Mycotoxins of Entomophthoraceous Fungi

Sothorn Prasertphon and Y. Tanada

Among the four entomophthoraceous species, *Entomophthora coronata, E. apiculata, E. thaxteriana,* and *E. virulenta,* only the first two species produced culture filtrates that were toxic when inoculated into the hemocoels of the larvae of seven insect species. The culture filtrates, however, produced no apparent toxicity when fed to the larvae of *Galleria mellonella.* The fungi produced mycotoxins in all media suitable for their growth *in vitro* and *in vivo.* The mycotoxins produced by the two fungi had similar properties and appeared to be identical, but *E. coronata* produced much more toxin than *E. apiculata* in the same period of time. More toxin were produced by both fungi in shake than in stationary cultures. The median effective doses (ED_{50}) of the culture filtrates (96-hour cultures) were estimated from data.

The mycotoxin was not chemically identified, but tests indicated that it was a protein of small molecular weight. The mycotoxin caused a characteristic blackening sign in inoculated larva of *Galleria mellonella;* it also inhibited metamorphosis and affected larval feeding. These signs and symptoms varied among the seven insect species. The mycotoxin also inhibited bacterial growth in dead *G. mellonella* larva.

In *Galleria mellonlela* larva, the mycotoxin apparently altered the staining properties of the hemolymph and caused the clumping and coagulation of the hemocytes. Silk gland cells also showed pathologies, but other tissues and organs appeared to be unaffected. Blood color changes and clumping of the hemocytes did not occur in fungus-infected larvae.

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INTRODUCTION

MICROORGANISMS are known to produce substances that are toxic to insects. Because some of these substances are not only specific, but nontoxic or only slightly toxic to warm-blooded animals, they offer promise for the control of insect pests. Interest in these toxic substances has increased greatly in the past ten years, as the inadequacies of highly toxic, nonspecific chemical insecticides have become apparent. The microbial toxins have been isolated from various microorganisms, such as fungi and bacteria. In the present study, we have investigated the production of toxins by entomophthoraceous fungi. A preliminary note of this study was published by Prasertphon (1967b).

"Toxin" has been defined by Ludwig (1960) as "a product of a microorganism or microorganism-host interaction which acts directly on living host protoplasts to influence either the course of disease development or symptom expression." "Mycotoxin" is a "toxin produced by fungi" (Forgacs *et al.*, 1962). The term "mycotoxicosis" has been defined as "poisonings of the host which follow entrance into the body of toxic substances of fungal origin" (Forgacs and Carll, 1962). According to Roberts (1964), some toxic substances of fungi are known to be nonproteinaceous and nonantigenic.

Although mycotoxins that affected vertebrates were known over a century ago, such toxins were reported for insects less than 50 years ago. In a culture filtrate of a fungus in the Aspergillaceae, Toumanoff (1928, 1931) detected a toxin that acted on honey bees. Burnside (1930) extracted an ether-soluble substance from a culture of *Aspergillus flavus* Link and found it toxic to bees. Dresner (1949) reported that germinating spores of *Beauveria bassiana* (Balsamo) Vuillemin secreted a substance which, when applied to house flies, produced a "knock-down" effect by rapid paralysis and caused death. This substance was also toxic to the dock beetle (*Gastroidea cyanea* Melsh.) and the potato tuberworm (*Gnorimoschema operculella* (Zeller)) (Dresner, 1950). This is the only known report of a contact or vapor poison being produced by an entomogenous fungus. Others have attempted to repeat Dresner's work with the same fungus strain, but failed to confirm his results (Steinhaus and Bell, 1953). Acetone extracts were obtained from the culture filtrates of nine species of fungi by Kodaira (1954). He found substances that were poisonous to silkworms from the cultures of *Aspergillus ochraceus* Wilhelm and *Sterigmatocystis japonica* Aoki. In 1959, he investigated seven species of muscardine fungi: *Beauveria bassiana, Spicaria pracina* (Maubli) Aoki, *Isaria farinosa* (Dickson) Fries (two strains), *Isaria rosea* Wize, *Metarrhizium anisopliae* (= *Oospora destructor*), *Aspergillus flavus* Link (two strains), and *Aspergil-*

lus oryzae Wehmer (three strains). Every strain of the seven species produced substances toxic for the silkworm. The strongest toxic substances were produced by *Metarrhizium anisopliae.*

Beard and Walton (1965) found that the culture filtrate of *Aspergillus flavus* var. *columnaris,* when mixed in the medium of the house fly, did not prevent the fly eggs from hatching, but the maggots soon died. They concluded that the fungus produced a toxic substance. Sussman (1952) found that an aqueous suspension of conidia of *Aspergillus flavus,* when injected into the pupa of the cecropia moth, produced a blackening color in the blood and integument, and concluded that the blackening was caused by polyphenols that reacted with the enzyme tyrosinase in the blood. He did not ascertain, however, whether the polyphenols were produced by the fungus or by the autolysis of the insect protein.

The entomogenous fungi are also capable of producing toxins *in vivo.* Schaerffenberg (1957) reported that *Beauveria bassiana* produced a mycotoxin in the infected larva of the cockchafer, *Melolontha melolontha* (Linnaeus). An acetone extract of the macerated tissues of the infected cockchafer larva also killed the larva of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), when it was fed potato leaves that had been dipped in the extract. In 1960, Kodaira investigated the *in vivo* production of toxins in silkworm larvae infected separately by nine species of fungi, including *Aspergillus ochraceus* and *Sterigmatocystis japonica.* In every case, he found that the chloroform or ether extracts of the blood of the silkworm in the state of death produced toxic symptoms when injected into a healthy silkworm and concluded that the toxic substances were produced in the blood.

If mycotoxins are to serve as insecticides (Tanada, 1967), then the toxins must be isolated and identified. Thus far, only a few attempts have been made. Kodaira (1961) extracted and purified the toxic substances from the fungus filtrates of *Metarrhizium anisopliae,* and named them "destruxin 'A' and 'B'." Apparently, a similar toxic substance was isolated from the culture of *Metarrhizium anisopliae* by Roberts (1964, 1966a, 1966b). This substance caused tetanic paralysis in the larvae of *Galleria mellonella* (Linnaeus) and of *Bombyx mori* (Linnaeus) when injected into the hemocoel. Cunningham *et al.* (1951) isolated cordycepin ($C_{10}H_{13}O_3N_5$) from the fungus filtrate of *Cordyceps militaris* (Linnaeus) Link, and observed that it inhibited the growth of many strains of *Bacillus subtilis.* Cordycepin formed needle-shaped crystals in alcohol and water. Bentley, Cunningham, and Spring (1951) attempted the isolation and structural determination of cordycepin. Kredich and Guarino (1961a, b) succeeded in isolating homocitrullylaminoadenosine from the fungus filtrate of *Cordyceps militaris* and studied its biosynthesis, and later its identification (Guarino and Kredich, 1963). Unfortunately, this material was not tested on insects.

Two compounds—$C_{29}H_{40}O_9$ (mol. wt. 492) and $C_{49}H_{64}O_{16}$ (mol. wt. 903)—were isolated from the culture filtrate of the fungus, *Myrothecium roridum* Tode, by Kishaba *et al.* (1962). These compounds inhibited the feeding activity of larvae of the Mexican-bean beetle, *Epilachna varivestis* Mulsant. According to White and Downing (1947) and Roberts (1964), *Myrothecium* and *Metarrhizium* are identical in morphology but may be distinguished by the fact that *Myrothecium* can digest cellulose.

MATERIALS AND METHODS

Fungi, insect species, and their culture

Four species of *Entomophthora* were used in the study, including three strains of *E. coronata* (Costantin) Kevorkian. The culture for the Hall strain was obtained from Dr. I. M. Hall who, along with Dietrick (1955), isolated it from the spotted alfalfa aphid, *Therioaphis maculata* (Buckton). Another strain, *NRRL No. 1912,* was sent to the Division of Invertebrate Pathology, University of California, Berkeley, by Dr. Hall, who obtained it from Dr. C. W. Hesseltine's culture collection, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois. It was apparently isolated by G. W. Martin in July, 1923, from dead wood and contaminated leaves being used in the isolation of myxomycetes (Martin, 1925, and personal correspondence).

Entomophthora apiculata (Thaxter) Gustafsson was received from the Northern Utilization Research and Development Division, and is listed in their collection as NRRL No. 1626. *Entomophthora thaxteriana* (Petch) Hall and Bell was originally isolated from the spotted alfalfa aphid, *Therioaphis maculata,* by Hall and Dietrick (1955) and was named *E. ignobilis* by Hall and Dunn (1957), but later found to be a synonym of *E. thaxteriana* (Hall and Bell, 1963). *Entomophthora virulenta* Hall and Dunn was isolated from the spotted alfalfa aphid. Both *E. thaxteriana* and *E. virulenta* were obtained from Dr. Hall.

All fungus stock cultures were established on slants made of Sabouraud's dextrose agar plus yeast extract, and stored at room temperature (22° to 27°C). The compositions of the fungus culture media used in the tests were as follows:

(1) Modified* Sabouraud's Dextrose with Yeast Extract (SDAY)

Neopeptone (Difco) .... 10 gm
Bacto-Dextrose .... 40 gm
Bacto-Agar .... 15 gm
Yeast Extract .... 2 gm
Distilled water (added until 1,000 ml) .... 1,000 ml

(2) Modified* Peptone Medium with Glucose (MPTG) (liquid medium)

Bactopeptone (Difco) .... 10.0 gm
Dipotassium phosphate .... 4.3 gm
Potassium biphosphate .... 3.4 gm
Glucose .... 5.0 gm
Distilled water (added until 1,000 ml) .... 1,000 ml

(3) Modified* Peptone Medium without Glucose (MPT) (liquid medium)

Bactopeptone (Difco) .... 10.0 gm
Dipotassium phosphate .... 4.3 gm
Potassium biphosphate .... 3.4 gm
Distilled water (added until 1,000 ml) .... 1,000 ml

* Formulas were modified from those provided by Difco (1953).

The cultures of *Entomophthora* species were stored in air-tight test tubes (Prasertphon, 1967a). Each tube, 18 × 150 mm, contained 10 ml of Sabouraud's dextrose agar with 0.2 per cent yeast extract (SDAY). A rubber "snap cap" (Pyrex), 18 × 25 mm, was used to cap the test tube instead of the usual cotton plug or metal cap; special precaution was taken to ensure that the inoculated tube was completely sealed and airtight. The tubes of fungus culture were stored vertically at room temperature. Viability was maintained for more than two years.

The strain of the greater wax moth, *Galleria mellonella,* had been carried through at least three generations from a single parental pair. The insects were reared at 30° ± 1.5°C on the medium developed by Roberts (1964). The larvae of the yellow Hungarian strain of the silkworm were reared on fresh leaves of mulberry, *Morus alba* Linnaeus. The codling-moth larvae, *Carpo-*

capsa pomonella (Linnaeus), and the corn-earworm larvae, *Heliothis zea* (Boddie), were reared on a modified-artificial diet (Ignoffo, 1963, 1965). The cecropia-moth caterpillars, *Hyalophora cecropia* (Linnaeus), were reared on fresh leaves of ceanothus. The larvae of the variegated cutworm, *Peridroma saucia* (Hübner), and of the armyworm, *Pseudaletia unipuncta* (Haworth), were reared on a modified-artificial diet (Shorey, 1963; Shorey and Hale, 1965).

The stationary cultures of *Entomoph-thora* were maintained in 50 ml of a modified peptone liquid medium without glucose (MPT) held in a 250-ml Erlenmeyer flask. The inoculated flask was capped with aluminum foil to prevent contamination, and incubated at room temperature without shaking.

The shake cultures were inoculated and handled in the same manner as those for the stationary cultures, except that they were incubated on a shaker (Gyrotory Shaker, Model G-10, New Brunswick Scientific Company, New Brunswick, New Jersey). The cultures were continuously shaken at 160 oscillations per minute for 96 hours at room temperature (22° to 27°C).

Mycotoxin production

The production of mycotoxin *in vitro* was investigated with shake fungus cultures. The fungus mycelia in the 96-hour shake cultures were separated from the fungus filtrate by filtering first through filter paper, and then through a filter membrane 4 mm in diameter, pore size 0.45 μ, under suction. The entire process was carried out in an ultraviolet-sterilized room. The fungus filtrate was stored in a 20-ml sterilized test tube covered with a bakelite screw cap and kept at 4°C.

The production of mycotoxin *in vivo* was established as follows: After the cultures of *E. coronata* and *E. apiculata* were grown on Sabouraud's dextrose agar plus yeast extract in petri dishes

for four days, five wax-moth larvae were introduced into each of the cultures for 24 hours. Then the larvae were transferred to petri dishes whose bottoms were lined with moist filter paper. After four days, 20 larvae in the moribund stage were washed in sterile, distilled water, dried, weighed (3.30 gm), and triturated in a sterile porcelain mortar. Seven ml of sterile distilled water were added to the triturated larvae, and the mixture was stored at 4°C for 24 hours. The suspension was then filtered first through a No. 2 Whatman filter paper, and subsequently through a millipore filter membrane, pore size 0.45 μ in diameter. The final filtrate (2 ml) was injected into the hemocoels of wax-moth larvae for the observation of toxic symptoms. The control larvae were treated in a similar manner, except that they were injected with the filtrate obtained from triturated uninfected larvae.

Inoculation of *Entomophthora* filtrates

The *per os* inoculation was made with a glass capillary tube attached to a 0.25-ml syringe with a Tuohy Luer-Lok catcher adapter (Martignoni, 1955; 1959). The whole unit was mounted on an Agla microinjector (Burroughs Wellcome & Co., London). The syringe was previously calibrated with mercury. The larvae were microfed under anesthesia in order to avoid serious traumatic lesions (Martignoni and Steinhaus, 1961).

The intrahemocoelic inoculation was performed with a No. 30 hypodermic needle attached to a 0.25-ml glass syringe mounted on an Agla microinjector. The needle was inserted through the planta of the left member of the third pair of prolegs of the wax-moth larva. The larvae were weighed prior to the inoculations, and ranged from 100 to 190 mgm.

Isolation and properties of mycotoxin

Roberts' (1964) method was used. Fifty ml of a 96-hour-old fungus culture were poured into a 200-ml separatory funnel, and an equal amount of anhydrous ether was added. The funnel was shaken vigorously for 30 minutes, and then the ether fraction was separated from the filtrate. The procedure was repeated three times with 50 ml of ether added each time to the fungus filtrate. The final volume of 150 ml of the ether extract was evaporated in a ventilation hood at room temperature for 24 hours. The residue of the ether extract was dissolved in 10 ml of sterile, distilled water for six hours before its injection into the larval hemocoel. The aqueous portion, remaining after ether extraction, was also evaporated in the same manner as the ether fraction, with the residue suspended in water before injection into the larva.

In a second attempt at isolation, the pH of the fungus filtrates was decreased. Fifty ml of the fungus filtrate required 45 ml of 0.1 N HCl to lower the pH to 2 from 6.9. Fifty ml of anhydrous ether were added and the mixture was vigorously shaken. This procedure was repeated three times. The residue of the ether extraction was suspended in 10 ml of sterile, distilled water and injected into the larvae. The aqueous filtrate portion was treated in the same manner as above and then injected into the larvae.

To separate the mycotoxin from the fungus culture filtrate, 10 ml of the culture filtrate were centrifuged at 1,400 *g* (3,100 rpm) for one and two hours in a clinical centrifuge. In a second attempt, 3 ml of the culture filtrate were centrifuged at 25,500 *g* (20,000 rpm) for one hour in an ultra-centrifuge, and in a third attempt, at 102,000 *g* (40,000 rpm) for two hours. The control filtrates were treated in the same manner in each experiment.

The thermal inactivation point of the mycotoxin in the culture filtrates was established by heating the filtrates at various temperatures in thermal tubes (Tanada, 1953). In the first experiment, the filtrates were heated at 40°, 60°, and 80°C for 10 minutes. Since the mycotoxin was inactivated between 40° and 60°C, the second experiment was conducted at 36°, 40°, 45°, 48°, 52°, and 60°C. The toxic activity of heated and unheated samples of the culture filtrates was tested by injection into wax-moth larvae.

Inasmuch as the mycotoxin in the culture filtrates became inactivated after prolonged storage at room temperature, the length of storage required for inactivation was investigated. Culture filtrates obtained after 96 hours were held at 25°C. Every two days a sample of the culture filtrate was taken from the incubator and injected into larvae.

Ten per cent trichloracetic acid (TCA) was mixed with the culture filtrates to denature and precipitate proteins in the mycotoxins. The following proportions were used:

(1) 9 ml of fungus-culture filtrate + 1 ml of TCA (9:1)
(2) 8 ml of fungus-culture filtrate + 2 ml of TCA (4:1)
(3) 9 ml of control MPT filtrate + 1 ml of TCA (9:1)
(4) 8 ml of control MPT filtrate + 2 ml of TCA (4:1)

In addition to the mixtures containing TCA, the fungus-culture filtrates were mixed with MPT filtrates as follows:

(1) 9 ml of fungus-culture filtrate + 1 ml of control MPT filtrate (9:1)
(2) 8 ml of fungus-culture filtrate + 2 ml of control MPT filtrate (4:1)

The toxic properties of these mixtures were determined by injecting them into the hemocoels of *Galleria mellonella* larvae.

Since the mycotoxin could not be extracted from the culture filtrates with ether, it was considered to be present in

the aqueous portion of the filtrate. Ten ml of the filtrate were evaporated in an open petri dish in a ventilated air chamber at room temperature for 24 hours. The control was the residue obtained from evaporating 10 ml of a modified peptone medium in the same manner. The residue of the culture filtrate was first examined under the binocular microscope to observe the type of crystallization, and then it was dissolved in sterile, distilled water before injecting into the larvae of *Galleria mellonella* to test for toxic reactions.

RESULTS

Production of mycotoxin in the fungus cultures

Of the four *Entomophthora* spp., the culture filtrates of only two species, *E. apiculata* and *E. coronata* (strains Hall and NRRL 1912), contained mycotoxin detectable by injection into the hemocoels of *Galleria mellonella* larvae. The presence of the mycotoxin was indicated by the blackening of the larval blood (plate 1). Also, growth and metamorphosis were inhibited; larvae did not reach the imaginal stage. The culture filtrate of *E. thaxteriana* and *E. virulenta,* however, apparently did not contain mycotoxins, since the larvae injected with these culture filtrates developed and emerged as adults, just as did the control larvae.

Entomophthora coronata and *E. apiculata* produced mycotoxin in stationary as well as in shake cultures. The stationary-culture filtrates, however, contained smaller amounts of the mycotoxin than the shake-culture filtrates. A 29.2 µl dose of each of the stationary-culture filtrates of *E. coronata* and of *E. apiculata* produced no response or blackening sign, but the same dose from shake cultures resulted in 90 per cent of the larvae showing the blackening sign (table 1). Detectable mycotoxin was produced by these two fungi after 36 hours of cultivation, but at 24 hours of cultivation, no toxin was detectable in the culture filtrates.

Entomophthora apiculata and *E. coronata* produced mycotoxin in all of the three media tested: modified peptone without glucose, modified peptone plus glucose, and Sabouraud's dextrose medium with yeast extract. Although *E. thaxteriana* and *E. virulenta* also grew in these media, they did not produce any detectable mycotoxin, even when a massive dose of their culture filtrates was injected into the larval hemocoels.

The physical appearance of the fungus mycelia was observed in wet-mount preparations with the compound microscope. Mycelia were still growing and showed no signs of autolysis after 36 hours of cultivation. After 48 hours, autolysis occurred in a small portion of the mycelia. At 60, 71, 84, and 96 hours, the mycelia showed increasing signs of autolysis, but some of the mycelia were

TABLE 1

RESPONSES OF *GALLERIA MELLONELLA* LARVAE TO INOCULATIONS OF STATIONARY AND SHAKE CULTURES OF *ENTOMOPHTHORA* SPP.

Type of culture*	Dose	Number† of larvae responding to filtrates:	
		E. coronata	*E. apiculata*
	µl		
Stationary	29.2	. .	. .
	43.8	8	7
	58.4	10	10
	73.0	10	10
Shake	29.2	9	9
	43.8	10	10
	58.4	10	10
	73.0	10	10

* Stationary and shake filtrates of a modified peptone medium were inoculated into the control larvae at a dose of 73.0 µl. None of the larvae showed symptoms.

† Ten larvae were inoculated at each dosage level. They weighed from 0.130 to 0.230 gm for those inoculated with the filtrates of *E. coronata*, and 0.130 to 0.240 gm for those inoculated with *E. apiculata*.
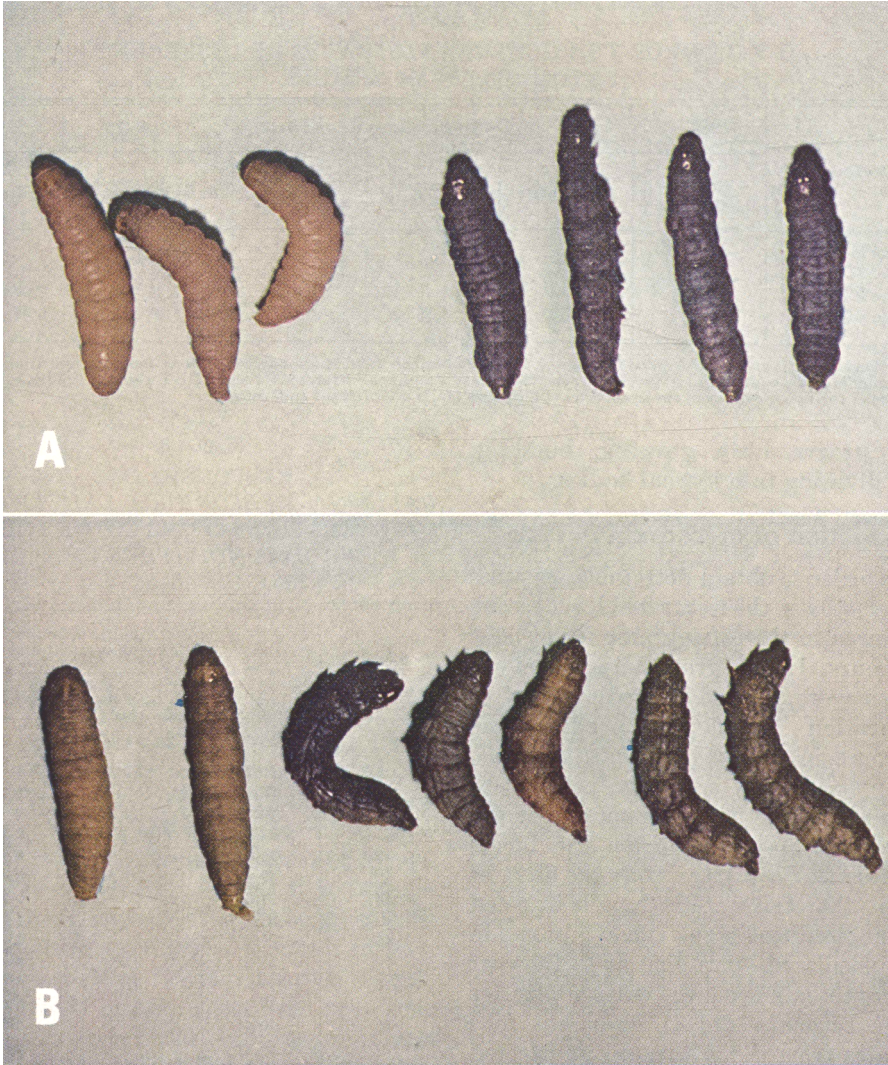
Plate 1. Effect of mycotoxin on larvae of *Galleria mellonella*. Black larvae in **A** (dorsal and lateral views) were injected with culture filtrates of *Entomophthora apiculata* and those in **B** (lateral view) with filtrates of *E. coronata*. Larvae intoxicated by the culture filtrates of both fungi exhibited similar symptoms of blackening and the backward bending of their bodies. Normal pale control larvae in **A** and **B** were injected with a modified peptone medium.

TABLE 2

pH VALUES OF THE CULTURE FILTRATES OF DIFFERENT
ENTOMOPHTHORA SPECIES

Medium*	pH values of culture filtrates of:					
	Control (No fungi)	*E. apiculata*	*E. coronata* strain Hall	*E. coronata* strain NRRL 1912	*E. thaxteriana*	*E. virulenta*
Control MPT.......	6.80	....	....	....	....	....
Control SDBY......	5.90	....	....	....	....	....
Filtrate MPT.......	....	8.05	8.15	8.10	7.85	7.90
Filtrate SDBY......	....	5.20	5.80	6.05	6.00	5.60

* Control MPT and control SDBY were the filtrates obtained from the modified peptone medium and from the Sabouraud's dextrose medium, respectively, after 96 hours of standing. Filtrates MPT and SDBY were the fungus-culture filtrates of the two media from shake cultures obtained 96 hours after fungus cultivation.

still progressively growing, budding, and dividing into hyphal bodies.

Production of mycotoxin *in vivo*

In order to obtain detectable amounts of mycotoxin, the triturated larvae must be stored in the refrigerator for at least 12 hours. If the ground-up larval remains were filtered immediately after trituration, the filtrate produced no response in *Galleria mellonella* larvae. It appeared that a storage period was required for sufficient amounts of the mycotoxin to diffuse from the larval tissues into the filtrate. Only the filtrates from the triturated fungus-infected larvae produced signs and symptoms in the inoculated larva similar to those of the mycotoxin from the culture filtrates of *E. coronata* and *E. apiculata*. The filtrates from the triturated uninfected larvae did not produce comparable toxic signs.

Properties of the mycotoxin

pH values of the culture filtrates varied with the type of culture medium used in growing the fungi (table 2). Use of the modified peptone medium (MPT) resulted in filtrates with higher pH values than with Sabouraud's dextrose medium (SDBY). When the four species of *Entomophthora* were grown in the same type of media, the pH values were nearly the same. Accordingly,

TABLE 3

RESPONSES OF
GALLERIA MELLONELLA LARVAE TO
INOCULATIONS OF MYCOTOXIN IN
TWO *ENTOMOPHTHORA* SPP.
STORED AT 25° C

Filtrate storage period (at 25°C)	Dose	Number of larvae* responding to filtrates of:	
		E. coronata	*E. apiculata*
days	*μl*		
4	29.2	8	9
	58.4	10	10
6	29.2	3	5
	58.4	10	10
12	29.2	0	0
	58.4	5	6
14	29.2	0	0
	58.4	6	4
16	29.2	0	0
	58.4	3	3
18	29.2	0	0
	58.4	2	2
20	29.2	0	0
	58.4	1	0
22	29.2	0	0
	58.4	0	0

* Ten *G. mellonella* larvae were inoculated at each dosage level.

there was no evident relationship between the pH value of the culture filtrates and the ability to produce mycotoxins by the different species of *Entomophthora*.

Inasmuch as the culture filtrates of *E. coronata* and *E. apiculata* were unstable when stored at room temperature, their storage at a lower temperature was investigated. At 4°C the mycotoxin in
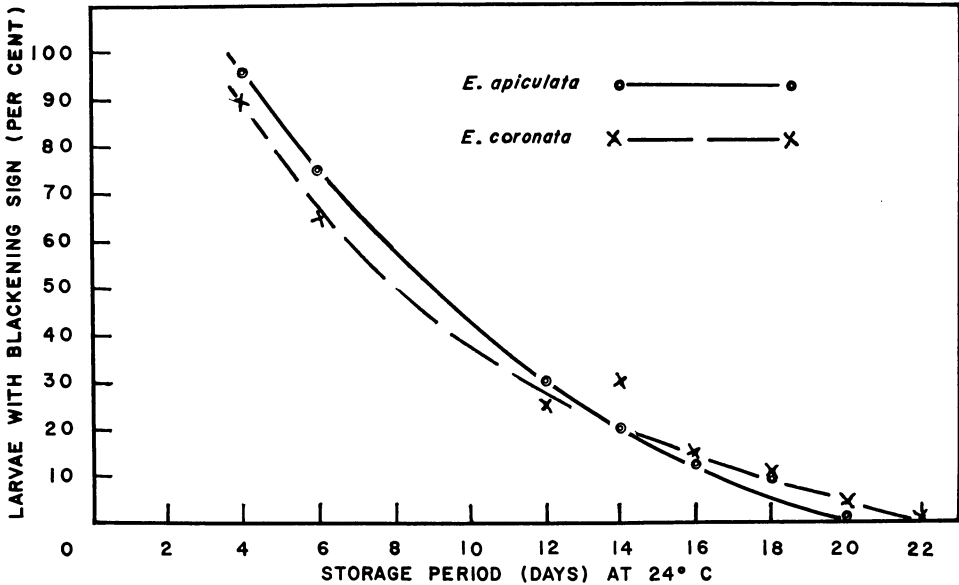
Fig. 1. Effects of storage at 25°C on the stability of the mycotoxin produced in the culture filtrates of *Entomophthora coronata* and *E. apiculata*. Mycotoxin activity was detected by inoculating the filtrates into the larvae of *Galleria mellonella*.

the culture filtrates of *E. coronata* and *E. apiculata* retained its activity for 52 weeks, when the test was concluded.

When stored at a constant temperature of 25°C, the mycotoxin of *E. coronata* was inactivated in 20 to 22 days, and that of *E. apiculata* in 18 to 20 days. The degree of activity of the mycotoxin in the culture filtrates is shown in table 3 and in figure 1. From figure 1, the percentage of inactivation of the mycotoxin could be estimated at different periods of storage at 25°C.

The results of the thermal tests indicated that the mycotoxin of *E. coronata* and *E. apiculata* was inactivated when heated between 48° and 52°C for 10 minutes.

When the culture filtrates of *E. coronata* and *E. apiculata* at the dose of 240 μl/gm larval weight were injected into the larvae of *G. mellonella*, they prolonged larval life beyond that of the control larvae and even affected metamorphosis. Silk production and pupation were also affected. Eventually the treated larvae died. (The signs and symptoms produced in these individuals are described later in this paper.) The two culture filtrates also inhibited metamorphosis of *Bombyx mori, Carpocapsa pomonella, Hyalophora cecropia, Peridroma saucia,* and *Pseudaletia unipuncta*. Larval feeding of some of these species also was affected.

The degree of inhibition of metamorphosis varied with the dose and insect species. The larvae of *Heliothis zea* could tolerate a dose of 501.9 μl/gm larval weight, and developed to adults. In contrast, the larvae of *G. mellonella* had a median effective dose (ED_{50}) of 72.3 μl/gm larval weight for the culture filtrate of *E. coronata*, and 281.0 μl/gm for the culture filtrate of *E. apiculata*.

Only a few of the black, intoxicated larvae of *G. mellonella* underwent putrefaction. Most became hard and mummified. This suggested that the fungus filtrates had an antibacterial property. To verify this, the culture filtrates were tested on bacteria isolated from the intestines of *G. mellonella* larvae. The fungus filtrates failed to inhibit the

growth of the bacteria when they were added to nutrient agar inoculated with the bacteria. This experiment should be repeated.

Methods used to determine nature of mycotoxin

An initial attempt was made with ether to extract the mycotoxin from the culture filtrates of *E. coronata* and *E. apiculata*. The ether extract and water portion were each injected into separate larvae of *G. mellonella*. The mycotoxin remained in the water portion and was not extractable by ether.

Since trichloroacetic acid (TCA) denatures proteinaceous substances and precipitates them out of solution, this acid was applied at 4:1 to the fungus-culture filtrates and to the modified peptone medium (MPT) to test for the presence of such substances. When the filtrates were separated from the precipitates and injected into the larval hemocoels, the typical blackening sign of intoxication in the blood was absent. On the other hand, the fungus-culture filtrate, when mixed with TCA at the ratio of 9:1, which did not cause precipitation, produced intoxication when injected into the larvae. These tests suggested that the proteinaceous substances precipitated by TCA contained the mycotoxin.

Centrifugation of the culture filtrates even at 25,500 *g* (20,000 rpm) for one hour, and at 102,000 *g* (40,000 rpm) for two hours, formed no apparent sediment—indicating that the mycotoxin had a small molecular weight.

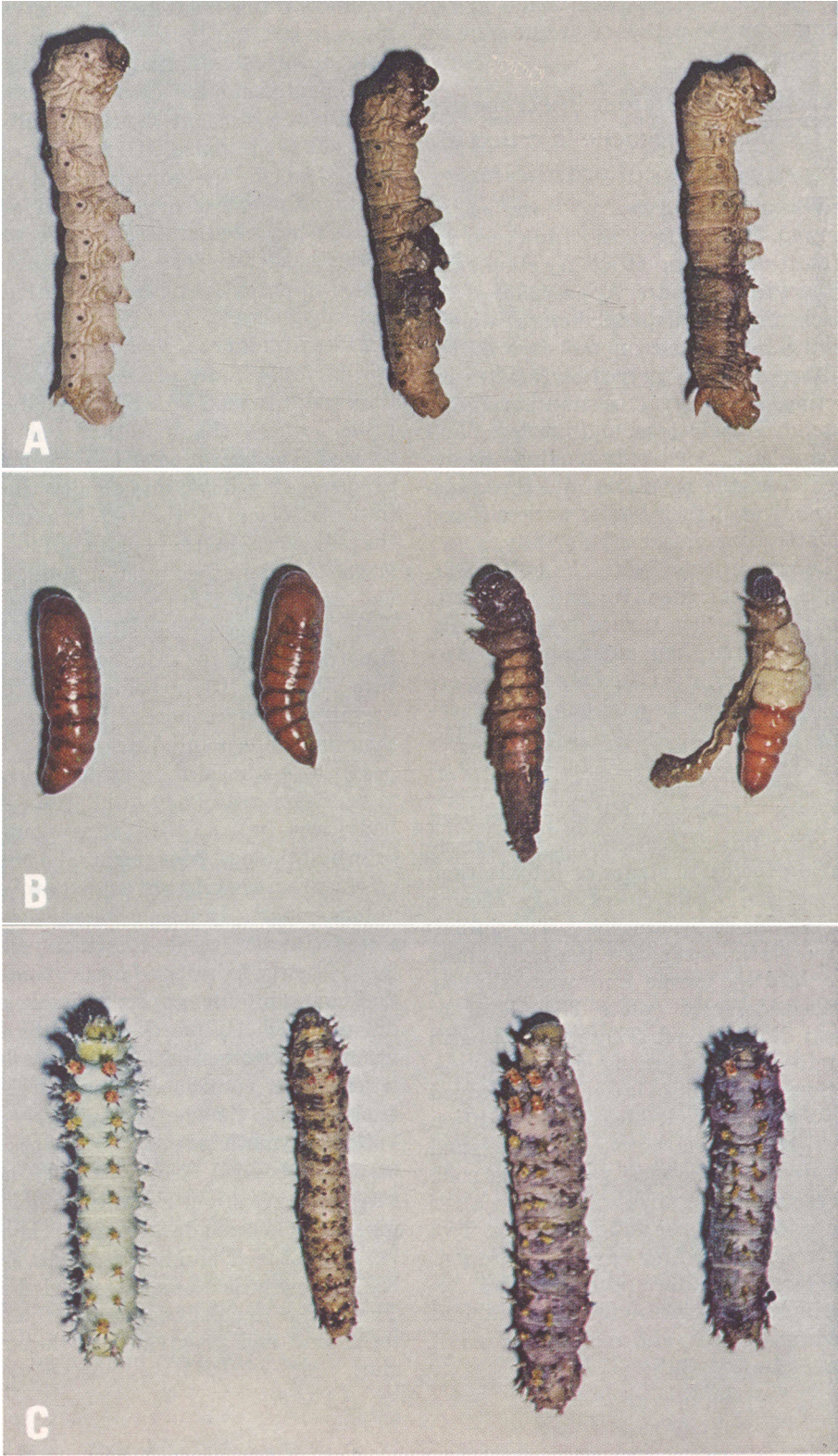Ten ml of the culture filtrates of *Entomophthora* species were completely evaporated in about 48 hours at room temperature. The residue was collected and dissolved in 5 ml of sterile, distilled water. The mixture obtained from the filtrates of *E. coronata* and of *E. apiculata*, when injected into the hemocoels of *G. mellonella* larvae, produced intoxication, but those of *E. thaxteriana* and *E. virulenta* caused no apparent toxicity. The mycotoxin, therefore, was not volatilized during the water evaporation.

Large numbers of long, needle-shaped crystals were noted when the culture filtrate residues of *E. coronata* and *E. apiculata* were examined under the binocular dissecting microscope. The crystals were transparent, straight, pointed at both ends, varied in diameter and length, and were visible to the naked eye. They were probably derived from material excreted by the fungi, and might be the mycotoxin. The needle-shaped crystals were rare, however, in the culture filtrates of *E. thaxteriana* and *E. virulenta* and in the modified peptone medium. Some crystals, smaller in diameter and shorter in length, were probably crystallized peptone.

Microfeeding of *G. mellonella*

Oral microfeeding of the culture filtrates of *E. coronata* and *E. apiculata* did not produce the blackening sign in the larvae of *G. mellonella*, and had no apparent effect, at doses of 29.4, 43.8, 58.4, and 73.0 μl per larva. However, these same doses, when injected into the hemocoels, did produce the blackening sign. Apparently the mycotoxin in these culture filtrates did not pass through the midgut epithelium into the hemocoel, or

Plate 2. **A.** Effect of mycotoxin on larvae of silkworm, *Bombyx mori.* Two brownish larvae on right were injected with culture filtrate of *Entomophthora apiculata.* The normal pale control larva was injected with the filtrate of a modified peptone medium. **B.** Effect of mycotoxin on pupation of corn earworm, *Heliothis zea.* Two abnormal prepupae on the right developed from larvae injected with culture filtrate of *E. apiculata.* Normal pupae (left) were from larvae injected with filtrate of modified peptone medium. **C.** Cecropia caterpillar, *Hyalophora cecropia,* showing the effect of *E. coronata* mycotoxin six hours after injection. Control larva (left) was injected with filtrate of modified peptone medium and was normal in color. The three intoxicated larvae developed black spots that spread and covered them entirely.

it was destroyed or inactivated in the midgut by the action of enzymes or by a change in pH.

Reactions of different insect species to the intrahemocoelic injection of *Entomophthora* culture filtrates

Wax-moth larvae, *G. mellonella,* showed blackening signs within ten minutes to 24 hours after injection, and often within 2 hours. Appearance of the blackening sign depended on the weight of the larvae and on the dose of the culture filtrates. Immediately after injection, some larvae became paralyzed, extended their legs and prolegs, and became stiff. Some only partially recovered from the paralysis, and the posterior halves of their bodies were stiff and slightly bent or arched dorsally.

Larvae injected with the culture filtrates became inactive; and with sufficient dosage they turned black and discharged from their anuses a dark gray coagulated liquid. Often the larvae were glued down at the anal end to the container by this liquid discharge. The blackening sign was first noticeable in the region of the dorsal blood vessel (heart), where it was more pronounced than at other locations in the larvae during the early stages of intoxication. Later, the whole insect body became evenly black (plate 1A). The intoxicated black larvae were less active than the healthy larvae and were slightly arched dorsally (plate 1B). Most of them did not feed. Those that fed still retained the blackening signs.

Some of the intoxicated larvae stayed alive even in their blackened condition until the control larvae had developed to adults. The longest period that such larvae remained alive was 25 days after the injection of the fungus filtrate. Two larvae lived for four days after the control larvae had developed to adults. Accordingly, the culture filtrates inhibited feeding, growth, and metamorphosis of the larvae.

Most of the blackened larvae could not spin silk, but those receiving a small dose of mycotoxin had the ability to spin small amounts of very thin silk. Such larvae died in their thin silk cocoons. If the intoxicated larvae pupated, they died in the pupal stage. After death, only some of the blackened larvae became putrefied; instead, their whole bodies hardened. The toxic culture filtrates apparently possessed antibacterial substances.

Silkworm larvae, *Bombyx mori,* were still alive 24 hours after injection, but their color turned brown, especially the proleg where the injection was performed. The brown color became darker in succeeding days, and finally turned into the color of the old exuviae of the silkworm larvae (plate 2A). The larvae attempted ecdysis, but the exuviae adhered to their bodies.

In some cases, the silkworms died three days after the injection of the culture filtrates. Their bodies were bent dorsally and were more or less stiff, and their anal ends pointed straight out. The posterior parts of the larvae appeared to be soaked with body fluid. The entire body was brown, with a pronounced brown in the posterior parts. The brown color progressively extended to the anterior part. In other cases, the injected larvae were brownish-yellow. Some newly-injected larvae vomited their gut contents, appeared weak, and did not feed. Unlike *G. mellonella* larvae, the silkworm larvae which received lethal doses continued, in general, to feed for some time before dying.

Codling-moth larvae, *Carpocapsa pomonella,* changed color within 20 minutes after injection. From their normal pink, they became darker pink. The larvae did not feed, and in some cases, their bodies were twisted and later dried out. The change in color began from the posterior end and progressed to the anterior.

Corn-earworm larvae, *Heliothis zea,* were much more resistant to the mycotoxin than were *G. mellonella.* Even a massive dose of the culture filtrates (about 320 μl/gm larval weight) did not cause any color change, as compared to the dose of 190 μl/gm of larval weight that was sufficient to produce the blackening sign in the *G. mellonella* larva. An *H. zea* larva, injected with a dose of 36 to 116 μl/0.363–0.590 gm of larval weight, developed normally and emerged to the adult stage, just as the uninjected larvae. However, when larvae were injected with a dose of 146 to 160 μl/0.363–0.590 gm larval weight, development was arrested; in some cases, the body became black, hard and brittle. The hardened skin, when touched with a pointed metal forceps, was easily broken.

Larvae injected with 146 to 160 μl transformed to the pupal stage about two days after the uninjected larvae, but their pupation was incomplete. The posterior part, especially the abdomen, formed pupal skin, but the region from the thorax to the head retained the larval characters. The body was shrunken abdomen (plate 2B). Part of the integument on the dorsal part of the body was that of the pupa, but the ventral integument remained larval in character. The abnormal individual had an enlarged abdomen, with head and throat of larval character, and retained the prolegs, but lacked adult wings, antennae, or legs. The incomplete pupa had white chalky skin at the thoracic region. All of these abnormal individuals died.

Cecropia-moth caterpillars, *Hyalophora cecropia,* developed the blackening sign from two to eight hours after injection. An injected larva discharged greenish-yellow, and at times reddish-brown, gut contents from its mouth and anus. When it was wounded, its blood was red-orange in color, and in a few minutes, became red-brown. The blood of an uninjected larva was green. Intox-

icated larva became more or less inactive, but still fed when the dose of mycotoxin was low.

The first blackening sign appeared at the base of the bristles as black spots, which spread and coalesced into larger spots (plate 2C). The entire bristle stem above the black spot also turned black. Then the prolegs became black. As intoxication progressed, the entire larval body became covered with black spots and left only small patches of green color. The body became flaccid. The larva stopped feeding and died within a day.

Variegated-cutworm larvae, *Peridroma saucia,* started to change color within five to seven hours after injection, stopped feeding, and died within two days. They became blackish-purple, and the dorsal vessel was only slightly different in color from that of an uninjected larva.

Armyworm larvae, *Pseudaletia unipuncta,* when injected with the culture filtrates (44 to 58 μl/0.280 to 0.590 gm of larval weight), changed from their normal light-brown to a purple color within one-half to two hours. The blood of the injected larva was bright purple in color. The color of the larva turned blackish-purple within three to four hours. The larva which showed purple signs died within one to three days after the injection of the culture filtrates. The dead larva was soft, unlike the dead larva of *G. mellonella.*

Pathology of mycotoxin

When paraffin sections of an intoxicated (and blackened) larva of *G. mellonella* were treated with Delafield's and counter-stained with Patay's stain, the hemolymph was stained dark-brown, as compared with the light-green color of the hemolymph of an uninjected larva. The blood cells of the injected larva were aggregated into clumps, with a clear zone surrounding each nucleus.

Fig. 2. Section through a larva of *Galleria mellonella* which had blackened after the injection of the culture filtrate of *Entomophthora coronata*, showing clumped, coagulated blood cells whose nuclei were stained darkly and surrounded by clear zones. Section stained with Delafield's hematoxylin and counterstained with Patay's stain. 960X.

TABLE 4

DATA FROM WHICH MEDIAN EFFECTIVE DOSES (ED₅₀) OF THE
CULTURE FILTRATES OF TWO *ENTOMOPHTHORA* SPP. WERE
ESTIMATED FOR LARVAE OF *GALLERIA MELLONELLA*

Dose*	*E. coronata* filtrate†			*E. apiculata* filtrate		
	Time lapse before larvae turned black	Larvae responding		Time lapse before larvae turned black	Larvae responding	
μl	*Hours*	*Number*	*Per cent*	*Hours*	*Number*	*Per cent*
25.55	.....	0	0	0.37 – 1.54	3	15
29.20	....	0	0	2.10 – 2.55	6	30
32.85	12.40	1	5	0.20 – 3.47	12	60
36.50	11.00	4	20	0.36 – 2.77	15	75
40.15	10.40	6	30	0.23 – 4.49	15	75
43.80	10.20	9	45	0.30 – 3.29	16	80
51.10	10.00	17	85	0.35 – 2.30	20	100
control‡	.....	0	0	....	0	0

    * Twenty larvae were inoculated at each dosage level. Larvae weighed from 0.120 to 0.180 gm for those inoculated with *E. coronata* filtrate, and from 0.130 to 0.180 gm for those inoculated with *E. apiculata* filtrate.
    † The filtrate of only *E. coronata* was diluted with the modified peptone medium at 1 part filtrate to 3 parts modified peptone medium.
    ‡ Inoculated with culture filtrate of modified peptone medium. Larvae did not show any color change.
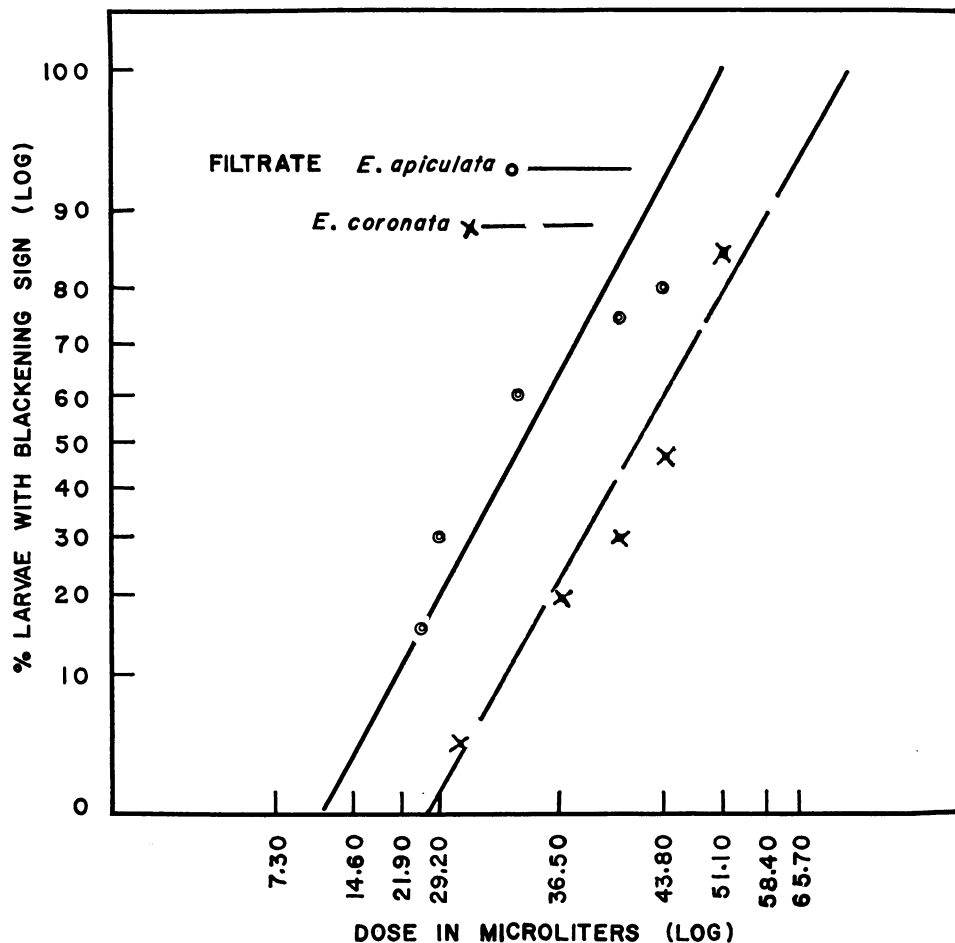
Fig. 3. Estimation of median effective dose (ED_{50}) of mycotoxin produced in culture filtrates of *Entomophthora coronata* and *E. apiculata*. Data were obtained from table 4. Filtrate of *E. coronata* was diluted with modified peptone medium at one part filtrate to three parts modified peptone medium (MPT).

The nucleus stained deeply with Heidenhain's iron hematoxylin. The cytoplasm of the blood cells stained dark-brown, and the borderline of each cell could not be differentiated in the clump of blood cells. The mixture of blood cells and coagulated hemolymph appeared to be semi-solid (fig. 2). Such aggregation, clumping, and coagulation of the blood cells and hemolymph did not occur in the uninjected larva.

The epithelial cells of the silk glands and reservoirs had a larger number of vacuoles than did those of the unin-

jected larva, especially in the areas around the nuclei, whose membranes were indistinct. The nuclear contents shrank and were attached to the vacuolar membranes or disintegrated. Remaining organs and tissues of the intoxicated larva had no detectable abnormalities.

Striking differences were noted between the histopathologies of *G. mellonella* larva injected with mycotoxin and those infected with the two *Entomophthora* species. The blood did not aggregate and clump or change color in

the hemolymph of the fungus-infected larvae as it did in the intoxicated larva described above. The fungus-infected larvae did not live for more than three to five days, while the intoxicated larvae lived up to 22 days.

Response to dosage of culture filtrates

In order to establish the median effective dose (ED_{50}), the standard culture filtrates of *E. coronata* and *E. apiculata* were injected at different dosages into the hemocoels of *G. mellonella* larvae. The data of table 4 were plotted on logarithmic paper (fig. 3). Since the original culture filtrate of *E. coronata* was very active (even a small dose of 25.55 μl/0.120 to 0.180 gm of larval weight produced a 100 per cent response), it was diluted one part of culture filtrate to three parts of MPT.

ED_{50} of the culture filtrates obtained by plotting dosage on logarithmic paper was very close to the ED_{50} obtained by probit analysis (Finney, 1952).

For *E. coronata* culture filtrates, the ED_{50} (diluted 1 part filtrate to 3 parts MPT) was estimated from the following:

(1) Figure 3 (the graph), 41.64 μl/ 0.120 to 0.180 gm larval weight;

(2) calculation, 43.65 μl/0.120 to 0.180 gm larval weight; and

(3) calculation (for ED_{50} range), 43.05 to 44.26 μl/0.120 to 0.180 gm larval weight.

For *E. apiculata* culture filtrates, the ED_{50} was estimated from the following:

(1) Figure 3 (the graph), 33.15 μl/ 0.130 to 0.180 gm larval weight;

(2) calculation, 32.81 μl/0.130 to 0.180 gm larval weight; and

(3) calculation (for ED_{50} range), 27.06 to 40.65 μl/0.130 to 0.180 gm larval weight.

Since the culture filtrate of *E. coronata* had been diluted 1:3, the ED_{50} of of the original culture should be $\dfrac{41.64}{4} =$ 10.41 μl/0.120 to 0.180 gm of larval weight obtained by plotting the dosages on logarithmic paper; and $\dfrac{43.65}{4} =$ 10.9125 μl/0.120 to 0.180 gm of larval weight by probit analysis. The ED_{50} range should be $\dfrac{43.50}{4}$ to $\dfrac{44.26}{4} = 10.76$ to 11.06 μl/0.120 to 0.180 gm of larval weight.

DISCUSSION

Out of four entomophthoraceous species which were examined, only two of them, *Entomophthora coronata* and *E. apiculata,* produced mycotoxins when cultured *in vitro* and *in vivo.* The mycotoxins produced by the two fungi appeared to be identical in every respect, but further study is necessary to establish whether only one mycotoxin was involved in our study. *Entomophthora coronata,* however, produced much more mycotoxin than *E. apiculata* when cultured in liquid medium for the same period of time.

The mycotoxin in the culture filtrates could be detected by injecting the filtrates into the hemocoels of wax-moth larvae (*Galleria mellonella*). The fungi produced more mycotoxin in shake than in stationary cultures, probably because they grew more rapidly under increased aeration and circulation of the medium. The mycotoxin appeared to be a natural excretory product rather than a product of autolysis of the fungus, because detectable amounts of the mycotoxin were found in fungus cultures after 36 hours, whereas autolysis did not begin until after 48 hours of cultivation. Smith (1953) also observed that autolysis oc-

curred at the end of 48 hours of culture. However, the peak of mycotoxin production in the filtrate occurred when autolysis was evident, so autolysis might be important in the accumulation of the mycotoxin in the filtrate. This aspect needs further investigation.

The mycotoxin possessed characteristics and properties similar to those of proteinaceous substance, e.g., it was precipitated by 10 per cent trichloroacetic acid. It was heat-labile and lost its activity during storage at room temperature or at 25°C for 22 days. Its thermal-inactivation point was between 48° and 52°C for 10 minutes. The mycotoxin could not be sedimented out of the culture filtrates by centrifugation at 102,000 *g* for two hours. It was non-ether-soluble, but water-soluble and nonvolatile, and appeared to form needle-shaped crystals in the residue of fungus-culture filtrates. When the residue was resuspended in water, it still caused intoxication in *G. mellonella* larvae. Thus far, we have not succeeded in establishing the chemical identity of the mycotoxin.

The mycotoxin, however, was different from "destruxin 'A' and 'B' " which Kodaira (1960) purified from *Metarrhizium anisopliae*. Destruxin A and B were heat-stable and could withstand heating for one hour at 100°C. They caused tetanic paralysis in injected larvae of *Galleria mellonella* (Roberts, 1964) and of *Bombyx mori* (Kodaira, 1960; Roberts, 1964). In our study, this form of paralysis did not occur in larvae inoculated with the mycotoxins of *E. coronata* and *E. apiculata*. Instead, larvae developed blackening signs. Moreover, the fungus-culture filtrates seemed to inhibit the growth of bacteria in dead *G. mellonella* larvae. This property is similar to that of cordycepin formed by *Cordyceps militaris* (Cunningham *et al.*, 1951).

When injected in sufficient amounts into the larval hemocoel, the culture filtrates inhibited metamorphosis and altered the feeding habits of some insect species. A lethal dose of the culture filtrates eventually caused the death of the insect in the larval stage or during pupation.

Microfeeding of the culture filtrates of *E. coronata* and *E. apiculata* to *G. mellonella* larvae did not produce any detectable signs or symptoms. The ineffectiveness of the mycotoxin when fed to larvae restricts the application of this toxin as a microbial pesticide on insect pests. Yendol (1964) also failed to intoxicate the eastern subterranean termite, *Reticulitermes flavipes*, by feeding it the culture filtrate of *E. coronata*. He speculated that (a) the mycotoxin might be a function of the specific host-parasite relationship, (b) the mycotoxin was diluted in the modified peptone medium or sequestered by compounds in the medium, with the result that the filtrates became inactive, or (c) the intestinal wall and integument acted as a barrier to the toxic product.

Intrahemocoelic injections of the culture filtrates of *E. coronata* and *E. apiculata* into seven different insect species showed that the mycotoxin inhibited metamorphosis, molting, and feeding, but there were differences in the reactions among the insect species to the toxin. The *Heliothis zea* larvae required a much larger dose than the larvae of the other species to produce these signs and symptoms. The blood-color change also varied among the different insect species.

Initially, change in the color of the blood was the most evident sign in the intoxication caused by the mycotoxin. A study of the histopathology of an intoxicated larva of *G. mellonella* showed that the mycotoxin severely affected the hemolymph, changed its staining property

and caused clumping of the hemocytes. Sussman (1952) found that an aqueous suspension of the conidia of *Aspergillus flavus* injected into a cecropia pupa caused blackening of the integument and blood. He believed that the blackening was caused by the enzyme tyrosinase, which is almost universally present in insects and is considered to play an important role in their respiration and structural development (Sussman, 1949).

Aside from the blood, the only other tissue or organ exhibiting detectable pathologies was the silk glands. This suggests that the pathological effect of the mycotoxin on the metabolism of the host consists primarily of causing the failure of the hemolymph to circulate the necessary nutrients and hormones to the tissues and organs involved in metamorphosis and molting. This aspect needs further study and clarification. Also, the mycotoxin should be compared with known poisons that act on the insect hemolymph.

Although the culture filtrate, and presumably the mycotoxin, when inoculated into the larva caused darkening of the blood and clumping of the hemocytes, the infection by *E. coronata* and *E. apiculata* did not cause blood color changes and clumping of the hemocytes. The histopathology of these different effects should be investigated further, because our study has shown that the mycotoxin is produced by the fungi in infected larvae.

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