A STUDY OF A SPECIES OF BEAUVERIA FROM DENDROCTONUS FRONTALIS

By

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INTRODUCTION

During the spring of 1939, collections of bark of the short leaf pine (Pinus echinata) from eastern Virginia were found to be infested with the larvae of the southern pine bark beetle (D. frontalis). Many of these larvae were found to be dead or dying from the action of some parasite. Upon examination it was found that both nematodes and fungi were present in the affected beetles. Subsequent collections from Virginia and North Carolina revealed a similar condition and diseased larvae were found regularly until fall.

Since the depredations of southern pine bark beetle annually causes losses of thousands of dollars, it is evident that any parasite which might conceivably control, or partially control them would be of great economic importance. It was decided to investigate further the nature of the disease of the larvae in an effort to determine the parasite involved. Accordingly a large number of cultural tests were performed in an effort to isolate the fungus present. This was accomplished and repeated many times and the fungus obtained was found to be a species of Beauveris which was subsequently shown to be highly pathogenic. Recently the pathogen has been identified as B. bassiana.

HISTORY AND REVIEW OF LITERATURE

The infestation of bark of woody plant by beetles has been repeatedly observed. Their role in disease transmission has been established in a number of instances, e.g., the Ips beetles and the blue stain of conifers and the Scolytus beetles and the Dutch elm disease. However, relatively little is known of parasites of such vector beetles, nor of those which merely invade and reproduce in bark. Nematodes as well as fungi have frequently been found in living and dead beetle larvae. However, often it is not possible to show and parasitic relationship between the organisms involved. There are, however, parasitic forms in both groups. Among the fungi, the Laboulbeniales and Entomophthorales are outstanding examples of such forms. Numerous other Ascomycetes are known to parasitize insects, as do many fungi imperfecti. Among the latter, the genus Beauveria is well established as containing entomogenous species. This genus, which belongs to the tribe Verticilleae of the Hyphomycetales, has been reported on a number of insect hosts.

The earliest work with the genus Beauveria was done in France during the latter half of the nineteenth century. During this period diseases of the silk worm focused attention on insect parasites and a number of genera were reported as containing insect parasites. Notable among these were Botrytis, Isaria, and Sporotrichum. Doubtless many of these earlier identifications

were in error as they are dissimilar to the known characteristics of members of these genera. In 1910 (4) Beauverie published a critical study of a fungus attacking the silkworm and suggested that it was not one of the previously described genera and should be given a different name. Subsequently in 1912 Vuillemin (9) described the genus Beauveria to which he transferred a number of species hitherto known by other generic appelations. Subsequent studies have led to the establishment of eight entomogenous species of Beauveria attacking a fairly large number of insects. Arnaud (1) in 1929 reported on the physiology of two species of this genus is France. Lefebvre (6 and 7) in 1931 reported two species of Beauveria as parasitizing the corn borer Pyraustra nubilalis and in 1934 he and Bartlett (2) carried on infection experiments with corn borer larvae in the field. They reported some control of this pest by means of spore dissemination in the field. Similar experiments were carried on in Canada by Stirrett et al., (8) in 1937, and by Beall, et al. (3) in 1939. Both groups reported partial control. In November 1939, Miss V. K. Charles (5) reported B. bassiana as a parasite of Anopheles quadrimaculatus, and B. globulifera as a parasite of the sweet potato weevil Cyclas formicarius.

It is apparent that the genus Beauveria represents a group of fungi of great economic importance as insect parasites. Preliminary reports by other workers indicate that members of this genus may be effective in the biological control of insect pests and

additional experimental evidence will be presented in this paper.

SOURCE OF MATERIAL

As previously stated, the original isolations were from bark collections in the early spring of 1939. During this period, 43 isolations of the fungus were made from seven bark collections. In January and February of 1940, two additional isolations were made, another in March, 1940, and one in April. All isolates upon comparison were found to be representatives of the same species.

EXPERIMENTAL RESULTS

Pure Culture Studies

In an attempt to identify the fungus isolated from larvae of the southern pine bark beetle single spore cultures were prepared by means of a micromanipulator. Single spores were picked up and planted on an hanging agar drop in Van Tieghem cells. Thirty-six hours later the agar drop was transferred to a tube of sterile potato-dextrose agar. Subsequently, plates were prepared from inoculum obtained from this tube. In examining this fungus it was observed that the hyphae are very small ranging from four to eight microns in diameter. The mycelium is profusely septate and hyalin although old cultures may become somewhat buff colored. The mycelium is densely aggregated, highly branching, and commonly anastomosing. The fertile hyphae are aerial and fructifications first appear as short bottle-shaped conidiophores bearing a single spore. These then elongate into typical zig-zag phialides in a verticilliate whorl. Eventually these phialides become conglomerate forming a capitate mass. The spores are hyalin continuous and round to obovate. Spores are produced in immense numbers and soon give cultures a dusty appearance. Spore size ranges from three to six microns in diameter. Spore germination is by means of one or more tubes and frequent anastomoses occur between germ tubes of different spores. (Figs. 1-6).

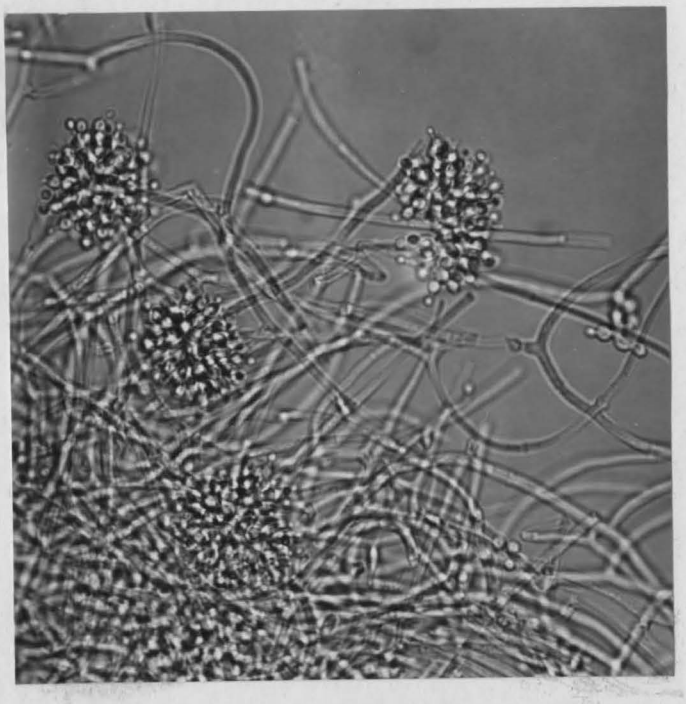

Fig. 1. Densely aggregated mycelium.

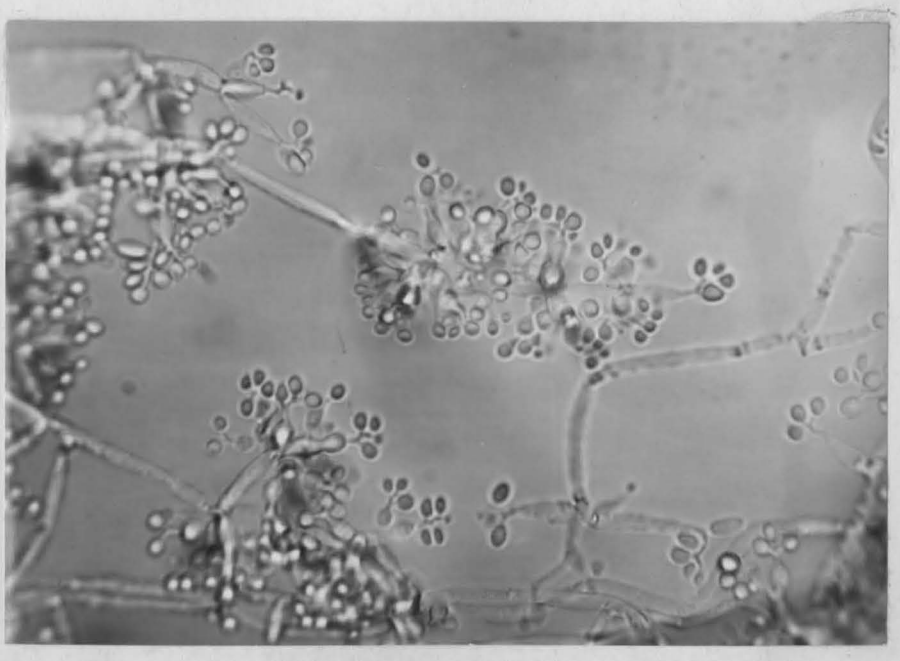

Fig. 2. Conidiophores, phialides and spores.

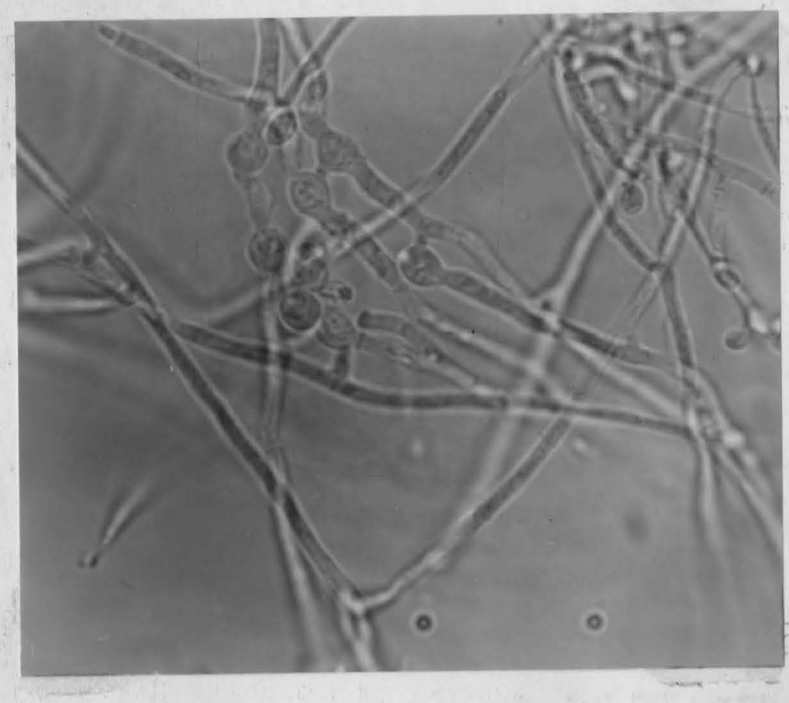

Fig. 3. Anastomosing hyphae.

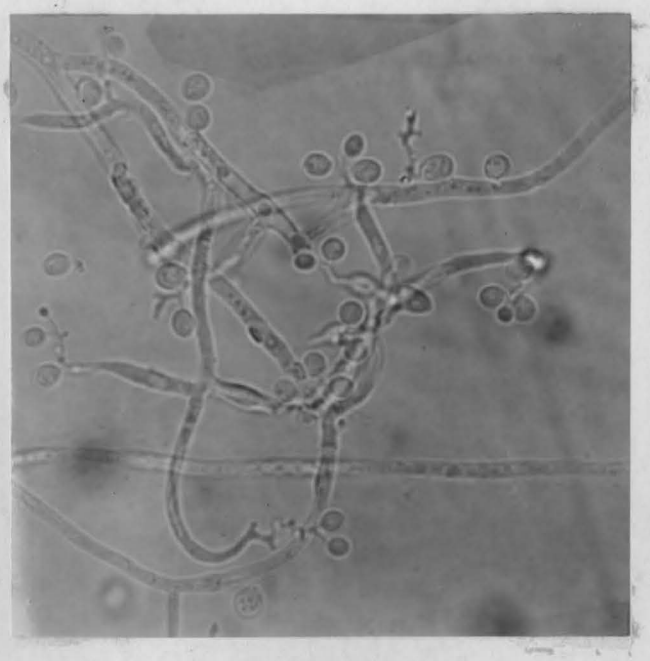

Fig. 4. Typical Conidiophores.

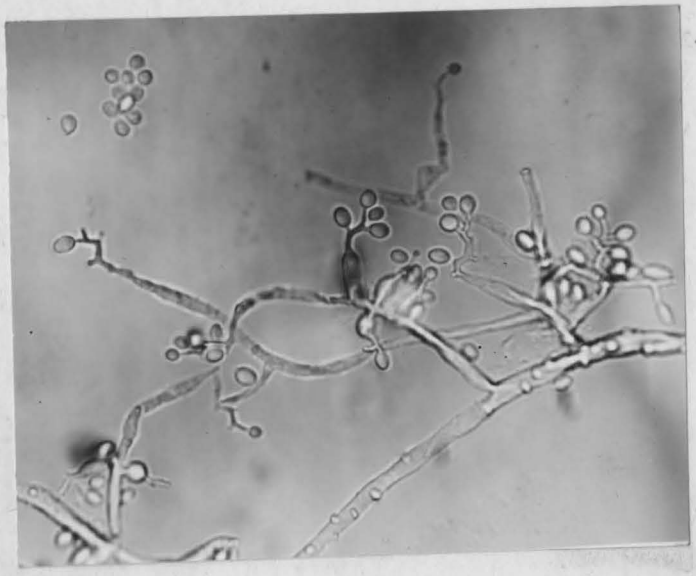

Fig. 5. Zig-zag phialides.

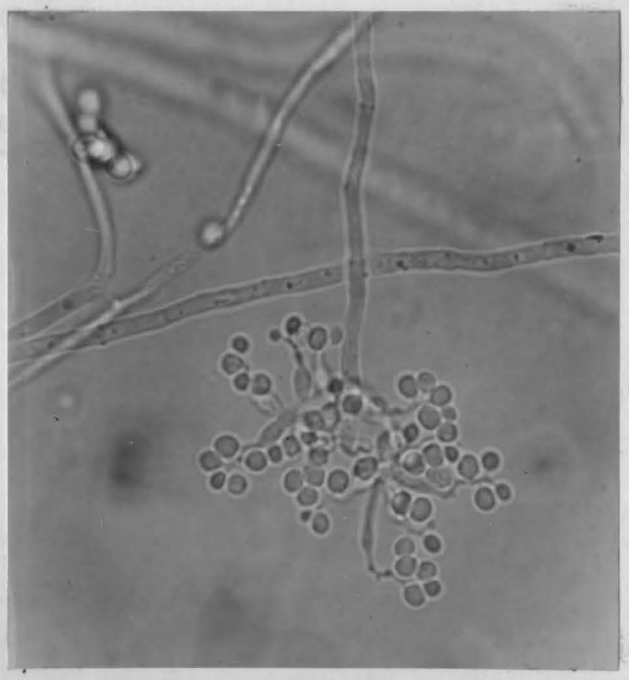

Fig. 6. Phialides in a verticillate whorl.

Cultural characters were observed on a number of media. The fungus grew readily on a rather wide range of nutrient materials. The colonies were at first white becoming buff or chalky or showing a tinge of pink. Colonies were raised with sometimes umbonate and sometimes umbillicate centers. On some media concentric growth zones were noted while on others these did not occur (Fig. 7).

The growth of characters of fungus are recorded in detail in Table I.

On March 29, 1940, it was noted that a sector had developed on one of the stock P. D. A. plates of the fungus (Fig. 8). Isolations were made from the sector and it was studied in pure culture. Resultant colonies differed in appearance from parent colonies in that typical concentric zonation did not occur. The sector and parent were compared as to pathogenicity and the sector was found to be much less virulent than its parent. Consequently it was eliminated from further tests. The comparison of the sector and parent is made in Table II.

Attempt was made to induce the pathogen to form a perfect stage. This was done by holding cultures and infected insects under a wide range of temperatures and humidities. However in no case was there any evidence that a perfect stage might be forming.

Temperature

The temperature requirements of the fungus were determined

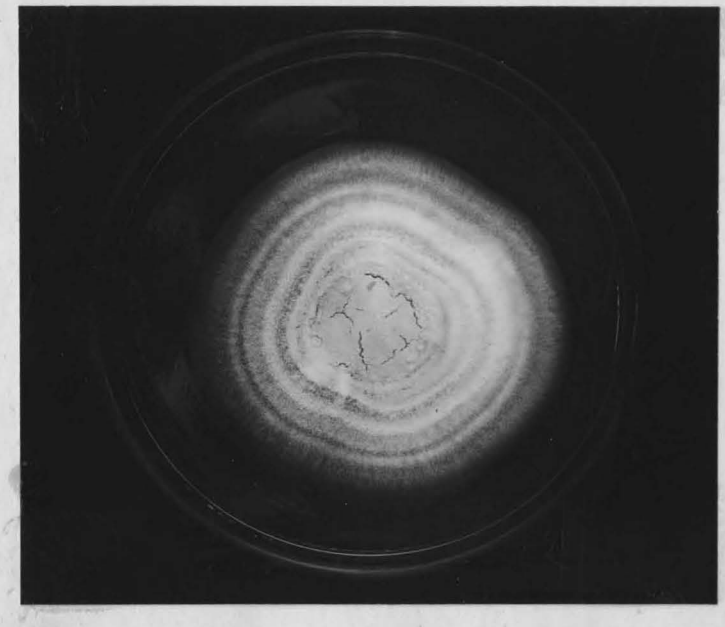

Fig. 7. A culture showing concentric growth zones.

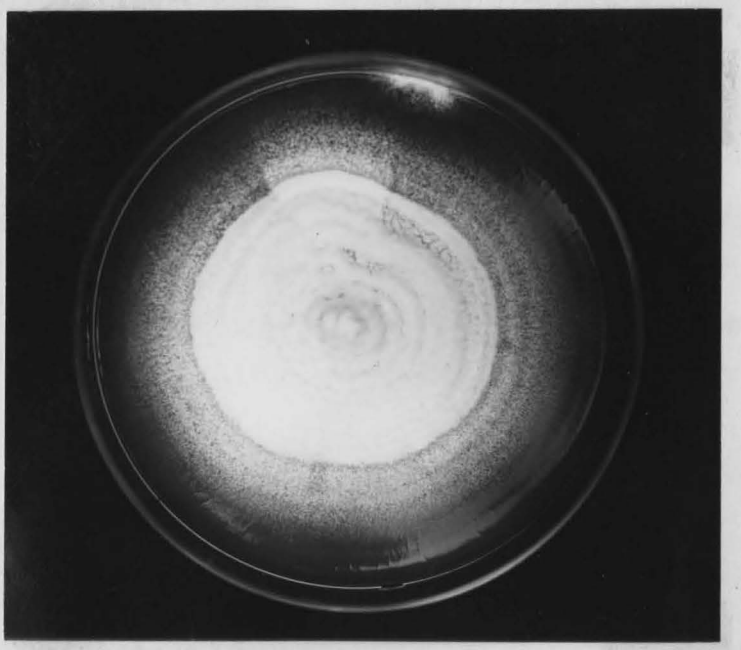

Fig. 8. Sector on potato-dextrose agar.

Table I

The Growth of Beauveria bassiana on Several Different Media at pH 5.5 and 27.5°C.

Medium	Colony Characteristics	Rate of Growth
Potato-dextrose agar	Elevated, flat cottony, umbilicate, with concentric rings, becoming purplish.	***
Glucose agar	White elevated cottony growth becoming purplish with spore formation.	**
Lead acetate agar	Sparse, mealy, chalky, flat.	*
Phosphate agar	Sparse, flat, chalky, mealy growth.	*
Bacto Nutrient agar	Flat, chalky, purple, cottony elevated, spores scarce.	**
Potato slant	Growth rapid, purple, cottony elevated, spores scarce.	***
Corn meal agar	Growth slow, chalky, spreading subilicate.	***
Egg Yolk	White cottony, yolk purple then orange becoming green. Colony surface convolute.	*
Egg White	Cottony, elevated, chalky, few spores.	*
Malt agar	Chalky, mealy, compacy, many spores.	**
10% Gelatin	Growth slow, cottony, elevated	*
Peptone agar	Flat, mealy pulverulent growth becoming yellowish by spore formation.	**

* slight growth
** good growth
*** abundant growth

Table II

Comparative Pathogenicity of a Sector of B. bassiana and its Parent

Pathogen	Host	Number	Number alive after 24hrs.	48hrs.	54hrs.	64hrs.	72hrs.
Sector	D. Larvae	25	25	24	20	12	4
	C. Adults	20	20	20	18	12	10
Parent	D. Larvae	25	25	6	0	0	0
	C. Adults	20	20	15	6	0	0

by placing triplicate plate cultures at each of the following temperatures: 5°C., 15°C., 25°C., 30°C., and 35°C. Growth was very slow at temperature below 15°C. and above 30°C. although fairly good growth was noted at temperatures as low as 15-18°C. Evidently the parasite is fairly tolerant of temperature fluctuation and may be active over a relatively wide range of temperatures. Unless otherwise stated all of the experiments were carried out at a temperature of approximately 25°C. Table III shows the affect of temperature variation on the action of B. bassiana.

Hydrogen Ion Concentration

Temperature and pH requirements were correlated by duplicating the temperature experiments with series of plates of the pathogen in which the pH had been adjusted at points from pH 3.5 to pH 8.5. It was found that the optimum pH was approximately 7.0 but that growth occurred at all values tested and that good growth occurred from pH 5.5 to pH 8.5 or higher (Fig.9). It was also found that maximum growth at pH 7.0 occurred at 25°C. Upon microscopic examination it was noted that the cultures at pH 6 and 6.5 sporulated most abundantly and that spores were not formed below pH 5.5.

From pH 3.5 - 5.0 growth at the end of ten days was small and very white and a depressed center was noticable. Spore

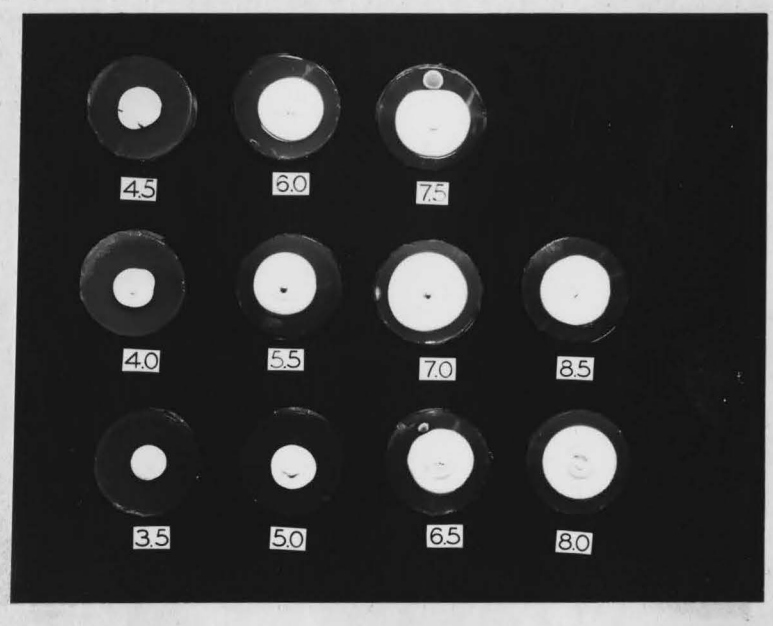

Fig. 9. Influence of pH on growth of Beauveria bassiana.

Table III

Affect of Temperature Variation on the Action of B. bassiana.

Temperature in °C.	Growth	Effect on 30 Dendroctonids
9-10°	none	none
15-16°	slow	killed in 21 days
23-27°	excellent	killed in 48-54 hours
35°	slight	killed in 26 days
45°	none	insects killed by heat after 5 days

production did not occur at these low pH values. From 5.5 - 8 there was a noticable increase in growth with profuse sporulation at from pH 6.0 - 7.0, 6.5 being best. Here the mycelial mass was a purplish creamy color. The depression at the middle of the culture although present was smaller as the pH increased. Here also, concentric growth rings were noticable. Colonies grown at pH 7.5 - 8.5 were very flat and appeared as a moist mycelial mass at 8 - 8.5. The colonies were dull creamy white and usually tended to become umbilicate. Replications of the pH experiments tended to demonstrate a definite response to pH variation. On acid media growth is very slow and spores are not formed as the pH rises to 5.5 and above the growth rate increases and spores are formed in abundance. The growth rate is greatest at pH 7.5 and sporulation at pH 6.5.

Infection Experiments

In order to determine whether or not the fungus isolated from dying bark beetle larvae was truly pathogenic, attempt was made to infect healthy larvae of the insect. A number of methods were tried, including direct inoculation with the spores, dusting with spores and spraying with spore suspensions. Infection was obtained by each method and as a result of observations made on these several methods the following technique was adopted: Larvae were picked from pine bark and were washed in sterile water. From

ten to twenty larvae were placed in a series of petri dishes containing moist filter paper. Check plates remained untreated while the larvae of test plates were dusted with spores of B. bassiana. In every test all the dusted larvae were killed in a maximum of fifty hours (Fig. 10). Death usually began after thirty-six hours. Adult Dendroctonus beetles, as well as pupae, were similarly tested, with positive results. Table IV gives composite results of a series of infection experiments.

In preliminary experiments the relative humidity was held at from 70 to 90 per cent as it was observed that infection would most readily occur under conditions of high humidity. In order to ascertain the moisture relationships of infection, 30 mealy bugs were dusted with Beauveria spores and placed in each of six moist chambers with relative humidity as follows: 90% - 70% - 50% - 30% - 20% - 10%. A series of untreated checks was also established. As shown by Table V the pathogen is most effective at high relative humidities. However it was also found that by raising humidity to the stauration point and holding it there that death of infected insects could be delayed for several days and that external symptoms were also delayed.

On the basis of preliminary infection experiments, it is evident that the fungus is definitely pathogenic to Dendroctonus frontalis in all stages of development. However, it was not known whether or not the organism was specific for this species or

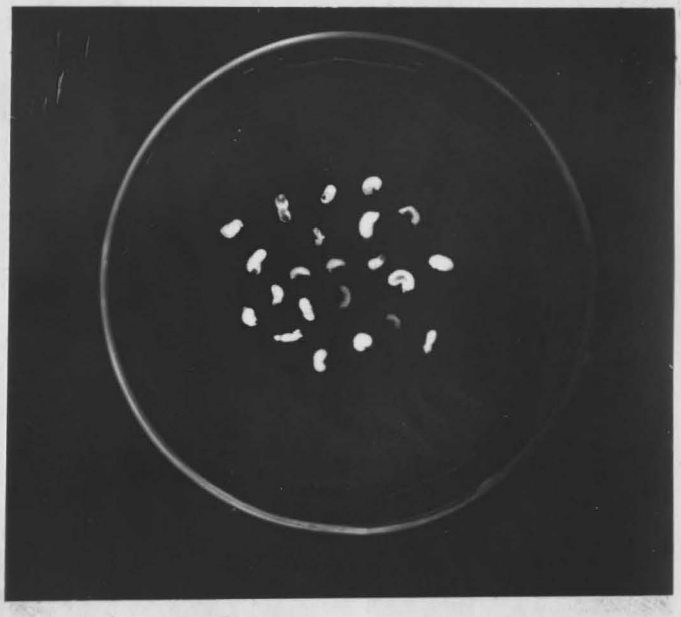

Fig. 10. Infected Dendroctonus Larvae.

Table IV

The Infection of Larvae of *D. frontalis* by *B. bassiana*

	Treatment	No. of Larvae	All dead after	No. of Adults	Time	Pupae	Time
Jan. 12	dusted	37	48 hrs.	16	54	7	36
Jan. 20	dusted	79	50 hrs.	24	60	25	28
Jan. 29	rolled	31	44 hrs.	12	63	14	35
Feb. 7	rolled	12	48 hrs.	18	55	20	34
Feb. 8	sprayed	83	54 hrs.	37	60	39	36
Feb. 20	rolled	64	50 hrs.	23	64	17	40
Feb. 29	sprayed	89	54 hrs.	45	60	32	40
Mar. 6	dusted	30	44 hrs.	12	54	4	32
Mar. 10	rolled	40	44 hrs.	20	54	7	32
Mar. 22	dusted	20	48 hrs.	20	60	--	--
April 6	rolled	37	44 hrs.	20	60	--	--
Totals		522	Av.50 hrs.	247	Av.50	165	Av.36

Table V

Effect of Humidity On Infection

Number of Insects	Humidity	Number killed in 60 hours	Checks
30	90%	30	None
"	70%	30	"
"	50%	19	"
"	30%	14	"
"	20%	8	"
"	10%	None	"

whether it had a wide host range. Consequently, it was decided to test its pathogenicity on other insect larvae. Accordingly, experiments similar to the foregoing one were performed with larvae of the oriental fruit moth, coddling moth, histerids and termites. Thirty of each of these insects were placed in moist chambers at 80% relative humidity after being dusted with spores of B. bassiana species (Figs. 11-14). Check plates were prepared and both series were held at 25°C. for eight days. The results of this experiment which are recorded in Table VI, demonstrate that the fungus is cosmopolitan and is able to parasitize a number of hosts and further suggests that it may occur rather commonly in nature.

Histology

From the rapid rate at which insects are killed by Beauveria it is evident that host penetration must occur rather readily. Avenues of entrance may be leg and antennae joints, spiracles and possibly mouth parts and setal membranes. Microscopic examination of large numbers of larvae and adults in all stages of infection afforded incomplete evidence as to the mode of infection by the fungus. While penetration was observed on several occasions it was decided to make histologic preparations in an effort to demonstrate the mode of infection. Altogether 145 infected adults and 210 infected larvae were prepared and

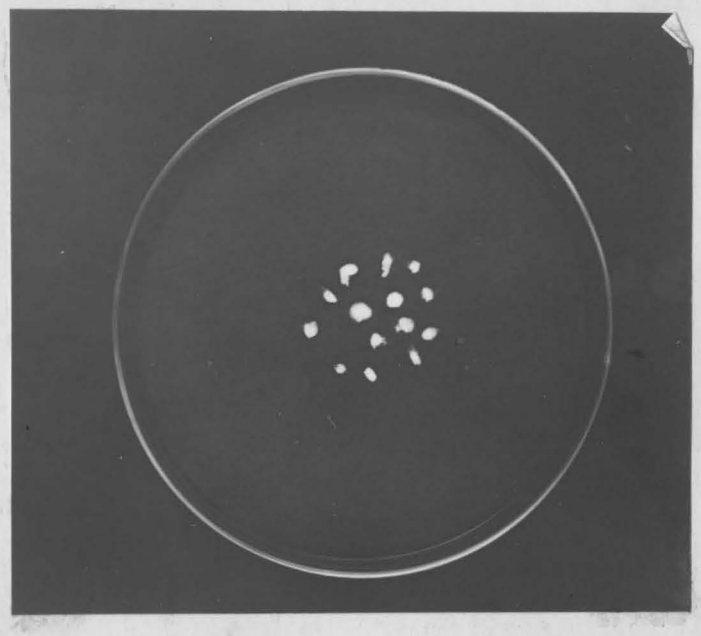

Fig. 11. Mealy bugs killed in 84 hours.

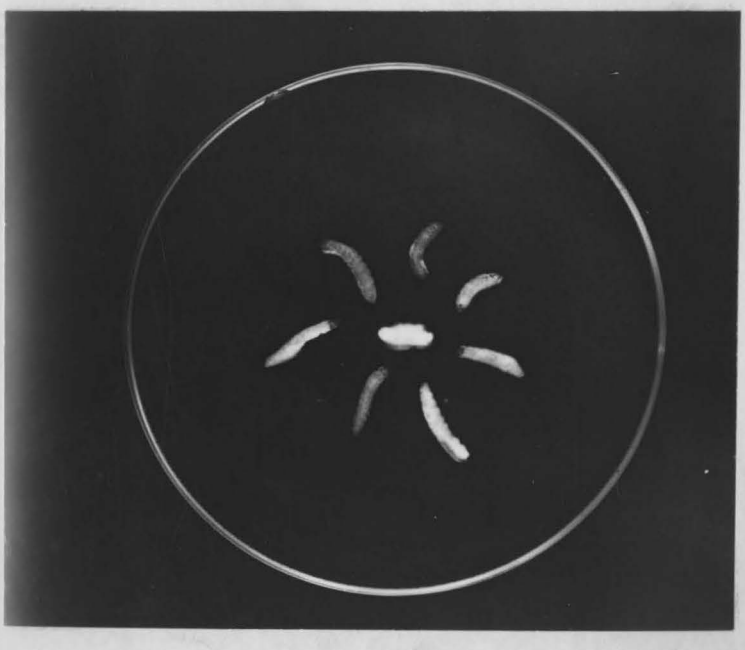

Fig. 12. Beauveria on coddling moth.

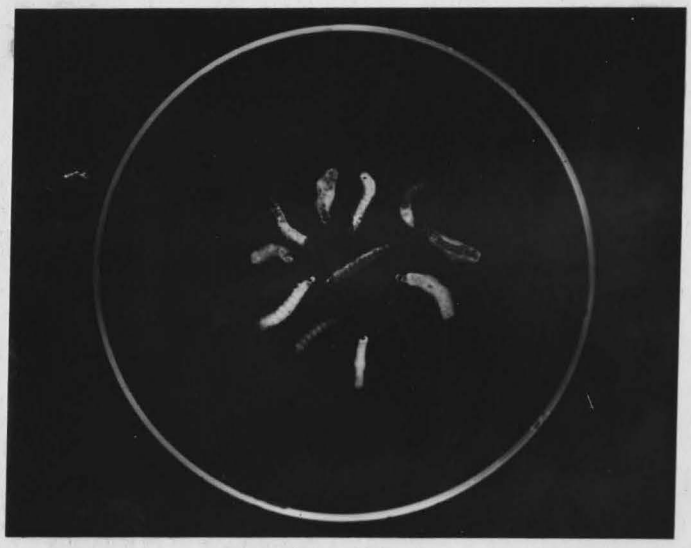

Fig. 13. Infected Histerid Larvae.

Fig. 14. Oriental Fruit Moth killed by Beauveria.

Table VI

Hosts of Beauveria sp.

Host	Number	Treatment	Number killed in 50 hours
Peach moth	50	Dusted	31
Coddling moth	50	Dusted	29
Histerids	50	Dusted	46
Termites	50	Dusted	50

studied and compared with healthy specimens.

Fixing, Dehydrating and Staining

All insects were fixed in Kahle's fixative for 12 hours then run through Zirkle's N-butyl alcohol method. They were then cleared in cedarwood oil for 24 hours, infiltrated and embedded in paraffin. It was found that the use of ethyl alcohol as a dehydrating agent made the insects brittle so N-butyl was used instead. Egg albumen was used as a fixative. A number of stains were tested including Orange G, Fast Green, Methylene blue, but Delafield's hematoxylin plus one drop of HCl per 100 cc. proved to be better than any of the several others tried. When this stain is applied and 70% ethyl alcohol used to destain, the mycelium is purple, the chitin becomes purplish yellow and the intestinal walls and other body structures become light purplish.

In addition to the dendroctonids, 19 hysterid, 30 peach moth, and 27 coddling moth larvae were infected and prepared for examination. The larvae were killed in Bouin's solution and run through Zirkle's N-butyl alcohol series and embedded. Subsequently they were sectioned and stained. The sections were then examined for evidence of mode and degree of infection.

From the examination of a large number of sections it is evident that the pathogen enters the host insects in one of several ways. Germ tubes from conidiospores may enter the host at appendage

joints, body segment joints, spiracles and setal membranes. The germ tube becomes compressed at the point of entrance and expands to a knob after penetration is effected. A branch or branches arise from this knob and ramify through the body of the host. Within the host the fatty portions of the body are most readily penetrated. This material is utilized by the fungus which spreads further and eventually peplaces the entire internal body mass. By this time the hyphae have become filled with food reserves and have begun to grow towards the surface of the host. These pointed hyphae break out all over the body of the host and form external knob-like swellings. From these structures primary conidia and/or conidiophores arise which soon produce characteristic phialides and conidiospores which serve to infect other insects.

From a study of 140 larvae and 140 adults it was determined that the initial stages of infection occur in from 24 - 36 hours after insects are dusted or sprayed with spores. Based on histologic examinations the process is as follows:

24 - 36 hours -- penetration through appendage abdominal joints (some direct penetration of larvae).

36 - 48 hours -- fungus hyphae ramifying through host. Fat first attacked and fatty structures act as foci for production of many new branches.

48 - 60 hours -- Intestinal tract filled with hyphae and head and muscles gradually filled.

60 - 72 hours -- Entire body cavity filled with mycelium (no spores). Mycelium, conidiophores and spores begin to appear on external surface of host.

Transmission

Attempt was made to infect healthy, dendroctonids, histerids, and peach moths by placing them in containers with an infected insect. Temperatures and moisture conditions were maintained at about the optimum for the insects and they were furnished an adequate food supply. Sixty of each of the three hosts were tested and ten of each were maintained as controls. From Table VII it will be seen that the presence of infected insects among healthy individuals may readily bring about infection of the latter.

A similar experiment was performed with white grubs and it was found that the grubs were susceptible and were killed in from 3 to 5 days. Subsequently, comparable experiments were performed with termites with similar results.

Table VII

Transmission of B. bassiana from Diseased to Healthy Larvae

Number	Host	Number killed in 144 hours	Check
60	Dendroctonid	50	0
60	Histerid	51	0
60	Peach moth	48	0

SUMMARY

A fungus was repeatedly isolated from dead and dying larvae of the southern pine bark beetle, Dendroctonus frontalis. After it had been obtained in pure culture the fungus was tested for its pathogenicity and was found to be highly pathogenic to larvae of D. frontalis.

Attempt was made to identify the pathogen and it was found to be a species of the Genus Beauveria of the Hyphomycetales. The fungus most closely resembles Beauveria bassiana and probably is a form of that species.

The fungus was studied in pure culture and its cultural characters as temperature, moisture, and pH requirements were determined. Further experiments were then undertaken in attempt to work out the etiology of the bark beetle disease. The pathogen was found to penetrate hosts through natural openings or through segment joints. After entrance has been effected the fungus rapidly invades the internal organs of the host and may cause death in from three to six days. Subsequently, the killed host may become covered with conidiophores and serve as a source of inoculum for the infection of healthy larvae.

The pathogen was found to be rather cosmopolitan and has been shown to be able to parasitize in addition to D. frontalis, the coddling moth, the oriental fruit moth, histerids, and termites.

B. bassiana has been reported as parasitizing many other hosts as well.

From results obtained it is evident that B. bassiana constitutes a natural parasite of Dendroctonus frontalis in Virginia and North Carolina. Under favorable weather condition, the pathogen may greatly reduce current populations of the beetle larvae. The habits of the host prevent the efficient use of the parasite artificially as a biological control.

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