

The Biology of *Harposporium anguillulae*

BY M. ASCHNER AND S. KOHN

Department of Microbiology, Technion-Israel Institute of Technology, Haifa, Israel

SUMMARY: *Harposporium anguillulae* Lohde was isolated in pure culture from an infected nematode and its behaviour on culture media under various conditions is described. The life history of the fungus in the nematode host has been investigated and the infection traced back to germinating conidiospores in the oesophagus. Two other *Harposporium* spp. were also isolated and it is shown that their mode of infection is similar to the one described for *H. anguillulae*. Because of these findings a new theory for the mode of infection is proposed and its significance in the ecology of the fungus discussed.

Among the predacious fungi which attack nematodes in soil, certain *Arthrobotrys* and *Dactylella* spp. have received much attention. These fungi can easily be studied in the laboratory since they grow well on simple nutrient agar media and their interesting mode of catching nematodes by means of sticky or constricting loops can be directly observed under the microscope. They have also been tested in experimental field studies as to their effectiveness in the biological control of certain root eelworms (Duddington, 1957). By contrast, no experimental work has been done so far with those fungi which infect nematodes by means of specialized conidiospores and kill them through the endozoic mycelium which develops from these spores within the host's body cavity. This is all the more surprising, as these fungi are not uncommon in soil and their parasitic relationship with soil nematodes was recognized long ago (Lohde, 1874).

A number of mycelial fungi with infective spores, most of them belonging to the genus *Harposporium*, have been described (Karling, 1938; Drechsler, 1941; Shepherd, 1955), but as far as we know, none of these has ever been studied in pure culture, and such fundamental questions as the mechanism of infection or the specificity of the parasite for the various soil eelworms still remain open. The present investigation, based on a detailed biological study of three *Harposporium* spp. is a contribution to this discussion.

METHODS

The strain of *Harposporium* used in the investigation was found in a sample of soil and manure which had served as substrate for the cultivation of mushrooms at Matzuba (northern Israel). A sample (0.1 g.) of this soil had been incubated at a temperature of 28-30° on a plate of tap-water agar, consisting of agar-agar strips (2%, w/v) in tap-water. After an incubation period of 14 days dead eelworms were seen on the agar surface, and septate hyphae with typically arcuate spores were seen sprouting from their bodies, indicating an infection with *Harposporium* sp. (Pl. 1, fig. 1).

Our fungus closely resembles *Harposporium anguillulae* Lohde as described by Karling (1938), and although there are some slight differences in the

measurements of the conidiospores (width 1μ , length $5-8\mu$ in our fungus as against 1.5μ and $5-14\mu$, respectively, in Karling's description) we do not feel justified in creating a new species for our organism. A complete identification will be possible only when strains of this parasitic fungus are compared on identical culture media or in the same species of host organism. As far as we know this has not been done yet.

The eelworms used in our experiments included several of the rhabditic species of nematode and one *Dorylaimus* sp. which had previously appeared in various soil cultures. None of these species was identified. The eelworm cultures were kept on tap-water agar at room temperature and *Escherichia coli* was added as food organism. Handling of the eelworms was facilitated by cutting holes of about 1 cm.^2 surface, so called lagoons, into the agar layer. These holes were filled with water, and large numbers of eelworms accumulated there and became infected when conidiospores of any of the examined *Harposporium* spp. were added to the water. For the microscopic examination of infections in living eelworms the following technique was developed. A drop of fluid from a lagoon with infected eelworms was mixed on a slide with a small amount (less than a loopful) of a 1% (w/v) aqueous solution of erythrosin. This preparation was then warmed gently by placing the slide without coverslip near an electric lamp until the edge of the drop began to dry. It was then covered with a coverslip and dried for 1 hr. more at room temperature until the coverslip exerted a slight pressure on the eelworms in the drop. The clearest pictures were usually obtained just before the preparation dried up entirely or the pressure of the coverslip burst the cuticle of the eelworm. The erythrosin stained the external hyphae and conidiospores of the *Harposporium* spp. and also had an anaesthetic effect on the movements of the eelworms.

RESULTS

Isolation of the infecting fungus

One infected eelworm was cut out of the agar medium, together with the small piece of agar adhering underneath and transferred to another plate which besides tap-water agar contained 100 p.p.m. penicillin and the same amount of streptomycin to suppress development of bacteria as far as possible; c. 0.1% (w/v) of 'Dithane' (a commercial preparation of zinc ethylene-bis-dithiocarbamate made by Rohm and Haas) was also added. This substance has a fungistatic action on many soil fungi but apparently not on a *Harposporium* sp. growing inside a nematode. After 2 days a small piece (about 0.25 cm.^2) of yeast-extract agar was cut from an agar plate and placed near the explanted nematode. The composition of the yeast medium was (g.): peptone, 0.5; glucose, 2; yeast extract (Difco), 0.2; agar-agar, 2; 100 ml. distilled water.

The growth of the fungus was enhanced by the nutritive material diffusing from the yeast-extract agar but the concentration was not high enough to support luxuriant growth of contaminating organisms which were still present on the surface of the eelworm. When the hyphae of the *Harposporium* sp. had spread somewhat and penetrated into the solid medium another small agar

block was cut out aseptically from the periphery of this growth and transferred to fresh yeast-extract agar. The transferred piece proved to be free from other micro-organisms and the excised hyphae continued to grow on this medium.

After about 14 days the growth was visible macroscopically as a dense snow-white dome-shaped colony, c. 0.5 cm. in diameter. No conidiospores were found in this culture and the only indication that our isolate was identical with the fungus in the eelworm was the appearance of 'endozoic mycelium' in the innermost part of the colony. This means that the mycelium in the colony looked like the growth inside an infected eelworm, and was composed of two morphologically different components: (a) strands of large thick-walled cells, comparable to the endozoic mycelium, in the centre of the colony; (b) thin-walled aerial hyphae in its outer parts. Sometimes the thick-walled mycelium showed a light green coloration. Subsequent cultures which have been grown now for several months on artificial media, showed a more luxuriant growth which covered the agar surface with a fluffy spreading mycelium.

In later experiments the method used for the isolation of strains of *Harposporium* was much simpler. A few drops from a lagoon with infected eelworms were spread over a plate of glucose yeast-extract agar containing about 0.1 mg./ml. of Aureomycin. The hyphae which emerged from the infected eelworms grew well on this medium while most bacteria were inhibited. After 3-4 days the small areas of mycelium which had formed around the infected eelworms were examined microscopically on the plate and when found to be free from contamination were cut out from the agar and transferred to a glucose yeast-extract agar slope.

Formation of conidiospores in cultures

The lack of conidiospores in these cultures made them unsuitable for experiments on the infection of eelworms. This difficulty was overcome in the following way. Colonies from glucose yeast-extract agar, together with some adhering agar medium, were cut into very small pieces (the size of a pinhead or smaller) and placed on tap-water agar plates; the intention was to imitate more closely the conditions of limited food supply which confront the fungus colony in nature after food material in the invaded eelworm has been used up. The validity of this principle has not been proven but the procedure worked satisfactorily and the fragmented mycelia developed hyphae which, with regard to spore formation, were identical with those observed in natural infections.

Growth requirements of the Harposporium sp.

Although *Harposporium anguillulae* in nature acts like an obligate parasite (at least it has never been reported to occur outside its nematode hosts), it does not seem to have special food requirements. Our organism was able to develop not only on the glucose yeast-extract agar medium mentioned above, but also on a simple glucose mineral-salts medium consisting of (g.): glucose, 1; $(\text{NH}_4)_2\text{SO}_4$, 0.2; K_2HPO_4 , 0.1; MgSO_4 , (anhydrous), 0.5; agar-agar, 2; 100 ml. distilled water. Growth on this medium, however, was much slower and less luxuriant than on the yeast-extract agar.

Our fungus grew on the surface of liquid yeast-extract medium and when this was agitated, as tiny pellets throughout the whole volume of fluid. The conditions in liquid media did not seem to be conducive to the formation of conidiospores; however, some were formed on those colonies which grew attached to the vessel-wall at the boundary between air and liquid.

Germination of the conidiospores

No germinating spores were found on the media commonly used for the detection of *Harposporium* spp. These media have to be low in food content; otherwise bacteria which always occur in the same environment would overgrow the more slowly growing *Harposporium* sp. This difficulty does not arise on nutrient media in which the development of bacteria is selectively inhibited. On glucose yeast-extract agar with 100 p.p.m. aureomycin some spores germinated and the process was followed under the microscope. The germination bud appeared always in the thickest part of the spore on the outer circumference of the crescent. It remained small and thin (less than 1μ in width) but the mycelium which developed without any transition from this tiny outgrowth had the appearance and dimensions of a normal-sized hyphomycete. The germination bud remained as a short neck-like piece between spore and mycelium. The direction of growth of the germ tube was opposite to that of the pointed horns of the spore (Pl. 1, figs. 3-5).

Not all the spores germinated under these conditions, many of them remained dormant even during an observation period of 2 weeks. Germination of spores occurred also on glucose mineral salts medium but, in accordance with the less favourable growth properties of this medium, the proportion of spores which germinated on this medium was rather small.

The penetration of the spore into the body cavity of the eelworm

Several opinions have been expressed about the manner of infection of the host eelworm by *Harposporium* spp. Drechsler (1941) for *H. oxycoracum* described crescent-shaped spores with a drop of sticky fluid adhering to one of the pointed tips. According to his view this fluid served to glue the spore to the outer integument of the eelworm, whence after germination the germ tube entered through the cuticle into the body cavity of the host. Duddington (1957) expressed the opinion that the conidia in *H. anguillulae* and most other species of *Harposporium* were sticky and that with all of them the mode of infection resembled that suggested by Drechsler for *H. oxycoracum*. Duddington rejected the possibility that such spores could enter the host by an oral route because of their too great size. Other authors (Zopf, 1888; Kostka, 1927) have assumed a connexion between spore shape and mode of infection. According to their view the spore pierced the outer cuticle of the nematode mechanically by means of the sharply pointed tips, the force required for this feat being provided by the momentum of the actively moving eelworm itself.

We attempted to decide the question of mode of entry by examining the earliest stages of infection. Hyphae which protruded through the cuticle of the eelworm always indicated a well-advanced infection, with the endozoic

mycelium occupying practically the whole of the body cavity. This stage was obviously too late to supply information about the mode of entry of the parasite. Consequently, we examined eelworms which had come in contact with spores but which were still living and without any external signs of infection. By using the microscopical technique described earlier it was possible to demonstrate the presence of germinating conidiospores inside the body of the eelworm. Such spores were invariably situated in the upper region of the digestive tract, between the oral opening of the buccal cavity and the median bulb of the oesophagus. They appeared to be firmly wedged in this narrow channel with the central part of the spore in close contact with the lining of the tract. The place of contact was also the place of germination of the spore and at the same time the place of entry of the fungus into the body cavity of the eelworm, with the tiny germination bud acting as the means of piercing the wall of the oesophagus. No incipient infection was ever found in or on any other part of the eelworm body, but the oesophagus contained sometimes more than one spore.

The picture of the early stages of infection with this fungus was identical in all the cases observed. The germ tube was situated in or near the muscle tissue of the oesophagus, growing towards the cardiac bulb (Pl. 1, figs. 6-8). After reaching this organ and encircling it, it usually grew in the direction of the head (Pl. 1, fig. 9). At these stages of infection the eelworm was still alive. Very soon afterwards side-branches pushed out from the encircling loop and entered the body-cavity of the host eelworm in the intestinal region. These branches completely dissolved the intestinal tissues, causing death of the host. The fungal growth inside the eelworm body then changed into the morphologically differentiated endozoic mycelium which finally formed typical chlamydospores (Pl. 1, figs. 2, 10).

The hyphae which sprouted from this mycelium pierced the cuticle and later gave rise to sickle-shaped conidiospores. At the point where hyphae protruded through the cuticle they were usually surrounded by a collar-like formation which somewhat resembled a hair socket in an insect cuticle. These sockets were clearly visible in stained preparations or in old infections after disintegration of the hyphae (Pl. 1, fig. 10). It was observed that with this species of *Harposporium* the piercing of the outer cuticle usually started in the head region and that formation of conidiospores was earlier on the hyphae in this region than elsewhere. It was indeed this observation which induced us to search for the start of infection in the upper region of the digestive tract.

Infectivity of the Harposporium strain for diverse species of eelworm

Among the numerous species of eelworms in the soil a large number belongs to the so-called *Rhabditis* group of nematodes. These eelworms feed on bacteria and other particulate matter which they take into the buccal cavity from the surrounding fluid and swallow as solid particles. Other eelworms, and among them many plant-parasites, are unable to swallow particulate food. They suck instead the fluid content of various living cells after piercing the cell wall by means of a protractile stylet in their mouth cavity. A genus of free-

living eelworms in this group is *Dorylaimus*. We had no difficulty in infecting rhabditic eelworms with our fungus. Several species were tested and all gave the same positive result. However, a species of stylet-bearing eelworm belonging to the genus *Dorylaimus*, when tested in the same way, remained free from infection during the whole period of observation, which lasted one month.

Comparison of Harposporium anguillulae with two other Harposporium spp.

While investigating the mode of infection of *Harposporium anguillulae* we isolated from eelworms found in garden soil (Haifa) two additional species of *Harposporium*. One of these organisms produced sickle-shaped spores which were considerably larger than those of *H. anguillulae*. They resembled spores of *H. helicoides* in their dimensions but lacked the mucus droplet described for this species. It was not possible for us, owing to lack of literature, to determine whether this species had already been described or not. We therefore called it, provisionally, species A (Pl. 2, figs. 11, 12). The spores of the other species had the form of short and rather small rods which were provided at their ends with two short thornlike processes. These processes were sharply bent at right angles to the long axis of the spore and pointed in opposite directions. A similar shape has been described for *H. diceracum* but our strain differed in some details from this species and could not be identified with it. We have called it provisionally, species B (Pl. 2, figs. 15, 16).

The infection of eelworms with species A presented a picture very similar to that observed in *Harposporium anguillulae*. Germinating spores were found in the lumen of the oesophagus and, again, in its upper region only. They were even easier to detect than the spores of *H. anguillulae* on account of their larger size. Occasionally double infections in the oesophagus were seen and in one case three spores were present in this organ. They were arranged in tandem fashion and covered practically the whole section from oral opening to median bulb. The development of the endozoic mycelium of this fungus differed from that characteristic for *H. anguillulae*. Instead of growing in close attachment to the musculature of the oesophagus the hyphae started their growth at some distance from this organ. This was due to a peculiar modification of the germination bud in this species. This bud grew as a fine filament (length 5–6 μ) which penetrated not only the intima of the oesophagus but the entire musculature of this organ. Thus the growth of regular hyphae started directly in the body cavity of the eelworm (Pl. 2, fig. 13). The filament-like germination bud was clearly discernible also when spores germinated on nutrient media (Pl. 2, figs. 19, 20).

Spores of species B in a very similar way developed, on nutrient media, a germination filament before the growth of regular hyphae started, but in the eelworm not a single infection among a large number examined could be traced back to a spore in the oesophagus. Spores were found only in the buccal cavity. This finding was rather surprising as the short and relatively small spores seemed to be much more suitable to enter the oesophagus than the large and unwieldy sickle spores of the other two species (Pl. 2, figs. 14, 17, 18). When the oral cavity of the eelworm was filled with mucus or food-debris it was sometimes difficult to detect the spores there. In these cases diagnosis

could be made by demonstrating the germination filament which originated in that locality and passed into the initial hypha. Species B grew less luxuriantly on glucose yeast-extract agar than the other two species of *Harposporium* but produced many conidiospores on this medium.

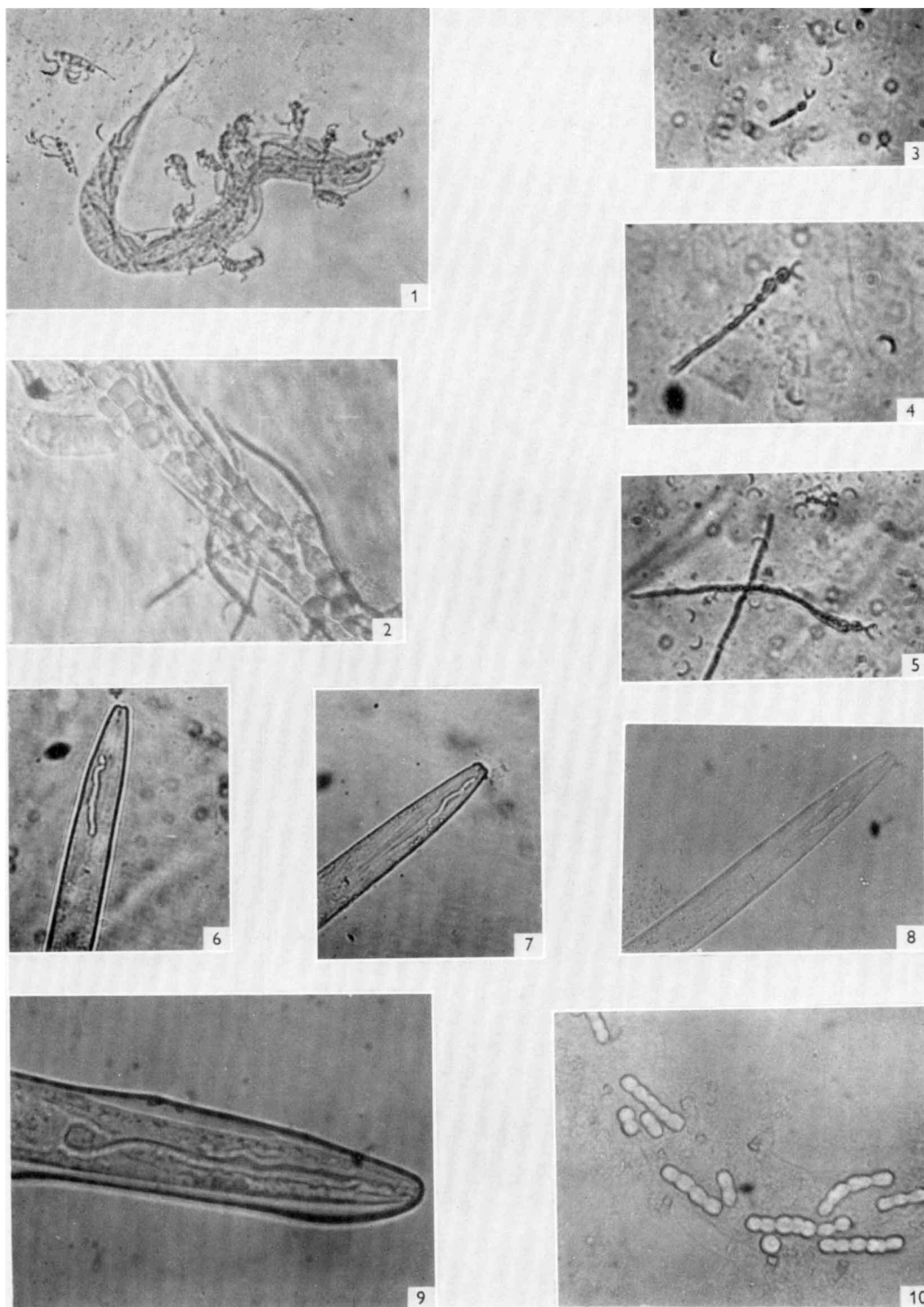
Species A and B, in contrast to *Harposporium anguillulae*, did not form rounded chlamydospores in the infected eelworm, and their external hyphae pierced the cuticula of the eelworm in the head and tail region at practically the same time. All three species of *Harposporium* were able to infect eelworms after prolonged growth on artificial media.

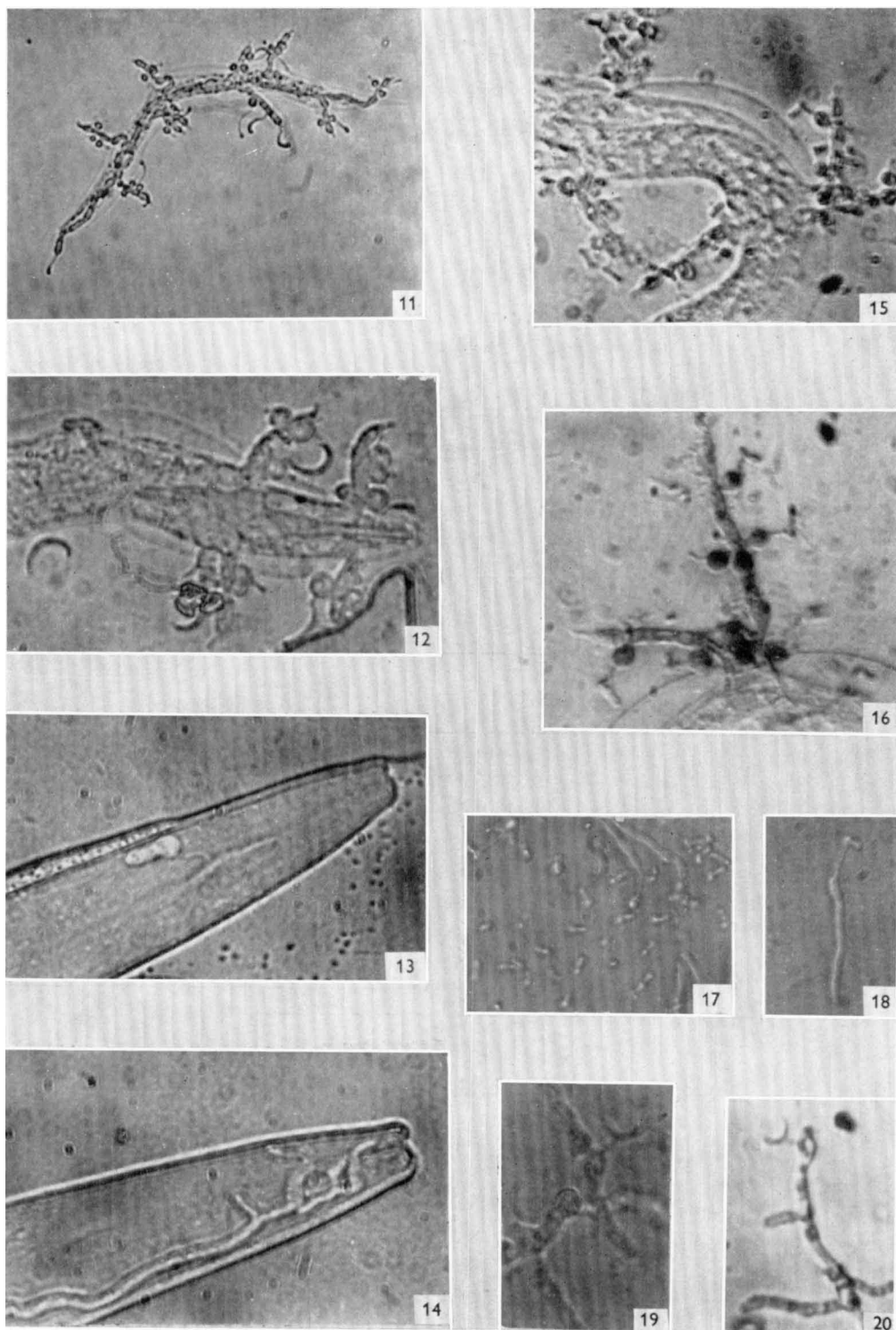
DISCUSSION

The conidiospores of *Harposporium anguillulae* and species A appear to enter the eelworm host by the oral route; otherwise their presence in the buccal cavity or oesophagus is difficult to account for. We must assume that they are taken up and swallowed by the eelworm, although their overall dimensions are larger than the lumen of the buccal cavity or the oesophagus. This would of course be impossible in the case of a straight and rigid channel, it seems however, that the eelworm can bend or twist its alimentary tract in the head region according to the (arcuate or helicoidal) shape of the particle which is to be swallowed. The *Harposporium* spores being very slender can thus be taken up whenever they enter the oral opening with one of their tips. It is not known what induces the spores to adhere to the upper region of the oesophagus. With their crescent-like curvature and pointed tips they resemble a fishhook, and their fixation in the oesophagus may be due to the hooklike function of one or both of these tips. It has, however, not been demonstrated that the oesophagus is actually pierced in that way.

The conidiospores of species B also enter the host by the oral route, but are found only in the buccal cavity and not in the oesophagus of infected eelworms. This may be due, obviously, either to a barrier at the entrance of the oesophagus which prevents spores of this shape to pass, or to inability of these spores to affix themselves in the lumen of this organ once they have entered it. In this case they are probably swept, together with the food, into the stomach to be digested there.

No signs were found which indicated with any of our fungi an invasion of the eelworm through the outer integument. We never observed spores adhering to a living nematode, not even after it had crawled through heaps of detached spores or had brushed against a spore-bearing hypha: furthermore, as mentioned already, incipient infections were seen only in the head region of eelworms. It may therefore be claimed that, with regard to the *Harposporium* spp. studied, infection of eelworms proceeds only through the oral route. The experiment with the *Dorylaimus* eelworm supports this claim: if the swallowing of a conidiospore is essential in order that host be infected with the fungus, then sucking species should be resistant to this type of infection since they do not swallow solid food. The unsuccessful attempt to infect the *Dorylaimus* sp. with *H. anguillulae* supports this assumption. A further conclusion to be





drawn from this experiment concerns the use of fungi for the biological control of eelworms in soil. Most economically important species of eelworm are found among the sucking nematodes and obviously no successful control may be expected from the use for this purpose of *H. anguillulae* or of other fungi with a similar mechanism of infection.

This work is dedicated to Professor Paul Buchner, Porto d'Ischia (Napoli) on his seventieth birthday.

REFERENCES

- DRECHSLER C. (1941). Some hyphomycetes parasitic on free-living terricolous nematodes. *Phytopathology*, **31**, 773.
 DUDDINGTON, C. L. (1957). *The Friendly Fungi*. London: Faber and Faber.
 KARLING, J. S. (1938). *Harposporium anguillulae*. *Mycologia*, **30**, 512.
 KOSTKA, G. (1927). Nematoden fangende Pilze. *Mikrokosmos*, **21**, 151.
 LOHDE G. (1874). Einige neue parasitische Pilze. *Tageblatt der 47. Versammlung deutscher Naturforscher und Ärzte in Breslau*. p. 203, (cited from Shepherd.)
 SHEPHERD, A. M. (1955). *Harposporium crassum* sp. nov. *Trans. Brit. mycol. Soc.* **38**, 47.
 ZOFF, W. (1888). Zur Kenntnis der Infektionskrankheiten niederer Thiere und Pflanzen. *Nova acta Leop. Carol. Deut. Akad. Naturf.* **52**, 315, (cited from Drechsler.)

EXPLANATION OF PLATES

PLATE 1

Different stages in the life history of Harposporium anguillulae

- Fig. 1. External hyphae emanating from the body of an eelworm. ($\times 250$).
 Fig. 2. The thick-walled cells of the endozoic mycelium in an infected eelworm. $\times 500$.
 Figs. 3-5. Different stages in the germination of the conidiospores on yeast-extract agar. Note the thin neck-like connexion between the spore and the initial hypha. $\times 450$.
 Figs. 6-8. Head parts of eelworms with germ tubes of the fungus originating in the oesophagus. $\times 250$.
 Fig. 9. Head region of an eelworm during an early stage of infection. The initial hypha grows inside the muscular layer of the oesophagus. $\times 500$.
 Fig. 10. An infected eelworm 1 month after death. The endozoic mycelium has been transformed into rounded chlamydospores. The external hyphae are already disintegrated, but their 'sockets', places of their former emergence from the cuticle, are still visible. $\times 500$.

PLATE 2

- Fig. 11. An eelworm infected with a *Harposporium* sp. strain species A. $\times 150$.
 Fig. 12. The same in higher magnification; note the arcuate shape and pointed tips of the conidiospores. $\times 720$.
 Fig. 13. Germination of a conidiospore of species A in the oesophagus of an eelworm. The germination filament penetrates the lining as well as the muscular layer of the oesophagus and the initial hypha starts its development in the body cavity of the eelworm. $\times 720$.
 Fig. 14. Development of the endozoic mycelium in an eelworm infected with species B. No involvement of the oesophagus. $\times 720$.
 Figs. 15, 16. Eelworms infected with species B. Note the rod-shaped spores. $\times 720$.
 Fig. 17. Spores of species B germinating on nutrient medium. $\times 450$.
 Fig. 18. A single spore of species B germinating on nutrient medium. A long thread-like germination tube connects the spore with the initial hyphae. $\times 450$.
 Figs. 19, 20. Conidiospores of species A germinating on a nutrient medium. The germination filament is visible. $\times 720$.

(Received 28 February 1958)

Since this article was written we have seen Drechsler, C. (*Bull. Torrey bot. Cl.* 1946, **73**, 557) describing *H. bysmatosporum* a species either identical with or very similar to our species B.