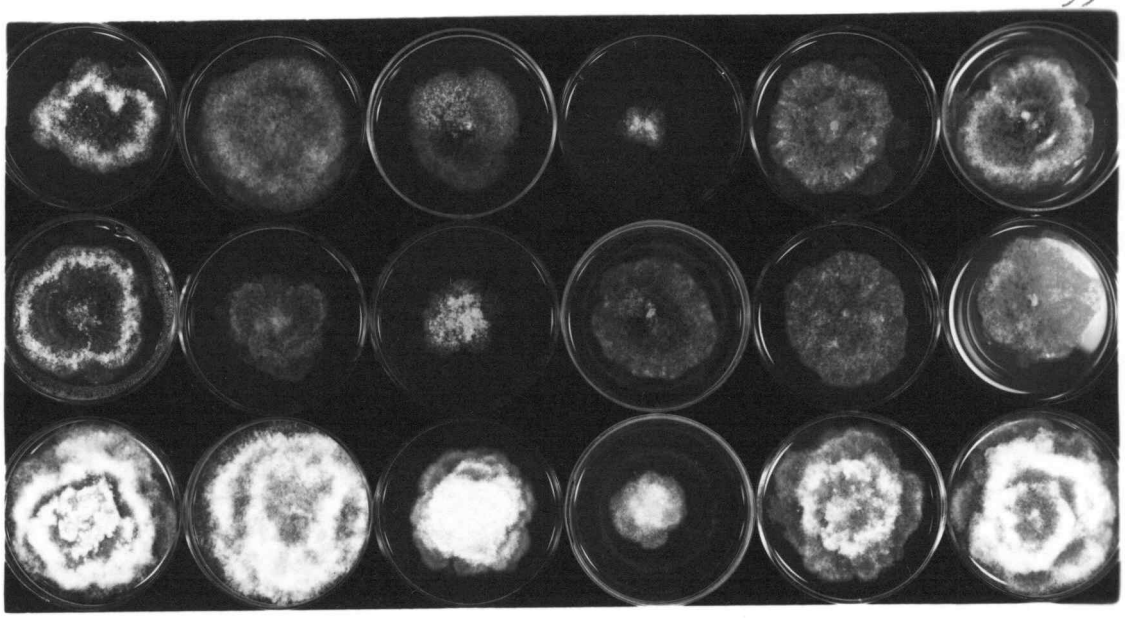
that these sectors resulted from the presence of more than one nuclear type in the hyphal tips that were transferred. The hyphal tip transfers were made six days after irradiation to minimize this possibility.

The mutants were altered in their microscopic characters on PDA. Spherical swellings were absent in mutants 1 and 8b, but present in 3a, 3b, 8a and 7 and in wild type. "Knobbiness" was present in mutants 1, 9 and 8b.

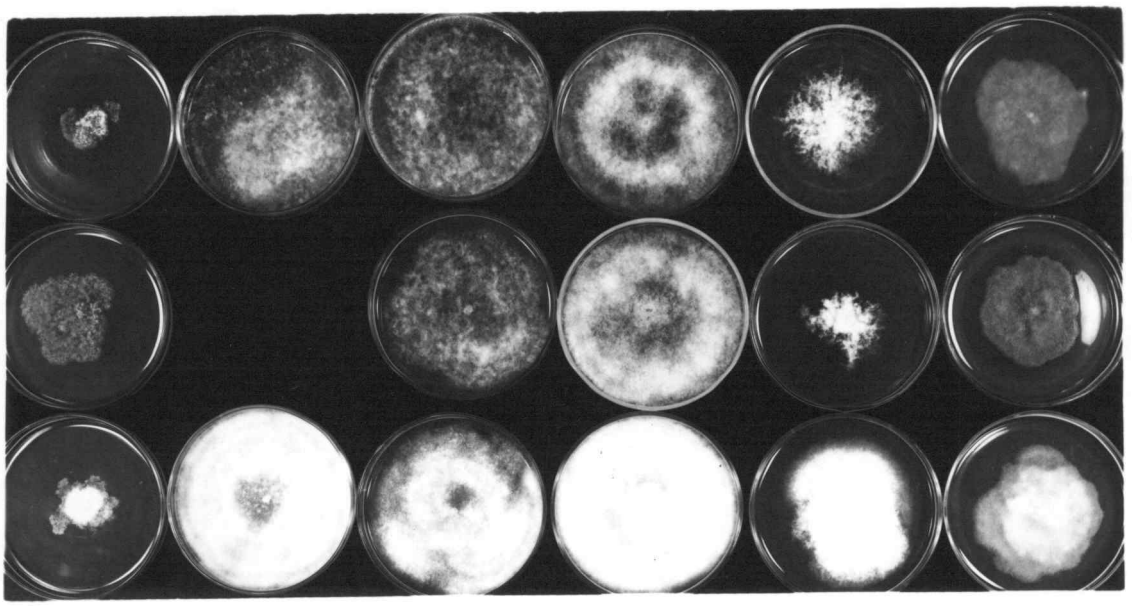
Morphological comparison on synthetic media

The mutants and wild type were compared on minimal medium A and on media supplemented with amino acids or vitamins (Figure 5). Characteristic differences in growth types were found among some of the mutants on the various media. "Twisted" growth was exhibited by mutant 5 on MM and MM plus vitamins. Spreading growth was exhibited by mutants 3a, 3b and 4 on all media, and the latter produced very dense growth on amino acid-supplemented medium. Mutant 9 showed reduced growth on MM and growth approximating wild type on MM plus vitamins. Mutant 1 showed reduced growth on all synthetic media.

Mutant 9 was retested in synthetic solutions and the amounts of growth in MM and in MM plus vitamins were not noticeably different.



7 8a 8b 9 10 wild type



1a 3a 3b 4 5 6

Fig. 5. Mutants of P. cinnamomi on three media after 8 days at room temperature. Media in each picture are (upper row) minimal medium; (middle row) MM plus vitamins; (lower row) MM plus amino acids.

Pathogenicity of mutants

Eight mutants were inoculated into apple fruits (Linton variety) and each produced a rotting of the fruits. The rotted areas varied from 2.5 to 4 cm., five days after inoculation. The mutants retained their characteristics after isolation from the rotted apples.

The pathogenicity of three mutants (1,3a,9) and the wild type was tested on cedar (Chamaecyparis lawsoniana Parl.) Small cedar trees growing in No. 10 cans were inoculated by pouring 25 ml. of mycelial suspension into holes made in the soil of each can. Ten cans were used for each mutant. Cans were kept in a warm chamber where soil temperature fluctuated from 18° to 25° C. Disease readings were taken after two months. Wild type and mutant 9 killed ten and nine trees respectively. Mutants 1 and 3a were not pathogenic. The roots of trees inoculated with mutants 1 and 3a were examined and no differences could be found between them and roots of uninoculated control trees. Isolations were attempted from the soil of each can four months after inoculation by placing soil in holes in apple fruits. Only wild type and mutant 9 cultures were recovered from the soil. Mutant 9 retained its mutant phenotype after recovery from the soil.

This experiment demonstrated that one mutant was pathogenic to cedar but that two were not. The non-pathogenic mutants were unable to survive in the soil. The two

non-pathogenic mutants grew more slowly on agar than did the pathogenic mutant.

Oospore production in paired cultures

Pairing studies to induce oospore formation were made with P. cinnamomi and other species. Twenty different isolates were paired in all possible combinations on oatmeal agar. Three isolates were placed on each plate. The procedure involved removing three 1-cm. strips of oat agar from each plate leaving three separated triangles of agar in the plate. Water agar was then poured into the empty strips. This facilitated observation in the area where the hyphae of the cultures intermingled. The isolates that were paired and the pairings that resulted in oospore formation after ten days are shown in Table 3. Oospores resulted in only 10 out of the 190 paired combinations. In each case P. drechsleri 114 was one of the parents. P. drechsleri did not form oospores in non-paired cultures.

In another experiment nine isolates of P. cinnamomi were paired with two isolates of P. cryptogea on corn meal agar. Oospores were formed in all but two pairings. This is in contrast to the complete absence of oospore formation when these two species were paired on oatmeal agar.

Several isolates were paired in agar plates containing a collodion membrane barrier. It was thought that the substances responsible for formation of antheridia and oogonia

TABLE 3. *PHYTOPHTHORA* SPECIES AND ISOLATES PAIRED ON OATMEAL AGAR AND PAIRINGS THAT RESULTED IN OOSPORES

Isolates paired in all possible combinations	Pairings that resulted in oospores
P. cinnamomi 39	*P. drechsleri* 114 x *P. cinnamomi* Pc17
P. cinnamomi Pc4	*P. drechsleri* 114 x *P. cinnamomi* Rhodo
P. cinnamomi Pc17	*P. drechsleri* 114 x *P. cinnamomi* Pc4
P. cinnamomi Rhodo	*P. drechsleri* 114 x *P. cinnamomi* 32
P. cinnamomi Yew	*P. drechsleri* 114 x *P. cinnamomi* Pc7
P. cinnamomi Rands	*P. drechsleri* 114 x *P. cinnamomi* 39
P. cinnamomi Pc13	*P. drechsleri* 114 x *P. cinnamomi* 5
P. cinnamomi 32	*P. drechsleri* 114 x *P. drechsleri* 113
P. cinnamomi 5	*P. drechsleri* 114 x *P. cryptogea* 21278
P. cinnamomi Pc7	*P. drechsleri* 114 x*P.palmivora* Cocos
P. boehmeriae	
P. cambivora 40512	
P. cryptogea 21278	
P. cryptogea P55	
P. drechsleri 113	
P. drechsleri 114	
P. lateralis P1121	
P. lateralis P188	
P. palmivora Cocos	
P. syringae 11302	

might diffuse through the collodion membrane which separated the two colonies, and induce their formation. Isolates were used that were known to produce oospores when paired. No oogonia or antheridia were formed when the collodion barrier separated hyphae of the two colonies, even though hyphae were appressed to both sides of the membrane. In two plates where the compatible strains of *P. drechsleri* were paired, the hyphae eventually grew around the barrier at the edges

of the plates, and mixed with the hyphae of the opposite strain. Oospores were then produced in abundance, greatly exceeding the numbers produced in control plates containing no barriers. This could indicate an accumulation in the hyphae or agar of a substance or substances effective in stimulating oospore formation.

PHYTOPHTHORA CACTORUM

Morphological variability among isolates

Eleven P. cactorum isolates were obtained from various sources (Table 4.).

TABLE 4. P. CACTORUM ISOLATES STUDIED, THEIR HOSTS AND GEOGRAPHICAL ORIGINS

Isolate	Host	Origin
Pc206	apple	Canada
1558	Saxifrage cotyledon L.	CBS*
634	Cox Orange apple tree	Netherlands
Pc204	unknown	Canada
Howbold	apple fruit	Canada
594	apple crown	Canada
var. applanata	Syringa vulgaris L.	Boston
APC	apricot tree	Canada
Syringa	Syringa vulgaris L.	CBS*
VAN	cherry tree	Canada
1620	Cox Orange apple tree	Germany
Skotland	soybean	North Carolina

* Centraal Bureau voor Schimmelcultures

In addition, the isolate Skotland, which was reported (60) to resemble P. cactorum, was included. This isolate was later found not to belong to this species.

These isolates were not identical in gross morphology when grown on WBA, although six isolates appeared to be very similar (Figure 6.). Morphological differences between isolates were reflected in differences in characters such as presence or abundance of oospores and sporangia and branching

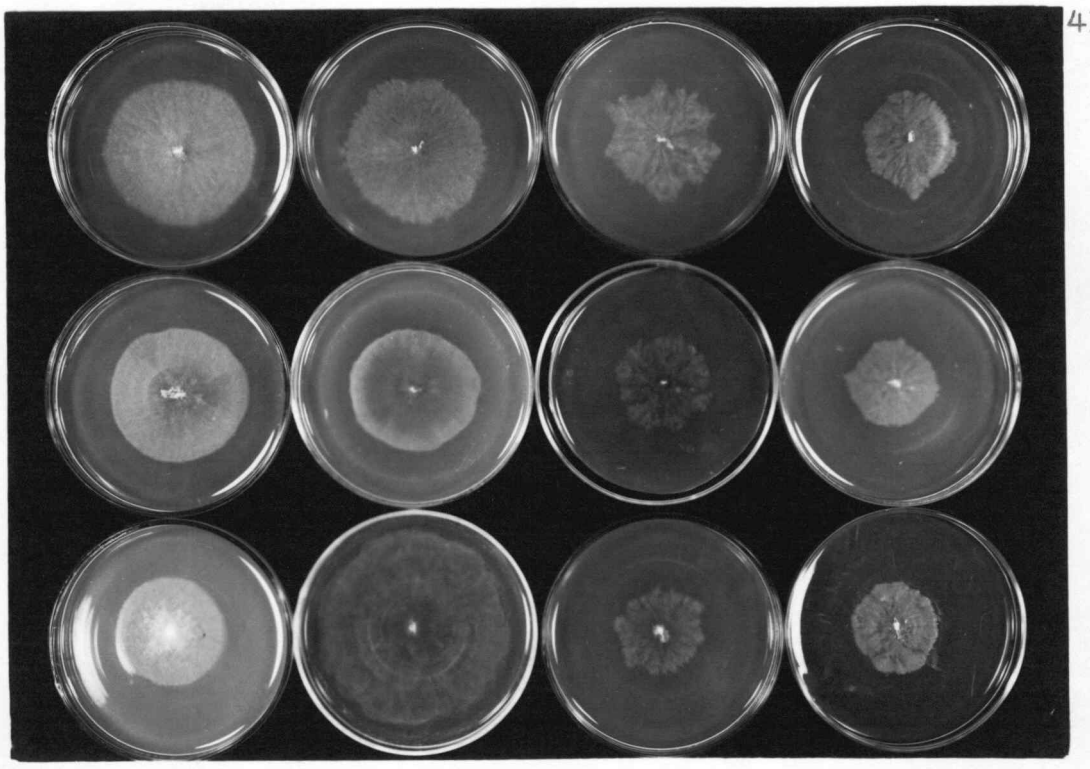


Fig. 6. P. cactorum isolates on WBA after 5 days at 20°C. (upper, l. to r.) Howbold, Syringa, VAN, 1620; (middle) 544, 1558, 206, 634II; (lower) Skotland, applanata, 204, APC.

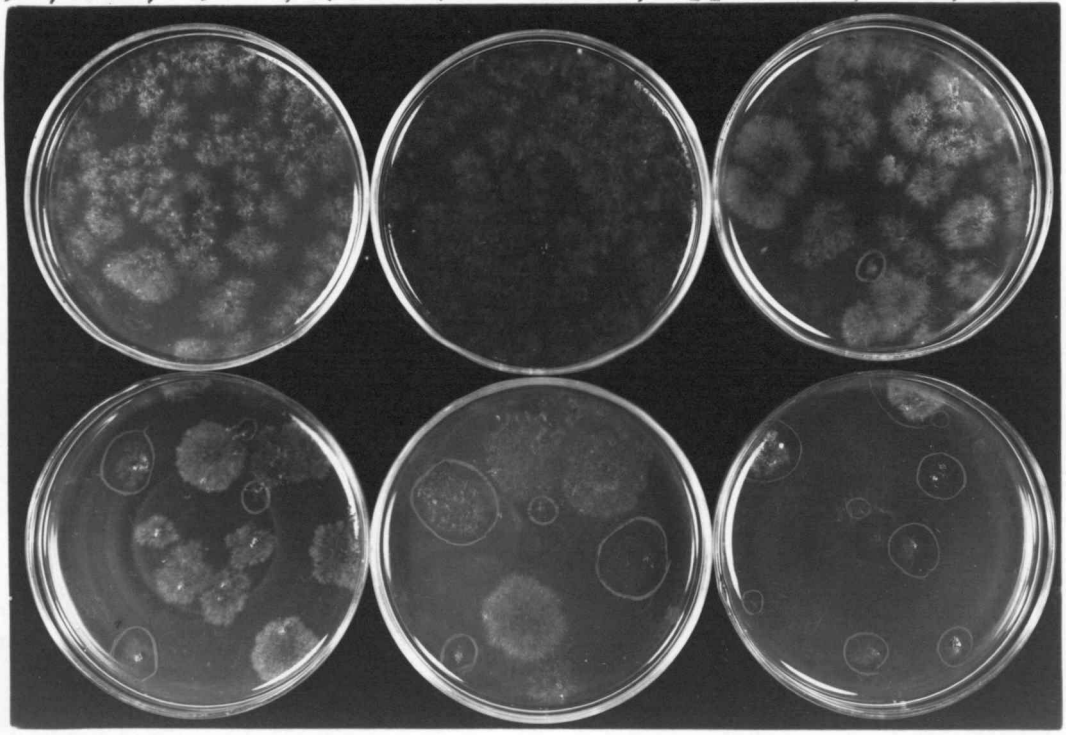


Fig. 7. Colonies of P. cactorum developing after ultraviolet dosages of (upper) 0, 15, 30; (lower) 45, 60, and 75 seconds. Sparse colonies are circled.

aspects of the hyphae. Each isolate remained constant when mycelial transfers were made in triplicate.

Variability within isolates

Zoospores were obtained from isolates 634, Howbold, Syringa, 1558, and Skotland. Fifty zoospore colonies of each of these isolates were transferred to WBGA and graded after they had grown out past the thickening stage (Table 5). Each of the isolates produced zoospore micro-colonies which were characteristic for that isolate. The branching and rapidity of development of the micro-colonies developing from the zoospores varied among isolates and it was possible to identify the isolate by microscopic examination of the developing micro-colonies.

TABLE 5. VARIABILITY OF ZOOSPORE COLONIES FROM FIVE ISOLATES

Isolate	Number picked / Number survived	Large	Medium dense	Small
634	50 / 48	48		
Howbold	50 / 49	49		
Syringa	50 / 50	50		
Skotland	50 / 49		47	2
1558	50 / 0			
1558 repeat	40 / 18	15		3*

* became large on new plates.

Zoospore colonies from three isolates exhibited no variability. Isolates Skotland and 1558 both produced two types of colonies. The two types from isolate Skotland were transferred to WBGA where they remained differentiable. The behavior of isolate 1558, which was treated in a similar manner to the other isolates, was striking in that no colonies survived the first transfer. The original zoospore colonies were reexamined and it was found that most colonies appeared to be dead and had not grown beyond a diameter of one millimeter but that a few had developed to three or four millimeters and a few had developed to 1.5 centimeters. Forty new zoospore colonies were picked and of the 18 which survived, three were sparse and small. These were retransferred to WBGA where they did not retain their atypical phenotype.

The death of the highly branched micro-colonies from zoospores of isolate 1558 has not been explained. A similar death of micro-colonies was later found in mutant C87S. The behavior of isolate Skotland indicated that it was either heterokaryotic or that it had been isolated as a multiple type.

A single zoospore colony (C78) of isolate 634 was selected for use in future mutation work. All of the experimental work carried out on P. cactorum was done with this culture and the mutants derived from it.

Variability of a single zoospore colony

The range of variation of the normal type, and the ease of grading that range were investigated. Zoospores were obtained from a colony of isolate 634 which originated from a single zoospore, and were plated on DPDA containing 25 ppm. streptomycin. Two days after plating, 100 zoospore micro-colonies were graded and transferred to WBGA, five per petri plate (Table 6).

TABLE 6. A COMPARISON OF 100 SINGLE ZOOSPORE COLONIES DERIVED FROM A SINGLE ZOOSPORE COLONY

Number in each group and rating when picked			Rating after two days on WBGA	Rating after three to five days on WBGA
large	medium	small		
16			x*	x
	45		x	x
	4		sparse	x
		12	x	x
		7	sparse	x
		4	-	sparse**
		12	-	-

* parental or wild type designated as x, no growth as -.
** rated as normal on new plates, two days after transferring.

The micro-colonies on the initial DPDA plate were not identical in size or aspect and were graded as small, medium or

large. The four colonies which showed only sparse development after three days on WBGA never became normal on those plates since neighboring colonies were in close proximity. However, these colonies developed as normal control colonies when transferred to new plates. Several interesting points developed from this experiment: (1) no permanent variability was encountered in the 88 zoospore colonies which were obtained; (2) delayed growth resulted in colonies that were much more sparse than normal, but this sparseness was not permanent. This point was found to be very important when obtaining mutant percentages from colonies developing from irradiated zoospores, since the many sparse colonies obtained did not differ initially from many mutants; (3) twelve percent did not survive the transferring operation.

In addition to the 100 colonies transferred, two very small colonies which did not appear on the original DPDA plate until three days after the others were picked, were transferred along with a control to new plates. After five days the three colonies were identical. This showed that even extreme delay in development was possible, but that it did not result in deviation from normal type once the colony was established.

DPDA was not very satisfactory as a plating medium since on this medium the colonies grew sparsely with a spreading growth habit. Some of the colonies soon grew together, and to obtain single colonies with assurance,

it was necessary to pick them before they became clearly macroscopic. For mutant selection this was a disadvantage since selection was much more reliable when colonies were visible and after the lag-phase had been evened out. It was later found that WBGA was a more suitable plating medium, since colonies thickened more rapidly and spread less extensively on this medium.

Oospore dormancy

Oospore germination in P. cactorum has been reported to occur at low rates in old cultures (8). An experiment was conducted to test the effect of 72 combinations of temperature, freezing, and glutathione concentrations on oospore dormancy. Glutathione is known to break dormancy of some seeds (69). Agar disks containing oospores from cultures one month old were used. Reduced glutathione was used at concentrations of 0.1 and 0.01 M. Agar disks were soaked in glutathione for periods of five and 15 minutes, and one and five hours. Temperatures of incubation in water after soaking in glutathione were 5°, 15°, 25°, and 30° C. In some treatments oospores were frozen before or after glutathione treatment. Controls, having no glutathione or freezing treatment, were run at different temperatures. Fifty oospores from each treatment were checked microscopically after one and four months. No certain germination was observed. Hyphae were observed to be produced from a few

spores after four months in three treatments. Whether these hyphae were originating from oospores or from vegetative oogonia in which no oospores had been produced could not be positively determined. The three treatments that resulted in hypha production were: (1) freezing, glutathione 0.1 M. for six hours, 25° C.; (2) freezing, glutathione 0.01 M. for five minutes, 5° C. and (3) freezing, glutathione 0.01 M., 30 minutes, 25° C. In many treatments the contents of all of the oospores had disintegrated after four months and even in untreated controls the percentage of oospores filled with protoplasm and presumably alive, was low.

Observations were made on developing oospores from several isolates. In isolates Syringa and APC a large number of oospores developed fully and remained filled with homogeneous protoplasm containing a refractive granule. In other isolates the number of oospores with disintegrating protoplasm was large. In some mutants ($Cl5_{103}$, $Cl5_{12}$, 145) oogonia and antheridia never produced fully differentiated oospores.

A very limited trial using dung solutions to break dormancy as reported by Gough (27) was conducted with isolate 634. Two and three month old agar cultures were flooded with a 1:25 dilution of the stock dung solution. Microscopic examinations were made after two and three weeks. Many oogonia were completely empty and appeared to have

hyphae growing from their bases. However, careful examination could not establish that germination of oospores had occurred.

IRRADIATION

Preliminary ultraviolet studies

Preliminary trials showed that a lamp-to-specimen distance of at least 50 centimeters was necessary to measure irradiation time accurately and still have viable zoospores. Short distances were employed at first and the suspensions were examined microscopically before and after irradiation. At a distance of 4.5 cm. and an exposure of three minutes all the zoospores were immobilized. At one and two minute exposures, 50 and 20 percent of the zoospores were estimated to remain motile. However, when these suspensions were plated out no colonies were obtained. Thus zoospores that were still highly motile immediately after irradiation at a high dosage did not survive to produce colonies. At a distance of 16 cm. and exposures of 10, 15, or 30 seconds, percentages of spores that survived to produce germination tubes were 77, 50, and 0, respectively. However, most of the germination tubes soon ceased growth. Approximately one percent of the zoospores that produced germination tubes ultimately developed into colonies.

The effect of different dosages of ultraviolet on zoospores was determined in a preliminary experiment. Irradiation distance was 59 cm., and exposure times of 0, 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, and 180 seconds were used. A standard dilution of 1:50 was used and four plates

were made at each dosage. Results of six of the dosages are shown in Figure 7. As the dosage increased, the number of colonies decreased, but their size and variability increased. The increase in size could be largely attributed to decreased colony numbers. The increase in variability was found upon subsequent transferring to be due largely to the production of phenotypic mutants. However, part of the variability was not permanent and was attributed to growth-lag. The variability due to growth-lag and colony density was superimposed upon that due to the presence of mutants and tended to make grading or selection for mutants from the initial plates unreliable. At exposures of 15 seconds and above, growth of germination tubes and development of the micro-colonies was delayed. Microscopic examination 20 hours after plating revealed that the degree of delay was proportional to the dose. When the delayed colonies were transferred to new plates, some retained their phenotypic differences but others did not.

With increasing dosages of irradiation the colony count did not decrease to zero as sharply as was expected. A colony would occasionally develop from suspensions which had received a three-minute dose. This was due in part to the survival of sporangia which were present in the zoospore suspension. Sporangia of some isolates were much more readily dislodged from their sporangiophores than those of other isolates. Isolates "Howbold" and 1558 had many

sporangia present in all zoospore suspensions prepared from them. Sporangia from colonies grown in liquid culture and rinsed in sterile water dehisced more readily than those from colonies grown on solid media. With increased irradiation dosage, the percentage of colonies originating from sporangia increased, since zoospores were killed preferentially. In the isolate used for mutant studies, sporangia were estimated to rarely exceed one percent, and colonies originating from sporangia were usually less than 0.1 percent when irradiation times of 30 seconds or less were used. With dosages of over 100 seconds the few colonies which developed usually originated from sporangia. Therefore, low dosages were used to: (1) maintain a low sporangium to viable zoospore ratio, and (2) to permit a sufficiently high dilution to minimize or eliminate the sporangia in aliquots taken from the dilutions.

A further difficulty in insuring that colonies were derived from single nuclei was the occasional presence of multiple zoospores. Multiple zoospores were examined with the phase contrast microscope and were found to be double, triple, or multiple in size and to carry four or more flagella. Multiple zoospores were readily determinable when motile at an enlargement of 100X with an ordinary microscope. However, it was difficult and sometimes impossible to determine whether a micro-colony originated from a single or multiple zoospore. The same isolate would produce

various frequencies of multiple zoospores under different conditions. They were at a minimum (sometimes absent) when zoospores were produced most easily and in greatest abundance. Many multiple zoospores occurred in older cultures, or in cultures in which it was difficult to induce zoospores to form. At least some arose through cleavage failure within the sporangium. Fusion of zoospores has been reported for two other species of Phytophthora (15,30) but was not observed in the isolates used here.

Dosage response to Ultraviolet

The effects of increasing dosages of ultraviolet on lethality and mutation rates were studied quantitatively. Lamp-to-specimen distance was 52 cm. and irradiation times of 0, 5, 15, 30, 45, 60, 75, 90, and 110 seconds were used. Four plates of zoospores were prepared for each dosage. Lethality counts were made directly on plated zoospore colonies. Mutation counts were made after transferring twice to WBGA. At the lower dosages 100 colonies were picked per dosage, but due to poor survival at the higher dosages of 60, 75, 90, and 110 seconds the number of colonies picked were 75, 42, 28, and 20, respectively.

The curves obtained (Figure 8) are similar to those published for Aspergillus terreus (33). Ultraviolet mutation curves characteristically rise to a maximum and then level off or even drop with higher dosages. In the present

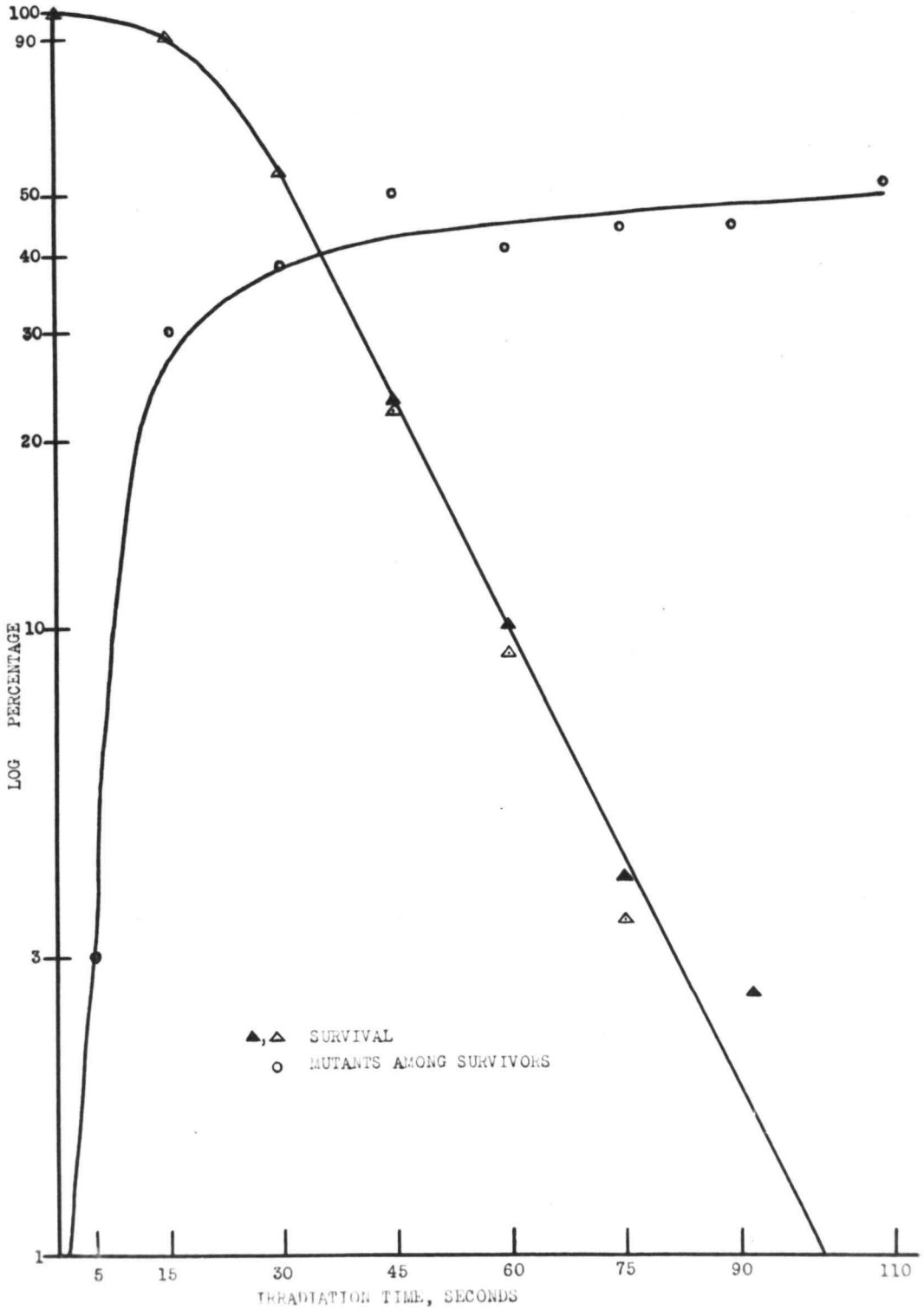


Fig. 8. Mutation and lethality response of P. cactorum to ultraviolet irradiation. Empty and solid triangles represent two different trials.

study a 30 percent mutation rate was obtained with only 15 seconds of irradiation. The maximum mutation rate of 50 percent is higher than most mutation rates which have been obtained with fungi.

No mutants were detected with zero irradiation time, and only 0.7 percent of the zoospores survived 110 seconds of irradiation. Motility of the zoospores immediately after irradiation, however, was not affected even at the 110-second dose. Longer irradiation times were applied and, even at five minutes of irradiation, no visible reduction in motility occurred. Apparently the systems responsible for motility were not affected by these dosages.

Response to X-rays

Only preliminary dosage response data were obtained with X-rays (Table 7).

TABLE 7. MUTATION RESPONSE OF P. CACTORUM TO X-RAYS

Irradiation time	Dosage in roentgens	Number picked	Number survived	Mutants	Percent mutation
0 min.	0	50	49	0	0
2	450	50	48	0	0
5	1125	50	48	0	0
10	2250	50	48	4	8.3
20	4500	50	48	14	29
30	6750	50	13	11	85

Lethality data were not obtained since dilutions were not sufficiently great to enable counts to be made. However, even at the highest dosage little kill was apparent, although colony development was delayed considerably. The small number of colonies which survived transferring in the highest dosage group may reflect the small size of picked colonies. In all experiments, even with non-irradiated spores, poor survival was obtained when zoospore colonies were picked when minute. The 85 percent mutation rate obtained at the highest dosage is not considered to be too reliable because the sample size was rather small. Also these mutants were not followed to check on stability. During the longer exposures the temperature of the suspensions raised several degrees in spite of a water bath.

X-ray irradiation is much less suitable than ultraviolet for the induction of mutations in zoospores because of the long irradiation time that is required.

MUTANTS OBTAINED BY ULTRAVIOLET IRRADIATION

Morphologic and microscopic characteristics

In a preliminary experiment 300 colonies that developed from irradiated zoospores were picked and graded for mutant characters. The colonies were graded when picked, and then regraded when developed on WBA. The 148 colonies which survived on WBA, including 48 graded as mutants, were tested for growth on minimal medium in agar plates. Grading results of a selected group of these colonies are presented in Table 8.

The colonies were graded into six classes based on gross morphology. Variation occurred within each of the classes and several of the colonies were later placed in a different class. After subsequent transfer to WBGA the mutant phenotypes remained separable, although their characteristics were somewhat altered. Nine colonies representing five of the colony types are shown in Figure 9. The intermediate class is not shown since this group was very similar to the wild type, and the colonies in this class were not definitely separable from the wild type. Of 17 colonies originally in this class, three were later transferred into other classes, and five others showed sufficient differences in microscopic characters to be retained as phenotypic mutants.

Four of the colonies sectored on WBA, but only in C87 was the sector sharply defined. Culture C146 grew only a

TABLE 8. GRADING OF A SELECTED GROUP OF COLONIES FOR MUTANT CHARACTERISTICS

Isolate no.	Growth type on WBA	Growth on MM	Sporulation after: 5 days S**	5 days O**	11 days* S	11 days* O	Remarks
C78c***	wild	xx	trace	x	x		
C81c***	"	xx	t	x	x		
C107	"	xx	x	-		t	
C172	"	xx	t	xx			
C176	"	xx	t	xx	xx		
C59	Intermediate	xx	t	x			
C252	"	xx	-	xx	x		some multiple antheridia
C163	"	xxx	x	xx			small oospores
C199	"	x	-	-	t		sporangia very small
C223	"	xx	x	-			
C220	Dwarf	xx	x	x			small spores, sectored
C209	"	x	-	-	t		abnormal sporangia
C143	"	x	-	-	t	t	snowflake morph. abnormal sporangia
C150	"	t	-	-			sectored
C2A	Twisted	xx	x	x	xx		sectored
C224	"	xx	x	-			
C87	"	x	x	-		t	sectored
C22	"	xx	xxx	t			parasitica type morphology
C225	"	xx	xx	t			sporangia large and round
C255	"	xx	x	x			
C6	"	xx	-	x		xx	many oogonia without antheridia
C145	Spreader	x	x	x			
C15	"	xx	x	-		t	hyphae dead in older parts of colony
C60B	"	xx	t	x			double antheridia common
C211	Circular Even	x	-	x	t		morphology close to *P. syringae*
C258	"	xx	-	-	x		sporangia resemble chlamydospores
C151	"	x	-	-			vigorous
C146	"	-	-	t		xx	oospores small
C190	"	xx	x	-		t	

* only changes are given
** S represents sporangia, O represents oospores
*** non-irradiated control

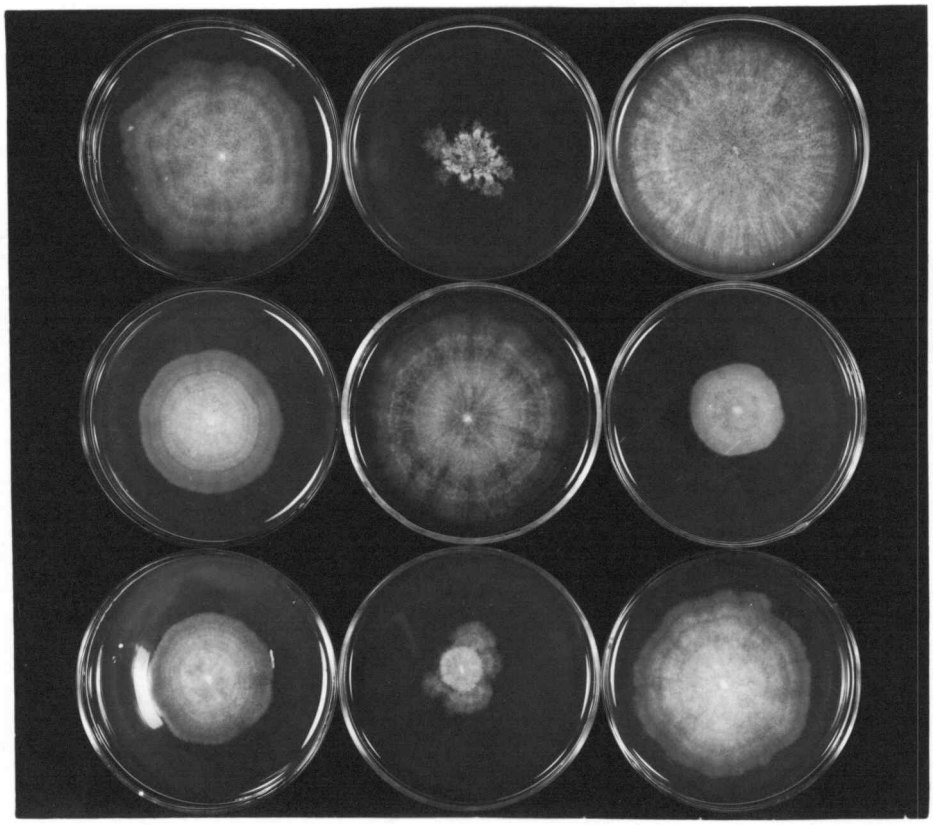


Fig. 9. Mutants of P. cactorum on WBGA after 14 days at room temperature; (upper, l. to r.) C87 smooth, C87 dwarf, C15; (middle) C190, C78 wild, C209; (lower) C146, C143, C211.

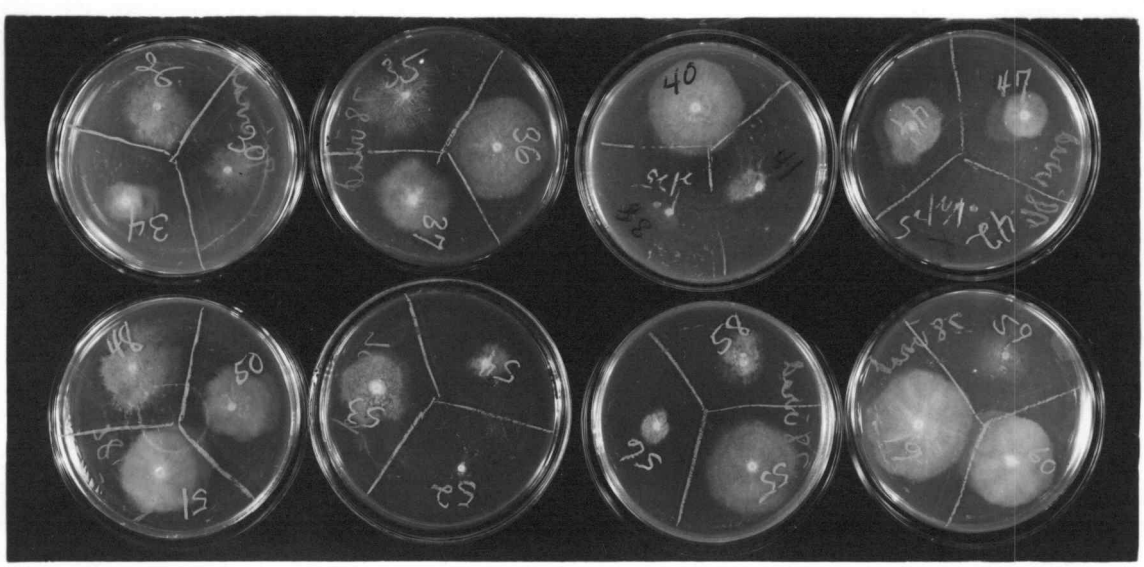
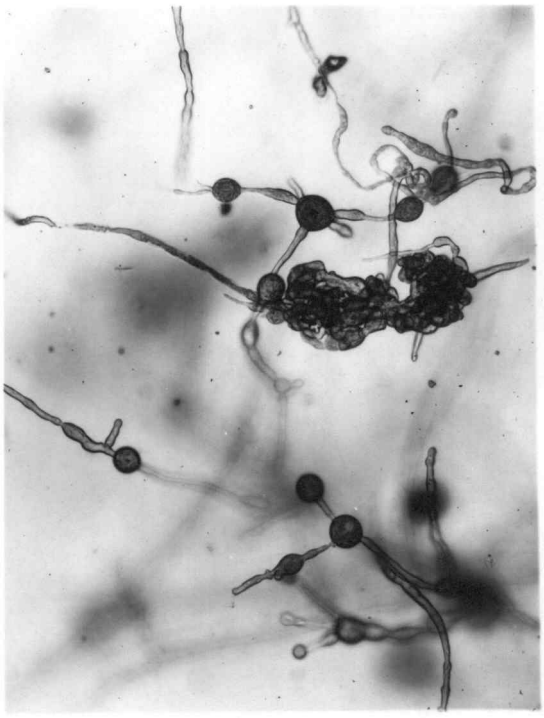


Fig. 10. Colonies of P. cactorum at the stage of rating for mutants; after 5 days on WBGA at 20° C.

few microns when first placed on minimal medium. Similarly, no growth was obtained on MM supplemented with either vitamins or amino acids. However, this isolate grew well on MM when retested after being on PDA for one month. Colonies at the grading stage on WBGA are illustrated in Figure 10. Various types of dwarf, sparse, and twisted mutant colonies are apparent.

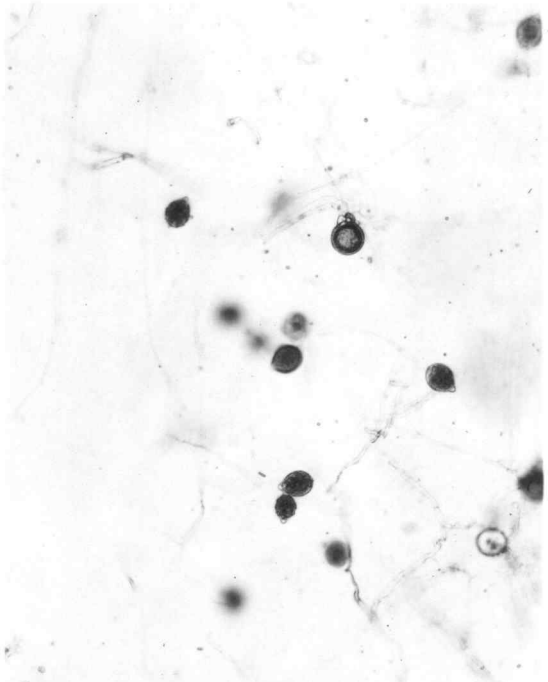
There was a profound effect upon sporulation in many of the mutants. In some cases the quantities of oospores and sporangia were increased, while in others one or both were delayed, reduced in number, or were absent. Differences in sporangial shapes and sizes were especially common. In some mutants these changes were to very small but normally shaped sporangia, to long, non-papillate sporangia, or to subspherical sporangia. Sporangia of one mutant resembled those of P. hibernalis (Figure 11B). In other mutants the sporangia graded into chlamydospores.

"Chlamydospores" and hyphae of one mutant resembled those of P. syringae (Figure 11A). In one mutant the sporangia were spherical and could not be distinguished from chlamydospores except for an occasional papilla, which was either lateral or terminal. Lateral papillae were common in other mutants also. Two subterminal papillae were present on a large proportion of the sporangia of one mutant. In one mutant the sporangiophores were very short, resulting in tight bunches of sporangia (Figure 11D).

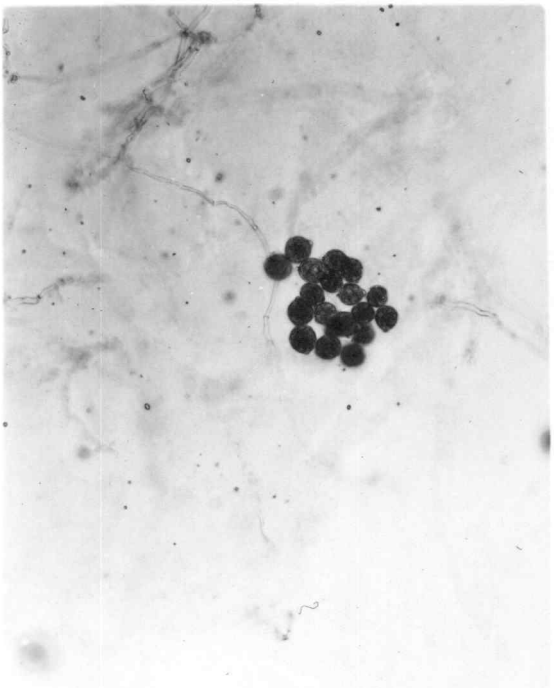


(A) "Chlamydospores" and hyphae of mutant 139. 140X.

(B) Long, non-papillate sporangia of mutant C87S1555. 140X.

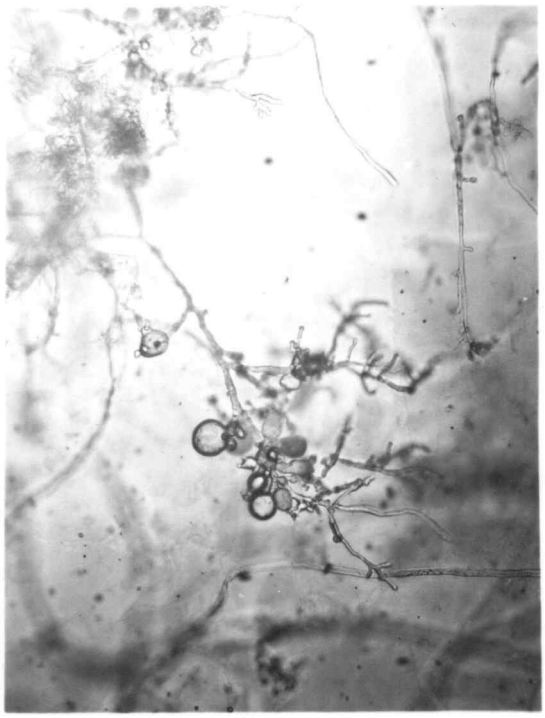


(C) Oospore and sporangia of the wild type. 140X.

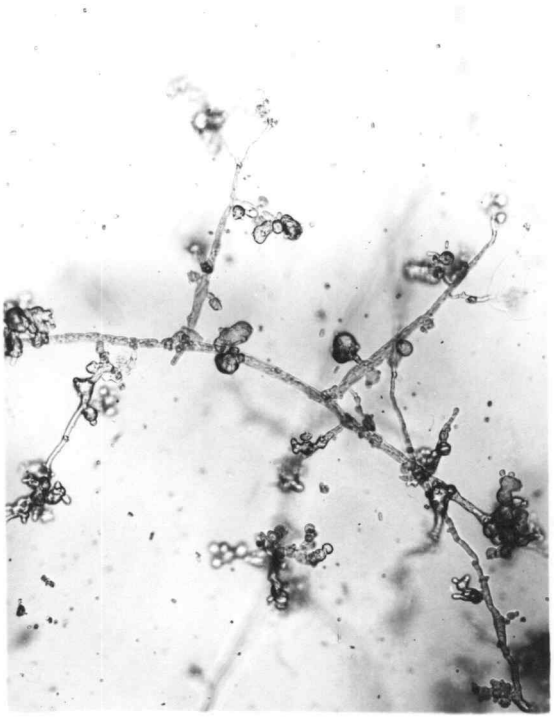


(D) Bunched sporangia of mutant 163. 140X.

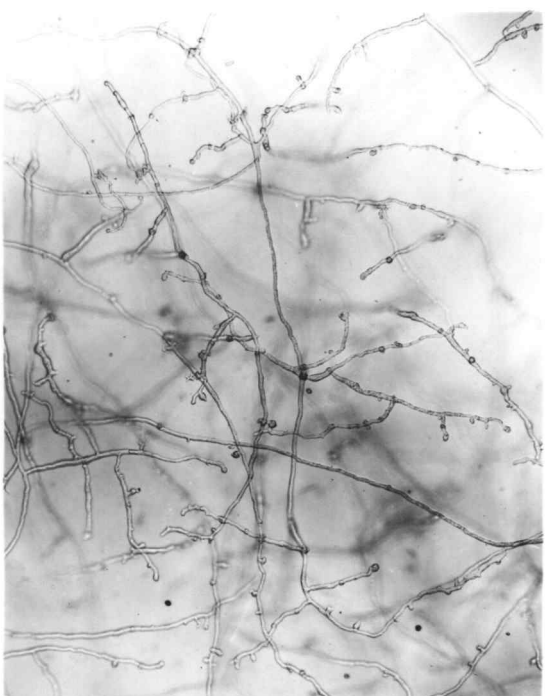
Fig. 11. Microscopic characters of three mutants and the wild type of P. cactorum; from WBGA cultures 16 days old.



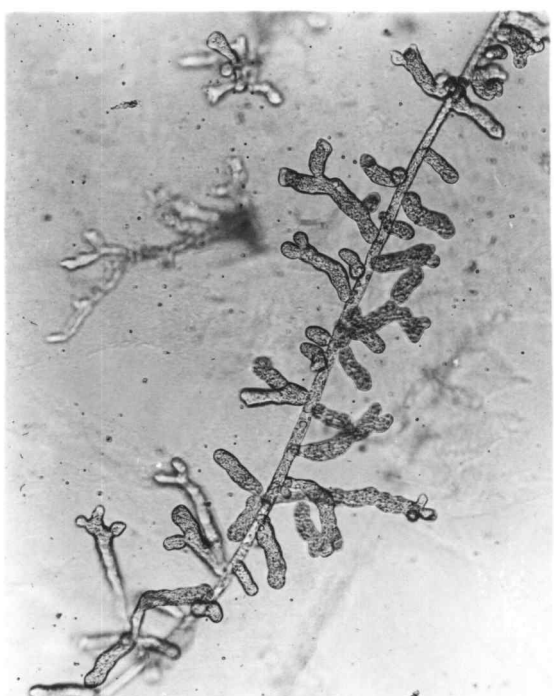
(A) Spherical swellings of mutant 93. 140X.



(B) Hyphal knobs of mutant 162. 140X.



(C) Wild type hyphae. 140X.



(D) Clubbed hyphae of mutant $C15_{12}$. 140X.

Fig. 12. Hyphal characters of three mutants and the wild type of P. cactorum; from WBGA cultures 8 days old.

Differences in oospores and in oogonia and antheridia occurred in a smaller number of mutants. A high proportion of oogonia had multiple antheridia in two mutants. The oospores were small (ave. 24 u) in two mutants when compared with those of the wild type (ave. 30 u). The oogonia of one mutant were very large (ave. 52.5 u) when compared with those of the wild type (ave. 34 u). However the oospores were the same size; those of the mutant were aplerotic.

Differences in hyphal characteristics were also very striking. The wild type has relatively smooth hyphae, but the mutants developed "clubs" or "knobs" characteristic for each mutant (Figure 12B, C, D). The swellings in mutant C93 resembled those of P. cinnamomi (Figure 12A). The clubbed hyphae of $C15_{12}$ resulted in colonies which were macroscopically graded as "ropy".

Screening for biochemical blocks and quantitative growth of selected mutants

Although many mutants grew very sparsely on agar plates of minimal medium, none of these approximated the wild type on WBGA. Three hundred colonies were screened for biochemical blocks on MM in agar plates and no replaceable blocks were found in this group. A few colonies were found which appeared to grow more than the wild type on MM plates. Differential growth of six mutants and the wild type in the three screening media are illustrated in Figure 13.

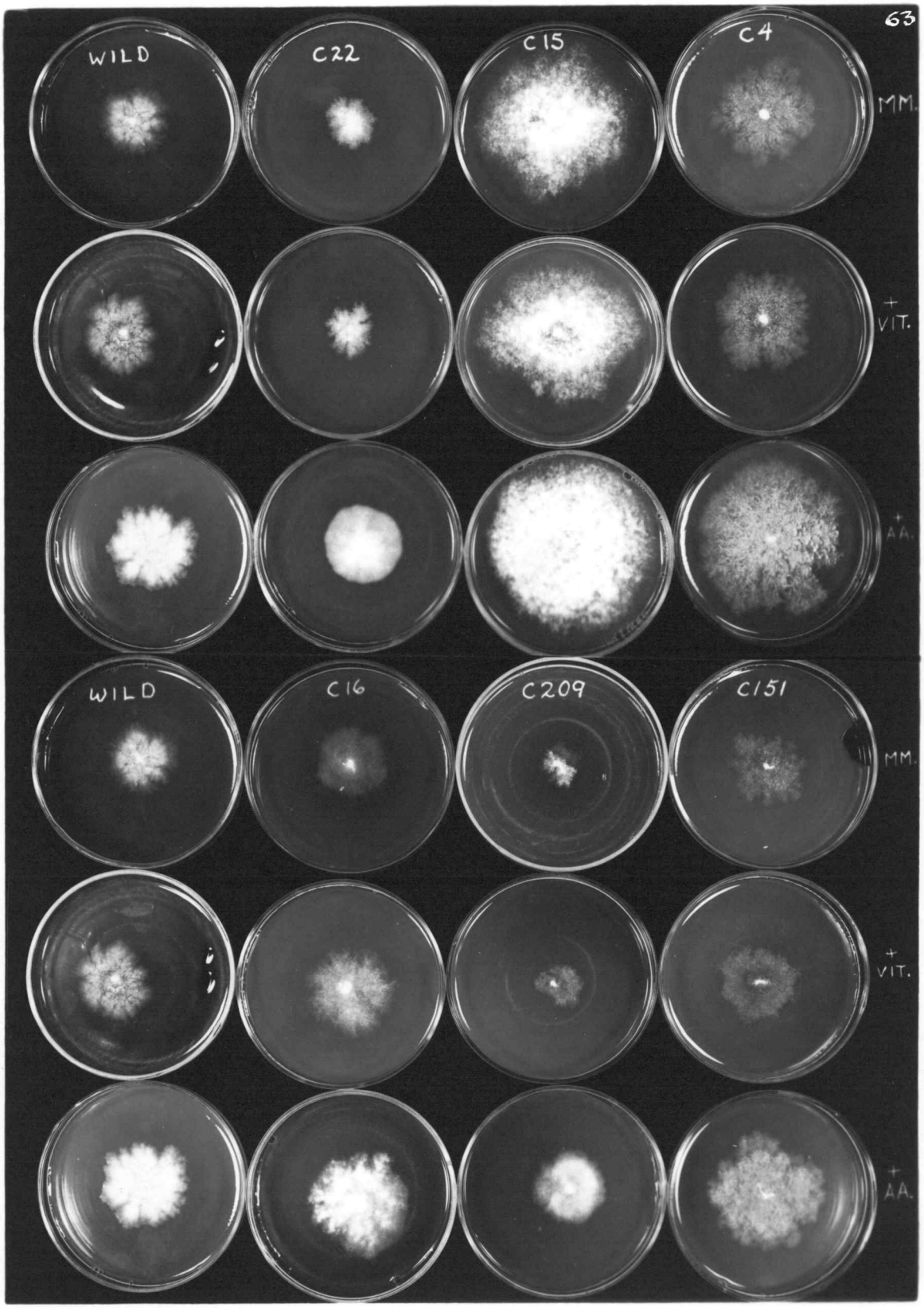


Fig. 13. Mutants and the wild type of P. cactorum on minimal medium, MM plus vitamins and MM plus amino acids; after 12 days at room temperature.

An experiment was designed to test quantitatively the effect of various supplemented media on growth of selected deficient mutants and on a few "vigorous" mutants. Quantitative growth differences among the isolates would reflect differing synthetic abilities and thus the degree of interference with wild type metabolism by the mutated "gene".

Procedure: Five media were prepared and 19 ml. aliquots were pipetted with an automatic pipette into 125 ml. Erlenmeyer flasks. Fifty flasks of each medium were prepared. All glassware was thoroughly cleaned and rinsed three times in distilled water. Media were minimal medium D (MM), MM plus vitamins, MM plus amino acids, and WBG solution. The WBG in this case was prepared with 30 g. glucose per liter of medium. The pH of each medium was 6.4. Fourteen cultures were used, with four replications in each medium. Inocula were equalized by growing each culture on MM in agar, transferring again to MM on agar and then transferring a very small amount of agar and hyphae to the flasks. The last transfer was done with the aid of a microscope so that amounts of hyphae transferred could be kept approximately equal. Flasks were kept at room temperature, randomized, and covered with brown paper to reduce light intensity. Colonies were removed from the flasks after 11 days, rinsed in water, and then hung on horizontal pins to dry. Pins were coated with silicone grease to reduce the sticking of the colonies to the pins. By employing the pin drying

board, it was unnecessary to weigh individual papers. Colonies were dried for 3.5 hours at 50° C., removed from the pins and weighed (Figure 14). Colonies were weighed at this time because it was considered that colonies in WBG and MM plus amino acid media were reaching a maximum size.

The mutants varied greatly in their ability to grow on the five different media. Growth on MM and MM plus vitamins was low for all isolates, including the wild type. This was surprising since good growth occurred on these media when solidified with agar. Only two mutants (154 and 37) approximated wild type growth on MM plus amino acids and on WBG. Mutant 154 produced three times as much growth on MM and MM plus vitamins as did the wild type, paralleling its behavior on agar plates of these media. Four mutants produced almost no growth on any medium. Two of these (162 and 203) produced growth only in WBG solution. Mutant 138 produced only a trace of growth in each medium and showed no response to any supplement. Five mutants showed a definite growth increase in WBG solution. Of this group, mutant 5 was especially interesting in that growth response to WBG solution was very marked, being much greater than response to amino acid-supplemented media.

Relative growth diameters reflected growth amounts as measured by dry weights, but did not accurately follow dry weight increases on supplemented media since colony densities were not graded. Thiamine and glucose levels were

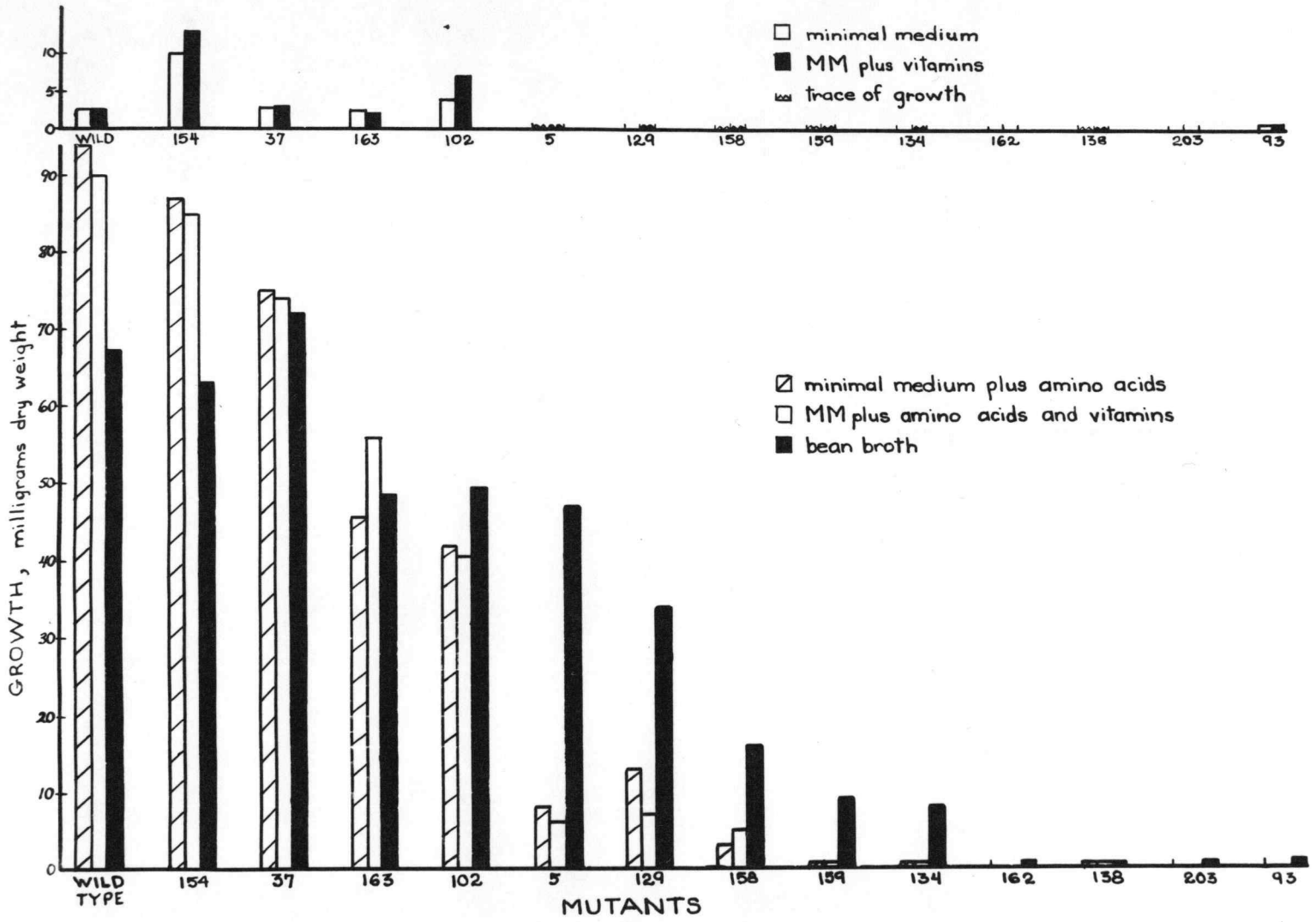


Figure 14. Quantitative growth of thirteen mutants and the wild type of P. cactorum in five media.

kept constant in the first four media; nitrogen levels were constant within each pair of the first four media but were much higher in the second pair. This means that growth increases in amino acid-supplemented media may in part be attributable to the increased N level. However, among the different mutants, the relative stimulation from the amino acid-supplemented media may be compared. Other workers (46) have found that growth of P. cactorum is higher when N is supplied in an organic form.

In evaluating this experiment several points must be kept in mind: (1) growth lag effects, (2) inocula equalization, and (3) physiological state of the hyphae. Growth-lag from hyphal transfers of Phytophthora is a serious problem which can give misleading results. For instance, good growth of mutant 159 occurred in the MM plus amino acid medium after four weeks, although none was evident after 11 days. Different media may exert their primary influence in increasing or decreasing growth-lag time.

Equalization of inocula by using small amounts may increase growth differences due to growth-lag.

The physiological state of transferred hyphae will vary with the medium, and cannot be entirely equalized for different mutants by growing on minimal medium. Growth on a complete medium before transfer would allow vitamins and amino acids to be transferred with the inoculum. The procedure used was considered to be the best one possible, but the above limitations were not entirely overcome.

VARIABILITY OF SELECTED MUTANTS

To determine whether selected mutants would produce identical colonies when single zoospores were derived from them was considered to be important. Information obtained would illustrate the stability of the mutants, and might indicate heterokaryosis or segregation which could then be investigated. Eight mutants were selected for this investigation (Table 9). Since their induction, these mutants had been transferred through four successive mycelial transfers and, with the exception of C87, had apparently remained constant.

TABLE 9. CONSTANCY OF SELECTED MUTANTS AS EXHIBITED BY THEIR ZOOSPORE COLONIES

Culture	Initial rating of plated colonies on DPDA	Rating of colonies transferred to WBGA			
		No. picked	Large 1.5-3cm	Small 2-5mm	No growth
C78 wild type	variable	30	24*	0	6
C2A	all very small	30	0	0	30
C87S(smooth)	10% large, 80% dwarf	30	12*	6	9
C87(sparse)	none	0	0	0	0
C255	all medium	30	16*	12**	2
C190	3 large, many small	30	3	18*	9
C22	all very small	30	6	18*	6
C15	all medium	30	27*	0	3
C209	variable	30	21*	0	9

* exhibited phenotype of source culture
** later grew into large colonies

None of the transfers of C2A survived and the original plated colonies did not grow further and appeared dead, as did many very small colonies of C87S and C190. Only cultures C78, C15, and C209 produced colonies of only one type, identical to the parent colonies. The other four mutants produced a larger number of colonies resembling the respective parent colony and a smaller number of colonies of a different phenotype. The different phenotype was a dwarf type for two mutants, and a large type for the other two. Dwarfs developed from the zoospores of C87S (Figure 16). These dwarfs had not changed when a second rating was made after four days. The three and six large colonies of C190 and C22 may have originated from sporangia rather than from zoospores.

In no case was a wild-type colony recovered from any mutant. Each of the mutants produced zoospore colonies which could be recognized as characteristic even when microscopic; even germinating zoospores of C15 were very characteristic, producing very long germ tubes with many short branches of approximately equal length. Zoospore colonies of the wild type were visible before those of any of the mutants, but after four days the colonies from C15 were consistently larger.

New zoospore colonies of mutant C255 were obtained and twelve were selected for further study. Seven labeled "normal" and five labeled "small" were transferred in

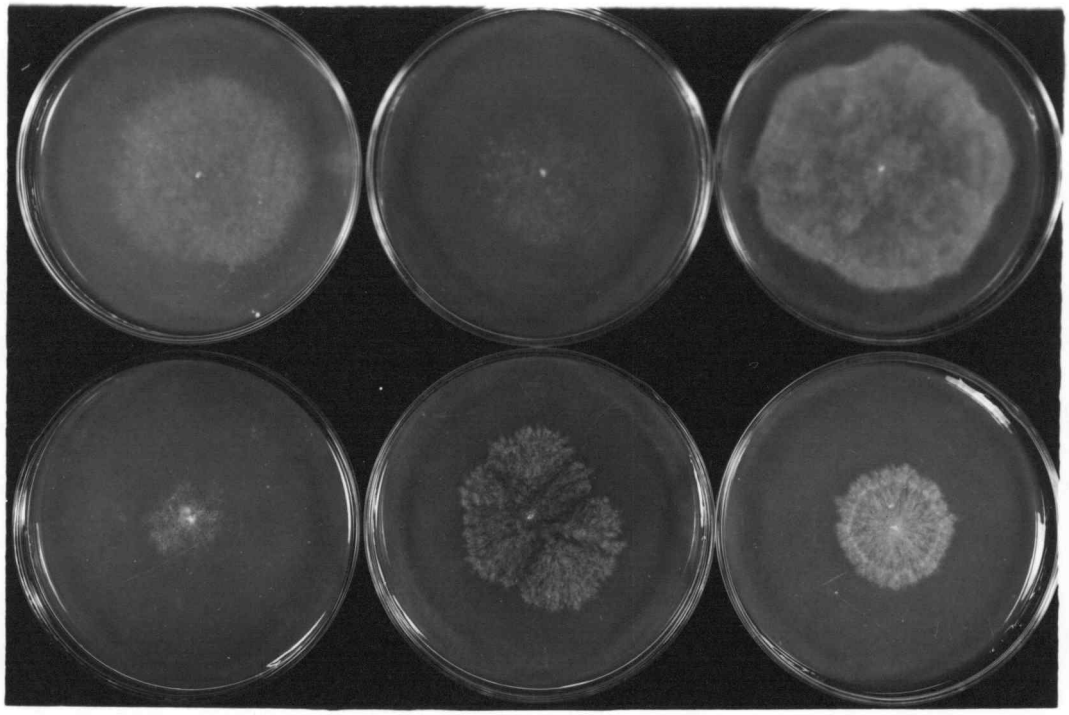


Fig. 15. Colonies derived from zoospores of C255 (upper) and C22 (lower); after 9 days on WBA at room temperature.

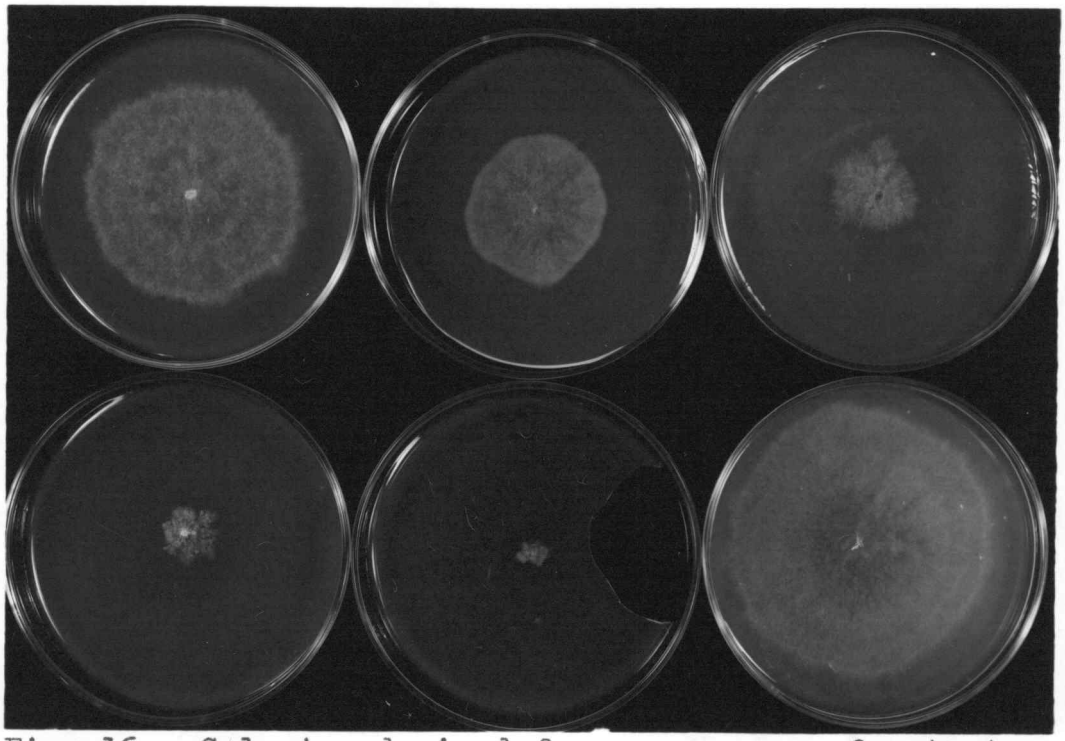


Fig. 16. Colonies derived from zoospores of mutant C87S, after 9 days on WBA at room temperature. (Upper, l. to r.) Class II, I, III; (lower) Class IV, V, wild type.

duplicate to WBA. After five days the colonies originally labeled "small" showed varying amounts of starvation-type growth. The colonies originally labeled "large" showed varying amounts of dense growth. After nine days the 12 colonies could be graded into four different groups, although they were not identical within each group. Mycelial transfers from each of these four were made and the resulting colonies retained their "parent" phenotypes. The replications of each colony were indistinguishable. Three of these colony types are shown in Figure 15. The variability exhibited by these zoospore colonies was not explained, and work was discontinued with C255 since none of the isolates derived from it produced sporangia readily, and it was decided that mycelial transfers could not give meaningful results.

Five subtransfers were made from new zoospore colonies of C22. Two of these became small regular colonies, one became a small irregular colony, one became a large irregular colony and one became a dwarf colony. Three of these colonies are shown in Figure 15. Work was likewise discontinued with C22.

Mutant C87 was chosen as a more favorable mutant for study since it had originally sectored into characteristic areas. In preliminary trials, zoospores, obtainable only from the smooth sector, gave two characteristic colony types, a colonial dwarf, and a smooth, fairly normal type

(Figure 9).

There was a possibility that mutant C87 was heterokaryotic. This could be tested, and if present, would be a useful tool in the study of nuclear movement in hyphae, identity of nuclei in a sporangium, etc. Mutant C15 was also chosen, since in preliminary trials zoospores derived from it produced identical colonies indistinguishable from the parent mutant. Mutant C15 was to represent a "control" for the behavior expected from C87.

Analysis of mutant C87S series

Mutant C87 was obtained from a group of zoospores which received ten seconds of ultraviolet irradiation at a distance of 59 cm. It was selectively picked as a mutant and was graded as a mutant in the liquid medium to which it was originally transferred. It produced about one-half as much growth on MM plates as did the wild type. On WBA it was classed as "twisted". It first sectored on WBA where one-half of the colony developed as a smooth, fairly normal type (C87S), and the other half developed as a sparse, gnarled type. On the sparse or gnarled portion the few sporangia produced were long and less papillate and the few oogonia produced were often without antheridia.

Mycelial transfers of C87-sparse produced colonies which varied from dwarf-gnarled to vigorous-smooth. Additional sectoring occurred, especially in the dwarf colonies.

Mycelial transfers of C87-smooth produced colonies which were more homogeneous but which were not identical. Since mycelial transfers did not behave in any predictable manner, further work was done with single zoospores. Zoospores were obtained from C87S and 30 zoospore colonies were transferred to DPDA and graded as either large or dwarf. Mycelial transfers in duplicate were then made to WBA. The resultant colonies could be graded into five classes (Table 10, Figure 16). The classes were described as follows:

Class I: Circular, even, medium. All those listed for this class appeared to be identical, macro- and microscopically.

Class II: Large, smooth, sometimes wiry. Not all identical.

Class III: Medium, smooth, sometimes wiry or erratic. Not all identical.

Class IV: Moderate dwarf, often ropy. Not all identical.

Class V: Dwarf. Not all identical.

Duplicate colonies of each culture appeared to be identical except for the colonies in which sectoring occurred. There the sectoring occurred at different times and the colonies subsequently developed differently.

All nine colonies in Class I appeared to be identical. In the other classes, variation occurred among some of the colonies. All colonies were transferred in triplicate to WBGA where all of them retained their phenotype except four colonies which had earlier exhibited sectoring, and

TABLE 10. COLONY TYPES ORIGINATING FROM SINGLE ZOOSPORES OF MUTANT C87, SMOOTH SECTOR

Culture number	Initial grading on DPDA	No. of colonies	Colonies on WBA in phenotypic classes I	II	III	IV	V
	Large	13	8	3	2	0	0
	Dwarf	16	1	4	8	4	3
	C78 wild type	1		1			
	Sectoring isolates		Class of sectors I	II	III	IV	V
87S3	Dwarf			x	x		
87S18	"			x		x	
87S22	"					x	x
87S23	"			x		x	
Colonies and phenotypic classes selected for further study							
87S15	Dwarf				x		
87S18	"					x	
87S23	"			x			
87S12	Large		x				

three dwarf colonies that sectored to a faster growing type for the first time.

This experiment showed that zoospores derived from

mutant C87S produced colonies which varied from colonial dwarf to large smooth types. The dwarf types frequently sectored to faster growing types and mycelial transfers from the dwarfs did not always retain the dwarf phenotype. Mycelial transfers from the other types retained their phenotypes. No significant variation between replications of the stable colonies was encountered. None of the colonies was identical to the wild type control, although some colonies in Class II resembled the wild type.

Four colonies were selected for further sampling of zoospores. Culture C87S15 was selected as a dwarf which developed into a colony of Class III. Culture C87S12 was selected as a large colony of Class I which showed no sectoring. Cultures C87S18 and C87S23 were selected as representatives of sectoring colonies of Classes IV and II.

Mutant C87S18

Results of colony types originating from zoospores of C87S18 are shown in Table 11.

The differences between first and second ratings are considered to reflect only developmental time lag. Data on the first rating are included to illustrate the misleading results obtainable if colonies were rated only once, and too early after transfer. Where differing types were apparent at the time of the second rating, these types were transferred to new plates for several successive transfers.

TABLE 11. MUTANT C87S18: COLONY TYPES ORIGINATING FROM ZOOSPORES

Number picked	Rating	Number surviving	Phenotype: Large, smooth, C87S18 type	Sparse	Colonial dwarf	Trace	Aerial
100 random on PDA	1st	77	67	10	0	0	0
	2nd	77	76	0	0	0	1
25 selected on DPDA	1st	23	5	15	0	3	0
	2nd	24	24	0	0	0	0
8 selected on WBGA	1st	6	2	0	2	2	0
	2nd	7	2	0	5	0	0
Totals*		108	102	0	5	0	1

* from second rating only

The "aerial" colony ($C87S18_{59}$) differed slightly but constantly from the large smooth type (Figure 20, middle and lower right). Three of the five colonial dwarfs grew into medium sized dense colonies and sectored, the other two remained dwarf (Figure 17, bottom center). The large class containing 102 identical colonies was considered to be the typical C87S18 type, and was very similar to the wild type.

Mutant C87S15

Colony types originating from zoospores of C87S15 are presented in Table 12.

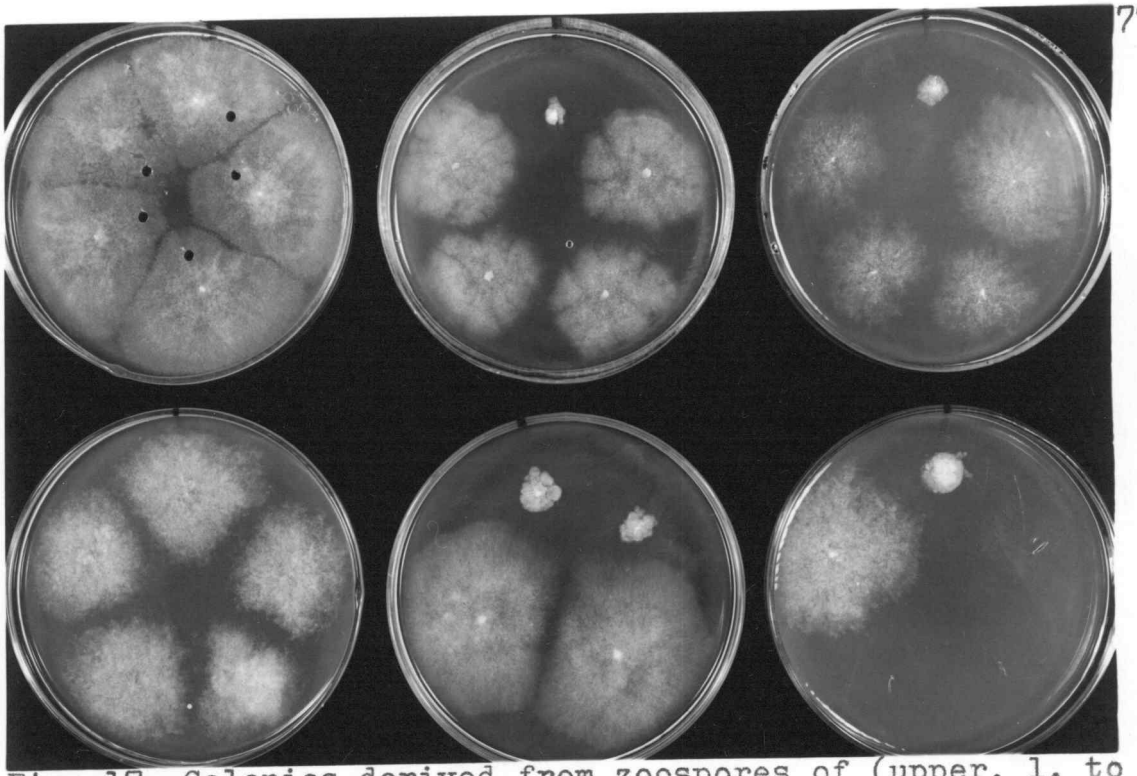


Fig. 17. Colonies derived from zoospores of (upper, l. to r.) wild type, C87S12, C87S15; (lower) C87S23, C87S18, C87S23; after 6 days on WBGA at room temperature.

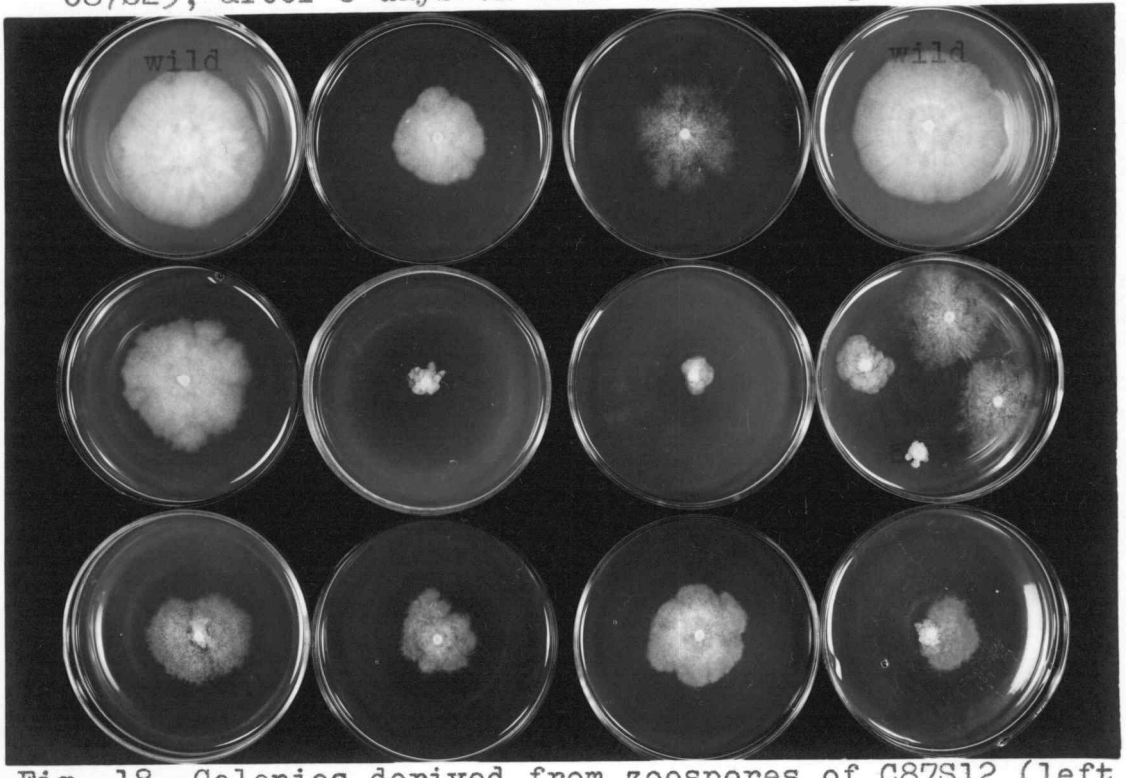


Fig. 18. Colonies derived from zoospores of C87S12 (left half) and C87S15 (right half); after 7 days on WBGA at room temperature. Typical C87S12 and typical C87S15 are at upper middle.

TABLE 12. MUTANT C87S15: COLONY TYPES ORIGINATING FROM ZOOSPORES

Number picked	Number surviving	Large C87S15 type	Large, dense, even	Colonial dwarf	Small, dense	Medium, dense
100	82	53	8	7	9	5

Only a final rating is presented. Again a large group of large identical colonies was obtained, with smaller numbers of dwarfs (Figures 17, top right, and 18) which became variable upon subsequent mycelial transferring. The large group of 53 was considered to be the typical C87S15 type. Only eight of these developed into a different type; all the others remained constant. However 16 colonies of 21 originally labeled "dwarf dense" produced sectors or developed into other types. Type classification for these sectoring colonies was meaningless since gradations occurred among all of the various types.

Microscopic differences were also noted among many colonies derived from C87S15. Sporulation ranged from none to abundant for either sporangia or oospores or for both. Knobs and swellings on hyphae ranged from none to abundant. Hyphal branching differences were also apparent and they reflected the macroscopic characters.

Mutant C87S12

Colony types originating from zoospores of C87S12 are presented in Table 13. Only a final rating is given.

TABLE 13. MUTANT C87S12: COLONY TYPES ORIGINATING FROM ZOOSPORES

Picked/ surviving	Large C87S12 type	Colonial dwarf	Medium dense	Small sparse	Medium twisted	Medium appressed
Culture C87S12:						
70/42	26	5	6	2	2	1
Culture C87S1218:						
50/42	36	6	0	0	0	0

All colonies of C87S12 were checked for single zoospore origin since zoospores were difficult to obtain from this culture and many were multiple. Again there resulted a large group of stable large identical colonies which were considered to be typical C87S12, and a small number of variable dwarfs (Figures 17, top center, and 18). One of the colonial dwarfs ($C87S12_{18}$) was selected for further zoospore work. Fifty zoospore colonies were obtained and again most colonies were of the large typical C87S12 type, with a fewer number of colonial dwarfs (Table 13). Two mycelial subtransfers were made of a colonial dwarf and a large C87S12 type and their phenotypes remained constant.

Mutant C87S23

One hundred and nineteen zoospore colonies of mutant C87S23 were transferred to WBGA. Of these 77 survived and

these ranged from colonial dwarfs to very large colonies (Figures 17 and 19). In contrast to the other mutants, no large group of constant colonies was obtained, although ten appeared to be identical and were considered to be typical C87S23. Grouping into classes was not possible since gradation occurred from colonial dwarf to large smooth colonies. Colonies were transferred individually to WBGA plates and the colonies still showed a complete range of variability.

Discussion of C87S data

Data derived from these experiments were not easily interpretable. A colonial dwarf was obtained from all of the mutants derived originally from C87S. The constant large type obtained in each case was recognizable as distinct for that mutant and none were identical to the wild type, although the large colonies of C87S18 were very similar (Figures 18 and 19). The dwarf colonies were often unstable and usually either grew out into other types, sectored into other types, or developed another phenotype upon transfer to new plates.

Analysis of mutant C15

Mutant C15 was selected as a comparison for the mutants of the C87S series, since in preliminary trials it showed complete stability, and it possessed a readily recognizable phenotype. Mutant C15 was characterized by a radiate growth habit, and a linear growth rate more rapid than the wild

type (Figure 20). In all, 116 zoospore colonies of mutant C15 were picked and graded (Table 14).

TABLE 14. MUTANT C15: COLONY TYPES ORIGINATING FROM ZOOSPORES

Number picked / number surviving	C15 type	Aerial	Wild type	Fluffy	Sparse	Ropy
100 random / 53	52	1	0	0	0	0
14 selected as different / 6	0	1	2	1	1	1
2 selected as C15 type / 2	2	0	0	0	0	0

Of the first 100 colonies chosen at random, 53 survived and all of these were identical except one ($C15_{52}$)(Figure 20). All 53 colonies were transferred (3 mm. disk inocula) singly to WBGA plates. The one colony which appeared different was transferred in quadruplicate. After four days, diameter readings were taken and colonies were graded. Again all 53 colonies had identical phenotypes except for $C15_{52}$. All four replicates of $C15_{52}$ were identical. After four days, colony diameters of the C15 type ranged from 2.6 to 2.9 cm. Diameters of $C15_{52}$ were 2.1 to 2.2 cm. Many sporangia of $C15_{52}$ had two papillae and a few even had three. Colony $C15_{52}$ was one of three that was labelled as small when picked and that survived. Thirty-five other

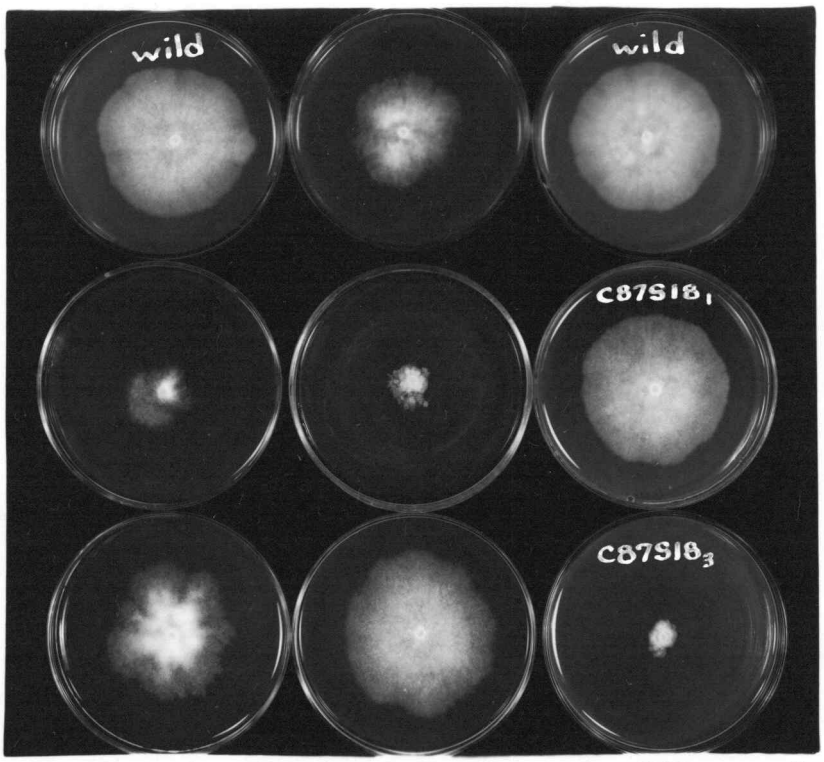


Fig. 19. Unlabeled colonies are from zoospores of C87S23; typical C87S23 at center in upper row.

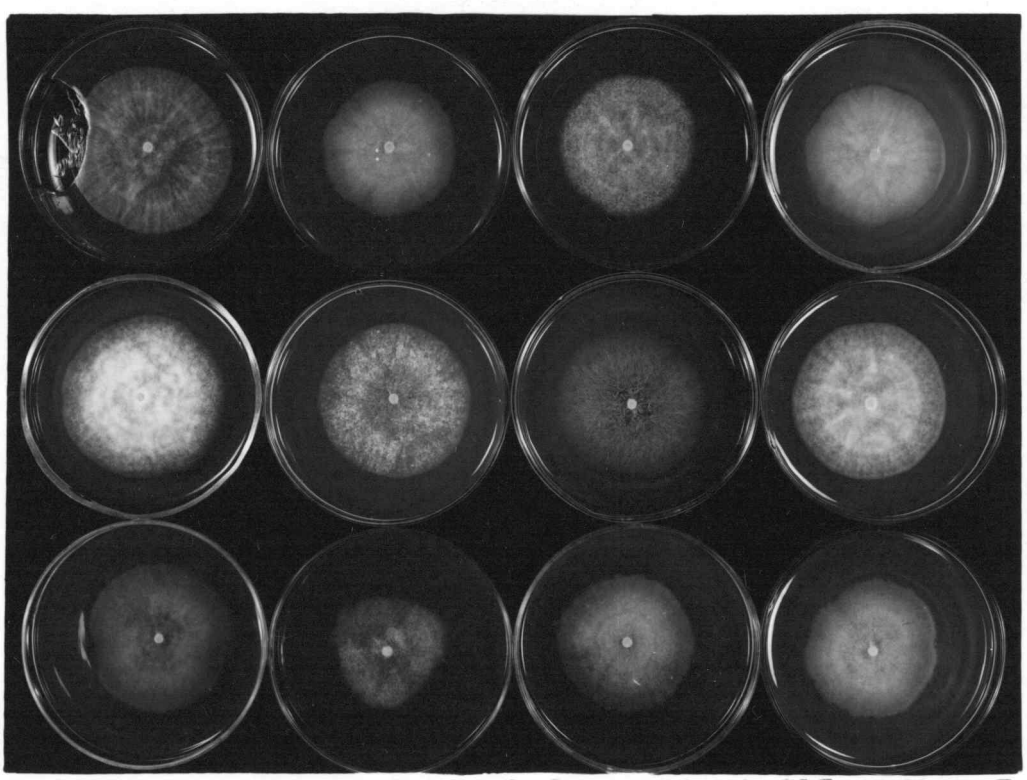


Fig. 20. Colony types derived from mutant C15, after 7 days on WBGA at room temperature; (upper, l. to r.) $C15_{20}$ (typical C15), wild type, $C15_{7}$, $C15_{103}$; (middle) $C15_{1}$, $C15_{52}$, $C15_{12}$, $C87S18_{59}$; (lower) $C15_{6}$, $C15_{6}$, $C15_{101}$, $C87S18_{9}$.

colonies labelled as small when picked did not survive. It was suspected that these small colonies might consist largely of different mutant types so the original DPDA plates were reexamined and 14 small or different colonies were picked, along with two normal-appearing colonies as a control. The two normal colonies that were picked developed into typical C15 colonies. Of the 14 small colonies picked only six survived, and none of these was of the C15 phenotype (Figure 20). Two ($C15_{101,103}$) were not separable from wild type. One ($C15_{12}$) produced "clubbed" strands of hyphae (Figure 12). One ($C15_{7}$) appeared very similar to $C15_{52}$ and one ($C15_{1}$) was even more fluffy. One colony ($C15_{6}$) was fairly sparse and later sectored into a faster growing type. These colonies retained their characteristic phenotypes after they were transferred to PDA tubes and re-transferred in replication to plates of WBGA.

Results with mutant C15 contrasted markedly with those obtained from the mutant C87S series. There were no dwarfs of any kind produced. The C15 phenotype was obtained from all but one of the surviving colonies from the first 100 zoospores picked. All but one of the seven different colonies obtained were stable upon subsequent mycelial transfer; and two colonies could not be distinguished from wild type.

NUCLEI OF ZOOSPORES

The unstable condition of mutants of the C87S series suggested that the uninucleate condition of the zoospores might stand in question. Nuclei of the wild type and of mutant $C87S15_{101}$ were stained with crystal violet and were found to be uninucleate (Figure 21).

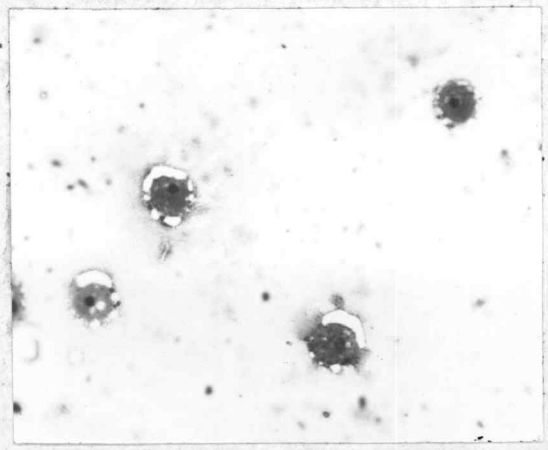


Figure 21. Uninucleate zoospores of a dwarf mutant ($C87S15_{101}$) of P. cactorum. 550X.

Several hundred zoospores were examined and in nearly all a round dark staining body approximately 1.7 microns in diameter could be seen. Nuclei in young hyphae were also found.

The procedure used was to invert an albumin-coated slide bearing a droplet of zoospore suspension over a bottle containing concentrated HNO_3 and scraps of copper, for 10 to 20 seconds. The slide was then air-dried and stained in one percent aqueous crystal violet for one to two minutes.

The slide was then rinsed in water and examined. The nuclei were stained a dark purple and the cytoplasm was stained pink. Details of nuclear structure were not differentiated by this staining procedure.

By following Smith's (36, p.90) modification of the gram stain, of which the above procedure is a part, loss of the nuclear stain occurred when the slide was immersed in alcohol (80 or 100 percent) either with or without an iodine-potassium iodide rinse. Examination of motile or killed zoospores with a phase contrast microscope did not reveal nuclei.

PATHOGENICITY OF ISOLATES AND SELECTED MUTANTS TO PEACH

Fifty-one mutants and eleven P. cactorum isolates were tested for pathogenicity to young peach trees. One-year-old peach trees were planted in a warm greenhouse in February. Preparatory to inoculation, each culture was transferred to a plate of PDA. Three stem inoculations were made on each tree after the trees had leafed out. Vertical slits one-half-inch long were made with a razor blade, and a three-millimeter disk of agar containing mycelium was inserted into the slit. Wounds were wrapped with cheesecloth and wet with sterile water. Six inoculations per isolate were made, two in each position on the tree. Inoculations were randomized throughout the block of trees. Canker sizes were measured after ten days. These data were analyzed by the analysis of variance and significant differences in canker sizes were found. Canker means were ranked by Duncan's multiple range test (Table 15).

The most striking result was that the pathogenicity of the different mutants ranged in a gradual series from no virulence to virulence equaling that of the wild type. Virulence, as measured by canker size, was in general correlated with colony size on WBGA. However there were some notable exceptions. One mutant (180) was only slightly pathogenic but produced large, vigorous colonies on WBGA. Five other mutants produced cankers less than one-half the

TABLE 15. RANGE OF PATHOGENICITY OF P. CACTORUM MUTANTS AND ISOLATES TO YOUNG PEACH TREES

Culture	Phenotype on WBGA	Mean canker length, mm.	Significance among means**
uninoculated control		0	
171	starvation	0	
203	"	0	
SKOT	small	0	
162	small or dwarf	0.8	
134	"	2.3	
159	"	2.5	
52	"	3.8	
93	"	7.6	
80	"	8.5	
124	"	8.5	
130	"	9.8	
144	"	10	
180	large	10.1	
C150*	medium	10.1	
42	medium, sparse	10.3	
3	medium	11.3	
C2A	--	12.1	
105	small	12.3	
112	dwarf	12.5	
139	dwarf	13	
82	trace	13.3	
128	medium	14	
81	large, fast, smooth	15	
76*	medium, twisted	15.1	
129	"	15.5	
158	very dwarf	15.5	
35	large, twisted	17.3	
182	medium, twisted	17.3	
48	large, twisted	17.6	
114	dwarf, dense	19	
163	large	19	
99*	medium, dense	19.1	
153	medium, twisted	19.6	
iso. HOW	large	21.2	
C255*	large	21.3	
189*	medium, dense	21.8	
179	large, wiry	22	
51	large, twisted	22.6	
iso.1558	large	23.3	
89	small, dense	23.6	
47*	small, dense	24.1	

TABLE 15. CONTINUED

Culture	Phenotype on WBGA	Mean canker length, mm.	Significance among means**
84	medium, dense	24.5	
34*	"	25	
iso.Pc206	large	26.3	
122*	medium, tufted	26.4	
72	medium	26.8	
154*	extra large	30.6	
C22*	large	32.3	
38*	medium, twisted	32.6	
applanata	large	33.3	
iso. 1620	large	34.8	
131	large, zonate	37	
164*	medium, dense	38.5	
113	large, wiry	39.2	
53*	large, tufted	39.5	
iso. VAN	large	41	
176*	medium	41.8	
iso. 574	large	42.3	
78 cont.	large	42.6	
iso. Pc17	large	45.2	
syringa	large	45.8	
C146*	large	47	
iso. APC	large	54.1	

** Any two means not scored by the same line are significantly different at the one percent level; any two means scored by the same line are not significantly different.

* Phenotypes after recovery from peach are shown in Figure 22.

Underlined culture numbers: Quantitative growth data were obtained for these mutants. See p. 66.

size of those of the wild type and yet produced colonies as large as wild type on WBGA. Conversely, two mutants which were small on WBGA produced cankers larger than those of six mutants which produced large colonies on WBGA. One mutant (82) produced only a trace of growth on WBGA and yet was more pathogenic than one large mutant (180). Only six mutants produced cankers as large as those of the wild type. Four of the ten natural isolates produced cankers of the same size as those of the natural isolate designated as wild type. One natural isolate, isolated from a cherry tree in Canada, produced cankers significantly larger than those of any of the other cultures. On the other hand, two natural isolates were much less pathogenic than both the wild type and many of the mutants.

Phenotypes of 24 cultures on WBGA after recovery from peach cankers are compared with the respective canker sizes in Figure 22. All except three of these colonies were indistinguishable from the respective parent colonies used in the inoculation trial. After reisolation from peach, the mutants 164 and 176 were much larger than the parent cultures. Mutant $C15_{20}$ was changed into a less radiate colony type resembling $C15_{6}$, a type previously obtained from a zoospore of $C15_{20}$.

Pathogenicity of single zoospore cultures of five mutant series was also tested (Table 16). The five mutant

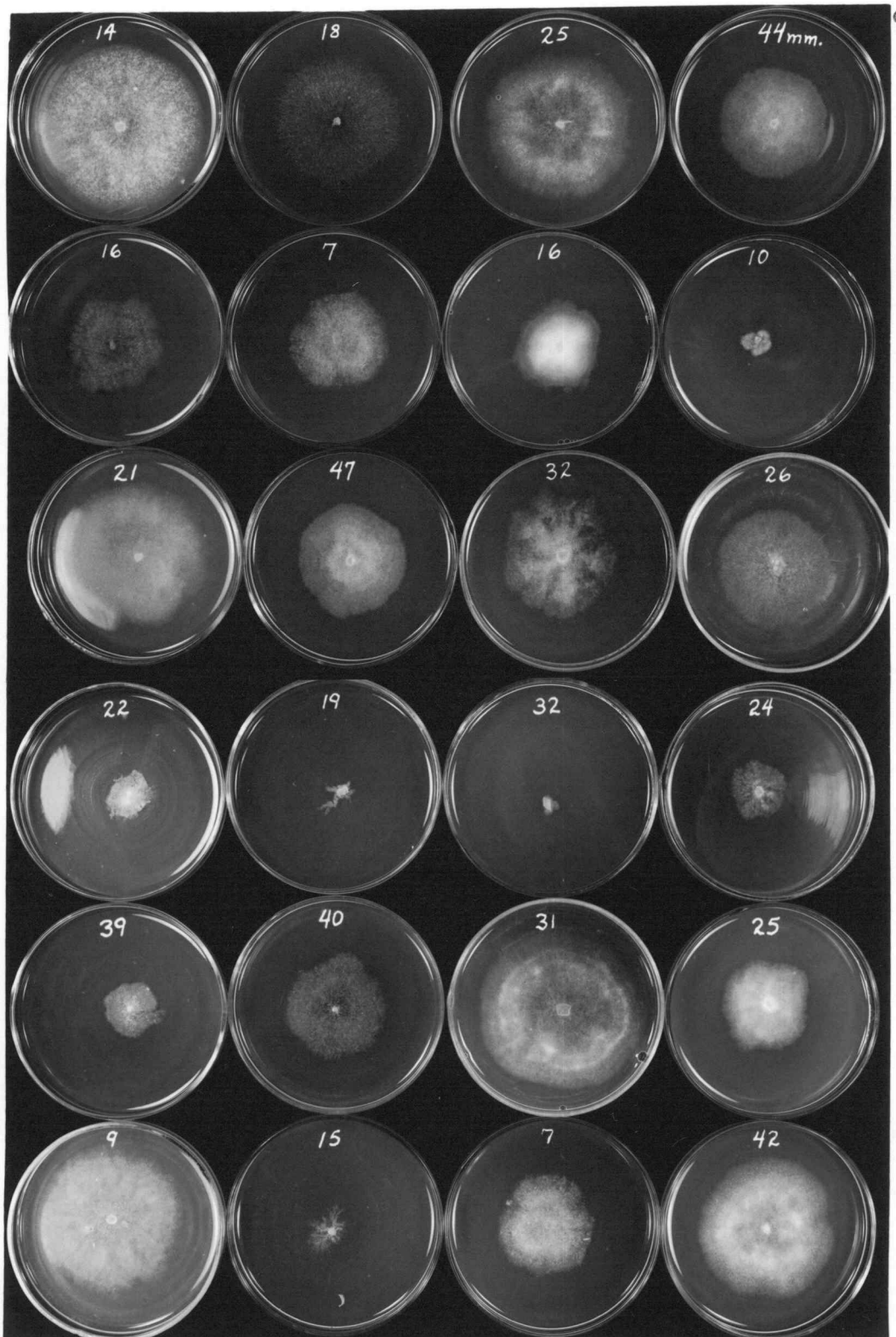


Fig. 22. Mutants recovered from peach (figures refer to canker lengths). (Row 1) $C15_{52}$, $C15_{12}$, $C15_{20}$, $C87S18_{9}$. (Row 2) $C87S15_{53}$, $C87S23_{18}$, C87Sparse, C150. (Row 3) C255, C146, C22, 122. (Row 4) 189, 99, 38, 47. (Row 5) 164, 53,154, 34. (Row 6) 124, 76, $C87S23_{18}$, 176.

TABLE 16. RANGE OF PATHOGENICITY OF SINGLE ZOOSPORE SUBCULTURES OF FIVE MUTANTS

Culture	Phenotype on WBGA	Mean canker length, mm.	Significance among means**
$C15_7$	aerial	9.3	
$C15_{52}$*	aerial	14.1	
$C15_{12}$*	ropy	18	
$C15_1$	fluffy	20.8	
$C15_6$	sparse	21.1	
$C15_{20}$*	C15 type	24.5	
C87 smooth large (original tube culture)		14.4	
C87 sparse* (original tube culture)	large when inoculated	15.6	
$C87S18_{59}$	large	39	
$C87S18_9$*	large	44	
$C87S12_{17}$	dwarf	1.3	
" 22	"	5.3	
" 33	"	8	
" 18	"	11	
" 63	large	11.6	
$C87S23_{86}$	large	3.8	
" 18*	large	6.8	
" 94	dwarf	8.3	
" 39	small	10	
$C87S15_{32}$	small	1.6	
" 106	dwarf	2.1	
" 23	medium	3.8	
" 66	small	5	
" 1	dwarf	7	
" 67	small	8.1	
" 86	large	8.8	
" 42	large	9.5	
" 36	dwarf---large	14.2	
" tube	C87S15 (large type)	15.6	
" 55*	dwarf---large	16.3	
wild type control	wild type	42.6	

* Phenotypes after recovery from peach are shown in Fig.22.

** Any two means not scored by the same line are significantly different at the one percent level; any two means scored by the same line are not significantly different.

series tested were those investigated earlier for constancy of morphological characters.

Only two of the cultures from the mutant series were as pathogenic as wild type. Both of these ($C87S18_9$ and $C87S18_{59}$) were large cultures of the series most closely resembling wild type on WBGA. Tube cultures of the original sparse and smooth sectors of mutant C87 were both equally and slightly pathogenic. When these two cultures were grown on PDA preparatory to inoculation, however, they did not differ in morphology.

Single zoospore cultures of the mutant series C87S12, C87S23, and C87S15 were only slightly pathogenic. Canker sizes ranged from 1.3mm. to 16.3mm. Significant differences in canker sizes were found within four of the five mutant series. No correlation between canker size and phenotype on WBGA was apparent.

Cultures of the C15 mutant series differed significantly in pathogenicity, with canker sizes ranging from 9.3 mm. to 24.5 mm. Each of these cultures produced large colonies of differing phenotypes on WBGA.

DISCUSSION

Phytophthora cinnamomi

Isolates of *P. cinnamomi* from Oregon, and from different parts of the world were found to differ in gross morphology. This is in contrast to a previous report where no differences were found among isolates (70).

Variation was not unexpected since *P. cinnamomi* in nature probably exists as a group of clones with separate histories and futures and with little or no gene flow among them. If hybrid oospores between *P. cinnamomi* and other species are produced in nature, as they were found to be in culture, further variability would result. If germination of such oospores occurs in nature, descendent isolates should result that are not referable to *P. cinnamomi*. Isolates are identified as *P. cinnamomi* on the presence of "typical cinnamomi" zonation, spherical hyphal swellings, absence of sporulation on common media, and pathogenicity to a cinnamomi host. These criteria do not result in unequivocal identification. That such is the case was demonstrated by the production of mutants that did not exhibit pathogenicity to a cinnamomi host, that lacked spherical swellings, and expressed non-cinnamomi colony morphology. Isolates from nature also varied in gross morphology so that identification of them must have been based mainly on the presence of spherical swellings, and the belief that the

isolate recovered in a particular situation would be P. cinnamomi. Mutants of P. cactorum were produced that had hyphal swellings closely resembling those of P. cinnamomi. Although the experienced person can identify with fair certainty the "central core" of P. cinnamomi isolates as P. cinnamomi, there are many isolates left unidentified that are probably mutants of P. cinnamomi. If the pathogenicity of such mutants is unchanged, the need for identification is not lessened. Similarly identification of non-pathogenic isolates as P. cinnamomi could be made on the basis of morphological criteria. It is not surprising that this situation had led to confusion. In spite of this confusion and the importance of accurate identification, no studies on variability have appeared. This is at least in part due to the fact that when more and more isolates of Phytophthora are considered, it is realized that there is no clear boundary that may be drawn about which one may say, "The variability within this group is that expressed by different isolates of P. cinnamomi; isolates outside this group are of other species." The possible gene flow between several other "species" and isolates of P. cinnamomi further complicates the situation.

Oospore production in cultures of P. cinnamomi paired with other "species" has been reported several times and was found in the present studies. Oospore formation has also been reported to occur in single cultures growing upon

appropriate media (77). Valid interpretation of these phenomena has been limited by an attempt to fit such behavior into one of the two categories, heterothallism or homothallism. P. cinnamomi is neither heterothallic nor homothallic; it exhibits features of both categories. Antheridia, oogonia, and oospores are formed on hyphae of P. cinnamomi even when such hyphae are derived from a single zoospore. These are only rarely produced in ordinary culture media, but are produced in abundance after stimulation by the proper chemical (77). Growth of P. cinnamomi in mixed cultures with certain other species results in formation of both hybrid oospores, and non-hybrid oospores. In non-paired cultures of the same age, no oospores are formed. It may be assumed that in nature oospores will be formed whenever the proper chemical stimulation occurs, whether the stimulation is from a chemical in the host or from chemicals in compatible Phytophthora species. The important point is that the oospores resulting in one case are non-hybrid oospores, and in the other case are mainly hybrid oospores. If both can germinate, they would play obviously different roles in the biology of the organism.

Phytophthora cactorum

Much needs to be known about nuclear behavior before an understanding of the biology in nature of a species such as P. cactorum can be obtained. However, certain theoretical

considerations may be of value in pointing to what needs to be known. It is known, and was reaffirmed in the present study, that oospores result readily from cultures derived from uninucleate zoospores. The antheridial and oogonial incepts may even arise adjacently upon the same hypha. P. cactorum is termed homothallic (homomictic) on the basis of this behavior. It should be recognized that such a conclusion is resting upon several unproved assumptions: (1) the mycelia are haploid, (2) the oospore is a zygote, (3) oospore germination occurs, and meiosis occurs in the oospore or shortly after germination, (4) the nuclei in the hyphae are identical, and (5) oospores are not formed in nature between mycelia of different thalli.

The assumption that the oospore is a zygote has probably been adequately satisfied (9). The other assumptions have not been satisfied as yet by experimental work, although there are many observations which have some bearing upon them. Experimental work to satisfy these assumptions must be based on having: (1) clearly recognizable stable mutants, and (2) oospores which can be germinated easily, and from which cultures can be established. In principle the procedure appears to be simple: (1) obtain stable mutants; (2) grow them in mixed culture with the wild type; (3) obtain and germinate oospores; (4) check for segregation of the mutant characters in cultures established from germinated oospores. In practice it is not simple.

Negative results for segregation in an apparently homomictic fungus such as P. cactorum would not establish the validity of the assumptions. Therefore success would appear to be more easily obtained for apparently heteromictic forms such as P. drechsleri or P. infestans. Different assumptions would have to be tested for these heteromictic forms. Determination of the assumptions for either the hetero- or homomictic forms would be of value. However, the most valuable information, the behavior of the species in nature, would not be answered conclusively from such studies. Such information could probably be obtained from carefully designed experiments in which known mutants of the proper species were used in localized areas. The difficulty of, and the time necessary for, oospore germination are the main factors limiting such an undertaking.

Knowledge of the nuclear behavior and biology in nature is important both from a theoretical and an economical standpoint. The confusion in the literature concerning identity of isolates, the significance of oospore production in paired cultures, and the application of the terms hetero- and homothallism to Phytophthora species, is in a large part due to the lack of such knowledge. The demonstration that oospores are formed between "species" pathogenic to different hosts should be evidence enough that such a knowledge is important.

The preliminary step of obtaining mutants has been carried out in this investigation. Mutants of many types were easily produced with both ultraviolet and X-ray irradiation. Known irradiation and screening techniques for other fungi were adapted for inducing mutants in zoospores and it was found that very low dosages of ultraviolet light produce a high mutation rate. A mutation rate of 50 percent was the highest obtained with ultraviolet light and the mutation-dosage response curve was similar to those reported for other organisms. Mutation screening with P. cactorum was found to be complicated by the small number of clear-cut characters usable in grading. Pigmented fungi such as Aspergillus that also have a definite colonial morphology are much more suited for mutant screening. A further complication was the growth-lag phenomenon which was especially apparent in colonies arising from irradiated zoospores. These difficulties were largely overcome by grading for mutant characters after two transfers had been made.

Zoospore motility is not immediately affected by ultraviolet light dosages which result later in complete mortality. Zoospores, motile uninucleate spores that are bounded only by a plasma membrane and containing no pigment, are well suited for studies on the mutating and physiological effects of measured dosages of radiation and of radiation quality.

Nearly all of the characters of the wild type culture of P. cactorum were altered in the various mutants obtained. Very long nonpapillate sporangia were produced that resembled those of P. hibernalis; sporangia were produced that were borne only in tight grape-like clusters; sporangia had double or triple papillae; sporangia graded into chlamydospores or could not be distinguished from them. Hyphal differences consisted in the production of various types of knobs, clubs or spherical swellings. The hyphal swellings of one mutant closely resembled those of P. cinnamomi. Colony characters were altered so that some mutant colonies greatly resembled layered isolates of P. parasitica. Oospores of one mutant were aplerotic. Sporulation varied in the different mutants from greater amounts than the wild type to complete absence of spores.

In Phytophthora there are not many more characters than these upon which a classification can be constructed. The differences found among the mutants cross species lines as they are now drawn. These mutants were only one irradiation removed from the wild type; further irradiation of already mutant types would probably change them into types even less resembling the wild type. Species separation in Phytophthora is believed, on the basis of these data, to be unsatisfactory in that it does not take into account the present or potential magnitude of variability of the species.

Nearly all of the mutants found grew less well in culture than did the wild type. However a few were found that produced larger colonies on WEGA, and one of these produced heavier colonies in minimal medium than did the wild type. This mutant could then be considered to be one of the rare mutant types that is "more successful" than the wild type. However, the pathogenicity of this mutant to peach was significantly less than that of the wild type. No mutants were found that were more pathogenic than the wild type, and most were less so. These data show that most mutants were less able to grow than the wild type, both in nature and in culture. However, a few mutants could grow better in culture. If such mutants arose spontaneously in culture, they could outgrow the wild mycelia and be selected for in subtransferring. If the pathogenicity of such mutated isolates were tested later, the isolates would be described as having lost their virulence in culture. Such loss of virulence has been found many times by plant pathologists.

Anomalous variability

Anomalous behavior was exhibited by four mutants that were investigated by the single spore technique. Six natural isolates did not exhibit this behavior. The anomalous behavior consisted of the production of non-identical colonies from single zoospores obtained from each mutant. The frequencies of colony types produced were unpredictable.

Similar anomalous behavior has been reported in a natural isolate of P. cactorum in the only other investigation of variability in this species. If such anomalous behavior is found to be common in Phytophthora, it will pose a serious limitation to genetic investigations.

An unspoken assumption in biology is that an organism produces identical nuclei following mitotic divisions except for a mutation frequency which is very low. The anomalous behavior found does not satisfy this assumption, or if it does, other ad hoc hypotheses must be constructed to explain the data. In either event, if such behavior occurred in genetic experiments with mutants, interpretation of crossing results would be confused.

Possible reservations concerning the anomalous behavior that was found might be that the phenomena observed were actually (1) due to the range of variation of genetically identical colonies on different plates; or (2) due to the growth-lag of the colonies--especially since grading was often done on colony size. That these reservations are not explanations was shown since (1) adjacent zoospore colonies on the same plate developed into radically different phenotypes, and the wild type colonies in different plates showed no variation; and (2) the growth-lag phenomenon was sufficiently investigated in wild type so that variation due to it was taken into account; and in the mutants, some "small" colonies never developed into the large type typical for

the parent mutant. In addition, the anomalous changes in one mutant were not unstable dwarf types but were colonies as large as the parent mutant differing in both macro- and microscopic characters. The classification based on colony size was used for convenience but in reality both macro- and microscopic characters were changed. Differences such as degree of branching of the hyphae, degree of "knobbiness", and amount of aerial mycelium, were difficult to describe accurately, but nevertheless, existed. Grading based on colony size was felt to be accurate when it was done after the growth-lag delay was evened out.

The anomalous variability of the mutants most extensively investigated was of two types. Single zoospore colonies derived from mutant C15 were nearly all identical to the large parent mutant. The few differing colonies were also large but exhibited differing morphological characters. Two among this group could not be separated from wild type. Descendants of mutant C87S, however, always produced a small number of dwarf colonies, and a larger number of one or more large type. In one descendant there was a complete range of colony types. The dwarf colonies often sectored into more vigorous types. Colonies derived from zoospores from a given colony type would produce the complete range of types. Although many colony types were produced, a certain pattern became evident in that each descendant produced a dwarf type and a large type characteristic for that

descendant.

Several *ad hoc* explanations can be suggested: (1) the mutants are not gene or chromosome-breakage mutants, but are the result of cytoplasmic changes; (2) a gene has been mutated which renders other loci much more susceptible to spontaneous mutation; (3) various degrees of aneuploidy could be occurring erratically, either with or without chromosome breakage; (4) unstable mitosis is occurring, resulting in unequal segregation and chromosome losses; (5) the spores are diploid and parasexual recombination is occurring; or (6) segregation of multiple mutants is occurring in the mycelia.

Proof for these or other explanations is of course not possible without oospore germination. It is difficult to see how proof could follow even then if this species is only homomictic, and if the mutant characters are not stable.

The term "mutant" is applied here without proof that a gene or chromosome mutation has occurred. "Mutants" are described in bacteria and imperfect fungi with a similar justification--that of analogy with conventionally sexually reproducing organisms such as *Neurospora*.

The anomalous behavior that was found cannot at present be explained, but it is probably not confined either to artificially induced mutants of *Phytophthora* or to species of *Phytophthora*. Such behavior, if widespread, merits further investigation.

SUMMARY

Phytophthora cinnamomi

Natural isolates of *P. cinnamomi* differed in such characters as amount of zonation and colony size. It was demonstrated that similar differences could exist within one culture, by starting cultures from hyphal tips. A colony originating from a hyphal tip did not yield all identical colonies when hyphal tips were grown from it. The variation found, however, was slight.

Mutants were easily induced in hyphae by 4000 r of X-ray irradiation. The mutants exhibited characteristic differences in colony type and growth rate on PDA and on four synthetic media. The hyphal swellings typical of *P. cinnamomi* were absent in two mutants. Mutant differences were greater than those of the isolates examined. If similar mutants were isolated from nature at least some would not be identified as *P. cinnamomi*.

All eight mutants tested were pathogenic to apple fruits and retained their mutant phenotypes upon recovery from the fruits. Two of three mutants tested, however, were not pathogenic to *Chamaecyparis* when tested by soil infestation. The two non-pathogenic mutants were not recoverable from the soil, whereas the pathogenic mutant was recovered.

No oospores were formed when compatible species were paired on agar plates when separated by a collodion membrane.

Oospore formation in paired cultures without collodion barriers was obtained in only 26 of 208 pairings made. At least some of the oospores were hybrid oospores. Similar results have been reported by other workers, but since oospores are also formed in single cultures with the proper chemical stimulation, the species cannot be said to be either heteromictic or homomictic. The implications of this behavior are discussed.

Phytophthora cactorum

Isolates of P. cactorum were compared and found to differ in gross morphology. Zoospore colonies derived from any isolate, however, produced identical colonies. Zoospores obtained from an unidentified species produced a few dwarf colonies among many large ones.

No lasting variability was found in colonies derived by zoospores from a single zoospore colony. However, developing colonies showed differences due to growth-lag.

Oospore germination was not obtained from combination treatments of glutathione, temperatures, and freezing. Nor was it obtained in a limited trial with dung solutions.

Techniques of ultraviolet irradiation of zoospores for the production of mutants were worked out and the difficulties caused by the presence of sporangia and multiple zoospores were overcome. Mutations were easily induced in the zoospores by ultraviolet light consisting mainly of the

wavelength 2537 A. Lethality and mutant dosage response curves were obtained. A 50 percent mutation rate was the highest obtained.

X-ray dosages of greater than 2250 r were necessary for obtaining many mutations. X-rays were not as satisfactory as ultraviolet for mutation induction.

A range of mutants was obtained which differed from the wild type in macro- and microscopic morphologic characters, and in growth rate. The majority of mutants were recognized by their reduced growth on white bean glucose agar. Many of the characters which were altered are those used in taxonomy. Among the mutants, sporangia differed in size and shape. Hyphal characters commonly varied, with the production of various degrees of knobbiness, spherical swellings and "clubs". Changes in colony morphology were especially common and were characteristic for each mutant.

The mutants varied greatly in their ability to grow on one natural and four synthetic media. Although most of the mutants grew less well than the wild type, one produced three times as much growth as did the wild type on minimal medium. No mutant having a specific biochemical block was found among the 300 tested.

The stability of several mutants was tested by comparing single zoospore colonies derived from them. Anomalous behavior was found, since such colonies were not identical. Thorough investigation of one mutant showed that descendants

of it always produced a small proportion of dwarf, dense colonies, and a large proportion of large, usually stable colonies, characteristic for each descendant. The dwarf colonies commonly sectored into faster growing types. Investigation of another mutant showed that most colonies derived from it were identical to the "parent", but that six other morphologic types were produced, one of which was not separable from the wild type.

Zoospores of the wild type and of one unstable mutant were found to contain only one nucleus per zoospore.

Pathogenicity of 50 mutants and 11 isolates to young peach trees was tested. A complete range of pathogenicity was obtained from the mutants, ranging from none, to pathogenicity equal to that of the wild type. Pathogenicity, as measured by canker size, was in general correlated with colony size on WBGA. However, there were some notable exceptions, since some large colonies were only slightly pathogenic. Pathogenicity of the least pathogenic isolates was less than that of many mutants.

Pathogenicity of zoospore subcultures of five mutants was also tested. Significant differences in pathogenicity occurred within three of the five subculture series. Pathogenicity in all but one series was reduced much below that of the wild type. No correlation between pathogenicity and phenotype on WBGA was apparent.

The ease of mutation induction combined with the differences found among the mutants demonstrates the great potential for variation in this fungus and the futility of drawing narrow species lines.

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