Presence of Toxic Substance in Fungus Bodies of the
Entomopathogenic Fungi, *Beauveria bassiana*
and *Verticillium lecanii*

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Beauveria bassiana and *Verticillium lecanii* have been isolated from the diseased pupae of the silkworm, *Bombyx mori* L., and both fungi were demonstrated to be pathogenic to the silkworm. The production of a toxic substance in the dead pupae infected with each fungus was confirmed through a bioassay by use of artificial diets containing powder prepared from the dead pupae. The toxic substance was also detected in the fungus bodies of both fungi cultivated in surface culture on chemically-defined media. The toxic substance was lethal to silkworm larvae by oral administration and caused atonic symptoms in the larval bodies at less than lethal doses. A toxin named bassianolide has been isolated from the fungus bodies of both fungi which accounted for the lethality and toxicity accompanying the atonic symptom detected in the dead pupae infected with each fungus.

INTRODUCTION

Various kinds of fungi have been isolated as causes of insect diseases. Until recently however, only a limited number of fungi have been known to produce toxins which may play an important role in mycosis. *Metarrhizium anisopliae* and *Aspergillus flavus* have been reported to kill diseased insects by production of toxins, destruxins (SUZUKI et al., 1971) and aflatoxins (OHOTOMO et al., 1975; MURAKOSHI et al., 1977), respectively. Aspochracin produced by *Aspergillus ochraceus* (MYOKEI et al., 1969) and beauvericin produced by *Beauveria bassiana* (HAMILL et al., 1969) were also toxic to insects.

In the course of screening for toxic metabolites of entomopathogenic fungi, we found that the dried fungus bodies of *Beauveria bassiana* and *Verticillium lecanii*, which were newly isolated from naturally infected corpses of silkworm pupae (*Bombyx mori*

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L.), were toxic to silkworm larvae when orally administrated with artificial diets, and that toxicity was unique in accompanying atonic symptoms in larval bodies. This fact suggested the possible production of a toxic substance in the fungus bodies of both fungi.

In this paper, we wish to report results which clarify the presence of a toxic substance in the fungus bodies of *B. bassiana* and *V. lecanii*.

MATERIALS AND METHODS

Isolation of the entomopathogenic fungi. The corpses of the diseased silkworm pupae were collected in a large scale rearing of the worm in the autumn of 1975. The isolation of fungi from the corpses was made by use of agar plates composed of Czapeck-Dox medium containing 2% yeast extract. Among over fifty strains isolated from the corpses, two kinds of fungi were predominant and taxonomically characterized to be *Beauveria bassiana* and *Verticillium lecanii*. *B. bassiana* No. 75-8 and *V. lecanii* No. 75-1 were subjected to further studies.

Pathogenicity test of the isolated fungi. For spore production, both *B. bassiana* No. 75—8 and *V. lecanii* No. 75—1 were grown on an agar plate composed of Czapeck-Dox medium containing 2% yeast extract at 25°C. After approximately 2 weeks, the fungi produced conidiospores and the spores of each fungus were harvested with distilled water. The harvested spores were suspended in water containing 0.05% Tween 40 at a density of 1.4×10^7 spores/ml. Then, 50 μ l of spore suspension were injected into the hypodermis of a silkworm pupa (*B. mori* cv. Shunrei $\times$ Shogetsu) and the pupa was kept at 25°C.

Cultivations. Spores of each fungus were inoculated into 20 ml of Czapeck-Dox medium containing 2% yeast extract in a 100-ml Erlenmyer flask. Each fungus was cultivated at 25°C. At intervals, the culture broth was sterilized at 120°C for 20 min. and was separated into culture filtrate and fungus bodies.

Toxicity test. The fungus bodies and diseased pupae were dried and pulverized into fine powder which was used for preparing artificial diets as test samples. Purified toxins were dissolved in acetone and added to the materials used for artificial diets.

An appropriate quantity of sample was added to a mixture composed of mulberry leaf power (50%), defatted soybean meal (24%), cellulose powder (15%), citric acid (4%), agar powder (5%), L-ascorbic acid (1.5%) sorbic acid (0.5%), and distilled water (2.5 ml/g of solid). The mixtures were steamed at 80—85°C for 15 min..

In the case of culture filtrates, water containing culture filtrate was used instead of distilled water.

Fourth or 5th-instar larvae of silkworm (*B. mori* L. cv. Shunrei $\times$ Shogetsu) were reared on the artificial diets prepared as above and their growth and symptoms were compared to those of the larvae fed on the diet lacking the test sample.

RESULTS

When the spores of *B. bassiana* No. 75—8 and *V. lecanii* No. 75—1 were injected into silkworm pupae, each fungus was very pathogenic, killing all pupae in three days. Four days after spore injection, *B. bassiana* formed thin films of mycelia and spores on the surface of dead pupa, whereas *V. lecanii* was not observed on the surface. After 14

Fig. 1. Silkworm pupae infected with entomopathogenic fungi. A) Control (no infection), B) *B. bassiana* No. 75—8 (after 14 days), C) *V. lecanii* No. 75—1 (after 14 days).

Table 1. TOXICITY OF SILKWORM PUPA INFECTED WITH *V. lecanii* No. 75-1 AND *B. bassiana* No. 75-8 TO THE LARVAE OF *B. mori*^a

Fungus	Content of pupal powder in solid ^b	Symptom of atony ^c	Mortality after a week
Nil.	5 %	—	0 %
	10	—	0
<i>V. lecanii</i>	5	±	0
	10	+	20
<i>B. bassiana</i>	5	‡	100
	10	‡‡	100

^a Ten larvae just after 3rd molting were used for each diet.

^b The corpses of pupae were collected 14 days after spore inoculation.

^c Observations were made on 2nd day of rearing.

days, as shown Fig. 1, both fungi grew well on dead pupae and the fungal mats of *B. bassiana* were much denser than those of *V. lecanii*. These corpses were powdered after drying and added to artificial diets for bioassay. As shown in Table 1, the powder prepared from the dead pupae was shown to be toxic to silkworm larvae by oral administration, suggesting the production of a toxic substance in the pupae by the fungi. Toxicity in the dead pupae infected with *B. bassiana* was appreciable even after 3 days of spore injection. Toxicity of the substance was unique in causing atony of larval bodies (Fig. 2).

In liquid culture, fungal mats were formed on the surface of the culture medium. Ten days after inoculation, each dried fungus body of *B. bassiana* No. 75—8 and *V. lecanii* No. 75—1 showed marked toxicity to silkworm larvae when fed with artificial diets. The atonic symptoms observed in the larvae fed on dried fungus bodies suggested that the toxic substance produced in the pupae infected with *B. bassiana* or *V. lecanii* was present in the fungus bodies harvested from the liquid culture. The activity of *V. lecanii* was somewhat stronger than that of *B. bassiana* and also revealed growth-inhibi-

Fig. 2. Poisonous symptom of fungus bodies of *B. bassiana* No. 75—8 and *V. lecanii* No. 75—1. Left: Control, Right: Poisoned worms.

Table 2. TOXICITY OF *V. lecanii* AND *B. bassiana* IN LIQUID CULTURES^a

Fungus		pH of filtrate	Yield of fungus bodies (Dry wt.)	Av. body wt. of surviving larvae after feeding ^b		Mortality after 6 days
				2 Days	6 Days	
Nil.		—	—	422mg	860mg	0%
Medium		5.6mg	—	376	650	0
<i>V. lecanii</i>	F	6.2	—	402	790	0
	B	—	336mg	177	333	80
<i>B. bassiana</i>	F	5.8	—	256	—	100
	B	—	324	189	401	20

^a Each fungus was cultured for 10 days in 20 ml of medium.

^b Ten silkworm larvae just after 3rd molting (average weight, 155 mg) were reared on each diet containing the powder of fungus bodies from 20 ml of culture and culture filtrate (1.5 ml/g solid).

Table 3. EFFECTS OF CULTIVATION PERIOD ON TOXICITIES OF *B. bassiana*^a

Cultivation period (Days)	pH of culture filtrate	Yield of fungus bodies	Toxicity after a week	
			Culture filtrate	Fungus bodies
Nil.	5.6	—	—	—
2	4.0	87mg	—	—
3	3.6	116	±	±
4	3.4	236	+	+
5	3.6	262	+	++
6	4.4	304	++	++
7	5.2	332	++	++

^a Each of culture filtrate and fungus bodies from 20 ml of culture was added to 10 g of solid in artificial diets. Ten silkworm larvae just after 3rd molting were reared on each diet.

tory activity to silkworm larvae. The results are summarized in Table 2.

As shown in Table 3, the time-course of toxicity in the fungus indicated that the production of the toxic substance by *B. bassiana* became marked for the first time when the pH values of culture filtrate began to increase gradually.

Experiments to date confirmed the presence of toxic substance in the fungus bodies

Table 4. TOXICITY OF BASSIANOLIDE TO SILKWORM LARVAE BY ORAL ADMINISTRATION^a

Dose (ppm)	Mean body weight (mg)				Number of survivors			Symptom of atony ^b
	Rearing period (days)				Rearing period (days)			
	0	2	4	6	4	6	8	
0	169	443	843	1000	10	10	10	—
1	170	460	830	993	10	10	10	—
2	173	315	601	914	10	10	10	±
4	173	221	390	721	10	10	10	+
8	174	202	221	369	8	2	1	⦿
12	176	201	221	—	4	0	0	⦿
16	179	198	189	—	2	0	0	⦿

^a Ten larvae just after 3rd molting were reared on each diet.

^b Observation was made on 2nd day of rearing.

of each *B. bassiana* and *V. lecanii*. Based on these results, we have isolated a toxic substance which causes atonic symptoms of the larval bodies from the fungus bodies of both fungi. We have designated this toxin bassianolide. The structure of bassianolide was determined as a cyclodepsipeptide composed of an alternative repeating sequence of four moles of L-N-methylleucyl-D- α -hydroxyisovaleryl (SUZUKI et al., 1977).

Bassianolide was lethal to silkworm larvae in the fourth instar (*B. mori* L. cv. Kinshū × Shōwa) when fed with an artificial diet at doses of more than 8 ppm (Table 4) and the symptom of atony was appreciable even at a dose of 4 ppm.

DISCUSSION

Until recently, several authors have reported that *B. bassiana* may produce a metabolite toxic to insects (DRESNER, 1950; KUCERA and SAMSINOKOVA, 1968; KUCERA, 1971; SCHAEFFENBERG, 1957; WEST and BRIGGS, 1968; WOJCIECHOWSKA, 1973), whereas, as far as we know, the toxic metabolite from *V. lecanii* has not been previously reported.

In our experiments, the newly isolated fungi, *B. bassiana* No. 75—8 and *V. lecanii* No. 75—1, were confirmed to be pathogenic to silkworm and to produce a toxic metabolite in their fungus bodies. The toxic metabolite in the fungus bodies caused the unique symptom of atony in silkworm larvae by oral administration even at less than lethal doses. Bassianolide (SUZUKI et al., 1977) isolated from the fungus bodies of both fungi was a cause of the atonic symptom. When fed with artificial diets, bassianolide was active to 4th-instar larvae of silkworm even at a dose of 4 ppm and lethal at doses of more than 8 ppm.

Since the production of a toxic substance was suggested in the infected silkworm pupae, the mycosis of insects due to *B. bassiana* or *V. lecanii* seemed to be related to an insecticidal toxin such as bassianolide.

Beauvericin has also been isolated from *B. bassiana* (KANAOKA et al., 1978) and was not toxic to silkworm larvae by oral administration even at doses of more than 1000 ppm, though it was reported to be toxic to brine shrimp and mosquito larvae (HAMILL et al., 1969).

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