

STUDIES ON GENETICALLY CONTROLLED
VARIATION IN THANATEPHORUS CUCUMERIS

by

A. ROY MCKENZIE,
B.Sc., M.Sc.(Agt.)

Department of Plant Pathology,
Waite Agricultural Research Institute,
University of Adelaide, South Australia.

A dissertation submitted to the University of Adelaide
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy.

JANUARY, 1966

STATEMENT

This dissertation has not previously been submitted for a degree at this or any other University, and is the original work of the writer, except where due reference is made in the text.

TABLE OF CONTENTS

	<u>Page</u>
SUMMARY	1
ACKNOWLEDGEMENTS	1v
INTRODUCTION	1
LITERATURE REVIEW	5
<u>Thanatephorus cucumeris</u>	5
(a) Taxonomy	5
(b) Life cycle	7
Nutritional Requirements of <u>T. cucumeris</u>	11
Mating System in <u>T. cucumeris</u>	13
Mutation	21
Anastomosis and Heterocaryosis	22
Variation in <u>Thanatephorus cucumeris</u>	31
GENERAL MATERIALS AND METHODS	36
Isolates of <u>T. cucumeris</u>	36
(a) Root strain	37
(b) Crucifer strain	38
General Media	40
(a) Potato-Vegemite-dextrose	40
(b) Soil agar	41
(c) Distilled water agar	41
(d) Nutritional studies	41
Cellophane	43
Maintenance of Cultures	44
Single Basidiospore Fecundity	44
(a) Formation of the perfect stage	44
(b) Isolation of basidiospores	45
(i) Collection of shed spores	45
(ii) Tetrad isolations	46
Hyphal Anastomosis and Heterocaryon Formation	51
(a) By opposition	51
(b) By maceration	52

Pathogenicity Tests	52
(a) Root strain	52
(b) Crucifer strain	53
Staining	55
Irradiation	57
EXPERIMENTAL	58
I Development of Self-Fertile Lines in <u>Thanatephorus cucumeris</u>	58
(a) Root strain	58
(i) Isolate 16 and progeny	58
(ii) Other field isolates	64
(b) Crucifer strain, isolate 48 and progeny	64
(c) Discussion	67
II Nutritional Studies	71
(a) Results	71
(i) Growth of 'ringed' type progeny of 16-8	72
(ii) Comparison of root and crucifer strain isolates	73
(b) Discussion	75
III Isolation and Characterization of Mutants of <u>T. cucumeris</u>	76
(a) Results	77
(i) Induction of mutants	77
(ii) Spontaneous mutants	79
(iii) Description of mutants	82
(iv) Stability of mutants	93
(b) Discussion	94
IV Anastomosis, Heterocaryosis and Recombination	99
(a) Study of anastomosis	99
(i) Between mutants and with P56	99
(ii) Between non-mutants	105
(iii) Between heterocaryons	107
(b) Formation of heterocaryons	108
(i) Stable heterocaryons	108
(ii) Unstable heterocaryons	112

(c) Analysis of heterocaryosis and recombination	115
(i) Stable heterocaryons	116
(ii) Unstable heterocaryons	128
(d) Discussion	132
GENERAL DISCUSSION	148
REFERENCES	175

SUMMARY

This investigation forms an initial part of a program to determine the extent and mechanisms of variation between and within different pathogenic strains of Thanatephorus cucumeris (Frank) Donk.

The crucifer-attacking strain of T. cucumeris is indigenous to soil at the Waite Institute and is widely distributed elsewhere. Variation within a field isolate, isolate 48, of this strain was expressed through the mechanisms of heterocaryosis by mutation and anastomosis, and sexual recombination. Evidence is presented to show that isolate 48 is homothallic, but heterocaryotic with sterility factors. These factors were overcome by spontaneous mutation and selection of selfed single basidiospore progeny. A third generation progeny was pathogenic to seedling stems of radish, the test host, was self-fertile and initially homocaryotic. Four induced and two spontaneous morphologic mutants were subsequently recovered as progeny of the G3 culture. Each mutant had a distinct set of cultural characters and has remained stable and

self-sterile. One mutant was pathogenic to radish stems, one produced hypersensitive flecks and four failed to penetrate the host.

Stable heterocaryons were formed from pairings of non-pathogenic mutants; each heterocaryon was indistinguishable from the field isolate and was pathogenic. Several heterocaryons were fertile and analyses of progeny from these crosses showed that cultural appearance, growth rate, pathogenicity and self-fertility were together controlled by a single gene mutation in each of the mutants involved and that the genes were unlinked. Results indicated that pathogenicity, which was dominant, was controlled by genes at several loci.

Unstable heterocaryons were formed from pairings of the pathogenic and a non-pathogenic mutant; each heterocaryon, which was initially indistinguishable from the field isolate, reverted to mutant type growth. Reversion in each heterocaryon was always in the same direction. One heterocaryon reverted to the non-pathogenic mutant component whereas in all others the reverted growth was that of the pathogenic mutant.

Reversion appeared to be due to factors common to the thallus rather than to localized cell conditions. Progeny analyses of two unstable crosses suggested non-Mendelian segregations for pathogenicity which may have been due to cytoplasmic influence.

Nuclear behaviour throughout the life cycle of the field isolate, single basidiospore progeny, mutants and heterocaryons, was studied in the crucifer strain of T. cucumeris. Vegetative cells were multinucleate but number of nuclei per cell varied considerably within a single thallus, and basidiospores were almost exclusively uninucleate. Similar studies with a field isolate and single basidiospore progeny of the root strain of T. cucumeris revealed that the basidiospores were either uninucleate (85-90%) or binucleate (10-15%), and that the production of binucleate spores, which appeared to be an inherited character, was due to migration of two nuclei from the basidium.

The results are discussed in relation to the influence of mechanisms of variation as they may operate in the field.

ACKNOWLEDGEMENTS

The writer wishes to express his sincere thanks and appreciation to Dr. M. Jean Mayo and Professor N.T. Flentje, under whose supervision this investigation was carried out, for invaluable discussion, criticism and guidance. The assistance of Mr. Bryan Palk with the photography and Mrs. M. Atkinson with the statistical analyses is gratefully acknowledged.

Financial support during these studies was provided through the Commonwealth Office of Education as an Australian Award of the Commonwealth Scholarship and Fellowship Plan.

INTRODUCTION

Since it was first recognised that micro-organisms cause plant diseases, plant pathologists have been concerned with the basic problem of control of these diseases. This concern has led, and is continuing to lead, to comprehensive investigations of the parasite, its hosts, the environment, and the interactions of each. Often such studies have been initiated in particular Laboratories as the result of disease surveys or the prevalence of a disease in the field and in time have been extended to become major investigations. Such has been the case in the Department of Plant Pathology at the Waite Agricultural Research Institute with the diseases caused by the soil-borne fungus Thanatephorus cucumeris (Frank) Donk.

The first studies in this Laboratory on the vegetative stage of T. cucumeris, known as Rhizoctonia solani Kühn, were initiated nearly 40 years ago (Samuel, 1928; Samuel and Garrett, 1932). These and subsequent investigations (Flentje, 1957; Flentje and Saksena, 1957) have shown that there

are a number of pathogenic strains of the fungus, some limited to hosts of a single family and others capable of attacking a wide range of hosts. Since that time various aspects of the pathogen have been studied to determine the most effective control measures. Included have been ecological and taxonomic studies (Kerr, 1955; Flentje, 1956; Warcup, 1959; Warcup and Talbot, 1962; de Beer, 1965; Talbot, 1965), studies on the life cycle (Flentje, 1956; Flentje, Stretton and Hawn, 1963*; Stretton, McKenzie, Baker and Flentje, 1964) and on the physiology of parasitism (Kerr, 1956; Kerr and Flentje, 1957; Flentje, 1957; Flentje, Dodman and Kerr, 1963*; Dodman, 1965). From some of these and other independent investigations it has been realised (Flentje, 1959; 1965) that present ecological control measures, such as crop rotation, chemotherapy, etc., are unlikely to be wholly effective for T. cucumeris.

* There are two papers published in 1963 by Flentje and co-workers. Since repeated reference will be made in this dissertation to one of these (Flentje, Stretton and Hawn) it will be designated as Flentje et al (1963) to distinguish it from the second paper which will be referred to as Flentje, Dodman and Kerr (1963).

In the past few years the importance of variation in T. cucumeris and its effect on possible methods of control, such as plant breeding for resistance, have been recognised and investigations were initiated to determine (a) the range of and mechanisms controlling variation both within and between different pathogenic strains, and (b) whether a particular strain is able to adapt by means of variation to meet new or different conditions, and the rate at which this adaptation may occur. These investigations were initiated with a comprehensive study of nuclear behaviour throughout the life cycle of several isolates of T. cucumeris (Flentje et al., 1963) and a preliminary study of the mechanisms of variation (Flentje and Stretton, 1964). In the present study the range and mechanisms of pathogenic and morphologic variation within strains are investigated in detail, using some new and modified techniques and morphological mutants derived from a third generation single basidiospore culture of a crucifer-attacking isolate of T. cucumeris. While this study represents a step forward towards the

style!

understanding of the mechanisms of variation and how they may influence control measures, it must still be considered preliminary, since it poses more questions than answers about the operation of variation in the field.

it/

LITERATURE REVIEW

Before reviewing the literature on aspects of nutrition, mating system, mutation, anastomosis and heterocaryosis, as they apply to the investigations reported in this thesis, it is profitable to examine the taxonomic position and life cycle of the organism studied.

Thanatephorus cucumeris

(a) Taxonomy

Rhizoctonia solani, the vegetative stage of the soil-borne fungus Thanatephorus cucumeris, was first reported as a parasite of crop plants in 1858 by Kühn. Since then many different pathogenic strains have been described, each strain attacking a narrow or wide range of host plants. Considerable confusion has followed both in the identification of the vegetative stage and the taxonomic position of the sexual stage. Isolates of R. solani and other species of Rhizoctonia have been identified, in the absence of a perfect stage, on the basis of 'rhizoctonia-like' hyphal morphology. However, the hyphal

morphology is not definitive and in recent years when a number of Rhizoctonia isolates have been induced to fruit, the sexual stages have proved to be quite different, some being Ascomycetes and some Basidiomycetes. The sexual stage of R. solani is a Basidiomycete, and under various specific epithets and in six different genera, its nomenclature has had a controversial history (Talbot, 1965).

Some other isolates of Rhizoctonia have been identified as Thanatephorus praticola (Kotila) Flentje [= R. praticola Saksena and Vaartaja]. Many authors now consider that T. praticola is indistinguishable from T. cucumeris while others claim they are distinct species. Talbot (1965) maintains this species separation, but de Beer (1965), who studied an isolate which culturally and pathogenically corresponded closely to T. praticola but was identified as T. cucumeris on the basis of its basidial stage, suggests that T. cucumeris should be regarded as a collective species which would include T. praticola. While de Beer's studies provide strong evidence, it

cannot be resolved at present that T. cucumeris, including T. praticola, is a single natural species. In this dissertation, the following nomenclature is used: T. solani for references where the sexual stage has not been reported; and T. cucumeris, 'praticola' or 'solani', where the sexual stage has been identified and the vegetative stage corresponds to T. praticola or T. cucumeris (sensu Talbot) respectively. This distinction is adopted to facilitate review of literature and discussion of the 'solani' strains used in this investigation.

(b) Life cycle

"praticola" or "solani" ?

T. cucumeris is typically parasitic on parts of susceptible host plants in or near the soil, but is often saprophytic on decaying plant roots and other soil debris or around roots of non-hosts and in the soil. The production of sclerotia in soil varies between strains of the fungus, with many strains maintained in the soil primarily as hyphae or hyphal fragments (Warcup and Talbot, 1962). There is no known conidial stage. Fructifications of basidia and basidiospores have been found under clods of soil (Warcup and Talbot, 1962) and on

plant parts (Exner, 1953). Evidence suggests that basidiospores fail to establish colonies in the soil (Flentje and Stretton, 1964), but they may be capable of infecting host tissue (Echandi, 1965).

All studies of vegetative cells indicate that they are multinucleate, the number of nuclei per cell varying with the strain studied. Tip cells usually contain (4-) 8 (-10) haploid nuclei (Hawn and Vanterpool, 1953; Sanford and Skoropad, 1955; Flentje et al., 1963) but the number is commonly reduced by secondary septation to two or three in older cells (Hawn and Vanterpool, 1953; Flentje et al., 1963). Hyphae in soil contain the same average number of nuclei per cell as when grown on agar (Flentje, unpublished).

Nuclear division in vegetative cells is conjugate (i.e. the grouping together and simultaneous division of all nuclei within a cell) followed by the separation of equal numbers of daughter nuclei into each new cell (Boidin, 1958; Flentje et al., 1963). Frequent aberrations in which either one nucleus failed to come into the group and divide or the daughter nuclei did not separate equally

resulted in different numbers of nuclei in successive cells.

The basic pattern of nuclear distribution and behaviour in the sexual stage has been described in detail (Hawn and Vanterpool, 1953; Saksena, 1961a; Flentje et al, 1963). The hymenium develops from short side branches a few cells behind growing tips. In cells of side branches which initially contain an even number of nuclei, the nuclei pair and septa develop between pairs. Each of these binucleate cells proliferates to form a clump of basidia. Nuclear fusion and meiosis to give four haploid nuclei occurs in the basidium prior to the typical formation of four sterigmata and spores. One meiotic nucleus then migrates into each basidiospore. When a cell of a short side branch initially contains an odd number of nuclei, one of the cells divided by septa contains three instead of a pair of nuclei; the trinucleate cell proliferates to form a clump of trinucleate basidia. One nucleus apparently does not take part in fusion and meiosis and the resulting basidia contain five nuclei (Flentje et al, 1963). Saksena (1961a) also

observed five-nucleate basidia and suggested a mitotic division in the basidium of one of the meiotic products to account for the extra nucleus.

Although it appears that basidiospores are typically uninucleate, bi- or trinucleate spores have been observed. This condition may have arisen either through mitosis in the basidiospore of a single nucleus from the basidium (Saksena, 1961a) or through migration of two or three nuclei into a spore from the basidium (Plentje et al, 1963). Evidence of the latter is the regular occurrence of basidia with four (or five) nuclei producing two or three (or four) basidiospores. There has been no evidence of degeneration of any nuclei in the basidium. Plentje et al (1963) found that the aberrant behaviour of nuclear migration into basidiospores was typical of certain field isolates. While insufficient isolates have been studied to generalize with certainty, it may be significant that of those so far examined in detail less than 15% of the spores of 'solani' isolates are binucleate while 35% or more of the spores of 'praticola' isolates are binucleate.

From the above discussion, it is apparent is it?
that the identification of T. cucumeris based only
on the 'rhizoctonia-state' is hazardous and leads to
confusion. It is essential that every attempt
should be made to obtain the sexual stage so that
studies of the vegetative stage can be soundly based
and adequate comparisons between isolates can be made.
In addition, any detailed investigations on varia-
bility within T. cucumeris must rest on extensive
cytological studies of nuclear behaviour in basidio-
spore formation.

Nutritional Requirements of T. cucumeris

Isolates of T. cucumeris usually have been grown
on common agar media, including potato-dextrose,
corn meal, soil, or Czapek-Dox, but very little is
known about specific nutritional requirements.
Kotila (1929) obtained good growth on an agar medium
consisting of a simple sugar, inorganic salts and
organic nitrogen. Whitney and Parmeter (1963)
showed a segregation of basidiospore progeny in
ability to grow on Czapek-Dox agar, which contains
only nitrate nitrogen.

In Rhizoctonia solani the source of nitrogen may be the most important factor in nutrition, some isolates requiring organic nitrogen supplied as peptone, gelatine, or casamino acids (Edwards and Newton, 1937; Ross, 1960) for satisfactory growth, while others are able to utilize inorganic nitrate or ammonium compounds (Tyner and Sanford, 1935; Steinberg, 1950; Butler, 1957). Requirements of R. solani for potassium, phosphorus, calcium, and molybdenum have been determined (Tyner and Sanford, 1935; Steinberg, 1950). Butler (1957) and Ross (1960) studied the ability of different isolates to utilize various sugars as carbon sources. While vitamins have been considered non-essential for growth of R. solani, Ross (1960) reported a thiamin requirement for one of the three isolates he studied.

When nutritional studies are carried out on a greater number of isolates of T. cucumeris, strains will likely be differentiated on the basis of their nutritional requirements. Isolates of strains with simple nutritional requirements would be most suitable for genetic studies using biochemical mutants.

Is this
that of
other
fungi?

Mating System in *T. cucumeris*

Homothallism is the ability of an organism to complete its sexual cycle starting from a single haploid nucleus (Korf, 1952), giving rise to progeny which, barring mutation, are self-fertile. Heterothallism applies when the sexual cycle can be completed only by the union of two haploid nuclei from compatible self-sterile thalli, and self-fertile recombinants do not appear among the progeny (Olive, 1958).

A homothallic condition may appear in an organism which is basically heterothallic when two nuclei of compatible mating type are included within a sexual spore before it is set free from the fruiting structure. This condition, termed secondary homothallism, occurs in the Hymenomyces with varying degrees of efficiency (Whitehouse, 1949) resulting in the production of both self-sterile and self-fertile progeny.

Published data for 230 species of Hymenomyces and Gasteromyces studied before 1949 indicate that, excluding those shown to be secondarily homothallic, about 10 per cent of the species are homothallic,

35 per cent heterothallic and bipolar and 55 per cent heterothallic and tetrapolar (Whitehouse, 1949). Included are two reports that Thanatephorus cucumeris is homothallic (Müller, 1924; Kotila, 1929), and four reports since have been published (Hawn and Vanterpool, 1953; Saksena, 1961a; Whitney and Parmeter, 1963; Flentje and Stretton, 1964). While the production of self-fertile basidiospore cultures among the progeny of isolates of T. cucumeris excludes heterothallism, the reports require re-examination to determine whether secondary homothallism is also excluded.

In T. cucumeris, homothallism can be demonstrated only from a self-fertile culture derived from a basidiospore which received a single haploid nucleus from the basidium and is proved if the progeny from that culture are uniform under optimum environmental conditions. If the progeny are not uniform but express variation with respect to basidiospore viability or fertility or both, either of which complicates an interpretation of mating system, it is likely that the original culture was heterocaryotic. Such a heterocaryon would arise

either from a basidiospore containing two (or three) genetically different nuclei or through mutation during vegetative growth. If the culture has arisen from a binucleate spore, secondary homothallism cannot be excluded.

From cytological studies on the sexual stage of an isolate of T. cucumeris the frequency of uninucleate spores and the probable origin of the nuclei in binucleate spores can be determined. This cytological information can then be used to estimate the probability that the fertile progeny had arisen from basidiospores which received single nuclei from the basidia.

Analysis of self-fertility of the basidiospores from four-spored basidia may also provide strong evidence of homothallism. Cytological studies of a four-spored basidium have shown that the spores produced will be initially either all uninucleate or that three will be uninucleate and one binucleate. The self-fertility of two or more single basidiospore cultures from a four-spored basidium would therefore include one or more cultures derived from a uninucleate

basidiospore. A note of caution is necessary, however, for if in a five-nucleate basidium the extra nucleus arose by mitosis of a meiotic nucleus, as suggested by Saksena (1961a), there could be more than one mitotic division, giving rise to basidia with more than five nuclei and thus more than one binucleate spore. Spores produced from these basidia could complicate any determination of homothallism based on tetrad analysis alone.

Saksena (1961a) claimed that his investigations provide cytological proof of true homothallism in *F. cucumeris* 'praticola'. He concluded that (a) mature basidiospores are regularly binucleate, and (b) the binucleate condition is chiefly due to the early division of the initial single nucleus received by each spore and to a limited extent due to the migration of more than one nucleus into a spore. He does not, however, account for the uninucleate condition that he observed in 34 of 108 discharged, mature basidiospores. Neither does he consider that the variation in number of spores per basidium and consequently the number of

initial nuclei per spore has any biological significance in this fungus.

Hawn and Vanterpool (1953) concluded that their 'praticola' isolate was homothallic on the basis of the self-fertility of four single basidiospore cultures from a total of 50 shed basidiospores, 34 of which were not visible. They observed predominantly uninucleate basidiospores from basidia bearing two to four spores, but did not present the data; a frequency of eight per cent binucleate spores could account for their self-fertile cultures.

No!

The cytology and fertility of T. cucumeris was studied by Müller (1924) who reported that the mature basidiospores were binucleate. The original paper was not available, but it would appear that the origin of the nuclei in the binucleate spores was not definitely determined, and if this was so, the evidence of homothallism was not conclusive.

which?

Kotila (1929) demonstrated homothallism in progeny of his 'praticola' isolate by tetrad analysis. In two instances he isolated from a fertile single basidiospore culture (B6-2) two basidiospores from

a four-spored basidium (basidia 89 and 91, Table 11, p. 1082); each basidiospore gave rise to a self-fertile culture. Kotila, however, did not appreciate the significance of these fertile cultures and did not specifically use them in support of his conclusion of homothallism; without them and considering the low viability of the basidiospores, his results could equally well be interpreted as an expression of secondary homothallism.

Whitney and Parmeter (1963) accepted the earlier reports of homothallism in T. cucumeris, and stated that their results with a 'praticola' isolate confirmed those observations. However, as the earlier reports do not present unequivocal evidence, the confirmation itself requires information on the nuclear condition and viability of the basidiospores before it can be fully assessed. Further, it will be shown below (Experimental Section IV (c)) that a sample of 20 single spore progeny obtained from shed basidiospores, the numbers used by Whitney and Parmeter, may not be (large enough to constitute a representative (sample.)

dl
dl.

Flentje and Stretton (1964) concluded that T. cucumeris is predominantly homothallic, their conclusions based on studies with 'solani' isolates 82 and 48 and 'praticola' isolate 42. Homothallism of isolate 82 was confirmed by the complete fertility and lack of variation of single uninucleate basidiospore progeny through five selfed generations. The evidence of homothallism in isolates 48 and 42 is not so conclusive. From isolate 48, only one first generation (G1) single spore culture (48-4) was fertile; while the second generation (G2) progeny were reported to be uniform in cultural characteristics and pathogenicity, G2 basidiospore viability was not indicated and only about 70 per cent of the cultures were fertile. The segregation for self-fertility in the G2 progeny suggests that 48-4 was heterocaryotic, and as the G1 basidiospores were one to two per cent binucleate, heterocaryosis in 48-4 could well have arisen from a binucleate spore. The analysis of progeny from isolate 42 was complicated by a high percentage of binucleate basidiospores (about 35 per cent of G1 basidiospores); 55 per cent of the G1 spores formed colonies and

verts?

half of these (or 28 per cent of the basidiospores) were fertile. No information was presented on the viability of G2 basidiospores, but it was reported that the progeny from fertile G1 cultures were either uniform or markedly variable with respect to fertility.

Thus the reports of homothallism in T. cucumeris have been based on observations which are not entirely conclusive, due to the abnormalities of nuclear behaviour which occur in the sexual stage. A state of secondary homothallism has been excluded only in Kotila's 'praticola' isolate and 'solani' isolate 82 of Flentje and Stretton. Both reports suggest that sterility factors not associated with mating type are responsible for the sterility of some single basidiospore cultures when grown singly or paired.

As T. cucumeris is now considered to be a collective species, it is not advisable at present to assume that all isolates or strains are homothallic.

Mutation

"A mutation is a more or less abrupt and stable change in a cell which can be transmitted in hereditary fashion to daughter cells" (Fincham and Day, 1963). Mutations occur spontaneously in all cell populations but usually with very low frequency. Although wild populations of fungi are probably heterocaryotic for many genes, most naturally-occurring genetic differences are not suitably marked in their effects on the phenotype to produce good material for experimental studies. Most genetical studies have been made on mutants recovered from uninucleate propagules after treatment with mutagenic agents.

Quote.

Mutations from the wild type to a non-wild, or mutant type, have been classified broadly into morphological, where the effect is on morphology or color but not on nutrition, and biochemical (auxotrophs) with effects on the capacity of the organism to carry out definable metabolic reactions.

In genetical studies of fungi, some use has been made of morphological mutants, particularly those which have vigorous but abnormally compact

growth. Their usefulness is limited by the difficulty of scoring two or more morphological effects in combination, and by the loss of viability usually encountered when several such mutants are introduced into the same individual. Auxotrophs, however, are more convenient for study; they fail to grow on a minimal medium, but when supplied with their growth requirements they commonly resemble the wild type in appearance. Auxotrophs can be scored more easily when in combinations and often the viability of such combinations is not reduced. However, a necessary prerequisite for the study of auxotrophs is a chemically-defined minimal medium which will support growth of the wild type.

Anastomosis and Heterocaryosis

Anastomosis is the vegetative fusion of two cells and occurs in the fungi between combinations of hyphae, spores and sexual structures. Anastomosis is important in that it can lead to the association of different types of nuclei or cytoplasm or both within a common cell or cells. The association of different types of nuclei may be essential for fertility, as in self-sterile

individuals, or may lead to heterocaryosis followed by recombination through the sexual or parasexual cycle.

Anastomosis is a complex phenomenon, the mechanisms of which are not clearly defined. It does not always occur whenever hyphae cross each other but is apparently influenced by chemotropic materials diffusing from hyphae which cause the hyphae to meet in a particular fashion (Köhler, 1930). All anastomoses are essentially end-to-end and hyphal tips alone are involved in the actual fusions; they have been classified as hypha-to-hypha, hypha-to-peg, peg-to-peg (bridging) and hook-to-peg (clamp connections) (Buller, 1933). A peg is a short branch-like protrusion from the side of a hypha produced in response to a stimulus from an adjacent hyphal tip. A successful anastomosis proceeds through several steps (Matsumoto et al., 1932; Buller, 1933) as follows: (a) hyphal contact, resulting from mutual attraction between two hyphae; (b) wall fusion, occurring at the point of contact between the walls of the two anastomosing hyphae; and (c) successful reaction, in which the fused walls break down and the two masses of

protoplasm become confluent without any adverse effect on the cells, i.e. subsequent death. Failure of successful anastomosis may involve any one of these steps (Flentje and Stretton, 1964).

A simple test, used with paired isolates of Thanatephorus cucumeris (Flentje and Stretton, 1964), will determine which of the steps have been reached in a presumptive anastomosis. It consists of puncturing with a fine glass needle one of the two cells involved; the punctured cell collapses and if the other cell of the presumptive anastomosis also collapses, it is accepted that there was a functional cytoplasmic connection between the two cells. Where no such collapse occurs, the hyphae of the presumptive anastomosis are teased apart with glass needles to determine whether there had been wall fusion or only contact between the hyphae. When cell death follows an apparently successful anastomosis (the 'killing reaction' in T. cucumeris) it occurs after a period of time and may include not only the anastomosed cells but also several adjacent cells. Cell death is determined by microscopic examination.

The phenomenon of cell death following anastomosis has been referred to in Podospore anserina as a 'barrage' (Riset and Schecroun, 1959) and in Neurospora crassa as an 'incompatibility reaction' (Garnjobst and Wilson, 1956). The choice of the terms 'barrage' and 'incompatibility' to describe this reaction is unfortunate as each term has been applied to a number of apparently distinct phenomena, and evidence is lacking to show whether these are due to similar mechanisms.

'Barrage', as originally translated (Brodie, 1936), implies a mutual aversion or repulsion with a gap between the mycelia of two opposed colonies; it therefore precludes anastomosis between the mycelia. A zone between two colonies, in which there is sparse abnormal hyphal growth and anastomosis but no cell death, characterizes the 'barrage' in Schizophyllum commune (Papazian, 1950). In P. anserina 'barrage' was first described (Riset, 1952) as an intermingling of hyphae at the line of juncture between two cultures with a failure of anastomosis and later (Riset and Schecroun, 1959) cell death was described.

'Incompatibility' is a term originally applied to flowering plants and to animals and its application to the fungi has led to some confusion. Certain incompatibilities as first described in the fungi are related to sex, i.e. the mating type factors (Burnett, 1956). Other more recently described incompatibilities appear to be related to vegetative stages. This type of incompatibility is manifested by the failure of certain strains of like mating types to form successful heterocaryons (Garnjobst and Wilson, 1956) or the failure of different nuclei to co-exist harmoniously in a heterocaryotic mycelium (Pittenger, 1964). In addition, degrees of incompatibility interaction have been described, i.e. compatible, hemi-compatible, semi-compatible, incompatible or noncompatible interactions; and the same interaction may be described by different terms or vice-versa. Thus unilateral migration of nuclei is a semi-compatible (Esser, 1959) or a hemi-compatible (Papazian, 1950) interaction, and one of the hemi-compatible (Raper and Raper, 1964) and the noncompatible (Papazian, 1950) interactions produce a 'barrage' (sensu Papazian).

The confusion in the use of the term incompatibility revolves around mating type factors on one hand and anastomosis and heterocaryosis on the other. Evidence has not been presented to determine whether the various incompatibilities are expressions of the same, similar or different phenomena. Until such evidence is obtained there is, therefore, a need for two terms, one applied to vegetative incompatibilities and the other to sexual incompatibilities; the two terms proposed are 'heterocaryon incompatibility' and 'dicaryon incompatibility'.

'Heterocaryon incompatibility' is the failure of heterocaryosis; it applies to the range of vegetative incompatibilities, including cell death following anastomosis and the formation of unstable heterocaryons which dissociate into homocaryotic components. 'Dicaryon incompatibility' is the failure at any stage from the initiation of the dicaryon (defined below) to the liberation of sexual spores after meiosis; the failure may or may not interfere with the formation and liberation of the sexual spores.

Heterocaryosis, the formation and development of a heterocaryotic state, is defined as the association of two or more genetically different nuclei in each of one or more cells of a thallus, the cells being capable of vegetative growth. Dicaryosis is redefined to apply to the sexual stage and is the process of change from either a uninucleate or a multinucleate cell to a binucleate cell (dicaryon), the nuclei of which are destined to fuse. Nuclei of the dicaryon may be genetically identical (as in a homothallic homocaryon) or different (as in either a homothallic or heterothallic heterocaryon). The dicaryon phase of the life cycle may be limited to a few cells (i.e. the ascogenous hyphae of Neurospora crassa) or extended due to environmental conditions (i.e. Melampsora lini).

Heterocaryosis is initiated from homocaryotic material in two ways (Fincham and Day, 1963); a mutation in a bi- or multinucleate cell yields a heterocaryotic cell, and anastomosis between hyphae of genetically different homocaryons may give rise to heterocaryotic cells. Once a heterocaryon is established it can be maintained by mycelial sub-

culturing and by single spore culturing of heterocaryotic asexual or sexual spores in which there has been no recombination. New heterocaryons may arise from heterocaryotic spores following mutation, anastomosis, or recombination via the parasexual or sexual cycle.

Heterocaryons may be classified as stable or unstable. A stable heterocaryon is one which is maintained more or less indefinitely by mycelial subculture. Mycelial growth of an unstable heterocaryon rapidly leads to hyphal dissociation into its homocaryon components; such dissociation is an expression of heterocaryon incompatibility. Other nuclear dissociations, which are not incompatibility reactions, occur through the production of sexual or asexual spores derived from single nuclei.

Demonstration of heterocaryosis involves proof that dissimilar nuclei are present together within single cells which are capable of vegetative growth, and cytological studies are necessary to determine whether the cells contain more than single nuclei. If the genetic differences of the

nuclei are associated with cultural characteristics (i.e. morphological or biochemical mutants) then heterocaryosis is demonstrated when the cultural characteristics of colonies of single cell origin taken from mixtures of mutant colonies are different from those of the homocaryotic mutant colonies. However, as most studies of heterocaryosis are concerned with the association of nuclei with specific and reliable genetic markers, it must be proved that the heterocaryons formed contain only nuclei from the contributing homocaryons. The components of the heterocaryon are determined by their dissociation from one another.

Basidiomycetes in general appear to maintain the heterocaryotic condition, and hyphal dissociation into homocaryotic components appears limited (Parmer et al., 1963). However, as with Thanatephorus cucumeris, there have been relatively few investigations of heterocaryosis using reliable genetic markers; dissociation has been reported in common-B heterocaryons of Schizophyllum commune, but it has been suggested (Papazian, 1958) that the cross may be heterohyphal rather than heterocaryotic. The

production of homocaryotic asexual spores is limited in Basidiomycetes, and in most species dissociation may be observed only through the basidiospores.

Isolation of single basidiospore cultures showing the characters of the component nuclei plus recombinants provide proof of controlled heterocaryosis.

Several factors which may complicate the demonstration of heterocaryosis, particularly the factor of cross feeding without heterocaryosis, are excluded by single cell isolations or tetrad analysis of the progeny or both.

Variation in *Thanatephorus cucumeris*

Variation is the expression of character deviations from a type; in the fungi, mutants or the wild type are commonly used as the reference. The wild type is an individual approximating to the more or less standard form of the species as found in the wild (Fincham and Day, 1963). Variation can occur by means of a range of mechanisms involving mutation, anastomosis, heterocaryosis, and recombination.

In *T. cucumeris* there appears to be two types, or levels, of variation; the variation among

different field isolates and among progeny from single field isolates. Much of the variation reported between field isolates has been based on observations of Rhizoctonia solani and Rhizoctonia species, but as stated earlier, there is no certainty that isolates described as Rhizoctonia sp. have the same sexual stage and differences between these isolates therefore cannot be accepted as evidence of variation in T. cucumeris. However, there is considerable variation both between and within 'solani' and 'pratricula' isolates which are known to have the same sexual stage. As there has been no evidence of hybridization between them (Plentje and Stretton, 1964) such isolates have generally been regarded as variants which may have progressed so far as to have been biologically isolated within the species. These variants differ from one another in one or more of a wide range of characters including cultural behaviour and host range.

Demonstration of variation in progeny from field isolates has been hampered by the difficulty of inducing the sexual stage. In recent years a number of field isolates have been fruited and

numerous workers have isolated single basidiospores and shown that they give rise to cultures which differ markedly from one another. However, much of this work is of limited value as evidence of variation from a single field isolate because the basidiospores have been derived from hymenia occurring in nature or in agar cultures derived from mycelia on infected plants. The hymenia may have been mixtures from hyphae from different thalli; the hymenium of T. cucumeris does not develop from a single pair of nuclei, as in the fruit body of Neurospora crassa or Coprinus lagopus (Fincham and Day, 1963), but is composed of many basidial clumps, each derived from separate pairs of nuclei. Unless isolates have been grown previously from single hyphal tips and then induced to fruit under conditions which exclude contamination, there is uncertainty about the results obtained with single basidiospore cultures.

A number of hyphal tip cultures of field isolates of T. cucumeris have formed the basidial stage in pure culture on agar (Daniels, 1963; Whitney and Parmeter, 1963; Plentje and Stretton,

1964; Papavizas, 1965) and on soil previously treated with aerated steam (Flentje and Stretton, 1964). (Flentje and Stretton did not record that the cultures were derived from hyphal tips; however Flentje (pers. comm.) confirmed this fact.) In each instance the single basidiospore cultures exhibited considerable variation in a wide range of characters. Although the range of variation within progeny from a single field isolate appears to be as wide as that between field isolates, there is one major difference; there is no evidence to date that progeny of a single field isolate differ from each other in host range.

There have been no detailed studies on variation in *F. cucumeris* to determine the limits of variation within the species, between field isolates of the same or different strains, or within the progeny of a single field isolate. Little is known about mechanisms controlling variability, whether the same mechanisms are responsible at each level or type of variation, or the effects of the mechanisms on the expression of variability. Anastomosis followed by the killing reaction has been observed

(Plentje and Stretton, 1964) between field isolates of the same and different pathogenic strains and between combinations of the progeny of certain field isolates. Combinations of the progeny of other field isolates anastomosed successfully with one another, but in no case was there satisfactory evidence of heterocaryosis. Whitney and Parmeter (1963) reported heterocaryosis by anastomosis between single basidiospore cultures from one parent. However, the lack of reliable genetic markers is a serious handicap to these investigations.

GENERAL MATERIALS AND METHODS

Certain general procedures which apply throughout this investigation are described here. Any modifications of them are considered with the experiment involved.

Isolates of *T. cucumeris*

The term 'strain', as used below, follows from Flentje and Saksena (1957) who used pathogenicity to widely different hosts as the basis for separation of isolates into strains. Each separate pure culture made by direct isolation from fresh material, whether from the soil, infected host tissue or from fructifications, is termed an 'isolate'; each isolate is an individual and mycelial subcultures are merely duplicates or replicates of that isolate. The original, or field, isolates were cultured from hyphal tips and assigned selection numbers; single basidiospore progeny are either grouped as G1, G2, etc., to designate the particular selfed generation or individually designated where necessary by the selection number for each generation in series.

(a) Root strain

Isolate 16 was isolated from wheat roots at Cuneana, South Australia, in 1953 and has been maintained in the stock culture collection of the Department of Plant Pathology at the Waite Institute.

This isolate has been described (Plentje et al., 1963): vegetative hyphae contain an average of eight nuclei per cell; growth rate is 0.5mm per hour on potato-Vegemite-dextrose agar (P.V.D.A.) at 25°C, producing a colony (Plate 1) which is initially white, later changing to dark brown in color; aerial hyphae are sparse, white to hyaline, and few sclerotia are produced. The colony grows from leading hyphae which produce one or two branches per cell in compound racemes. Fructifications of this isolate can be induced on the surface of treated soil. About 88 per cent of the basidiospores are uninucleate, the remainder are binucleate, and only about half the spores shed are viable, producing vigorous colonies. Isolate 16 is pathogenic to roots of a wide range of hosts.

G1 progeny were obtained in December, 1961, and G2 progeny of isolate 16-8 were first isolated

in April, 1962, and again in April, 1963. Some of the latter 62 progeny were used in anastomosis studies (Flentje and Stretton, 1964). All isolates were maintained as stock cultures.

(b) Crucifer strain

The crucifer strain is indigenous to the soil at the Waite Institute, a heavy red-brown silt loam. Isolate 48 was isolated from this soil in 1961 by J.H. Warcup, and has been described (Flentje et al., 1963): vegetative hyphae commonly contain five to eight nuclei per cell; growth rate is 0.7mm per hour on P.V.D.A. at 25°C, producing a white colony (Plate 1) with few aerial hyphae, white to hyaline, and small, loose sclerotial bodies at or near the edge of the Petri dish. Fructifications can be induced regularly on the surface of treated soil, producing basidiospores which are almost exclusively (98-99 per cent) uninucleate. Isolate 48 is pathogenic to the stems of cruciferous hosts, producing infection pegs from infection cushions which penetrate the stem directly and cause spreading lesions followed by death of the plant.

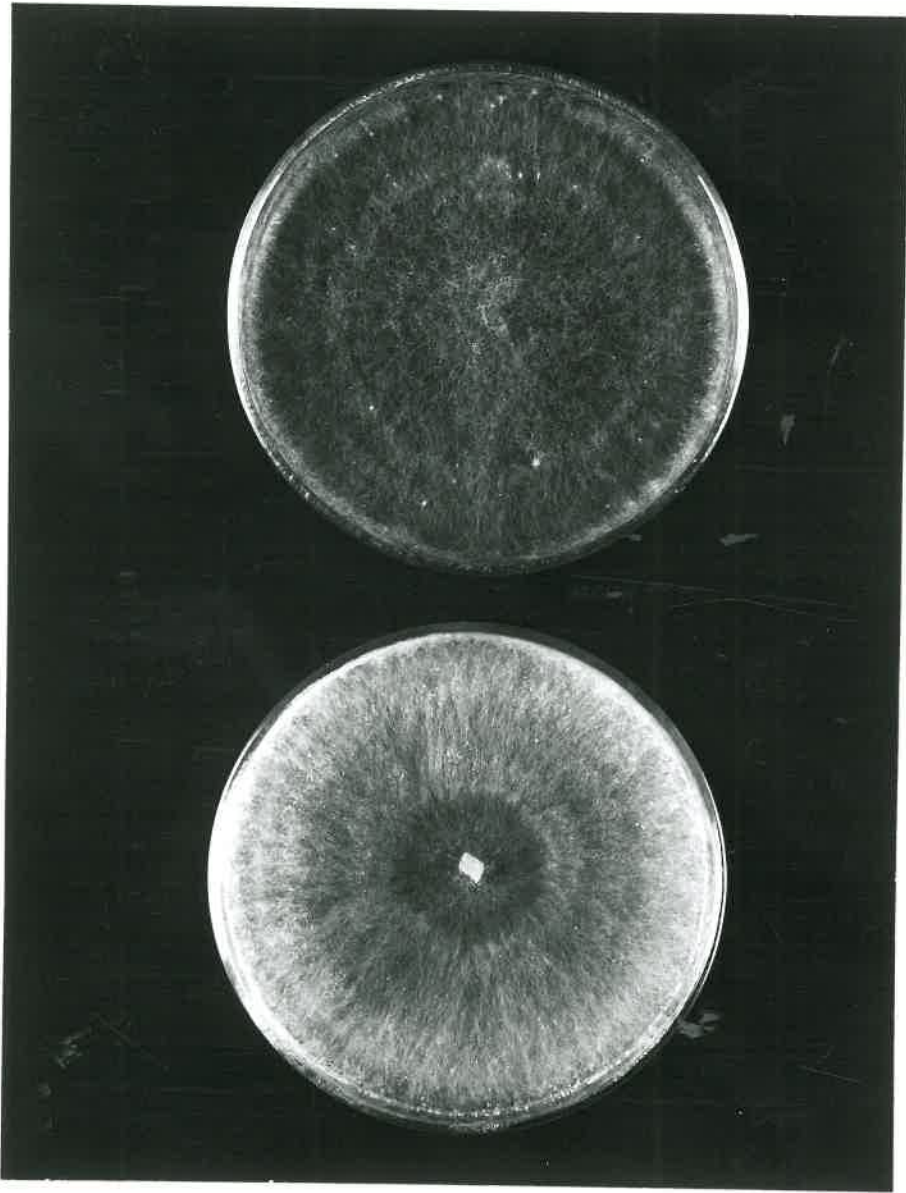
G1 progeny were isolated in December, 1961, and all isolates were maintained as stock cultures.

PLATE 1

Field isolates of Thanatephorus cucumeris

Top: root strain, isolate 16

Bottom: crucifer strain, isolate 48



General Media

All media were sterilized in a gas-fired, self-generating steam autoclave at 15psi exhaust pressure. The sterilizing period was 15 minutes for 200-1000ml volumes and eight minutes for volumes of less than 200ml.

(a) Potato-Vegemite-dextrose (P.V.D. and P.V.D.A.)

P.V.D. was used as the control liquid medium in nutritional experiments. P.V.D.A. was the general medium used for determination of cultural characteristics of all isolates for cytological studies, fruiting and pathogenicity experiments, and for studies of heterocaryosis.

P.V.D.A. prepared from instant mashed potatoes (Lacy and Bridgman, 1962) showed greater uniformity between batches than when prepared from fresh potatoes and was used throughout these investigations. It consists of:

'Deb'* instant mashed potatoes	22.0g
'Vegemite'**	1.0g
dextrose	20.0g
distilled water	1000ml
agar (Davis) in solidified medium	15.0g

-
- * 'Deb' brand, Rosella Foods Pty. Ltd., Melbourne.
** 'Vegemite' concentrated yeast extract, Kraft Foods Ltd., Melbourne.

(b) Soil agar

This medium was used for maintenance of stock cultures and for germination of basidiospores. It was also used in some experiments on heterocaryon formation. Its composition is:

Filtrate of 1000g air-dried Waite soil in 1000ml distilled water, obtained by autoclaving for one hour, and filtering after allowing it to settle overnight.

KH ₂ PO ₄	2.0g
Yeast extract (Difco)	1.0g
dextrose	1.0g
distilled water	to 1000ml
agar (Davis)	15.0g

When there was a risk of bacterial contamination, as with the collection of basidiospores from treated soil, antibiotics (aureomycin, 10 p.p.m.; neomycin, 10 p.p.m.; and streptomycin, 10 p.p.m.) were added to the sterilized and cooled soil agar immediately prior to pouring into Petri dishes.

(c) Distilled water agar

Distilled water	1000ml
agar (Difco)	15.0g

(d) Nutritional studies

A synthetic, 'minimal' medium (pH 6.8) was used in the nutritional studies. It had the following composition, with trace elements (Steinberg, 1950) added as chlorides:

KH_2PO_4	0.005M	Fe	0.8 p.p.m.
K_2HPO_4	0.005M	Zn	0.4 p.p.m.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.0005M	Cu	0.05 p.p.m.
NH_4NO_3	0.02M	Mn	0.1 p.p.m.
Sucrose	0.025M	Mo	0.04 p.p.m.
Distilled water to make 1000ml		Ca	4.0 p.p.m.

All chemicals were A.R. grade, and the distilled water used throughout was twice glass-distilled rain water. Sucrose and ammonium nitrate were replaced in some experiments by equivalent amounts of other carbon and nitrogen sources.

A vitamin supplement, used in some experiments, was prepared in 20 per cent ethyl alcohol and added to the sterilized and cooled medium at the time of adding the inoculum. The supplement consisted of the following (expressed as μg per liter of medium):

Thiamine	100
biotin	10
riboflavin	10
nicotinic acid	50
P-aminobenzoic acid	100
pyridoxine hydrochloride	100
calcium pantothenate	100

Experiments were conducted using 250ml Erlenmeyer flasks containing 50ml of medium and the total yield of mycelium only was determined. Inoculum was added initially as aliquots of a

washed mycelial mat homogenized with sterilized distilled water and cultures were incubated as still or shake cultures. In later experiments inoculum consisted of plugs of agar or discs of Cellophane 5mm in diameter cut from peripheries of colonies growing on soil agar or solidified synthetic medium. The inoculum was held at the surface of the medium by means of a glass rod with a bent and flattened tip, the rod being held in position by the cotton wool stopper of the flask. Submerged inoculum in general gave inferior growth. All cultures were incubated at 25°C.

Mycelial growth was determined as oven-dry weight. The mycelial mats were harvested on previously dried and tared filter papers, washed thoroughly with distilled water and dried for 24 hours at 90°C.

Cellophane

The value of Cellophane for cultural studies of microorganisms was first recognised by Flemming and Smith (1944). In the present studies Cellophane facilitated the isolation of tetrads of basidiospores, the collection and movement of shed spores, the growth and isolation of anastomosed hyphae, and

the handling of vegetative hyphae for staining.

Pieces of Cellophane (British Cellophane Ltd., non-moisture proof cellulose film 22 μ in thickness) were boiled in distilled water for 30 minutes to remove the surface film, and then sterilized by autoclaving in distilled water for 20 minutes.

Maintenance of Cultures

Duplicate stock cultures of each isolate of T. cucumeris were maintained on soil agar slants in test tubes and were transferred yearly. One set of cultures was stored at 5°C, the other at room temperature which ranged from 15-29°C over the year. Subcultures were normally taken from the 5°C stocks with the other set held in reserve.

Single Basidiospore Progeny

(a) Formation of the perfect stage

Basidia and basidiospores of self-fertile isolates of T. cucumeris developed on the surface of treated soil. The technique (Stretton et al., 1964) consisted of growing a culture of an isolate on agar, usually P.V.D.A., in a 9cm Petri dish; after 7-10 days when the culture had reached the

edge of the dish, the lid was removed and the culture covered to an approximate depth of 1cm with soil which previously had been treated with aerated steam at a temperature of 71°C for 30 minutes. The dishes were then placed in environment cabinets with day length of 14 hours, light intensity of 100-400 foot candles at the surface of the soil, day temperature at 22°C and night temperature at 20°C. Relative humidity was not effectively controlled, but 40-60 per cent appeared to be optimum for all isolates which fruited during the course of these investigations. The hymenial layer developed on the soil after one to two weeks. The soil was watered with distilled water one to three times daily to keep it moist.

(b) Isolation of basidiospores

(1) Collection of shed spores

Single basidiospores were obtained by inverting fructifications over Cellophane on agar plates (soil agar plus antibiotics); the shed spores were incubated at 25°C for one to three days, depending on the rate of germination, before they were transferred to separate P.V.D.A. plates.

Under a dissecting microscope (x48 magnification), a small piece of agar was picked up on the curved tip of a fine glass needle and touched to the Cellophane over a single, germinating basidiospore. The spore would usually adhere to the agar and could be transferred to the surface of a fresh agar plate.

Plentje and Stretton (1964) described a similar method but the spores were shed directly on agar. The Cellophane method, however, was less time-consuming, allowed the separate transfer of spores touching one another, and there was less risk of damage to the spores through handling.

(ii) Tetrad isolations

Mature but undischarged basidiospores are easily dislodged from their sterigmata by the slightest contact (Kotila, 1929). However, it is important to prevent the spores from drying since this causes an inhibition of germ tube formation and eventual death of the spore.

Several methods for isolating single tetrads were tried with varying degrees of success; a dissecting microscope was used throughout.

Micropipettes on a Leitz micromanipulator gave only limited success when spores were collected in the meniscus of a droplet of water in the tip of the pipette. Invariably, by the time the last spore of the tetrad had been collected, the droplet had evaporated and the spores were either sucked into the pipette or stuck to the outer glass wall and were lost.

Small pieces of agar held on the end of needles in the micromanipulator were not suitable for collecting tetrads. An agar cube ($2-3\text{mm}^3$) could not be held rigidly on the needle end, because of this, manipulation of a flat surface of the agar to the plane of the spores was virtually impossible; a smaller piece of agar which could be held firmly on the needle dried out rapidly and a flat surface was not maintained.

A number of tetrads were collected on cubes of agar ($2-3\text{mm}^3$), each cube being held on the tip of a scalpel blade and manipulated by hand. The four spores were immediately separated from one another on the agar and later transferred. It was observed that after 15 minutes on agar, the spores

adhered to the agar and, once adhered, could not be moved without being damaged. This method is satisfactory if the tetrads of spores are collected when the hymenium is beginning spore production. However, with an active hymenium there is a great risk of collecting either more than one tetrad per agar cube or four spores but from two or more adjacent basidia. The risk of contamination by this method is high.

The method finally adopted was the collection of tetrads of basidiospores on the surface of small pieces of Cellophane (illustrated in Fig. 1).

A small lump of soil carrying fructifications was set on a block of agar on a slide and examined for mature tetrads. The soil lump was manipulated so that four spores, clearly arising from a single basidium, were in a vertical plane.

The apparatus for removing the tetrads consisted of a piece of brass tubing (4mm internal diameter, 25mm long) with an attached brass rod. A thick disc of agar was cut and held in the brass tubing; the rod was fixed in the needle holder of the micromanipulator and the unit was adjusted to

a horizontal position. A small piece of sterilized Cellophane, the shape of an obtuse angled triangle, was placed by means of small forceps on the vertical surface of the agar disc so that one corner of the Cellophane extended below the agar. The agar acted as a support and provided a water film for the Cellophane. The manipulator was then moved horizontally until the protruding corner of the Cellophane came in contact with the tetrad of spores, observed at x96 magnification. Upon contact, the mature spores came away freely from the sterigmata and were maintained in the water film on the Cellophane. The manipulator was then moved away; the Cellophane was removed by forceps and placed on the surface of an agar plate. The four spores, usually resting in a clump, were separated in a water film by a series of manipulations with a flexible glass needle. When the needle (0.05mm diameter, 20mm long drawn from one end of a glass rod) was lightly pressed along its length on the Cellophane a film of water formed around it. The needle was then moved across the Cellophane until the spores floated in the water film. As the needle was

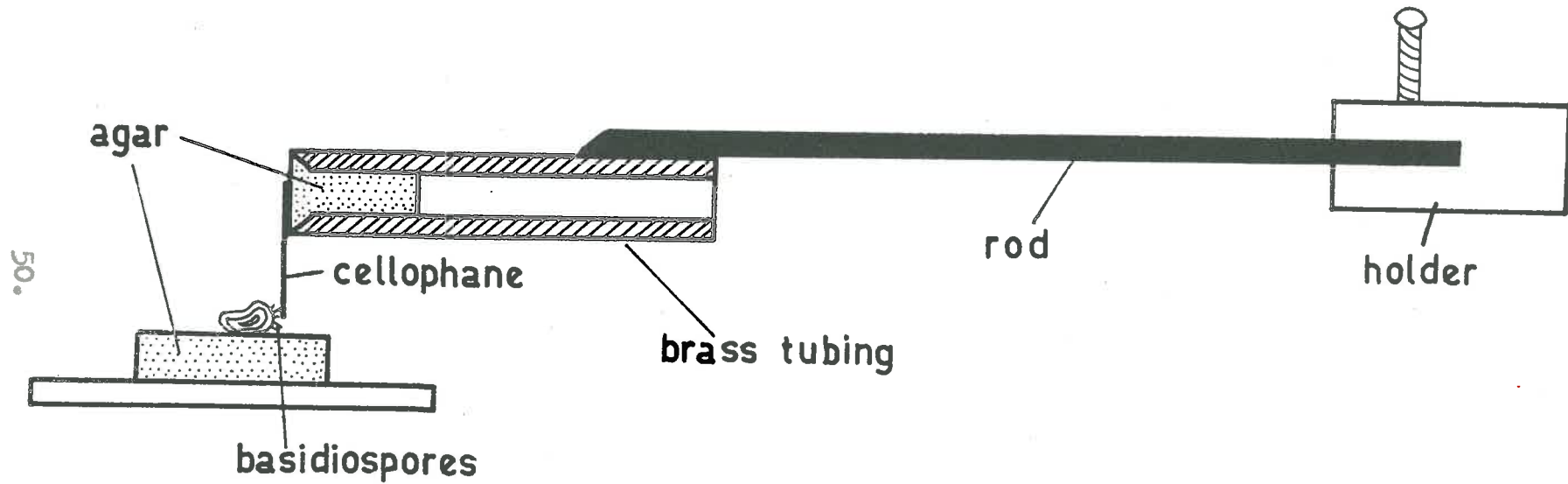


FIGURE 1. Diagram of apparatus for removing a tetrad of spores from a basidium

slowly lifted from the Cellophane, the spores separated from one another and remained on the Cellophane. After germination, the spores were transferred to individual Petri dishes.

Hypheal Anastomosis and Heterocaryon Formation

(a) By opposition

Isolates were opposed in pairs on Cellophane overlying either D.W.A. or soil agar in Petri dishes. Inocula, taken from peripheries of colonies of isolates actively growing on P.V.D.A., were placed 25mm apart on the Cellophane and incubated at 25°C until the two mycelia had come into contact. Anastomoses between paired cultures were usually hypha-to-hypha or hypha-to-peg. The anastomosis reaction between the cultures was determined by puncturing one of the two cells involved in a presumptive anastomosis and observing the other cell (Flentje and Stretton, 1964).

When functional cytoplasmic connections between cells of opposed hyphae had been demonstrated, other presumptive anastomoses were cut out and, together with the underlying Cellophane, transferred to P.V.D.A. plates. Each transfer consisted

of only the two opposed cells, all other cells having been punctured. Each pair was carefully observed and any new cells forming at the free extremity of either cell were punctured while new cells forming from the vicinity of the anastomosis were allowed to grow, to ensure that, if a functional anastomosis had occurred, the resulting colony would be heterocaryotic. This was a laborious method and not too successful, as many of the presumptive anastomoses did not form heterocaryons and a number of anastomosing cells died as a result of handling.

(b) By maceration

Isolates were grown in pairs by macerating together two pieces of inocula (2mm^3) with a sterile glass rod in a Petri dish and placing the macerate either as a unit or a streak on a fresh P.V.D.A. plate. Heterocaryon formation by this method was more reliable and less time-consuming. However, actual anastomoses between hyphae in the macerate were not observed.

Pathogenicity Tests

(a) Root strain

No reliable method for determining the extent

of pathogenic variation in the progeny of isolate 16 was developed. The basic methods consisted either of adding inoculum to a substrate at seed level or mixing it throughout the substrate, at the time of seeding. Wheat (Triticum vulgare, variety Gabo) was used as the reference host. Surface sterilized seeds were either germinated on agar before planting or planted directly. The substrates tested were vermiculite, perlite, sterilized sand, natural soil, and soil treated by aerated steam. Constant or fluctuating moisture levels in the substrate were obtained by adding water or a nutrient solution (Hoagland and Arnon, 1950) at varying time intervals to bring the substrate to constant weight. Experiments were carried out in an environment cabinet with a day length of 14 hours, light intensity of 2500 f.c., day temperature of 22°C, and night temperature of 20°C. Variation in disease reaction of a single isolate was observed in consecutive experiments using the same method and between replicates within the same experiment.

(b) Crucifer strain

Radish (Raphanus sativus, variety Long White)

101c1e) was used as the test host for the pathogenic reactions of the progeny of isolate 48 following the method of Plentje, Bodman and Kerr (1963) modified as described below.

Seedlings were grown in washed, coarse sand in earthenware pots placed in the environment cabinet and watered daily with nutrient solution. After one week, the seedlings were removed from the pots and washed free of sand in distilled water. Healthy seedlings with straight stems were laid on microscope slides, two seedlings per slide about 5mm apart, and secured with rubber bands about 10mm from each end of the slide. Free water was removed from around the seedlings and slide by touching with absorbent tissue. A piece of inoculum (25x5x2mm) cut from close behind the growing edge of a young culture was placed between the stems on each slide. Slides were then transferred to specimen jars (100x38x100mm), three per jar with the radish roots resting on filter paper moistened with nutrient solution. Jars were placed in the environment cabinet. The pathogenic reaction of an isolate to the radish stems was determined after four to seven days by microscopic examination.

Staining

Vegetative hyphae, basidia and basidiospores were stained with HCl-Giemsa (Robinow, 1945), adapted from the method described by Hrushovetz (1956). For vegetative hyphae a section about 15mm wide was cut through the radius of a colony growing on Cellophane over P.V.D.A.; this section, which included the inoculum plug, together with the underlying Cellophane was placed in a Petri dish lid for fixing and staining. For basidia and basidiospores, fructifications on small lumps of soil were teased apart, to free them from most of the soil particles, before fixing and staining.

The material was fixed in Carnoy's fixative (3:1 mixture of 95 per cent ethyl alcohol and glacial acetic acid) for 10 minutes, immersed in cold 1N. HCl for five minutes, hydrolyzed in 1N. HCl at 60°C for eight minutes, and washed for five minutes in three changes of distilled water. The material was then immersed in phosphate buffer for five minutes and placed in the Giemsa staining solution for 10 to 30 minutes for vegetative hyphae and up to 48 hours for fructifications. The time

in the stain depended on the age of the vegetative culture and the amount of soil adhering to the fructifications.

The phosphate buffer (0.1% KH_2PO_4 and 0.2% Na_2HPO_4) was adjusted to pH 6.8. The Giemsa stock solution contained 3.8gm Giemsa's stain powder in 125ml glycerine and 375ml methyl alcohol. The staining solution was made by mixing 20 drops Giemsa stock solution with 10ml phosphate buffer.

The material was removed from the stain, placed in buffer on a slide, and a cursory microscopic examination revealed whether the nuclei were clearly stained.

Vegetative mycelium usually was separated from the Cellophane in buffer on a slide by means of dissecting needles. The inoculum plug was then removed and the mycelium was cut and teased apart as required before mounting. Stained clumps of basidia and basidiospores were teased apart and transferred several times from one drop of buffer to another to free them from any remaining soil particles.

Temporary mounts were made in phosphate buffer and the coverslip sealed with a colorless nail

varnish. Care to ensure proper sealing gave mounts which were useful for up to three months.

Irradiation

Basidiospores were shed from fructifications on Cellophane over soil agar plus antibiotics and were immediately irradiated in situ in open Petri dishes. A Phillips brand TUV 15W germicidal lamp, with an intensity of 70.8 ergs per second per mm² at a distance of nine inches, was used as the source of irradiation. Most of the output was of wavelength 2,537Å. Immediately after irradiation, the Petri dishes were incubated at 25°C until the basidiospores had germinated or died.

EXPERIMENTAL

I. Development of Self-Fertile Lines in Thanatephorus cucumeris

Investigations of the mechanisms of variation require genetically pure individuals which form uni-nucleate cells and are capable of sexual reproduction at some stage of the life cycle. In T. cucumeris the basidiospores are the only known cells which may contain single nuclei. The following investigations were designed firstly to obtain genetically pure cultures through basidiospores and secondly to determine the mating system of the several 'solani' type field isolates.

(a) Root strain

(1) Isolate 16 and progeny

The general procedure used was to fruit isolate 16 and obtain a range of basidiospore cultures for study. The G1 cultures were then tested for fruiting and G2 progeny were collected from those which did fruit. This was continued to G5 progeny. The observations on the characteristics of basidiospores and single basidiospore progeny of selected lines arising from field isolate 16 are summarized in Table I.

TABLE I

**CHARACTERISTICS OF BASIDIOSPORES AND SINGLE BASIDIOSPORE PROGENY FROM
T. CUCUMERIS ROOT STRAIN ISOLATE 16**

Generation	Isolation Number	4-spored Basidia* %(of 100)	Basidiospores		Basidiospore Progeny		
			Uninucleate %(of 400)	Viability %(of 200)	Number Cultured	Cultural Variation	Self-fertile %
P	16	52	90	47	92	Wide	27
G1	-8	57	-	-	52	2 types	20
G2	-28e	54	85	45	91	Wide	15
G3	-28	-	-	-	14	Wide	16
G3	-64	59	90	35	101	Wide	58
G4	-85	-	-	22	11	Wide	-

* Remainder were 3-spored basidia

The frequency of three- and four-spored basidia was determined from active hymenia on clumps of soil. In each generation the four-spored basidia comprised about one half of the total. The remaining basidia were three-spored; approximately half of these produced four sterigmata, one of which did not give rise to a spore, and the rest produced only three sterigmata. Observations on the living material were confirmed in stained preparations. When three spores developed from a three-sterigmate basidium, one of the spores was binucleate. When three spores developed from a four-sterigmate basidium, again one spore was binucleate and the sterigma which did not produce a spore contained no nucleus, suggesting that the binucleate condition was due to the migration of two meiotic nuclei into one spore. In addition, occasional cases of one binucleate and three uninucleate spores arising from a single basidium which had produced four sterigmata were seen. Two basidia were observed, one from living and one in stained material of isolate 16, each of which had produced five sterigmata and five spores.

The percentage of viable basidiospores was determined 10 days after the spores had been shed. Twenty-two to 47% of the spores produced colonies, approximately 10-15% failed to germinate, and the remaining 38-68% formed germ tubes before growth ceased. A spore which had not germinated and produced one or two branches within six days rarely produced a vigorous colony.

Cultural characteristics of the single basidiospore progeny were recorded when each first subculture had developed to a diameter of 3-4cm. The characters included growth rate, color of mycelial mat, presence or absence of zonation, distribution and abundance of sclerotia, and presence or absence of tufts of aerial hyphae. Wide differences, particularly in growth rate, color, and zonation, were observed in the progeny of each isolate. In the progeny of isolate 16-8 there were two quite distinct types of growth habit which were described as 'smooth' and 'ringed'. The 'smooth' colony was similar to that of isolate 16, developing at a uniform rate of growth. The 'ringed' colony was characterized by an initially

slow growth rate with the production of a dense surface and subsurface mat. After several days when the colony diameter reached 2-4cm, growth appeared to cease and no increase in diameter was recorded for the next six to ten days; during this period the advancing hyphae curled back into the colony. New growth originated from branch hyphae and was not uniform, appearing more like numerous sectors arising from the dense central mat; this mycelial growth was sparse. The 'ringed' growth habit has remained stable in cultures maintained for over four years.

The fruiting ability of isolates through to the G₄ was extremely variable; most isolates, including all of the 'ringed' type, were not induced to fruit. Among the self-fertile isolates, there was considerable variation in regularity and amount of hymenial development; most isolates only occasionally produced small scattered wefts of basidia, while one or two isolates from each generation fruited profusely almost every time they were tested.

The extent of pathogenic variation in the progeny was not determined, due to the lack of a

suitable method.

The variation in cultural characteristics, fertility of progeny and viability of basidiospores even in G₄ isolates indicated that genetically pure lines had not been obtained. This failure could be due primarily to binucleate basidiospores, since if one assumes that the percentage of binucleate spores is similar in stained material and discharged spores, and that a binucleate spore is more likely to be viable than a uninucleate spore, then there is a bias toward progeny derived from binucleate spores. Many of the single spore cultures from each generation failed to fruit indicating that either there were sterility factors present or possibly that field isolate 16 was heterothallic. In either case cultures derived from binucleate spores would be more likely to fruit. The variation, then, in the progeny of any generation could be due to genetically different types of nuclei segregating, either in pairs or individually, in the basidiospores. The extent of this variation was not determined from tetrad analyses because only two (or rarely three) spores of any tetrad isolated were viable.

The percentage of binucleate spores could account for all the self-fertile progeny of each isolate except 16-8-28c-64, from which the number of self-fertile cultures is twice the probable number of cultures derived from binucleate spores. On the above basis, it was expected that about one-half of the fertile G4 cultures from 16-8-28c-64 had been derived from uninucleate basidiospores and that the progeny from any one of these cultures would be uniform. However, progeny from each of six cultures (isolation no. 85 and five others) were as variable as the progeny of the field isolate. It appears then, that the progeny from a G4 isolate were still variable, whether the isolate was originally derived from a uninucleate or a binucleate spore. One possible explanation of this is that isolate 16 and its progeny mutate readily and in the time between shedding of a spore and fruiting of the culture derived from that spore sufficient mutations had accumulated to account for the variation. There is, however, insufficient experimental evidence to support this or any other hypothesis.

Because of these complications, further investigations with isolate 16 and its progeny were suspended.

(11) Other field isolates

A number of isolates of the root strain were obtained from soils in different areas of South Australia by de Beer (1965) and of these, isolates 97, 98, 99 and 100 each readily formed the sexual stage in fruiting trials in 1965. The nuclear condition of 300-400 basidiospores was determined from stained material of each isolate; each produced almost exclusively uninucleate basidiospores (isolate 97, 98.8%; 98, 98.7%; 99, 98.7%; and isolate 100, 98.5%). A number of the G1 progeny of isolate 99 have been induced to fruit, suggesting it is homothallic, but further studies are required to provide the supporting evidence.

(b) Crucifer strain, isolate 48 and progeny

The work described in this section and summarized in Table II was done in collaboration with Miss Helena M. Stretton and Professor N.T. Plentje. The same general procedure as described for isolate 16 was used and isolates from each generation

TABLE II

CHARACTERISTICS OF BASIDIOSPORES AND BASIDIOSPORE PROGENY FROM T. CUCUMERIS
CRUCIFER STRAIN ISOLATE 48

Generation	Isolation Number	4-spored Basidia* %(of 100)	Basidiospores		Basidiospore Progeny			
			Uninucleate %(of 400)	Viability %(of 200)	Number Cultured	Cultural Variation	Pathogenic Variation	Self-fertile %
P	48	99	99	99	24	Narrow	None	65
G1	-11	100	-	30**	31	None	None	83
G2	-14	97	-	80	71	None	None	94
G3	-56	100	99	99	100	None	None	100

* Remainder were 5-spored basidia

** 48-11 count of 100

were selected for their ability to fruit regularly.

In all isolates the basidia regularly gave rise to four sterigmata, each producing a basidiospore. One five-spored basidium was observed in the initial count of 100 basidia from the hymenium of isolate 48, and three from 48-11-14. Two other five-spored basidia were noticed during scanning of later fructifications of isolate 48; no three-spored basidia were observed in any of the fructifications. Basidiospores of isolates 48 and 48-11-14-56 were almost exclusively (99%) uninucleate; no counts were made for isolates 48-11 and 48-11-14.

The cultural characteristics of the G1 progeny of isolate 48 were less variable than those of isolate 16, the variation being limited to color of mycelium and distribution and abundance of sclerotia; no cultural variation was observed in the progeny of successive generations.

All cultures derived through successive generations from isolate 48 were pathogenic to stems of radish seedlings, causing seedling death within four or five days.

Subsequent to the published data (Flentje and Stretton, 1964) about two-thirds of the G1 progeny

have been induced to fruit. Isolate 48-11 developed one discrete hymenial patch on the soil in one of four plates and from this patch basidiospores were shed and a number of prebasidial cells isolated. Only 30% of the spores were viable and 83% of the resulting single spore cultures were fertile. All the colonies arising from the single prebasidial cells of 48-11 fruited prolifically whereas the stock culture of isolate 48-11 could not be induced to fruit when tested again. With successive generations the percentages of basidiospore viability and self-fertility increased to a maximum in the fourth generation.

Anastomosis reactions of paired cultures were determined for various combinations of progeny: cell death following anastomosis occurred between 48-11 and isolate 48, other G1 isolates, 48-11 prebasidial cell cultures and G2 isolates (48-11 single spore progeny), and between paired G2 isolates; successful anastomoses with no cell death occurred between two subcultures of any single isolate, between 48-11 prebasidial cell cultures paired one with another and with isolate 48, between paired G3

isolates and between G4 isolates paired one with another and with isolate 48-11-14-56.

The progeny of a self-fertile single basidiospore culture (i.e. 48-11-14-56) were uniform in cultural characteristics (Plate 2), pathogenicity, fertility, and ability to anastomose with one another, indicating that the culture was homothallic and had been derived from a uninucleate basidiospore. It was therefore concluded that isolate 48 of the crucifer strain was homothallic.

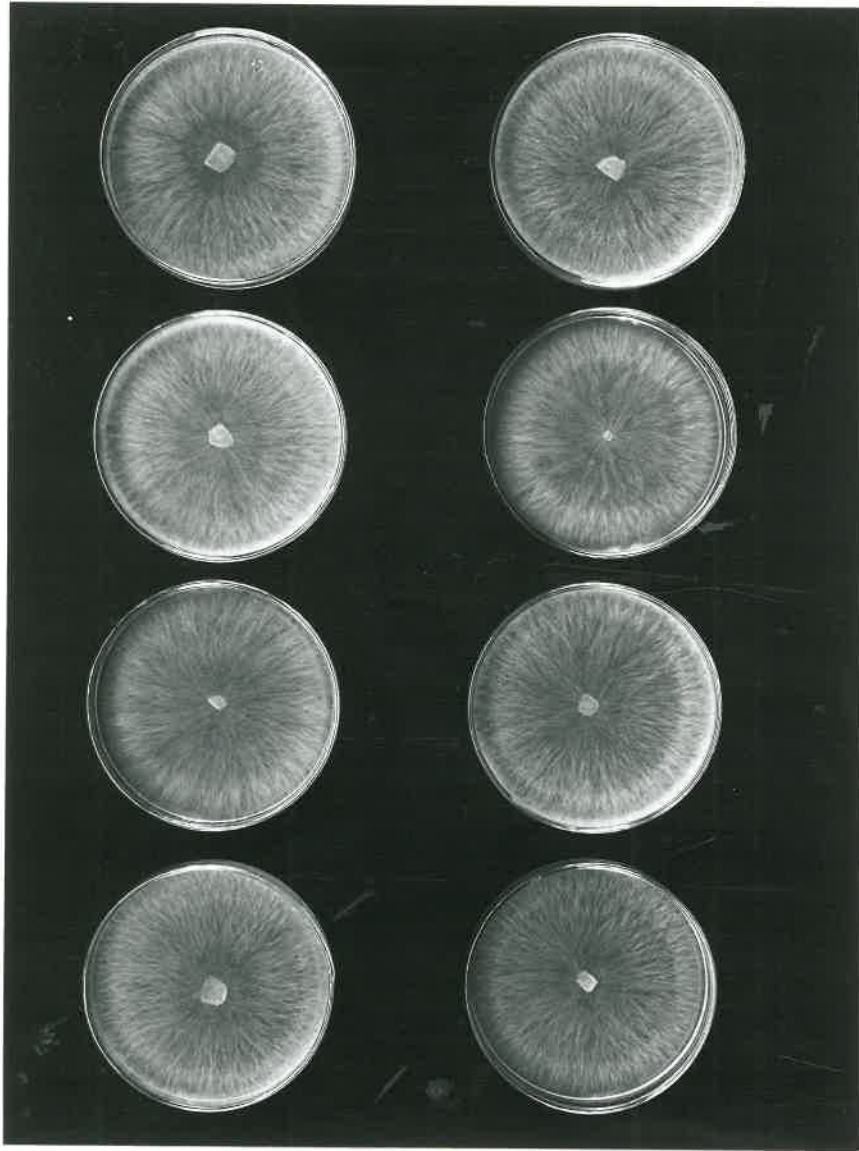
(c) Discussion

Observations on the number of basidiospores per basidium, the nuclear condition and viability of basidiospores, and the cultural variation of progeny of isolates 16 and 48 confirmed preliminary reports on G1 cultures (Flentje et al., 1963; Flentje and Stretton, 1964). The investigation of successive generations has provided important new information.

It appears from the results with G4 cultures that isolate 16 may be homothallic, but because of the complication of binucleate basidiospores and the considerable variation of the progeny in all

PLATE 2

Progeny of P56,
showing uniform cultural appearance



generations, the possibility of secondary homothallism has not been completely eliminated. Flentje et al (1963) reported that aberrant nuclear behaviour in the formation of basidiospores is typical of certain isolates; the present findings suggest that in isolate 16 this behaviour is inherited unchanged through four generations. No such aberrancy was observed in four other field isolates of the root strain of I. cucumeris, 'solani' type, so it cannot be regarded as a characteristic of the root strain.

The investigations provide conclusive evidence of the homothallic nature of isolate 48, and in parallel studies (Flentje and Stretton, unpublished) homothallism has been demonstrated in isolate 69, another isolate of the crucifer strain. The results of the fruiting experiments show that sterility factors, as suggested by Kotila (1929) and Flentje and Stretton (1964), do occur. The behaviour of 48-11, where one discrete hymenial clump arose on one plate in a fruiting trial could be explained by a mutation from sterility to fertility. This hypothesis is supported by the following evidence:

(a) other stock subcultures of 48-11 could not be

induced to fruit; (b) all cultures arising from single prebasidial cells from the hymenial clump in 48-11 formed prolific hymenial layers; (c) anastomosis between individual prebasidial cell cultures of 48-11 and stock subcultures of 48-11 gave a killing reaction whereas those between prebasidial cell cultures or between stock subcultures did not result in cell death; and (d) 83% of the progeny of 48-11 were fertile which suggests that the mutation was inherited. It is concluded from the number of G1 single spore cultures which have fruited during the course of these investigations that isolate 48-4, studied by Flentje and Stretton (1964), probably did not arise from a binucleate basidiospore as was suggested in the Literature Review.

In his list of heterothallic Hymenomycetes and Gasteromycetes, Whitehouse (1949) included certain species twice when they were recorded as bipolar by one person and tetrapolar by another. Takamaru (1961) suggested that the discrepancies between different investigations were due to lack of understanding of the mechanisms involved; he found that all isolates of a given species have a

similar mating system. Following Takemaru's suggestions, if Thanatephorus cucumeris is a single natural species then any isolate of T. cucumeris will be homothallic and the binucleate condition of basidiospores is not an expression of secondary homothallism. However, it is not clear at this stage that T. cucumeris is a single natural species and it would be preferable to investigate the mating system for many more field isolates until a more substantial background is developed.

II. Nutritional Studies

Experiments were designed to compare the growth of different isolates of T. cucumeris, and in particular to determine (a) whether a simple chemically-defined medium suitable for use in genetical studies could be developed; and (b) what nutritional factors, if any, control the growth habit of the 'ringed' progeny of isolate 16-8.

(a) Results

None of the preliminary experiments, initiated by Arnold (W.N. Arnold, unpublished) and extended during the course of these investigations, has been presented here. However, these experiments

disclosed that at 25°C the yields of isolate 16 on the synthetic minimal medium (described in Methods and Materials) and on P.V.D. were not significantly different, and no significant increase in yield was obtained by an increase in any constituent of the synthetic medium.

(1) Growth of 'ringed' type progeny of 16-8

Growth of 16-8-4 ('ringed') was significantly less than that of either 16-8-10 ('smooth') or 16 on any carbon source (Table III). Six sugars — sucrose, maltose, lactose, glucose, ribose, and xylose — were used as sole carbon sources (C equivalent 3.6g per liter) in the synthetic medium; two other levels of sucrose, half concentration (C = 1.8g) and twice concentration (C = 7.2g) were also tested. Inoculum consisted of plugs from the edge of colonies growing on the solidified synthetic medium.

The influence of the source of nitrogen on growth was studied (Table IV). In this experiment the inoculum was from colonies growing on Cellophane over the solidified synthetic medium. Potassium nitrate, ammonium sulphate, caseamino acids (Difco,

vitamin-free) and peptone (Difco, proteose peptone) were substituted for ammonium nitrate as nitrogen source (N equivalent 0.56g per liter) in the synthetic medium. The yield from 16-8-4 was significantly less on any nitrogen source as compared with the yield of isolate 16, 16-8, or 16-8-10.

The data in Tables III and IV indicate that although isolates 16, 16-8 and 16-8-10, which are similar in cultural characteristics, differ significantly in yield when grown on the different carbon or nitrogen sources, the differences are of a small order. Isolate 16-8-4, however, produces only half (or less) the yield of the above isolates when grown on the same medium. In no case did the yield of 16-8-4 approximate that of 16 and it seems unlikely that the relatively low yield of 16-8-4 is due to simple C-N nutrition. Use of the vitamin supplement (described in Materials and Methods) did not significantly alter the yield of isolate 16-8-4.

(11) Comparison of root and crucifer strain isolates

The results of a typical experiment (Table V) summarize the main differences in the nutrient requirements of the root and crucifer strain isolates. The inocula were from cultures of isolates

TABLE III

GROWTH OF ROOT STRAIN ISOLATES OF T. CUCUMERIS ON A
SYNTHETIC MEDIUM WITH DIFFERENT SUGARS
AS CARBON SOURCE

Carbon Sources	Yield dry weight (mg)*		
	16	16-8-10	16-8-4
Sucrose	132.0	129.0	36.75
Maltose	116.0	99.0	30.25
Lactose	63.5	56.25	19.0
Glucose	124.25	107.5	59.0
Ribose	44.0	37.25	12.25
Xylose	79.5	71.75	24.25
Sucrose ($\frac{1}{2}$ x conc.)	84.5	77.5	22.25
Sucrose (2x conc.)	138.25	124.75	48.0

* Mean of four replicates after 13 days growth

L.S.D. 5% = 9.92

1% = 13.16

0.1% = 17.07

TABLE IV

GROWTH OF ROOT STRAIN ISOLATES OF T. CUCUMERIS ON A
SYNTHETIC MEDIUM WITH DIFFERENT NITROGEN SOURCES

Nitrogen Source	Yield dry weight (mg)*			
	16	16-8	16-8-10	16-8-4
Ammonium nitrate	157.5	142.0	150.0	53.25
Potassium nitrate	122.5	114.5	109.5	37.75
Ammonium sulphate	121.25	117.75	115.5	43.75
Casamino acids	165.0	153.75	163.25	57.5
Peptone	183.25	168.0	175.25	63.5

* Mean of four replicates after 16 days growth

L.S.D. 5% = 9.28

1% = 12.34

0.1% = 16.05

TABLE V

GROWTH OF ROOT STRAIN AND CRUCIFER STRAIN ISOLATES
OF L. CUCUMERIS ON LIQUID MEDIA

Medium	Yield dry weight (mg)*			
	Root strain		Crucifer strain	
	16	16-8	48	48-11
Synthetic medium	175.7	157.8	18.4	14.6
Synthetic + vitamins	175.0	157.4	21.8	18.0
Synthetic + peptone - ammonium nitrate	200.6	184.6	221.4	233.4
Potato-Vegemite- Dextrose	181.6	166.6	222.4	223.2

* Mean of five replicates after 16 days growth

L.S.D. 5% = 12.14

1% = 16.13

0.1% = 20.96

16, 16-8, 48, and 48-11 growing on Cellophane over soil agar. None of the isolates showed a vitamin requirement. The outstanding difference between these isolates of the root and crucifer strains was the much poorer growth of the crucifer strain on synthetic medium with ⁱⁿorganic nitrogen, whereas when supplied with peptone as the nitrogen source at the same N equivalent the yield was increased tenfold and was significantly better than the root strain. The restricted ability of the crucifer strain to use inorganic nitrogen was also evident when it was subcultured on solidified synthetic medium. The first subcultures of isolates of the crucifer strain failed to grow on this medium whereas isolates of the root strain maintained active growth for more than three months of weekly serial subculturing.

Results of other experiments indicated that the growth of the crucifer isolates in response to peptone as the sole nitrogen source was directly proportional to added nitrogen and that casamino acids were less effective on a nitrogen basis than peptone; no artificial mixture of individual amino acids was found which compared with peptone as a nitrogen source.

(b) Discussion

Results of preliminary experiments with field isolates of T. cucumeris agree with the earlier reports of requirements by isolates of Rhizoctonia solani for potassium, phosphorus, magnesium and trace elements (Tyner and Sanford, 1935; Steinberg, 1950). There was no indication of vitamin requirements by the isolates under the experimental conditions employed.

The experiments suggest that growth of the 'ringed' isolates, G2 progeny of the root strain isolate 16, is not due to simple nutritional factors. While significant increases in yield of 16-8-4 were recorded with glucose replacing sucrose as carbon source and with peptone replacing ammonium nitrate as the source of nitrogen, no medium tested supported yields of 16-8-4 which approached that of the field isolate 16 on the synthetic minimal medium. Yields of progeny which were similar to isolate 16 in cultural characteristics were equal on P.V.D. and the synthetic minimal medium. However, individual isolates differed in ability to utilize the various carbon or nitrogen sources, indicating heterogeneity in the progeny additional to that of cultural

appearance and self-fertility.

Crucifer strain isolates required peptone as the nitrogen source to obtain growth equal to that on P.V.D. However, peptone may contain something in addition to organic nitrogen which is necessary for growth since the amount of growth by the crucifer isolates was not duplicated on artificial mixtures of amino acids. No chemically-defined medium was found suitable as a minimal medium for use with isolates of the crucifer strain.

III. Isolation and Characterization of Mutants of *T. cucumeris*

In the preliminary experiments it was not possible to obtain genetically pure lines of the root strain isolate 16, but such lines were readily obtained from isolate 48 of the crucifer strain. It was therefore decided that the main genetical studies should be concentrated on isolate 48 and its progeny. However, as isolate 48 would not grow on media without an organic nitrogen supply, a chemically-defined minimal medium was not developed. For the initial genetical studies, as described below, attention was concentrated on morphological

and pathogenicity mutations.

The single basidiospore culture of the crucifer strain, isolate 48-11-14-56, subsequently referred to as P56 because it is the parent isolate from which all mutants were derived, was selected. P56 is homothallic, indistinguishable from the wild type field isolate 48, self-fertile producing viable uninucleate basidiospores and uniform progeny, and anastomoses with its progeny and with other G3 cultures. In addition to P56, a non-pathogenic field isolate 82, which fruits on soil agar and is homothallic (Plentje and Stretton, 1964) was used as parental material.

(a) Results

(1) Induction of mutants

Different levels of ultraviolet irradiation were required for P56 and isolate 82 to give approximately 95% killing of basidiospores with the expectation of several per cent mutants among the survivors. The intensity for 82 was between 2,147 and 3,717 ergs per mm^2 and for P56 between 10,620 and 16,992. At the lower intensity for each isolate, about 50% of the basidiospores

died within 12 hours of irradiation, another 30% were viable but germination was retarded, and the remainder had neither germinated nor collapsed after 14 days. At the upper levels of intensity, about 50% of the spores had collapsed and died within 12 hours, and of the remainder, a few germinated but most remained dormant; none formed colonies. At intensities of 3,186 ergs per mm² for isolate 82 and 12,744 for P56, 50% of the spores again died within 12 hours, another 40 to 45% did not germinate, but collapsed and died over a period of five weeks, and the balance formed colonies but some colonies died before they exceeded 20 to 30mm in diameter.

Basidiospores were shed from fructifications to single Petri dishes for periods of up to one hour to provide heavy concentrations of spores for irradiation. These time intervals were sufficient for mitosis to have occurred in those spores which were shed at the beginning of the periods. Binucleate spores did not complicate the isolation of irradiated mutants and would have been screened out by virtue of growth habit. A mutant culture would develop from a binucleate spore only if both nuclei

mutated and the mutants were at the same locus in each nucleus or if one nucleus mutated and the second nucleus was inviable. Any other combinations of mutated and non-mutated nuclei within single binucleate spores would result in wild type colonies or spore death.

Almost every basidiospore in the non-irradiated controls was viable and the progeny from each isolate were uniform in cultural characteristics.

None of the single spore cultures which developed from irradiated basidiospores of isolate 82 showed variation in cultural appearance and self-fertility.

From irradiated basidiospores of P56, 150 colonies were recovered including 37 which appeared to be mutants. Many of the mutant colonies sectored, reverted to wild type or died. All wild type colonies were similar in cultural appearance and pathogenicity, and each was self-fertile. Four stable mutant colonies (sparsa, stumpy, fleecy, and curly), each distinct in morphology, are described below.

(11) Spontaneous mutants

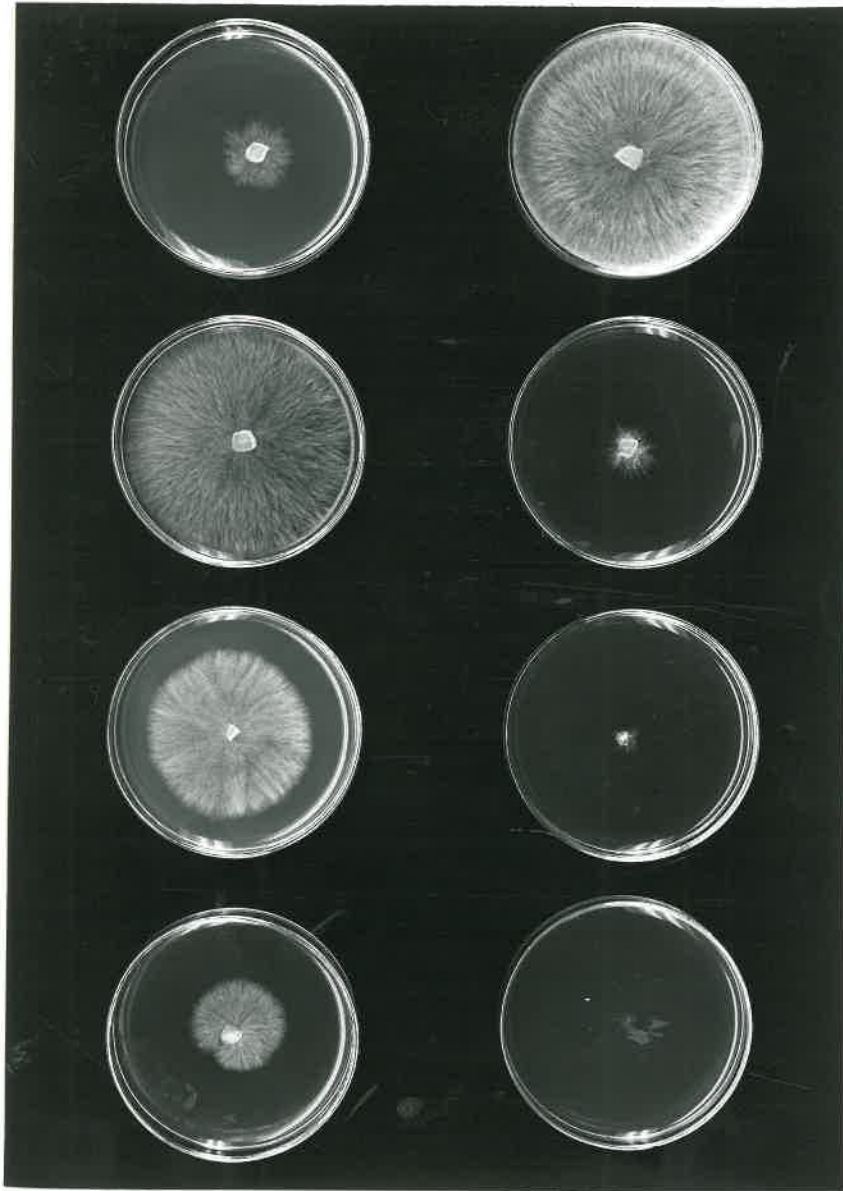
Single basidiospore cultures from P56 isolated from the initial fruiting trials were similar

in cultural appearance (see Plate 2), pathogenicity and self-fertility. Three months later, after weekly serial subculturing on P.V.D.A., a culture of P56 was fruited, and a sample of 50 shed basidio-spores was isolated prior to further irradiation trials for additional mutants. However, these single spore cultures varied in growth rate and cultural appearance (Plate 3). Two stable and morphologically distinct single spore cultures (rusty and ropy) were selected for further study and each is described below.

The variation in progeny of P56 revealed that in the three month period of vegetative subculturing, spontaneous mutation had occurred and that P56 was no longer homocaryotic. Comparisons were made between the first P.V.D.A. subculture from the stock culture, the twelfth serial subculture and the original description of isolate P56, and each was indistinguishable in appearance, growth rate, pathogenicity and ability to fruit, indicating that accumulation of mutant nuclei in the mycelium of P56 did not visibly alter its characteristics.

PLATE 3

Progeny of P56,
showing variability in cultural appearance
resulting from spontaneous mutation



(iii) Description of mutants

The main characteristics of each mutant, together with the parent P56 (Plates 4 and 5) are given in tabular form in Tables VI, VII, and VIII. Further details of cultural characteristics are given in the text. Growth is expressed as the average colony diameter after six days on F.V.D.A. at 25°C. Hyphal branching and septation were determined by microscopic examination of living and stained material. The number of nuclei per cell were counted in three types of cells (Table VII), namely: (a) hyphal tips at the periphery of an actively growing colony ('peripheral tip cells'); (b) cells other than tip cells, three to five days old and adjacent to the inoculum plug ('older non-tips'); and (c) the terminal cells of branch hyphae which arose adjacent to the inoculum plug ('branch tips'). Other counts (Table VIII) were made of nuclei in consecutive cells from the peripheral tips.

P56 is indistinguishable from the field isolate 48. The wild type growth is typically racemose branching with normally one branch developing per cell, the newest branches being closest to

PLATE 4

Cultures of isolate 48, P56 and mutants

[Left to right]

Top: isolate 48 P56

Center: sparse stumpy fleecy curly

Bottom: rusty ropy

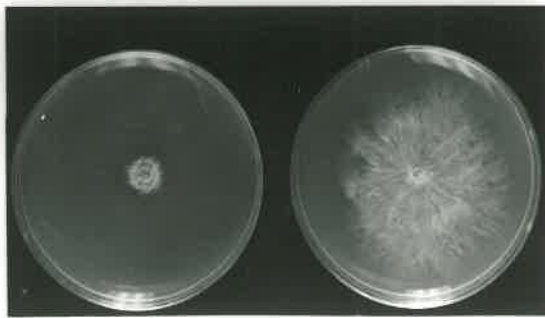
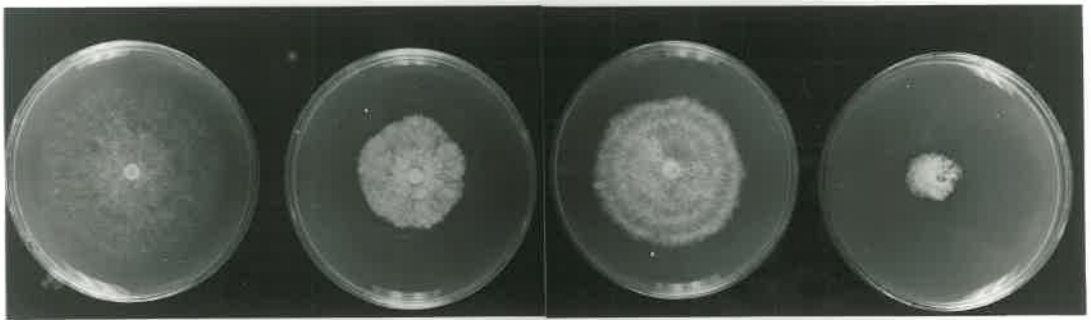
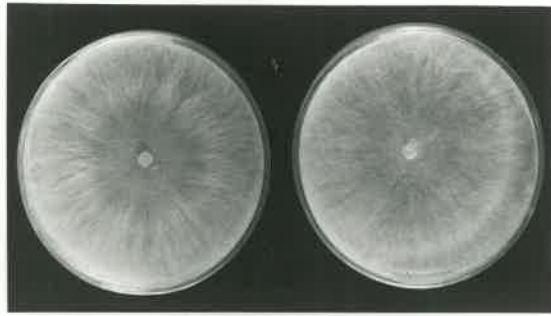


PLATE 5

Hyphae of P56 and mutants

[Left to right]

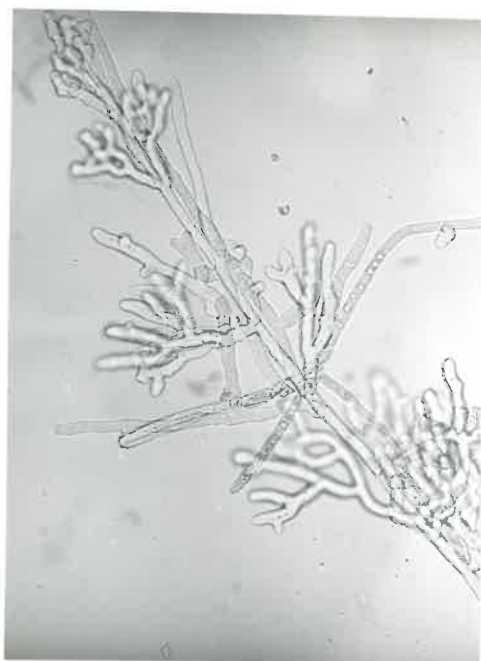
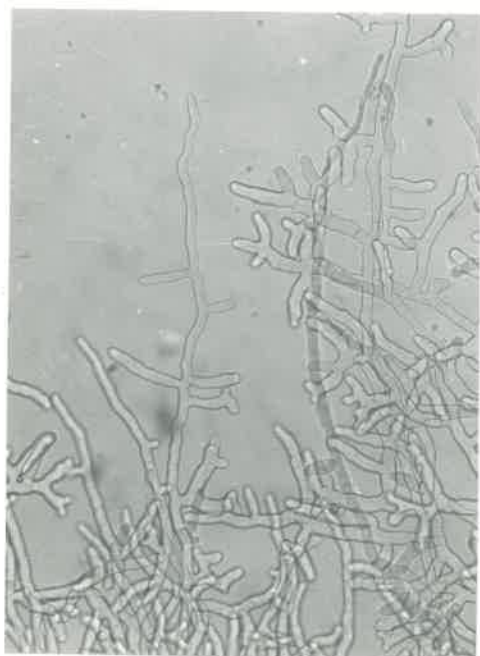
Top: P56 sparse
 (x60) (x160)

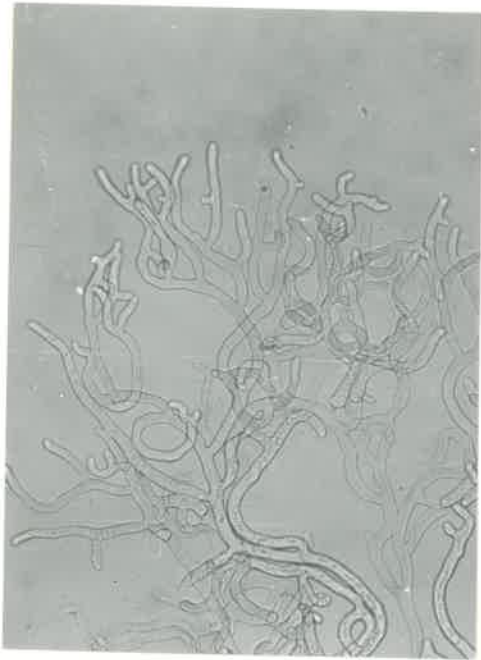
Bottom: stumpy fleecy
 (x160) (x160)

[Continued on 2nd page]

Top: curly rusty
 (x160) (x160)

Bottom: ropy
 (x60)





the hyphal tip. A septum is formed in a dividing cell in the region of and immediately following the mitotic nuclear division, and separates the two groups of daughter nuclei of that division. Surface mycelium extends beyond the subsurface mycelium in a growing colony.

Sparse is similar to P56 in branching and septation, but the growth is much less dense than that of P56 and the surface and subsurface mycelium grow equally at the periphery of the colony.

Stumpy appears banded around its periphery due to the subsurface mycelium extending beyond the surface mycelium. One to four side branches, each one to three cells in length, develop from each cell of the main hyphae and the tips of the branches are often swollen, appearing club-shaped. Septation is regular but the cells are shorter than those of P56.

The growth habit of flescy is superficially similar to that of Rhizopus nigricans. Advancing colony growth is by unbranched, aerial 'runner' hyphae which curve back to the agar surface ahead of the colony and grow for a short distance along the agar. Where a runner hypha touches the agar

it branches prolifically producing 'feeder' branches and subbranches, each one to four cells long, growing along the surface of and into the agar. Many new runners develop from the feeder hyphae, and a dense colony is produced. Cells of the runner hyphae are 60-100 times longer than wide, whereas cells of the feeder hyphae, which are four to eight times wider than the runner hyphae, are only 5-15 times longer than wide. Runner hyphae take up the HCl-Giemsa stain more readily than do feeder hyphae to the extent that when the nuclei in the feeder hyphae are slightly understained the nuclei in many cells of the runner hyphae are overstained and the cell contents of some cells are obliterated by overstaining.

The growth of curly is characterized by loss of apical dominance, and as a consequence there are no typical leading hyphae. Many terminal branches have forked tips and the radial extension of growth is transferred from branch to branch. Self-anastomosis between the apex and base of single tip cells is common as is anastomosis between tip cells and between tips and branches. The branches are curled or twisted in spirals and the curling is

most pronounced in aerial hyphae. White to hyaline chains of barrel-shaped cells (monilioid bodies) regularly develop as extensions of the aerial hyphae which curl together in tufts. Septation is irregular. In some instances a septum is laid down forming a cell which is wider than long and normally contains from 0-7 nuclei with very little cytoplasm. In other instances septa do not form and a single cell may consist of many branches, subbranches and anastomosed branches, and contain from 6-50 nuclei, usually in groups of 6-9. It appears that nuclear division is regular but the control of septation is disrupted. In one hypha the numbers of nuclei in each of five consecutive cells were 15, 43, 3, 18, and 29; the first and last two cells were multibranched, curled and anastomosed structures whereas the third cell was wider than long. Due to the growth habit of this isolate, the counts of nuclei per cell were not divided into the three groups as with the other mutants, but the range in number of nuclei was similar in young cells at the periphery and in older cells near the inoculum plug.

Rusty is superficially similar to curly in cultural appearance, and growth occurs in two stages.

Leading hyphae, usually unbranched, radiate along the surface of the agar for distances up to 3mm from the colony. Apical growth then terminates and branching and aerial growth begins from the base of the leading hypha, extending towards its apex. The aerial hyphae are as curled as those of curly and sclerotial development is similar. The surface and subsurface branches show a slight tendency to curl and as they develop the area between adjacent leading hyphae becomes filled in with dense growth. Branching begins at the base of a leading hypha and extends to its apex; septation is regular. New leading hyphae then develop from branch cells or rarely as extensions of the previous leading hyphae.

The leading hyphae of ropy grow out from the colony as surface or subsurface mycelium, usually with considerable distance between the hyphae. The short side branches (1-5 cells in length) develop profusely from a leading hypha as it extends, giving the appearance of rope-like strands; very little mycelial growth occurs away from or between the strands.

Counts of number of nuclei per cell of the above isolates revealed some hitherto unsuspected variation. The differences between means within isolates and between means of corresponding cell types and totals between isolates (Table VII) were analysed (t-test) and all differences were significant at the 1.0% level except the following: peripheral tips and older non-tips of ropy (5.0% level), older non-tips and branch tips of sparse and of ropy, peripheral tips between sparse and fleecy, older non-tips between sparse and fleecy, branch tips between sparse and ropy, and between fleecy and rusty, and totals between sparse and fleecy (5.0% level), between sparse and ropy, and between P56 and fleecy. The hyphal tips at the periphery of a colony contained greater numbers of nuclei than either the non-tips or the tip cells of hyphae from an area of the colony which was three to five days old, but there was no relationship between the nuclear numbers of the non-tip cells and the branch tips. The non-significant difference between the total means of sparse and ropy or of P56 and fleecy was due to the balancing effect of

the means of the three types of cells making up the totals. No obvious relationship exists between the number of nuclei per cell and other observed characteristics of any isolate except that of irregular septation of curly.

All counts of nuclear numbers for each isolate were from the same stained material; the 100 cells counted to give the mean number of nuclei in peripheral tip cells of each isolate (Table VII) included the 20 tip cells of Table VIIIa and 80 others, and the differences in mean between Tables VII and VIIIa for P56 and stumpy are considered as sampling errors. The distribution of the mean number of nuclei in consecutive cells of each isolate (Table VIIIa) was analysed for linear, quadratic, and cubic trends; the only positive result was a cubic trend significant at the 0.1% level in curly. Table VIIIb shows the types of distribution patterns of nuclei in individual hyphae of P56. The same sort of variation within and between hyphae was recorded in each mutant. In addition to the counts made of nuclear numbers, about 300-400 hyphae of each isolate were scanned

TABLE VIII

NUMBER OF NUCLEI IN CONSECUTIVE CELLS FROM THE TIPS
OF HYPHAE OF P56 AND MUTANTS

(a) Mean number of nuclei (and range)

Isolate	Nuclei per cell*						
	Tip Cell	2nd	3rd	4th	5th	6th	7th
P56	10.1 (7-15)	9.6 (7-14)	9.6 (8-12)	9.4 (7-14)	10.1 (7-14)	10.2 (7-15)	9.8 (7-14)
<u>sparse</u>	7.5 (5-9)	8.4 (6-11)	8.6 (7-11)	8.3 (6-11)	7.7 (6-11)	8.2 (6-11)	8.3 (6-11)
<u>stumpy</u>	9.0 (6-12)	9.6 (7-13)	9.9 (7-14)	10.1 (5-13)	9.6 (6-12)	9.8 (5-14)	9.9 (5-12)
<u>fleecy</u>	7.7 (3-12)	8.4 (6-12)	8.4 (6-11)	7.6 (5-10)	8.2 (6-10)	7.9 (6-12)	8.2 (6-11)
<u>curly**</u>	12.7 (5-29)	12.4 (2-43)	9.4 (1-25)	12.1 (0-27)	14.4 (7-29)	15.4 (3-28)	10.0 (1-20)
<u>rusty</u>	6.4 (3-9)	7.6 (4-14)	7.3 (5-9)	6.9 (4-13)	7.8 (4-14)	7.4 (5-15)	7.8 (5-11)
<u>ropy</u>	7.2 (4-10)	7.6 (5-10)	8.7 (5-18)	8.8 (5-18)	9.1 (5-14)	7.9 (6-12)	8.1 (6-13)

* Mean of 20

** Mean of 11

TABLE VIII

(b) Number of nuclei per cell in
five individual hyphae of P56

Hypha No.	Number of nuclei per cell						
	Tip Cell	2nd	3rd	4th	5th	6th	7th
1	15	11	10	7	7	11	11
5	8	8	8	8	8	8	7
6	12	11	9	12	14	15	14
8	7	7	8	8	9	7	13
11	10	10	11	11	11	11	11

and in stumpy, 15 hyphae were observed which each contained a nucleus wedged in the septal pore between either the third and fourth or fourth and fifth cells; none of the cells of these hyphae had burst which suggests that the nuclei either were in the process of migration from one cell to the other at the time of fixing and staining, or had been trapped in the pores as the septa formed.

Nuclear division in tip cells of P56 appears to be conjugate. In one stained preparation of mycelium of P56, the nuclei in nearly all the tip cells had divided but septation had not occurred. The number of nuclei per cell were counted in tip and penultimate cells of each of 25 hyphae; in each hypha selected the branch developing from the penultimate cell had not grown sufficiently for a mitotic division of the nuclei of the penultimate cell to have occurred. The tip cells contained even numbers of nuclei (14-28) of approximate diameter of 1.6μ while the penultimate cells contained 7-14 nuclei of about 3.0μ diameter. The size and even number of nuclei in the tips indicate that all nuclei in each cell had divided. However, the

observed frequencies and relationships of nuclei in the tip cells to 'n' nuclei per cell in the corresponding penultimate cells are as follows: $3(2n)$, $10(2n+2)$, $5(2n+4)$, $6(2n+6)$, and $1(2n-8)$.

The pattern of division and separation of the nuclei in the division prior to the one actually in progress at the time of fixing can be determined by dividing the number of nuclei per tip cell by two and comparing with the number in the penultimate cell of the same hypha, and is based on the assumption of no nuclear migration after septation between the tip, penultimate and third cells. On this assumption, only three of the 25 divisions would have included all nuclei of the cell followed by even segregation, and there were eight in which one nucleus had not taken part in division, seven in which there was unequal segregation of the daughter nuclei and seven in which both abnormalities occurred together. This would mean that in 15 out of 25 divisions, one nucleus had failed to divide and in 14 divisions there was an abnormality in segregation of daughter nuclei. However, as stated above it appeared that, in the divisions in progress at the time of fixing, all nuclei in each of the 25

cells were taking part; in phase contrast studies of P56 mycelium, in only a small percentage of divisions had a nucleus failed to participate in the division. Thus it seems that the assumption of no nuclear migration between cells is incorrect.

(iv) Stability of mutants

The growth rate and cultural appearance of each mutant and P56 were compared on a number of different agar media. None of the isolates grew on media containing only inorganic nitrogen with or without the vitamin supplement. The growth habit of each isolate on soil agar or media containing organic nitrogen was similar to that described on P.V.D.A. and although the growth rates of the isolates varied slightly on the different media the relative rates of growth were the same. No differences from the original descriptions of the mutants have been observed after 15 months, either from stock cultures or cultures maintained on P.V.D.A. and subcultured regularly.

Each mutant and P56 was tested for pathogenicity on the seedling stems of tomato (Lycopersicon esculentum Mill.), lettuce (Lactuca sativa L.), and subterranean clover (Trifolium

subterraneum L.), and none was pathogenic to or produced infection cushions on any of these reference hosts.

(b) Discussion

Since variation of field or single basidiospore isolates in laboratory culture rarely has been recorded (Kernkamp et al, 1952; Flentje and Stretton, 1964) it has been generally assumed that the mutation rate in T. cucumeris is extremely low. However, the fact that P56 yielded uniform progeny at one stage and three months later yielded a number of mutant progeny indicates (a) that mutation certainly occurs in some isolates, and (b) that a homocaryotic culture derived from a uninucleate basidiospore may rapidly become heterocaryotic through such spontaneous mutation. As there was no difference observed between homocaryotic and heterocaryotic colonies of P56 it appears that the wild type growth habit is stable and dominant. However, because the vegetative cells are multinucleate and nuclear division is conjugate, mutant nuclei could be carried forward in vegetative hyphae but would only be expressed in the progeny of sexual reproduction.

This would readily account for the lack of variation and sectoring in various field isolates of T. cucumeris.

In contrast to P56, no spontaneous or induced mutant cultures were recovered from basidiospores of the non-pathogenic isolate 82. The factors which control cultural appearance, growth rate and self-fertility in this isolate either are not readily mutable or were not expressed under the conditions of these experiments.

Basidiospores of P56 were four times more resistant to ultraviolet irradiation than those of isolate 82, but mutants were recovered only from P56. Each of the four induced and two spontaneous mutants selected for further study had a distinct set of cultural characteristics and a particular pathogenicity reaction with radish stems and each has remained stable and self-sterile.

Flentje (1957) developed the hypothesis that there are stages in the establishment of effective invasion of the host by T. cucumeris and that the process may fail at one or other of four stages, namely: (a) no attachment of the hyphae growing

loosely over the host to the surface of the host tissue; (b) no infection cushion formation, or cushions formed but loosely attached to the tissue surface; (c) failure of infection pegs to penetrate the surface of the tissue; and (d) occurrence of a hypersensitive reaction immediately after penetration, resulting in small localized necrotic lesions and death of the invading hyphae. The pathogenic reactions of the six mutants provide support for this hypothesis and suggest that the different stages may be under separate genetical control. Sparse is blocked at stage (a), ropy at (b), rusty at (b) or (c), and curly at (d). Fleecy is not blocked at any stage and progressively invades the stem tissue, causing seedling death. The reaction of stumpy suggests there may be another stage where failure can occur, namely, inhibition of growth of the hyphae by the host prior to contact between hyphae and host tissue.

The data suggest that pathogenicity in the crucifer strain is not a function of the wild type growth rate and habit; sparse, with growth rate and cultural appearance approaching the wild type,

does not form cushions, whereas fleecy which is slower growing than and culturally distinct from the wild type, after making contact with the seedlings, infects and kills them as rapidly as P56.

Analyses of the data from cytological studies revealed that P56 and each mutant behave independently with respect to number of nuclei per cell in three different types of vegetative cells (Table VII). These results are at variance with those of Sanford and Skoropad (1955) on two isolates of different strains of Rhizoctonia solani. Analysis of their data (t-test) shows that the mean number of nuclei per cell of each cell type did not differ between isolates and that the mean numbers in hyphal tips are significantly less than those in other cell types. The irregular distribution of nuclei in six successive cells from the tip (Table VIII) corresponds to figures presented by Boidin (1958) and Flentje et al (1963) for isolates of T. cucumeris. However, this distribution is not easily accounted for simply on the basis of uneven nuclear segregation or the failure of single nuclei to divide during the formation of new cells,

as believed by Flentje et al., and raises again the important question of nuclear migration. Comparison of nuclear numbers in tip and penultimate cells of hyphae of P56, together with observations of nuclei wedged in septal pores between cells of non-damaged hyphae of stumpy, suggests that nuclear migration may well occur. Saksena (1961b) previously had suggested this because he found nuclei wedged in septal pores in stained material and Sanford and Skoropad (1955) reported observations of nuclear migration in living cells. The evidence is unsatisfactory, however, as movement during fixing and staining may have brought about the effects observed by Saksena and those reported above, and it is unlikely that Sanford and Skoropad would have seen nuclei in living cells with ordinary illumination. Further critical investigation is needed before a satisfactory answer can be given to the question of nuclear migration in T. cucumeris.

IV. Anastomosis, Heterocaryosis and Recombination

The mutants from P56 as described above, appeared to be stable and were sufficiently different in cultural characteristics and pathogenicity to serve as useful tools in a study of anastomosis, heterocaryosis and recombination. In addition some non-mutant single basidiospore cultures, G1 and G2 progeny, were included in the study for specific purposes. A range of different techniques and combinations of mutants were used for the study.

(a) Study of anastomosis

(1) Between mutants and with P56

The six mutants (sparse, stumpy, fleecey, curly, rusty, and ropy) and P56 were opposed in pairs in all possible combinations on Cellophane, four plates of each combination, to determine their ability to anastomose with one another. Fifty presumptive anastomoses from each pairing were tested and cytoplasmic connections were demonstrated between each pairing. (Due to the time required for testing, all Petri dishes were transferred to 5°C when the opposed hyphae had begun to intermingle,

to retard growth so that anastomosing hyphae could be easily traced and their origins determined.) Pairings between P56 and any mutant resulted in successful anastomosis (Table IX). Between mutants only two combinations resulted in successful anastomosis and in all others cell death occurred within 24 hours of the demonstration of cytoplasmic connections. The killing reaction was either complete, where virtually every anastomosis resulted in cell death, or intermediate with 70-90% of the anastomoses resulting in cell death while the remainder were apparently unaffected. Four to five days later, a zone of dead cells was clearly visible at the line of junction between these paired colonies where the killing reaction had occurred. As no growth developed from the anastomoses of these pairings, it appeared either that heterocaryosis had not occurred or that the heterocaryons which formed were not culturally different from the contributing colonies.

P56 and curly were again paired on Cellophane to determine whether heterocaryosis followed anastomosis. Twenty-four colonies derived from single

TABLE IX

ANASTOMOSIS REACTION OF MUTANTS AND P56 WHEN OPPOSED
ON CELLOPHANE OVERLYING AGAR

<u>Culture</u>	<u>spars</u>	<u>stumpy</u>	<u>fleecy</u>	<u>curly</u>	<u>rusty</u>	<u>ropy</u>
P56	S*	S	S	S	S	S
<u>spars</u>		K	S	I	I	K
<u>stumpy</u>			K	I	K	K
<u>fleecy</u>				I	I	K
<u>curly</u>					I	I
<u>rusty</u>						S

- * S - Successful anastomosis without adverse effects on the cells
- K - Anastomosis followed by collapse and death of all anastomosed cells
- I - Intermediate, 70-90% of anastomosed cells died, remainder anastomosed without adverse effects on the cells.

presumptive anastomoses were tested for pathogenicity and self-fertility. Five colonies were similar to curly in cultural characteristics and caused hypersensitive flecks on radish stems; none was induced to fruit. The other 19 colonies were wild type in appearance but with some initial variation in growth rate; each was pathogenic and self-fertile. Three were selected to represent the initial range in growth rate and basidiospores were shed and isolated from each. The progeny of each colony exhibited wide variation in growth rate and pathogenicity. Most of the total of 375 single basidiospore cultures were wild type in appearance, pathogenic, and after subculturing were uniform in growth rate; 32 were slow growing mutant types but none was similar to curly in cultural characteristics. As no curly type cultures were recovered, the variation in progeny of these presumptive heterocaryons was attributed not to recombination of P56 and curly nuclei, but rather to the expression of spontaneous mutation within P56 itself. It was at this time that variation in progeny of P56 was observed in an independent test (Plate 3). Thus it was concluded that heterocaryons

probably were not formed as the result of anastomoses between P56 and curly.

Since heterocaryosis was not detected and the killing reaction occurred in most combinations between mutants, attempts were made to observe the nuclear behaviour in anastomosing cells by phase-contrast microscopy. Mutants were again paired, eight Petri dishes per pair, and incubated at 25°C until opposing hyphae had begun to intermingle. Four replicates of each pairing were then transferred to 5°C to retard growth; the other four were maintained at 25°C, and from some of these plates areas of anastomosing hyphae, together with underlying Cellophane, were mounted on slides in sterile distilled water, covered with coverslips and examined. Such preparations, however, proved unsuitable for phase-contrast microscopy. The nuclei were not easily observed due to the distortion of light by the Cellophane, and the hyphae soon ceased growth and died. All intact plates were examined six days after initial contacts between the opposed hyphae and the same reactions as recorded previously (Table IX) were observed. However, one or two

sectors had developed from the zone of dead hyphae in every plate at 25°C, each sector consisting of mycelium which was wild type in appearance (Plate 6). The plates stored at 5°C were returned to 25°C and subsequently, small wild type sectors developed only in those plates containing sparse and fleecy or ropy and rusty, the pairings which gave successful anastomosis reactions. It was not determined whether the origin of a sector was an anastomosis between single cells of each mutant, but subsequent studies (described below) proved that the wild type growth was heterocaryotic and composed of nuclei of each mutant. It is thus apparent that heterocaryons can be formed between mutants which give a killing reaction when anastomosed and further it seems that temperature has an effect on anastomosis and heterocaryon formation. Although the factors responsible for the killing reaction are not known, the effect of temperature might be explained as follows: heterocaryosis follows anastomosis and a heterocaryotic hypha of several cells in length is produced from the anastomosed cells before the visible onset of the killing reaction; cell death is observed

PLATE 6

Heterocaryotic sectors formed from the
zones of cell death between mutants

Top: [left] sparse + ropy
 [right] sparse + curly

Bottom: rusty + curly



from 12 to 24 hours after anastomosis and as it involves not only the anastomosed cells but also up to six cells on either side, it may also include one or more of the cells of the developing heterocaryon, the cytoplasm of which was contributed by both mutant colonies; growth of the wild type heterocaryon at 25°C must be sufficient for the terminal cells to escape the killing reaction and develop the sector, whereas at 5°C growth may be sufficiently retarded for the terminal cells of the heterocaryon to be killed. Such an explanation is supported by the observations that in an established wild type colony, growth at 5°C is limited to approximately five new cells per hypha in 48 hours, whereas at 25°C, 150 to 200 consecutive new cells are produced per hypha in 48 hours.

(11) Between non-mutants

Since the killing reaction occurs within and between G1 and G2 wild type progeny of field isolate 48, additional experiments were carried out to determine whether heterocaryons develop from anastomoses between paired wild type colonies by escaping the killing reaction. Two G1 and two G2

cultures were paired in each combination. Observations are presented on the pairings of the G2 isolates.

Two G2 isolates were paired in each of 12 Petri dishes and incubated at 25°C until opposed hyphae met. Eight plates were transferred to 5°C, four for eight hours and four for 72 hours and then returned to 25°C. Each plate was examined three times daily for one week. In all 12 pairings, cell death was observed from 12 to 30 hours after initial anastomosis and occurred only after hyphae had anastomosed. There was no indication of antagonism between non-anastomosed hyphae and no sectors were detected from the zone of dead hyphae between two colonies.

Pairs of opposed hyphal tips were transferred from these plates to separate Petri dishes of Cellophane over soil agar; each transfer consisted of a hyphal segment, including the tip and three to ten cells, from each isolate, the tips not yet having made contact. The tip cells continued to elongate and anastomose in only one of 16 transfers; however no new cells developed from the anastomosis

and cell death extended to all four cells of the one hyphal segment. In the other transfers, each of the hyphal segments branched and formed a small colony before anastomosis occurred, and by the time cell death was first observed the hyphae had so intermingled that it was not always possible to identify the anastomosing hyphae. Dead cells of three hyphae were associated in a single anastomosis in two separate instances but it could not be determined whether two hyphae of one colony had anastomosed with a hypha of the other colony or if one hypha were a developing heterocaryon. The experiments were not conclusive and were discontinued.

(iii) Between heterocaryons

Four wild type heterocaryons of curly + rusty, each derived from a hyphal tip, two from separate sectors arising from the paired mutants on Cellophane and two from the wild type mycelium produced from macerates (described below), were paired on Cellophane. All combinations anastomosed successfully.

Fifteen heterocaryons, formed by macerating the six mutants together in pairs, were themselves

paired in all combinations on Cellophane. In two combinations, stumpy + fleecy with stumpy + ropy, and stumpy + fleecy with fleecy + ropy, the killing reaction occurred, affecting about 50% of the anastomoses. Successful anastomoses with no cell death occurred in the other 103 combinations.

(b) Formation of heterocaryons

(1) Stable heterocaryons

Curly and rusty were macerated together as described under Materials and Methods. Within 24 to 48 hours, fast growing hyphae emerged from the macerate to produce colonies on agar or on Cellophane over agar which were wild type in appearance and growth rate. Numerous hyphal tips, taken from the periphery of each colony and subcultured separately, produced wild type colonies which were uniform in appearance and growth rate; one of these cultures has been maintained by weekly serial subculturing for six months with no change in cultural characteristics.

Colonies derived from macerates of P56 alone, and of curly and rusty separately and together, were compared for cultural characteristics (Plate 7), for pathogenicity to radish seedlings (Plate 8) and

PLATE 7

Colonies resulting from maceration of P56,
and of curly and rusty separately and together

P56

curly

rusty

heterocaryon
(curly + rusty)

109.

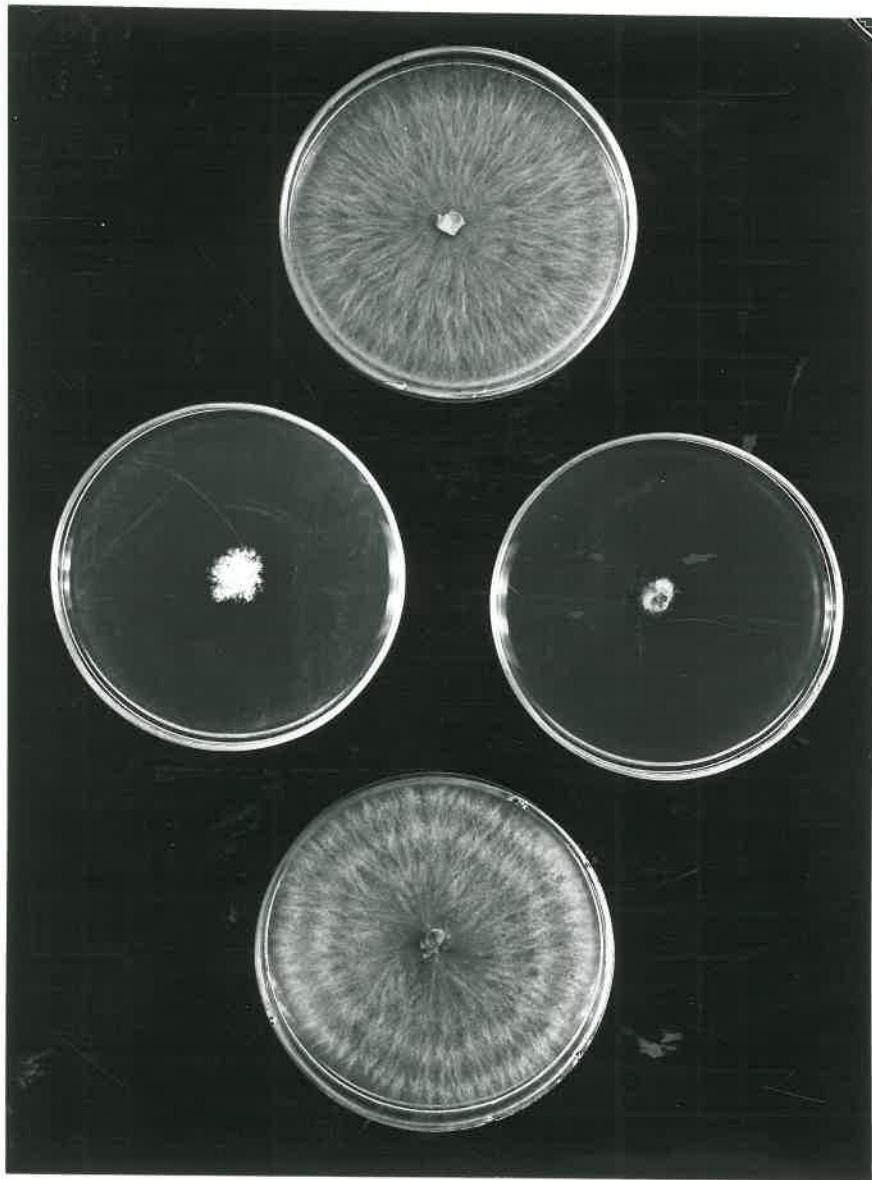


PLATE 8

Pathogenicity test on radish stems

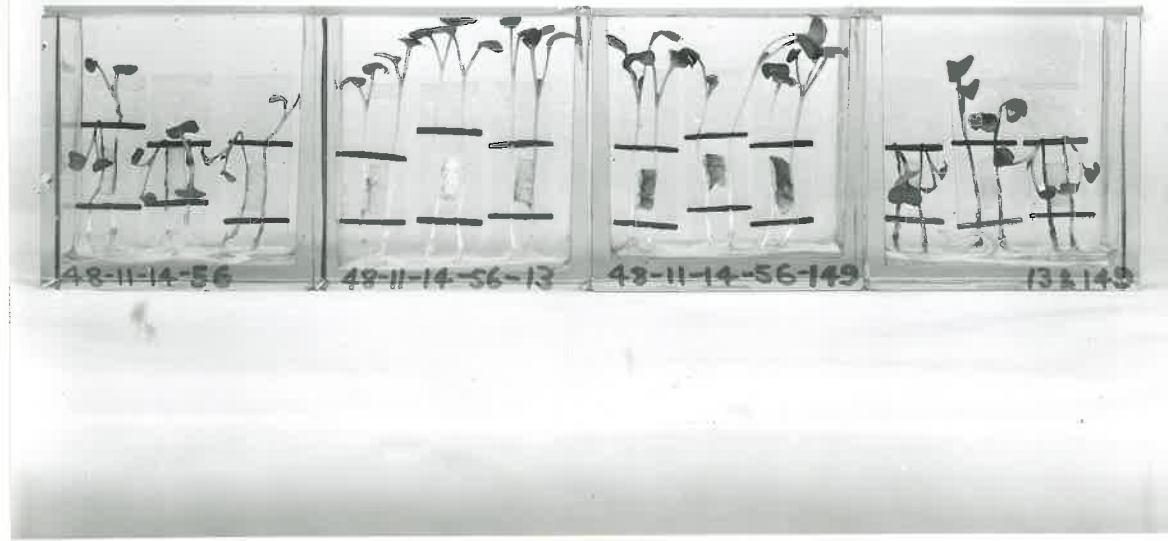
[Left to right]

P56

curly

rusty

heterocaryon
(curly + rusty)



48-11-14-56

48-11-14-56-13

48-11-14-56-149

13 & 149

for fruiting ability. No wild type growth resulted from the mutant colonies when macerated separately, and the colonies of P56, curly, and rusty were the same as described previously (Tables VI and VII), except for an initial lag of 36 hours before either mutant commenced growth on P.V.D.A. The wild type growth from the macerate of curly + rusty was indistinguishable from P56 in cultural appearance, growth rate and pathogenicity. It seemed probable that this wild type growth was a heterocaryon between curly and rusty and the evidence to substantiate this is described below in the section on recombination. Nuclear numbers in cells of this heterocaryon were counted (Table X); the differences between means within the heterocaryon, and between the corresponding means of the heterocaryon and P56, curly or rusty (Table VII) were analysed (t-test) and all differences were significant at the 1.0% level except in the peripheral tips between P56 and the heterocaryon and in the branch tips between rusty and the heterocaryon. Branching and septation of the heterocaryon were regular, but there was a suggestion of a significant linear trend at the 5.0% level in the mean numbers of nuclei

TABLE X

NUMBER OF NUCLEI IN VEGETATIVE CELLS OF A STABLE
(curly + rusty)

AND AN UNSTABLE (curly + fleecy) HETEROCARYON

(a) Number of nuclei per cell in three types of cells

Heterocaryon	Nuclei per cell (mean, S.E., and range)			
	Peripheral tip Cells	Older non-tips	Branch tips	Total
<u>curly</u> + <u>rusty</u>	8.90±0.19 (5-13)	7.17±0.13 (3-12)	5.90±0.11 (4-9)	7.28±0.10 (3-13)
<u>curly</u> + <u>fleecy</u>	9.92±0.16 (7-19)	7.30±0.12 (4-13)	6.58±0.15 (4-11)	7.78±0.10 (4-19)
Number of cells counted	100	200	100	400

(b) Number of nuclei in consecutive cells from the tips

Heterocaryon	Nuclei per cell (mean and range)*						
	Tip Cell	2nd	3rd	4th	5th	6th	7th
<u>curly</u> + <u>rusty</u>	8.7 (6-13)	8.8 (6-12)	9.2 (5-14)	9.6 (7-14)	9.7 (6-13)	9.7 (5-15)	10.4 (6-16)
<u>curly</u> + <u>fleecy</u>	10.6 (7-19)	10.1 (5-19)	11.1 (7-21)	11.4 (7-19)	12.8 (7-18)	11.6 (6-22)	12.2 (6-18)

* counts of 20 hyphae

in consecutive cells from the tip.

Wild type heterocaryons were also recovered from the macerates of sparse, stumpy, curly, rusty and ropy in paired combinations (Table XI). After hyphal tip subculturing, the heterocaryons were indistinguishable from one another and from P56, and each was pathogenic. Results of fruiting studies are reported below.

(ii) Unstable heterocaryons

Macerations of fleecy with sparse, stumpy, curly, rusty, or ropy gave rise to mycelial growth which was initially wild type in appearance but after reaching diameters of 5-7cm, the peripheral growth of each colony reverted to mutant type (Table XI). The colony of stumpy + fleecy reverted to stumpy whereas all other colonies reverted to fleecy, and in each the reversion occurred abruptly, with one half or more of the periphery of a colony reverting at the same time and the remainder following rapidly.

A number of experiments were conducted to demonstrate that functional but unstable heterocaryons had been constituted and that wild type growth was not due to cross-feeding between mutants. Curly

TABLE XI

TYPES OF HETEROCARYONS FORMED FROM MACERATIONS OF
MUTANTS IN PAIRS

Mutants	Induced				Spontaneous	
	<u>sparse</u>	<u>stumpy</u>	<u>fleecy</u>	<u>curly</u>	<u>rusty</u>	<u>ropy</u>
<u>sparse</u>	O*	+	u--fl.	+	+	+
<u>stumpy</u>		O	u--st.	+	+	F--+
<u>fleecy</u>			O	u--fl.	u--fl.	u--fl. F-
<u>curly</u>				O	F--+	F--+
<u>rusty</u>					O	+
<u>ropy</u>						O

*O No heterocaryon

+ Stable heterocaryon

u--fl. Unstable heterocaryon reverting to fleecy

u--st. Unstable heterocaryon reverting to stumpy

F- Fertile

and fleecy when macerated separately and placed one over the other, separated by Cellophane, on soil agar, formed typical mutant colonies, whereas when macerated together and placed either on or under Cellophane on soil agar, produced wild type growth which subsequently reverted to fleecy.

When curly and fleecy were macerated together, hyphal tips were taken at daily intervals from the periphery of the developing wild type growth and plated out separately. All wild type hyphal tips gave rise to wild type colonies and all the colonies reverted to fleecy after attaining diameters of 6-7cm. The colony size when reversion occurred was not influenced by temperature, amount of agar, type of agar medium or size of Petri dish used. Macerates of the reverted fleecy with curly reconstituted the heterocaryon but with fleecy gave rise only to fleecy. The heterocaryotic wild type and the reverted fleecy were each pathogenic to radishes.

Curly and fleecy were then macerated together in different proportions from a ratio of 1:1 volume size of inoculum to 100:1. Irrespective of the ratio used the results were the same and the

unstable heterocaryon developed.

Weekly samples were taken for 15 weeks from single colonies of the curly + fleecy heterocaryon growing on P.V.D.A. or soil agar. Each sample consisted of plugs, 3mm diameter, taken at 5mm intervals along a radius of the heterocaryon and subcultured separately on P.V.D.A. or soil agar. The type of growth from plugs taken at the same distances from the center over the 15 weeks did not differ; unstable heterocaryotic colonies formed from all P.V.D.A. plugs taken at distances of 30mm or less and from all soil agar plugs of 25mm or less, while fleecy colonies developed from all plugs from greater distances.

Similar results were obtained for each unstable heterocaryon formed from the macerates of fleecy and another mutant, except that the stumpy + fleecy heterocaryon always reverted to stumpy growth which, when tested for pathogenicity, was repelled by the radish stems.

The mean numbers of nuclei per cell in the different types of cells of the wild type mycelium of curly + fleecy (Table X) were significantly

different at the 1.0% level from those of the curly + rusty heterocaryon or of P56, fleecy or curly (Table VII), except for the means of older non-tip cells between the two heterocaryons. There was an increasing linear trend, significant at the 5.0% level, of the mean number of nuclei in consecutive cells from the tips.

(c) Analysis of heterocaryosis and recombination

Each of the induced mutants (sparse, stumpy, fleecy, and curly) and the spontaneous (rusty and ropy) mutants of P56 has a distinct cultural appearance and pathogenic reaction. Macerates of pairs of these mutants always give rise to wild type mycelial growth, which indicates that, if each heterocaryon contains only nuclei of the two component mutants, the six mutants are non-allelic. In T. cucumeris the only way in which the components of a heterocaryon can be determined is by recovery of the components as progeny from sexual reproduction. This in turn depends on reliable genetic markers.

Although a fungus is homothallic it is still possible to make crosses by combining two

genetically different individuals in a heterocaryon and inducing that to fruit. The sexual spores are of either selfed or hybrid origin and, if progeny of hybrid origin only are selected, then a genetic analysis is possible. A single gene mutation is usually determined by crossing mutant to wild type and recovering only mutant and wild type progeny with equal frequencies. Such a cross was attempted with curly and P56, as described in the previous section, but the particular culture of P56 used was itself heterocaryotic because of spontaneous mutation and there was no indication that curly had been a component in the cross. Another way of determining the genotype of a mutant is by crossing the mutant to a second mutant and analysing the resultant progeny. This type of cross may also give information on linkage. Presented are the results of several experiments in which paired mutants gave rise to stable or unstable, but fertile, heterocaryons.

(1) Stable heterocaryons

Each fertile heterocaryon obtained from macerates of the paired mutants fruited on the surface of treated soil within seven days of the

addition of soil, the same interval as for P56. Shed basidiospores were isolated from each cross. The basidiospore culture types are listed in the Tables in one or more batches from each cross. Each batch consisted of every basidiospore shed in a single area on Cellophane. Of the basidiospores listed as dead, most did not germinate, but a few formed short germ tubes before growth ceased.

Each single spore culture was scored for cultural appearance, growth rate, pathogenicity, and fertility. In addition, heterocaryon tests were used to confirm identification of mutant type progenies. Every mutant progeny was macerated together with each of the parental types of that cross. If wild type growth was produced from the macerate of the progeny and one parent, and mutant type from the macerate of the progeny and the second parent, it was accepted that the progeny was similar to the second parent. If, however, mutant type growth resulted from both macerations, and wild type growth developed from a maceration of the progeny and a third mutant, the progeny was considered to be the double mutant recombinant. All wild type heterocaryons produced from macerations of single mutant

progeny with parental mutant were indistinguishable and each was pathogenic. The genotypes of the mutant progenies were determined on the basis of comparisons with the parental mutants for the several characteristics.

From the cross curly x rusty four cultural types of single basidiospore progeny (Table XII) were recovered, wild type, the two single mutant types curly and rusty, and a slower growing type (button). All wild type cultures were indistinguishable from P56; each was subsequently induced to fruit and the resultant basidiospores were 95-99% viable, yielding only wild type progeny. The wild type was presumably the non-mutant recombinant. The curly and rusty progeny were identical in characteristics to curly and rusty respectively. Each curly progeny formed the wild type heterocaryon with rusty but not with curly and each rusty progeny formed the wild type with curly but not with rusty. Macerations of each button culture with either curly or rusty did not produce wild type growth, but with a third mutant (sparse, stumpy, or ropy) a wild type heterocaryon was established, suggesting that button was the double mutant recombinant.

The button colony on P.V.D.A. is small (2.0cm diameter in six days) and dense, initially white but rapidly turning dark brown in color. Hyphal branching is irregular and similar to that of curly but septation is regular like that in rusty. Button is non-pathogenic to radishes, with few hyphae growing over the stems, and is self-sterile.

Batch 1 shed basidiospores of the cross curly x rusty yielded only curly progeny (Table XII) and presumably resulted from selfed basidia. Batch 2 gave rise to the four phenotypes which occurred with frequencies not significantly different from equality ($\chi^2_3 = 6.138$, $P = 0.1-0.05$); these may all have been the result of crossing or may have included some selfed progeny. From these data it was postulated that curly and rusty phenotypes are each due to a single gene difference and these segregate independently. As a result, the genotypes of the progeny of the cross were assumed to be: wild type $\pm \pm$, curly $cu \pm$, rusty $\pm ru$, and button $cu ru$.

Two groups of tetrads were also isolated from fructifications of this cross to verify the hypothesis. Group 1 tetrads, from the same fructifications as

TABLE XII

SINGLE BASIDIOSPORE PROGENY OF THE CROSS curly x rusty
Shed spores

Phenotype	Wild type	<u>curly</u>	<u>rusty</u>	<u>button</u>	Dead	Total
Genotype	± ±	<u>cu</u> ±	± <u>ru</u>	<u>cu ru</u>		
Batch 1	0	21	0	0	48	69
Batch 2	12	19	19	8	9	67

Classification of tetrads

	Group 1	Group 2	Total
Selfed <u>curly</u>	10	6	16
<u>rusty</u>	0	0	0
Hybrid Parental ditype	1	2	3
Non-parental ditype	2	3	5
Tetratype	3	3	6
Incomplete (2 or 3 dead per tetrad)	10	0	10
Non-fit*	5	0	5
			<u>45</u>

* Observed pattern of non-fit tetrads

Wild type	<u>curly</u>	<u>rusty</u>	<u>button</u>	Dead
2	-	1	1	-
-	1	1	2	-
-	3	1	-	-
1	2	1	-	-
1	2	-	-	1

the shed basidiospores, were picked off on agar blocks held on a scalpel blade. Group 2 tetrads were removed on Cellophane from fructifications when the cross was reconstituted seven months later. Of the total of 45 tetrads examined (Table XII), 16 had resulted from selfing of curly; no selfed tetrads of rusty were recovered. There were 14 complete tetrads which must have resulted from hybrid basidia and these were classified as (a) parental ditype, two of each parental type, (b) non-parental ditype, two of each recombinant type, or (c) tetratype, one of each parental and recombinant type. Analysis of hybrid tetrads, according to the null hypothesis, independent segregation, which predicts equal numbers of parental ditype and non-parental ditype tetrads (Perkins, 1953), confirmed that curly and rusty phenotypes are due to non-linked, single gene differences, and that wild type and button respectively are the non-mutant and double mutant recombinants. In addition there were 10 incomplete tetrads and five tetrads which did not fit the hypothesis. Each incomplete tetrad contained two or three non-viable basidiospores and

the missing progeny were not limited to a particular progeny type. The non-fit tetrads may have resulted from errors in isolation since they were picked off on blocks of agar when the hymenium was actively producing basidiospores and may not have been single tetrad isolations.

Two sets of isolations were also made from the first fructifications of the cross curly x rusty, each set consisting of basidia with sterigmata, prebasidial cells and a vegetative runner hypha, with a continuous hyphal connection from basidia to hypha. The material was freed from soil particles in sterile distilled water; one set was divided into sections, the other left intact, and plated on Cellophane over soil agar plus antibiotics. Mycelial growth arising from basidial and prebasidial cells was that of curly while wild type growth developed from each vegetative runner hypha; no rusty hyphae were detected. These observations suggest that the heterocaryon contained a greater proportion of curly nuclei than rusty nuclei.

From each of the crosses curly x ropy and stumpy x ropy, only one batch of basidiospores was

isolated due to sparse hymenial development. The cross curly x ropy yielded four cultural types (Plate 9, Table XIII); recovery of a high frequency of curly progeny suggests some selfing of curly. Each single spore culture was identified and tested. The wild type progeny were possibly the non-mutant recombinants, and curly and ropy progeny were identical with curly and ropy respectively. Heterocaryon tests suggested that tight button may be the double mutant recombinant. Similar results were obtained from the cross stumpy x ropy (Plate 10, Table XIV). In this cross it appeared that some selfed progeny had been recovered. Identification of the cultural types again suggests that wild type may be the non-mutant recombinant and flat button the double mutant recombinant. However, no tetrads were isolated from either cross and because of the apparent recovery of selfed progeny, no statistical analyses were attempted.

Button, the double mutant recombinant of the cross curly x rusty, was itself separately macerated with ropy and stumpy. Each resultant cross yielded single basidiospore cultures of eight types, namely:

PLATE 9

Types of single basidiospore progeny
of the cross curly x ropy

Wild type

curly

ropy

tight button

123.

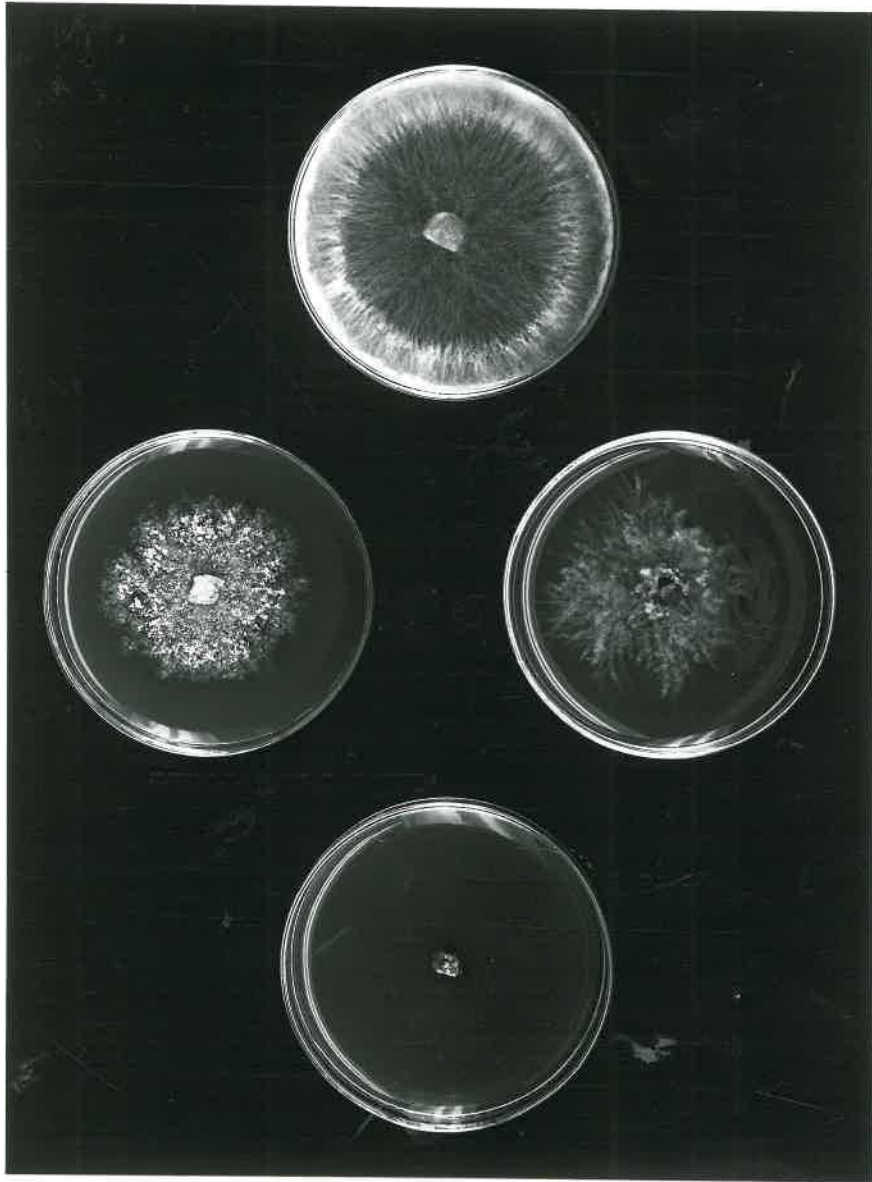


TABLE XIII

SINGLE BASIDIOSPORE PROGENY OF THE CROSS curly x ropy

Shed spores

Phenotype	Wild type	<u>curly</u>	<u>ropy</u>	<u>tight button</u>	Dead	Total
	17	82	4	7	36	146

PLATE 10

Types of single basidiospore progeny
of the cross stumpy x ropy

Wild type

ropy

stumpy

flat button



TABLE XIV

SINGLE BASIDIOSPORE PROGENY OF THE CROSS stumpy x ropy

Shed spores

Phenotype	Wild type	<u>stumpy</u>	<u>ropy</u>	<u>flat button</u>	Dead	Total
	18	37	18	7	54	134

wild type, three single mutants, three double mutants and triple mutant (Tables XV, XVI). All the wild type cultures, with one exception discussed below, yielded uniform wild type progeny. The single, double, and triple mutants were identified phenotypically and verified by heterocaryon tests. No mutant progeny was self-fertile. Pathogenicity was not determined for the triple mutants or double mutants other than button due to their slow growth. The 'pincushions', whether triple or double mutants, reached maximum diameters of less than 6mm after three weeks growth on P.V.D.A., and the 'modified buttons' (i.e. tight button, etc.) formed colonies of 8-12mm diameter.

Progeny from shed basidiospores of the cross ropy x button appear to be the result both of selfing of button and of crossing (Table XVa). Batch 1 progeny are not significantly different from Batch 2 progeny (contingency $\chi^2_5 = 5.417$, $P = 0.5-0.3$). The six recombinant types of progeny occurred with frequencies not significantly different from equality in the pooled data ($\chi^2_5 = 1.657$, $P = 0.9-0.8$), indicating independent segregation of three single genes

TABLE XV

SINGLE BASIDIOSPORE PROGENY OF THE CROSS ropy x button

(a) Shed spores

<u>Phenotype</u>	<u>Genotype</u>	<u>Batch 1</u>	<u>Batch 2</u>	<u>Total</u>
<u>Wild type</u>	<u>± ± ±</u>	7	6	13
<u>curly</u>	<u>cu ± ±</u>	7	3	10
<u>rusty</u>	<u>± ru ±</u>	11	3	14
<u>ropy</u>	<u>± ± ro</u>	6	7	13
<u>button</u>	<u>cu ru ±</u>	15	15	30
<u>tight button</u>	<u>cu ± ro</u>	6	5	11
<u>pin-cushion</u>	<u>± ru ro</u>	5	8	13
<u>curly pin-cushion</u>	<u>cu ru ro</u>	6	3	9
<u>Dead</u>		32	35	
<u>Total</u>		95	85	

TABLE XV

(b) Tetrads

Phenotype	Genotype	Observed tetrad patterns					
		1	2	3	4	5	6
Wild type	<u>± ± ±</u>	-	1	2	-	1	-
<u>curly</u>	<u>cu ± ±</u>	1	1	-	1	-	1
<u>rusty</u>	<u>± ru ±</u>	1	-	-	-	-	2
<u>ropy</u>	<u>± ± ro</u>	-	-	-	1	(1)	-
<u>button</u>	<u>cu ru ±</u>	-	-	-	1	1	-
<u>tight button</u>	<u>cu ± ro</u>	1	-	-	-	-	1
<u>pin-cushion</u>	<u>± ru ro</u>	1	1	-	1	-	-
<u>curly pin-cushion</u>	<u>cu ru ro</u>	-	1	2	-	1	1
Frequency		2	2	1	1	1*	1**

* 3-spored basidium (see text)

** Non-fit tetrad

Classification of tetrads

Pairs of genes	Parental ditype	Non-parental ditype	Tetratypes
<u>cu/ro</u>	1	1	5
<u>cu/ru</u>	2	2	3
<u>ru/ro</u>	0	3	4

differences. The spores of each of seven four-spored basidia and one three-spored basidium were removed on Cellophane from the fructifications. The colonies produced from the spores of the three-spored basidium (Table XVb) were one wild type, one button (cu ru ±) and one curly pincushion (cu ru ro). The wild type culture fruited, yielding a sample of 41 wild type and seven ropy progeny plus five non-viable spores; this segregation suggests that the wild type culture had been derived from a basidiospore which contained two nuclei, one $\pm \pm \pm$ and one $\pm \pm ro$. For the purpose of tetrad analysis, the nuclei produced in the three-spored basidium were considered as four types, the fourth being ropy. All eight tetrads appeared to have been derived from hybrid basidia, and when classified according to tetrad types, seven fitted the hypothesis of three single gene differences. With the small number of tetrads for analysis, recovery of non-parental ditypes with equal or greater frequency than parental ditypes is evidence against linkage. The possibility that the spores of the non-fit tetrad came from more than a single basidium is remote due to the method of isolation.

The frequencies of phenotypes in the progeny of the cross stumpy x button again suggests some selfing of button (Table XVI). The batches are not significantly different (contingency $\chi^2_5 = 8.04$, $P = 0.2-0.1$). In the pooled data the six recombinant types occurred with frequencies not significantly different from equality ($\chi^2_5 = 2.00$, $P = 0.9-0.8$) which suggests they arose from hybrid basidia in which there was independent segregation of three single gene differences.

All but one of the single basidiospore cultures from the above crosses have remained stable in morphology. A wild type sector developed from a single hypha in a week-old subculture of a rusty progeny derived from the curly x rusty cross. The wild type growth was maintained in several hyphal tip subcultures and when one was induced to fruit, 67 wild type and six rusty progeny plus four non-viable spores were recovered. From the non-sectoring area of the rusty colony, 50 subcultures were taken and each was serially subcultured for five weeks; no further sectors were observed.

The data from the five crosses are consistent with the hypothesis that appearance, growth rate,

TABLE XVI

SINGLE BASIDIOSPORE PROGENY OF THE CROSS stumpy x button

Shed spores

<u>Phenotype</u>	<u>Genotype</u>	<u>Batch 1</u>	<u>Batch 2</u>	<u>Total</u>
<u>Wild type</u>	<u>± ± ±</u>	6	3	9
<u>stumpy</u>	<u>st ± ±</u>	2	1	3
<u>curly</u>	<u>± cu ±</u>	2	9	11
<u>rusty</u>	<u>± ± ru</u>	4	3	7
<u>flat button</u>	<u>st cu ±</u>	4	2	6
<u>button</u>	<u>± cu ru</u>	20	24	44
<u>hairy pin- cushion</u>	<u>st ± ru</u>	5	2	7
<u>loose pin- cushion</u>	<u>st cu ru</u>	3	5	8
<u>Dead</u>		56	42	
<u>Total</u>		102	91	

pathogenicity, and self-fertility are together controlled by a single gene mutation in each of the mutants stumpy, curly, rusty, and ropy, and that the genes are unlinked. The higher frequencies of curly, stumpy, or button progenies from different crosses suggest either a tendency of these nuclei to fuse or greater proportions of these nuclei in the heterocaryons.

(11) Unstable heterocaryons

Two unstable heterocaryons which were induced to fruit, formed basidia and basidiospores five weeks after being covered with treated soil. Cultures derived from shed basidiospores of the cross fleecy x ropy were almost exclusively fleecy (Table XVII). The one wild type culture was pathogenic and fertile, yielding only wild type progeny, and the single ropy culture was indistinguishable from ropy in all observed characteristics. After producing a colony 20mm in diameter in 10 days, the third culture, a possible double mutant, ceased growth and died with the peripheral hyphae rupturing and extruding protoplasm. The fleecy cultures developing from basidiospores varied in growth rate and slightly in appearance, but the first

TABLE XVII

SINGLE BASIDIOSPORE PROGENY OF THE CROSS fleecy x ropy

Shed spores

Phenotype	Wild type	<u>fleecy</u>	<u>ropy</u>	Others	Dead	Total
Batch 1	0	10	0	0	28	38
Batch 2	0	19	0	0	34	53
Batch 3	1	28	1	1	20	51

Pathogenicity reaction of fleecy progeny

	Lesions and seedling death	Hyper-sensitive reaction	Non-pathogenic no cushions
Abundant aerial hyphae on stems	18	6	1
No aerial hyphae	21	9	2

subcultures were uniform. Minor microscopic differences were detected in the number and length of feeder hyphae produced per cell of the aerial runner hyphae and the amount of subbranching; although the extremes could be easily distinguished on this basis, different cultures presented a gradation between the extremes. Each fleecy progeny formed an unstable pathogenic heterocaryon when macerated with ropy, but no heterocaryon at all with fleecy. When tested for pathogenicity the fleecy progeny varied in their reactions to radish stems. The reactions were either (a) pathogenic, with infection cushions producing spreading lesions and seedling death, (b) hypersensitive reaction, with cushions producing small necrotic flecks on the stems, or (c) non-pathogenic with the mycelium growing over the stems without adhering or producing cushions, and within each of these categories, some cultures produced abundant aerial mycelium from the hyphae growing on the stems and others did not, giving a total of six classes. Pathogenicity tests were repeated a total of six times over a period of several months and the reaction of each fleecy progeny was constant.

These results do not fit the hypothesis of segregation of single gene differences although it was earlier established that ropy is a single gene mutant; neither ropy nor fleecy produces masses of aerial hyphae on radish stems and neither mutant produces the hypersensitive reaction.

Similar results were obtained from the progeny of the second cross (fleecy x button). Spores from this cross gave rise only to fleecy cultures (Table XVIIIa) and of 13 tetrads removed on Cellophane, only three were completely viable (Table XVIIIb). Three of the tetrads apparently resulted from the selfing of fleecy, two from the selfing of button and one was possibly a parental ditype. The other seven tetrads each contained two or more non-viable basidiospores and no other cultural types were recovered. The button types were verified as button by heterocaryon and pathogenicity tests. The fleecy progeny formed unstable heterocaryons when macerated with button, rusty, curly or ropy, but no heterocaryon at all with fleecy. The same six reactions in pathogenicity tests as recorded with the progeny of the

TABLE XVIII

SINGLE BASIDIOSPORE PROGENY OF THE CROSS fleecy x button

(a) Shed spores

Phenotype	<u>fleecy</u>	Other types	Dead	Total
Batch 1	64	0	99	163
Batch 2	22	0	45	67

Pathogenicity reaction of fleecy progeny

	Lesions and seedling death	Hyper-sensitive reaction	Non-pathogenic no cushions
Abundant aerial hyphae on stems	19	11	1
No aerial hyphae	35	15	5

TABLE XVIII

(b) Tetrad

Phenotype	Observed tetrad patterns									
<u>fleecy</u>	4	3	-	-	1	2	1	1	-	-
<u>button</u>	-	-	4	3	2	-	1	-	1	-
Other types	-	-	-	-	-	-	-	-	-	-
Dead	-	1	-	1	1	2	2	3	3	4
Frequency	2	1	1	1	1	1	1	3	1	1

Pathogenicity reaction of fleecy progeny

	All <u>fleecy</u> progeny from tetrads	Individual selfed tetrads		
		No. 1	2	3
<u>Lesions and seedling death:</u>				
Abundant arial hyphae	3	2	-	-
No arial hyphae	6	1	2	1
<u>Hypersensitive reaction:</u>				
Abundant arial hyphae	3	1	-	-
No arial hyphae	3	-	1	-
<u>Non-pathogenic, no cushions:</u>				
Abundant arial hyphae	2	-	1	1
No arial hyphae	1	-	-	1
Totals	18	4	4	3

previous cross occurred among these fleecy cultures; however, no segregation pattern was evident either from the cultures derived from shed spores or from the apparently selfed tetrads, but the recovery of button cultures from both selfed and crossed basidia provides proof that button was involved in this cross.

These results neither completely confirm nor reject the hypothesis that fleecy is a single gene mutant. The recovery of predominantly fleecy progeny from each cross initially suggests a large degree of selfing of fleecy. The single wild type culture from the cross fleecy x ropy was probably the non-mutant recombinant, since if a back mutation of a single nucleus from mutant to wild type had occurred a greater number of wild type progeny would have been expected unless, of course, the back mutation occurred after meiosis in the basidium. The absence of types other than ropy or button respectively from the two crosses would suggest that the contributing mutants were homocaryotic and that the subsequent heterocaryons contained only nuclei of the contributing mutants. However, since the same six pathogenic reactions of the fleecy cultures

occurred from the progeny of both crosses, it would appear that these effects were due to fleecy alone and not to recombination and segregation in the heterocaryons. It is possible that fleecy contained nuclei isogenic for morphology but differing at several genes for pathogenicity. However, the segregation patterns of the three apparently selfed tetrads of fleecy do not appear to fit a simple Mendelian segregation.

(d) Discussion

Several workers (Schultz, 1936; Richter and Schneider, 1953) have attempted to anastomose different field isolates of Rhizoctonia solani and divide them into closely related groups, on the assumption that success or failure of anastomoses indicated a close or distant genetic relationship respectively. Whitney and Parmeter (1963) reported the formation of heterocaryons in Thanatephorus cucumeris from anastomoses between single spore progeny of one parent and suggested that heterocaryosis between progeny was controlled by a 'bipolar compatibility mechanism'. However, none of these investigations actually demonstrated

that cytoplasmic connections had occurred between anastomosing cells nor did they record any cell death following anastomosis. Attempts by Flentje and Stretton (1964) to anastomose different field isolates of *T. cucumeris* resulted in either no reaction, wall fusion without cytoplasmic connection, or cell death following cytoplasmic intermingling (i.e. the killing reaction).

All isolates used in the present investigation were single basidiospore progeny of different selfed generations derived from field isolate 48 of the crucifer strain of *T. cucumeris*. These results showed that in all pairings of progeny cytoplasmic connections were formed, but in a number of cases the killing reaction followed. Uniform single spore isolates (G3 or G4 progeny) from a pure genetic line all anastomosed successfully, whereas between progeny of a heterocaryon (G1 progeny of isolate 48) the anastomosis reaction was variable with many pairings resulting in widespread killing of anastomosing hyphae. The killing reaction also occurred between morphological mutants derived from the same G3 single basidiospore culture.

These results are broadly similar to those reported for Podospora anserina and Neurospora crassa.

The anastomosis killing reaction in both P. anserina (Rizet and Scheeroun, 1959) and N. crassa (Garnjobst and Wilson, 1956; Wilson et al., 1961) is a genetically controlled and irreversible reaction which is due to the interaction of some protein constituents of the cytoplasm; this reaction prevents heterocaryosis but not dicaryosis. Other genetic factors have been described in N. crassa which affect the vigour, rate of growth and nuclear dissociation in vegetative mycelia of heterocaryons (Holloway, 1955; Pittenger, 1964). In P. cucumeris, the killing reaction which occurred in most combinations of paired mutants (Table IX) was irreversible for the cells affected but it did not prevent the formation of heterocaryons. The nature of the reaction is not known but it appears to vary in severity; some pairings of the mutants resulted in the death of every anastomosis while in others the killing reaction affected only 70-90% of the anastomoses.

The development of heterocaryons following anastomosis was investigated in a range of paired

cultures. In pairings of wild type cultures, it was not possible to recognise presumptive heterocaryotic hyphae because of the lack of cultural differences between the anastomosing cultures and presumptive heterocaryons. Similarly in pairings of a mutant and its wild type parent, the presumptive heterocaryons were not culturally different from the parent, and when fruited they yielded progeny, some of which were mutants but none of which were related to the original mutant. The accumulation of spontaneous mutations in the mycelium of a wild type culture complicates the proof of heterocaryosis in which the wild type is a component. The need for suitable definitive markers in a study of heterocaryosis is shown by these observations and emphasized by the results obtained from the use of distinct mutants.

Heterocaryons were formed between paired mutants by different methods; it was demonstrated that heterocaryosis occurred in spite of the anastomosis killing reaction between the mutants. The influence of temperature on the formation of heterocaryons appears to be its effect on the rate

of growth of the developing heterocaryotic hyphae before the onset of the killing reaction, since heterocaryotic sectors were produced from pairs of mutants which were opposed on Cellophane and maintained at 25°C but not from the same pairs maintained at 5°C until after the killing reaction had occurred and then transferred back to 25°C. Heterocaryons from paired mutants were easily formed by macerating the two mutants together and plating out the macerate. Each developing heterocaryon, which was subcultured from hyphal tips, was wild type in appearance and pathogenic. It was therefore concluded that wild type growth and pathogenicity are dominant characteristics. Of the four induced (sparse, stumpy, fleecy, and curly) and the two spontaneous mutants (rusty and ropy), only fleecy was not mutant for pathogenicity. Many of the heterocaryons derived from mutants when paired in all combinations were stable and each was maintained by serial subculturing for six months with no apparent change in cultural characteristics or pathogenicity. However, six heterocaryons, each

with fleecy as one component, were unstable and each reverted to mutant type growth before the mycelium had reached the edge of a Petri dish. The reversion of an unstable heterocaryon was always in the same direction; in the fleecy + stumpy heterocaryon the reverted growth was stumpy, whereas each of the other five heterocaryons reverted to fleecy. The mechanisms responsible for reversion in the unstable heterocaryons cannot be determined on the basis of evidence now at hand. However, the reversion does not appear to be due to nuclear migration nor to abnormalities in nuclear behaviour during cell division, either of which could segregate the nuclei of the component mutant types in the developing heterocaryon (Parmeter et al, 1963). The fact that the reversion occurred almost simultaneously over large sections of the periphery of a colony suggests that reversion is governed by factors common to the thallus rather than by localized cell conditions.

The wild type growth which resulted from pairings of the mutants was shown to be heterocaryotic by hyphal tip subculturing. Proof that the wild

type contained only the nuclei of the contributing mutants was obtained from the results of fruiting experiments. Single basidiospore progeny were recovered from each of the five stable heterocaryons which were induced to fruit, and identified by cultural characteristics and by heterocaryon, pathogenicity and fruiting tests. From each cross of single x single mutant the progeny segregated into four distinct types: the non-mutant wild type, two single mutants, and the slow-growing double mutant. The two crosses of double mutant x a third single mutant each yielded the theoretically-expected eight progeny types: wild type, three single mutants, three double mutants, and the triple mutant. Tetrad and random spore analyses showed that the four mutants involved in these crosses, stumpy, curly, rusty, and ropy, were each due to single gene mutations which were located either on separate chromosomes or at wide intervals on the same chromosomes. The fifth non-pathogenic mutant, sparse, was not a component of any of the fertile heterocaryons.

Two heterocaryons, each of which was unstable and reverted to fleecy growth, were also induced

to fruit. The recovery of progeny of each which were predominantly fleecy type in cultural appearance suggests that fleecy nuclei are able to self. However, the fleecy cultures from each cross exhibited the same range of variation in pathogenicity reactions. This variation might be due to pathogenicity mutations within the fleecy nuclei, but the progeny from three apparently selfed basidia of fleecy do not appear to be the products of simple Mendelian segregation for pathogenicity reactions.

When tested separately, none of the mutants has been induced to fruit or to form structures recognisable as basidia, whereas in some paired combinations they give rise to fertile heterocaryons. The frequency of curly, stumpy, fleecy, or button (cu ru) progeny in some crosses indicates a tendency of each to self in those crosses, and tetrads resulting from the selfing of curly were recovered from the cross curly x rusty. This suggests that there are a number of stages, similar to those in the Ascomycetes (Raper, 1960), in the sexual progression from vegetative hyphae to the release of

mature basidiospores. The developmental sequence in T. cucumeris has been described (Flentje et al, 1963) and the most probable stages at which blockages might occur are as follows: (a) dicaryosis, the change from a multinucleate cell to several binucleate cells by septation; (b) proliferation of the binucleate cells leading to basidia; (c) karyogamy; (d) meiosis; (e) sterigmatal and basidiospore formation, including the migration of nuclei into the spores; and (f) basidiospore maturation and release. The recovery of tetrads of curly basidiospores, of only curly hyphae from the basidial and prebasidial cells and of only wild type hyphae from the vegetative runner hyphae which gave rise to the basidia in the cross curly x rusty, is evidence that the sexual progression of curly is blocked at the stage of dicaryosis and that rusty nuclei trigger septation which results in the binucleate cells. Rusty appears to be blocked at some later stage which requires the presence of a curly nucleus in the binucleate cells. However, there is not sufficient information at present to determine the specific stages of blockage in rusty

and the other mutants, but since the wild type recombinants are self-fertile and recovered mutant progenies have not been induced to fruit, the blockages are more likely genetic than cytoplasmic factors. The failure of some of the wild type heterocaryons to fruit also suggests that there may be more than six stages at which the sexual cycle may be blocked. None of the heterocaryons between pairs of irradiated mutants have been induced to fruit even though the mutants (i.e. stumpy and curly) are non-linked single gene mutations, whereas a heterocaryon between a single irradiated mutant (stumpy) and the double mutant, carrying the irradiated curly gene and the spontaneous rusty gene, fruited readily. This failure in some heterocaryons appears to be inherent, but the environment may also have an influence; high relative humidity is known to affect the development of prebasidial cells (Stretton et al., 1964). The fertile and non-fertile heterocaryons were maintained under similar environmental conditions during the fruiting trials, but it has been a frequent occurrence in these trials that not all replicates of a single culture,

whether a synthesized heterocaryon or a wild type single basidiospore culture, formed fructifications.

The high frequency of one of the parental types amongst the progeny from each of several fertile heterocaryons may also be a reflection of the ratio of the component nuclei in those heterocaryons. If it were, different frequencies of progeny types in individual batches from the same cross would suggest that adjacent parts of a single thallus contain different ratios of the same two mutant nuclei. It is more likely, however, that the differences between some batches are due to different stages of maturity of individual clumps of basidia and if spores were collected from the same clumps over a number of successive days, the progeny type frequencies of different batches would be similar and perhaps more representative of the nuclear ratio in the heterocaryon. The differences between batches show that single small sample sizes, such as the 20 progeny per heterocaryon used by Whitney and Parmeter (1963), may not be representative samples and could lead to misinterpretation.

Additional information on nuclear ratios can

be obtained from cytological studies, particularly on anastomoses. The cytological studies have provided a great deal of information on nuclear numbers in vegetative cells of the mutants (Tables VII and VIII) and of heterocaryons (Table X), but this information does not help to explain the nuclear behaviour during heterocaryosis and critical information is not available. For instance, if a heterocaryon is formed through anastomosis between two mutants, each containing 6-10 nuclei per cell, the anastomosed cells contain 12-20 nuclei. How are these nuclei sorted out in the cells developing from the anastomoses to return rapidly to a complement of 6-10 nuclei per cell in heterocaryotic hyphae, and is the number of nuclei per cell critical, related to cell size, cytoplasmic volume or other characteristic? The number of curly nuclei in a cell which is anastomosing to produce a heterocaryon could be as high as 50 and yet the heterocaryotic cells not far removed from the site of anastomoses contain 3-13 nuclei (Table X). In one heterocaryon (curly + rusty) the curly nuclei seem to predominate, whereas in a second, unstable heterocaryon (curly +

fleecy) the curly nuclei appear to be drifted out as the heterocaryon develops. Critical studies on the nuclear behaviour during heterocaryosis may help to answer some of these questions.

Studies on the behaviour of the unstable heterocaryons may also provide some leads on nuclear behaviour in heterocaryosis. The present data suggest that mutation and cytoplasmic factors both could be mechanisms responsible for reversion and for variation of pathogenicity in the progenies of the unstable heterocaryons, but they do not distinguish between these or other alternatives. It is probable that more than a single mechanism is involved, and in order to determine this and to determine the genotype of fleecy, additional data are required, particularly from tetrad analyses, on progeny from (a) the two fertile heterocaryons, (b) other heterocaryons in which fleecy is a component, and (c) crosses of the fleecy progeny which produce each of the different pathogenic reactions, with mutants of known genotype. Such experiments should eventually make it possible to interpret the phenomenon of unstable heterocaryons.

Recovery of wild type and ropy progeny from a wild type culture which was derived from one of three basidiospores, the products of a single basidium from the cross ropy x button, is the first evidence in T. cucumeris of a culture derived from a binucleate basidiospore. This evidence is proof that two nuclei may migrate from the basidium and enter a single basidiospore, as claimed by Flentje et al (1963). It emphasizes the need to examine critically single basidiospore progeny of isolates of T. cucumeris in any investigation on variation, since a wild type single basidiospore culture could be the result of either a single wild type nucleus or of two (or more) nuclei, one or both being mutants, in the basidiospore. Cultures derived from basidiospores containing mutant nuclei could well account for the wide number of colony types recovered by Whitney and Parmeter (1963) from their crosses of C.D.A.-negative and C.D.A.-positive cultures.

The results of this investigation purposely have not been compared with results of the heterothallic, higher Basidiomycetes, and in particular

of Schizophyllum commune, as there appears to be some doubt about the mating system and the actual function of the mating type factors in these fungi. A complete review is beyond the scope of this dissertation, but it would appear that at least some of the investigations are more readily explained on the assumption that S. commune is homothallic and that the 'A and B' factors are associated primarily with heterocaryon incompatibility and are not mating type factors. The tetrapolar mating pattern was delineated by Kniep (1920, cited by Mounce, 1921), who claimed that the difference in nuclear behaviour during the formation of basidiospores between a monocaryotic and a dicaryotic fruiting body is that in a monocaryotic fruiting body the four nuclei in a basidium are the products of two mitotic divisions and not the products of karyogamy and meiosis. This writer is not aware of other published reports which would support or refute Kniep's claims. However, since that investigation there have been a number of reports of self-fertile monocaryotic cultures and Raper (1960) suggested that this self-fertility is probably a mutative anomaly which

results in 'functional homothallism'. More recently, Middleton (1964a, b) synthesized heterocaryons of S. commune between biochemical mutants containing the same A and B factors, induced them to fruit and demonstrated sexual recombination of the biochemical mutant genes; he concluded that the occurrence of sexual recombination was not directly affected by the A or B factors, although he apparently did not consider his results as a demonstration of homothallism in S. commune. It would seem, therefore, that an examination of the cytology of a monocaryon fruiting body and a re-evaluation of interpretations of the published material on the basis of a homothallic mating system could be very rewarding.

GENERAL DISCUSSION

Most references in the literature on Thanatephorus cucumeris simply catalogue variation or attempt to correlate one type of variation with another (i.e. pathogenicity and cultural characters), but this approach has yielded little progress. Only a few recent studies have been concerned with the mechanisms underlying variation, which was the central theme of the present study.

Uninucleate cells are essential for a study on variation and, as other workers had found, basidiospores are the only cells in T. cucumeris which may be uninucleate. In order to utilize these cells effectively, it was necessary to obtain much fuller information than previously reported on the mating system and this in turn involved study of fertility. In most previous investigations of the mating system, only small percentages of single basidiospore progenies formed normal basidia and basidiospores. Of the remainder, a few progenies formed abnormal basidia, with or without spores (Hawn and Vanterpool, 1953; Daniels, 1963), but the majority failed

even to form basidia (Kotila, 1929; Hawn and Vanterpool, 1953; Flentje and Stretton, 1964). There seems little doubt that fertility is controlled genetically but the mechanisms could be either sterility factors in a homothallic organism, or secondary homothallism with fertile single spore cultures derived originally from binucleate basidiospores.

From the present investigation and those earlier studies in which fertility was studied through several selfed generations (Kotila, 1929; Flentje and Stretton, 1964), it is clear that some isolates of *T. cucumeris* are homothallic. However, it cannot be assumed that all isolates are homothallic, since *T. cucumeris* may not even represent a single natural species and investigations have covered only some half-dozen isolates. But it can be assumed that failure to fruit of some progenies of homothallic isolates is due to sterility factors. Results of fruiting trials with heterocaryons formed from paired morphological mutants suggests that a number of non-allelic genes may control fertility. The report of Daniels (1963) of a range of abnormal

basidia and spores formed from single spore cultures could be regarded as supporting evidence, but many of the 'abnormal' basidia illustrated by Daniels are better interpreted as shed basidiospores which had landed on hyphae and were germinating by repetition. Further investigation of factors controlling fertility is required but will depend upon appropriate genetic markers and more intensive study into the influence of environment on fertility.

It now appears that variation can occur by mechanisms involving mutation and anastomosis, both leading to heterocaryosis, and recombination. Before discussing these mechanisms and their possible operation in nature, it should be pointed out that many of the relevant technical terms (isolate, strain, etc.) have been used with different meanings by different workers. As confusion with terms can seriously affect interpretation, the writer considers it important that terminology be discussed. Early literature on Rhizoctonia included such terms as varieties, strains, groups, anastomosis groups, and isolates, when referring either to individual field isolations or to numbers of field isolations

which were considered similar in certain characteristics. Whitney and Parmeter (1963) refer to their field isolation of T. cucumeris as a clone and to single basidiospore progeny as strains. Flentje and Saksena (1957) grouped their field isolations (isolates) into pathogenic strains. Exner (1953) described field isolations as isolates, single basidiospore progeny as cultural strains and went so far as to divide T. cucumeris into formae speciales, basing the divisions on differences in the vegetative stage, diseases produced, and specificity to particular parts of hosts.

The term 'clone' — "a genetically uniform population derived from a single nucleus by mitotic divisions" (Fincham and Day, 1963) — is applied to vegetative propagation. It should not be used with T. cucumeris because the vegetative propagules are multinucleate hyphae, and they are not likely to remain genetically pure, although phenotypically they may not change. 'Isolate', in its strict sense, applies only to the first isolation of one individual; hence, one isolate cannot be equated to a second even if they are genetically identical,

and serial subcultures from a single first isolation are not actually included within the meaning of this term. A 'strain' is equated to a 'physiologic race' (Ainsworth, 1961). Applying the term 'strain' to progeny of a single parent (Whitney and Parmeter, 1963) forces the pathogenic groupings of Flentje and Saksena (1957) into a higher subdivision, where they become bound up with Exner's (1953) formae and this in turn leads to further confusion. Thus, the terms 'clone', 'strain', and 'isolate', are unsatisfactory and should be redefined or else completely new terms should be introduced. However, much more information may be required before this can be accomplished satisfactorily. In the meantime, the writer has followed the practice of this Laboratory and used the terms 'strain' and 'isolate' (sensu Flentje and Saksena, 1957).

Returning to the discussion of mechanisms of variation, the concept of the wild type serves as a useful starting point. The wild type is an individual without known genetic abnormalities which approximates the standard form of the species in

nature (Fincham and Day, 1963). The wild type thus represents the sum of available genetic factors advantageous for growth and survival in a situation which involves all the components of the environment. For a particular soil over a period of time, the components of the environment will include soil type, moisture relations, temperature, oxygen and carbon dioxide tensions, food substrates including host and non-host plants, and competition and parasitism from other microorganisms. The wild type is that type which from the available genetic material is best able to cope with these components. There can be little doubt that mutations occur in nature, but, according to the definition of the wild type, any mutation is likely to be deleterious for this situation. As shown in the present investigations, mutations may accumulate in the wild type although they are unlikely to affect either its cultural characters or behaviour. However, the particular situation may change, either by the organism spreading to new areas or by modifications in the original soil through changed cropping or management practices. In this changed environment

the original wild type may no longer be the best adapted, and as a result of the new selective forces a new wild type may eventually emerge. On this basis, one could account for the very limited number of cultural types of T. cucumeris which so far have been isolated from any one soil under more or less constant conditions, and for the quite different cultural types which are found in different soils or in the same soils under different conditions.

The wild type is therefore dynamic and can change with differing selective forces. Thus, one would eventually expect to recover a single wild type from different field isolates brought together under the same conditions. However, field isolates of different pathogenic strains of T. cucumeris which have been brought together and studied under uniform conditions in various Laboratories have maintained their distinctive characters. The information in Table XIX, tabulated for simplicity from results of the present investigation and from published data (Flentje et al., 1963; Flentje and Stretton, 1964), shows that no single cultural type has evolved from the three field isolates

TABLE XIX

SUMMARY OF CHARACTERISTICS OF THREE FIELD ISOLATES
OF T. CUCUMERIS

	Isolate No.		
	16	48	82
Pathogenic specificity	roots, unspecialized	crucifer stems	non-pathogenic
Infection cushions formed	none	+	cushion-like but producing basidia
Cultural appearance: color sclerotia	brown few	white many, small	white many and basidial clumps
Nitrogen requirement	inorganic	organic	not determined
Homothallic	not proved conclusively	+	+
Fruiting substrate	soil	soil	agar and soil
Variation (range) in progeny	wide	narrow	narrow
Binucleate basidio-spores %	10-12	1-2	0-1
U.V. intensity for 95% kill, ergs/mm ²	not determined	12,744	3,186

studied. Within a single pathogenic strain, the crucifer strain, isolate 69 is similar to isolate 48 in many respects, including host range, physiology of parasitism, and fertility, but has remained culturally distinct from isolate 48 (Dodman, 1965; Flentje and Stretton, unpublished). Further, there has been no obvious change in cultural appearance or pathogenicity of these or other field isolates maintained by regular subculturing for more than 12 years (Flentje and Stretton, 1964). It is thus apparent that the concept of a single wild type for T. cucumeris is not valid. The reasons may be that different isolates of different or even the same pathogenic strains may not have the same genetic material available to respond to the selective forces of a particular situation. These differences in genetic material may be due in part to the fact that individuals may belong to different species or subspecies of Thanatephorus and have different ancestries, or if they have a common ancestry they may have evolved through different sets of selective forces which have isolated them from one another. For the present it is mere

useful to consider wild type as an isolate corresponding to the form of a pathogenic (or cultural) strain as found in nature, and to study various wild types to determine the extent of variation in, and the genetical makeup of each. Such studies will determine whether the mechanisms and ranges of variation between wild types of the same, similar, or different pathogenic strains overlap or are distinct. This will provide a sounder basis for subdividing T. cucumeris into subspecies, formae speciales, etc., on reasonably stable differences.

All field isolates studied have been heterocaryotic, as shown by variation in single spore progenies of original hyphal tip cultures. As it is now known that mutation readily occurs, it is likely that any and every field isolation of T. cucumeris will be heterocaryotic. However, the variation in single spore progeny of a heterocaryotic field isolate either may cover a wide range of characteristics including cultural appearance, saprophytic ability, pathogenicity and self-fertility (i.e. isolate 16) or may be restricted to minor differences in pigmentation and amount

of sclerotial development (i.e. isolate 48 or 82). There is no direct evidence to explain these differences but again they could be due to differences in genetic material available in different isolates. In his ecological studies in cereal growing areas, de Beer (1965) found that the root strain of T. cucumeris was of limited distribution either in any one soil or over a range of soil types, whereas the crucifer strain and a non-pathogenic strain were fairly widespread. This suggests that although the crucifer and non-pathogenic strains are restricted in pathogenicity, each is apparently well adapted for saprophytic growth, having genetic material able to adjust to a range of selective forces, whereas the root strain is more dependent on the parasitic phase for survival and has genetic material less able to adjust to the selective forces of the saprophytic environment. Selective forces on a strain with little available genetic material (i.e. few genes controlling saprophytic ability) could result in a high mutation rate of that material as the strain attempts to reach an equilibrium with the environment. However, to test

such a hypothesis, much more critical information is required, firstly on the components of the environment which comprise the selective forces, and secondly on the extent or limits of mutation itself.

Mutation does affect a range of physiological characteristics and they may affect cultural appearance, growth, fertility, basidiospore visibility, and pathogenicity and virulence. Except for a mutation from sterility to fertility in the single spore culture of 48-11, mutations were only detected as a result of passage through the sexual stage and recovery from uninucleate propagules, the basidiospores. The heterocaryotic field isolate 48 yielded first generation progeny varying only in ability to fruit and in minor cultural characteristics; no non-pathogenic mutants were detected. However, P56, the third generation single spore culture, was initially homocaryotic but rapidly accumulated mutants which, when recovered as single spore progeny, were culturally distinct and non-pathogenic (e.g. the mutants rusty and ropy).

The occurrence of non-pathogenic mutants in P56 and their apparent absence in isolate 48 is

of considerable interest. As P56 and 48 are genetically similar, it is probable that similar mutations had occurred in 48 but were selected against and lost as 48 reached equilibrium with its environment. This would mean that the mutants in P56 would probably be lost if P56 was grown for an extended period in the field.

Mutants from P56 were readily obtained as either the spontaneous mutations mentioned above or induced mutations. Experiments with these mutants demonstrate the importance of reliable genetic markers in studies of variation in pathogenic and saprophytic behaviour. Investigations of these mutants in relation to infection and disease progress in radish seedlings showed that each mutation affected a different stage and that pathogenicity is controlled by at least four independent genes.

The general understanding of factors influencing anastomosis and heterocaryosis is poor. Most information has come from studies on Neurospora crassa, where heterocaryon incompatibility was shown to have a genetic basis (Garnjobst, 1955;

Holloway, 1955; Garnjobst and Wilson, 1956), but most subsequent investigators avoided the problem either by using mutants induced in the same genetic background or following a backcrossing program to obtain the necessary homogeneity between isolates (Pittenger, 1964). The present investigation deals with anastomoses between closely related progeny of T. cucumeris isolate 48. The results show that although pairs of closely related cultures anastomose and form cytoplasmic connections, in some combinations the participating cells died. Death of the participating cells of paired mutants, however, did not necessarily prevent the formation of heterocaryons. Other work (Flentje and Stretton, 1964) indicates that pairings of single spore cultures from different isolates of the same pathogenic strain commonly resulted either in wall fusions without cytoplasmic connections or in complete killing of anastomosing cells, which did prevent heterocaryon formation. There is little known about what mechanisms are involved in anastomosis or how these mechanisms operate, and what their limits are.

Heterocaryons were formed from combinations

of the non-pathogenic mutants and were stable, producing cultures indistinguishable from the wild type both in cultural appearance and pathogenicity. This work with reliable genetic markers confirms the earlier work of Whitney and Parmeter (1963) and provides more extensive evidence of the effects of heterocaryosis on pathogenicity. The heterocaryons formed between the pathogenic mutant, flescy, and single non-pathogenic mutants were also wild type in appearance but each of these heterocaryons was unstable and rapidly reverted to mutant type growth. This work provides the first significant evidence of heterocaryon instability in T. cucumeris.

Tetrad and random spore analyses of progenies from fertile heterocaryons provide genetical evidence that the basic pattern of nuclear behaviour in the formation of basidiospores in T. cucumeris, namely karyogamy and meiosis, is similar to that described for many Basidiomycetes, and supports the cytological evidence (Hawn and Vanterpool, 1953; Saksena, 1961a; Flentje et al. 1963). The analyses suggest that both mutants in a heterocaryon were necessary to initiate fruiting, but that both

were not necessarily required to continue to the formation of spores. In some crosses the majority of spores represented only one of the mutants and evidence of selfing was obtained by tetrad isolations. These results support the suggestion that there are several genetically controlled steps in the sexual process. The recombination studies also show that, in the stable heterocaryons, genetic variation occurs through the sexual cycle. Crosses of two single mutants yielded the two parental and two recombinant progeny types and crosses of single x double mutant yielded eight progeny types. The fertile but unstable heterocaryons, each containing the pathogenic mutant fleecy as one component, also yielded progeny which were variable, although this variation did not appear to follow the Mendelian pattern of segregation. Progenies were predominantly fleecy in cultural characteristics but varied in pathogenicity to radish seedlings. This variation in pathogenicity apparently was not due to genetic exchange with nuclei of the second component in each of the crosses.

Some aspects of variation in number of nuclei per cell in vegetative hyphae of T. cucumeris have been discussed previously, but there are several important questions arising from these studies, the answers to which may help in the understanding of the mechanisms of variation. Firstly, does the influence of a particular nucleus extend beyond the cell which contains it? In many organisms there appears to be a relatively constant nucleo-cytoplasmic ratio and it is thought that a nucleus cannot extend its control over an indefinitely large amount of cytoplasm (Swanson, 1960). However, cytoplasmic streaming occurs in many plant cells including fungal cells, and in the fungi a cell is not entirely enclosed by cell walls. It seems likely that nuclei may extend their influence at least into contiguous cells. Three different sets of observations in T. cucumeris suggest that the influence of a nucleus may extend beyond the limits of a single cell: (A). A few stained cells in the mycelium of the mutant curly were without stained nuclei. The uniformity of the stain in the cells and in the nuclei of the mycelium indicated that

these few cells were living but contained no nuclei.

(B). Isolations from fructifications of the curly x rusty cross were made progressively from the binucleate basidial and prebasidial cells back to the multinucleate vegetative cells. All the binucleate cells tested contained curly nuclei, and only in the multinucleate cells were both mutants commonly represented. This suggests that the initiation of septation and dicaryosis in the side branches by rusty was controlled by rusty nuclei in cells other than these side branches.

(C). The abrupt reversion from wild type to mutant type growth in each of the unstable heterocaryons might suggest that the last few wild type cells contained only nuclei of the reverting mutant, that those cells were wild type because of the influence of nuclei of the second mutant which were present in cells further back from the hyphal tips, and that when this influence was diluted reversion to mutant growth occurred.

The second main question arises from the first; what proportion of two different mutant nuclei is required for the mutants to complement

one another. In their review of this topic, Par-
meter et al (1963) concluded that in the Ascomycetes,
heterocaryons synthesized from complementary de-
ficient mutants were maximal in growth rate over
a wide range of nuclear ratios as determined by
conidial plating. However, in the Basidiomycetes
studies have been limited to those organisms contain-
ing two nuclei per cell in vegetative heterocaryons,
and ratio changes have not been exhibited. In the
stable heterocaryons of T. cucumeris, it would appear
that the component nuclear types are not in equal
proportions, as judged from the frequencies of
mutant progenies.

Nothing is known about nuclear behaviour dur-
ing and immediately following a cytoplasmic connec-
tion and the initiation of a heterocaryon. It is
probable, however, that approximately equal numbers
of the component nuclei are present in the initial
cells of a heterocaryotic hypha and that the pro-
portion changes as the hypha grows. The reversion
to mutant type in the unstable heterocaryons supports
this suggestion, as do the two observations of
wild type heterocaryons containing wild type and

mutant nuclei. The first, a wild type single spore culture, derived from a basidiospore containing one wild type and one ropy nucleus, fruited to yield approximately 80% wild type and 20% ropy progeny. The second, the wild type sector arising in a rusty progeny was presumably due to a single mutation to wild type in one of about six nuclei in a single cell; the wild type mycelium was induced to fruit and the progeny were 93% wild type and 7% rusty. These figures are undoubtedly overestimates of the final proportions of wild type nuclei in the heterocaryons, as the wild type progenies would have arisen both from selfed and crossed basidia whereas the mutant progenies probably had been derived only from hybrid basidia. Nevertheless, it appears that the final ratios of the component nuclei were different from the initial ratios.

There are several possible ways in which the nuclear ratio in a heterocaryon could change, namely: degeneration of nuclei; differential rates of division of the component nuclei in single cells; differential segregation of daughter nuclei in hyphal tips during septum formation; or nuclear

migration. Nuclear degeneration has not been observed in T. cucumeria, but if it occurs in a heterocaryon it seems likely that it would affect either all the nuclei and result in death of the heterocaryon, or all the nuclei of a particular genotype. Furthermore, in subcultures made over an extended period there was no indication, even in the unstable heterocaryons, of different rates of survival of the component nuclear types. With regard to differential rates of division, the cytological evidence in T. cucumeria shows that generally all the nuclei in a cell divide simultaneously. In other fungi there is no good evidence to suggest that nuclei in the same hyphal tips divide at different rates (Parmeter et al., 1963). Uneven segregation of pairs of daughter nuclei could account for localized changes in nuclear ratios, but would require a complex system of control. In a stable heterocaryon, differential segregation would involve initial differentiation between mutant nuclei up to a point of balance and then a mechanism to maintain the balance by even segregation of pairs of daughter nuclei. In an unstable heterocaryon, segregation

would have to be controlled so that one of the two types of nuclei always segregated toward the growing hyphal tips. The reverted growth of a stumpy + fleecy heterocaryon always is stumpy whereas the heterocaryons with fleecy and each of the other four single mutants and one double mutant always revert to fleecy. Stumpy, however, forms stable heterocaryons with each of these other mutants. It cannot be easily explained, on the basis of one or several mechanisms controlling differential segregation, how the same mutant nuclei behave differently in different heterocaryons.

Migration of nuclei could account for different numbers of nuclei in successive cells of the hyphae, as discussed earlier, and there is evidence (Bracker and Butler, 1964) that the septal pore in Rhizoctonia solani is similar to that in other Basidiomycetes where nuclear migration has been demonstrated. However, the evidence for nuclear migration in the Basidiomycetes (Snider, 1963; Giesy and Day, 1965) has been based on observations of unilateral migration of one nuclear type through pre-existing hyphae of a second

nuclear type; this may be an entirely different situation from that of migration within an established heterocaryon of one of the nuclear types making up that heterocaryon. Further, in the anastomosis experiments with mutants of T. cucumeris, heterocaryotic hyphae were observed only at the line of junction of two mutant colonies. Failure to recover heterocaryotic hyphae from around the periphery of either mutant could be taken as evidence against extensive unilateral migration in these cultures.

It has now been established that variation in T. cucumeris can occur by the mechanisms of heterocaryosis by mutation and anastomosis, and recombination. There is, however, little definitive evidence as to which mechanisms are operative in nature and how they operate. The concept of wild type in T. cucumeris, as discussed above, offers an explanation of how mutation may be induced by selective forces in a natural environment, but the critical information to test such a hypothesis has not yet been obtained. Studies to date have shown that mutation can affect growth and

pathogenicity, but there are some indications that mutation may not enable, in time or extent, individuals to meet certain changes. Flentje and Stretton (1964) showed that field isolates introduced to new soil may die out completely, and so far there has been no evidence to show changes in pathogenic specificity to new hosts.

It could be postulated that to meet certain selective forces, such as a new soil type, new genetic material must be introduced by anastomosis and heterocaryosis. Such introduction could result in hybrids formed from existing strains, and pose additional problems in disease control measures. Information on this aspect is very restricted although considerable speculation has been published and there is an urgent need for investigations before speculation becomes accepted as fact. Heterocaryosis can occur between single spore cultures from one parent and affect cultural characteristics, pathogenicity and fertility, but this is little different from variation by mutation as the differences between progeny from a single parent arose initially by mutation. But even during heterocaryosis between

such closely related cultures, the anastomosis killing reaction may occur, indicating that this mechanism of variation may be limited because of some type of heterocaryon incompatibility. The evidence that field isolates which differ in a range of characteristics including pathogenicity, either will not form cytoplasmic connections or form cytoplasmic connections followed by the killing reaction (Flentje and Stretton, 1964), suggests that the factors affecting heterocaryon incompatibility are widely distributed. No successful heterocaryons have been formed between cultures pathogenic to different hosts and it is probable that hybridization between two such strains would be rare in nature.

Studies on recombination have been limited and concern only crosses involving closely related individuals. It should be emphasized that in considering the effects of recombination under natural conditions through the sexual cycle, there is an important practical consideration. For serial types of T. cucumeris in which the spores are airborne, there is evidence that the spores

can survive and infect new plants (Echandi, 1965). However, for soil-borne forms there is no reliable evidence that spores can survive and produce new colonies in soil (Flentje and Stretton, 1964).

There is at present no information as to whether recombination occurs through a parasexual cycle (Pontecorvo, 1956) in T. cucumeris. The simultaneous division in close proximity of the nuclei in vegetative cells would appear to present easy opportunity for genetic exchange, but, because of the absence of asexual spores, such interchange of genetic material may not be detected readily.

The suggestions put forward here could account for much of the variation within single field isolates of T. cucumeris, but they do not account for the variation between field isolates and in particular for the origin of distinct pathogenic strains. If further evidence supports the suggestion that mutation and recombination do not immediately yield new pathogenic strains, it is probable that a series of mutations may be involved and that at one or more points in the evolutionary sequence, incompatibility factors

operate to prevent heterocaryosis, resulting in isolation of particular genotypes. Isolated genotypes would then be subjected to further mutations in different directions and thus evolve different pathogenic strains. Evolution of a new pathogenic strain would be due to the effects of particular selective forces, and while there is little knowledge of what these forces might be, they are likely to be related to a potential new host plant.

The present investigation has utilized some new and modified techniques for tetrad and shed basidiospore isolations, for induction of mutants, and for the identification of mutant progenies by the heterocaryon test. There is now a need for screening techniques, particularly for use in the detection of pathogenicity mutations occurring either spontaneously or in irradiated material. A suitable minimal medium would be useful for recovering and utilizing biochemical mutations in further studies of variation in T. cucumeris. Recombination studies have been hampered by the failure to induce fructifications consistently, and investigations of the effects of the

environment on fruiting would greatly facilitate more extensive variation studies.

The importance of the environmental selective forces and of the factors associated with anastomosis and heterocaryosis have been discussed. Determination of these effects is necessary to understand the limit or extent of variation, and indirectly will have an important bearing on the development of suitable control measures. Perhaps the most promising lines of investigation into this aspect are heterocaryon studies with mutants from each of several field isolates of the same pathogenic strain and subsequently with mutants from each of several isolates of different pathogenic strains.

The six mutants used in the present study are ideal tools for studying the mechanisms of penetration, and together with the purification of the active material from radish seedlings which induces the formation of infection cushions (Dodman, 1965), should contribute significantly towards an understanding of the physiology of the fungus and of parasitism.

REFERENCES

- AINSWORTH, G.C. (1961). Ainsworth and Bisby's dictionary of the fungi. 5th Edit. Commonwealth Mycological Institute, (Kew).
- de BEER, J.F. (1965). Studies on the ecology of Rhizoctonia solani Kühn. Ph.D. Thesis, University of Adelaide.
- BOIDIN, J. (1958). Essai biotaxonomique sur les Hydnes résupinés et les Corticiés. Theses No. 202. Univ. de Lyon :99-102.
- BRACKER, C.E., and BUTLER, E.E. (1964). Function of the septal pore apparatus in Rhizoctonia solani during protoplasmic streaming. J. Cell. Biol. 21: 152-7.
- BRODIE, H.J. (1936). The barrage phenomenon in Leucispora betulina. Genetica. 18: 61-73.
- BULLER, A.H.R. (1933). Researches on fungi. Vol.V. Longmans Green, (London).
- BURNETT, J.H. (1956). The mating systems of fungi. I. New Phytol. 55: 50-90.
- BUTLER, E.E. (1957). Rhizoctonia solani as a parasite of fungi. Mycologia. 49: 354-373.
- DANIELS, Joan (1963). Saprophytic and parasitic activities of some isolates of Corticium solani. Trans. Br. mycol. Soc. 46: 485-502.
- DODMAN, R.L. (1965). Studies on plant exudates and the mode of penetration by Thanatephorus cucumeris. Ph.D. Thesis, University of Adelaide.

- ECHANDI, B. (1965). Basidiospore infection by Pellicularia filamentosa (= Corticium microsclerotia), the incitant of web blight of common bean. Phytopathology. 55: 698-9.
- EDWARDS, H.I., and NEWTON, W. (1937). The physiology of Rhizoctonia solani Kühn. V. The activity of certain enzymes of Rhizoctonia solani Kühn. Scient. Agric. 17: 544-9.
- ESSER, K. (1959). Die Incompatibilitätsbeziehungen zwischen geographischen Rassen von Podospora anserina. II. Die Wirkungsweise der Semi-Incompatibilitäts-Gene. Z. VererbLehre. 90: 29-52.
- EXNER, Beatrice (1953). Comparative studies of four Rhizoctonias occurring in Louisiana. Mycologia. 45: 698-719.
- FINCHAM, J.R.S., and DAY, P.R. (1963). Fungal genetics. Blackwell Scientific Publications, (Oxford).
- FLEMMING, A., and SMITH, G. (1944). Some methods for the study of moulds. Trans. Br. mycol. Soc. 27: 13-19.
- FLENTJE, N.T. (1956). Studies on Pellicularia filamentosa (Pat.) Rogers. I. Formation of the perfect stage. Trans. Br. mycol. Soc. 39: 343-356.
- FLENTJE, N.T. (1957). Studies on Pellicularia filamentosa (Pat.) Rogers. III. Host penetration and resistance, and strain specialization. Trans. Br. mycol. Soc. 40: 322-336.
- FLENTJE, N.T. (1959). The physiology of penetration and infection. In Plant Pathology Problems and Progress 1908-1958. pp.76-87. (Eds. Holton, C.S., et al), Univ. of Wisconsin Press.

- FLENTJE, N.T. (1965). Pathogenesis by soil fungi. In Ecology of Soil-borne Plant Pathogens. pp. 255-266. (Eds. Baker, K.F., and Snyder, W.C.), Univ. of California Press.
- FLENTJE, N.T., DODMAN, R.L., and KERR, A. (1963). The mechanism of host penetration by Thanatephorus cucumeris. Aust. J. biol. Sci. 16: 784-799.
- FLENTJE, N.T., and SAKSENA, H.K. (1957). Studies on Pellicularia filamentosa (Pat.) Rogers. II. Occurrence and distribution of pathogenic strains. Trans. Br. mycol. Soc. 40: 95-108.
- FLENTJE, N.T., and STRETTON, Helena M. (1964). Mechanisms of variation in Thanatephorus cucumeris and T. praticolus. Aust. J. biol. Sci. 17: 686-704.
- *FLENTJE, N.T., STRETTON, Helena M., and HAWN, E.J. (1963). Nuclear distribution and behaviour throughout the life cycles of Thanatephorus, Waitea, and Ceratobasidium species. Aust. J. biol. Sci. 16: 450-467.
- GARNJOBST, Laura (1955). Further analysis of genetic control of heterocaryosis in Neurospora crassa. Am. J. Bot. 42: 444-8.
- GARNJOBST, Laura, and WILSON, J.F. (1956). Heterocaryosis and protoplasmic incompatibility in Neurospora crassa. Proc. natn. Acad. Sci. U.S.A. 42: 613-8.
- GIESY, R.M., and DAY, P.R. (1965). The septal pores of Coprinus lagopus in relation to nuclear migration. Am. J. Bot. 52: 287-293.

* Since repeated reference has been made in this dissertation to this paper, it has been designated as Flentje et al (1963) to distinguish it from the paper referred to as Flentje, Dodman and Kerr (1963).

- HAWN, E.J., and VANTERPOOL, T.C. (1953). Preliminary studies on the sexual stage of Rhizoctonia solani Kühn. Can. J. Bot. 31: 699-710.
- HOAGLAND, D.R., and ARNON, D.I. (1950). The water-culture method for growing plants without soil. Circ. Calif. agric. Exp. Stn. No. 347.
- HOLLOWAY, B.W. (1955). Genetic control of heterocaryosis in Neurospora crassa. Genetics. 40: 117-129.
- HRUSHOVETZ, S.B. (1956). Cytological studies of Helminthosporium sativum. Can. J. Bot. 34: 321-7.
- KERNKAMP, M.F., de ZEEUW, D.J., CHEN, S.M., ORTEGA, B.C., TSIANG, C.T., and KHAN, A.M. (1952). Investigations on physiologic specialization and parasitism of Rhizoctonia solani. Tech. Bull. Minn. agric. Exp. Stn. No. 200.
- KERR, A. (1955). Studies on the parasitic and saprophytic activities of Pellicularia filamentosa (Pat.) Rogers and Sclerotinia homeocarpa Bennett. Ph.D. Thesis, University of Adelaide.
- KERR, A. (1956). Some interactions between plant roots and pathogenic soil fungi. Aust. J. biol. Sci. 9: 45-52.
- KERR, A., and FLENTJE, N.T. (1957). Host infection in Pellicularia filamentosa controlled by chemical stimuli. Nature, Lond. 179: 204-5.
- KNIEP, H. (1920). Über morphologische und physiologische Geschlechts-differenzierung. Verh. phys.-med. Ges. Würzb. 46: 1-18. (Cited by Mounce, 1921.)

- KÖHLER, E. (1930). Zur Kenntnis der vegetativen Anastomosen der Pilze. II. Planta. 10: 495-522.
- KORF, R.P. (1952). The terms homothallism and heterothallism. Nature, Lond. 170: 534-5.
- KOTILA, J.E. (1929). A study of the biology of a new spore-forming Rhizoctonia, Corticium praticola. Phytopathology. 19: 1059-1099.
- LACY, M.L., and BRIDGMON, G.H. (1962). Potato-dextrose agar prepared from dehydrated mashed potatoes. Phytopathology. 52: 173.
- MATSUMOTO, T., YAMAMOTO, W., and HIRANE, S. (1932). Physiology and parasitism of the fungi generally referred to as Hypochnus Saasakii Shirai. I. Differentiation of the strains by means of hyphal fusion and culture in differential media. J. Soc. trop. Agric. (Taiwan). 4: 370-388.
- MIDDLETON, R.B. (1964a). Evidences of common-AB heterokaryosis in Schizophyllum commune. Am. J. Bot. 51: 379-387.
- MIDDLETON, R.B. (1964b). Sexual and somatic recombination in common-AB heterokaryons of Schizophyllum commune. Genetics. 50: 701-710.
- MOUNCE, Irene (1921). Homothallism and the production of fruit-bodies by monosporous mycelia in the genus Coprinus. Trans. Br. mycol. Soc. 7: 198-217.
- MÜLLER, K.O. (1924). Untersuchungen zur Entwicklungsgeschichte und Biologie von Hypochnus solani P.u.D. (Rhizoctonia solani K.). Arb. biol. Reichsanst. Land- u. Forstw. 13: 197-262. (Rev. appl. Mycol. 4: 184. 1925. Abstr.)

- OLIVE, L.S. (1958). On the evolution of heterothallism in fungi. Am. Nat. 92: 233-251.
- PAPAVIZAS, G.C. (1965). Comparative studies of single-basidiospore isolates of Pellicularia filamentosa and Pellicularia praticola. Mycologia. 57: 91-103.
- PAPAZIAN, H.P. (1950). Physiology of the incompatibility factors in Schizophyllum commune. Bot. Gaz. 112: 143-163.
- PAPAZIAN, H.P. (1958). The genetics of Basidiomycetes. Adv. Genet. 9: 41-69.
- PARMETER, J.R., Jr., SNYDER, W.C., and REICHLER, R.E. (1963). Heterokaryosis and variability in plant-pathogenic fungi. A. Rev. Phytopath. 1: 51-76.
- PERKINS, D.D. (1953). The detection of linkage in tetrad analysis. Genetics. 38: 187-197.
- PITTINGER, T.H. (1964). Spontaneous alterations of heterokaryon incompatibility factors in Neurospora. Genetics. 50: 471-484.
- PONTECORVO, G. (1956). The parasexual cycle in fungi. A. Rev. Microbiol. 10: 393-400.
- RAPER, Carlene A., and RAPER, J.R. (1964). Mutations affecting heterokaryosis in Schizophyllum commune. Am. J. Bot. 51: 503-512.
- RAPER, J.R. (1960). The control of sex in fungi. Am. J. Bot. 47: 794-808.
- RICHTER, H., and SCHNEIDER, R. (1953). Untersuchungen zur morphologischen und biologischen Differenzierung von Rhizoctonia solani K. Phytopath. Z. 20: 167-226.
- RIZET, G. (1952). Les phénomènes de barrage chez Podospora anserina. I. Analyse

génétique des barrages entre souches
S et s. Revue Cytol. Biol. vég. 13: 51-91.

- RIZET, G., and SCHECROUN, Janine (1959). Sur les facteurs cytoplasmiques associés au couple de gènes S-s chez le Podospora anserina. C.R. Acad. Sci. (Paris). 249: 2392-4.
- ROBINOW, C.F. (1945). Nuclear apparatus and cell structure of rod-shaped bacteria. Addendum to Dubos, R.J., The bacterial cell. pp. 355-377. Harvard Univ. Press, (Mass.).
- ROSS, D.J. (1960). Physiological studies of some common soil fungi from grassland soils. N.Z. Jl. Sci. 3: 219-257.
- SAKSENA, H.K. (1961a). Nuclear phenomena in the basidium of Ceratobasidium praticolum (Kotila) Olive. Can. J. Bot. 39: 717-725.
- SAKSENA, H.K. (1961b). Nuclear structure and division in the mycelium and basidiospores of Ceratobasidium praticolum. Can. J. Bot. 39: 749-756.
- SAMUEL, G. (1928). Two "stunting" diseases of wheat and oats. J. Dep. Agric. S. Aust. 32: 40-3.
- SAMUEL, G., and GARRETT, S.D. (1932). Rhizoctonia solani on cereals in South Australia. Phytopathology. 22: 827-836.
- SANFORD, G.B., and SKOROPAD, W.P. (1955). Distribution of nuclei in hyphal cells of Rhizoctonia solani. Can. J. Microbiol. 1: 412-5.
- SCHULTZ, H. (1936). Vergleichende Untersuchungen zur Ökologie, Morphologie und Systematik des 'Vermehrungspilzes'. Arb. biol. Reichsanst. Land- u. Forstw. 22: 1-41.

- SNIDER, P.J. (1963). Genetic evidence for nuclear migration in Basidiomycetes. Genetics. 48: 47-55.
- STEINBERG, R.A. (1950). Growth on synthetic nutrient solutions of some fungi pathogenic to tobacco. Am. J. Bot. 37: 711-4.
- STRETTON, Helena M., MCKENZIE, A.R., BAKER, K.F., and FLENTJE, N.T. (1964). Formation of the basidial stage of some isolates of Rhizoctonia. Phytopathology. 54: 1093-5.
- SWANSON, C.P. (1960). The cell. Foundations of Modern Biology Series. Prentice-Hall, Inc., (N.J.).
- TAKEMARU, T. (1961). Genetical studies on fungi. X. The mating system in the Hymenomycetes and its genetical mechanism. Biol. J. Okayama Univ. 7: 133-211.
- TALBOT, P.H.B. (1965). Studies of 'Pellicularia' and associated genera of Hymenomycetes. Persoonia. 3: 371-406.
- TYNER, L.E., and SANFORD, G.B. (1935). On the production of sclerotia by Rhizoctonia solani Kühn in pure culture. Scient. Agric. 16: 197-207.
- WARCUP, J.H. (1959). Studies on Basidiomycetes in soil. Trans. Br. mycol. Soc. 42: 45-52.
- WARCUP, J.H., and TALBOT, P.H.B. (1962). Ecology and identity of mycelia isolated from soil. Trans. Br. mycol. Soc. 45: 495-518.
- WHITEHOUSE, H.L.K. (1949). Multiple-allelomorph heterothallism in the fungi. New Phytol. 48: 212-244.

- WHITNEY, H.S., and PARMETER, J.R., Jr. (1963).
Synthesis of heterokaryons in Rhizoctonia
solani Kühn. Can. J. Bot. 41: 879-886.
- WILSON, J.R., GARNJOBST, Laura, and TATUM, E.L. (1961).
Heterocaryon incompatibility in
Neurospora crassa — micro-injection
studies. Am. J. Bot. 48: 299-305.

TABLE VII

DESCRIPTION OF MICROSCOPIC CHARACTERISTICS OF P56 AND MUTANTS

Isolate	Hyphal branching*	Septation*	Nuclei per cell (mean, S.E., and range)**			
			Peripheral tip cells	Older non-tips	Branch tips	Total
P56	Regular	Regular	8.77±0.20 (6-15)	5.36±0.10 (3-10)	4.79±0.12 (3-8)	6.07±0.11 (3-15)
<u>SPARSE</u>	Regular	Regular	7.63±0.16 (4-11)	5.92±0.12 (2-11)	6.42±0.12 (4-9)	6.48±0.08 (2-11)
<u>STUMPY</u>	Irregular	Regular	10.06±0.23 (5-17)	7.96±0.13 (4-14)	7.86±0.15 (5-11)	8.46±0.10 (4-17)
<u>FLEECY</u>	Irregular	Regular	7.63±0.16 (3-12)	5.90±0.10 (3-12)	5.27±0.12 (2-8)	6.18±0.08 (2-12)
<u>CURLY</u>	Irregular	Irregular	***	***	***	11.46±0.39 (0-50)
<u>FUSIX</u>	Irregular	Regular	6.36±0.13 (3-10)	4.89±0.14 (1-11)	5.64±0.17 (2-9)	5.44±0.09 (1-11)
<u>POXY</u>	Irregular	Regular	7.02±0.16 (4-11)	6.56±0.12 (1-11)	6.39±0.13 (4-9)	6.64±0.08 (1-11)

* For details see text

** Number of cells counted — peripheral tip cells, 100; older non-tips, 200; branch tips, 100; total, 400

*** Due to branching habit, cells not differentiated

TABLE VI

DESCRIPTION OF MACROSCOPIC CHARACTERISTICS OF P56 AND MUTANTS

Isolate	Colony Diameter cm	Colony Color	Cultural Appearance	Pathogenicity to radish seedlings	Self-fertility
P56	10.0	off white	wild type	Pathogenic; infection cushions, spreading lesions, seedling death	Yes
<u>Sparse</u>	9.0	off white	similar to wild type but sparse; few aerial hyphae	Non-pathogenic; no cushions, grows freely over stems without adhering	No
<u>Stumpy</u>	8.5	grey	dense, even radial growth; abundant aerial hyphae	Non-pathogenic; growth inhibited on stems	No
<u>Fleshy</u>	6.3	white	dense, even radial growth with dentate periphery; abundant aerial hyphae	Pathogenic; similar to P56	No
<u>Curly</u>	5.0	light brown to brown	dense, irregular shaped colony; aerial hyphae and sclerotia in tufts	Hypersensitive reactions; cushions produce localized necrotic flecks	No
<u>Rusty</u>	3.8	light brown to red brown	dense, irregular shaped colony; aerial hyphae and sclerotia in tufts	Non-pathogenic; few superficial cushions, sparse growth on stems	No
<u>Ropy</u>	6.5	white to light brown	'Ropy' irregular shaped colony; few aerial hyphae	Non-pathogenic, no cushions, grows over and adheres to stems	No