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Biology of toxigenic anamorphs

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Abstract - The competition for food between toxigenic fungi and small mammals, and the interactions that result, are explored as paradigms for the biological and evolutionary implications of mycotoxin production. The life cycles, relationships and substrate preferences of the principal toxigenic mold genera are discussed, and some evidence, largely drawn from fungus-invertebrate interactions, is presented in support of the hypothesis that toxin production may be much more widespread among ascomycetous anamorphs than has generally been thought.

Biologists are accustomed to discovering that biological structures, substances or phenomena, at first thought to lack significance, actually play an important role in the life of the organism giving rise to them. The raw materials from which some mycotoxins are manufactured are amino acids. Is it reasonable to suppose that these key raw materials, the building blocks of all proteins, are being squandered in throwaway substances by fungi? When we remember that the fungi are justifiably famous as snappers up of unconsidered trifles, humble recyclers of what other organisms discard, the answer must surely be an unqualified no. Mycotoxins may be secondary metabolites, removed from the mainstream of cell growth and maintenance, but I find it intellectually unsatisfying to label them as lacking in practical significance to the organism that makes them.

Let's examine the case of mold versus mouse. A grain falls to the ground. It is found by a small mammal, and stored against the long winter ahead (note the influence of the Canadian climate on my thinking -- perhaps I should add 'or against the long dry season ahead'). The grain is taken underground and kept in a situation where it neither freezes nor dries out too much. In these conditions it becomes or remains accessible to the molds. But there is also a threat. Suddenly one day, as the fungus is exploring and assimilating its food in a leisurely way, the mammal remembers, or finds, its cache, and decides to make a snack of the grain. End of story. Fungus loses out, falling prey to mammalian digestive enzymes.

But if, by chance, during genetic recombination mediated by the parasexual cycle, a new compound is synthesized that makes the fungus and its food unpalatable, or has emetic or toxic effects on the mammal, the fungus is more likely to survive, because one of four things will happen. (A) The mouse will sniff the grain and reject it in favour of other food. (B) The mouse will taste but then reject the grain. (C) The mouse will eat it, be sickened by it, and learn to avoid it in future. (D) The mouse will be poisoned by it and die. In each case, the fungus is more likely to survive than before, and to continue its slow progress through the grain, successfully producing enormous numbers of spores as it does so.

Even the names we have given to some mycotoxins imply such a mechanism: vomitoxin, refusal factor, slobber factor. So **Aspergillus** and not the mouse gets the peanut, **Fusarium** and not the mouse gets the corn or wheat, and **Penicillium**, not the mouse, gets the cheese. These three genera are the most successful and numerous of all toxigenic fungi, and I don't want to give you the impression that they rely solely on their mycotoxins for their success. Some species of **Aspergillus** are extremely drought-tolerant, being able to grow in substrates so dry -- water activities as low as 0.62 -- that almost nothing else can compete with them. And while some aspergilli are relatively thermotolerant, most penicillia are mesophiles, and some fusaria are psychrotolerant, growing at temperatures below 0 C, so they cover the full spectrum of temperatures at which life can thrive (ref. 1).

In the world of toxigenic fungi I am intrigued by one particularly complex case, which may also involve mice, and perhaps I can enlist your help in cracking this one. I refer to the ergot fungus, **Claviceps purpurea**, the mycotoxin producer longest known to Man. Here we have an organism with a complicated life history, an organism that goes through several fascinating performances, at least one of which may have a significance that to the best of my knowledge has not been discussed in the literature. If I may begin in Spring, the first thing that happens is the germination of what we call the ergot, a fungal sclerotium which has lain on the ground all winter. From the hard, purplish sclerotium arise several small stromata (Fig. 1). Each of these has a fleshy stalk, and a round head containing many perithecial cavities (Fig. 2). Within each cavity many long meiosporangia called asci develop, each containing 8 extremely long ascospores. Each ascospore becomes divided up into perhaps 100 part-spores (Fig. 3).

Eight hundred spores per ascus; perhaps 50 asci per perithecial cavity; 100 perithecia per stroma; 5 stromata per ergot. A total of 20 million propagules per ergot. Astronomical numbers of spores shot into the air. But the reason for this behaviour is no mystery. It evolved because the target of those spores is very small, relatively distant, and available only during a brief time-window. The target is the short-lived stigma of the grass flower. This is probably as difficult to hit as the moon was for the U.S. space program. The difference is that instead of guiding one launch vehicle precisely, the fungus sends out millions of timely but disposable, unguided launch vehicles in the hope that at least one of them will reach the target. Since the fungus is still with us, we know that hope to be justified.

Why such a small target? Because through it the fungus can gain access to one of the richest and most concentrated supplies of food to be found in any plant, the ovary of the developing seed. The grass goes ahead and channels food to the ovary just as if a normal fruit was being developed. But instead, the alien fungus commandeers these supplies and uses them to produce a mass of fungal tissue that releases spores in a sweet-smelling nectar. Insects spread these spores to other grass plants. All these activities have been beautifully tailored by evolutionary pressures, and we think we understand what they are all about. I have told you these things to show you how finely tuned, how specialized, this fungus is, and to suggest that we should be able to find comparable rationales for other phases in the life cycle. Now the fungal tissue dries up and hardens to form the ergot, with which you may be familiar. This is a sclerotial anamorph from which, as we have already seen, the teleomorph arises. But with the formation of the sclerotial anamorph the mystery deepens, at least for me. The ergot doesn't look like a grain. It is larger, longer, darker, and sticks out further (Fig. 4). This means that it is instantly distinguishable from the normal fruits of the plant. Might there be a good reason for this? I believe that evolutionary forces have shaped most biological phenomena, so I suppose I must now try to provide a heuristic explanation of the evolutionary pressures involved. I don't know whether you will agree with my reasoning, but I hope you will find it worthy of some consideration.

A grazing cow is **not** going to notice the difference between an ergot and a normal grain, and will swallow the lot. So the mechanism can't be aimed at cows, But remember that the ergot spends far more time on the ground, where cows are unlikely to eat it. Down there it is likely to be discovered by much smaller, but still marginally intelligent animals -- mice. The world has many more mice than cows, and the visible differences between an ergot and a regular grain will be obvious to a mouse. Perhaps this visual clue saves ergots from being eaten, so they will survive to produce millions more spores next Spring. But there's another complicating feature. As you know, the ergot contains an incredible cocktail of alkaloids -- about 100 of them I believe -- which are biologically active, but whose presence cannot be visually detected. Some of them cause vasoconstriction in mammals, some of them have interesting effects on the mammalian central nervous system. This may all be pure chance. But these compounds are secondary metabolites made from expensive primary metabolites. Could they have a role to play in the world of the mouse? Perhaps. But for this to be true, we must assume that the mouse, when it first encounters an ergot, eats at least some of it in order to find out what it tastes like.

Prolonged vasoconstriction and the subsequent gangrene are bad, but LSD-induced hallucinations are good, if those people who habitually use LSD are to be believed. Is the fungus giving mixed messages? I have two hypotheses. The first suggests that the pleasurable mental state induced by the lysergic acid derivatives encourages the mouse to eat more, and distracts it from the onset of the more ominous symptoms: so the occasional ergot is thus sacrificed in order

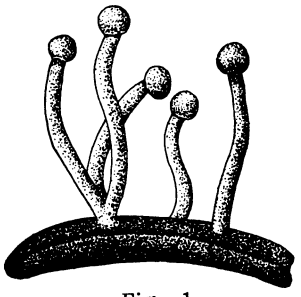

Fig. 1

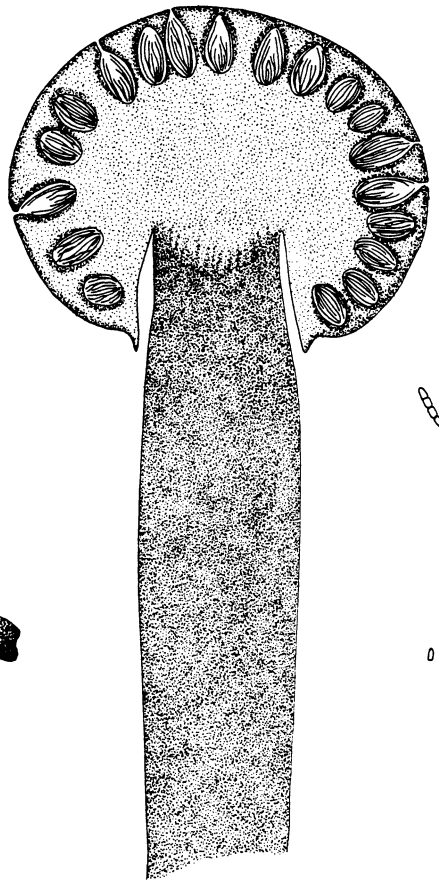

Fig. 2

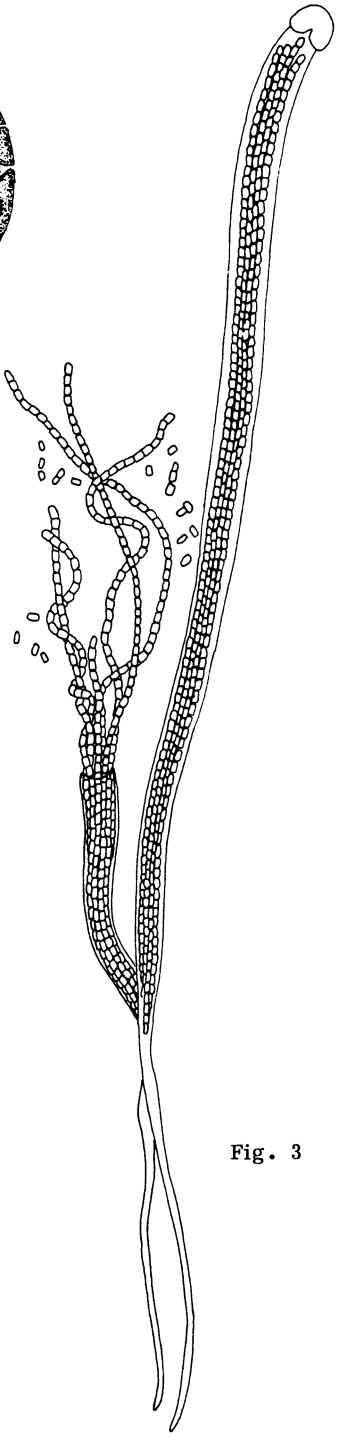

Fig. 3

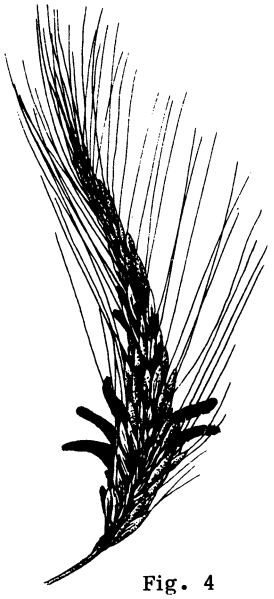

Fig. 4

Fig. 1. Germinated ergot bearing teleomorphic fructifications.
Fig. 2. Section through teleomorph showing perithecial cavities.
Fig. 3. Asci showing long ascospores fragmenting into part-spores.
Fig. 4. Ear of rye showing projecting ergots.

to remove the pressure of mouse predation. My second hypothesis is based on the knowledge that there are good trips and bad trips, the nature of any particular trip depending largely on the state of mind of the individual immediately before the drug is taken. What is the state of mind of a mouse likely to be? I suggest that it is paranoid, perennially frightened, on the alert for danger, because it is subject to so many predators. Hallucinogenic compounds may well heighten those feelings. I put it to you that a mouse will always have a bad trip, seeing hawks, owls, foxes and cats lurking behind every blade of grass. This mouse will quickly learn not to nibble on those big, dark grains. So perhaps the alkaloids have evolved because they discourage mice from eating ergots, each of which can then go ahead and produce its 20 million spores.

I don't have any experimental evidence for either of these hypotheses, but such evidence might not be too hard to obtain. It would certainly further our understanding of the biology of ergot, and might provide us with one rational explanation for the existence of its alkaloids.

Now I'll move on from the relationships of mycotoxins and small mammals to the relationships of mycotoxin-producing fungi with each other. This is an area that only mycologists seem to think about. I've seen papers that compared specific properties of such fungi as **Saccharomyces**, **Neurospora**, **Mucor** and **Allomyces**, while apparently ignoring the fact that these fungi belong to very different groups (different Classes, even different Phyla), which means that any comparisons among them are going to be loaded with phylogenetic implications. How does this kind of thing work out in the toxigenic molds? Well, to begin with, almost all of them produce only asexual spores called conidia. But despite the fact that most of them don't develop the diagnostic sexual stage, we can tell that all of them belong to Phylum Dikaryomycota, which comprises the Classes Ascomycetes and Basidiomycetes (ref. 2).

Let's look at some members of the big three -- **Fusarium**, **Aspergillus** and **Penicillium** (Fig. 5). **Fusarium graminearum**, the mold that produces zearalenone, has **Gibberella zeae**, an ascomycete of the Order Hypocreales, as its sexual stage (ref. 3) This fungus is unusually cooperative in that the asexual form and the sexual form, which we call the anamorph and teleomorph, respectively, often develop together on moldy corn. But the anamorphs causing Alimentary Toxic Aleukia: **Fusarium sporotrichioides** and **Fusarium poae**, have never been found to produce teleomorphs. This means one of two things. Either we have not yet found the conditions under which these anamorphs produce their teleomorphs, or they no longer produce them. Comparisons with other fusaria that have teleomorphs in the ascomycete genera **Gibberella**, **Nectria** and **Nectriopsis** convince us that all fusaria belong to the Order Hypocreales. So we confidently assume that if teleomorphs of the ATA fungi turned up, they would belong to the Hypocreales. And if they don't turn up, we will still assume that these two anamorphs once had hypocrealean teleomorphs, but lost them along the way.

The other two major genera of toxigenic molds, **Aspergillus** and **Penicillium**, are closely related to each other, less so to **Fusarium**. Although most species of both genera have not been connected with a teleomorph, some species of **Aspergillus** have been connected to teleomorphs in twelve ascomycete genera, including **Eurotium**, **Emericella** and **Neosartorya**. Some **Penicillium** species have been linked to species of four ascomycete genera, including **Eupenicillium** and **Talaromyces** (ref. 3). All these teleomorphs have something in common, they belong to the same family, the Trichocomaceae, of the Order Eurotiales. So there's no question about it: **Aspergillus** and **Penicillium** are cousins.

The next order of business is to compare the Eurotiales with the Hypocreales. An important part of this comparison rests on their sexual fructifications (Fig. 6). In both, the asci are enclosed within a surrounding wall, but there the similarity ends. The ascoma of the Hypocreales is called a perithecium. It has a narrow opening at the top, and its cavity is lined with a palisade of laterally packed asci. Note that these are narrow and tubular. This is because they are spore guns, which take turns at lining up with the narrow opening in the roof of the ascoma and discharging their spores through it into the outside air.

Let's compare this with the ascoma of the Eurotiales, which is called a cleistothecium. Here the outside wall is complete: there is no way out for the spores, at least in the short run. Since there is now no point in shooting the spores, the mechanism has been lost: the asci are not tubular, and they aren't arranged in a hymenial layer. Instead, the asci are spherical, randomly arranged, and their walls break down when the spores are mature. In other words, the asci of the Eurotiales are as different from those of the Hypocreales as they could be and still be considered asci.

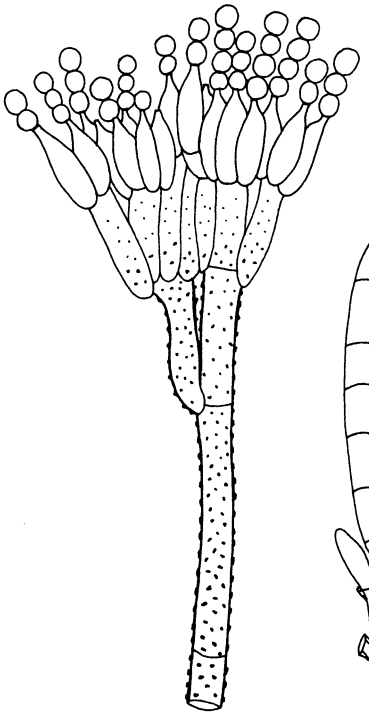

Fig. 5a

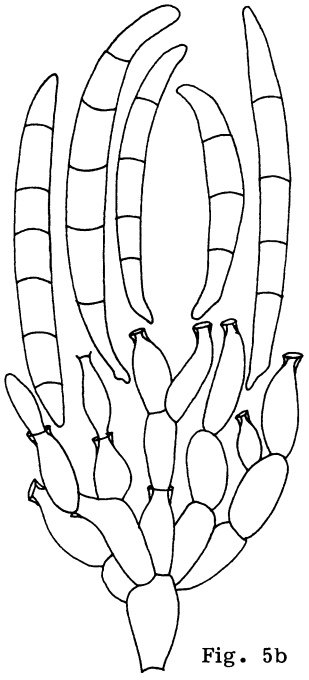

Fig. 5b

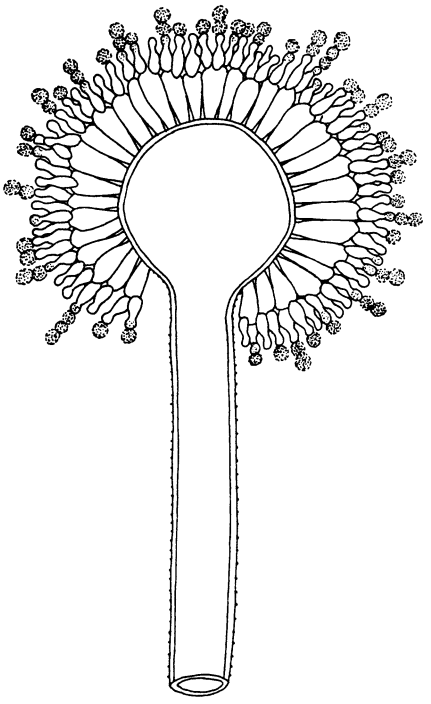

Fig. 5c

Fig. 5. (a) Penicillium; (b) Fusarium; (c) Aspergillus.

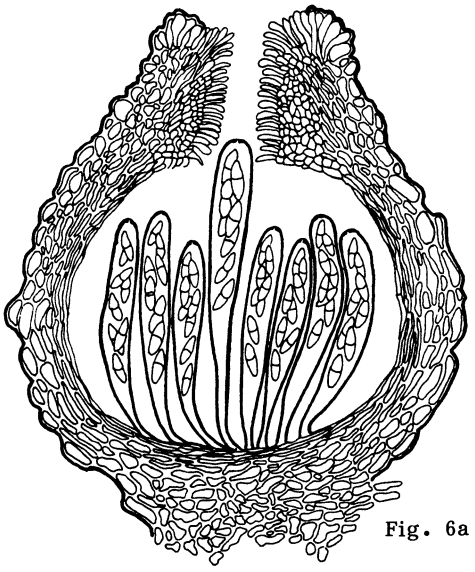

Fig. 6a

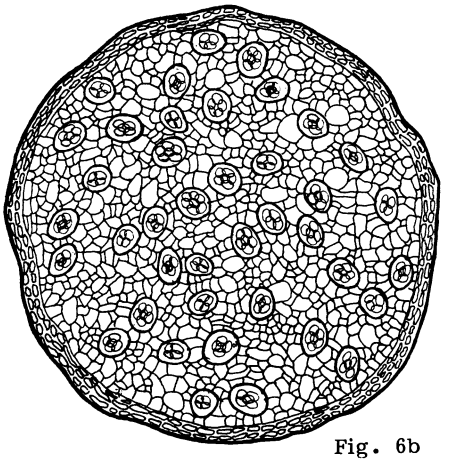

Fig. 6b

Fig. 6. (a) Perithecial ascoma; (b) Cleistothecial ascoma.

Yet are the differences so fundamental that we have to start looking for two different origins for asci, wondering whether asci are actually polyphyletic? Or is there some common ancestry underlying their present differences? We now think that the morphological and functional differences may be rooted in the different ecological niches currently occupied by these fungi. The perithecial ascomata of the Hypocreales develop superficially on wood or other resistant plant parts where shooting their spores will get them into the atmosphere at large, to be carried away, perhaps on epic journeys in the upper air, perhaps to fall to earth quite close by. This spore shooting makes evolutionary sense; it will have selective value.

The cleistothecial ascomata of the Eurotiales, on the other hand, often develop in hidden places: inside the bark of trees, in animal burrows, in the soil. All places where spore shooting would be a waste of time and energy. So evolutionary selection pressure has favoured strains that didn't waste material on such effete pastimes. So perhaps we don't need to postulate different origins for the Eurotiales and the Hypocreales. We may, in fact, be looking at two end points of an evolutionary radiation similar to that which took place in the mammals. There, it led to such functionally different forms as whales and bats which, beneath their very different exteriors, still have basically similar skeletons, perhaps the most obvious shared pattern being that of the pentadactyl limb.

And, in fact, current mycological theory does not consider the Eurotiales primitive, as was once thought, but rather derived from spore-shooting ascomycetes like the Hypocreales or Sphaeriales by secondary simplification. Malloch and Cain (ref.4) and Malloch (ref. 5) suggested that similarities between the anamorphs of the Trichocomaceae and the Hypocreales constitutes evidence that the two groups are related. Certainly **Penicillium** and **Aspergillus** are anything but primitive. One of the secrets of their success is their spore-producing mechanism, the phialide, which they share with the anamorphs of the Hypocreales. The phialide, a specialized single cell which produces a potentially endless succession of conidia from an open end, without itself changing in length or shape, is probably the most sophisticated and efficient way of producing conidia ever evolved (ref. 6).

Although the conidiophores and conidiomata of eurotialean and hypocrealean anamorphs vary widely in their degree of complexity, an issue explored elsewhere by Kendrick and DiCosmo (ref. 3), and Samuels and Rossman (ref. 7), they all have phialides as their conidiogenous cells. Although the phialide has probably evolved more than once, I consider the phialides of these fungi to have a common origin, to belong to the same genetic line. So our main toxigenic molds are related. But does this really matter?

Certainly if what I have said about the ecological importance of mycotoxins is true, it will be one factor contributing to the success of these ascomycetes. But why are mycotoxins sought for, detected, analyzed? That's easy: because people or animals have suffered adverse effects after eating specific foods, feed, or forage crops (refs. 8, 9). What are the principal fungi that occur on these substrates? What about fungi that occur on other substrates that are never eaten by us or our animals? It so happens that a recently published book with the title 'Introduction to Food-borne Fungi' (ref. 10) lists the main fungi found contaminating food. There are just over 100 species. Does it surprise you when I tell you that 8 belong to **Fusarium**, 17 to **Aspergillus**, and 21 to **Penicillium**? **Almost half of the fungal taxa commonly isolated from food belong to our three principal toxigenic genera.**

Now this can be interpreted in one of two ways. Either as an indication that molds that grow on food tend to evolve toxins. Or, and this is the main thrust of my argument, as an indication that we have tended to detect mycotoxins in these fungi because they constantly draw themselves to the attention of food microbiologists. I suggest that if we scrutinized a different subset of molds, species that occur on substrates that are never eaten by us or our animals, we might well find that many of them, too, produced mycotoxins. Of course, it hardly matters to us, personally, or to our domestic animals, if a fungus growing on dead leaves or dead wood produces a mycotoxin. But there may be many ecological implications. Let me explore just one of them.

We know that fungi are important as early colonizers of dead leaves, softening them up, or as we say, 'conditioning' them so that they become palatable and digestible to the small detritivorous animals that are second in line (ref. 11). And we have found that the mycelia of some of these conditioning fungi are very nutritious to the animals (ref. 12). In our early studies of this

conditioning phenomenon, we tended to think of the fungi simply as leaf-processing agents, basically unaffected by the animals. But once it became clear that the animals eagerly sought out the spore-producing structures of these fungi and fed on them preferentially, we saw that the fungi and the animals were not just elements of a succession, but also that the animals preyed upon the fungi during the crucial reproductive phase. We did not know what countermeasures the fungi took, nor indeed if they had any at their disposal. But it now seems more and more likely to me that some at least of those fungi probably produce mycotoxins as a way of discouraging their animal nemeses.

I would like to present evidence, from four sources, in support of my prognostications. First, from studies carried out in my laboratory on leaf colonizing fungi. It is true, as I said earlier, that some of these fungi are excellent food sources for the animals; but it is also true that animals fed certain other species suffer significant mortality. Since the thesis we were trying to establish at the time was that fungi were indeed important intermediaries in energy flow in woodland streams (ref. 13), we did not stress the species which, though readily eaten, generated unpleasant consequences. I now understand that those results may indicate production of mycotoxins, though that hypothesis needs to be tested experimentally.

My second intimation of trouble is gleaned from our studies of molds which attack and kill insects. In my laboratory, we have been experimenting with some molds that have demonstrated the ability to kill spruce budworm larvae. If some of these fungi are injected into the haemocoel of the larvae, they often kill the larvae in 24-48 hours. Examination of the entrails of the dead larvae reveals some fungal hyphae, but often not nearly enough to have caused major physical disruption of the insect's inner workings. It has been demonstrated that these molds produce toxins that hasten the death of the larvae (ref. 14).

My third indicator is that when some of the fungi that exploit tiny soil animals such as nematodes, rotifers and amoebae make contact with their prey, the animal, though capable in other circumstances of vigorous and protracted attempts to escape, submits meekly to its fate, making little or no fuss about being killed (G.L. Barron, pers. comm.). We suspect from this behaviour that the fungi must have some potent, fast-acting toxin, narcotic or anaesthetic at their disposal.

My fourth warning sign is in the form of **Chaetomium cellulolyticum** or **Chaetomium virescens**, isolated from a compost heap, which was found to have high cellulolytic activity, to grow extremely fast at 37 C on cellulosic substrates, and to produce very high yields of apparently edible protein. The fungus was being grown on a pilot plant scale at various places, highly touted as a way of converting cellulosic wastes such as bagasse and corn stover into animal feed (refs. 15, 16). But then it was discovered, rather belatedly, that this wonder fungus produced unacceptable levels of mycotoxins, especially sterigmatocystin, along with the protein.

These four examples suggest to me that mycotoxins will in future be found to be far more widely distributed than has been widely believed. I hope that some of you will be involved in testing that hypothesis. It will be interesting to see which groups of fungi, if any, are completely innocent of any involvement in the great mycotoxin plot. In this context, it is fascinating to note that those moulds domesticated in the Orient as processors of various fermented foods seem to have been selected at least in part, though certainly unconsciously, for their non-production of mycotoxins (ref. 17).

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