STUDIES IN THE ENTOMOPHTHORACEAE

I. Observations on the genus *Conidiobolus*[1]

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INTRODUCTION

While in Cuba during the summer of 1933 the writer encountered a fungus growing on living termites which had been placed in damp chambers for observation. Upon examination, the fungus proved to be a species of *Conidiobolus,* one of the Entomophthoraceae. This circumstance immediately aroused the writer's interest, since, so far as can be ascertained from the literature, no members of this genus have been reported as infesting termites. The fungus is of further interest since it was found to be identical with a saprophytic species described by Martin (**6**) as *Conidiobolus villosus,* which appeared as a contaminant on a plate of nutrient agar containing a bit of decaying wood harbouring a species of *Hypochnus.* Moreover, since the genus *Conidiobolus* is separated from *Empusa* and *Entomophthora,* among other reasons because of its saprophytic mode of existence, the writer was led to make further studies of the nature and affinities of the fungus in question in the hope that a comparison with other species of the Entomophthoraceae might help to elucidate the relationships of the various genera within the family. At the same time it was hoped to gain some understanding of the biology of the species concerned and to ascertain by experiment its ability to infect living insects, as well as to grow on various substrata.

MATERIALS AND METHODS

The Cuban fungus was isolated directly from termites of the genus *Nasutitermes*[3] inhabiting aerial termitaria by allowing spores

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[1] Contribution from the Laboratories of Cryptogamic Botany, Harvard University, No. 148, and No. 10 from the Department of Botany and Plant Pathology, University of Puerto Rico, College of Agriculture.

[2] This investigation was undertaken while the writer was the holder of an Edwin F. Atkins Scholarship, stationed at the Harvard Botanical Gardens at Cienfuegos (Soledad), Cuba.

[3] The writer has recently collected from termites of the same genus in Cabo Rojo and Mayagüez, Puerto Rico, material which, when cultured, was found to have a life history similar to that of the Cuban fungus, although the former possesses slightly smaller spores (average diameter of smooth type 28–34 u). This is the first report of the occurrence of this fungus from the island.

from conidiophores on infected insects to be discharged against the lid of damp chambers and by transferring these spores to Blakeslee's agar. Subcultures of Martin's[2] type material of *Conidiobolus villosus* and an undetermined culture of *Conidiobolus,* isolated by Derx, from an unknown origin, as well as an unnamed species of Empusa described by Sawyer (**10**) were grown under similar conditions for comparison.

For the purpose of studying the life-cycle of these fungi, van Tieghem cells were for the most part employed; petri dishes proved most useful in studying the growth of the fungi on different media. Furthermore, in order to ascertain whether the fungi were truly saprophytic or facultative parasites, inoculation experiments were conducted. Petri dishes of the fungi were first allowed to grow luxuriantly and to sporulate abundantly, and then the insects were allowed to crawl over the surface of the cultures for varying periods of time, at the end of which the insects were removed to sterile petri dishes containing paper moistened in order to maintain and insure optimum conditions of humidity for the development of these fungi.

Observations

Since the Cuban strain, unlike the other members of the genus, was found to be parasitic on termites, it first became necessary to establish its identity by working out its morphology, development and life-cycle in pure culture. When placed on artificial media, the subspherical, to ovoid basally apiculate spore, sends out from any point on the periphery except the rounded apiculus, a short germ tube which may bear a secondary conidium (fig. 2, I) similar to the first but slightly smaller, or may elongate, branch, and produce extensive hyphae which later, by the appearance of cross-walls, form irregularly shaped hyphal bodies. Within the host, however, the mycelium very soon begins to break up into hyphal bodies which subsequently give rise to mycelia that penetrate the insect body and emerge as external conidiophores which later bear the smooth or primary conidia (fig. 1, E-F). These are discharged in a manner similar to that described by Martin (**6**) for *Conidiobolus villosus* and by Thaxter (**12**) for *Empusa* (*Triplosporium*) *Fresenii.* Some of the primary conidia may then produce capillary or villose outgrowths (fig. 1, G-H), usually densely covering the entire surface of the

[2] Martin's type strain from the Thaxter culture collection at the Farlow Herbarium; Derx strain from the Centraalbureau voor Schimmelcultures in Holland; Cuban strain from Harvard Botanical Gardens, Cienfuegos, Cuba; and *Empusa* sp. from Miss C. M. Jacobs of Hunter College, New York City.

spore as in *Delacroixia coronata*, Gallaud (4), fig. 1, E, or occasionally restricted to small areas of the spore surface (fig. 2, A) thus approaching *Empusa* (*Triplosporium*) *Fresenii* (Thaxter l. c.), *Entomopthora fumosa* Speare (11), and *Entomophthora pseudococci* Speare (11). Under favorable conditions, that is with an abundance of moisture, nutriment and warmth (70–75° F), the hair-like appendages may swell at their terminal extremities (fig. 2, C) and give rise to minute uninucleate piriform or subglobose conidia (fig. 2, D-G) within 36–48 hours after the villose spores have been discharged from the conidiophores.

The life-history and morphology of this Cuban fungus, as just described, show certain similarities to those of another member of the Entomophthoraceae namely, *Delacroixia coronata* (Cost.) Sacc. and Syd., agreeing not only in the size and shape of the conidia but also in the production of villose spores which in turn may bear minute conidia at the ends of the capillary projections. The fungus agrees also with *Conidiobolus villosus* except that, in Martin's (6) original description no mention was made of the small conidia. It should be noted, however, that Martin's description gives an excessive range of size for the conidia including some so small as to suggest that the latter had originated, unobserved, as micro-conidia. Furthermore, while studying the type culture of *C. villosus*, the writer was able to obtain micro-conidia under the conditions mentioned above. Martin (l. c., p. 314) states that "A few resting spores have been seen in which the hairs were slightly swollen at the tips. In one culture a very few of the resting spores were provided with distinctly conical, spine-like processes. Intermediate stages were present in all cases, and such aberrant spores seem to represent minor variations due to unknown causes." Had the spores been examined in a saturated atmosphere, it seems probable that the significance of the variations might have been evident, and that these aberrant spores would have been found to be proliferating into micro-conidia, the "conical, spine-like processes" representing the former point of attachment of these minute spores. Under unfavorable moisture conditions, that is, absence of water of condensation, the echinate conidia or "resting spores" become thick-walled and after a period of rest when transferred to a suitable substratum, do not produce the micro-conidia, but germinate by putting forth a germ tube similar to that described by Martin (l. c.)

Like Martin (6) and Gallaud (4), the writer was unsuccessful in all his attempts to secure the sexual phase of the fungus, even by contrasting the various strains in artificial culture. However the appearance in the Cuban material of certain thick-walled structures closely resembling zygospores, needs further critical study to determine their true nature.

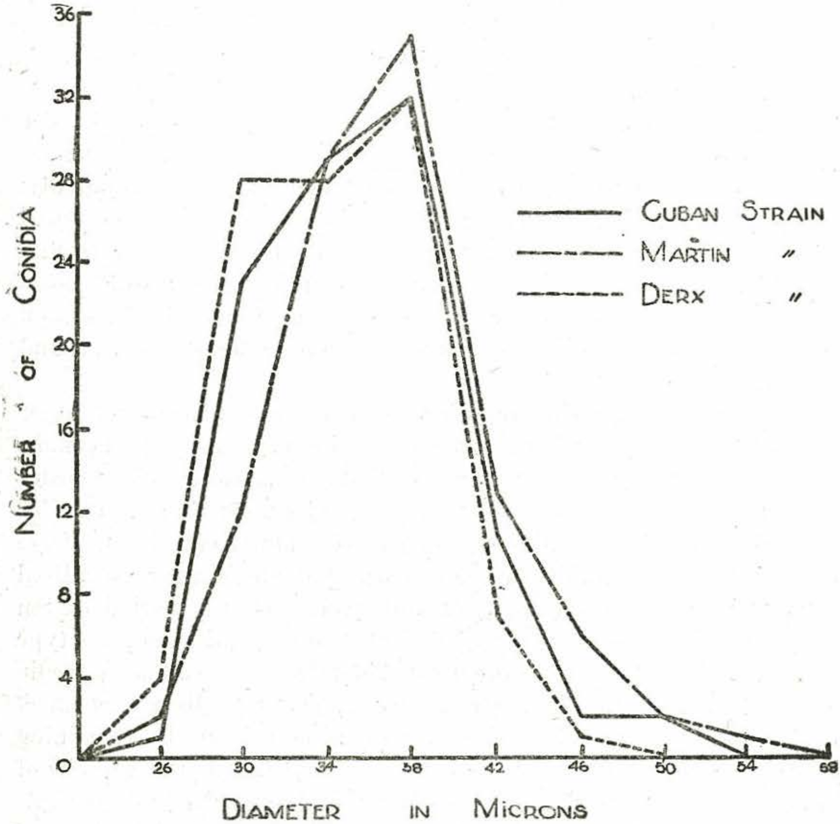
The presence of micro-conidia in *Conidiobolus villosus* not only indicates a general relationship of this fungus with members of Thaxter's (**12**) sub-genus *Triplosporium,* of *Empusa,* but also brings into question the status of the species. In 1897 Constantin (**3**) described *Boudierella coronata,* a fungus which he had isolated from the gills of *Psalliota campestris,* as a new genus of the Entomophthoraceae. This fungus, which is known only in the conidial stage, is characterized not only by the formation of villose conidia or "resting spores", but also by the production of micro-conidia as noted above. Later Saccardo and Sydow renamed the fungus *Delacroixia coronata* (Cost.) Sacc. and Sydow, since the former generic name had been preëmpted. In 1905 Gallaud (**4**) isolated the same fungus from orchid seeds, but since he only rarely obtained the "spores en couronne" of Costantin, he referred the material to the original author for identification.

Since *Delacroixia coronata* and *Conidiobolus villosus* are so similar in their life histories and morphological details, the writer feels that there is no necessity of maintainng the two names unless it is possible eventually to demonstrate differences in the sexual phases of the two genera. Furthermore since the genus *Delacroixia* was based only on the appearance of "spores en couronne" or micro-conidia, induced by an abundance of moisture as in *C. villosus,* the writer agrees with Lakon (**5**) that this is not a sound basis for establishing a new genus, since Thaxter (l. c.) had already shown that a somewhat similar proliferation takes place in the sub-genus *Triplosporium* of the genus *Empusa.*

In view of the fact that *Conidiobolus* has been separated from *Entomophthora* and *Empusa,* primarily because of its saprophytic mode of life, experiments were undertaken to test the ability of the three strains of *C. villosus* to parasitize various insects. The writer, therefore, in triplicate series, exposed thirty-five termites and ten corn-borer larvae to infection by allowing them to crawl about on three sets of petri-dish cultures representing the three strains of the fungus that have been discussed above, and then placed the insects in damp chambers. This experiment showed that not only the Cuban strain isolated from termites but also the type material of *C. villosus* originally from rotten wood, was able to infect 60% of the termites within ten days, whereas in the case of the Derx strain whose source of isolation is not known, no infection took place even at the end of twenty-one days. These results immediately suggested that there was a physiological difference which distinguished the latter strain from the other two. In contrast, only one corn-borer was killed by the Cuban strain after ten days, while the others remained free from infection even after a longer period of exposure. In a similar experiment however *Empusa* sp., attacked approximately 40% of the larvae within the same length of time and under similar environmental conditions. The present experiments with the Cuban fungus on termites, together with the earlier work of Costantin (3) who was successful in infecting *Musca domestica* with his organism, demonstrates the fact that these reputedly saprophytic fungi may live parasitically upon the proper hosts. Gallaud (4), however, using a variety of insects, was unable to find any evidence of parasitism, although when he placed dead cockroaches (Blatta) in contact with the spores he found that infection took place most readily in the muscles of the abdomen, thorax, and appendages.

Because the Derx culture behaved so differently from the other two in regard to parasitism it became necessary to study the morphological and physiological aspects of the various strains in order to determine what differences, if any, existed among them. In order to do this, the three strains of *Conidiobolus* and *Empusa* sp. were inoculated on the following substrata: Blakeslee's agar, sterilized carrots, egg yolk, swordfish, pork and liver. After a period of ten days, growth at room temperature, the Cuban strain and the type material of *C. villosus* were practically identical on the various media used. Growth of the Derx strain on egg yolk and liver was much more vigorous than the other strains, although on the remaining substrata it was noticeably more meager. Although the growth of *Empusa*, a recognized parasite, was less abundant on Blakeslee's agar, it was comparable in vigor with the development of *Conidiobolus* on the other substrata. It seems clear from the foregoing that the so-called saprophytic *Conidiobolus* is able to parasitize termites and conversely that the parasitic *Empusa* may be grown saprophytically on a variety of artificial media. Hence the existing differences between these two genera on this basis alone loses much of its weight.

Since differences in character of growth are so slight in all but the Derx strain of *Conidiobolus*, and since the organisms lack morphological distinctions, the writer made measurements of one hundred spores of the smooth type of each of the three strains in an endeavor to find a biometrical means of distinguishing them. The spores measured were produced on the same type of media and under identical environmental conditions. It was noted that the spores of *Conidiobolus* from the three different localities were practically indistinguishable (Graph I), although those of the type strain



were 3 u. larger than the others. In view of our present knowledge of the fungi in question, it is doubtful if the Derx strain should be considered other than as a variant. Furthermore, the aforementioned organisms agree in nuclear condition as well as in their morphological and physiological behavior. Gallaud (**4**) has shown that *Delacroixia coronata* possesses multinucleate conidia while Martin (**6**) found the same to be true in *Conidiobolus villosus*. Moreover, the writer has confirmed Martin's findings not only in the type material but in the Cuban and Derx strains as well. In the genus *Empusa* a similar multinucleate condition has been shown in the conidia of a number of species investigated by Riddle (**9**), Cavara (**2**) and Olive (**8**).

The fact that *Conidiobolus villosus* and *Delacroixia coronata* have been shown to be similar morphologically, cytologically, and to a lesser extent physiologically, indicates that the confusion as to their taxonomy should be clarified.

Conclusions

As has already been stated, the genus *Delacroixia* was established to include a species of Entomophthoraceae with "spores en couronne". Gallaud, in 1905 encountered a fungus with spinose resting spores which Constantin identified as his *D. coronata* deviating from the type in only rarely producing the spores typical of the genus. Later, Martin (l. c.) described *Conidiobolus villosus* characterized by the villose appendages borne on the resting spores. The present writer has shown that these appendages are capable of giving rise to minute conidia similar to those of *D. coronata* and furthermore that the two fungi are similar morphologically and physiologically. There seems, therefore, no need of maintaining the later species, *C. villosus* as distinct.

Moreover, the genus *Conidiobolus,* founded by Brefeld (**1**) in 1884 was at that time thought to be readily distinguished from *Empusa* and *Entomophthora* by its saprophytic mode of existence. This, together with the fact that the strongly branched mycelium broke up into hyphal bodies early in the life cycle of the fungus, were the criteria upon which the genus was established. It has been shown elsewhere in this paper that at least certain strains of *C. villosus* may lead a parasitic mode of life in addition to a saprophytic one.

Furthermore other investigators, such as Speare (**11**), Molliard (**7**), and Sawyer (**10**), have shown that in the genera *Empusa* and *Entomophthora* a number of species which were considered obligate parasites, in reality are able to grow saprophytically. Undoubtedly other known species will be found to have a similar facultative saprophytism if investigators interested in growing them in artificial culture observe the cultural requirements revealed by Sawyer's work. The distinction supposed to exist between *Entomophthora, Empusa* and *Conidiobolus* is, therefore, lessened; as a matter of fact, material from artificial cultures of these genera, when examined under the microscope, cannot be distinguished morphologically with any degree of certainty until the resting spores appear. In addition, the microconidia in Thaxter's subgenus *Triplosporium* is another structural characteristic commonly found in certain species of each of the three genera in question. In view of these facts, the writer believes that he is justified in making the new combination **Entomophthora coronata**[1] (Cost.) with the following as synonoms: *Boudierella Coronata* Cost., *Delacroixia coronata* (Cost.) Sacc. and Syd., and *Conodiobolus villosus* Martin.

In conclusion the writer wishes to express his indebtedness to Professor W. H. Weston, Jr., under whose direction this work was conducted, for his unstinted interest and kindly criticism, and to Doctor D. H. Linder for many helpful suggestions throughout the progress of this problem.

Summary

A comparative morphological and biometrical study on artificial media, has been made of strains of *Conidiobolus villosus* obtained from the Farlow Herbarium, the Centraalbureau voor Schimmelcultures, and isolated directly from termites in Cuba. Furthermore, infection experiments have shown that the fungus which has hitherto been considered a saprophyte may adapt itself to a parasitic habit, especially on termites. The Derx strain from Holland, in contrast to the others, appears to be strictly saprophytic. Moreover, in the life-history of the species in question an additional stage has been observed which consists of the production of minute conidia borne at the tips of the spiny appendages of the villose conidia or "resting spores". For these reasons a new combination, *Entomophthora coronata* (Cost.) has been made and *Delacroixia coronata* and *Conidiobolus villosus* have been reduced to synonomy.

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[1] The writer prefers to use the generic name *Entomophthora* rather than *Empusa* since the latter has been used earlier (1824) for a genus of orchids and is untenable as a genus of the Entomophthoraceae according to Article 65 of the International Rules of Botanical Nomenclature, which reads, "A name of a taxonomic group is illegitimate and must be rejected if it is a later homonym, that is if it duplicates a name previously and validly published for a group of the same rank based on a different type. Even if the earlier homonym is illegitimate, or is generally treated as a synonym on taxonomic grounds, the later homonym must be rejected."

EXPLANATION OF TEXT FIGURES

All drawings were made with the aid of a camera lucida, from material mounted in lactophenol. Each division of the scale represents 10u.

Text Figure No. 1.

A-D. Martin or type strain.

A and B. Smooth-walled conidia showing range in size. X 500.

C and D. "Resting spore", or echinulate conidia. X 500.

E-H. Cuban strain.

E and F. Smooth-walled conidia. X 500.

G and H. "Resting spore", or echinulate conidia. X 500.

Text Figure No. 2.

A-G. Martin or type strain.

A. "Resting spore" showing development of minute conidia only on portion of peripheral area. X 500.

B-G. Successive stages in the development of the minute conidia. X 500.

B. Villose appendage.

C. Appendage swollen at distal end, a primary step in the formation.

D-F. Further changes in development.

G. Minute conidium just prior to discharge.

H. J. Derx Strain.

H. "Resting spore" with minute conidia on portion of peripheral area as in Martin strain, Fig. A. X 500.

I. Primary conidium giving rise to secondary by means of a germ tube. X 500.

J. "Resting spore" showing minute conidia on only portion of periphery as in figures A and H. X 500.

K. N. Cuban strain.

L and N. Germinating minute conidia. X 500.

M. "Resting spore" showing conidial development on entire peripheral area prior to septum formation and discharge. X 500.

PLATE IX

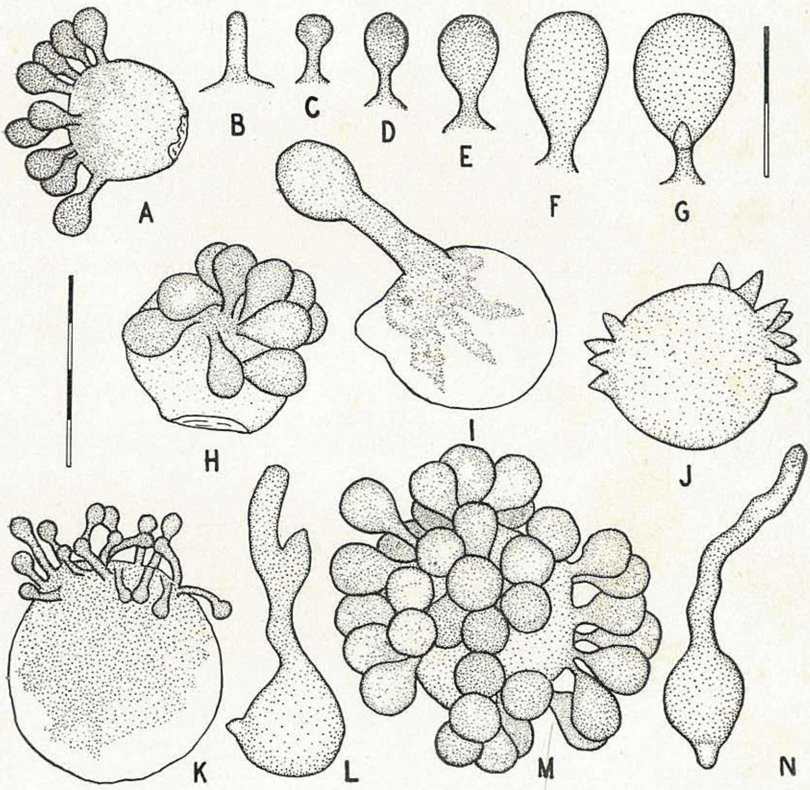


PLATE X

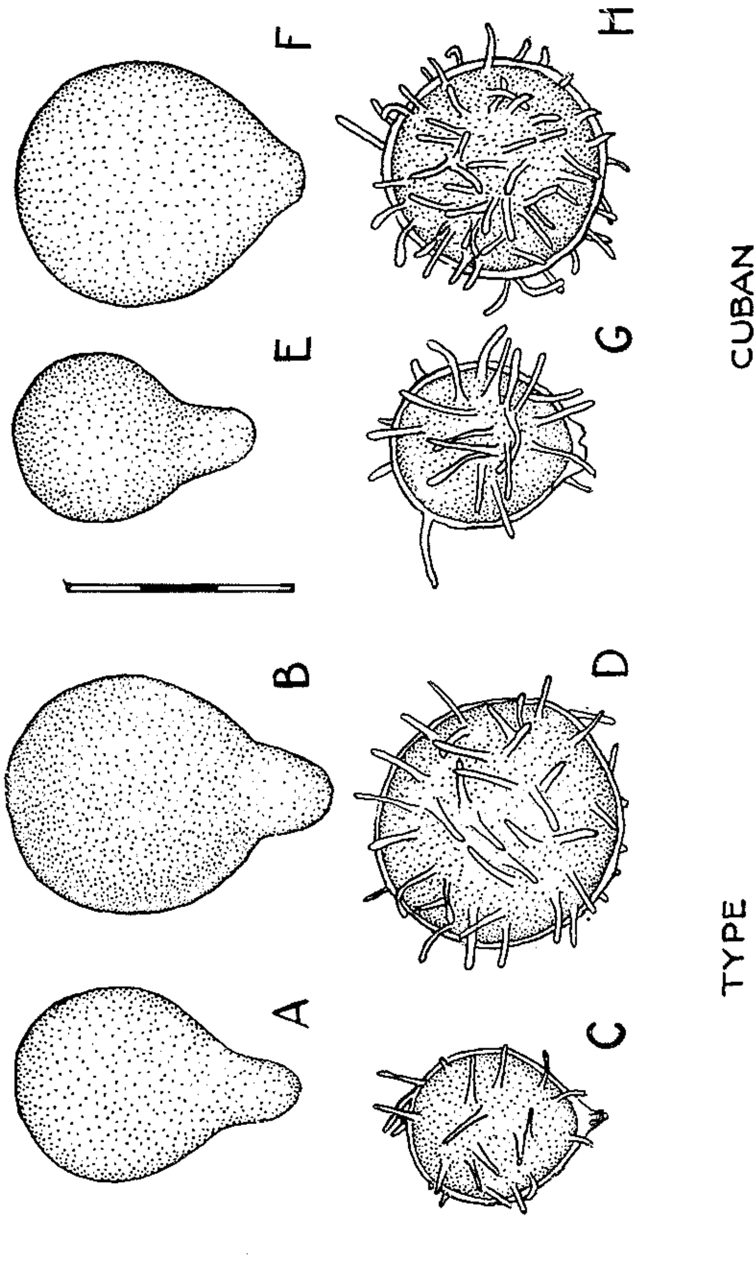
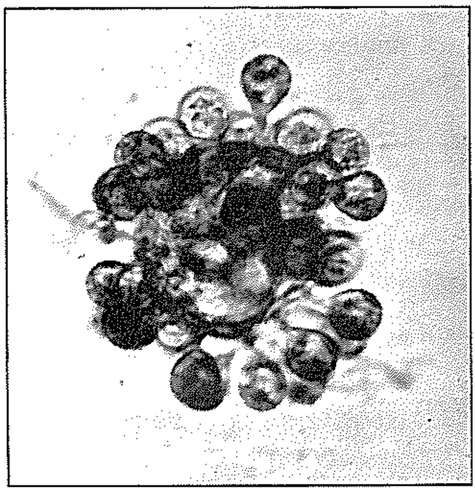


PLATE XI



Photomicrograph showing villose type of conidium bearing the smaller micro-conidia at the apical extremities of the hair-like projections.