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The Effect of Nematode Age on Trap Formation in the Nematophagous Fungus Arthrobotrys Conoides

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THE EFFECT OF NEMATODE AGE ON TRAP FORMATION IN THE NEMATOPHAGOUS FUNGUS *ARTHROBOTRYS CONOIDES*

by

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VITA

Mary Lou Tortorello is the daughter of Joseph Anthony Tortorello and Mary (Fabiano) Tortorello. She was born and raised in Chicago, Illinois where she received her elementary and secondary education.

In 1969, she entered Northern Illinois University in DeKalb, Illinois, majoring in Biological Sciences/Microbiology. She was elected a member of the Phi Sigma Society and participated in a research project sponsored by the National Science Foundation. The Bachelor of Science degree was awarded to her in 1973.

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INTRODUCTION

There exists in nature an unusual relationship between certain species of fungi and small worms belonging to the class Nematoda. This relationship would not appear so unique if described simply as a predator - prey association. In this case, however, the familiar roles of animal as predator and plant as prey have been reversed.

These particular fungi ordinarily exhibit a saprophytic mode of nutrition, deriving their food from decaying matter, since they possess no photosynthetic capabilities. In this respect, they are not unlike many other species of fungi. However, when the tiny nematodes come into their vicinity, they will form traps in their mycelia, enabling them to catch the nematodes as prey. Here, then, is an instance of a predatory plant, and the term "nematophagous fungi" quite appropriately describes these nematode - eating fungi.

Generally, research on this fascinating relationship has been limited to the efforts of a comparatively few enthusiasts. This is undoubtedly due to their being, for the most part, unheard of in conventional courses of biological study.

The subject of this research is an exploration of only one aspect of the nematode - fungus interaction. It is an investigation into the effect that the age of the nematode has on eliciting the response of trap formation in the fungus.

REVIEW OF THE LITERATURE

General Remarks

Nematode - trapping fungi are not a recent discovery. Woronin (1870) first reported that the fungus Arthrobotrys oligospora formed networks of loops in its mycelium. The purpose of these networks was not known at the time. Their function was made clear when the first recorded observation of a fungus actively trapping nematodes was made by Zopf (1888). Since then, over fifty species of fungi that prey on nematodes have been described, primarily through the independent efforts of Dreschler and Duddington. There are probably many more species yet to be described.

The fungi known to capture nematodes do not belong to any one taxonomic class. Using the taxonomic terminology of Alexopoulos (1962), some belong to the Oomycetes, some to the Zygomycetes, at least one is a Basidiomycete and many belong to the Fungi Imperfecti, those fungi for which no sexual stage in the life cycle has been found.

The capture of the nematodes is quite an extraordinary feat. Duddington (1957) has aptly stated,

> It must be remembered that nematodes are, for their size, powerful and enormously active; they move from place to place by means of rapid threshing of their bodies . . . To capture such an animal is no mean task for a fungus that is itself composed of threads so delicate that the finest gossamer would in comparison be as a steel hawser is to a piece of string . . .

The trapped nematodes struggle violently but seldom escape. X-ray diffraction analysis by Winkler, Douglas and Pramer (1960) has shown that the great tensile strength exhibited by the fungal mycelium is due to

chitin being a major component of the cell wall.

The special organs of capture evolved by the fungi vary in design and detail from species to species. In some species, networks of rings or loops are formed by hyphal branching and anastomosis. Drechsler (1937) reported that these ring complexes are coated with a sticky substance of unknown chemical composition, enabling the fungi to capture their prey by adhesion and entanglement. Other fungi produce spherical knobs on short stalks which rise up out of the mycelium. These knobs are also coated with an adhesive. There are a number of fungi that form traps of a constricting ring design. Each ring is composed of three cells. When a nematode tries to crawl through, the cells of the ring suddenly inflate, gripping the nematode so that it cannot escape.

The type of trapping organ produced is not a generic characteristic, i.e., a particular genus may contain species exhibiting all three types of traps. For example, within the genus Dactylaria, D. psychrophila produces the sticky network of rings, D. eudermata produces the sticky knobs and D. brochopaga exhibits the constricting ring trap. Usually, however, the type of trap is a specific characteristic. One notable exception is Dactylaria candida, which produces both sticky knobs and constricting rings.

Whatever the mechanism of capture, the fate of the nematode is the same. The fungal hyphae penetrate its body, absorbing and digesting its content. Balan and Gerber (1972) have suggested that the fast death exhibited by the captured prey is caused by production of ammonia by the fungus. This is a possibility that accounts for the observation that nematodes frequently die before their bodies are penetrated by the fungal

hyphae.

Nematodes are not the only animals to be preyed upon by these fungi. As diverse an assortment of organisms as amoebae, rotifers and an insect, besides nematodes, have been reported to be captured by various species of the fungi. The reader is referred to the excellent reviews of Dreschler (1941, 1944), Duddington (1955, 1956), Pramer (1964, 1965) and Soprunov (1966) for a more detailed description of the various species of predatory fungi and their diversity of prey.

Morphogenetic Stimuli

The fact that the fungi ordinarily do not form the traps, but do so in the presence of nematodes, suggests that some type of stimulus is produced by the nematodes and received by the fungus as a signal to form the traps. Comandon and De Fonbrune (1938) demonstrated that even the water in which the nematodes had been suspended induced trap formation; when boiled, the water was no longer active. Roubaud and Deschiens (1939) found that trap formation was inducible by addition of human blood serum. Later, Deschiens and Lamy (1942) observed that serum from a wide variety of animals was also active. Lawton (1967) separated horse serum by electrophoresis and showed that the fungi will produce rings in response to beta and gamma globulin fractions. The albumins had no stimulatory effect. Bartnicki-Garcia, Eren and Pramer (1964) have demonstrated that trap formation in one fungus is dependent on the presence of carbon dioxide.

Pramer and Stoll (1959), observing the trap - inducing abilities of worm - free broth culture filtrates in which nematodes had developed, were the first to propose "nemin" as the name for this chemical messenger. Investigations into the chemical nature and isolation of nemin

were aided by the description of a nemin assay procedure by Winkler, Kuyama and Pramer (1961). In this procedure, nemin was measured by the reciprocal of the highest dilution of the preparation being assayed that induced traps per milligram of the preparation. Kuyama and Pramer (1962) extracted a substance obtained from nematode homogenates which caused trap formation. Through electrophoresis and ion exchange chromatography, they purified it and determined it to be protein in nature. The active principle was not dissolvable in a variety of organic solvents, and it was not dialyzable. Pramer and Kuyama (1963) suggested that nemin was a complex substance of high molecular weight rather than a simple molecule. Rudek (1972) found similarities in inorganic chemical composition of various animal sera and a nematode water filtrate. He also demonstrated that peptone in the medium promoted spontaneous trap formation. Nordbring-Hertz (1973), however, provided evidence that simple peptides obtained from casitone were able to induce trap formation.

Feder, Everard and Duddington (1960) proved that trap formation could be stimulated by a single, dried nematode. This indicated that nemin was present in the body of the nematode and was possibly released in very small amounts. They suggested that nemin may be a general product of metabolism or may be actively released by contact between the nematode and the fungus. Trap formation was shown to be completely repressed by the RNA synthesis inhibitor, 6-methyl purine, in the report by Galsky, Monoson and Williams (1973). They postulated that the action of nemin may reside at the level of transcription. The chemical nature of nemin, be it a single molecule or perhaps a complex of a

variety of molecules, remains to be determined.

It is evident from the work of Monoson and Ranieri (1972) that the nematode - trapping fungi also produce a substance that attracts the nematodes. The investigations of Monoson, et al. (1973) have indicated that the nematode attraction substance (which they have named "NAS") was concentration dependent, inactivated by treatment with acid or base and was not a common plant growth regulator (gibberellic acid, indole-3-acetic acid, kinetin) or cyclic AMP.

Economic Aspects

There has been some speculation that the nematode - trapping fungi may be used as biological control agents for nematodes that are pathogenic in plants (Soprunov, 1966). Experimentation in this area has involved addition of organic matter to soil, leading to an increase in numbers of free - living nematodes and their fungal predators and a subsequent decrease in numbers of parasitic nematodes (Linford, 1937; Linford, Yap and Oliveira, 1938); addition of nematode - trapping fungi to the soil (Linford and Yap, 1939; Deschiens, Lamy and Vautrin, 1943; Duddington, Jones and Williams, 1956; Mankau, 1961); or addition of both organic matter and fungi to the soil (Duddington, Jones and Moriarity, 1956). Iffland and Van Burgh Allison (1964) demonstrated that although trap formation was induced by a plant pathogenic nematode, the roots of the parasitized plant did not induce their formation.

Some investigations into the control of nematodes parasitic in animals by the predaceous fungi have also been done. Descazeaux and Capelle (1939) fed cultures of fungi to horses and guinea pigs having nematode infections, but the fungal spores did not survive the passage

through the gastrointestinal tract. Roubaud and Deschiens (1941) described an interesting experiment on the control of Strongyloides and Bunostomum parasites of sheep. Two enclosures were infected with the nematodes; one was treated with spores of several predaceous fungi, and the other was left untreated. Two healthy lambs were kept in each enclosure. It was found that the lambs from the fungus - treated enclosure showed very little Bunostomum and no trace of Strongyloides in their dung. The lambs from the untreated enclosure showed dung infested with both nematodes.

The results of all these biological control experiments have been variable, with control being effected in some cases, in others, little or none. When the complexity of ecological considerations that are involved in biological control methods is imagined, it must be concluded that much information concerning the physiologies and interactions of nematodes and fungi, along with other soil organisms, needs to be gathered before this speculation is realized.

Ecological Considerations

Studies on the nutritional requirements of several nematophagous fungi have been reported. Cooke (1962) showed that although nematodes are necessary to initially stimulate trap formation, the fungi cannot remain actively predaceous without an organic energy source. Olthof and Estey (1966) confirmed this by demonstrating that low levels of carbon and nitrogen in media resulted in less predaceous activity of the fungus. Since there was little difference in the amount of mycelial growth, these carbon and nitrogen levels were assumed to have a direct effect on trapping activity. It was suggested that there is a critical

point in nutritional levels which determines whether the fungus will assume a saprophytic or predatory mode.

Satchuthananthavale and Cooke (1967) tested the vitamin requirements of several species of nematophagous fungi. All tested required a thiamine supplement, and all except one required biotin.

Utilization of nitrogen sources by several predatory fungi was analyzed by Satchuthananthavale and Cooke (1967a). They found differences in ability to use nitrite between fungi forming networks of adhesive loops and those forming traps of the constricting ring design, the former being able to utilize nitrite, the latter being unable. It was postulated that the fungi forming constricting rings are more advanced toward an obligatory predaceous mode of life with a reduction in their competitive saprophytic ability. Possibly most of their nitrogen nutrition must be obtained from their nematode prey. On the other hand, the fungi forming networks of adhesive loops may be good competitive saprophytes, being able to utilize inorganic nitrogen compounds and may not depend so much on the predaceous habit.

Dobbs and Hinson (1953) demonstrated that spores of many fungi when placed in natural soils are unable to germinate under conditions normally considered to be favorable to germination. Cooke and Satchuthananthavale (1968) studied germination abilities of several species of nematophagous fungi. There were no general differences between fungi with the same type of trap or with different trapping mechanisms. They concluded that attempts to establish the fungi for purposes of biological control were unlikely to meet with success because of the many variables and inconsistencies encountered in the results of these ex-

periments.

Several studies have been done to elucidate what effect differences in environmental factors might have on trap formation. Feder (1963) found a decrease in nematode capturing efficiency of several fungi as temperature became more favorable for growth. Monoson (1971) reported that trapping efficiency increased as temperature decreased. However, Gorlenko, Kondakova and Pletneva (1959) have shown that the efficiency of some nematode - trapping fungi is not significantly affected by temperature, as long as it is within the limits of normal growth. Couch (1937) suggested that the pH of the medium played a part in trap formation. Rudek (1972), however, found that pH had no effect on trap formation; rather it had a more pronounced effect on mycelial growth. Olthof and Estey (1965) showed that different environmental factors do have an effect on mycelial growth of several species of the fungi. However, no consistent similarity in environmental requirements was found among fungi belonging to the same genus or between those possessing the same type of trapping organ.

Feder, Everard and Wootton (1963) analyzed the sensitivities of several different species of fungi in forming traps in response to a substance derived from nematodes. They concluded that each fungus has a different reactive threshhold to nemin. Lawton (1957) demonstrated that trap formation was not due to starvation of the fungus. Recent evidence by Balan and Lechevalier (1972), however, indicated that traps were induced by adverse conditions of culture resulting in lack of nutrients and/or water.

Purpose

Nowhere in the literature is there found an analysis of the

effect of nematode age on trap formation in the fungus. Pramer and Stoll (1959) provided some evidence that nemin was produced as the nematode population reached its maximum level and when death and disintegration of the nematodes had begun.

Bird (1971) has associated age in nematodes with molting, which occurs normally in four stages before the adult stage is reached. The first larval stage (L_1) takes place within the body of the adult female. The second larval stage (L_2) is the first free - living stage. The nematode then goes through two more molts (L_3, L_4) before being classified as an adult (L_5).

Chow and Pasternak (1969) have determined that various enzymes in the nematode become more evident at different stages in the life cycle. They concluded that a precise regulatory mechanism exists that controls the sequential appearance of the enzymes.

It became apparent to this researcher that nemin, like various enzymes, may also be produced at a specific stage in the life cycle. This was noted especially in light of the work of Pramer and Stoll (1959) who suggested that nemin was produced as the nematode culture grew older. Their analysis, however, was done on various ages of the entire culture of the nematodes. At all times, the culture used would have contained all of the nematode larval stages in different proportions as it grew older.

The purpose of this research is the determination of the stage in the nematode life cycle in which nemin is elaborated. By first achieving a separation of the nematode larval stages, their individual effects on trap formation in the fungus can be analyzed.

MATERIALS AND METHODS

Culture of Arthrobotrys conoides

The nematophagous fungus used in this research was Arthrobotrys conoides[1]. This fungus produces simple, unbranched conidiophores, 150 - 400 u high, bearing whorls of conidia (Figure 1). It captures nematodes by means of complexes of loops that are coated with an adhesive (Figure 2). Since a sexual stage in its life cycle is not known to exist, it is classified in the Fungi Imperfecti.

Stock cultures of the fungus were maintained at 25°C on Difco Corn Meal Agar in 15 x 100 mm plastic Petri plates. Mycelial growth was abundant and relatively rapid on this medium.

Experimental plates of the fungus were obtained by aseptically transferring approximately 1 cm^3 blocks of the stock fungus culture to the center of corn meal agar plates. These were then incubated at 25°C for ten days, at which time the fungus had grown over the entire plate. It had been determined in the earlier stages of experimentation that the fungus would not form traps unless the mycelium had completely covered the agar plate. This fact is supported by the report of Cooke and Pramer (1968), who observed the same condition.

Culture of Panagrellus silusiae

The free - living nematode selected for use in this research was

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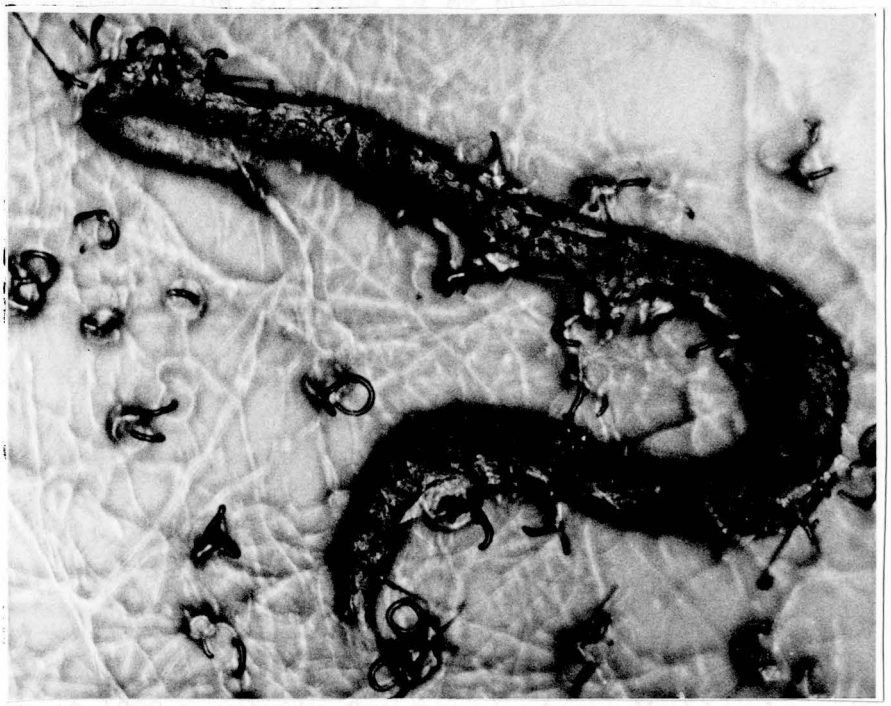
[1]ATCC #15594, obtained from the American Type Culture Collection in Rockville, Maryland.

Magnification, 440 x

Figure 1. Characteristic Conidia of

Arthrobotrys conoides



Magnification, 100 x

Figure 2. Nematode Entangled in Characteristic Trap Complexes of Arthrobotrys conoides

Panagrellus silusiae[1]. The culture method is a modification of that described by Tarjan (1960). Stock cultures of the nematode were maintained at 20 - 25°C on the commercially available baby food preparation, Gerber Mixed Cereal, in glass culture dishes 10 cm in diameter and 5 cm in depth. Panagrellus silusiae is by no means a fastidious organism; however, the culture was most vigorous if the weight/volume proportion of cereal to water was approximately 1:5.

After four to five days of incubation at 20 - 25°C, the entire culture would be swarming with the nematodes, and the cereal would be hydrolyzed. The worms would climb up the sides of the culture dish, and they would be scraped off with a rubber spatula for the experimentation.

Separation of Nematode Larval Stages

After the nematodes were harvested from the culture dishes, they were washed four times in distilled water in a Damon HN-S table top centrifuge at 1000 x gravity for two minutes. Washing by centrifugation removed most of the organic matter still present from the cereal culture. The final washed nematode suspension was then poured onto a column of glass Microbeads[2], approximately 15 - 20 cm in height. The separation procedure is a modification of the method reported by Samoiloff and Pasternak (1969).

Theoretically, the beads when packed together form openings between each other tiny enough so that only the youngest and smallest free-

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[1]Obtained from the laboratory collection of Dr. B. J. Jaskoski, Loyola University, Chicago, Illinois.

[2]Obtained from the Ferro Corporation, Cleveland, Ohio.

living larval stage (L_2) will be able to pass through the column. In reality, this is not entirely true. Initially, only the L_2 nematodes will pass through. However, after about 20 minutes, larger worms will also be able to make their way through the beads. It was necessary, therefore, to make the collections of L_2 nematodes as quickly as possible and also to microscopically examine each fraction collected to be certain that no larger worms had passed through.

Because of this limitation, it was advantageous to set up several columns, so that once the older stages started coming through, a new column could be employed. Separatory funnels, or any type of column equipped with a stopcock may be used. However, ordinary titration burettes were employed as columns in this study, so that a maximum height might be achieved using a minimum amount of beads. This was necessary because the amount of beads available was not unlimited. Also a greater number of columns could be set up using less beads.

After each use, the beads were washed by vigorous agitation in concentrated HCl and rinsed thoroughly with water. This was repeated until the washes ran clear, usually four to five times. The beads were then dried in a glass beaker over a low bunsen burner flame to facilitate pouring into the columns for use later. It was imperative that the beads were washed after each use; otherwise, organic debris from the worm suspensions would accumulate and dry on them. This would in effect make the beads and the spaces between them larger, resulting in the older and larger stages of the nematode easily passing through.

Larval stages of the nematode were identified according to average lengths of the worm as reported by Gysels and van der Haegen

(1962). The L_2 nematodes measured on the average 350 u; L_3, 500 u; L_4, 850 u; and L_5, 1600 u. An optical micrometer, set in the ocular of a microscope and calibrated to a stage micrometer, facilitated these measurements.

L_2 nematodes were collected in fractions of 0.5 ml from the column. Each fraction collected was examined microscopically to be certain that a pure L_2 population resulted.

The later larval stages (L_3, L_4 and L_5) were obtained by allowing L_2 stage collections to molt at 25°C on Difco Corn Meal Agar plates for the appropriate period of time. L_3 nematodes were obtained after approximately 24 hours, L_4 nematodes were obtained after approximately 48 hours, and L_5 nematodes after approximately 72 hours. Molting to the successive larval stages was almost entirely synchronous for all individuals. Any inconsistent individuals that were observed were removed manually with a Pasteur pipette before performing the bioassay.

The most difficult stage to collect by this successive molting technique was, surprisingly, the L_5 or adult nematodes. By the time this stage was reached, there would invariably be some gravid female worm present who would contaminate the pure adult population with a brand new batch of L_2 stage worms. For the most part, then, the L_5 stage was collected by manually selecting adult worms from relatively old stock cereal cultures.

Often the collections of L_2 nematodes from the columns were very scant to begin with. The main factor that seemed to determine abundance of L_2 nematodes collected was the age of the stock nematode culture. It seemed that if nematodes were harvested from cultures more than ten days

old, very few or no L_2 nematodes were collected. This observation is supported by the report of Elmiligy and Norton (1973), who reported that the accumulation of organic acids in the culture was inhibitory to nematode survival or reproduction. For this reason the small culture dishes described earlier were employed rather than very large containers enabling mass culture of the nematode. Also, cultures ranging from five to eight days old (as soon as the worms crawled up the sides of the dish) were generally employed for a maximum harvest of L_2 nematodes.

It also seemed that if the height of the column of beads was more than 20 cm, very few or no L_2 nematodes were collected. If the column was less than 10 cm in height, a mixture of the stages was collected.

Another variable that might or might not have determined the bounty of the L_2 harvest was the quality of the original worm inoculum into the cereal culture. Generally it seemed that if a stock culture was very old, inocula taken from it and placed into new culture would hydrolyze the cereal, but the worms would not climb up the sides of the dish. Therefore, no worms could be harvested.

On the other hand, sometimes when the conditions of culture age, type of culture dish, height of the column and care in washing the beads were adhered to, either a mixture of stages or no nematodes at all resulted in the collection. Again, there were times when the above conditions were not all met, and a pure population of L_2 nematodes was collected. The collection of L_2 nematodes often seemed at best not easily regulable and at worst somewhat fortuitous. Indeed this is an area where considerable research could be directed in the future.

However, if at any time not enough L_2 nematodes were harvested,

those that were collected could be concentrated by centrifugation, placed on corn meal agar plates and refrigerated. This prevented molting to the successive stage until more L_2 nematodes were collected. The worms remained dormant in the refrigerator without molting for at least one week. When brought back to room temperature, they resumed normal activity within a few minutes, retaining their ability to molt and also to elicit trap formation in the fungus.

When enough individuals were finally collected, a few milliliters of water were poured onto the agar plate. The live nematodes immediately moved into the liquid phase. This suspension was then poured off and, since it was usually contaminated with debris from the molting process and with bacteria, was washed with distilled water by centrifugation, as described above.

Design of the Bioassay

The analysis of trapping ring formation for each nematode larval stage was done on ten day old cultures of the fungus. A number 13 cork borer was used to punch out discs in the fungus cultures approximately equidistant from the center of the plate. This ensured that the analysis would be done on mycelia of approximately the same age.

A 0.1 ml aliquot of the appropriate live nematode larval suspension was placed on each disc. The plates were then incubated at 25°C.

Two main problems were encountered in using live nematode suspensions in the bioassay. Although the suspensions were shaken each time before an aliquot was removed, the worms would precipitate out, resulting in a different number of individuals being inoculated. To reduce this error, aliquots were removed and worms were inoculated as

quickly as possible after shaking the suspension. The second main problem encountered was that of the worms crawling off of the disc before the fungus could respond. To help alleviate this problem, the areas of agar around each disc were removed. In this way, the worm suspension would not run off of the disc onto an outside area. Rather, the surface tension between agar and water kept the inoculum on the disc. Also, the more concentrated the nematode suspension was, the greater chance there was that more worms would remain on the discs to elicit trapping ring formation. However, variations in results appeared, mainly due to these two factors. For this reason, twenty experimental replicates were done for each stage.

Control discs on each fungal plate were also made. The inoculum for control discs was a 0.1 ml aliquot of the nematode suspension, devoid of any nematodes. This provided a determination of any "background" trap formation, due to factors other than the particular nematode larval stage being observed.

After an incubation time of 24 hours, each disc was examined microscopically. The number of fungal trap complexes produced on each disc was counted in five sweeps of the microscopic field. The traps were not at all difficult to recognize. They were differentiated from the rest of the mycelium in that they formed ring complexes which rose up three - dimensionally out of the substratum (Figure 2).

In order to quantitatively ascertain the amount of nematode material being inoculated, some of the nematode suspension for each larval stage was reserved for protein determination by the Lowry method (Lowry, et al., 1951). This determination of total protein provided a

comparative basis of analysis for all the stages. The Lowry method is preferable to other quantitative methods, e.g., dry weight or Biuret protein determination, in that it is more accurate and many times more sensitive. This was important because of the minute amount of nematode material being used.

The first step in the protein determination procedure was the homogenization of the reserved worm suspension in a Waring blender. Often the nematodes would appear to be lifeless after treatment in the blender, only to resume their vigorous activity within a few minutes of their removal. It was determined that they should be kept in the blender for at least 15 minutes. This surprisingly long period of time was necessary for the complete rupture of the remarkably hardy nematodes.

One ml of the nematode homogenate and 1 ml of Reagent I (see Appendix A) were put into a test tube and left at room temperature for exactly ten minutes. Then 3 ml of Reagent II (see Appendix A) were immediately added, mixed vigorously and incubated at 50°C for ten minutes. After cooling to room temperature, the percent transmittance of the mixture was read at 540 mu on a Coleman Junior II Spectrophotometer.

Percent transmittance was converted to optical density by the formula:

$$\text{optical density} = \log \frac{100}{\% \text{ T}}$$

Optical density was interpreted as milligrams of protein by reading directly from a bovine serum albumin standard curve. The standard curve had previously been done by the same Lowry procedure.

The complete data for each larval stage were then recorded as number of fungal traps produced per milligram of nematode protein added.

RESULTS

Counts of trap complexes of the twenty experimental replicates for each nematode larval stage were averaged. These mean counts are given in Table I.

Table I. Mean Number of Trap Complexes Produced for Each Larval Stage

Larval Stage	Mean Number of Traps Produced
L_2	18.3
L_3	321.1
L_4	505.1
L_5	334.1

The data were obtained in five sweeps of the microscope field for each of twenty experimental replicates of each stage and represent the results of 0.1 ml inocula of nematode suspension. At no time were traps produced on any of the control discs, i.e., the results tabulated here are due only to the presence of the nematodes added, not to any "background" trap effectors.

Protein determinations were read as percent transmittances from a spectrophotometer and converted to optical densities. Optical densities for each stage were compared to a bovine serum albumin standard curve. The standard curve is given in Appendix B. Protein determinations obtained for each larval stage from the standard curve are illus-

trated in Figure 3 and tabulated in Table II.

Table II. Protein Determination for Each Larval Stage

Larval Stage	Percent Transmittance	Optical Density	Protein Concentration (mg/ml)
L_2	61.0	0.2147	0.121
L_3	80.2	0.0958	0.054
L_4	74.9	0.1255	0.071
L_5	83.2	0.0799	0.045

The combined data of fungal traps produced per milligram of nematode protein are given in Table III. Since the original inoculum on each disc was 0.1 ml of nematode suspension, but protein determination is given in milligrams per milliliter, the Mean Number of Traps Produced that are tabulated in Table III have been corrected by increasing original counts ten-fold.

Table III. Trap Complexes Produced per Milligram of Nematode Protein Added

Larval Stage	Mean Number of Traps Produced	Nematode Protein Added (mg/ml)	Traps Produced per Mg Protein
L_2	183	0.121	1,512
L_3	3211	0.054	59,463
L_4	5051	0.071	71,134
L_5	3341	0.045	74,244

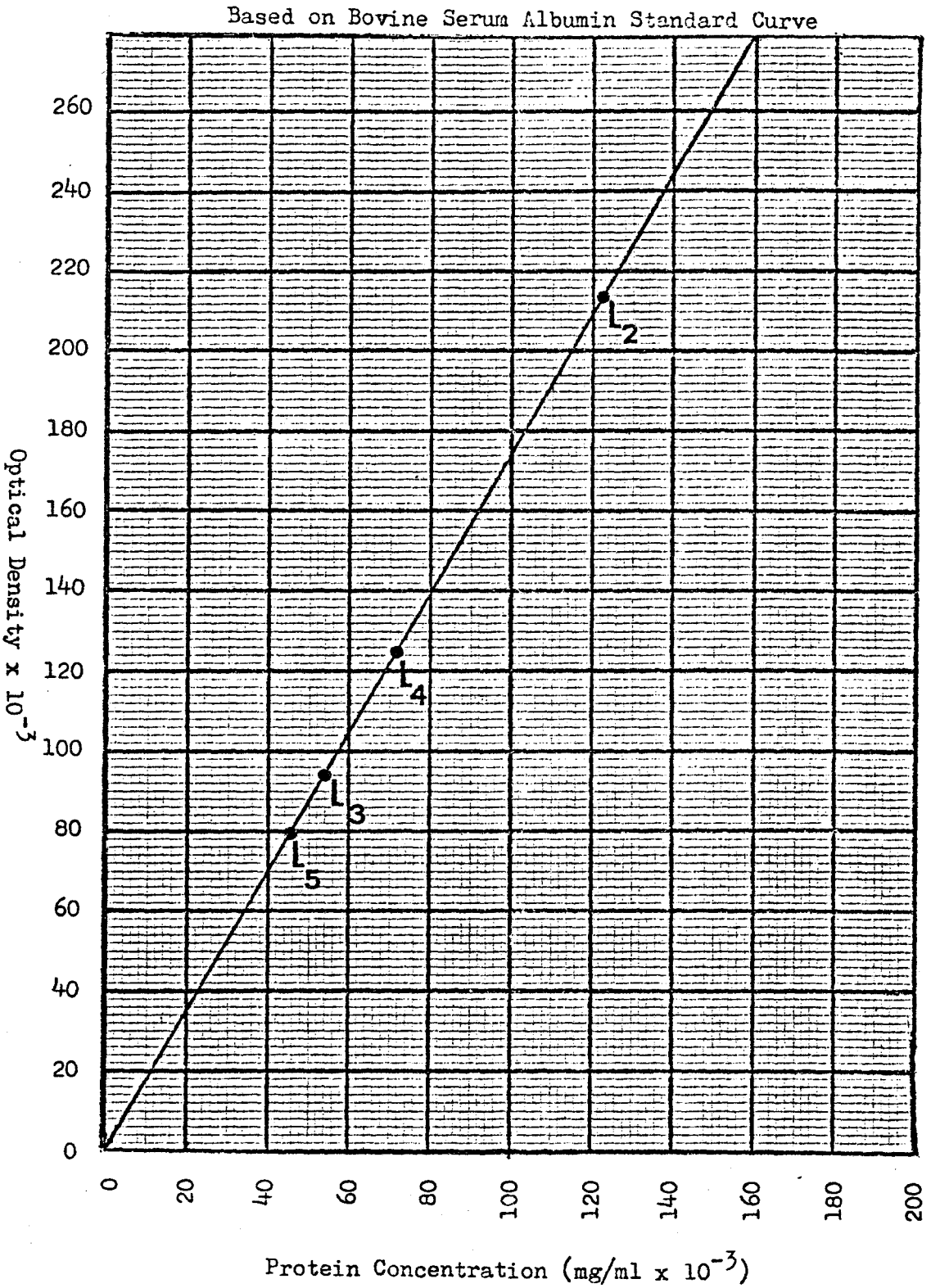

Figure 3. Total Protein Determination for Each Larval Stage

The same data given in Table III are illustrated graphically in Figure 4, which also shows the 95% confidence limits for the mean of each larval stage. The computation of confidence limits was done according to the procedure outlined by Sokal and Rohlf (1969).

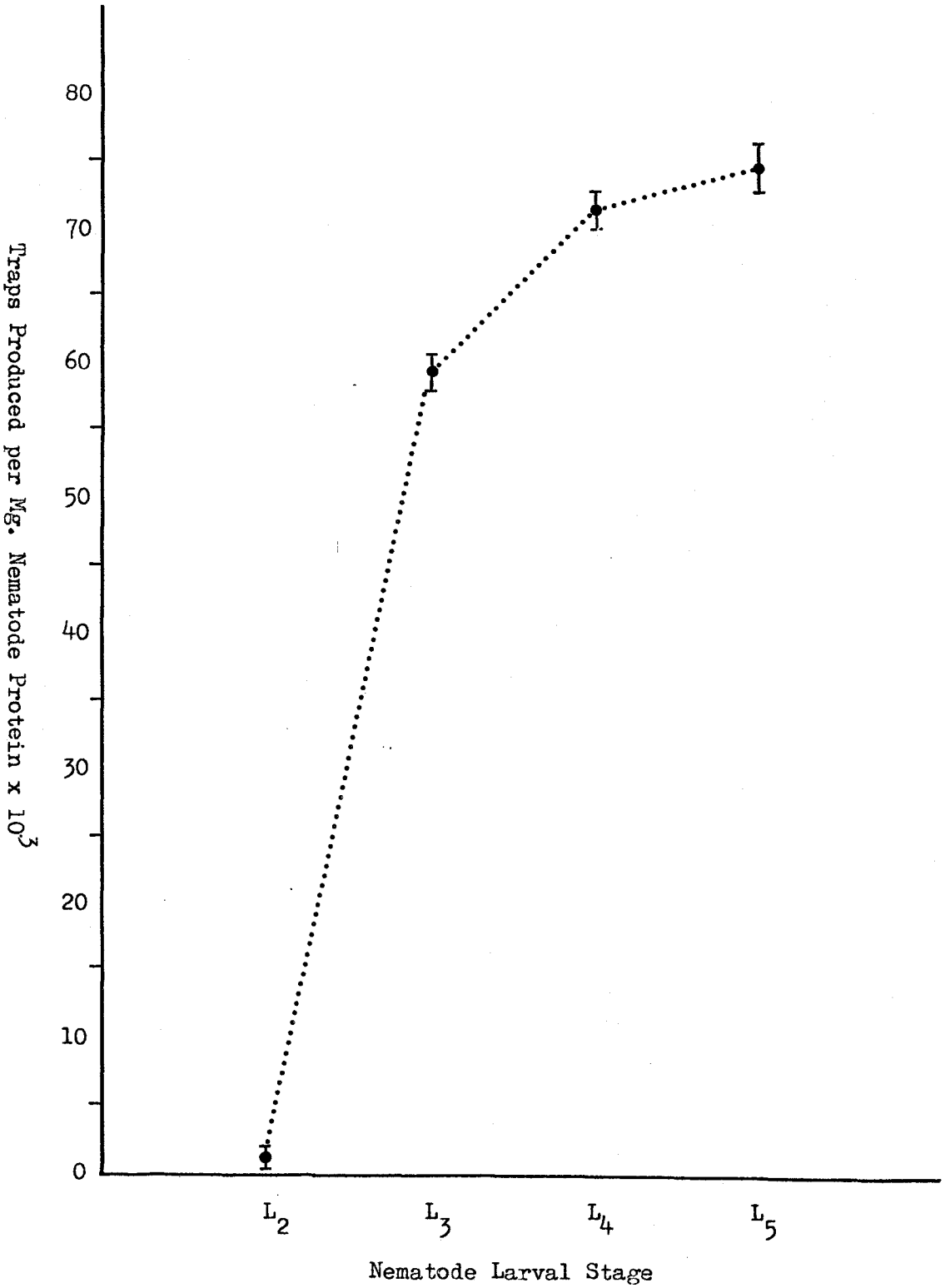

Figure 4. Fungal Traps Produced per Milligram of Nematode Protein for Each Larval Stage

DISCUSSION

Production of nemin occurs in all stages of the nematode. This is readily evident since there was a measurable response by the fungus to each nematode larval stage.

Furthermore, the data indicate that nemin production increases as the nematode matures. The question that must be asked now is if this increase is significant, or if the differences between the larval stages are due only to chance variation. This question can only be answered through a statistical analysis of variance. The completed analysis computations are contained in Appendix C. The procedure followed was that described by Sokal and Rohlf (1969) for a single classification analysis of variance with equal sample sizes.

The preliminary overall analysis of variance shows there to be a significant difference in nemin production between the different larval stages within 95% confidence limits. This statement, however, is not a sufficient analysis of the results. It is necessary to analyze the data in greater detail, showing which means are significantly different from each other; for example, is there a significant increase in nemin production between L_2 and L_3 nematodes, between L_3 and L_4, and so on.

This comparison among stages is also contained in Appendix C. Initial considerations determined that there were six comparisons to be made: $L_2 - L_3$, $L_2 - L_4$, $L_2 - L_5$, $L_3 - L_4$, $L_3 - L_5$ and $L_4 - L_5$. All tests of significance were analyzed within 95% confidence limits.

The first comparison showed that there is a significant increase

in nemin production by the L_3 stage from the L_2 stage ($L_2 - L_3$). Since this was proven, and since the L_4 and L_5 stages show even greater nemin production than the L_3 stage, the next two comparisons ($L_2 - L_4$ and $L_2 - L_5$) were eliminated. It was unnecessary to go through the computations to prove that their differences would also be significant.

The next comparisons are not so evident. The statistics show that nemin production is not significantly increased between the L_3 and L_4 stages, nor is there a significant increase between L_4 and L_5 stages. There is, however, a significant increase from the L_3 stage to the L_5 stage.

The statistics of these last comparisons may be misleading. There is an increase in nemin production between L_3 and L_4 stages, but not enough for it to be designated as significantly increased. Similarly, there is an increase between L_4 and L_5 stages, but not enough to be considered significant. The increases, however, finally become significant when the L_3 and L_5 stages are compared. Considered on the whole, it is probably not erroneous to state that nemin production increases with age of the nematode.

Pramer and Stoll (1959) reported that nemin is produced when the nematode culture reached a maximum level and when death of the nematodes commenced. They were unable to detect any production of nemin by young cultures of nematodes. In fact, in the early stages of experimentation, this researcher could not detect any response from the fungus upon inoculation of L_2 stage worms, either. It initially seemed that Pramer and Stoll (1959) were correct in relating nemin production only with older worms. It was only after a more concentrated suspension of L_2 stage

worms was inoculated that any trap formation was noted. Nemin must be excreted in minute amounts by younger worms, and this is probably why Pramer and Stoll (1959) could not detect it in their young cultures.

Nemin is some type of metabolic substance present in all stages, but which increases with age. It may be compared to the acid phosphatases that have been associated with the aging process. Chow and Pasternak (1969) found acid phosphatases to be present in all stages of the nematode; however, Epstein, Himmelhoch and Gershon (1972) showed that acid phosphatase pigment granules increasingly accumulate in the intestinal epithelium of nematodes as they grow older.

Given the scant amount of knowledge regarding the properties and chemical structure of nemin, one might speculate that nemin is some type of general metabolic intermediary or enzyme or hormone. It has protein - like properties. It has morphogenetic capabilities. In one case, it has been shown to be highly antigenic (Pramer, 1964). And its production increases with maturity.

Surely this is an area that can be further researched. Even when the huge amount of research that has been done on aging is considered, it still must be concluded that relatively little is known about the process. Perhaps further investigations into the nature of nemin will help shed some light on the subject.

SUMMARY

Nemin, the term referring to the substance or substances responsible for eliciting the trap formation response in nematophagous fungi, was thought to be produced by nematodes as the population grew older and when death and disintegration of the nematodes had begun.

It has been shown in this research, by separating the nematode larval stages and analyzing their individual effects on the fungus, that nemin is produced by all stages of the nematode. It is also evident that nemin production increases as the nematodes mature.

REFERENCES CITED

Alexopoulos, Constantine John. 1962. Introductory Mycology. John Wiley and Sons, Inc. New York.

Balan, J. and Nancy N. Gerber. 1972. Attraction and killing of the nematode Panagrellus redivivus by the predaceous fungus Arthrobotrys dactyloides. Nematologica 18: 163-173.

Balan, J. and Hubert A. Lechevalier. 1972. The predaceous fungus Arthrobotrys dactyloides: Induction of trap formation. Mycologiya 64: 919-922.

Bartnicki-Garcia, S., Jacob Eren and David Pramer. 1964. Carbon dioxide dependent morphogenesis in Arthrobotrys conoides. Nature 204: 804.

Bird, Alan F. 1971. The Structure of Nematodes. Academic Press, Inc. New York.

Chow, H.H. and J. Pasternak. 1969. Protein changes during maturation of the free-living nematode, Panagrellus silusiae. J. Exp. Zool. 170: 77-84.

Comandon, J. and P. De Fonbrune. 1938. Recherches experimentales sur les champignons predateurs du sol. Comp. Rend. Soc. Biol., Paris 129: 619-625.

Cooke, Roderic C. 1962. The ecology of nematode-trapping fungi in the soil. Ann. Appl. Biol. 50: 507-513.

Cooke, Roderic C. and David Pramer. 1968. Interactions of Aphelencus avenae and some nematode-trapping fungi in dual culture. Phytopathology 58: 659-661.

Cooke, R.C. and V. Satchuthananthavale. 1968. Sensitivity to mycostasis of nematode-trapping Hyphomycetes. Trans. Br. Mycol. Soc. 51: 555-561.

Couch, J.N. 1937. The formation and operation of the traps in the nematode catching fungus Dactylella bembicoides Dreschler. J. Elisha Mitchell Scient. Soc. 53: 301-309.

Descazeaux, J. and L. Capelle. 1939. Contributions a l'etude des Champignons predateurs de larves de Nematodes parasites des animaux domestiques. Bull. Acad. Vet. France 12: 284-288.

Deschiens, R. and L. Lamy. 1942. Sur les facteurs determinant l'apparition des pieges chez les Hyphomycetes predateurs de Nematodes. Comp. Rend. Acad. Sci., Paris 215: 450-452.

Deschiens, R.L., L. Lamy and E. Vautrin. 1943. Essais de practiques de prophylaxie de l'anguillulose des vegetaux par l'emploi d'Hyphomycetes predateurs. C.R. Acad. Sci., Paris, 216: 534.

Dobbs, C.G. and W.H. Hinson. 1953. A widespread fungistasis in soil. Nature, Lond. 172: 197-199.

Dreschler, Charles. 1937. Some Hyphomycetes that prey on free-living terricolous nematodes. Mycologiya 29: 447-552.

Dreschler, Charles. 1941. Predaceous fungi. Biol. Rev. 16: 265-290.

Dreschler, Charles. 1944. A species of Arthrobotrys that captures springtails. Mycologiya 36: 382-399.

Duddington, C.L. 1955. Fungi that attack microscopic animals. Bot. Rev. 21: 377-439.

Duddington, C.L. 1956. The predaceous fungi: Zoopagales and Moniliales. Biol. Rev. 31: 152-193.

Duddington, C.L. 1957. The Friendly Fungi. Faber and Faber. London.

Duddington, C.L., F.G.W. Jones and F. Moriarty. 1956. The effect of predaceous fungus and organic matter upon the soil population of beet eelworm Heterodera schactii Schm. Nematologica 1: 345.

Duddington, C.L., F.G.W. Jones and T.D. Williams. 1956. An experiment on the effect of a predaceous fungus upon the soil population of potato root eelworm Heterodera rostochiensis Woll. Nematologica 1: 341.

Elmiligy, Ismail A. and D.C. Norton. 1973. Survival and reproduction of some nematodes as affected by muck and organic acids. J. Nematol. 5: 50-54.

Epstein, J., S. Himmelhoch and D. Gershon. 1972. Studies on aging in nematodes: III. Electron microscopical studies on age-associated cellular damage. Mech. Ageing Dev. 1: 245-255.

Feder, William A. 1963. A comparison of nematode-capturing efficiencies of five Dactylella species at four temperatures. Mycopath. Mycol. Appl. 19: 99-104.

Feder, William A., C.O.R. Everard and C.L. Duddington. 1960. Heterocaryotic nature of ring formation in the predaceous fungus Dactylella doedycoides. Science 131: 922-942.

Feder, William A., C.O.R. Everard and L.M.O. Wootton. 1963. Sensitivity of several species of the nematophagous fungus Dactylella to a morphogenic substance derived from free-living nematodes. Nematologica 9: 49-54.

Galsky, Alan G., Herbert L. Monoson and Shirley A. Williams. 1973. Inhibition of nemin-induced trap formation in a predaceous fungus by 6-methyl purine. Life Sciences 12: 219-224.

Gorlenko, M.V., E.I. Kondakova and O.I. Pletneva. 1959. On the biology of predaceous fungi. Bull. Moscow Assoc. Exptl. Biol. 64: 89-97.

Gysels, H. and W. vander Haegen. 1962. Post embryonale ontwikkeling en vervellingen van de vrijlevende Nematode Panagrellus silusiae (de Man 1913) Goodey 1945. Natuurvetensch. Tijdschr. 44: 3-20.

Iffland, Donald W. and Patricia Van Burgh Allison. 1964. Nematode trapping fungi: evaluation of axenic healthy and galled roots as trap inducers. Science 146: 547-548.

Kuyama, Shimpei and David Pramer. 1962. Purification and properties of a protein having nemin activity. Biochim. Biophys. Acta 56: 631-632.

Lawton, June R. 1957. The formation of constricting rings in nematode catching Hyphomycetes grown in pure culture. J. Exp. Bot. 8: 50-54.

Lawton, June R. 1967. The formation and closure of the constricting rings in two nematode catching Hyphomycetes. Trans. Br. Mycol. Soc. 50: 195-205.

Linford, M.B. 1937. Stimulated activity of natural enemies of nematodes. Science 85: 123-124.

Linford, M.B. and F. Yap. 1939. Root-knot injury restricted by a nematode trapping fungus. Phytopathology 28: 14-15.

Linford, M.B., F. Yap and J.M. Oliveira. 1938. Reduction of soil populations of the root-knot nematode during the decomposition or organic matter. Soil Sci. 45: 127.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mankau, R. 1961. The use of nematophagous fungi to control root-knot nematodes. Phytopathology 50: 645.

Monoson, H.L. 1971. Growth relationships between Aphelenchus avenae and two species of nematophagous fungi. Trans. Ill. State Acad. Sci. 64: 38-42.

Monoson, Herbert L. and Gene M. Ranieri. 1972. Nematode attraction by an extract of a predaceous fungus. Mycologiya 64: 628-631.

Monoson, Herbert L., Alan G. Galsky, Judith A. Griffin and Eleanor J. McGrath. 1973. Evidence for and partial characterization of a nematode attraction substance. Mycologiya 65: 78-86.

Nordbring-Hertz, Birgit. 1973. Peptide-induced morphogenesis in the nematode trapping fungus Arthrobotrys oligospora. Physiol. Plant 29: 223-233.

Olthof, Th. H.A. and R.H. Estey. 1965. Relation of some environmental factors to growth of several nematophagous hyphomycetes. Can. J. Microbiol. 11: 939-945.

Olthof, Th. H.A. and R.H. Estey. 1966. Carbon and nitrogen levels of a medium in relation to growth and nematophagous activity of Arthrobotrys oligospora Fresenius. Nature 209: 1158.

Pramer, David. 1964. Nematode trapping fungi. Science 144: 382-388.

Pramer, David. 1965. Symposium on Microbial Insecticides. III. Fungal Parasites of Insects and Nematodes. Bact. Rev. 20: 382-387.

Pramer, David and Shimpei Kuyama. 1963. Nemin and the nematode trapping fungi. Bact. Rev. 27: 282-292.

Pramer, David and N.R. Stoll. 1959. Nemin: A morphogenic substance causing trap formation by predaceous fungi. Science 129: 966-967.

Rohlf, F. James and Robert R. Sokal. 1969. Statistical Tables. W.H. Freeman and Co., San Francisco.

Roubaud, E. and R. Deschiens. 1939. Sur les agents de formation des dispositifs de capture chez les Hyphomycetes predateurs de Nematodes. C.R. Acad. Sci., Paris 209: 77-79.

Roubaud, E. and R. Deschiens. 1941. Essais relatifs a la prophylaxie de l'Anguillulose du mouton par l'usage des Hyphomycetes predateurs du sol. Comp. Rend. Soc. Biol., Paris 135: 687-690.

Rudek, Walter Thomas. 1972. The formation and constriction of the trapping rings in the nematophagous fungus Dactylaria brochopaga Dreschler. Master's Thesis, Loyola University, Chicago, Illinois.

Samoiloff, M.R. and J. Pasternak. 1969. Nematode morphogenesis: fine structure of the molting cycles in Panagrellus silusiae (de Man 1913) Goodey 1945. Can. J. Zool. 47: 639-643.

Satchuthananthavale, V. and R.C. Cooke. 1967. Vitamin requirements of some nematode trapping fungi. Trans. Br. Mycol. Soc. 50: 821-828.

Satchuthananthavale, V. and R.C. Cooke. 1967a. Nitrogen nutrition of some nematode trapping fungi. Trans. Br. Mycol. Soc. 50: 423-428.

Sokal, Robert R. and F. James Rohlf. 1969. Biometry. W.H. Freeman and Co., San Francisco.

Soprunov, F.F. 1966. Predaceous hyphomycetes and their application in the control of pathogenic nematodes. Academy of Sciences of the Turkmen, SSR.

Tarjan, A.C. 1960. Induction of traps of nematophagous fungi using Panagrellus redivivus. Nature 185: 779-780.

Winkler, Eleanor J., Lowell A. Douglas and David Pramer. 1960. X-ray diffraction analysis of cell walls of nematode trapping fungi. Biochim. Biophys. Acta, 45: 393-395.

Winkler, E.J., Shimpei Kuyama and David Pramer. 1961. A Nemin Assay Procedure. Nature 191: 155-156.

Woronin, M. 1870. Sphaeria lemnaereae, Sordaria coprophilia, S. fimiseda, Arthrobotrys oligospora. In: A. de Bary and M. Woronin, Beitr. Morph. Physiol. Pilze III. Abhandle. Senckenbergische Naturf. 7: 325-360.

Zopf, W. 1888. Zur kenntnis der infections - Krankheiten neiderer thiere und pflanzen. Nova Acta Leop. Carol. 52: 314-376.

APPENDIX A

APPENDIX A

Formulas for Reagents of the Lowry Protein Determination

Reagent I:

a. 2% copper sulfate

b. 4% sodium potassium tartrate

c. 10% anhydrous sodium carbonate in 0.5 N sodium hydroxide

Mix 1 ml a and 1 ml b with 2 ml water. Add 1 ml diluted a and b to 10 ml c.

Reagent II:

Dilute 1 ml Folin Phenol Reagent with 10 ml water.

APPENDIX B

APPENDIX B

Bovine Serum Albumin Standard Curve

Concentration (mg/ml)	Percent Transmittance	Optical Density
.008	96.5	.0154
.016	93.0	.0315
.032	86.8	.0615
.060	77.3	.1118
.080	70.5	.1518
.160	53.5	.2716
.200	46.1	.3363

APPENDIX C

APPENDIX C

List of Abbreviations and Symbols in Appendix C

ANOVA	-	analysis of variance
df	-	degrees of freedom
F_s	-	calculated F value
MS	-	mean square
SS	-	sum of squares

Overall ANOVA Table

Source of Variation	df	SS	MS	F_s
Among groups	3	69,299.2371	23,099.7457	31.70
Within groups	76	55,380.4701	728.6903	
Total	79	124,679.7072		

Since F_s is greater than critical F^*, 2.74, within 95% confidence limits, there is a significant difference in nemin production between groups.

*Critical F value obtained from Rohlf and Sokal (1969).

Comparisons Among Means

Comparison	SS	F_s	Result*
$L_2 - L_3$	33,358.1840	45.78	significant
$L_3 - L_4$	1,362.1224	1.87	not significant
$L_4 - L_5$	96.7210	0.13	not significant
$L_3 - L_5$	2,184.7796	3.00	significant

*Based on critical F value, 2.74, within 95% confidence limits

APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

5/5/1975
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