HETEROKARYON FORMATION AND GENETIC RECOMBINATION BETWEEN AUXOTROPHIC AND MORPHOLOGICAL MUTANTS OF *THANATEPHORUS CUCUMERIS*

By R. L. DODMAN*

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Abstract

Two auxotrophic mutants of *T. cucumeris*, one requiring nitrite and the other nicotinic acid, were obtained by irradiation of basidiospores with ultraviolet light. The deficiency was caused by a single-gene mutation in each case. Both mutants were fertile.

Fertile heterokaryons were formed between auxotrophic and morphological mutants. Random-spore analyses indicated that the genetic factors responsible for the mutations segregated independently of each other.

I. INTRODUCTION

Heterokaryosis and recombination in *Thanatephorus cucumeris* (Frank) Donk have been studied by McKenzie *et al.* (1969) with a range of spontaneous and induced mutants, differing from each other in morphological characters. It was demonstrated that each aberration resulted from a non-linked, single-gene mutation. Heterokaryons formed between pairs of mutants were wild type in appearance and recombination was demonstrated by the recovery of wild types and double mutants from the progeny of these heterokaryons. Further studies are limited by the range of morphological mutants that can be conveniently recognized. A search for nutritional markers was begun, because of the proven advantages of this type of mutant with other organisms.

Nutritional investigations with *T. cucumeris* have indicated that, in general, this fungus is prototrophic, although there is evidence of a requirement for vitamins by some isolates (Sherwood 1970). In studies of four distinct groups of *T. cucumeris*, Sherwood (1970) found that all isolates of group T had an absolute requirement for thiamine. The suitability of these deficiencies for genetical studies has not been investigated.

Whitney and Parmeter (1963) made use of an unknown growth requirement in their investigations on heterokaryosis. They found that half of the progeny of isolate R43 (anastomosis group 1, Parmeter, Sherwood, and Platt 1969) made poor growth on Czapek–Dox agar, while the other half grew normally. Heterokaryosis was demonstrated by recovering isolates which made poor growth on Czapek–Dox agar among the progeny of an isolate synthesized from positive and negative types.

* Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide; present address: Queensland Wheat Research Institute, Toowoomba, Qld. 4350.

In further work with isolate R43, Meyer and Parmeter (1968) induced mutation by ultraviolet irradiation and obtained isolates unable to form sclerotia on a defined minimal medium; these isolates were also intolerant to pentachloronitrobenzene. Again, heterokaryosis was demonstrated with the aid of these markers.

This paper describes the production and isolation of mutants with defined requirements, and the use of these mutants for studies of heterokaryosis and recombination.

II. Materials and Methods

The wild-type culture, 48 G3–56, and the mutants *stumpy*, *curly I*, *rusty*, and *ropy* have been described previously (Flentje, Stretton, and McKenzie 1967). The parent culture, isolate 48, belongs to anastomosis group 2 as defined by Parmeter, Sherwood, and Platt (1969). The mutant *curly II* (isolate 48–25) was obtained as a single-basidiospore culture from irradiated basidiospores of isolate 48.

In the different aspects of these studies five media were used. For the development of fructifications, the fungus was grown on potato–Vegemite–dextrose agar (P.V.D.A.) (McKenzie *et al.* 1969). Basidiospores were shed on and germinated on peptone–glucose–yeast extract agar (P.G.Y.A.), which contains all the inorganic constituents of the minimal medium (see below) and, in addition, 1 g proteose peptone (Difco), 0·1 g yeast extract (Difco), and 5 g glucose per litre. The glucose was autoclaved separately and the appropriate amount added aseptically to the remainder of the medium. The defined minimal medium contained 3·4 g $NaNO_3$, 21·05 g glucose, 0·68 g KH_2PO_4, 1·07 g K_2HPO_4, 4 mg $CaCl_2.2H_2O$, 0·5 g $MgSO_4.7H_2O$, 0·8 mg $FeSO_4.7H_2O$ complexed with 1·0 mg of the disodium salt of ethylenediaminetetraacetic acid, 0·4 mg $ZnSO_4.7H_2O$, 100 μg $MnSO_4.4H_2O$, 50 μg $CuSO_4.5H_2O$, and 40 μg MoO_3 dissolved in 1 litre of distilled water and containing 1·5% agar (Difco Bacto). As before, the glucose was autoclaved separately and added aseptically.

A sorbose medium was used to restrict the size of cultures developing from basidiospores. This contained all the constituents of the minimal medium except that the glucose was reduced to 0·42 g/l, the sodium nitrate to 1·7 g/l, and sorbose was added at the rate of 19·6 g/l.

A "complete" medium contained all the ingredients of the minimal medium (except where indicated) and, in addition, 20 μg H_3BO_3, 3 g ammonium tartrate, 1 g $NaNO_3$ (instead of 3·4 g) 2 g proteose peptone (Difco), 4 g casamino acids (Difco), 2·5 g yeast extract (Difco), 10 g sucrose, 10 g glucose (instead of 21·05 g), 5 g malt extract (Difco), 0·1 g ribose, 0·5 g ribonucleic acid type VI (Sigma Chemical Co.), 200 μg each of adenylic acid, guanylic acid, cytidylic acid, and uridylic acid, 1 mg thiamine, 2 mg nicotinamide, 2 mg pantothenic acid, 500 μg pyridoxin, 500 μg riboflavin, 5 mg inositol, 500 μg *p*-aminobenzoic acid, and 10 μg biotin dissolved in 1 litre of water and containing 1·5% agar (Davis). The sucrose, glucose, malt extract, and ribose were combined and autoclaved separately and added as required. The mixture of vitamins was treated in a similar way.

Fructifications of isolate 48 G3–56 were produced on soil (Stretton *et al.* 1964). Basidiospores were then shed on Cellophane covering P.G.Y.A. in Petri dishes and irradiated with ultraviolet light from an Oliphant Germicidal lamp, filtered to provide a wavelength of 2537 Å, and producing an intensity of 100 μW/cm² at a height of 23 cm. Heterokaryons between mutants were produced as described by McKenzie *et al.* (1969). Single-hyphal-tip cultures were made from all putative heterokaryons because of the possibility of cross-feeding with auxotrophic mutants.

III. Experimental

(a) *Isolation of Mutants*

Basidiospores of 48 G3–56 (and similar fourth or fifth generation wild-type cultures) were irradiated with various dosages of ultraviolet light. Dosages ranging

from 300 to 1200 ergs/mm^2 were obtained by maintaining the light at a constant height of 23 cm from the spores and varying exposure times from 30 to 120 s. The isolation of auxotrophic mutants was then carried out by two different methods.

(i) *Total Isolation Procedure*

Irradiated spores were incubated in the dark at 25°C and germinated spores picked off singly with a needle carrying a small piece of agar. The sporelings were transferred to complete medium and subsequently serially subcultured twice to distilled-water agar and finally to minimal medium. Any cultures which failed to grow or made poor growth during these transfers were examined further for possible nutritional requirements.

Over 500 cultures were examined in this way, the majority (over 90%) being able to grow on minimal medium. A few cultures made rapid and dense growth on the initial transfer to complete medium, but subsequently grew poorly or failed to grow on distilled-water agar. When transferred to fresh plates of complete medium, either from water-agar cultures or the original complete medium, little or no growth occurred. It appears that these cultures either required some factor not present in the complete medium or, due to mutation, released an autotoxin. One culture was found (48 G4–40) which made dense growth on the complete medium, but sparse growth on water agar. Despite this difference in density, growth across both media occurred at the same rate and the culture continued to grow sparsely on water agar, even after several serial transfers.

(ii) *Rescue Procedure*

Basidiospores were shed, irradiated, and incubated as previously described. However, after 24 hr incubation on P.G.Y.A., the Cellophane and spores were transferred to sorbose medium to restrict colony size. The developing colonies were examined daily at ×50 magnification and those showing slow or sparse growth were transferred to complete medium. The ability of these cultures to grow on minimal medium was then assessed.

In contrast to the previous method, a much lower proportion of the cultures obtained was nutritionally wild type. Of 70 cultures, 53% were able to grow on minimal medium, while the remaining 47% made very sparse growth, often on the original transfer to complete medium. The method thus appears to increase the frequency of recovering mutants. However, only one culture (48 G6–30) was obtained which would grow on serial transfer to complete medium; unlike the mutant 48 G4–40, this mutant showed a reduced growth rate on minimal medium and was unable to grow on the second serial transfer.

(*b*) *Characterization of Mutants*

The two isolates 48 G4–40 and 48 G6–30 were examined to determine what factor(s) would allow wild-type growth on agar media. Quantitative studies were then carried out in liquid culture to show that these factors completely satisfied the nutritional requirements of each mutant.

(i) *Characterization on Agar*

To provide some general indication of the type of deficiency involved, the mutants were grown on the following four media:

A, minimal plus vitamins.

B, minimal plus ammonium tartrate and vitamin-free casamino acids.

C, minimal plus casamino acids and vitamins.

D, complete medium.

The materials added to A, B, and C were at the same levels as in the complete medium.

It was found that isolate 48 G4–40 grew sparsely on A, but made dense growth on B, C, and D. This suggested an inability to use nitrate as a source of nitrogen and thus further tests were carried out with nitrite-, ammonium-, and amino-nitrogen as separate sources of nitrogen. Good growth was obtained on all forms of reduced nitrogen, including sodium nitrite (Fig. 1), suggesting that this mutant is unable to reduce nitrate to nitrite, because it lacks the enzyme nitrate reductase.

With the other mutant, isolate 48 G6–30, good growth was obtained on A, C, and D, but not on B, indicating a requirement for one or more of the eight vitamins present in medium A. Further testing to determine the specific vitamin(s) involved was carried out with minimal medium containing all vitamins, but omitting each one in turn. In this way it was found that this mutant failed to grow only in the absence of nicotinic acid (Fig. 2), suggesting that it was unable to synthesize nicotinic acid and that this was the sole deficiency of this isolate.

(ii) *Quantitative Responses in Liquid Culture*

The yields of mycelium of a wild-type isolate (48 G3–56) and the nicotinic acid-requiring mutant (48 G6–30) were compared in liquid minimal medium, with and without nicotinic acid (100 μg/l).

To minimize pH changes in the medium the phosphate levels were increased to 6·4 g potassium dihydrogen phosphate and 3·5 g dipotassium monohydrogen phosphate per litre, giving an initial pH of 6·4. Although this did not eliminate pH changes, it at least maintained conditions of near neutrality during the period of growth. Preliminary studies indicated that higher phosphate levels reduced yields of mycelium. Both isolates were transferred from P.V.D.A. to water agar and 6-mm disks of this water-agar culture were used to inoculate the liquid medium (50 ml of medium per 250-ml conical flask). The inoculum disk was placed on the bent and flattened end of a glass rod projecting down through the cotton plug to the liquid surface. In this way, the inoculum was maintained above the surface of the liquid and new mycelial growth was across the surface, resulting in a more rapid attainment of maximum yield. Preliminary studies indicated that yields were as high with this method as in shake culture. After 7, 10, 12, 14, 18, 22, and 26 days, mycelium was removed from five replicate flasks. The culture filtrate was retained for pH deter-

Fig. 2.—Wild type (48 G3–56) (*A* and *C*) and nicotinic acid-requiring mutant (48 G6–30) (*B* and *D*) on minimal medium with (*C* and *D*) and without (*A* and *B*) nicotinic acid.

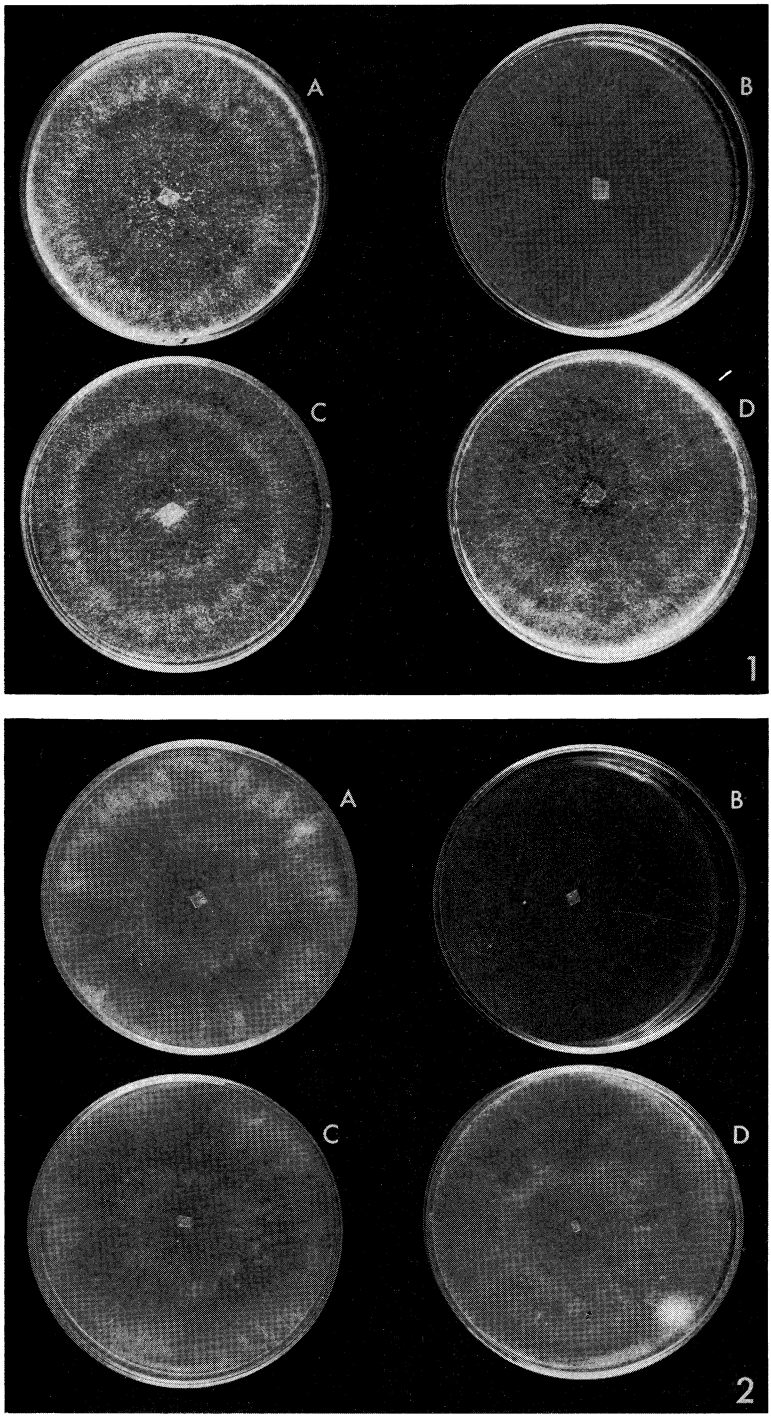
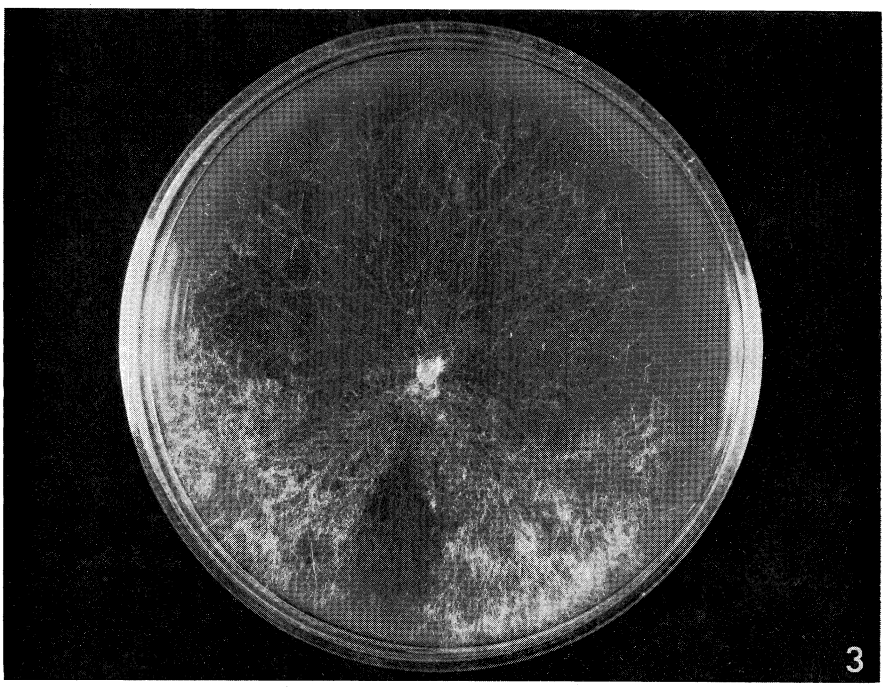


Fig. 1.—Wild type (48 G3–56) (*A* and *C*) and nitrite-requiring mutant (48 G4–40) (*B* and *D*) on minimal medium (sodium nitrate) with (*C* and *D*) and without (*A* and *B*) sodium nitrite.



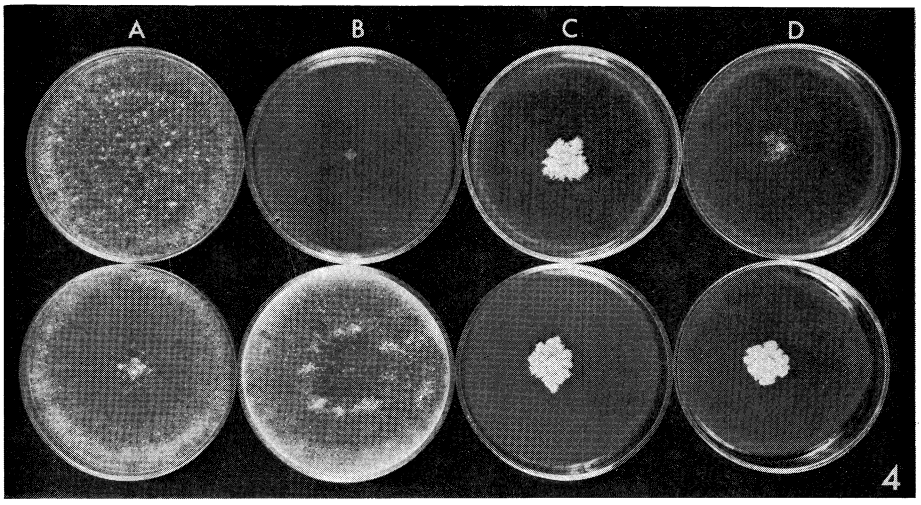


Fig. 3.—Sectors of wild-type mycelium formed from the pairing of a morphological mutant (48–25) and an auxotrophic mutant (48 G4–40). The morphological mutant has made no detectable growth, the auxotrophic mutant has grown sparsely, and the heterokaryon has grown densely and rapidly.

Fig. 4.—Single-basidiospore progeny from heterokaryon 48 G4–40+48–25 showing four classes of progeny (top, sodium nitrate as nitrogen source; bottom, sodium nitrite as nitrogen source): *A*, wild type; *B*, nitrite-requiring progeny; *C*, *curly-II*; and *D*, double mutant recombinant (*nitr cu-II*).

mination and the mycelium was washed with distilled water on a Buchner funnel with a sintered-glass filter (porosity 2). The mycelium was then dried at 80°C and its dry weight determined.

The yields of the two isolates on both media are shown in Figure 5, as well as pH changes during growth. It can be seen that the growth of the wild type and mutant in the presence of nicotinic acid is very similar, while the mutant is unable to grow at all in the absence of nicotinic acid, indicating an absolute requirement for the vitamin.

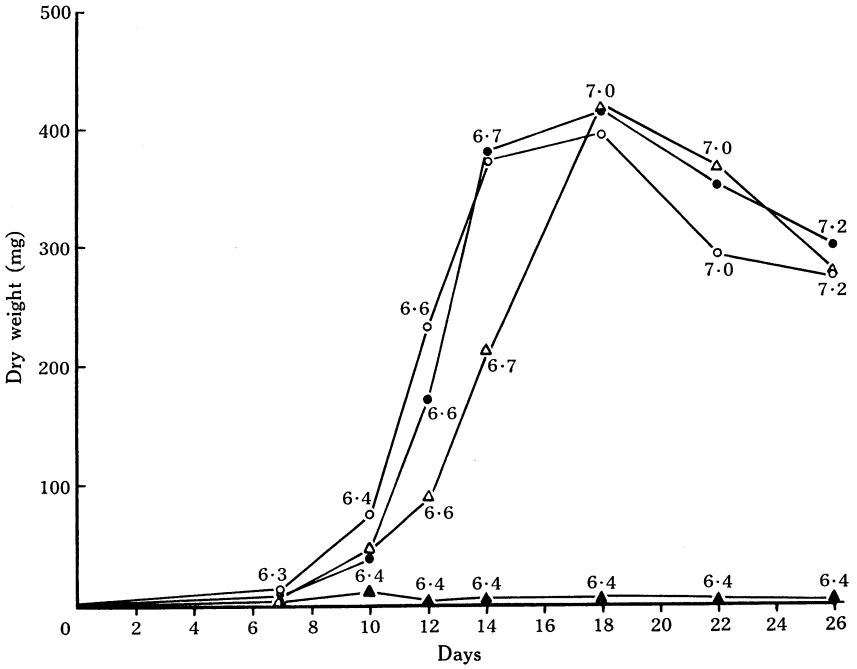


Fig. 5.—Yields of mycelium of wild type (48 G3–56) (○, ●) and nicotinic acid-requiring mutant (48 G6–30) (△, ▲) on minimal medium with (●, △) and without (○, ▲) nicotinic acid. pH changes in the medium during growth are shown.

The growth of the nitrite-requiring mutant (48 G4–40) was compared with that of the wild type in liquid medium containing either sodium nitrate or sodium nitrite as the nitrogen source (nitrogen content 0·56 g/l) but similar in other respects to the minimal medium. In this case, it was found that the additional phosphates in combination with sodium nitrite proved inhibitory to the growth of both wild type and mutant, necessitating the use of the normal phosphate levels; considerable changes in pH thus occurred during growth. An extra harvest was also made at day 16.

The yields obtained on both nitrate and nitrite, as well as pH changes, are shown in Figure 6. Mycelial yields of the mutant and wild type were similar on nitrite, but no growth of the mutant occurred on nitrate. A longer lag phase was found on nitrate than nitrite, with maximum yield being attained about 4 days later on the latter nitrogen source.

(c) *Heterokaryon Formation and Recombination*

Attempts were made to form heterokaryons between the two auxotrophic mutants and a range of morphological mutants, including 48 G4–9 (*stumpy*), 48 G4–13 (*curly I*), 48 G4–149 (*rusty*), 48 G4–150 (*ropy*), and 48–25 (*curly II*). All cultures, prior to maceration, were grown either on minimal medium or distilled-water agar to reduce transfer of metabolites with the maceration plug. After maceration of the two partners, the mixture was transferred to minimal medium. The developing cultures were examined regularly and small mass hyphal transfers made to water agar from any fast-growing, dense areas of the culture (Fig. 3). Single-hyphal tips were obtained from the water-agar culture and transferred to minimal medium. Putative heterokaryons were considered to be fast growing and dense on minimal medium.

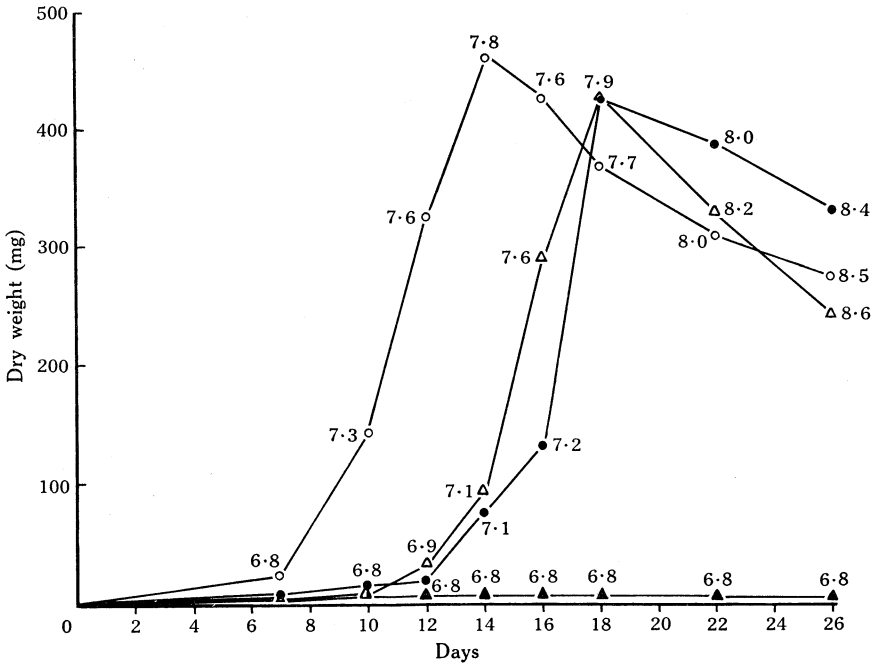


Fig. 6.—Yields of mycelium of wild type (48 G3–56) (○, ●) and nitrite-requiring mutant (48 G4–40) (△, ▲) on minimal medium containing either sodium nitrate (○, ▲) or sodium nitrite (●, △) as nitrogen source. pH changes in the medium during growth are shown.

From 11 attempted pairings, six putative heterokaryons were obtained but fructifications were obtained with only two of these combinations (48 G4–40+48–25 and 48 G6–30+48–25). Single-basidiospore cultures were obtained in each case from a random sample of spores. By growing the progeny on minimal medium with and without the specific nutritional requirement of each auxotroph, it was possible to group the progeny into various classes. Each putative heterokaryon yielded four classes of progeny representing the two parental types in each pairing and the two recombinant types, one of these being a wild type and the other a double mutant.

The four classes of progeny obtained from one heterokaryon are shown in Figure 4. The recovery of four classes of progeny from each pairing demonstrated that each was a true heterokaryon, containing nuclei from both partners of the combination, and also, that recombination of factors within these nuclei had occurred.

The frequencies of the various classes among the progeny of the two heterokaryons are listed in Table 1. Analysis of the data indicated that the four classes in each heterokaryon were recovered in frequencies not significantly different from equality (Table 1). Furthermore, the frequencies of parental and recombinant types from each heterokaryon were not significantly different from equality ($\chi_1^2 = 1{\cdot}57$ and $0{\cdot}086$, $P = 0{\cdot}3$–$0{\cdot}2$ and $0{\cdot}8$–$0{\cdot}7$ for 48 G4–40+48–25 and 48 G6–30+48–25, respectively), suggesting that there is no linkage between the genes involved in each cross.

TABLE 1

CLASSIFICATION AND FREQUENCIES OF SINGLE-BASIDIOSPORE PROGENY FROM TWO HETEROKARYONS

Heterokaryons	Progeny					χ_3^2
	Wild type	Parental types		Double mutant	Total	
48 G4–40 (*nitr*) +48–25 (*cu-II*)						
Genotype	++	*nitr*	*cu-II*	*nitr cu-II*		3·30
Frequency	22	30	22	18	92	$P = 0{\cdot}5$–$0{\cdot}3$
48 G6–30 (*nic*) +48–25 (*cu-II*)						
Genotype	++	*nic*	*cu-II*	*nic cu-II*		2·93
Frequency	29	32	22	22	105	$P = 0{\cdot}5$–$0{\cdot}3$

IV. DISCUSSION

Auxotrophic mutants have been widely used in genetical studies with other fungi. In this study, two stable, auxotrophic mutants of *T. cucumeris* were obtained by irradiation of basidiospores with ultraviolet light. The analysis of the progeny types from heterokaryons involving these mutants indicates that each resulted from a single-gene mutation.

Each auxotroph was apparently capable of forming heterokaryons with a range of morphological mutants, although heterokaryosis was only proven in two cases. However, in view of the complete complementation that occurred in the remaining putative heterokaryons it seems likely that these were true heterokaryons, which were infertile. McKenzie *et al.* (1969) encountered this problem in a number of heterokaryons between different morphological mutants. The mutants they obtained were self-sterile and crosses between irradiated mutants were infertile; in addition, crosses involving 48 G4–149 (*rusty*) were infertile. The auxotrophic mutants were found to be fertile and readily produced large numbers of basidiospores. The two heterokaryons which fruited both involved combinations of irradiated mutants, but other heterokaryons involving only irradiated mutants or irradiated and natural

mutants did not fruit. Both heterokaryons with 48 G4–149 were infertile, supporting the suggestion by McKenzie *et al.* (1969) that this mutant may carry a blockage which affects basidial development.

The number of auxotrophs obtained is still insufficient for the type of work envisaged, i.e. linkage studies and chromosome mapping. However, considering the number of cultures which have been processed, and the methods which have been used, the proportion of mutants obtained is reasonable. Nevertheless, the methods used in these studies involve much time and labour and some improvements involving efficient screening methods to remove a greater proportion of the wild types are required to enable recovery of larger numbers of auxotrophs. The availability of fertile auxotrophic mutants means that it is now possible to investigate screening methods and determine the effects of different treatments on the early growth of mutant spores.

V. Acknowledgments

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VI. References

Flentje, N. T., Stretton, H. M., and McKenzie, A. R. (1967).—Mutation in *Thanatephorus cucumeris*. *Aust. J. biol. Sci.* **20**, 1173–80.

McKenzie, A. R., Flentje, N. T., Stretton, H. M., and Mayo, M. J. (1969).—Heterokaryon formation and genetic recombination within one isolate of *Thanatephorus cucumeris*. *Aust. J. biol. Sci.* **22**, 895–904.

Meyer, R. W., and Parmeter, J. R. (1968).—Changes in chemical tolerance associated with heterokaryosis in *Thanatephorus cucumeris*. *Phytopathology* **58**, 472–5.

Parmeter, J. R., Sherwood, R. T., and Platt, W. D. (1969).—Anastomosis grouping among isolates of *Thanatephorus cucumeris*. *Phytopathology* **59**, 1270–8.

Sherwood, R. T. (1970).—Physiology of *Rhizoctonia solani*. In "*Rhizoctonia solani*, Biology and Pathology". (Ed. J. R. Parmeter.) pp. 69–92. (Univ. Calif. Press.)

Stretton, H. 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.

Whitney, H. S., and Parmeter, J. R. (1963).—Synthesis of heterokaryons in *Rhizoctonia solani* Kühn. *Can. J. Bot.* **41**, 879–86.