MUTATION IN *THANATEPHORUS CUCUMERIS*

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Summary

Fertile, homothallic, homokaryotic cultures selected from the progeny of two field isolates were used to study the occurrence and significance of mutants in *Thanatephorus cucumeris* (Frank) Donk. Mutations in the crucifer stem-attacking isolate occurred spontaneously and were also induced by ultraviolet irradiation. Six of the mutants showing stability in culture were compared with one another and with their parent culture. The mutants differed in cultural appearance, growth rate, general morphology, mean number of nuclei per cell, and pathogenicity to crucifer stems, but none could be induced to fruit. Comparison of their pathogenic reactions on radish stems suggests that a series of steps, each controlled by a different genetic factor, is involved in the invasion of stems by the fungus. In the other isolate, which was non-pathogenic, no mutants were detected.

The minimum lethal dose of ultraviolet irradiation for the spores of each isolate differed considerably.

I. Introduction

Thanatephorus cucumeris (Frank) Donk is regarded as having a stable vegetative stage (*Rhizoctonia solani* Kühn) in culture (Kernkamp *et al.* 1952) although it is now well known that it occurs in a wide variety of different strains and that these exist in nature as heterokaryons (Flentje and Stretton 1964; Garza-Chapa and Anderson 1966). The most likely expression of variation in culture would be the occurrence of sectors, but this had seldom been recorded until Kernkamp *et al.* (1952) showed that sectors frequently appeared in field isolates grown in the presence of certain chemicals. These workers regarded the sectors as mutants but their assumption that the cultures, grown from hyphal tips, were genetically pure, was unjustified. Cultures from hyphal tips would have been heterokaryotic and the sectors may therefore have been due to a selective action of the medium on the heterokaryotic culture.

The common occurrence of sectors in single-basidiospore cultures (Flentje and Stretton 1964) offers more reliable evidence of mutation, but even here the occurrence of binucleate single basidiospores casts doubt on the interpretation.

Although no conclusive investigations on mutation in *T. cucumeris* have been carried out it is important to investigate whether mutation does occur and what part it plays in the biology of the fungus, particularly in relation to the questions of host range, virulence, survival ability, and the origin of new strains. This paper describes such an investigation.

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II. Materials

Two isolates of *T. cucumeris* were used: isolate 48, isolated from soil at the Waite Agricultural Research Institute and pathogenic on stems of Cruciferae; and isolate 82 from soil, Cungena, S.A., non-pathogenic to all hosts tested (Stretton, Flentje, and McKenzie 1967).

Previously (Stretton, Flentje, and McKenzie 1967) isolates obtained directly from the field have been designated field isolates. As these investigations are moving more into genetical fields the appropriate terminology will now be adopted. A field isolate is a "wild type" as defined by Fincham and Day (1963), as is also any individual derived from and approximating to the field isolate.

First, second, and third generation single-basidiospore cultures are designated by the isolation number for each generation in series. The isolation numbers have no particular significance, but are used for brevity. Further, for brevity G1, G2, etc. are used to designate the particular generation, e.g. 48–11 is a G1 culture.

III. Methods

Basidiospores of the non-pathogenic isolate 82 formed readily on low-nutrient agar. Isolate 48, however, did not fruit on agar, but fruited readily on aerated, steam-treated soil (Stretton *et al.* 1964). Basidiospores were shed on Cellophane covering agar in Petri dishes. Half the plates were irradiated with ultraviolet light sufficient to kill 95% of the spores. The remaining plates were untreated. The spores were irradiated either immediately after being shed or after having been stored at 5°C; no spores showed visible sign of a germ tube at the time of irradiation. A Philips TUV 15-W germicidal lamp, 2537 Å, intensity 37 μW/cm at a distance of 1 m (=17·7 ergs/mm^2/sec at 18 in.) was used to induce mutations.

Pathogenicity was determined by the method described by Flentje, Dodman, and Kerr (1963) except that the slides with seedlings attached were placed vertically in glass jars. This lessened the rise of nutrient solution creeping up along the stems and the Vaseline was no longer required to prevent this. Furthermore, after inoculation the seedlings were incubated under the same temperature and light conditions as used for raising the seedlings.

IV. Experimental and Results

(*a*) *Isolate 82*

This non-pathogenic isolate of *T. cucumeris* has so far proved unusually stable in culture throughout five selfed generations by yielding single-basidiospore cultures in each generation, indistinguishable from one another and from those of the other generations, in growth rate, cultural appearance, ability to fruit on low-nutrient agar, and inability to attack plant tissue. Spores of this isolate were irradiated for various times with ultraviolet light at a distance of 18 in. An optimum dosage for mutation, aimed at killing 95% of the spores, thereby increasing the possibility of mutation in the remaining 5%, was found difficult to assess. At a dosage of 2124 ergs/mm^2 (2 min exposure) all spores germinated and developed into cultures indistinguishable

from the parent. At a dosage of 4248 ergs/mm^2 (4 min exposure) all spores were killed although not all immediately. At a dosage of 3186 ergs/mm^2 (3 min exposure), 50% of the spores were killed immediately, 45% remained apparently dormant or

TABLE 1

DESCRIPTION OF MACROSCOPIC CHARACTERISTICS OF THE SIX G4 MUTANTS COMPARED WITH THEIR G3 PARENT CULTURE OF ISOLATE 48

Culture	Colony Diameter* (cm)	Colony Colour*	Cultural Appearance	Pathogenicity (on radish stems)
G3 parent 48–11–14–56†	10·0	Off white	Wild type; dense, even growth, aerial hyphae	Virulent pathogen; produces infection cushions, spreading lesions, seedling death
G4 mutants *sparse*	9·0	Off white	Similar to wild type but sparse; few aerial hyphae	Non-pathogenic; grows freely over stems without adhering. Occasional superficial cushions
stumpy	8·5	Off white, gradually browning	Dense growth, even at first, tendency to show zonate bands after 1 week. Aerial hyphae	Non-pathogenic; growth inhibited on stems. Growth inhibited by exudate from radish stems
fleecy	6·3	White	Dense, fluffy in concentric rings, giving "fleecy" appearance. Dentate periphery, abundant aerial hyphae	Virulent pathogen; produced infection cushions, spreading lesions, seedling death
curly	5·0	Dark brown on agar, aerial hyphae white	Dense, irregular-shaped colony, clumpy or nobbly appearance due to curling of aerial hyphae	Hypersensitive reaction; infection cushions, localized necrotic lesions
rusty	3·8	Light brown at growing edge to dark rusty-brown at centre	Dense, irregular-shaped colony; much of hyphae subsurface	Non-pathogenic; occasional superficial cushions, sparse growth on stems
ropy	6·5	Off white	Irregular-shaped colony; deeply dentate periphery giving "ropy" appearance. Few aerial hyphae	Non-pathogenic; grows freely over stems without adhering. No obvious response to exudate

* On potato–Marmite–dextrose agar, after 6 days.

† Indistinguishable from original field isolate 48 in all characteristics listed here.

died over a period of some 5 weeks, and 5% produced cultures indistinguishable from the parent. One possible mutant was obtained at this dosage, but before the colony reached a substantial size it reverted to wild-type growth and, on fruiting, produced

TABLE 2

DESCRIPTION OF MICROSCOPIC CHARACTERISTICS OF THE SIX G4 MUTANTS COMPARED WITH THEIR G3 PARENT CULTURE OF ISOLATE 48

Culture	Morphology	Hyphal Branching	Septation	Peripheral Tip Cells	Average No. of Nuclei per Cell ± S.E. (range in parenthesis)*		
					Older Non-tips	Branch Tips	Total
G3 parent 48–11–14–56	Growth and cell elongation follows a regular pattern; branching occurs each side of a runner hypha to give a "fishbone" appearance	Regular	Regular	8·8±0·20 (6–15)	5·4±0·10 (3–10)	4·8±0·12 (3–8)	6·1±0·11 (3–15)
G4 mutants							
sparse	Growth and cell elongation regular as with the parent, but network more open due to decrease in branching	Regular	Regular	7·6±0·15 (4–11)	5·9±0·12 (2–11)	6·4±0·12 (4–9)	6·5±0·08 (2–11)
stumpy	Short side branches, giving "stumpy" appearance. Some loss of apical dominance	Irregular	Regular	10·1±0·23 (5–17)	8·0±0·13 (4–14)	7·9±0·15 (5–11)	8·5±0·11 (4–17)
fleecy	Extensive branching around advancing runner hyphae, side-branch cells often truncated	Irregular	Regular	7·6±0·16 (3–12)	5·9±0·10 (3–12)	5·3±0·12 (2–8)	6·2±0·08 (2–12)
curly	Extensive curling of hyphae and side branch development leading to loss of apical dominance	Irregular	Irregular	†	†	†	11·4±0·39 (0–50)
rusty	Wavy hyphae, and frequent anastomosis of tip cells. Close weave due to increased side-branch development of short cells	Irregular	Regular	6·4±0·13 (3–10)	4·9±0·14 (1–11)	5·6±0·17 (2–9)	5·4±0·09 (1–11)
ropy	Open network, profuse branching along a few prominent faster-growing runner hyphae, giving "ropy" appearance	Irregular	Regular	7·0±0·16 (4–11)	6·6±0·12 (1–11)	6·4±0·13 (4–9)	6·6±0·08 (1–11)

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

† Due to branching habit, cells not differentiated.

single-spore cultures all of which were wild type in cultural appearance, non-pathogenic, and able to fruit on agar. This failure to produce mutants further demonstrated the stability of this non-pathogenic isolate.

(*b*) *Isolate 48*

More success was achieved in inducing mutations in basidiospores of the pathogenic isolate 48. A G3 single-basidiospore culture (48–11–14–56) was used as the parent. This culture was indistinguishable from the wild type (isolate 48) in growth rate, cultural appearance (Plate 1, Fig. 1), fruiting ability, and pathogenicity (Plate 1, Fig. 2), but differed from the wild type in being homokaryotic (Stretton, Flentje, and McKenzie 1967) and produced single-spore cultures which were genetically identical.

(*c*) *Mutants Induced by Ultraviolet Irradiation*

Large numbers of single basidiospores of 48–11–14–56, shed on Cellophane over agar, were irradiated with ultraviolet light at a distance of 9 in. A 4-min exposure (dosage $\doteq$ 16,992 ergs/mm^2) resulted in 50% of the spores dying within 12 hr, the remainder lying dormant or eventually dying over the next few weeks. At an exposure of 2 min almost all spores germinated and all those isolated formed colonies identical with the parent. At an exposure of 3 min (dosage = 12,744 ergs/mm^2) approximately 30% died within 12 hr. After that time approximately 200 germinating spores were isolated and, although a high percentage subsequently died, 24 developed into colonies showing variations from the parent in one or more characters relating to colour, growth rate, density of growth, branching, extent of aerial growth, prevalence of sectors, curling of hyphal tips, zoning, septation, and regularity of growth. Of these mutants, four subsequently died, and five were unstable and reverted to wild type. Of those remaining, four (viz: *sparse*, *stumpy*, *fleecy*, and *curly*) were selected which showed no tendency to sector, and which were sufficiently different in cultural characteristics—colony colour, growth rate, and morphology—from the parent and from one another, to be distinguished readily (Plate 1, Fig. 1; Tables 1 and 2). The regular pattern of hyphal elongation and branching illustrated previously (Flentje, Stretton, and Hawn 1963) was disrupted to some extent in each of these mutants (Plate 2). The disruption was most extreme in *curly* where septation was irregular and greatly reduced, resulting in a variation of 0–50 nuclei between successive septa. Where there were large numbers of nuclei they occurred in groups of 6–9 which appeared to represent cells unseparated by septa. The colony characteristics, hyphal morphology, and numbers of nuclei in vegetative cells of all mutants are listed in Table 2. None of these mutants could be induced to fruit.

(*d*) *Mutants Occurring Spontaneously*

As stated earlier, single-spore cultures obtained from the G3–56 culture were identical (Plate 3, Fig. 1). However, after repeated subculturing over a period of 3 months, this G3–56 culture was again induced to fruit and basidiospores were shed on to agar and isolated. The spores now gave rise to cultures which exhibited a range of variation in growth rate and cultural characteristics (Plate 3, Fig. 2) showing that

mutations had apparently occurred spontaneously in G3–56, thereby rendering it heterokaryotic. Two of these spontaneous mutants (*rusty* and *ropy*) were selected for further testing. Their cultural (Plate 1, Fig. 1) and morphological (Plate 2) characteristics are listed in Tables 1 and 2.

(*e*) *Pathogenicity of Mutants*

Of the six mutants only *fleecy* was as virulent as G3–56 on radish seedlings. *Curly* reacted the same way as G3–56 prior to penetration but then produced only small black hypersensitive lesions. The four remaining mutants failed to penetrate radish stems. The results of the pathogenicity tests are illustrated in Plate 1, Figure 2, and Plate 3, Figures 3–5. The reaction of each mutant to radish stem exudate was compared with that of G3–56 by the method described by Flentje, Dodman, and Kerr (1963). The growth of *stumpy* was completely inhibited by the exudate; *ropy* grew across the exudate with no obvious reaction; *curly*, *sparse*, and *rusty* grew across the exudate occasionally forming infection cushions; *fleecy* and G3–56 formed abundant infection cushions above the exudate.

V. Discussion

Despite the fact that the spores of the non-pathogenic isolate 82 were four times more susceptible to ultraviolet irradiation than the spores of G3–56, we have found no conclusive evidence for the occurrence of mutation in isolate 82, although, of course, mutations may have occurred without being detected by our methods. The lack of spontaneous mutants could be due to selection against such mutants during vegetative or reproductive growth, so they did not participate in spore formation, but on the other hand the lack of induced mutants amongst the irradiated spores would support the hypothesis of a low mutation rate for this isolate. We have no satisfactory explanation to offer, however, for such a low mutation rate.

It was, however, possible to obtain both spontaneous and induced mutants from G3–56. As far as we are aware this is the first unequivocal demonstration of mutation in *T. cucumeris*, although it is very likely that some of the variation in single-spore cultures reported by other workers was due to mutation. The recovery of spontaneous mutants after 3 months of repeated transfers of G3–56 shows that this culture, originally known to be homokaryotic, had accumulated mutants and become heterokaryotic. The mutant nuclei were presumably carried forward in the multinucleate hyphal tip cells as the result of conjugate division of nuclei followed by even segregation of daughter nuclei. No change in the wild-type cultural appearance, growth rate, pathogenicity, or fruiting ability was detected in the culture over the 3-month period in which the mutants accumulated in the heterokaryon; thus the mutants did not modify significantly the expression of the wild-type nucleus.

The six stable, spontaneous, and induced mutants in homokaryotic culture, however, each affected different morphological and cultural characters and each was apparently self-sterile. Five of the six were non-pathogenic to radish stems and the detailed analysis of infection and disease progress showed that in different mutants

pathogenicity was blocked at different stages which could be arranged in a series. *Fleecy*, although relatively slow growing, was as virulent as wild type. It would seem unlikely, therefore, that lack of pathogenicity of other mutants was due simply to slow growth rate. *Curly*, slower growing than *fleecy*, was blocked at the disease progress stage by a hypersensitive reaction after apparently normal infection had occurred; *curly* freely formed infection cushions in response to radish stem exudate. The slow-growing *rusty* and the faster-growing *sparse* occasionally formed loose superficial cushions on the stems and also in response to radish stem exudate, but produced only occasional vestigial infection pegs and did not penetrate radish stems. The blockage in these mutants then occurs prior to infection and is concerned either with the formation of infection cushions themselves, or with penetration from the cushions. *Stumpy* failed to grow over radish stems and when tested against exudate, growth was completely inhibited. The blockage here is apparently concerned with the abnormal response to the exudate. *Ropy* grew over stems without attaching or forming any cushions and when tested against exudate showed no reaction. In this instance the blockage is at the earliest stage of infection and any attachment to the stem and formation of infection initials is prevented.

The fact that different genetic factors appear to control different stages of infection and disease progress, and these can be correlated with such processes as the formation of infection cushions in response to stem exudates supports earlier work by Flentje (1959) and Flentje, Dodman, and Kerr (1963).

The mutants described above, because of their stability, the fact that they are self-sterile, and because they carry distinctive morphological and pathogenicity markers have been used subsequently to study heterokaryosis and genetic recombination in this fungus. This work will be described in a further paper.

VI. Acknowledgments

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EXPLANATION OF PLATES 1–3

PLATE 1

Fig. 1.—Cultural characteristics of parent isolate 48 (*A*), of identical G3 single-basidiospore culture 48–11–56 (*B*), of G4 spontaneous mutants (from G3–56) *rusty* (*C*) and *ropy* (*D*), and of G4 ultraviolet mutants (from G3–56) *sparse* (*E*), *stumpy* (*F*), *fleecy* (*G*), and *curly* (*H*).

Fig. 2.—Pathogenicity tests on radish stems, showing variation in host–fungus reaction after 7 days of parent field isolate 48 (*A*), of G3 single-basidiospore culture (*B*), and of mutant basidiospore cultures *rusty* (*C*), *ropy* (*D*), *sparse* (*E*), *stumpy* (*F*), *fleecy* (*G*), and *curly* (*H*).

PLATE 2

Figs. 1–7.—Hyphae showing morphological characteristics at the growing edge of cultures (on potato–Marmite–dextrose agar) of G3–56 (*1*), of spontaneous mutants *rusty* (*2*) and *ropy* (*3*), and of ultraviolet mutants *sparse* (*4*), *stumpy* (*5*), *fleecy* (*6*), and *curly* (*7*).

PLATE 3

Fig. 1.—Single-basidiospore cultures from G3–56, showing lack of variation in cultural characteristics.

Fig. 2.—Single-basidiospore cultures from G3–56, showing variation in growth rate and morphology after mutations have occurred in G3–56.

Fig. 3.—Superficial cushions of *sparse* mutant on surface of radish stem. No penetration or rotting. Stained with trypan blue.

Fig. 4.—Localized lesion on radish stem, developed under infection cushion of *curly* mutant.

Fig. 5.—Infection cushion development and penetration of radish stem tissue by *fleecy* mutant, causing spreading lesions and extensive rotting.

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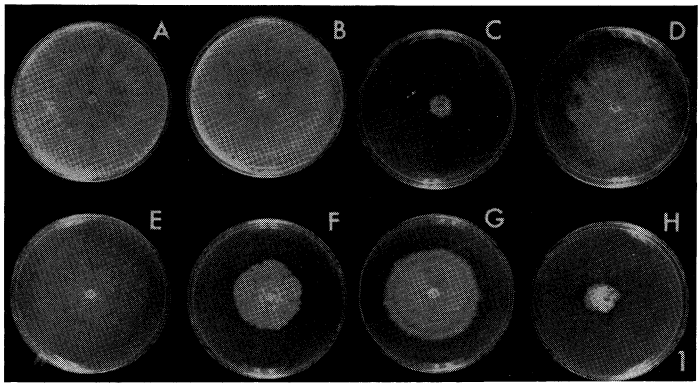

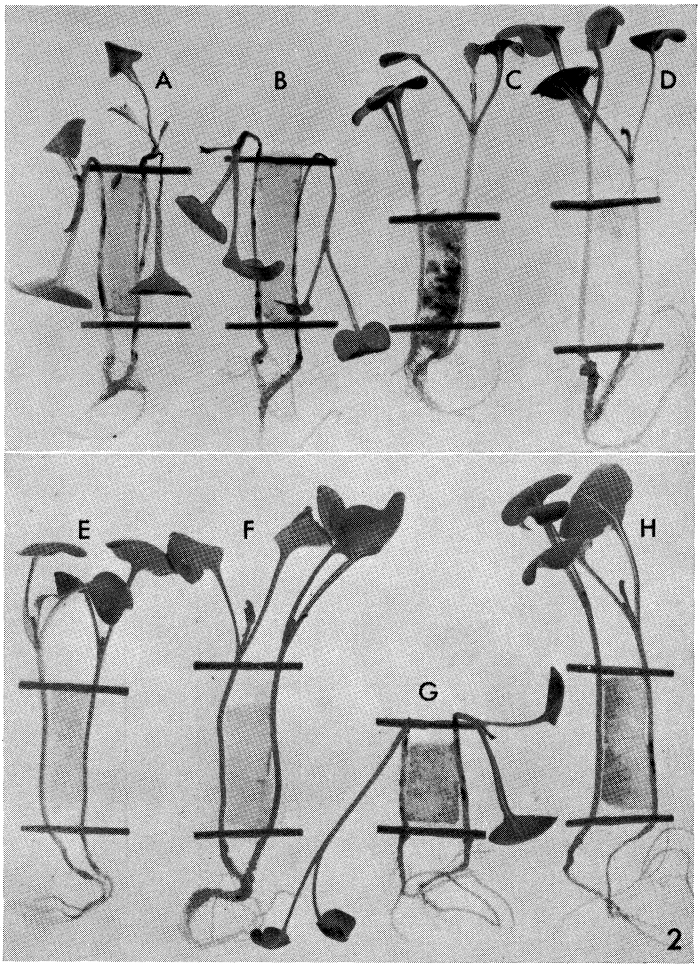

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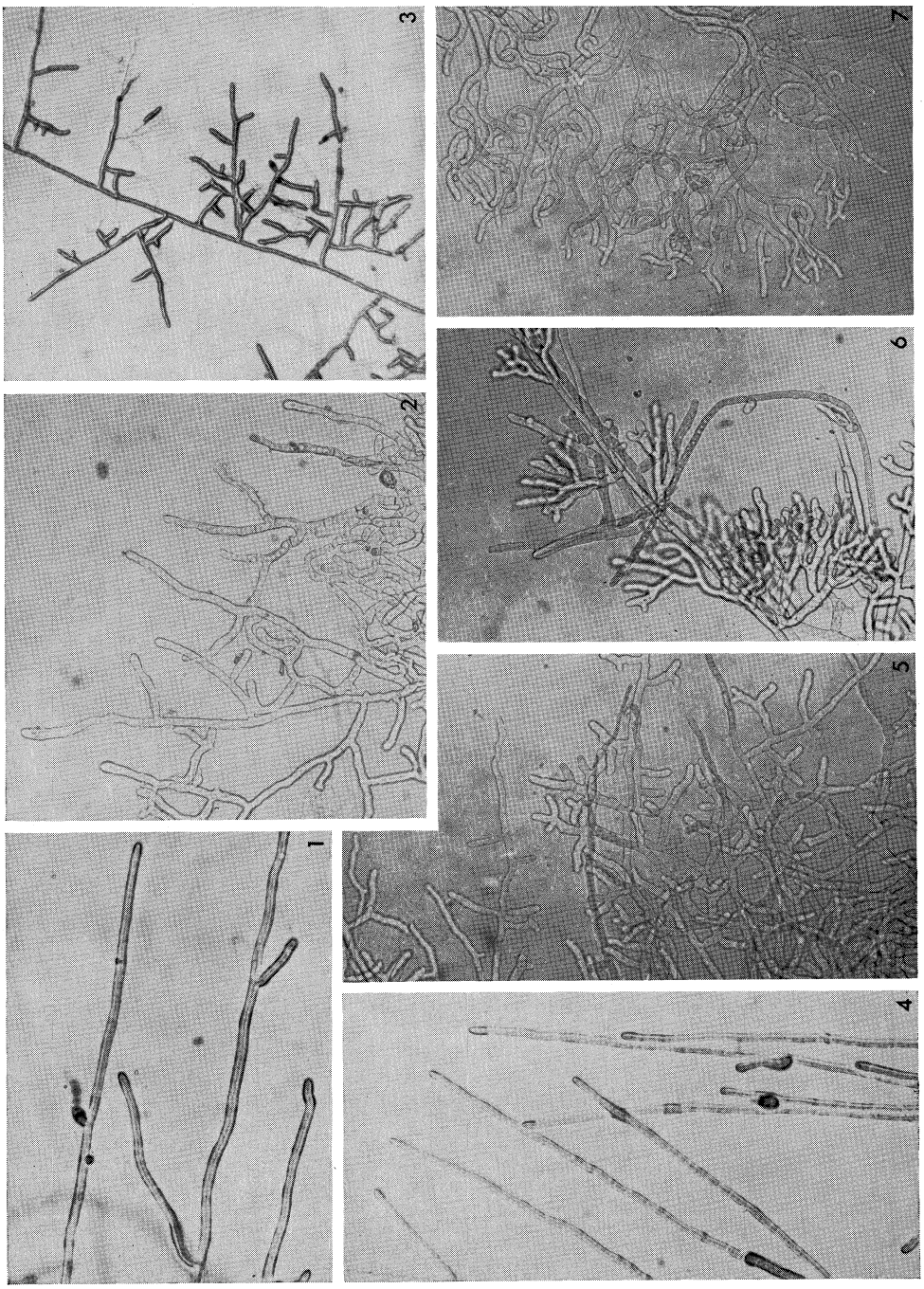

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