PATHOLOGY AND BIOLOGY OF *DITYLENCHUS* SP. IN RELATION TO A DISEASE OF *AGARICUS CAMPESTRIS* L.

By

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INTRODUCTION

In 1906 there was published by Newstead (28) in England what may be the first report of a mushroom crop loss attributed to nematodes. Enormous numbers of "'eel-worm' (Tylenchus sp.), very similar if not identical with the eel-worm (Tylenchus devastatrix)," were found. A book by Falconer in 1910 (17) contains a description of a disease of mushrooms called "black spot." This disease was attributed to nematodes found in diseased spots on the mushroom caps. Steiner in 1931 (34) reported identifying Rhabditis lambdiensis Maupas 1919 from badly diseased mushrooms submitted for examination from Missouri. Another report by Steiner in 1933 (35) gives more complete details of the nematode and a discussion of its possible role as a carrier of a bacterial disease pathogen of mushrooms. In 1934 Austin and Jary (3) reporting results of a mushroom pest survey in England, tell of frequently finding a Rhabditis nematode in mushrooms showing typical "brown spot" disease. Goodey identified the nematode as Rhabditis teres Schnr., a form closely related to that described by Steiner as being a carrier of bacterial pathogens of mushrooms. Haseman and Ezell (22) published, in 1934, details of their findings of nematodes in commercial mushroom houses at Leeds, Missouri. The nematode involved was that identified by Steiner as Rhabditis lambdiensis.

Events which lead to the discovery, in 1949, of a new species of Ditylenchus as a pest of commercial mushrooms in Pennsylvania are described in a paper by Lambert, Steiner, and Drechsler (24). As

information of the above findings began to spread throughout the mushroom industry of this country and abroad, more reports of nematodes affecting mushrooms began to appear in print. Atkins (2) in England wrote of instances dating back to 1947 in which mushroom crop losses were associated with the presence of nematodes. Ditylenchus intermedius (de Man 1880) Filipjev, 1936 and a species of Rhabditis were identified as occurring in great numbers in separate cases of crop damage in 1949 from England (1). Cairns and Thomas in 1950 (13) described three types of mushroom diseases associated with nematodes, and outlined a program for control of the nematode pests. A non-technical paper for growers was prepared by Thomas and Mitchell (38) based largely upon cooperative work of the Pennsylvania State Experiment Station and the Division of Nematology, U. S. Department of Agriculture. Cairns (9) in 1951 reported that species of Aphelenchoides and Aphelenchus were also associated with mushroom losses. Details of a control program for all the known nematode pests of mushrooms were presented. The paper by Thomas and Mitchell was republished in England in 1951 because of the concern abroad about nematodes causing mushroom crop losses.

Goodey (20) in 1950 identified Ditylenchus nematodes from various diseased mushroom beds in England. He considered these nematodes to be similar to Ditylenchus intermedius.

Seinhorst and Bels (32) in 1951 reported finding a species of Ditylenchus, they considered to be D. destructor Thorne, 1945, in mushroom beds in Holland. They also reported finding Rhabditis species and Diplogaster lheritieri Maupas, 1919 in infested compost. Bovien (7) in 1951 reported results of examination of mushroom compost in

in Sweden not producing normally. Species of Rhabditis, Cephalobus and, in some samples, enormous numbers of Ditylenchus thought to be D. destructor were found. In 1951, D. destructor was reported in numerous samples of infested compost in England (21). Rasmussen in 1952 (29) reported that occasionally nematodes seem to be the cause of reduced yields of mushrooms in Denmark.

Personal communication with pathologists from France, Belgium, and Switzerland tell of the presence of nematodes associated with decreased mushroom crops in these countries. Communication with phytonematologists in widely separated areas of the United States of America tell of finding nematodes of various species in samples of compost examined when mushroom crop losses were detected. In some instances nematodes involved were considered to be morphologically identical to D. destructor.

Sarazin in 1952 (31) reported that successful eradication of nematodes in mushroom beds without injury to the mushroom can be accomplished by application of 1:10,000 hydroxyquinoline sulfate. This initiated a new method for nematode control in the infested bed, but also involves need for toxicology studies in regards to health of consumers of treated mushrooms.

Cairns in 1952 (10) reported results of a quantitative and qualitative study on the ability of Ditylenchus sp. to survive in the dormant and resistant state of anabiosis. This property is a factor of importance in spread and control of the nematode. Cairns (12) also reported results of experiments which indicate that environmental moisture is an important factor in determining thermol-death responses of this species of Ditylenchus. A recent note (11) by the same author

points out that the fungus-feeding habit of Ditylenchus sp. from mushrooms can be turned to benefit when the nematode is cultured in the laboratory for use in research, teaching, and nematocide screening.

The subject matter of this thesis pertains to certain relationships of one particular nematode pest of the commercial mushroom, Agaricus campestris L. The nematode studied is one of apparently several species of Ditylenchus associated with mushroom crop losses, and is considered to be a new species. Recommendations for control of nematode diseases of mushrooms have proven to be very satisfactory when carefully followed. Effectiveness of this program is wide in scope; eradication is possible not only of the various nematode genera and species involved, but also of most other mushroom pests and pathogens borne in compost or casing soil. The present work is therefore not a matter of how to control the nematode, but rather to understand why the control measures are effective. Knowledge of this and certain phases of biology of the nematode in relationship to factors in production of mushrooms, may eventually lead to improvement of present control practices and to devising new ones. Use of Ditylenchus sp. as the test nematode for these experiments was considered particularly advantageous. Its morphology and life history present all the features that make buccal-stylet bearing nematodes such difficult pests to exclude and to eradicate.

MATERIALS AND METHODS

I. Origin and types of populations of Ditylenchus sp. used.

Nematodes used throughout this work originated from a single sample of mushroom compost collected April 1949 from a diseased mushroom bed in the commercial mushroom plant of Mr. Brent Hood, West Grove, Chester County, Pennsylvania. It was considered that these nematodes represented a mixed-population; a population consisting of progeny descended from more than one female. This collection of nematodes served as the initial inoculum for many subsequent laboratory-reared cultures.

For some experiments it was considered desirable to have a population of nematodes which had its origin from a single female. To obtain this type of population, single larvae were placed in small culture tubes together with one mature male. Of several separate colonies resulting in those tubes in which the larva developed into a gravid female, only one colony was selected. As soon as the female in this colony was observed to be gravid, the male nematode was removed. Thus, all the progeny of this female were considered siblings, being progeny of the same parents. Numerous subcultures have been made from this population which originated in May 1951. These subcultured populations were also considered sibling populations as all were descended from the single female ancestor.

II. Types of nematode cultures used.

Cultures of nematodes were maintained in all cases in mixed culture with one or more fungi which served as a source of food for

the nematodes. Of the culture methods used, only one is likely to be unfamiliar enough to warrant explanation. This is the use of mushroom-spawn cultures[1] for rearing large populations of Ditylenchus sp.

Carefully made, inexpensive mushroom-spawn cultures growing in glass bottles (Figure 1) are available from mushroom-growers supply houses. Cultures consist of a substrate of manure-compost or of cooked grain, usually wheat or rye. The substrate was permeated with white mycelium of fungus at the time of delivery of the spawn. Manure-compost type of spawn culture was used most frequently because it provided a substrate of the same nature as that found in commercial mushroom beds. Grain-spawn culture was used to provide uniform amounts of mushroom inoculum since each kernel of grain is covered and penetrated by hyphae of the fungus.

Combinations of host fungus, substrate, and type of culture container, as well as the general uses of each are presented in Table 1.

III. Methods of isolating nematodes.

The simplest and most direct method of obtaining individual nematodes from various substrates was to add water to a small sample in a dish and with aid of a low power dissecting microscope to mechanically remove nematodes with a needle or a pipette. Isolation of large numbers of nematodes or examination of large samples of infested material for presence of nematodes required the use of other methods.

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[1]This method was suggested by Dr. E. B. Lambert, Division of Fruit and Vegetable Crops and Diseases, U. S. Department of Agriculture.

TABLE 1. Types of cultures of nematodes and their general uses.

Type of culture container	Host fungus and culture media		General uses
	Agaricus campestris	Other fungi	
Spawn bottles	Manure-compost grain-compost	----	Production and study of large populations. Production of nematodes in dormant, "curd" masses.
Petri dishes	Potato-dextrose agar Manure-compost	Potato-dextrose agar	Quick population build-up. Easy removal of individual nemas for study.
Test tubes Micro-tubes (6 mm. x 30 mm.)	Potato-dextrose agar Manure-compost	Potato-dextrose agar	Experiments involving single or few individuals or eggs.
Depression-slides	Potato-dextrose agar	Potato-dextrose agar	Detailed microscopic observation of nematodes.

A. Baerman funnel method.--Six-inch glass funnels supported in a rack were fitted with short lengths of rubber tubing and tubing clamps. Squares of closely woven cheese cloth were clipped in place on the rim of the funnel with four spring-type clothes pins. Samples to be processed were reduced to small pieces and placed on the cloth. After the tubing was clamped shut the funnel was filled with sufficient tap water to bring the water level over the sample. Active nematodes become free of the substrate and move through the cheese cloth and settle to the bottom of the funnel. Small amounts of water, containing these nematodes, are drawn off into Syracuse watch glasses or beakers.

B. Decantation plus sieving method.--Infected material was mixed with water and agitated, then the mixture was allowed to settle until heavier particles had gathered at the bottom of the container. The suspension above this sediment was then decanted into another container. If the decanted suspension contained nematodes, it was saved for further processing and the sediment was discarded. Sieves used in conjunction with decantation provided the quickest way of isolating nematodes from certain types of samples. Sieves used in these experiments were of the type designated as National Bureau of Standards, eight-inch diameter, Fine Series, Nos. 30, 100, and 270. These sieves possess, respectively: 27.62, 101.01, and 270.26 openings per linear inch.

C. Waring Blendor -- decantation -- sieving method.--Use of the Waring Blendor in isolation of nematodes from infested plant material was devised by Taylor and Loegering and described in a report by the former (37). In order to obtain more accurate analysis of plant materials for their nematode content, the original method was modified to the following procedure: Plant samples were cut to short lengths of about two cm. and placed in the blendor with 50 ml. of tap water. Then blendor was operated about 20 to 40 seconds. The resulting mass was poured into a beaker and material remaining in the blendor rinsed into the beaker with an additional 100 ml. of water. Beakers containing the plant material were set aside overnight to allow nemas to move out of the tissues. The following day the mixture was stirred, then poured on to a number 30 sieve, the liquid being caught in a beaker. An additional 50 ml. of tap water was used to rinse out the

first beaker onto the screen and to rinse the residue retained on the screen; this residue was then discarded. Material in the beaker was allowed to settle for about one hour and examined. If too much plant debris was present, the contents were agitated and then passed through a number 100 sieve. Residue on the screen was rinsed with a few milliliters of water and discarded. Material was again allowed to settle and examined.

Applications of the isolation methods are summarized in Table 2.

TABLE 2. Methods of nematode isolation from various types of materials.

Material	Method of nematode isolation		
	Baermann funnel	Decanting plus sieving	Waring Blendor -- decanting -- sieving
Compost	+	+	
Soil	+	+	
Spawn cultures		+	
Agar cultures		+	
Plant tissue	+	+	+

IV. Methods of manipulation of nematodes.

Because of their small size, all manipulations of individual nematodes were carried out under binocular, stereoscopic, dissecting microscopes. Individual nematodes and eggs were picked up and transferred by means of a finely pointed bamboo needle inserted in a dissecting needle handle. Numerous nematodes or eggs when in water were more quickly picked up and transferred by drawing them into medicine droppers having tips of small diameter. For convenience,

these fine-tipped medicine droppers will be referred to as capillary pipettes.

In order to change the liquid surrounding a number of nematodes or to concentrate them into a very small volume of liquid a special type of glass dish was used (Figure 2). These dishes are not available commercially, but are easily made with simple glass-working equipment. The dimensions are not critical; dishes with cone diameters of 15 to 20 mm. are satisfactory. The dish is filled with a suspension of nematodes and/or their eggs. Because of the small volume of the dish nematodes and eggs soon settle and come in contact with sides and bottom. Most of the liquid is withdrawn leaving nematodes covered with only a thin film of liquid. They can then be withdrawn as a concentrated mass from the apex of the cone with a capillary pipette.

Further freeing of nematodes from materials or organisms present is done as follows: Refill the conical dish with water or other desired liquid. This serves to agitate the nematodes which are again allowed to settle and the surrounding liquid is then withdrawn. Serial changes of the liquid can be made to accomplish any desired degree of dilution or isolation without loss of nematodes.

V. Methods of counting nematodes.

Relatively few nematodes (1 to 500) were counted directly, larger numbers (500 or more) were counted indirectly by examination of representative samples. Counting was facilitated by use of ruled Syracuse watch glasses in conjunction with an ocular disc ruled into squares.

VI. Methods of inoculation with nematodes.

A variety of methods were used to inoculate various cultures, plants, or plant parts. Each method is described below and its particular applications are shown in Table 3.

A. Inoculation with bamboo pick or capillary pipette.--Individual adults, larvae, or eggs, covered with a minimum amount of water or substrate, were transferred on the tip of a bamboo pick or few to many hundreds of nematodes were transferred as a suspension in a small amount of water drawn into a capillary pipette. Immersion of the bamboo pick or pipette into boiling water between inoculations removed the possibility of introducing undesired nematodes into the subsequent culture being inoculated.

B. Foliar bud and axil inoculation.--This method consisted of drawing nematodes from a water suspension into a capillary pipette and placing droplets in or on foliar buds and in axils of plants. Inoculated plants were then placed for seven days in a moist chamber.

The next three methods were used to inoculate fleshy, underground plant parts such as storage roots, bulbs, and tubers.

C. Cork-borer method.--This method, as used in these experiments, was essentially the same as described by Baker (4) although some modifications were necessary.

The species of Ditylenchus from mushrooms, being a form which feeds on fungi, necessitated making inoculations free of fungus contaminants if nematode infection was to be shown. The following procedure was used.

1. Plant parts were scrubbed with a brush under running water so that all loose outer tissues were removed.

2. Plant parts were submerged in 1:1000 mercuric chloride solution for five minutes, then were transferred to an inoculating chamber, rinsed in sterile water and dried on sterile towels.

3. Cores of plant material were removed by inserting a sterile, 10 mm. diameter cork borer to a depth of about one-half the diameter of the plant part.

4. The plant part was inoculated by putting into the resulting hole several droplets of previously sterilized water containing large numbers of disinfested nematodes.

5. The core of plant material was replaced; forcing it out of the cork borer by means of an alcohol dipped and flamed glass rod, and the cut surface sealed with a square of waterproof adhesive tape.

6. The plant part was enclosed in a cellophane produce bag to prevent dessication and contamination.

D. Inserted tube method.--This method for inoculation of fleshy plant parts was devised by O. Thorne of the Division of Nematology. Use of the following modified procedure resulted in inoculation sites having little contaminating fungus growth.

1. An area of the plant part about one inch in diameter was wiped with clean, moist toweling and disinfested with 70 percent ethyl alcohol. After about 3 minutes, traces of the remaining alcohol were removed by wiping with a cloth moistened with sterile water.

2. The sharpened end of a short length of metal tubing was inserted to a depth of about 1 cm. in the center of the disinfested spot (Figure 3). The tubing used was of aluminum, 1 cm. in diameter, 2.5 cm. in length, and having the edge of one end sharpened.

3. Several drops of water containing nematodes were placed in the exposed end of the inserted tube and covered with a small beaker or vial.

4. After the water content of the tube had disappeared (1-3 days), the metal tube was removed and the wound sealed with a square of waterproof adhesive tape.

E. Tuber-eye inoculation method.--This method was devised as a laboratory means of testing ability of Ditylenchus sp. to establish infection where the method of inoculation did not involve wounding of the potato tuber.

A glass specimen vial, 23 mm. in diameter and 38 mm. in length, was filled with nematode infested manure-spawn, and inverted over the site of a lateral bud or eye of the tuber and held firmly in place with a strip of waterproof adhesive tape (Figure 4). Continued activity of nematodes was maintained by occasional additions of water to substrate in the vials. Lateral shoots and adventitious roots developed within the vial where they were continuously exposed to the nematodes and could be observed under the dissecting microscope.

F. Planting in nematode infested soil.--Another means of exposure of plants to action of nematodes was to plant seeds on other propagules directly in nematode infested material.

Contents of twenty manure-spawn cultures of the nematode were mixed with an approximately equal amount of steam-sterilized sand and used as potting medium.

Potato tubers were planted, one per pot, in 6-in. pots containing the infested medium. Seeds were sown and covered with the medium in

thumbpots. Seedlings that grew too quickly to be retained in the small thumbpots were transferred to 5-in. pots. All seedling plants were kept for a period of 7 days in a moisture chamber, beginning with the first appearance of seedlings. The plants were then kept in the greenhouse until time of observation for nematode infectivity.

Applications of the above described methods of inoculation of plant materials tested are shown in Table 3.

TABLE 3. Applications of methods of inoculation of Ditylenchus sp. to various plant materials.

Plant material	Method of inoculation					
	Bamboo pick or capillary pipette	Foliar bud and axils	Cork-borer	Inserted tube	Tuber-eye	Infested rotting medium
Algal cultures	+					
Fungus cultures	+					
Seedlings		+				+
Tubers			+	+	+	+
Bulbs			+	+		+
Fleshy roots			+	+		

A moisture chamber was constructed in the greenhouse to provide an atmosphere of high humidity where droplet inoculations would not evaporate too quickly from buds and axils and where developing seedlings planted in infested medium could be kept covered with a moisture film. The chamber (Figure 5) contained two nozzles which provided a

very fine mist of water. A layer of sphagnum moss in the bottom helped to maintain high humidity about the plants when the water was turned off.

VII. Methods of temperature measurement.

Two types of thermometry instruments were used: conventional sealed-in-glass mercury thermometers and thermistors, a new type of resistance thermometer. All were corrected or calibrated to a National Bureau of Standards certified standard thermometer with a scale of -5 to 101° C. in 0.1° C. intervals.

Western Electric Thermistors,[1] Model 27A, were used to determine temperatures under conditions where mercury thermometers could not be used. Two advantages of the thermistor over either a thermocouple or a platinum resistance element are: (1) its high resistance eliminates for practical purposes resistance problems in circuit design, thus permitting any length lead wires, and (2) there is no need for a junction maintained at a reference temperature.

The Model 27A thermistor bead is a glass-coated spheroid about 0.25 mm. in diameter with two very fine platinum alloy leads. The associated circuit design (Figure 6) which translates thermistor values into temperature is essentially a conventional Wheatstone bridge with the thermistor in one arm.

The thermistor beads were mounted for use in certain of the experiments in two different ways. One was mounted at the tip of a

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[1]Thermistors were obtained from Western Electric Company, Inc., Allentown, Pa.

probe for measuring temperature changes within sealed vials immersed in a hot water bath. Another bead was mounted in the specimen chamber of a slide constructed for microscopical observation of effects of heat upon nematodes.

The thermistor-probe (Figure 7) consisted of a wooden handle with a strip of celluloid mounted in a slot at one end. A thermistor bead was cemented at the distal end of the celluloid and its leads were extended back on each side to leads of heavier gauge connected to terminal screws on the wooden handle.

The thermistor equipped micro-observation slide (Figure 8) consisted of a brass plate with a small cell constructed of cover glasses cemented to it and positioned over an opening in the metal to allow use of transmitted illumination for microscopic observation. The thermistor bead was contained within the cell and its leads connected to the terminal posts. The entire observation chamber was cemented with Varno-cement[1] and a well of this material was formed at each open end of the cover-glass chamber to hold water to counteract rapid evaporation from within the chamber. A shallow well made of cement on top of the observation chamber kept microscope objective immersion liquids from mixing with water containing nematodes within the chamber.

VIII. Method of direct observation of heat effects upon nematodes.

The previously described micro-observation slide was centered on a warming stage to permit observation of Ditylenchus sp. while under

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[1]A thermolabile, waterproof, dielectric cement obtained from Varniton Co., 416 N. Varney Street, Burbank, California.

influence of known temperatures. This warming stage (Figure 9), made by E. Leitz Co., consisted essentially of a hollow metal block with a condensing lens and centigrade thermometer built into it. It was placed on the regular stage of the microscope so that the condensing lens served to replace the top lens element which had been removed from the substage condenser of the microscope. The built-in thermometer was not used since it did not indicate the exact temperature existing within the chamber of the micro-observation slide. Two rubber tubes connected the warming stage to an electrically heated water bath which was thermostatically regulated. A rubber-bulb hand pump in one of the tubes caused circulation of water through the system. In use the entire apparatus was set up as illustrated in Figure 10.

IX. Method of thermal death determination.

Nematodes were placed in small cork-sealed vials which were submerged in a water bath at room temperature. Nematodes in the active state were contained in 1 ml. of water while those in anabiosis were not moistened. The water bath was then uniformly increased to predetermined values and held constant. Samples of nematodes were removed at definite intervals timed from the minute the water bath attained the desired temperature and were observed and tested to determine whether alive or dead.

The hot water bath was a commercially made apparatus with a capacity of about 40 liters. Heat for the bath was supplied by electric immersion heating elements and a variable speed mixer served to circulate the water. Constant water temperatures to within $\pm$0.1° C.

were maintained by a mercury-type thermostat and relay unit. A closeable wire basket held the corked vials completely submerged and allowed free circulation of water around all of the vials. Containers used to hold nematodes for submersion in the water bath were clear glass specimen vials, short form, with lip; 15 mm. diameter, 45 mm. height, and of approximately 4 ml. capacity.

X. Methods of nematode viability determination.

Determination of whether or not a nematode is dead is usually not a simple matter as inactivity does not necessarily indicate death. In experiments where necessary to determine viability or death of nematodes, more than one method was employed. Each method alone may be subject to some criticism, but when used in conjunction with the other methods, little doubt remained in declaring a nematode dead. Procedure in the present work was as follows:

Treated nematode samples and checks left at room temperatures were placed in Syracuse watch glasses and the water content was brought to 3 ml. by addition of tap water which had been aerated by exposure to air in an open vessel for 24 hours. Nematodes in shallow water in watch glasses were allowed to stand overnight before examination. One or two drops of a saturated aqueous solution of gentian violet were added and contents of each dish agitated to disperse the stain. Nematodes which had become permeable accumulated the stain from the diluted concentration in the water and became a conspicuous violet color. Observational experience and confirmation with the other methods of viability testing have shown that individuals which absorb gentian violet stain are either dying or dead. Live nematodes have

been left in this stain for more than 4 months without lethal effects and without staining to an extent apparent under the dissecting microscope.

Occasionally a few nematodes which did not stain but which were not moving were found at the time of examination. Internal morphology of these nematodes was so altered that it was obvious they were dead. Forms that appeared normal but not active and that responded to stimulus of a light touch in the neck region with a bamboo pick were alive and most frequently showed complete recovery of activity after varying lengths of time. Inactive forms that appeared morphologically normal but did not respond to tactile stimulus were either dead or in state of coma. If pressing with a bamboo needle on their bodies showed a lack of elasticity in recovery of the depressed area to normal configuration, the nematodes were considered dead. The final check was to cut across the body of the nematode; if its contents did not extrude from the cut surfaces, the nematodes were considered dead. If the body contents did flow from the wound, the nematode was considered to have been alive, but in state of coma.

All nematode samples were held in Syracuse dishes for observation and testing for a maximum period of 14 days. By the end of this period determination of the condition of nematodes was completed. The staining reaction or changed morphology of dead nematodes was usually so obvious that very few required the expedient of depressing with a needle to test turgor. Cutting was used only to confirm decisions made on the basis of the other tests.

II. Method of determination of pathogenicity of nematodes.

Determination of the role of Ditylenchus sp. as a pathogen of the mushroom, Agaricus campestris, required construction of a special sterile observation chamber. This was necessary in order to prepare and maintain for long periods cultures consisting only of nematodes and fungus; all other organisms being excluded. History and principles governing design and operation of sterile chambers have been comprehensively reviewed and discussed by Reynier (30).

The apparatus used (Figure 11) consisted of an internally sterile, wood and glass box sealed against contamination and containing three chambers accessible to each other through sliding doors. Entry and exit of materials was by means of a germicide trap containing a 1:500 solution of Zephiran Chloride.[1] Manipulation of materials and instruments within the chambers was possible by means of permanently installed long rubber gloves worn by the operator. One chamber contained a binocular, dissecting microscope, another chamber contained a monocular microscope. The ocular tubes of the microscopes, mounted in flexible plastic diaphragms, extended to the exterior. Each chamber was supplied with an ultraviolet germicidal lamp which was interchangeable with a standard fluorescent lamp used for general illumination. An intense source of illumination that could be directed through the glass into either microscope chamber was provided by a Spencer microscope lamp.

The preparation chamber, which connected with the germicide trap, and the dissecting microscope chamber were each supplied with an

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[1]Zephiran Chloride is the trademark used by Winthrop-Stearns, Inc., New York 18, N. Y., for a mixture of high molecular, alkyl-dimethyl-ammonium chlorides.

electrically sterilized inoculating instrument. This eliminated the necessity of using an open flame within the apparatus which might have had deleterious effects on cultures maintained within the chamber. The inoculating instruments (Figure 12) consisted of a doubled nichrome heating element wire fastened to two heavy gauge copper wires in a short length of insulated electrical cable. The other end of the cable was connected to a standard 2-pronged electrical plug. The cable was wrapped with plastic dielectric tape to provide a smooth handle surface easily disinfested. Inserting the plug into an electrical outlet for a few seconds caused the nichrome wire to become red hot. Outlet receptacles were connected to a small transformer with an input of 115-120 volts AC and an output of 6 volts AC.

Sterile air was supplied to the apparatus by a continuous-duty aquarium pump connected to a Cambridge Absolute Filter[1] which removed all particles 0.3 microns in diameter or even smaller. A slight positive atmospheric pressure was thus maintained within the chamber to prevent inward air movement through possible leaks.

To put the sterile chambers into use all internal surfaces and equipment were first sponged with a detergent, such as Dreft, and then with a solution of 1:500 Zephiran Chloride. The only exceptions were those parts of the microscopes which should not be moistened. The germicide lamps were then turned on for 24 hours and all equipment

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[1]The Cambridge Absolute Filter used was obtained from the Cambridge Corporation, 350 South Geddes Street, Syracuse, N. Y. The filter was a very high efficiency air cleaning filter with an air flow resistance that causes a pressure drop of only one inch of water at rated capacity.

was moved about at intervals to assure exposure of all surfaces to ultraviolet rays.

Materials to be used were passed through the germicide trap which lead to the first, or preparation, chamber where the metal containers and wrapping were removed. Containers not previously sterilized in an autoclave or hot-air steriliser were surface disinfested in this preparation chamber by sponging with a solution of 1:500 Zephiran Chloride and exposure to the germicide lamp. Only after these precautions were objects passed into the observation chambers.

A separate inoculation chamber was also frequently used for routine inoculation of culture media or other work in which the hazard of contamination had to be reduced.

XII. Method of disinfesting the nematodes.

The nematode concerned in this study exists in an environment containing bacteria and fungi and although the surface of its body may have these organisms on it, normally none are found within the nematode. Freeing nematodes from all other associated organisms was accomplished by two separate processes: (1) Reduction of the number of contaminants by successive washings of the nematodes. (2) Disinfestation of the surface of the nematodes for possible contaminants. The procedures used were as follows:

1. A large quantity of nematodes was isolated from a manure-spawn culture bottle by decantation and sieving and finally collected in a relatively clean condition in tap water in a beaker of about 800 ml. capacity.

2. Water in the beaker was brought to a volume of about 500 ml. and then twice passed thru a number 270 sieve to remove the nematodes which were washed from the sieve and collected in about 10 ml. of tap water in a beaker. The water volume was again brought to about 500 ml. with tap water and the process was repeated.

3. Washing nematodes as described in No. 2 was repeated for 7 changes with tap water and then for 3 more times with sterile water. The nematodes were then brought to a concentrated mass in about 10 ml. of sterile water.

4. Surface disinfestation of the nematodes was accomplished by placing them in a 1:20,000 solution of Zephiran Chloride for 20-30 minutes. During this treatment the nematodes were transferred into the sterile chamber or inoculating chamber depending upon their intended use.

5. Nematodes were removed from the disinfestant, washed thoroughly in sterile water and were then ready for use.

The above procedure resulted in a dilution of more than $5X10^{n}$ of the first concentrated sample of nematodes. This reduced the amount of soluble toxic materials and any non-adsorbed virus particles that might have been present to an extremely small amount. Action of the disinfestant upon viruses was not known, nor could the presence of toxic substances or viruses within the nematodes be discounted. Therefore, controls were incorporated in the pathogenicity experiment to detect these possible contaminants.

XIII. Method of obtaining mushroom cultures free of bacteria.

Mushroom fruiting bodies of small size, free of discoloration and with unopened veils were selected as a source of inoculum. Numerous

small fragments were aseptically removed from the cap and were inoculated into potato-dextrose agar slants. Resulting cultures were incubated at room temperature and only those free of contaminants were retained.

XIV. Methods of testing for contamination of nematodes and special cultures.

It was necessary to test completeness of the nematode disinfestation process and to test for presence of contaminating organisms in mushroom and mushroom-nematode cultures. Three general methods were used: preliminary examination, dark-field microscopic examination, and inoculation of various test media.

Preliminary examination consisted of viewing the surface of a culture with the dissecting microscope for structures or colonies of contaminating organisms.

Dark-field microscopic examination of liquid smears from the surface of cultures or suspensions of nematodes served as a quick way to determine presence of otherwise invisible contaminating organisms.

Final criteria for absence of contaminating organisms were negative findings in tubes of test media inoculated from smears of cultures on the last day of incubation.

Test media used for detection of bacterial contaminants were: Difco brand Fluid Thioglycollate medium, Difco brand Nutrient Agar and Difco brand Nutrient Agar plus 2 percent glucose (for anaerobic cultures). Media used for detection of fungal contaminants were: Difco brand Potato-Dextrose Agar and Ben Venue brand Czapek-Dox Agar tablets. All media were prepared with distilled water. Bacterial

test media were adjusted with 1 N HCl or 1 N NaOH to give pH values of approximately 7.0 as determined by an electric pH meter. Fungal test media were adjusted to pH 5.6 for Potato-Dextrose Agar and pH 7.3 for the Czapek-Dox medium. All media were placed in 20 X 150 mm. pyrex culture tubes stoppered with cotton plugs and autoclaved at 15-17 pounds pressure for 20 minutes on each of three successive days with the exception of the Fluid Thiogylcollate Medium which was autoclaved only one time.

After inoculation, tubes were incubated at 28° C. If examinations were negative tubes were held for two weeks before discarding.

Deep inoculation into tubes of Fluid Thioglycollate Medium exposed bacteria present to a variable range of oxidation-reduction potentials. Another method[1] used for anaerobic bacterial cultures was that employing Nutrient Agar plus 2 percent glucose. The agar slant was prepared and inoculated in the usual manner and the cotton plug was pushed down into the tube but not so deeply that it touched the agar slant. About 2 gm. of pyrogallic acid crystals were dropped into the tube onto the cotton plug, 10 drops of 40 percent NaOH solution were added and the tube was closed with a rubber stopper. The tube was incubated in a horizontal or inverted position so that the chemicals did not run through the cotton plug and onto the culture. The chemical mixture resulted in absorption of oxygen from the atmosphere within the sealed tube causing conditions favorable for growth of anaerobic organisms.

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[1]Suggested by Dr. N. R. Smith, formerly Senior Bacteriologist in the Division of Soil Management and Irrigation, U. S. Department of Agriculture.

Presence of toxic agents or virus within the nematodes was checked by inoculating mushroom cultures with droplets of water containing fragments of macerated, disinfested nematodes. With a steel needle, several cuts were made by drawing the needle through the droplets and into hyphae beneath to provide wounds for virus penetration.

XV. Method of evaluating nematode diseased condition of mushroom spawn.

A method for obtaining quantitative data of the condition of mushroom-spawn when acted upon by the nematode was devised. This was based upon measurement of the mushroom mycelium content present on the surface of manure substrate within the clear glass culture bottles. Figure 13 shows how progressive reduction in the amount of visible mycelium is dependent upon the extent of damage caused by nematodes. The amount of white mycelium visible on the surface was found to parallel the amount of spawn remaining within the compost substrate which was also being attacked by nematodes.

The amount of visible mycelium present within spawn culture bottles was measured in terms of its reflectance of a standardized light. Photometric readings of reflectance were translated into relative values of percentage destruction of mushroom-spawn. At the time of inoculation of a spawn-culture the mycelium was at or near its highest reflectance value which constituted zero percent destruction. Decreases in amount of spawn remaining were detected photometrically as lowered reflectance values. When all of the spawn was destroyed only dark manure-compost substrate was visible. This gave the lowest reflectance value obtainable for the culture and constituted 100 percent destruction of the mushroom spawn.

The standardized reflectance apparatus was made of three units: the illumination-reflectance box, the photoelectric photometer (Photovolt, Model 500), and a variable voltage regulator. The illumination-reflectance box (Figure 14) was made of wood and contained in one end an opening with a removable cover for insertion of a manure-spawn culture bottle. Directly in front of the bottle, towards the opposite end of the box, was an opaque diaphram with two slots. The position of the slots was such that no highlights due to the curved surface of the culture bottles were detected by the light sensing unit of the photometer. The source of illumination was two frosted glass, 40 watt, 115 volt AC, incandescent lamps located behind panes of ground glass which served as light diffusers. These lamps were wired in parallel and provided with lead wires which plugged into an outlet on the voltage regulator unit. The light sensing element of the photometer, attached by a metal clip on the outside of the box, was centered directly over an aperture in the end of the box which opened between the two light sources. The angle of acceptance of the sensing element was restricted by a metal tube to include only light reflected from contents of the spawn bottle showing through the two slots as the bottle was slowly rotated.

The photometer scale used was calibrated in arbitrary units from zero to 100 in intervals of one. This scale was linear over its range of response to illumination received by the photoelectric tube. To reduce the intensity range of the meter to values existing in the illumination-reflectance box, a Wratten ND-2 neutral density filter was fitted in front of the aperture of the photoelectric tube housing and the instrument operated on a low sensitivity range.

The voltage regulator was used to adjust intensity of illumination within the illumination-reflectance box. Fluctuating supply line voltages made it necessary to frequently check reflectance of a standard grey colored card. The amount of light reflected was brought to a standard value by means of the voltage regulator. The circuit design of the photometer unit provided automatic compensation for power variations.

To use the apparatus, it was allowed to warm-up, the light intensity was standardized, a culture bottle was inserted in the illumination-reflectance box and slowly rotated. Maximum and minimum reflectance values were read from the photometer scale and were recorded.

OBSERVATIONS AND EXPERIMENTAL RESULTS

I. Description of the nematode.

The nematode (Figure 15) serving as subject of this thesis is considered to be a new species of Ditylenchus (Nematoda: Tylenchidae). Since it is an undescribed form, it is herein referred to only as Ditylenchus sp. or by the proposed common name, mushroom-spawn nematode. Morphologically, this form is most similar to D. destructor Thorne, 1945, the potato-rot nematode, but is is distinguished from it by certain routine measurements (Table 4).

Of particular diagnostic value, in view of the overlapping ranges of some of the characteristics presented in Table 4, is appearance of the lateral fields. Two lateral areas, called lateral fields, in the cuticle of Ditylenchus nematodes have longitudinal grooves or striae. Mushroom-spawn nematodes have lateral fields containing 6 easily distinguishable striae and the elements lying between striae are each bisected longitudinally with a single fine striation. The lateral fields, therefore, contain 11 striae of which 6 are distinct and 5 usually less distinct. D. destructor, as characterized by Thorne (39), has lateral fields composed of 6 distinct striae with no further subdivision of the elements lying between them.

II. Life history studies.

A. Reproduction by the nematodes.--

1. Progeny production by isolated females. Adult males and females normally occurred in populations of the mushroom-spawn

TABLE 4. Comparison of diagnostic features of mushroom-spawn nematode and potato-rot nematode.

Diagnostic character	Sex	Mushroom-spawn nematode	Potato-rot nematode
Length (mm.)	Female	0.6-0.9	0.8-1.4
	Male	0.6-0.8	0.8-1.3
Total length ÷ diameter	Female	23.1-44.4	30-35
	Male	27.7-32.2	34-40
Total length ÷ oesophagus	Female	6.4-9.5	8-10
	Male	6.8-8.7	7-8
Total length ÷ tail length	Female	12.2-16.8	15.20
	Male	11.6-15.0	12-16
Head to vulva ÷ total length x 100	Female	73.9-86.9	78-83
Testes length ÷ total length x 100	Male	69.0-78.0	73-80
Striae in lateral field		11	6

nematode. As spermatozoa were usually found in the female genitilia indicating transfer from males present, it was considered likely that reproduction involved both sexes, although no characteristic manner of copulation was observed. The following experiment was performed to determine whether isolated females could produce progeny capable

of surviving and multiplying in the absence of male parents.

Single, third, or fourth stage larvae were placed in each of 110 micro-tubes (7 mm. X 30 mm.) containing potato-dextrose agar inoculated with single kernels of grain mushroom-spawn. Tubes were sealed with cotton plugs, placed in a moist-chamber, and frequent observations made for the production of progeny.

Of the 110 isolated larvae 54 developed into females, of which 7 laid eggs. Only three of these gravid females produced eggs that continued development and which eventually multiplied into apparently normal colonies containing both sexes. Examination of four of the isolated females did not reveal presence of spermatozoa or alteration of gonads. As no males were present at the time of the last larval molts into females, it was considered possible for this species of Ditylenchus to reproduce occasionally by facultative-parthenogenesis.

2. Deposition of eggs. The purpose of these observations was to determine the time interval between attainment of adulthood and the first deposition of eggs; the number of eggs laid per day, and the number of eggs a nematode can lay.

Data summarized in Table 5 were gathered from observations of micro-tube spawn cultures each inoculated with one third or fourth-stage larva plus one male.

All cultures were maintained under laboratory conditions with temperatures ranging from 20 to 28° C.

The difficulty of making accurate observations for longer periods terminated the experiment at the end of six days.

These data show that, under conditions of this experiment, eggs were laid within 24 hours after last larval molt. Daily rate of egg

TABLE 5. Deposition of eggs by nematodes of various ages.

Days after last molt	Eggs per day per female	Total eggs per female
1	0-3	0-3
2	0-3	0-4
3	0-4	0-14
4	0-4	0-16
5	0-7	0-21
6	0-7	0-23

deposition was variable, but showed an increase after the first two days. Although not shown in Table 8, successive daily rates of egg deposition were variable with individual female nematodes.

3. Time to hatching of nematode eggs. Observations of nematode cultures used in the preceding experiments showed that when eggs apparently were fertilized, 2-4 days were required from the time of deposition until hatching. Time intervals of 2-3 days were required for non-fertilized eggs.

4. Time from egg deposition to adulthood. Observations of nematode cultures used in the preceding experiments showed that 10-13 days were required from time of egg deposition to development of adult male and female nematodes.

B. Host Range.--The mushroom-spawn nematode was tested on a wide range of plant life to determine whether its host range was limited or extensive.

1. Algae. Culture tubes containing the algal cultures listed below were inoculated with disinfested suspensions of nematodes and maintained 40 days for observation. These algae were selected because they may be found in soil.

Ankistrodesmus falcatus (Corda) Rolfs

Chlamydomonas proteus Pringsheim

Hormidium flaccidum (Kütz.) Br.

Scenedesmus sp. Meyen 1829 Van Neil No. 14.4.1

Selenastrum minutum (Naeg.) Collins

Nematodes did not multiply in any of the cultures and within 10 days most of them were found at the edges of the agar slants or on walls of the tubes in the condition of anabiosis. Algae were spread to some extents on the surface of the substratum by nematode movements but apparently were not injured by presence of nematodes. There was no evidence of feeding by nematodes on any of these algae.

2. Fungi. Fungus cultures were inoculated with suspensions of washed nematodes and maintained 40 days for observation. Nematodes were observed to survive in all the cultures; to multiply to a limited extent in some, and to multiply considerably in others. Cultures tested are listed below under three headings based upon the degree of multiplication of nematodes on each:

a. Cultures on which nematodes continued to multiply and increased greatly in numbers.

Colletotrichum phomoides (Sacc.) Chester

Pythium debaryanum Hesse

Rhizoctonia solani Kühn (3926 USDA Kreitlow)

Sclerotinia homeocarpa Bennett

Sporendonema sp.

Tuber melanosporium Vitt.

b. Cultures in which nematodes continued to multiply but at a reduced rate.

Choanephora cucurbitarum (B. & Rav.) Thax.

Fusarium oxysporum f. batatas (Wr.) Snyder and Hansen

Polyporus compactus Overh.

c. Cultures in which nematodes ceased multiplying, but continued to exist in active state.

Mycogene perniciosa Magn.

Thielaviopsis basicola (Berk.) Ferr.

3. Seed plants. A number of seed plants were selected to represent garden and field crops as well as ornamentals likely to be exposed to Ditylenchus sp. in compost used as fertilizer when discarded from mushroom houses. All tests to determine whether nematodes could cause apparent damage to the plants and become established within the plant and multiply were negative. Plants tested are listed in groups according to the method of inoculation.

a. Seeds planted in nematode-infested potting medium, then seedlings were exposed 7 days to high humidity in a moist chamber, and all plants grown for 58 days in the greenhouse. Plants were checked for visible symptoms on roots and above-ground parts and then examined by Waring Blendor-decantation-sieving method for evidence of multiplication of nematodes within the tissues. Plants tested were as follows:

*Allium Porrum, L. (Leek, Extra large)

*Allium cepa L. (Onion, White Silverskin)

Amaranthus sp. L. (Amaranthus, Molten-fire)

Anaethum graveolens L. (Dill)

Antirrhinum majus L. (Snapdragon, Maryland Pink)

Apium graveolens var. rapaceum D. C. (Celeriac, Large Smooth Prague)

Apium graveolens dulce Pers. (Celery, Giant Pascal)

*Arachis hypogaea L. (Peanut, Virginia Jumbo)

Ambrosia artemisifolia L. (Ragweed)

Asparagus officinolis var. altilis L. (Asparagus, Mary Washington)

*Avena sativa L. (Oats, Arlington; Oats, Fulgrain)

Beta vulgaris L. (Beet, Detroit Dark Red Perfected)

Beta vulgaris L. (Sugar Beet, U. S. 1177)

Beta vulgaris L. (Mangels)

Beta vulgaris var. cicla L. (Swiss Chard, Fordhook Giant)

Brassica oleracea italica Plenck. (Broccoli, Italian Green Sprouting)

Brassica oleracea gemmifera Zenker (Brussels Sprouts, Perfection)

Brassica pekinensis Rupr. (Chinese Cabbage, Chihili)

Brassica oleracea capitata L. (Cabbage, Late Dutch Flat)

Brassica oleracea botrytis L. (Cauliflower, Snowball)

Brassica oleracea acephala D. C. (Collards, Southern or Georgia)

Brassica oleracea fimbriata Mill. (Kale, Tall Green Scotch)

Brassica caulorapa Pasq. (Kohl Rabi, Early White Vienna)

Brassica juncea crispifolia Bailey (Mustard, Southern Giant Curled)

Brassica Napus L. (Rape, Dwarf Essex)

Brassica Napobrassica Mill. (Rutabaga, American Purple Top)

Brassica Rapa L. (Turnip, Purple Top White Globe)

Bromus brizaeformis Fisch. and Mey. (Quake Grass)

Capsicum frutescens L. (Pepper, California Wonder)

Cichorium Intybus L. (Chicory, Witloaf)

Cichorium Endivia L. (Endive, Green Curled)

*Citrullus vulgaris Schrad. (Watermelon, Tom Watson)

*Crotalaria spectabilis Roth (Rattlebox)

*Cucumis sativus L. (Cucumber, A and C)

Cucumis Anguria L. (Gherkin)

Cucumis Melo L. (Muskmelon, Long John)

Cucurbita Melopeppo Alf. (Squash, Yellow Prolific Straightneck)

Cucurbita Pepo Mill. Duchesne (Squash, Improved Hubbard)

Cucurbita Pepo L. (Pumpkin, Large Yellow Field)

Cynara Scolymus L. (Artichoke, Green Globe)

Cynoglossum anabile Stapf. and Drummond (Chinese Forget-me-not)

Dactylis glomerata L. (Orchard grass)

Daucus Carota L. (Wild carrot)

Daucus Carota L. (Carrot, Imperator)

Fagopyrum esculentum Gaertn. (Buckwheat)

Festuca elatior L. (Alta Fescue, Blue Tag)

Glycine max Merr. (Soybean, Bansei)

Gossypium hirsutum L. (Cotton, Stoneville-2B)

Hibiscus esculentus L. (Okra, Long Green)

Hordeum vulgare L. (Barley, Calhoun 3)

Lactuca sativa L. (Lettuce, Great Lakes)

Lactuca sativa var. longifolia Lam. (Lettuce, Dark Green Cos)

*Lespedeza stipulacea Maxim (Korean Clover)

*Lolium perenne L. (Rye Grass)

Lycopersicon peruvianum (L.) Mill.

*Lycopersicon esculentum var. commune Bailey (Tomato, Marglobe)

*Medicago sativa L. (Alfalfa)

Nasturtum officinale R. Br. (Water-Cress)

Nicotiana tabacum L. (Tobacco, Maryland Broadleaf)

Pastinaca sativa L. (Parsnip, Hollow Crown)

Petroselinum hortense Hoffm. (Parsley, Triple Curled)

Phaseolus vulgaris L. (Bean, Best Yet Wax; Bean, Kentucky Wonder Pole)

Phaseolus humulis Alef. (Bean, Bush or Dwarf Top Crop)

*Phaseolus limenanus Bailey (Lima Bean, Henderson's Bush)

*Phleum pratense L. (Timothy)

Pisum sativum L. (Pea, Telephone)

*Poa pratensis L. (Blue Grass, Kentucky)

*Oryza sativa L. (Rice, Calrose)

Raphanus sativus L. (Radish, Early Scarlet Globe)

Rheum Rhaponticum L. (Rhubarb, Victoria)

Solanum melongena L. (Egg Plant, Black Beauty)

Sorghum vulgare Pers. (Sorghum)

Spinacia oleracea L. (Spinach, Domino)

Tragopogon porrifolius L. (Salsify, Mammoth Sandwich Island)

*Trifolium hybridum L. (Clover, Alsike)

*Trifolium incarnatum L. (Clover, Crimson)

*Trifolium repens L. (Clover, Ladino)

*Trifolium pratense L. (Clover, Red)

*Triticum aestivum L. (Wheat, Lee)

Tropaeolum majus L. (Nasturtium, Golden Globe)

*Zea mays var. rugosa Bonaf. (Sweet Corn, Golden Cross Bantam)

Zinnia elegans Jacq. (Zinnia, California Giant Mixed)

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*Plants marked with asterisk were droplet-inoculated in foliar buds and axils.

b. Propagules planted in nematode-infested potting medium and grown 58 days before examination for external symptoms and evidence of multiplication of nematodes within the tissues. Plants tested in this manner were:

Gladiolus sp. (Gladiolus)

Iris Xiphium L. (Iris, Wedgewood)

Solanum tuberosum L. (Potato) of the following varieties: Chippewa, Green Mountain, Irish Cobbler, Katahdin, Kennebec, Marygold, Pontiac, Russet Rural, Sequoia, Sebago, Teton, and White Rose.

c. Propagules inoculated by cork-borer method and the inserted tube method and kept 60 days before examination for evidence of multiplication of nematodes within the tissues. Plant parts tested in this way were:

Allium cepa L. (Onion, White Silversides)

Beta vulgaris L. (Sugar beet)

Daucus carota L. (Carrot, Imperator)

Dioscorea batatas Decne (Yam)

Iris Xiphium L. (Iris, Wedgewood)

Solanum tuberosum L. (Potato) of the 12 previously listed varieties.

d. Potato tubers of the preceding 12 varieties were also inoculated by the tuber-eye method and examined after 50 days for evidence of injury to developing roots and shoots and for multiplication of nematodes within the tissues.

Checks were made of the nematode inocula used in each method to determine approximate numbers, viability, and infective capacity on mushroom-spawn at the beginning of each set of inoculations. Infested potting medium contained Ditylenchus sp. at the approximate rate of not less than 3000 active nematodes per pot. Approximately 5000 nematodes were introduced per plant by means of the cork-borer, inserted-tube, and tuber-eye inoculation methods. Examination of the infested potting medium at the end of the experiment revealed the presence of living nematodes, although in reduced numbers.

None of the plants inoculated by any of the methods described showed any symptoms which could be attributed to the nematodes. Multiplication of nematodes was only observed to occur where fungal

contaminants had become established in inoculation wounds. In these instances, the nematodes were restricted to tissues containing fungi and were never observed in tissue not contaminated by fungi.

C. Anabiosis.--The mushroom-spawn nematode was found to be capable of surviving in the state of anabiosis. This state of being is considered a form of dormancy in which some nematode species can exist during periods of adverse environmental conditions. Importance of anabiosis in biology of Ditylenchus sp. and in considerations of control warranted making some observations and quantitative studies of the phenomenon. Large populations of nematodes reared in culture presented opportunities for observations that would have been very difficult or impossible in the field or mushroom house.

1. Migration and aggregation. When their fungus-host supply began to be depleted, nematodes were seen in increasing numbers on inner surfaces of culture containers. In culture bottles, they began migrating in the moisture film, moving upward toward the cotton-plugged end. At time of complete food depletion, the inner surface would become, in many instances, completely covered with migrating, undulating nematodes. Nematodes would then begin to aggregate (Figure 16) into groups of various sizes. Aggregations became consolidated and took on a dendritic appearance (Figure 17). These masses, composed of thousands of nematodes, continued to move upwards until reaching the neck of the bottle and the cotton plug. Migration over surface of the mushroom-depleted compost within culture bottles similarly occurred, but was less apparent than on glass container walls. In cultures containing millions of nematodes kept

under conditions of high external relative humidity (90 percent), aggregations penetrated around the cotton plugs and came out and over rims of the bottles. These masses of nematodes, which eventually ceased migrating, but continued to increase in size as more nemas collected, were called "curds." Curds appeared in the neck of the bottle, and on uppermost portions of the compost, coating fragments of straw and soil with pale yellow masses of nematodes (Figure 18). The nematodes in the curds were either in anabiosis or entering that condition.

Curds on the compost substratum gradually became less apparent as they turned darker yellow or brown in color and more reduced in size because of evaporation. Curds on the glass surfaces did not become dark brown color, but did lose their whitish or yellow color to become light brown or tan. Aggregation into curds was accompanied by a fairly strong and characteristic odor, not present before curd formation. The odor was not that of putrifaction which is itself characteristic for decaying mushrooms. This odor gradually diminished, but curd material moistened after several months again gave off detectable amounts of the odor. Curds were composed of nematodes in all stages which were coated with slime-like substance. Although many bacteria were continuously present about the nematodes, the advent of the odor only during curd formation suggested this substance was, therefore, a product of the nematodes and not of bacteria.

Preparation by nematodes for entry into the state of anabiosis was somewhat different in cultures containing relatively few or isolated nematodes. Migration and aggregation were less evident. Often, only individuals or groups composed of a few nemas formed;

the nematodes coiling together. It was not possible to decide whether or not the majority of nematodes in glass containers moved off the media and onto the glass and there entered anabiosis for any reason other than chance. Once on the drier glass surfaces motility was more quickly restricted so that the nematodes would be trapped there and thus enter anabiosis whereas nematodes on the moist substratum would remain in the active condition for longer periods.

The variable conditions under which nematodes enter and are dispersed in the state of anabiosis makes counts of them in curd masses and in infested compost a matter of interest. A curd fragment about the size of a pin-head (Figure 19) contained approximately 6600 nematodes, 86 percent of which revived when moisture was added. A 2.0 mg. sample of curd material contained nematodes at the rate of 17,445,000 per gram. Compost from a diseased mushroom bed, which had been allowed to dry out, contained nematodes capable of reviving from anabiosis at the rate of 960 per gtam of air-dry compost.

2. Factors inducing anabiosis. Observations of nematodes in cultures gave no definite information regarding conditions influencing entry into the state of anabiosis. Lack of sufficient food seemed the most obvious factor, but nematodes were often found migrating, aggregating, and in anabiosis while what appeared to be ample food supplies were still present. Drying of culture substrate appeared to indicate entry into anabiosis in some instances, yet the phenomenon was also observed to occur directly on moist media and wet surfaces. In tubing closed at each end with cotton and laid horizontally migration was upwards as well as towards the ends and the usual type of curds was produced.

3. Recovery from anabiosis. Moisture is a prime factor in recovery of activity by dormant nematodes. Curd masses placed in water gradually disintegrated, releasing individual nematodes as the curd matrix dissolved (Figure 20). Nematodes later began to uncoil and to slowly regain movement (Figure 21) and eventually returned to normally active condition. As long as nematodes were kept completely submerged in liquid water they never entered the condition of anabiosis.

Samples of compost containing nematodes were moistened to activate the nematodes then dried gradually to induce anabiosis, and moistened again. This was repeated five times over a period of two months to demonstrate the possibility for survival under widely fluctuating environmental moisture conditions. Dry curd masses placed under conditions of high humidity were found to absorb moisture as indicated by an increase in weight and some of the nematodes revived to activity.

4. Quantity and life stage of nematodes revived from anabiosis. The purpose of this experiment was to determine the number of nematodes and stages in the life history that could revive from increasingly longer existence in anabiosis.

Pieces of compost covered with masses of nematodes in process of aggregating to form curds were removed from a spawn-culture of Ditylenchus sp. and were broken into smaller fragments for more uniform drying when they were spread out in uncovered petri dishes for 24 hours in the laboratory. Dried fragments were crumbled, passed through a number 30 sieve, put in a 1 oz. screw-top vial closed with a bakelite cap, and kept in a cupboard. At intervals, four 0.4 gm. samples of

the material were removed from the thoroughly mixed vial contents, placed in ruled Syracuse watch glasses containing 5 ml. of tap water, and held for observation and counting of revived forms. Counts obtained from the four samples at each interval were averaged and are plotted in Figure 22 along with observations of which stages in the life cycle of the nematode revived.

The data indicate that, quantitatively, there was a uniform decline in the total numbers of nematodes revived with increase in time. After being kept in a state of anabiosis for 15 days, very few larvae within eggs and a reduced number of young hatched larvae were revived. After 40 days in anabiosis, no eggs showed active larvae, and very few second stage larvae were revived. After 100 days only third and fourth stage larvae and young adults of both sexes were revived, the majority being fourth stage larvae and adults. It was observed that no single life stage contributed the only individuals capable of anabiotic survival, as in the case of the "Dauer" form in other nematode species and genera.

5. Duration of survival in anabiosis. Nematode infested compost from a commercial mushroom house was allowed to dry slowly in a perforated cardboard carton and kept in the laboratory. At intervals, compost samples were withdrawn and moistened to revive nematodes surviving. The maximum period for survival under these conditions was found to be about 3 years to 4 months at which time a few third and fourth stage larvae revived. After 4 years there were no survivors.

III. Temperature studies.

A. Temperature effects upon nematodes.--The mushroom-spawn nematode is subjected to a wide range of temperatures if it becomes involved in cultural practices used in commercial production of mushrooms. The purpose of the following sets of experiments was to determine whether or not exposure of nematodes to these temperatures would be detrimental to their activity, survival, and ability to reproduce.

1. Thermal-death range of nematodes. In commercial mushroom houses nematodes may be exposed to temperatures higher than those existing at the time mushrooms are growing. The duration of exposure of nematodes to these higher temperatures is always for relatively long periods, that is, hours or days. The following experiments were designed to determine the thermal death range of active and dormant specimens of Ditylenchus sp. under temperature conditions occurring in commercial mushroom production.

a. Relatively high temperatures, short time exposures. The purpose of this experiment was to determine the thermal-death range of Ditylenchus sp. when exposed to relatively high temperatures for periods of time ranging from a few minutes to 24 hours. A factor existent in commercial practice is that nematodes are most likely to be brought to a particular temperature gradually. Therefore, to simulate this condition, a uniform, gradual approach to and descent from the thermal levels studied was incorporated in the experiment.

A sibling population of Ditylenchus sp. reared on a manure mushroom-spawn culture provided quantities of nematodes in the state of anabiosis. Equal weighed samples of curds, placed in vials, were

exposed to three different moisture levels and allowed to reach equilibrium as evidenced by attaining constant weight, measured on an analytical balance. One lot was held in liquid water, restoring the nematodes to activity. A second lot was held in a moist chamber. A third lot was held in a dessicating chamber. Heat treatment involved bringing nematode samples uniformly and gradually to required temperature by submergence in the electrically controlled hot water bath. After varying intervals of exposure, nematode samples were placed in paper insulation and brought back gradually to laboratory temperatures and put in water in Syracuse watch glasses. This was followed by a period of observation and testing to determine whether nematodes were alive or dead.

Table 6 presents data showing rates at which temperature within vials came to equilibrium with temperature of the water bath. The thermistor-probe sealed into a vial was used to detect temperature changes.

TABLE 6. Rates of temperature change within nematode-sample vials transferred to hot water bath.

Location of thermistor bead	Rate of change
Suspended in vial	.2° C./sec.
Touching bottom of vial	.3° C./sec.
Immersed in 1.0 ml. of water in the vial	.07° C./sec. (4.2° C./min.)

The average rate of temperature change for the water bath was .5° C./min. These data show that temperatures within the vials

would quickly come to equilibrium and be maintained with the water bath as it was heated.

Temperature changes within a vial removed from the heated water bath and placed in an insulated box were also measured using the thermistor probe. These data are graphed in Figure 23 along with the average time-temperature data of the water bath as it was heated. These temperature curves were considered to represent changes within vials as they were brought from room temperature to test temperature levels or vice versa. It was considered reasonable to assume that active nematodes were at the temperature of the 1.0 ml. of water in immersed vials. Time-temperature curves for the water bath and for the air and bottom surface in the vials were considered to closely approximate temperature changes of the curd samples because of their small size and the long time intervals involved.

Results of this experiment to determine thermal-death range of nematodes at relatively high temperatures are presented in Figure 24. These data indicate that lethal temperatures vary with condition of the nematodes. Nemas active in water are killed at the lowest temperatures; nemas in anabiosis conditioned to high humidity required higher temperatures; nemas in anabiosis conditioned to low humidity required highest temperatures. The amount of moisture to which nematodes had been exposed prior to heat treatment was shown to be a factor in determining thermal death ranges. It was observed that developing embryos and larvae within eggs were killed at all exposures above 40° C. No definite conclusions could be made concerning relative heat tolerance of nematodes in various developmental stages.

b. Relatively low temperatures, long time exposures. The purpose of this experiment was to determine the thermal-death range of the Ditylenchus sp. when exposed to relatively low temperatures lasting from one to several days. Physical and physiological conditions of the mushroom-spawn nematodes used in this experiment were those of active nematodes on and within manure-compost substrate. This experiment was considered to represent conditions in commercial production of mushrooms in which there is long exposure of active nematodes to temperatures slightly above that at which the nematode can thrive 30° C. (86° F.).

Twenty-eight test tubes containing approximately equal amounts of manure mushroom-spawn were each inoculated with about 1000 nematodes from a sibling population. Cultures were incubated at 22-26° C. (72-78° F.) until all showed evidence of reproduction as indicated by presence of eggs and young larvae. The tubes were then placed at 32.5° C. (90° F.) for 24 hours. Twenty-one tubes were then placed at 35° C. (95° F.) for 24 hours and the remainder were transferred to incubators at 37.8° C. (100° F.) and 40.6° C. (105° F.), each receiving seven tubes. Daily thereafter, for a period of 7 days, one tube was removed from each incubator and retained for observation to determine if nematodes were dead or alive and able to multiply.

Table 7 presents results of this experiment.

At 32.5° C. (90° F.) and 35.0° C. (95° F.) nematodes remained active, eggs were not killed and multiplication continued. At 37.8° C. (100° F.) nematodes became inactive within six hours; eggs were killed; multiplication ceased, and no survivors could be revived after 3 days of exposure. Some nematodes survived 37.8° C. (100° F.) for 3 days and regained activity and multiplied upon return to

laboratory temperatures. There were adults of both sexes and larvae of second, third, and pre-adult stages. At 40.6° C. (105° F.) nematodes became inactive within six hours; eggs were killed and multiplication ceased. No survivors exposed for one day or more revived upon return to laboratory temperatures. Results indicate long exposures (4 days or more) of active nematodes in compost to temperatures above 35° C. (95° F.) can be lethal.

TABLE 7. Observations of effect of relatively low temperatures for long exposures on survival of Ditylenchus sp.

Temperature	Days of exposure to temperature indicated						
	1	2	3	4	5	6	7
32.5° C. + 1° C. (90° F.)	Alive	Alive	Alive	Alive	Alive	Alive	Alive
35.0° C. + 1° C. (95° F.)	Alive	Alive	Alive	Alive	Alive	Alive	Alive
37.8° C. + 1° C. (100° F.)	Few alive	Few alive	Few alive	Dead	Dead	Dead	Dead
40.6° C. + 1° C. (105° F.)	Dead	Dead	Dead	Dead	Dead	Dead	Dead

c. Microscopic examination of nematodes subjected to heat. Observations were made using the thermistor-observation slide to determine if visible changes in behavior and appearance occurred in active nematodes in water subjected to heat. Results of these observations were as follows:

20-32° C. Nematodes remained alive but became less

active as temperature was raised. They showed sensitivity to rapid temperature rises as small as 1° C. as evidenced by a brief period of quick, jerky movements before again adjusting to the new temperature level. Nematodes exposed to these temperatures revived upon return to room temperature.

33-38° C. Nematodes ceased all movements and gradually straightened out. These nematodes revived within a few minutes upon return to lower temperatures.

39-43° C. Nematodes remained in the straightened body attitude but began to rapidly tremble. This action continued for a few minutes then nematodes again became quiescent. Upon return to lower temperatures some revived.

44-45° C. All movements abruptly ceased even when nematodes were brought to these temperatures before the rapid trembling had stopped. Some nematodes revived if returned to lower temperatures within a short time (15 min.).

Above 46° C. Coalescence of clear oil globules within intestinal cells was observed to occur (Figure 25). Nematodes did not revive upon return to lower temperatures.

2. Effect of low temperatures on nematodes. The purpose of this experiment was to determine effect of low temperatures upon activity and survival of the mushroom-spawn in an active state and in

anabiosis. Temperatures selected represent a range from a few degrees below mushroom cropping temperatures to temperatures representative of winter conditions in the field.

Manure mushroom-spawn culture bottles inoculated with approximately 8200 nematodes from a sibling population and cotton-plugged test tubes containing curd masses about one week old were transferred to temperature storage rooms. To avoid possible effect from abrupt temperature-change, cultures were transferred on successive days to storage rooms of increasingly lower temperatures. These successive temperatures were 18° C. (65° F.), 13° C. (55° F.), 7° C. (45° F.), 1.7° C. (35° F.), and -3.9° C. (25° F.). In each of the last three mentioned temperature rooms, three inoculated spawn bottles and three test tubes were left as each temperature was successively reached. Three inoculated spawn bottles and three test tubes were kept at 23.9° C. (75° F.) as checks. A thermometer inserted into manure compost within one bottle showed that temperature of the compost came to equilibrium with temperature of the storage room within the 24 hours alloted for each successive transfer. At 40-, 80-, and 120-day intervals one bottle and one tube from each storage room were removed and passed at daily intervals to storage rooms of successively higher temperatures until reaching 23.9° C. (75° F.). Curd masses in the tubes were moistened and inoculated into mushroom-spawn. All cultures were maintained at this temperature for observation to determine if survivors were capable of multiplication.

Results of this experiment showed that nematodes in the active state withstood exposures for as long as 120 days at 7° C.(45° F.) and 1.7° C. (35° F.) and 80 days at -3.9° C. (25° F.). Exposure of

120 days at -3.9° C. (25° F.) was lethal. Survivors of all other temperatures were able to reproduce and renew their normal activities upon return to 23.9° C. (75° F.).

Nematodes while at 7° C. (45° F.) remained very sluggishly active, did not reproduce nor cause any apparent destruction of the mushroom mycelium. Nematodes at 1.7° C. (35° F.) and -3.9° C. (25° F.) were quiescent, did not reproduce nor cause destruction. At all three temperatures there was no evidence of the nematodes migrating or aggregating or curd formation. Ice formed within the cultures at -3.9° C. (25° F.). Survivors when returned to temperatures of 12.8° C. (55° F.) and above regained activity within 24 hours. Active nematodes in check cultures maintained at 23.9° C. (75° F.) did not cease normal activities.

Nematodes in curd masses survived all the low temperature conditions tested and regained activity within 24 hours after addition of water. Reproduction was observed in all spawn cultures inoculated with the nematodes revived from anabiosis after cold temperature exposures.

Results of this experiment indicate that low temperatures can reduce activity or completely inactivate Ditylenchus sp. not in anabiosis and can cause death of these nematodes under conditions of long exposure (over 90 days) at below-freezing temperatures. Nematodes in state of anabiosis survived all the exposures tested. No apparent damage was done to mushroom mycelium by nematodes in any of the time intervals at low temperatures.

B. Temperature effects upon nematode disease of mushroom.--The purpose of this experiment was to determine effects of various temperatures upon rate of destruction of mushroom-spawn by Ditylenchus sp. Temperatures tested included the entire temperature range in which nematodes are likely to encounter mushroom mycelia in compost of mushroom beds.

Bottles of manure mushroom-spawn, which contain the same bacterial flora as commercial mushroom compost, were selected for uniformity in amount of mycelial growth apparent, were inoculated either with approximately 8200 nematodes suspended in 5.0 ml. of water or with 5.0 ml. of the same water but with nematodes removed as checks. The nematodes were from a sibling culture reared on manure mushroom-spawn. Inoculated culture bottles were transferred from the laboratory to controlled-temperature storage rooms. To avoid possibility of detrimental effects due to sudden temperature changes, cultures were conditioned by leaving them for one day at each temperature, beginning at 23.9° C. (75° F.), as they were transferred through successive temperatures to desired temperature levels. Each temperature-controlled storage room contained three nematode-inoculated bottles and one check bottle. Results of the experiment are presented in two parts.

1. Temperature effect upon rate of destruction of mycelium. Effect of temperature upon rate of destruction of mushroom mycelium was quantitatively determined by use of the standardized photometric procedure. A reduction in reflectance value was the result of destruction of the white-colored mycelium leaving the dark-colored compost substratum beneath to register on the photometer. Reflectance values were converted to percent mushroom mycelium destroyed. At

0 percent destruction reflectance of the spawn was at its highest, at 100 percent destruction, reflectance was at its lowest as only compost remained. This correlation was found to exist by microscopic examination which showed destruction of the hyphae to result in change of its white color to brown and its almost complete elimination as a recognizable fungus structure.

Results of the experiment are presented in a graphic form (Figure 27). Lines representing each temperature are the average data obtained from three nematode-inoculated cultures at each temperature tested. Values within temperature groups were so similar that averages for each group were considered representative. Similarly, check values between temperature groups were so similar that the single average value was considered representative.

Results show that temperature was an important factor influencing both the first evidence of destruction of mycelium and the subsequent rate of destruction. Higher temperatures resulted in a more rapid appearance of symptoms and a more rapid rate of destruction.

2. Temperature effect upon nematode population. During the previous experiment, differences were noted in number of nematodes developing in bottles kept at different temperatures. These differences were particularly apparent when nearly all mycelia had been destroyed and nematodes were migrating and aggregating to form "curds." The purpose of this work was to determine effects of different temperatures upon nematode populations as evidenced by total count of nematodes and the ratio of males to females at time of complete elimination of mycelium.

Living and dead nematode eggs, larvae, and adults from one bottle held at each temperature were counted to obtain total numbers of nematodes present. Living adults only were counted for determining sex ratios. Data of this experiment are summarized in Table 8.

TABLE 8. Effect of temperature upon nematode population increase and sex ratio.

Temperature	Days to elimination of mycelium	Total nemas in culture	Multiples of increase of original population	Sex ratio ♂ : ♀
29.5° C. (85° F.)	41	642,500	78	1:1.66
23.9° C. (75° F.)	41	12,050,000	1465	1:1.48
18° C. (65° F.)	54	27,900,000	3390	1:1.23
13.0° C. (55° F.)	85	10,945,000	1332	1:1.29
7° C. (45° F.)	100	10,050,000	1238	1:1.25

Results indicate that at time of complete destruction of the mushroom hyphae, different total amounts of nematodes were present at different temperatures. Optimum temperature for nematode population increase was not the temperature at which most rapid destruction of mycelia occurred. Variation in sex ratio occurred at different temperatures; in general, there was an increase in proportion of females at temperatures above optimum for total population increase. No conclusions can be induced from the data as regards to rate of population increase because time intervals required for depletion of good supply

were different.

IV. Role of Ditylenchus sp. in causing mushroom disease.

Manure compost substrate of commercial mushroom beds contains many organisms along with mushroom mycelium. When a diseased condition of mycelium develops it is difficult to determine what has been the cause and whether it produced damage directly or indirectly. The purpose of the following observations and experiments was to determine the role of the mushroom-spawn nematode in relation to the diseased condition of mushroom mycelia associated with it.

A. Observation of feeding.--Ditylenchus sp. in agar culture with A. campestris was observed to feed upon the hyphae. Nematodes migrating through the medium frequently contacted hyphae, pressed their heads at about right angles to the hyphae, and made a brief succession of rapid thrusts with the buccal-stylet (Figure 29). It was not possible to see stylet tips actually protruding into hyphae due to the curved surface of hyphae. Closeness of the appressed heads and the extended position of the stylet shafts indicated penetration had occurred. After penetration, the nematodes either remained quiescent and in position for several minutes or moved actively away. In either case, at sites of feeding slow, gradual oozing out of hyphal contents was often noted when the stylets were withdrawn. Nematodes were observed to remain at one location for as long as 30 minutes before retracting stylets and moving away. Other nematodes were observed to move along hyphae or to go from one to another making punctures, as evidenced by extrusion of hyphal contents. Random punctures were made as frequently as 6 in 10 minutes

by individual nematodes which remained at each site for only brief times. No distinct changes or movements of materials within the stylet-punctured hyphae were observed. Nothing was seen to be injected into the hyphae. Results of these observations show that the mushroom-spawn nematode does actually make numerous wounds in mushroom mycelia exposing the cytoplasm to bacteria normally always present.

B. Number of nematodes and rate of mycelial destruction.--The purpose of this experiment was to determine relationship of number of Ditylenchus sp. present to rate of destruction of mushroom mycelia in compose substrate.

Manure-spawn culture bottles were used in this experiment because their contents are very similar to the mushroom compost in the commercial beds and contain the same bacterial flora. The culture bottles were inoculated with 5 ml. water suspensions containing known quantities of nematodes from a sibling culture. Two controls were inoculated with 5 ml. of the same water, but with nematodes removed. Inoculated cultures were incubated at 18.5° C. (65° F.). This temperature was considered representative of the average temperature existing in commercial beds from time of spawn introduction until casing soil is applied. Reflectance readings were made at intervals with the photometric apparatus. Minimum and maximum reflectance values were averaged for each reading, converted to percentage mycelium destroyed, and are presented in graphic form (Figure 30). Curves representing initial inocula of 0, 10, 100 and 1000 nematodes are based on average of data from 2, 4, 3 and 3 cultures, respectively.

Results of the experiment show that the number of nematodes initiating an infection determines the time required for symptoms to first appear and that, when once initiated, rates of destruction of mycelia are about the same regardless of number of nematodes initiating infestation. This was correlated with the observation that nematode populations increased to such an extent as to be readily visible to the naked eye before general decline of the spawn became apparent. The number of nemtoades at time of complete destruction of the mycelia were considered about equal regardless of initiating number, as determined by the quantity of curd masses formed.

C. Proof of nematode pathogenicity.--The purpose of this experiment was to determine if Ditylenchus sp., in the absence of contaminants, can cause a disease of mushroom mycelia.

Sixteen tubes containing Agaricus campestris growing on potato-dextrose agar were inoculated with water droplets each containing about 100 living, disinfested nematodes from a manure-spawn culture. Four sets of control cultures of 8 tubes each were inoculated with the following:

1. Droplets containing about 100 living, non-disinfested nematodes and bacteria from the same manure-spawn culture.

2. Droplets of water containing contaminants, but without nematodes from the same nematode infested culture. Cuts with a sterile needle were made through these droplets into mycelia.

3. Droplets containing macerated, previously disinfested nematodes from the same culture. Cuts with a sterile needle were made through these droplets into mycelia.

4. Droplets of sterile rinse water used in nematode disinfesting procedure. Cuts with a sterile needle were made through these droplets into mycelia.

The 16 tubes inoculated with disinfested, living nematodes all showed destruction of the mushroom hyphae. Two of the colonies were found to contain bacterial contaminants, the remaining 14 tubes were free of contamination. Tubes inoculated with nematodes plus contamination all showed destruction of the mushroom hyphae. Tubes inoculated with contaminants but without nematodes showed injury restricted to the cuts made in the mycelia. Extensive destruction of hyphae did not occur although bacterial contamination was present. Tubes inoculated with macerated, disinfested nematodes showed no destruction of mycelia and contained no bacterial or fungal contaminants. The remaining tubes inoculated with sterile rinse water showed no destruction of mycelia, although one tube contained a fungus contaminant.

Data indicate that no toxic substance or virus was associated with the macerated nematodes, assuming such agents could cause a disease and were not inactivated by the treatment. Contaminants alone did not cause extensive damage to mycelia even though wounds were present. Sterile water used to disinfest the nematodes did not cause damage to mycelia even though wounds were present. Therefore, results of the experiment were considered to demonstrate that Ditylenchus sp. alone caused destruction of mycelia of A. campestris.

It was noted that non-disinfested nematodes cause pronounced disease symptoms within a short time (4-7 days) as contrasted with a longer period (3 weeks) required by disinfested nematodes.

Bacteria always accompanied non-disinfested nematodes and were rapidly spread and greatly multiplied in the cultures. Bacteria in cultures not containing nematodes did not spread rapidly, greatly multiply, nor cause extensive damage to hyphae. This suggests a relationship existed between activities of the nematodes and the bacteria which resulted in a more rapid rate of mycelia destruction.

DISCUSSION

The mushroom-spawn nematode, although considered a new species, is similar enough to Ditylenchus destructor, the potato-rot nematode, to be mistaken for it. It is possible that a casual observer would notice only the 6 distinct striae in the lateral field and overlook the remaining 5 very fine striae and would consider a specimen so examined to be the potato-rot nematode. Comparison of standard measurements would not necessarily serve to distinguish between D. destructor and the mushroom-spawn nematode, if the striations of the latter were not correctly counted. Measurements of some individuals of mushroom-spawn nematodes extend int the limits set by Thorne (39) for D. destructor. There is also the possibility that if the potato-rot nematode were living on mushrooms its measurements would be different. There is a report by Thorne and Fielding (40) in which they state that considerable variation in size and morphology resulted in D, destructor when transferred from potato to certain other seed plants. Transfer from a seed plant to a fungus host could similarly cause changes in appearance of the nematode.

Testing Ditylenchus obtained from diseased mushroom beds for ability to multiply when inoculated into potato tubers has been used as a criterium for considering such nematodes D. destructor. Standard inoculation procedures make no provision to avoid fungus contamination of tubers when they are inoculated with nematodes. None of the authors reporting successful transfer of Ditylenchus from mushrooms into potatoes have mentioned the point of establishing infection in

absence of such contaminants. In view of the preceding possibilities for incorrectly identifying the mushroom-spawn nematode, it is possible that it is to be more widely found as a pest in commercial mushroom culture than has been reported.

Reports of D. destructor associated with the mushroom-spawn disease abroad and in this country necessitated cross-infection tests. Ditylenchus sp. was therefore inoculated into known hosts of the potato-rot nematode and the latter inoculated into mushroom-spawn. It was not possible to establish the mushroom-spawn nematode in any of the hosts of D. destructor that were tested although several different methods were used, particularly, for inoculations of numerous varieties of potato tubers tested. Multiplication of this nematode occurred only in instances where fungus contaminants were present. D. destructor obtained from potatoes grown in Idaho, in the region of the type material for this nematode, failed to multiply in mushroom-spawn under conditions of experiments reported here. This is additional evidence that differences exist between these nematodes and therefore the identifications should be very carefully made.

Inability of D. destructor from Idaho to become established on mushroom-spawn does not eliminate the possibility that D. destructor cannot thrive on a fungus host. Dr. A. Baker in Canada (in personal communication) reports success in rearing some populations on fungus cultures but not others. The whole issue involving induced size and morphological variations, different host ranges, and existence of so-called strains serves to emphasize need for better microscopical observation and more thorough descriptions of Ditylenchus nematodes. The possibility of incorrectly identifying the mushroom-spawn

nematode as D. destructor has particularly serious implications in this country, because of the quarantine established against the potato-rot nematode.

Occurrence of females that can produce viable, reproducing offspring in the absence of a male parent was of such interest that special precautions were taken to verify the observation. Spermatozoa of Ditylenchus apparently are not released into or motile in water. Also, the fact that larvae do not possess genital openings and shed their cuticle at each molt would seemingly eliminate chance of impregnation of the isolated females. Examination of females that did produce progeny in absence of males did not show presence of spermatozoa. Therefore, this mode of reproduction is considered to be "facultative parthenogenesis" as bisexual reproduction is apparently the usual method. Ability to reproduce parthenogenetically would be of value for maintenance of the species. Availability of a Ditylenchus capable of parthenogenesis makes it possible to experiment with populations of definite ancestry and could lead to a better understanding of the extent of genetic variations possible within populations.

The observations regarding egg production and time for development to adulthood, although under culture tube and laboratory conditions, did give some concept of time and quantity of nematode increase. As environmental factors, particularly temperature, are extremely variable in commercial mushroom production, a more applied approach to considerations of number of nematodes present and of temperature effect upon them is discussed later in relationship to pathogenicity.

Studies of host range of Ditylenchus sp. were of value in several general respects. They served to demonstrate how the parasite can exist in soil; its degree of host specificity; its role as a possible parasite of other crops; and, as previously discussed, its value as a species diagnostic character. Of plant forms tested only fungi were found to be suitable hosts. The possibility is not eliminated that there may be algae, other than those tested, existing in soils that could serve as a food source. Perhaps, filamentous algae would be more readily attacked than unicellular forms. Failure to establish the nematode on any of the numerous seed plants suggests that the parasite is limited to lower forms of plant life. Fungi serving as suitable hosts represent the Phycomyceteae, Ascomyceteae, Basidiomyceteae, and Fungi Imperfecti. Despite the wide variety of fungi on which the nematode was able to thrive, there were species on which it could survive but did not greatly increase in numbers, and others were apparently unsuitable for the nematode's active exixtence. Detailed study of these variations could, perhaps, add to our understanding of the complex problem of host-parasite relations. A result of host testing is the conclusion that the mushroom-spawn nematode probably does not live in seed plants but exists as a parasite on various fungi in soil. Failure of the nematode to infect test plants representative of those used as straw in preparation of compost substrate for mushrooms, suggests presence of nematodes in compost is not due to use of infected plants. It is more likely that nematodes are present because of invasion of dead or dying plants when in the field or in the course of usage as straw for bedding, or in preparation and handling mushroom growing centers.

Ecological aspects of mushroom-spawn nematodes in soil would be a worthwhile subject for investigation. Tests showed that among the wide variety of fungi that can serve as suitable hosts, several important plant pathogenic forms are to be found. Since none of the crop plants were infected or showed evidence of deleterious effects being produced by these nematodes, the use of Ditylenchus sp. as a form of biotic control is a possibility. Large numbers of the nematodes easily reared on spawn cultures could be applied as inoculum to soil and would, perhaps, eventually become established as a more or less permanent member of the rhizosphere. Field surveys in the Pennsylvania mushroom growing region inidcated that the nematode may already be a commonly found inhabitant of the soil. The fact that the nematode can probably live as a parasite on various fungi in the soil is not regarded as a favorable characteristic by mushroom growers. This means that once a soil becomes infested there are good chances for its persistence.

Inability of Ditylenchus sp. to infect and become established in any of the crop and ornamental plants tested suggests the practice of utilizing compost from mushroom houses for fields and gardens is reasonably safe. This statement applies only to compost known to be free of other parasitic nematodes or where the mushroom-spawn nematode is the only stylet-bearing nematode present. There is, of course, always the possibility of seed plants existing which may serve as suitable hosts and variations within the large populations of the nematode may be selected for and result in "strains" pathogenic to higher plants. These same possibilities are applicable in considerations of using the nematode as a biotic control measure.

The phenomenon of anabiosis was considered primarily in its relation to control of the nematode. Some of the observations made, however, serve to illustrate that use of mushroom-spawn cultures of Ditylenchus sp. make available study material in quantities perhaps never before attained and in a manner unique in that it permits continuous observation. The increased possibilities for research on factors influencing anabiosis and on cytological and physiological phases of this interesting and important characteristic of certain nematodes cannot be overstressed.

The role of anabiosis as an important factor in planning control of the mushroom-spawn nematode was recognized early and became even more fully appreciated with increased field experience. It was desirable, therefore, to learn certain facts about this capacity for surviving periods of adversity. Ditylenchus sp. may exist in mushroom beds in enormous numbers and, as the experiments and observations showed, all the stages in the life history of the organism are able to exist in the dormant condition for varying lengths of time. Eggs were first to succumb, later the young larval stages; and most enduring were the 3rd and 4th stage larvae and adults of both sexes. It was discovered that there is gradual loss of viability in nematodes in anabiosis, but that survival periods were considerably in excess of time intervals between mushroom crops. Extended periods with fluctuating conditions of moisture and dryness causing repeated revival and dormancy do not result in death of all nematodes. Long exposures in the revived active state accompanied with lack of food do not result in death. Also long exposure of nematodes in shallow water (less than 1 inch) were not lethal. Such conditions would not

ordinarily be encountered in situations or materials related to mushroom culture.

The observation that there is apparently no one stage in the life history of Ditylenchus sp. that is best able to exist in the condition of anabiosis is at variance with observations of some other nematode species and genera. In these, one of the larval stages apparently is the most enduring or "Dauer" stage. Review of literature revealed that aggregation of the potato-rot nematode to form curds has not been reported, although this is known to occur with D. dipsaci, a related species. Dr. Baker (in personal communication) stated that he has never seen curd formation and knows of no such observation having been made with D. destructor. This may prove of some diagnostic value in distinguishing closely related species and may also serve as a basis for determining why rather similar nematode forms differ in capacity to enter anabiosis.

Observations of mushroom-spawn nematodes during curd formation suggested that a substance was secreted by them. This material coating the nematodes may be of protective value, particularly, when large numbers are aggregated and the amount of this substance present becomes appreciable. The quantity produced is often sufficient for chemical analysis which, if performed, might assist in devising better nematocides. It is fortunate that this material is water-soluble and that nematodes can be revived and maintained in the active state by the presence of liquid water, because they are then forced into a condition less resistant to heat and other means for nematode eradication.

Commercial production of mushrooms is different in many respects to production of other crops and is characterized especially by great variations in environmental factors, particularly temperature. Like other organisms, nematodes are affected by heat, therefore, experiments involving temperatures within the range encountered by these parasites were performed. Of principle concern to the pathologist is determination of thermal-death ranges. On the basis of available knowledge and a few preliminary experiments recommendations for control of the mushroom-spawn nematode were quickly evolved and found to be satisfactory. However, availability of unusually large amounts of nematodes in convenient form presented opportunities for research the results of which could be applicable to situations other than those found in mushroom culture.

Preliminary experiments indicated that nematodes in the state of anabiosis were more resistant to heat than those in an active state as was expected. However, finding that humidity apparently influenced degree of heat resistance of nematodes while in anabiosis was something new and required further consideration. Belehradek (6), in his comprehensive monograph about effects of heat on living organisms, points out that sudden temperature changes are different in action than are gradual changes. In general, quick changes introduce the factor of "heat shock" which, being in itself harmful to the organisms, constitutes another factor to be considered in determining thermal death ranges. In mushroom pest eradication, rapid temperature rises are encountered only in steam treatment of casing soil and temperatures usually attained are well above minimal values for nematode death. However, actual testing of commercial soil

treatments as well as studies within mushroom houses showed that a variety of moisture and temperature conditions exist. It was evident that nematodes were submitted to gradually increased temperatures while in the active state in water and in the state of anabiosis following previous exposures to varying humidity levels. The final experimental design therefore, endeavored to incorporate the factors of gradually increasing temperatures and the different thermal responses of active nematodes and of dormant nematodes conditioned to extremes of humidity content. Consideration of these factors in determination of thermal-death ranges apparently was done for the first time in these experiments.

Results (Figure 24) suggest that action of short exposures of heat may be different in active nematodes than in nemas in anabiosis. The former show a more or less level thermal-death curve but with an initial abrupt drop to this level indicating that a certain narrow temperature range exists above which death of the nematodes result regardless of the briefness of exposure (under conditions of the experiment). Use of the thermistor-observation slide confirmed this critical temperature to be close to 46° C. at which point coalescence of reserve oil globules occurred. Exposures for over 1/4 hour at 45° C. also resulted in death of the nematodes, but were not correlated to any visible cytological change. However, temperatures above 39° C. produced rigor and rather pronounced tremors which quickly ceased when temperatures reached 44-45° C. This suggests that something else had happened to nerves transmitting the rapid and strong stimuli producing muscular actions evident during rigor and tremor behavior. Death of active nematodes exposed for increasingly longer

periods at temperatures from below 44-45° C. to about 39-40° C. may be due to other causes. Rigor and tremors brought about in this temperature range when prolonged could conceivably result in harm to the nerve and muscle systems, or they may be symptoms of damage to centers of nervous control, the ganglia.

Thermal-death curves for nematodes in anabiosis differ from that for active nematodes in that they show a less abrupt drop in lethal temperatures as periods of exposure are increased. This suggests that death in this phase of the curves is not due to a melting point or a surface phenomenon except, perhaps, at the 1 minute exposure period but is due to some change or reaction in which time is a factor. The more or less level phase of the curves indicates particular temperature levels exist which when once reached and maintained for more than certain minimal times result in death. No attempt will be made to explain the reasons for death in nematodes in anabiosis as so little is known about physiological and cytological conditions existing when the animals are in this state. It can be inferred that water content of nematodes is a factor involved as evidenced by the lethal-temperature range being lower for nematodes in anabiosis preconditioned to high humidities. The degree of hydration may be of significance in altering melting points, for example, reserve oil globules which are also present in nematodes in anabiosis do not coalesce at 46° C. as occurs in active forms. It may be necessary to point out that observation at high magnifications of internal organization of nematodes in anabiosis is very difficult because they are examined in the dry state and are not as transparent as when in the moistened, revived condition. Addition

of water begins almost immediately to produce changes in the nematodes so that they cannot be considered to be in the same condition. Perhaps, use of a substance such as paraffine oil would improve optical resolution without causing rehydration and still not in itself be detrimental to the nematodes. There are certain similarities between nematodes in the state of anabiosis and organisms preserved by freezing-dessication techniques. Preliminary attempts to do this with the mushroom-spawn nematode have been unsuccessful, probably due to the greater complexity of nematodes in comparison to other organisms surviving such drastic treatment. The author is of the opinion, however, that there is a chance for accomplishing this.

The previously discussed thermal-death ranges and microscopical observations show similarity to findings of other workers. Hoshina and Godfrey (23) working with one of the root-knot nematodes found a plateau in the thermal-death curve for larvae at 50-51° C. and for eggs at 53-56° C. but did not attempt to interpret the results. Chitwood (14) states that reserve oil droplets in eggs and larvae of Meloidogyne javanica (Treub 1885) Chitwood 1949 coalesce at 53-54° but not at 52° C.

The experiment in which active nematodes were maintained at temperatures from 32-41° C. (95-115° F.) for extended periods was to obtain extension of the thermal-death curve (Figure 24) for active nematodes. This experiment was conducted to determine if long exposures to relatively low temperatures could also be lethal. Active mushroom-spawn nematodes are subjected to these conditions in preparation of compost for use in mushroom beds. Results showed that such exposures do cause death and this fact indicates that efforts by

growers to take advantage of the natural heat of composting can be a worthwhile low-cost means of eradication. The nature of lethal action by heat in these temperature ranges is evidently not that of physical alteration but is, perhaps, that of deleterious upsets of metabolic processes.

After about 3 months exposure to below freezing temperatures nematodes not in curd masses were killed, while nematodes in anabiosis survived more than 4 months. These results suggest that Ditylenchus sp. can exist through winter-time conditions in the field or in other locatinns. The reason for death when it does finally occur was not determined, but the prolonged exposure periods required suggest that again metabolic processes may be affected rather than direct physical damage being involved. Anabiosis provides the nematodes with greater resistance to cold conditions due, perhaps, to reduced metabolic rates or to reduced amount of unbound water which presumably characterizes this state of being.

Determination of nematode viability by various methods has been reviewed by Smith (33). Boyd (8) in 1941 seems to have been one of the first to apply a staining technique to indicate death of nematodes, although other workers had made observations regarding staining properties of living and dead nematodes. Boyd using iodine in potassium iodide solution found dead larvae of Heterodera rostochiensis Wollenweber, 1923 stained yellow, but the test had a serious detriment in that iodine is itself lethal. Fenwick and Franklin (18) found that a 1:10,000 solution of neutral red would not stain living Heterodera larvae and could be used as a measure of injury and of death under certain conditions of heat and possibly chemical action. Smith (33)

tested a number of stains to determine viability of Anguina tritici (Steinbuck, 1799) Filipjev, 1936, the wheat-nematode, after exposure to nematocides. The stain most used was gentian-violet which he said gave excellent results with the wheat-nematode but was unsatisfactory with other nematodes including the stem and bulb nematodes Ditylenchus dipsaci (Kühn, 1857) Filipijev, 1936, the root-knot nematode, and vinegar eel nematodes. His observation that living, stylet-bearing nematodes of the family Tylenchidae stained in the manner to be expected for living, non-stylet bearing nematodes such as the vinegar eelworm, suggests that conditions of the nematodes were not normal or that misidentification of the stylet-bearing forms had occurred. This view is further substantiated by results of tests to determine a reliable staining procedure to distinguish living and non-living mushroom-spawn nematodes. This Ditylenchus species never showed obvious staining in the region of the head or vulva with any of 25 biological stains tested unless the animals were dying or dead; this has been found to be true with other genera in the Tylenchidae and Aphelenchidae. Stain uptake by dead nematodes was apparently due to a change in permeability of the cuticle or hypodermis, but considerable differences in reactions were also noted between stains. Dyes showing best penetration and intensity of staining were in the basic quinone-imide and phenyl-methane groups. Stains used to indicate presence of reducing agents, such as methylene blue and 2,3,5-triphenyl tetrazolium chloride, were not found successful when used for this purpose in order to determine viability. Gentian violet was the stain finally selected for routine use because it rapidly and distinctly stains dead and dying nematodes, does not lose color or

staining ability with prolonged exposure, and is non-toxic to the stylet-bearing nematodes.

Mushroom-spawn nematodes may be present in compost containing mushroom spawn in varying stages of development from time of inoculation to fruiting body formation. Temperature conditions may also be variable, ranging from warm temperatures existing shortly after pasteurization treatment at the time of inoculation of spawn into the compost to the cool growing temperatures of refrigerated mushroom houses. In order to measure and thus better understand effects of varying temperatures upon rate of destruction of mushroom mycelia and upon the nematodes themselves, it was necessary to start with equal amounts of mushroom mycelium already present and with equal numbers of nematodes. Manure-spawn cultures inoculated with nematodes were used to fulfill these requirements and to provide conditions in the substrate (bacteria and other factors) similar to those that exist in mushroom beds. Results obtained in this manner represent the condition of mushroom beds already permeated with mycelium. Time for effects of destruction would probably be shorter if the spawn in the beds was not as extensively developed.

Increased temperatures resulted in quicker destruction of mushroom-mycelia, as might be expected. However, although the rates of destruction were similar at 24° C. (75° F.) and 30° C. (85° F.), the number of nematodes present at the end of the test period were considerably different. Relatively fewer nematodes in cultures at 30° C. indicated temperature affected some factor besides rate of multiplication of the nematodes or their activity (which appeared to be the same as that of nematodes at 24° C.). It is suggested that this

factor is the increased rate of multiplication and metabolic activity of bacteria at the higher temperatures. Bacteria were present in compost of the spawn-cultures just as they always are present in compost of mushroom beds. Rates of mycelium destruction at the other temperatures was also found not to be determined alone by the amount of nematodes present. For example, the nematode populations present and the time of complete destruction of the spawn at 24° C. and 13° C. were about the same but only 40 days were required for the former as contrasted to 85 days for the latter. Although maximum nematode increase occurred at 18° C., the rate of spawn destruction was not as rapid as at 24° C. and 30° C. with less rapid nematode multiplication. Absence of detectable destruction of spawn at 7° C. apparently was due primarily to the inactivating effect of temperature upon movement and feeding by nematodes. The general conclusion to be drawn is that temperature is an important factor in determining rate of destruction of mycelium. Its effect is upon activity and reproductive rates of the nematodes present and reproductive and metabolic rates of bacteria, which as secondary invaders are dependent upon the activity and number of nematodes present. The observation of decreased numbers of females in proportion to males with decrease in temperature is interesting in its genetical implications. Also to be noted is the fact that a higher proportion of females did not evidently correlate with a higher rate of nematode increase. Results of this experiment show that mushroom crops raised under cooler temperature conditions have the potential advantage of a retarded rate of mycelium destruction.

At the time of discovery of Ditylenchus sp. in commercial mushroom houses there was little doubt that it was living as a parasite

on mushroom mycelium which usually became totally destroyed. Microscopic observation revealed that nematodes did make frequent punctures of hyphae with their stylets which exposed hyphal contents to action of other organisms, especially bacteria. The periods of prolonged contact by nematodes with stylets inserted into hyphae suggested that they also feed on hyphal contents. Thus, two possible explanations for destruction of mycelia were available. Attempts to resolve the relative importance of each was largely academic in interest, for it should be realized that under ordinary circumstances bacteria are always present in compost medium in which mushrooms are grown. There seemed no reason to doubt that the extrusion of cytoplasm from numerous punctures and its exposure to bacterial action could cause serious harm to hyphae. There was less evidence regarding importance of feeding action, which may itself produce two kinds of deleterious effects: introduction of a salivary secretion and withdrawal of cytoplasmic contents. Although actual flow of granules and other cellular inclusion in hyphae to the point of stylet insertion was not observed, the occasional movements of the muscular, oesophageal bulb, several minutes after insertion of the stylet, suggested sucking of hyphal contents. No injections of a secretion into hyphae were observed.

Feeding action of the mushroom-spawn nematode is apparently somewhat different than that reported by Christie and Arndt (15) for Aphelenchoides parietinus (Bastian, 1865) Steiner, 1932 and Aphelenchus avenae Bastian, 1865. These nematodes, while feeding on fungi, were observed to exhibit rapid muscular movement of the oesophagal bulb immediately after insertion of the stylet. Withdrawal of hyphal contents was so forceful that various cellular inclusions were seen

moving to the stylet.

Although no salivary secretion was seen to be injected by Ditylenchus sp., it is believed to occur. Use of lapse-time photomicrography with phase-contrast illumination might make this study possible. Ditylenchus sp. is provided with a well-developed dorsal oesophageal gland, the duct of which opens into the lumen of the oesophagus close to the base of the stylet. Goodey (19) summarized evidence for the flow of pathogenic secretions from the stylet of nematodes, but refrained from referring to the oesophageal glands as salivary in the absence of adequate evidence. Linford (25) as a result of later observations said, "It now appears, however, that the dorsal gland, in esophaguses of the Aphelenchus and Ditylenchus types is definitely salivary in function." The long feeding period of the mushroom-spawn nematode, the delayed action of its muscular oesophagal bulb, and the fact that destruction occurs as a result of feeding in the absence of bacterial contaminants are all considered good, but indirect evidence for injection of a salivary secretion. This probably aids in liquefaction of host cytoplasm in preparation for uptake through the minute stylet orifice.

As stated before, it was desired to determine the effect of Ditylenchus sp. upon mushroom mycelia without the complicating factor of contamination, although this situation probably never exists in mushroom beds. The experiment was designed to test the pathogenic nature of the nematode on its host, A. campestris, and is apparently the first record of proof of pathogenicity of a plant-parasitic nematode in demonstrable absence of contaminating organisms. Metcalf (27), who pioneered the use of agar as a culture medium for nematodes,

may have been the first person to raise nematodes germ-free. Dougherty and Calhoun (16), who do not recognise Metcalf's claim of pure-culturing on the basis of their own experiments and because he did not record any specific tests for sterility, have succeeded in rearing saprophytic nematodes in germ-free cultures. Other workers have utilized methods of surface sterilization of nematodes for various purposes but have not attempted establishment of infection under germ-free conditions.

Results of this experiment showed that Ditylenchus sp. could cause destruction of mushroom mycelia although no contaminants were present. Destruction was considered to be due to the dibilitating effect of the nematodes' feeding actions and possibly due to deleterious effect of their metabolic products. The relatively long time required for elimination of the mycelium under germ-free conditions suggests that the nematode is weakly parasitic. This is substantiated by observations of its effects on other fungi which even in the presence of bacteria, may show slow decline although the nematodes thrive and multiply. The experiment showed that presence of contaminants is an important factor in accelerating rate of destruction of mycelium attacked by the mushroom-spawn nematodes.

One other experiment was performed in regards to pathogenic effects of the nematode. Its purpose was to determine rate of mycelial destruction at a constant temperature when the numbers of nematodes initiating infestation were different. As expected, the more nematodes present the shorter the time to complete elimination of mycelium. Times from first appearance of spawn destruction to total elimination of mycelium were about the same regardless of numbers of nematodes

initiating the infestations. This correlated with observations that about equal numbers of nematodes were evident at the times spawn destruction was first apparent. Results of this experiment demonstrate that local infestations initiated by relatively few nematodes can also result in total destruction of mycelium within the growing and harvesting period for cultivated mushrooms. It should be noted that decline in production of mushroom fruiting bodies in mushroom beds becomes evident at about the time destruction of mycelium is first detectable. Also, the nematodes may be present when the mushroom spawn is just beginning to develop and spread and would probably cause crop failure to appear earlier in these instances.

In addition to the mushroom-spawn nematode, other forms exist which have actually been observed to feed on fungi and could conceivably cause similar damage to cultivated mushrooms. In addition to specific forms already mentioned, there are also Aphelenchoides limbari Steiner, 1936 (3) and Ditylenchus dipsaci (Kühn, 1857) Filipjev, 1936 (26).

SUMMARY

The purpose of this work was to study the biology and pathogenicity of the mushroom-spawn nematode, Ditylenchus sp., in relation to a disease it produces in cultivated mushrooms.

The mushroom-spawn nematode, which is very closely related to the potato-rot nematode, Ditylenchus destructor, was shown to differ from it morphologically and physiologically. The principal distinctions are that Ditylenchus sp. possesses: 11 striae in the lateral fields (6 distinct, 5 often less distinct); generally smaller size; capacity for collecting into aggregates of nematodes in anabiosis called "curds;" and inability to become established in potato tubers or other seed-plants.

The mushroom-spawn nematode is able to reproduce parthenogenetically although bisexual reproduction is apparently the common method. Under experimental conditions, development from egg to adulthood was 12-13 days; eggs may be laid within 24 hours after the pre-adult molt. Egg production is variable between individuals and with individual females and reaches 7 eggs per day within 5-6 days after egg laying begins.

Ditylenchus sp. was not able to sustain itself in an active condition on various algae tested as possible hosts. Multiplication did not occur on any of a large number of seed plants inoculated with the nematode, nor were harmful effects observed as a result of its presence. No multiplication occurred in 12 varieties of potato tubers or in other plant parts known to be hosts of D. destructor, except

when contaminating fungi were present. Ditylenchus sp. parasitizes a wide variety of fungi, including plant pathogenic species, and exhibits varying degrees of success in becoming established on different fungi.

This nematode has the capacity of existing in a condition of dormancy called anabiosis. Eggs were least able to survive, existing about 50 days; 1st and 2nd stage larvae ceased reviving after 100 days; and older larvae and young adults survived for over 3 years. Formation of "curds" was accompanied by presence of a substance, presumably a secretion of the nematode, which is water-soluble and possesses a characteristic odor. Nematodes in anabiosis were more resistant to extremes of cold and heat.

Thermal-death curves were obtained for active nematodes in water and nematodes in anabiosis as curd masses previously conditioned to high and low humidities. Under conditions of the experiment, the thermal-death range for active nematodes occurred along the following points: 50° C./1 min.; 45° C./15 min.; 40° C./24 hrs. Nematodes in curd masses previously exposed to high humidity were killed at 60° C./1 min.; 55° C./30 min.; 50° C./3 hrs.; and 45° C./24 hrs. Nematodes in curd masses exposed to low humidity were killed at 75° C./1 min.; 70° C./15 min.; 65° C./1 hr.; 55° C./3 hrs.; and 50° C./5 hrs. Active nematodes were found to be sensitive to changes as small as 1° C., ceased all activity at 33-38° C.; entered a condition of rigor at 39-43° C.; and underwent coalescence of reserve oil globules at 46° C. or above. Recovery to apparently normal activity was possible up to 45° C. depending upon duration of exposure to heat. Active nematodes in mushroom-spawn cultures on manure compost were killed

after 3 days exposure to 38° C. and after 1 day at 41° C. Nematodes in anabiosis withstood 120 days exposure at -4° C. and active nematodes were killed between 90-120 days at the same temperature. Nematodes were reduced in activity at 7° C., and showed no activity at 2° C. and lower. Within 13° C. to 30° C. the proportion of females to males increased with increased temperature.

Increased temperature shortened time to complete destruction of mushroom-spawn and increased rate of destruction after first appearance of symptoms. At high temperature the rate of destruction was not correlated with increase in nematode population. Optimum temperature for nematode population development was 18° C.

The mushroom-spawn nematode was observed to make numerous punctures and to feed on mushroom hyphae. Reasons are presented to show that these resulted in exposure of hyphal contents to bacterial action and to debilitation due to withdrawal of cytoplasm and probable injection of a salivary secretion. Number of nematodes present and rates of mycelium destruction were correlated. Pathogenicity of the nematode on mushroom mycelium in germ-free cultures was proven, and is possibly the first demonstration of pathogenicity of a plant parasitic nematode in absence of contaminating organisms. Results of the germ-free experiments and observations of the effects of the nematode in culture, indicate that it is weakly parasitic. Rapid destruction of mushroom mycelium is dependent upon presence of secondary invaders, especially bacteria, which are always present in mushroom compost.

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Figure 1. Types of mushroom-spawn culture bottles used for rearing large populations of Ditylenchus sp. and for quantitative measurements of spawn destruction under various conditions.

Figure 2. Conical dishes used for changing solutions covering nematodes and for concentrating nematodes in a small volume of liquid.

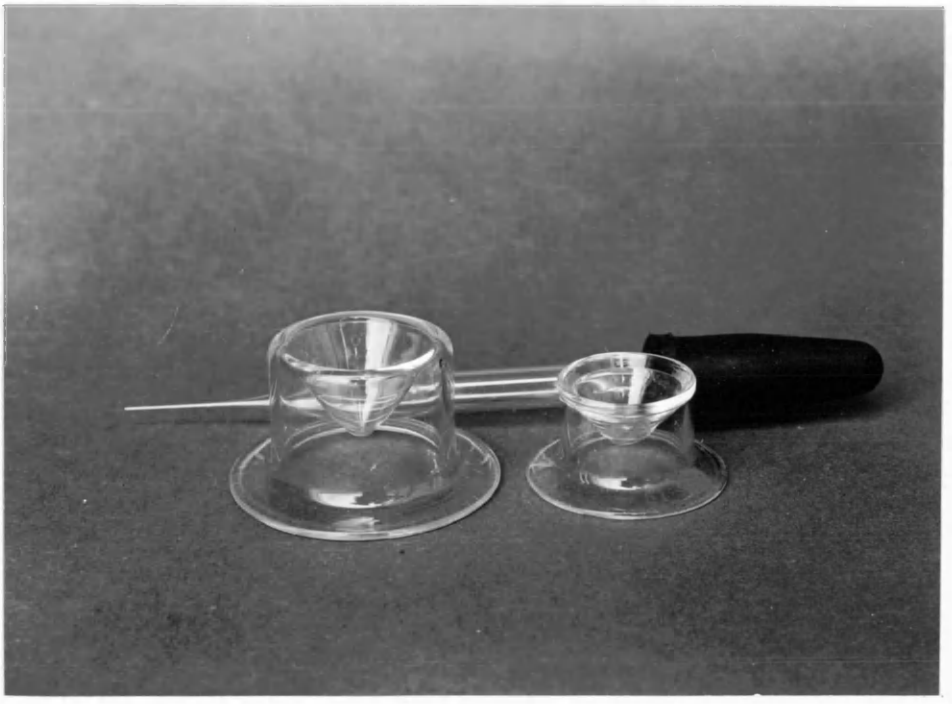

Figure 3. Inserted-tube method of inoculation used to determine ability of Ditylenchus sp. to become established and multiply on potato-tubers and other fleshy plant parts.

Figure 4. Tuber-eye inoculation method used to determine ability of Ditylenchus sp. to enter potato-tubers without artificial wounding and to determine its ability to infect and injure shoots and adventitious roots which develop within the tube.

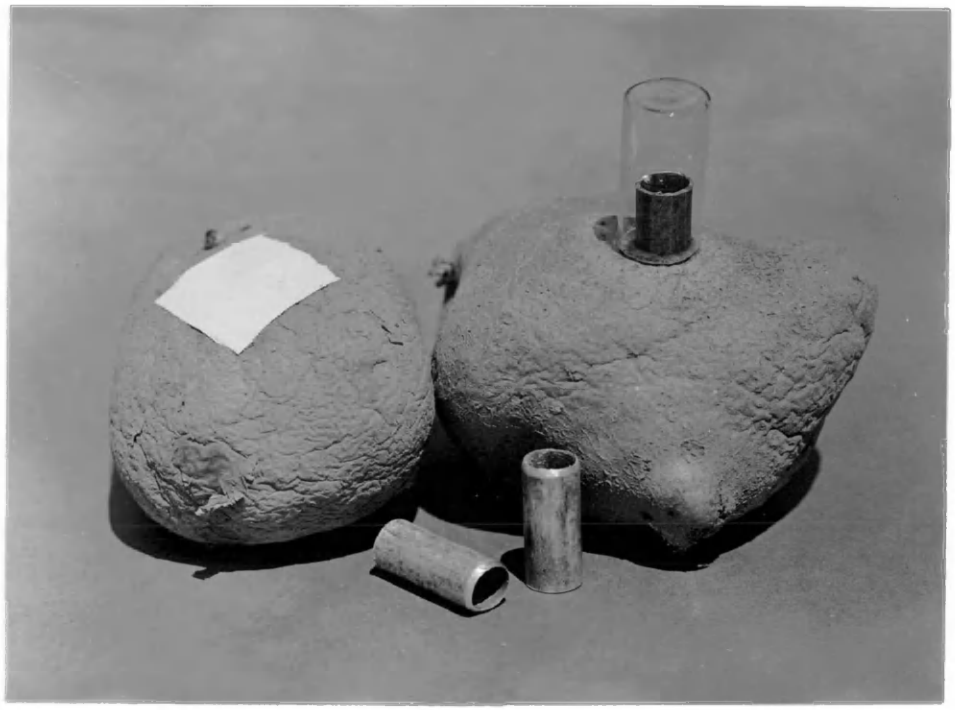

Figure 5. Moist-chamber used to provide high humidity and to maintain a moisture film on seedlings planted in nematode-infested potting medium and inoculated in buds and axils with suspensions of the nematodes.

Figure 6. Schematic circuit diagram of the thermister thermometers used in experiments to determine the effects of heat upon the mushroom-spawn nematode.

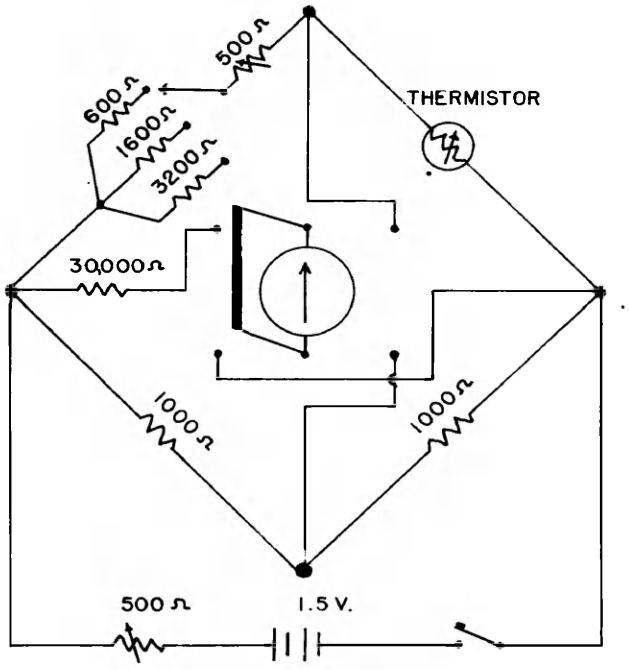
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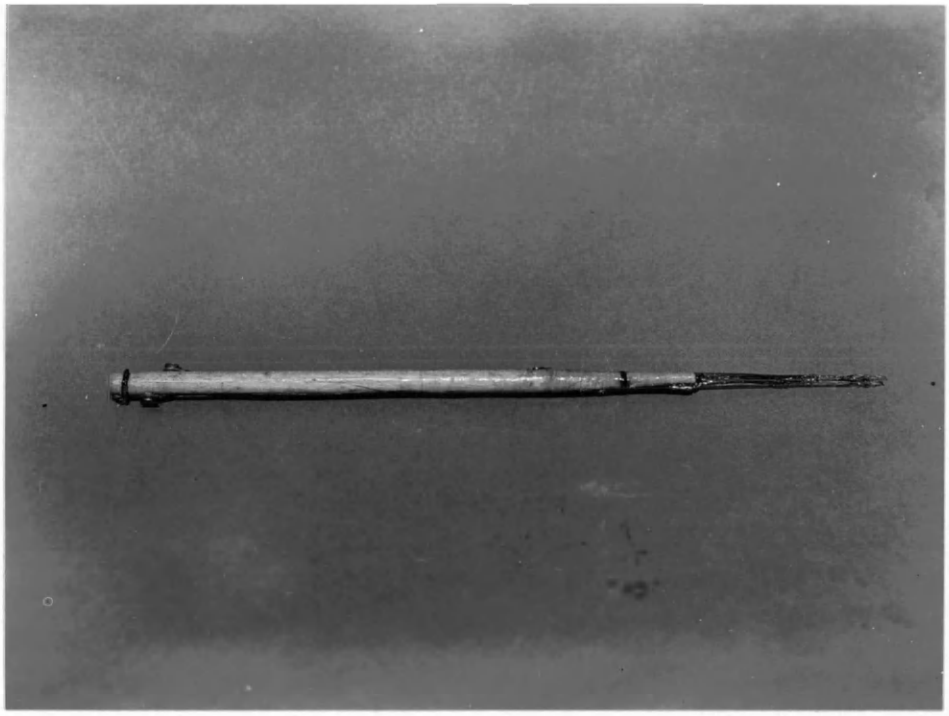
Figure 7. Probe with thermistor bead mounted at its tip which was used to measure temperature changes within sample-vials utilized in thermal-death range studies of the mushroom-spawn nematode.

Figure 8. Micro-observation slide with thermistor bead incorporated into it to measure temperatures to which nematodes were exposed while being observed microscopically.



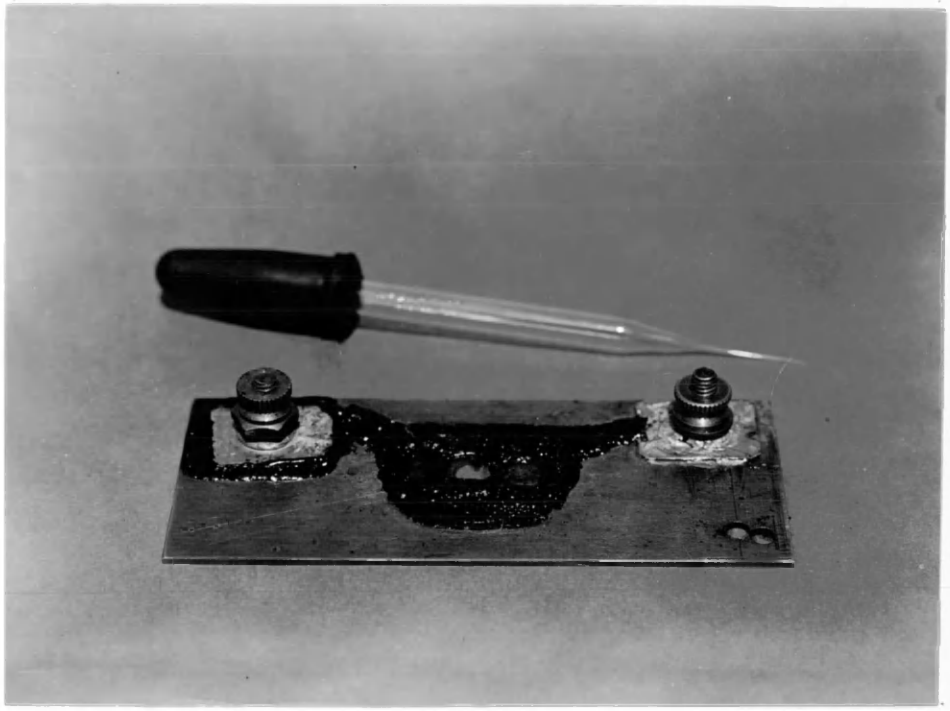


Figure 9. Warming-stage with built-in condensing lens and thermometer and the rubber-bulb pump used to circulate heated water through the stage.

Figure 10. Complete assembly of equipment used to make microscopical observations of effects of heat at measured temperatures upon mushroom-spawn nematodes.

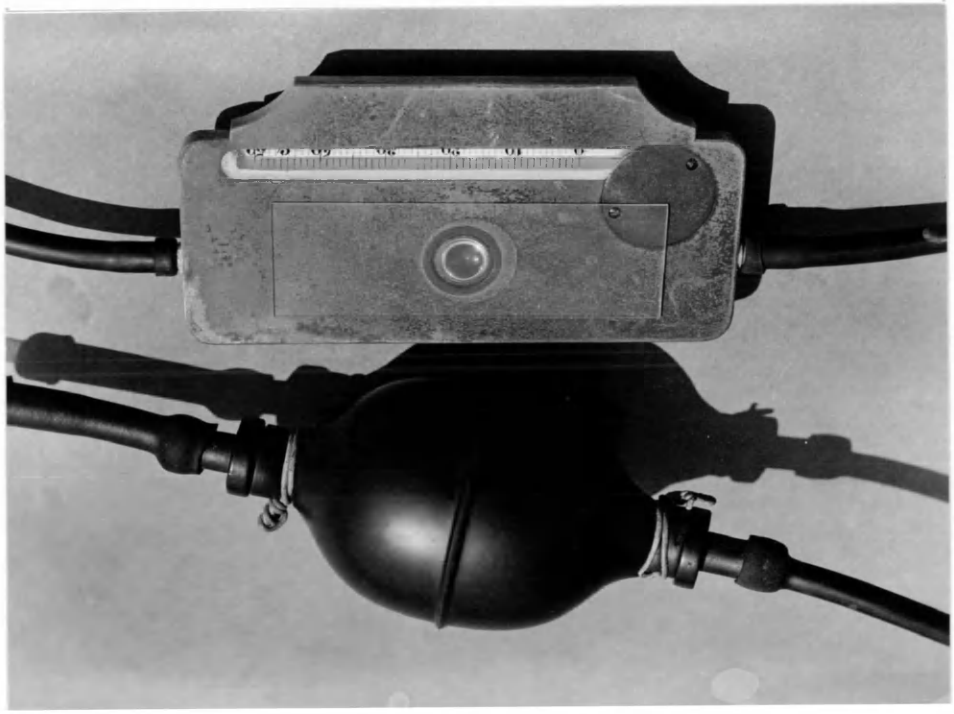
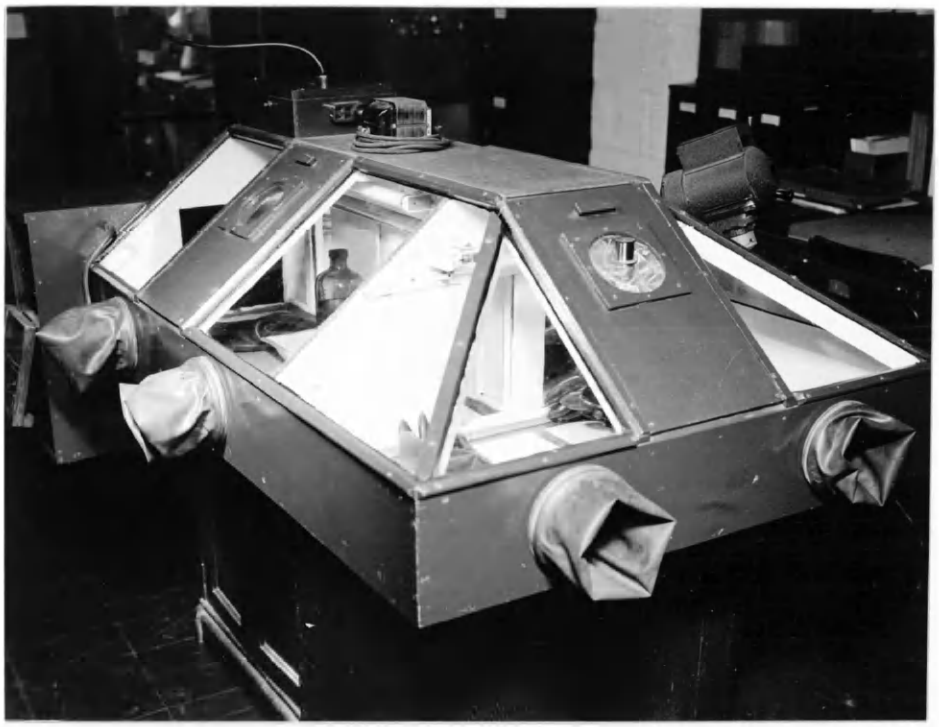


Figure 11. Sterile-chamber apparatus used in experiments to determine pathogenicity of Ditylenchus sp. on mushroom mycelium under germ-free conditions.



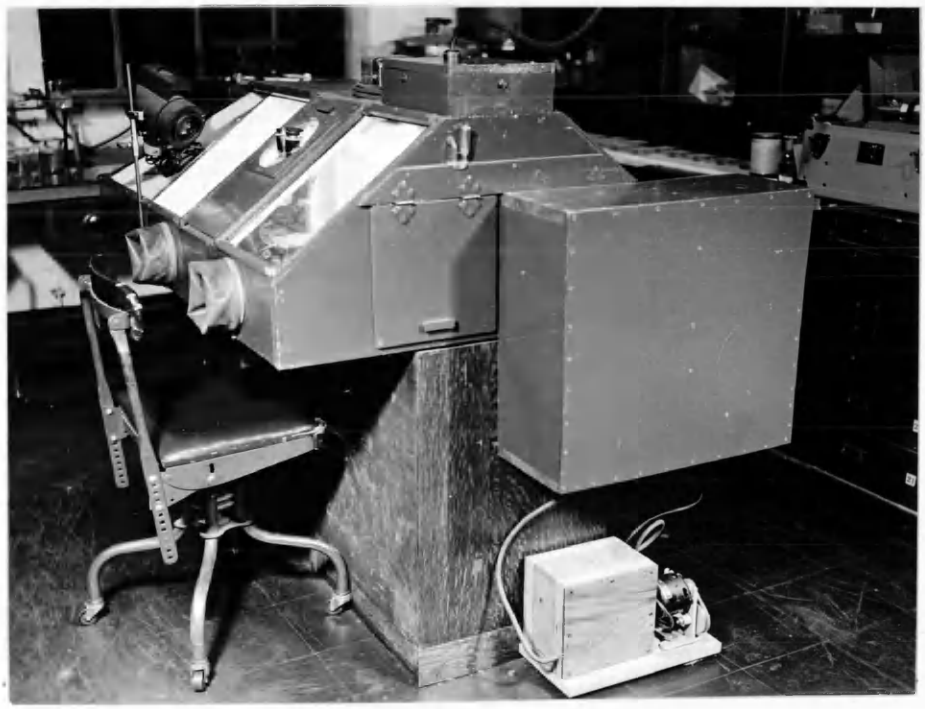
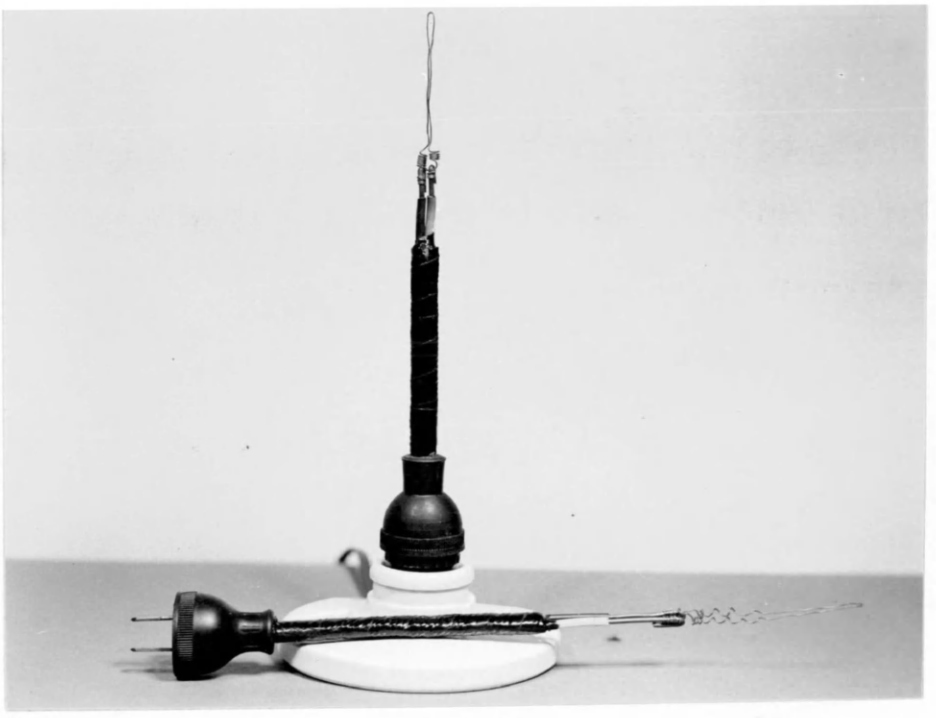


Figure 12. Electrically heated inoculating instruments used in sterile-chamber to eliminate need of open flames for inoculating instrument sterilisation.

Figure 13. Series of nematode inoculated manure-spawn culture bottles showing symptoms of progressively increased destruction of mushroom-mycelium. These cultures had been inoculated at the same time but with the different indicated numbers of Ditylenchus sp.



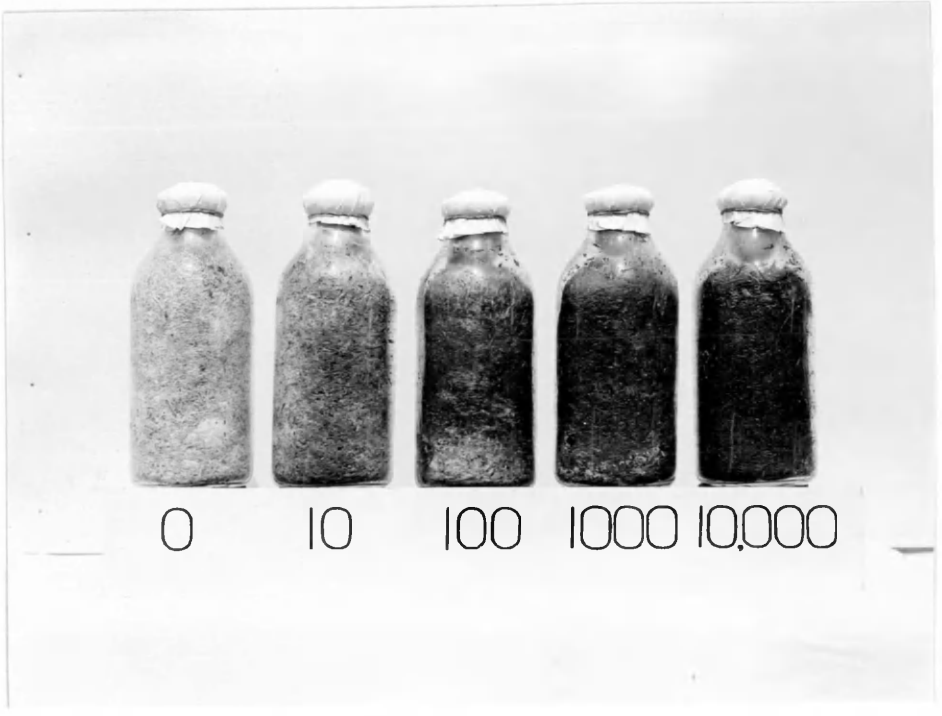

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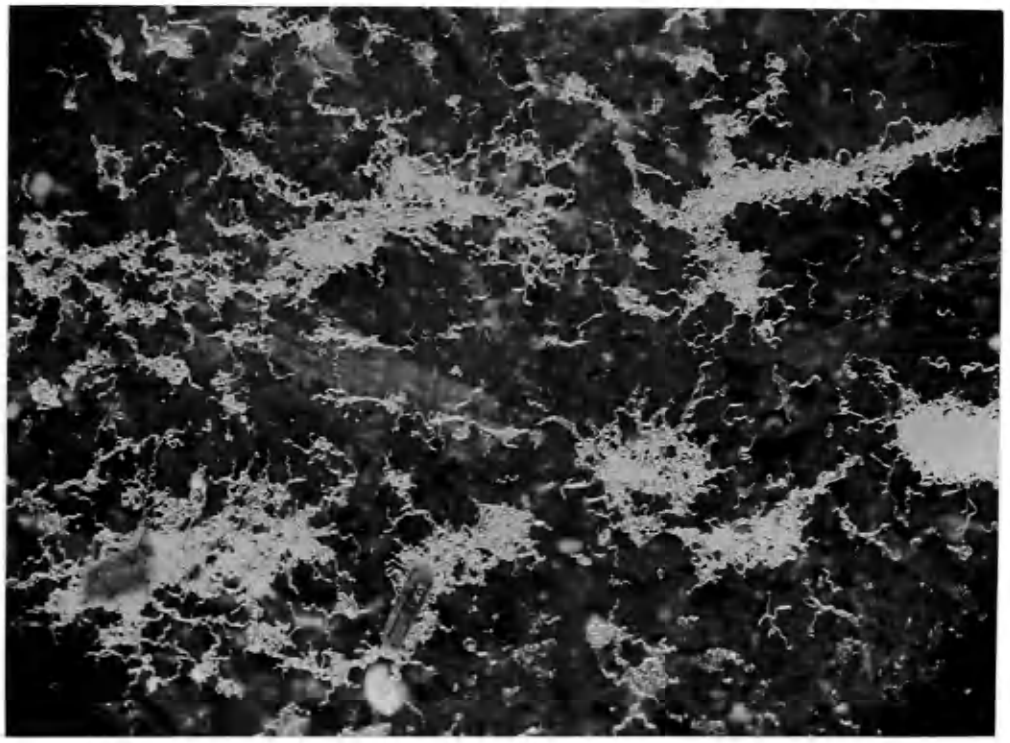
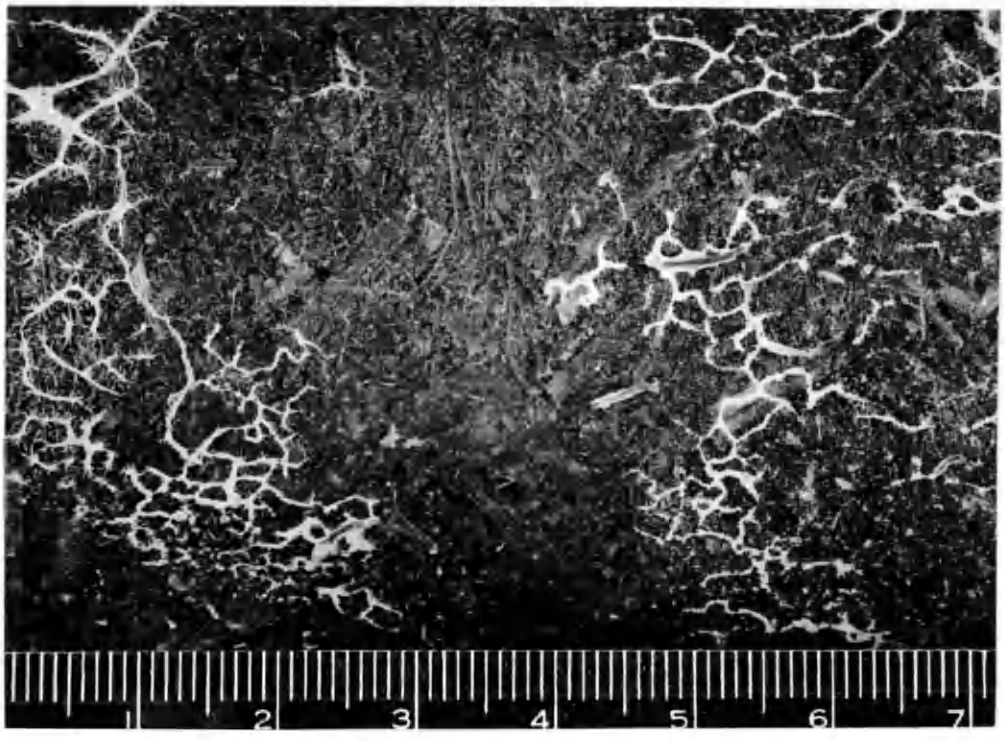
Figure 14. Apparatus used to provide standardized illumination for photometric determination of mushroom-spawn destruction. A section of the box cover was removed to show placement of light bulbs, diffusing glasses, and the glare-eliminating mask through which the surface of the spawn culture appears for reflectance measurement as the bottle is rotated. The photoelectric cell unit is clipped in place over the aperture in the lamp-end of the box and reflectance is read on the photometer to which it connects.

Figure 15. Photomicrographs of female (left) and male (right) Ditylenchus sp. nematodes. Each picture is a composite of serial photomicrographs and presents a median longitudinal optical section of the nematode as seen with an oil immersion objective

Figure 16. Macrophotograph of Ditylenchus sp. migrating and aggregating on inner surface of a spawn-culture bottle following extensive destruction of the mushroom mycelium.

Figure 17. Macrophotograph of Ditylenchus sp. in characteristic dendritic aggregations prior to curd formation. The scale is in millimeter and centimeter units.

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6
7

Figure 18. Aggregations of Ditylenchus sp. coating compost fragments on the surface at the top of a manure-spawn culture.

Figure 19. Macrophotograph showing size comparison of a common pin to a curd fragment which contained over 6,600 mushroom-spawn nematodes in anabiosis.

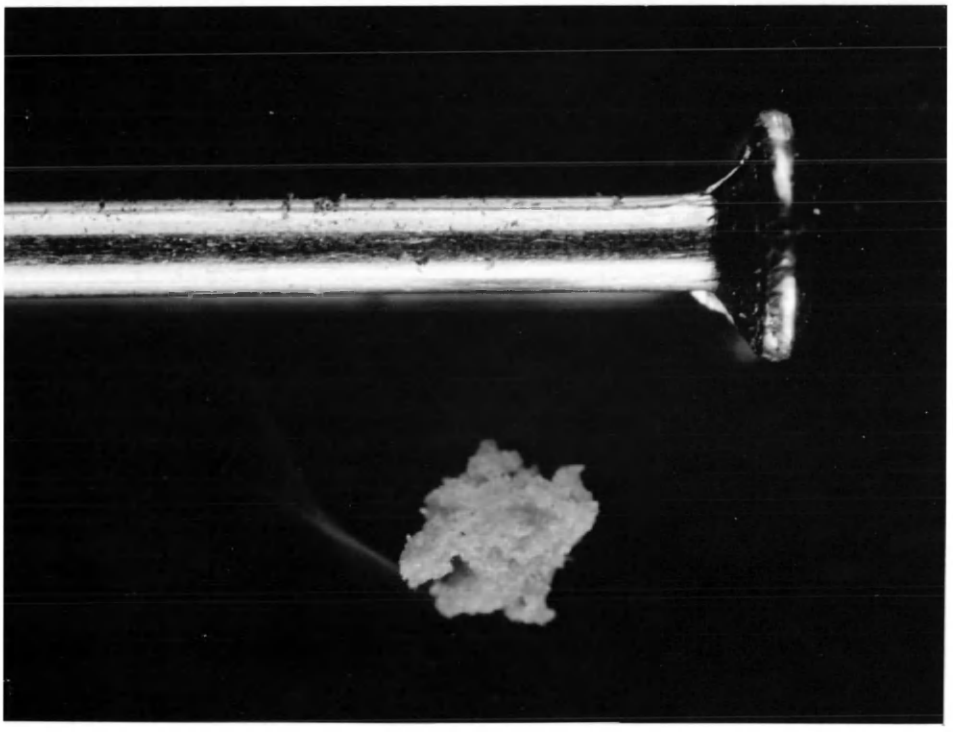
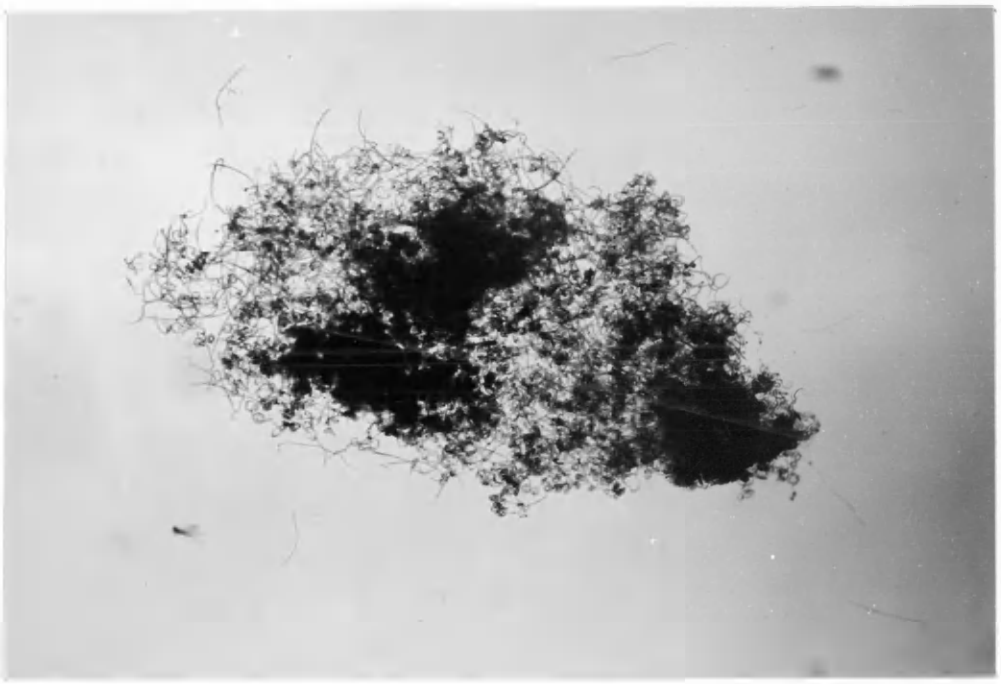


Figure 20. Progressive dissolution of the curd matrix in water and release of the inactive mushroom-spawn nematodes.



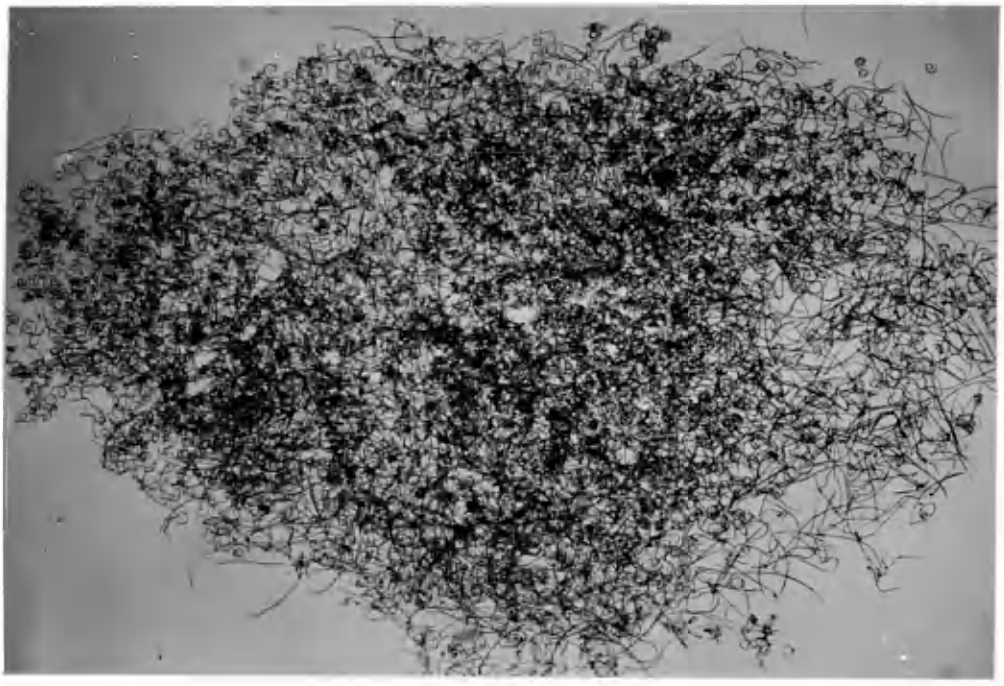
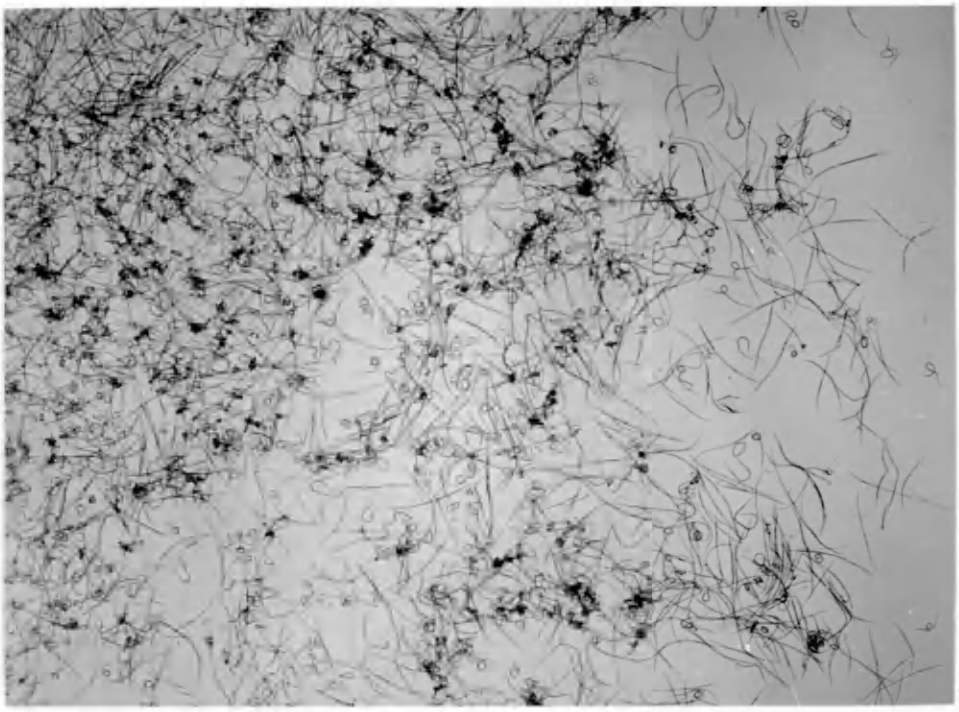
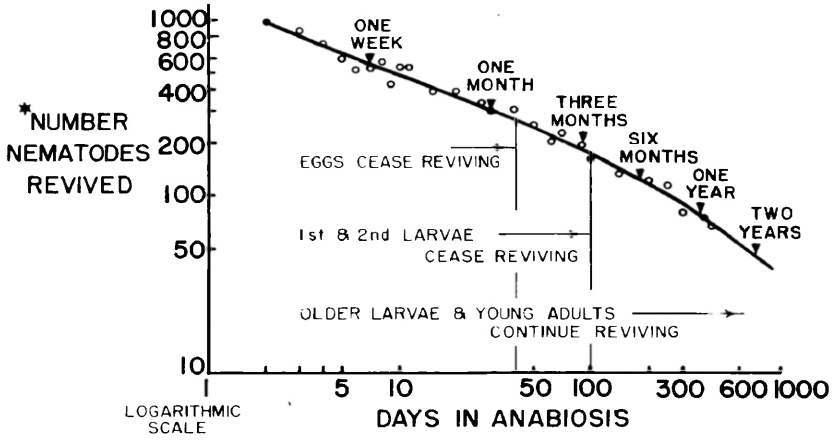


Figure 21. Mushroom-spawn nematodes in various stages of recovery to normal activity after release from the curd mass.

Figure 22. Comparison of the number of mushroom-spawn nematodes and their life history stages revived after increasingly longer durations in the condition of anabiosis.



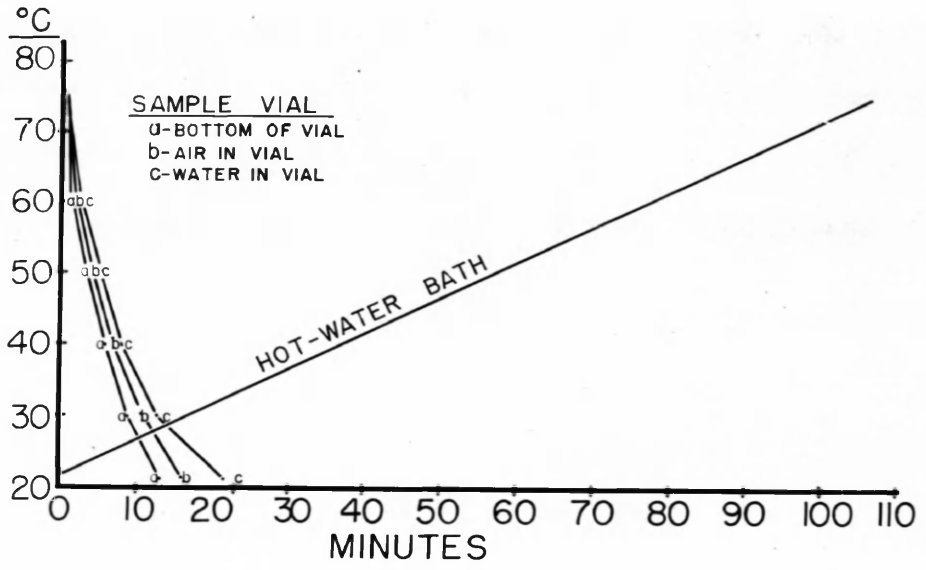
SURVIVAL OF DITYLENCHUS SP. IN ANABIOSIS



*AVERAGE OF FOUR .04 GM. SAMPLES

Figure 23. Temperature change of the hot-water bath as it was heated and temperature changes of different locations within sample vials after being placed in insulation for gradual return of heat-treated nematode samples to room temperature.

Figure 24. Comparison of thermal-death curves of Dity lenchus sp. in the active condition and in the state of anabiosis following previous conditioning to high and low humidity.


°C
80
70
60
50
40
30
20
SAMPLE VIAL
a-BOTTOM OF VIAL
b-AIR IN VIAL
c-WATER IN VIAL
HOT-WATER BATH
0 10 20 30 40 50 60 70 80 90 100 110
MINUTES

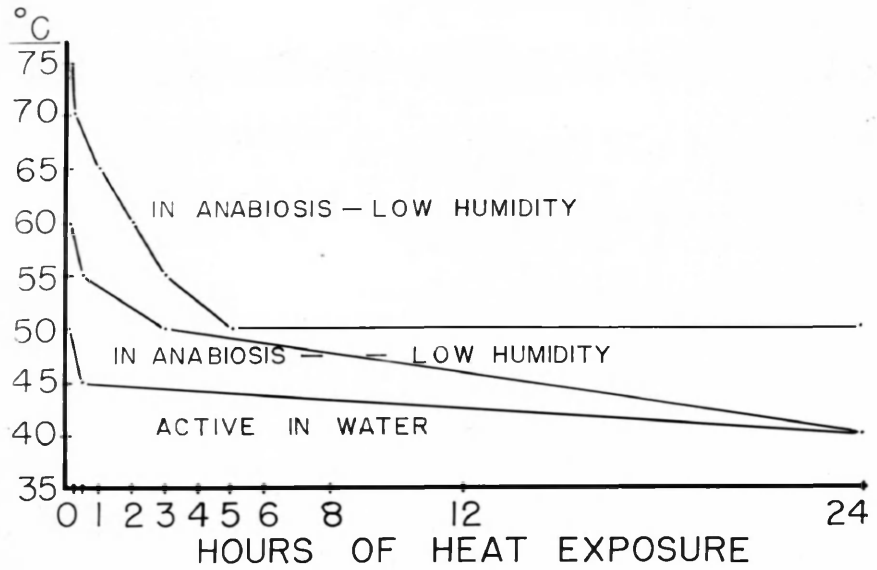

°C
75
70
65
60
55
50
45
40
35
IN ANABIOSIS — LOW HUMIDITY
IN ANABIOSIS — — LOW HUMIDITY
ACTIVE IN WATER
0 1 2 3 4 5 6 8 12 24
HOURS OF HEAT EXPOSURE

Figure 25. Photomicrographs showing comparison of a normal, untreated mushroom-spawn nematode (upper) and a treated nematode (lower) submitted to heat at temperatures causing coalescing of oil droplets in the cells.

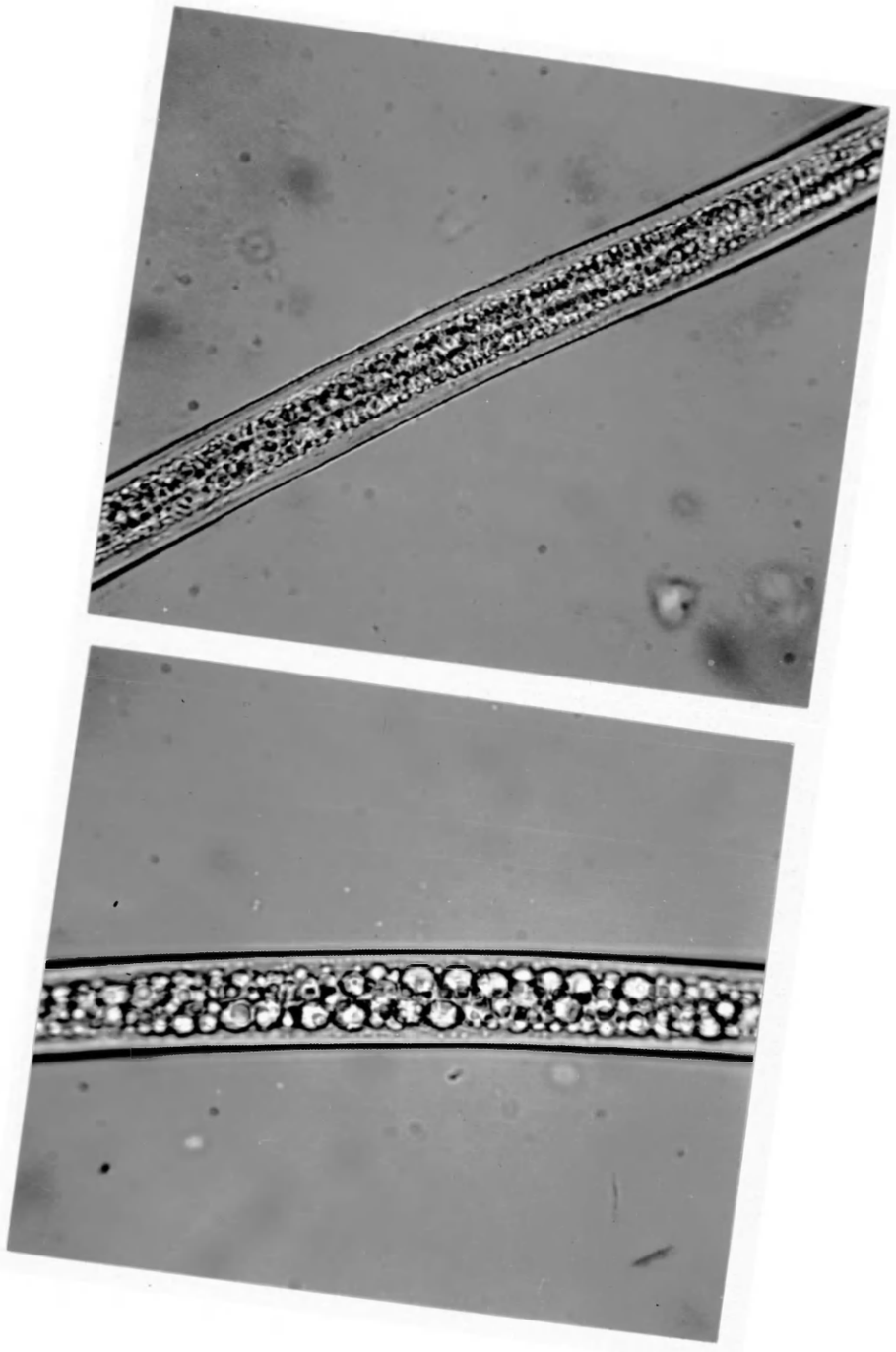


Figure 26. Comparison of temperature effects upon the time to first appearance of destruction and the subsequent rate of destruction of mycelium in manure-spawn cultures inoculated with equal numbers of Ditylenchus sp.

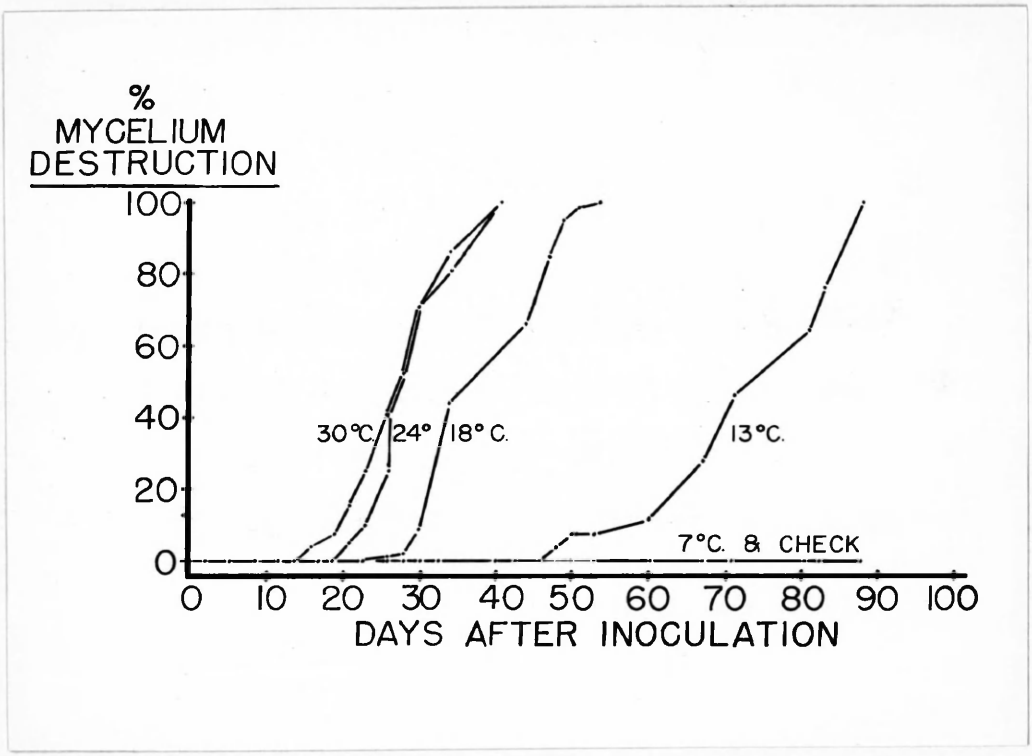
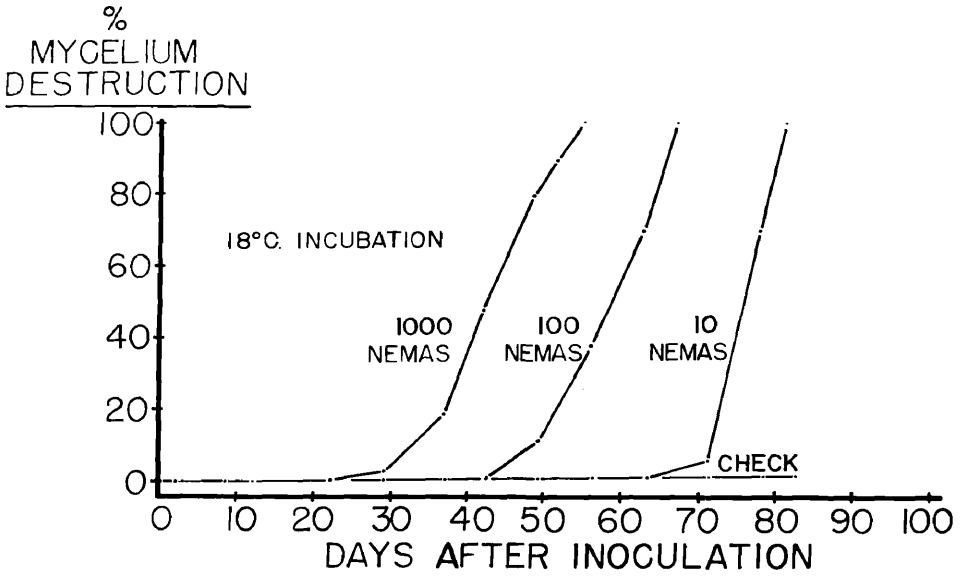

%
MYCELIUM
DESTRUCTION
100
80
60
40
20
0
30°C.
24°
18° C.
13°C.
7°C. & CHECK
0 10 20 30 40 50 60 70 80 90 100
DAYS AFTER INOCULATION

Figure 27. Photomicrographs of Ditylenchus sp. feeding upon mushroom hyphae. Note that head and neck regions of the nematodes are oriented to be about perpendicular to the hyphae.

Figure 28. Comparison of effects of varying numbers of Ditylenchus sp. upon the time to first appearance of destruction and the subsequent rate of destruction of mushroom mycellium in manure-spawn cultures incubated at 18° C. (65° F.).


%
MYCELIUM
DESTRUCTION
100
80
60
40
20
0
18°C. INCUBATION
1000
NEMAS
100
NEMAS
10
NEMAS
CHECK
0 10 20 30 40 50 60 70 80 90 100
DAYS AFTER INOCULATION

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Publications:

1. Cairns, E. J., and C. A. Thomas. 1950. Nematodes causing mushroom crop losses. Mushroom Science 1: 89-91.

2. Cairns, E. J. 1952. Nematode diseases and their control in mushroom crops. (Abs.) Phytopathology 42: 4.

3. Cairns, E. J. 1952. Anabiotic survival of a new species of Ditylenchus nematode. (Abs.) Phytopathology 42: 464.

4. Cairns, E. J. 1953. A culture-reared, plant parasitic nematode suitable for teaching and research. Phytopathology 43: 105-106.

5. Cairns, E. J. 1953. Moisture conditions and control of the mushroom-spawn nematode, Ditylenchus sp. (Abs.) Phytopathology. In press.

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