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Title: THE PARASITE ASPERGILLUS FLAVUS LINK AND OTHER FUNGI OF THE BIOSPHERE OF THE ALKALI BEE NOMIA MELANDERI CKLL., IN EASTERN OREGON AND WASHINGTON

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The pathogenicity of A. flavus on alkali bee larvae was studied using sample materials from three nesting sites of these soil inhabiting bees along the Oregon-Washington state line. Soil saprophytes of the biosphere of the alkali bee were also studied.

Field observations and disection of soil cores from bee beds provided a basis for estimates of A. flavus disease at the three sites. Fungi, including A. flavus and soil saprophytes were isolated from: soil samples, the surface of adult alkali bees, pollen balls, and larval feces.

In vitro disease induction experiments were carried out comparing uninoculated larvae and larvae surface inoculated with conidia of A. flavus. The tests were conducted at five levels of relative humidity within each of three temperature treatments.

Field observations and soil cores revealed A. flavus as the predominante fungus pathogen in two of the three sites studied. One site was free of the pathogen.

Studies of soil dilution plates and fungal isolations from adult bees, pollen balls, and larval feces disclosed propagules of A. flavus in the biosphere of the bees in two of the three beds. Saprophytic fungi of the biosphere did not vary significantly over a three year period. These fungi provided a useful index of soil contamination of adult bees, pollen balls, and larval feces.

Studies of the effect of relative humidity and temperature on disease induction in larvae surface inoculated with A. flavus conidia revealed that temperatures of 25° to 30°C and relative-humidities from 90 to 100 percent favored disease development while lower temperatures and/or lower humidities inhibited disease induction.

The increased incidence of A. flavus disease of larvae during the spring and summer is considered to result from increasing soil temperatures and relative humidities within the brood cells. Conversely, a reduced incidence of disease during the fall and winter results from lower temperatures.

Higher incidence of A. flavus disease in one of the beds (the Garbe bed) was attributed to its age and higher bee population. Soil cores taken from the Garbe bed to establish the Harris bed account for the prevalence of disease in the latter. The lack of A. flavus

disease in the Wallace-Key bed was not clear, but its younger age and lower population density offer a partial explanation.

Control of A. flavus within the nesting site might logically aim at elimination of the fungus from the soil. Sub-irrigation of the beds with fungicide is one possible approach.

The Parasite *Aspergillus flavus* Link and other Fungi of the Biosphere of the Alkali Bee *Nomia melanderi* Ckll., in Eastern Oregon and Washington

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THE PARASITE *ASPERGILLUS FLAVUS* LINK AND OTHER FUNGI OF THE BIOSPHERE OF THE ALKALI BEE, *NOMIA MELANDERI* CKLL., IN EASTERN OREGON AND WASHINGTON

INTRODUCTION

The alkali bee, *Nomia melanderi* Ckll., is endemic to arid western United States and is a highly efficient pollinator of alfalfa. Bohart (1950) has noted its contribution to high yields of alfalfa seed in Utah, while Menke (1954) has attributed increased alfalfa seed production in the Yakima area of Washington and in southern Idaho to the presence of the alkali bee. Stephen (1959) indicates that while the national average for alfalfa seed production in 1956 and 1957 was 181 and 179 pounds per acre respectively, in the areas of Washington and Oregon where the alkali bee is common yields of 1,200 pounds per acre are not uncommon.

The alkali bee is a soil nesting species and until the work of Stephen (1960), it inhabited only natural nesting sites. Alfalfa seed producers desiring to benefit by the efficiency of this pollinator were, therefore, dependent upon the occurrence of natural nesting sites in the vicinity of alfalfa fields. The publications of Stephen (1960) described a method for construction of artificial nesting sites which allowed seed producers to establish nesting sites throughout the alfalfa growing area.

N. melanderi overwinters as a prepupa within its cell. The

adult emerges in the late spring (May or June) depending on conditions of soil moisture and temperature. Multiple mating occurs upon emergence and may continue periodically until the female bee selects a suitable nesting site and burrows into the soil. A typical nest is from 6 to 10 inches deep and consists of a vertical main burrow, a chamber, and 1-15 cells. The main burrow leads from the soil surface downward and ends in a chamber of variable size excavated by the bee. Cells are excavated below the chamber floor and each is provisioned with alfalfa pollen and nectar. Each cell is sealed with a layer of soil after an egg is deposited on top of its provision. One female will construct as many as 24-26 cells in her life time (Stephen, 1959). In the Pacific Northwest there is typically only one generation per year; however, in California, where soil temperatures typically run higher for a longer period of time, several generations may be produced in a single summer (Torchio, 1967). After several days the 1st instar larva emerges from the egg and begins feeding on the pollen ball (Hackwell and Stephen, 1966). Feeding continues through five instars. At the conclusion of the fifth instar the larva defecates once and the fecal material is deposited at the bottom of the chamber. The postdefecated 5th instar larva is known as the prepupa, and it is in this form that it overwinters in the cell.

The alkali bee is subject to a number of predators and diseases. Stephen (1959) reported the occurrence of Aspergillus ustus and A.

terreus in association with diseased prepupae collected from bee beds along the Oregon-Washington state line. He reported that diseased prepupae rarely exceeded 5 percent in any core sample taken from an infected bed. However, prepupae from one natural nesting site, the Garbe site reported in this thesis, had infection rates as high as 35 percent in 1957 and 1958. In the years immediately following Stephen's report of the fungus associates of the alkali bee, disease became more prevalent. Recently, artificial nesting sites have become infested with disease producing fungi two to three years after construction. The occurrence of fungus diseases in natural nesting sites has also become widely recognized.

The present study was initiated in 1962, as essentially no work had been done on fungus parasites of the alkali bee. The object of the study was to determine the principal fungus parasites occurring in selected bee nesting sites in the Milton-Freewater area of Oregon and Washington. In addition information was obtained on the mode of infection by the primary pathogen, and other fungus associates of the alkali bee were studied.

Early in the investigation it became apparent that the primary fungus parasite of alkali bee prepupae in these sites belonged to the Aspergillus flavus Link group of species as defined by Raper and Fennell (1965). It was decided, therefore, to concentrate this study around A. flavus and certain fungi associated with it.

LITERATURE REVIEW

Aspergillus flavus has long been known as a parasite of insects. Among those insects parasitized are: the desert locust (Lepesme, 1938; Misna, 1952), the cecropia moth (Sussman, 1951a, 1951b, 1952a, 1952b, 1952c), Malacosoma (Gee and Massey, 1912), mealybugs (Boyce and Fawcett, 1947), termites (Beal and Kais, 1962), the rice moth (Hedge and Shanmuggasundarm, 1968), and the honey bee (Betts, 1951; Burnside, 1930; Howard, 1896; Turesson, 1917; Vincens, 1923). The earliest report of A. flavus parasitism of insects is that of Howard (1896). Because of the economic importance of the honey bee, the fungus has received attention under the names Stonebrood and Bee Paralysis.

The most comprehensive review of A. flavus pathogenesis of honey bees and other insects was provided by Mandelin (1963). He reported that A. flavus infection of adult honey bees is usually initiated through the alimentary canal. However, death resulted in some before the body cavity was invaded by the mycelium. He suggested that death results from a toxin produced by germinating conidia in the digestive tract. The early symptoms of infection include a partial paralysis which can be induced by feeding healthy bees on filter-sterilized extracts from A. flavus colonies. These results lend further credence to the view that toxins are involved.

In other experiments death of the adults was attributed to blockage of the gut by a mycelial mass.

The work on aflatoxins, reviewed by Austwick (1965), supports the hypothesis that death of adult honey bees was a result of toxins produced by the fungus. However, the results of the present study indicate that toxin production is not a key factor in the death of alkali bee larvae.

Bailey (1963) reported that in honey bee larvae infection may take place through penetration of the cuticle by germ-tubes produced from conidia germinating on the cuticular surface. However, he suggested that infection is usually initiated via the gut. In the case of the alkali bee larva current evidence indicates that infection occurs via conidia germinating on the cuticle of the larva.

The relationship of fungi and wild bees has been recognized but has not been studied extensively. The earliest report of A. flavus parasitism of the alkali bee is that of Steinhaus (1951) who isolated A. flavus and A. flavipes from diseased alkali bee larvae collected in Utah in 1950. Subsequently, Steinhaus and Marsh (1962) reported the isolation of A. flavus from several alkali bee "specimens" sent to them from Washington. They also reported four additional collections of diseased adults and larvae, but did not provide specific diagnosis.

Stephen (1959) reported A. ustus and A. terreus from diseased

alkali bee larvae collected from beds along the Eastern Oregon-Washington border. This constitutes the only other report of fungus parasitism of the alkali bee. However, these identifications are of questionable certainty.

The work reported here is the first attempt to elucidate the relationship of A. flavus to various aspects of the alkali bee under field and laboratory conditions.

MATERIALS AND METHODS

Five sites were studied in the Touchet-Milton-Freewater area along the Oregon-Washington state line. Three of these were studied repeatedly: the Henry Garbe site, 3 miles from Touchet on the Washington side of the line; the Archie Harris site, 2 miles south on the Oregon side of the line; and, the Wallace-Key site, one mile from Umapine, Oregon in Oregon.

The Garbe and Wallace-Key locations were natural nesting sites, bees from the former having the highest incidence of fungus disease and those from the latter having the lowest incidence of disease. The Harris location was an artificial nesting site constructed between the years 1956-1958. Bees from this site had a moderate incidence of fungus disease. The sites were within 10 miles of each other.

The study was divided into three phases. The first phase consisted of field observations on the prevalence of fungus mortality and of laboratory identification of fungi associated with the bees. The second phase consisted of a series of studies on the fungi of the alkali bee biosphere. Isolations of fungi were made from the soil of beds, the surface of adult bees, the pollen balls provisioning the cells, and the feces of bee larvae. The final phase was devoted to laboratory study of disease induction in prepupae using conidial inoculum of

A. flavus. The investigation was carried out between the years 1962 to 1968.

The Garbe site covers about one acre of exposed hillside adjacent to the Garbe home in Washington. The site is sub-irrigated by a natural spring which provides moisture into the early summer. This site was used by Stephen for some of his early studies and provided cores used to populate many of the artificial beds set up nearby. Larval mortality has been unusually high in this bed.

The artificial nesting site on the Harris property was constructed adjacent to a dirt road which passed through alfalfa fields. The bed itself consists of section I constructed in 1956 and section II completed in 1958. Each section is sub-irrigated by means of pipes reaching into the sub-surface gravel layer. The soil is considerably less compact than that of the Garbe bed and during the course of this study both sections varied from exceedingly moist to slightly so. Larval mortality has been moderate in relation to other beds studied. Soil core studies of the Harris bed carried out between 1962 and 1968 indicated a uniform incidence of fungus mortality between sections I and II. Therefore, this bed was treated as a unit in subsequent studies.

The Wallace-Key natural nesting site is a two acre pasture area which slopes gently into a marsh at one end. Cells are constructed nearer to the surface than in the other two sites studied.

Larval mortality has been very low.

Field Observations

To obtain a general idea of the incidence of fungus mortality and of the variety of fungi involved in the three beds selected for study, cells were exposed by digging at randomly selected spots in the beds. Observations were noted and photographs taken. When necessary for identification or other purposes the infected prepupae and fungus mycelia were placed in sterile test-tubes and brought into the laboratory for further study. However, with experience it became possible to distinguish A. flavus infections by visual inspection in the field.

Core Samples

In order to obtain information on seasonal variations in the incidence of fungus caused mortality 4 cores, 4" in diameter and 12" in length, were taken from each of the study sites on each of 11 dates over the period from July 1962 to July 1963. As stated above the Harris bed was divided into two parts for this study. The soil coring device was constructed from an 18" steel pipe having an inside diameter of 4 1/2". By means of a gradual taper the lower 12" of the pipe was sharpened to facilitate driving it into the soil. Cylindrical sleeves were constructed of heavy gauge galvanized metal to fit inside the pipe. They were designed so that the long edges slightly

overlapped when the sleeve was fitted in place in the corer. The 12" long sleeves were with a recurved leading edge that fitted over the basal edge of the corer and prevented slippage upward as the sleeve and corer were driven into the ground by a heavy sledge-hammer. After withdrawal from the soil the corer was removed from the sleeve. The sleeve and its enclosed soil core were held together by means of a heavy rubberband which was slipped around the sleeve before the corer was completely removed. Core samples were then transported within the sleeve to the laboratory, and were stored in a cold room at 40°F. In no case was the storage period longer than one week.

In the laboratory the soil cores were removed from the sleeves, dissected, and examined. The numbers of infected and healthy larvae per core were recorded.

Isolations from the Soil

These studies involved fungal isolations from soil. Since the female alkali bee prefers soils of a nesting site with a high sodium salt content (Stephen, 1959), it was initially felt that salinity might have a strong influence on the fungus populations isolatable with standard laboratory techniques. A preliminary study was therefore conducted using three media, Potato-Dextrose Agar with streptomycin (SPDA), Czapek's with streptomycin and Peptone-Dextrose Agar plus Rose Bengal and Aureomycin each with three concentrations

NaCl, 0, 3, and 10 percent. Results of this study indicated little difference among the three media and salt concentrations. Potato-Dextrose Agar with streptomycin was, therefore, selected for use in all subsequent studies.

Soil samples were taken directly from the bee beds and also from some of the core samples. Soil dilution plates were made after 25 grams of soil was suspended in 250 ml. of sterile distilled water by shaking the mixture in a wrist action mechanical shaker for one hour. A dilution series of 1:100, 1:1,000, 1:10,000 and 1:50,000 was prepared. One ml. of each dilution was added to each of three Petri-plates and swirled with 20 ml. of sterile molten Streptomycin-Potato-Dextrose Agar at 45°C. The plates were incubated at room temperature and read for colonies of A. flavus and other fungi on the sixth day after preparation.

Isolations from Adult Bees

Adult bees were collected and brought into the laboratory. They were placed in separate test-tubes containing 10 ml. sterile distilled water. The test-tube was shaken for a one minute period after which 2 ml. was removed by means of a sterile pipette and placed in a sterile Petri-plate. Molten SPDA, at 45°C. was then added to the Petri-plate and the contents swirled to insure uniform mixing. The plates were incubated at room temperature and read for A. flavus

and associated fungi after five days.

Isolations from Pollen Balls

Isolation of fungi from pollen balls was complicated by the tendency of the delicate walls of the surrounding cell to crumble allowing contamination of the surface of the pollen ball by soil particles. As a result, pollen balls certain to be free of surface contamination were not obtained. These initial difficulties were overcome by placing the contaminated pollen ball in a sterile Petri-plate, breaking it open with sterile dissecting needles and removing for plating, particles from the freshly exposed center by means of a sterile dissecting needle. The pollen, presumably free from contamination, was transferred to Petri-plates of sterile SPDA. The plates were incubated at room temperature and read at the end of five days for the presence of A. flavus and other fungi.

Isolations from Larval Feces

Predefecating, 5th instar larvae were collected in the field and placed in sterile Petri-plates for transportation to the laboratory. In the laboratory each larva was transferred to a sterile Petri-plate where it was allowed to defecate, a process which took from one to three days. Two samples from each fecal mass were transferred by means of a sterile dissecting needle to the surface of solidified sterile

SPDA in a Petri-plate. The plates were incubated at room temperature and read at the end of five days for the presence of A. flavus and other fungi.

In Vitro Disease Induction

Two laboratory experiments were carried out to test the pathogenicity of A. flavus on prepupae of the alkali bee, and to determine moisture and temperature conditions most favorable to pathogenicity of the fungus.

The cultures of A. flavus used were obtained from a dead prepupa. Plates of PDA were inoculated in the center with conidia and incubated until conidial production had taken place over the entire agar surface.

In each of the two experiments 300 prepupae were surface sterilized by three 15 second washings in 70 percent ethyl alcohol. Each of the first two washings was followed by a sterile distilled water rinse while the final washing was followed by two rinses.

The prepupae were then divided into two groups of 150 each. Those to be inoculated were transferred aseptically in groups of 25, a single group being placed in each of 6 Petri-plate cultures of conidia-bearing A. flavus. Each plate was shaken, to produce an even distribution of conidia over the moist prepupal surface, until each prepupa had a greenish cast due to adhering conidia. The

prepupae were then transferred individually by means of sterile forceps into 15 mm. × 75 mm. culture-tubes.

The 150 prepupae serving as controls were similarly treated except that they were shaken on the surface of sterile PDA without A. flavus conidia.

Both the inoculated and control prepupae were divided into sub-groups of 10, each sub-group of 10 tubes being placed upright in a large jar containing an inch layer of a sulfuric acid-water mixture in a ratio calculated to provide a selected relative humidity. Humidities employed in the closed jars were 30, 50, 80, 90 and 100 percent. Incubation of each humidity series was for 30 days at 20°C., 25°C., and 30°C., the temperatures most closely covering the range of soil temperatures encountered in the field during the spring and summer.

RESULTS

Field Observations

Field observations provided information on the rise and decline of bee populations and on some of the contributing factors. Populations of alkali bees differed notably at different stages of this study. Emergence of adult bees in 1962 began in late May and continued through mid-June, whereas, in 1965 because of the wet and cool spring emergence did not begin until late June. It continued through mid-July. A downward trend in population was noted from 1962 to 1965 in the Garbe bed. Similarly in the Harris bed the population was high in 1962 but in 1963 nesting was confined almost exclusively to the edges of the bed. In 1964 and 1965 the population in this bed rose again to the 1962 level. The population of the Wallace-Key bed remained fairly low and uniform throughout this study.

Variation of the adult population during the months of July and August was in part due to summer thunder showers, since a heavy thunder shower during the day sometimes killed the greater part of the adult population. This in turn influenced the total number of eggs deposited during that particular season.

In July, 1962, one hundred cells from each of the beds were randomly excavated and examined. More than one-half of the one

hundred cells removed from the Garbe bed contained fungi. Forty-seven contained A. flavus hyphae and conidia, 9 contained a white fungus complex, which later laboratory examination demonstrated to consist of A. flavus, Cunninghamella sp., and Chaetomium sp., and 3 contained an orange stibellaceous form possibly in the genus Stilbum. Unfortunately, the inoculum of the latter fungus did not grow on various media tried, so it was not possible to fully identify the species.

In the Harris bed Section I, 19 cells contained A. flavus, 3 cells contained the white fungus complex and, 1 the orange stilbellaceous form. In Harris bed Section II, 17 cells contained A. flavus, 4 cells contained the white fungus complex and the orange stilbellaceous form was absent.

In the Wallace-Key bed no fungi were observed although several cases of apparent bacterial infection occurred.

In the summer of 1964, one week after excessively heavy rains, investigation of cells at the Garbe and Harris beds demonstrated numerous cells to be provisioned but without eggs. The pollen balls were partially submerged in water, and were within the week covered with a heavy growth of A. flavus. In a few cases A. flavus infestations were noted on pollen balls which contained living eggs or young larvae. In other cases the egg or young had died. It was not clear that mortality was due solely to the activities of the fungus.

Field observations indicated that mortality of bee larvae due to A. flavus was initiated as soil temperatures rose in the early spring, continued throughout the summer, and declined towards late summer as soil temperatures declined. During this higher temperature period in both the Garbe and Harris beds, it was always possible to find at least a few cells in which A. flavus was attacking the egg, young larva, or the pollen ball. In the latter case death of the egg or larval stage always resulted. In the Wallace-Kay bed larval mortality was always low or absent. In all beds infections that could be clearly recognized as recent were much less common or absent during the late fall and winter months.

Adults which had been drowned by rain while in the burrows frequently became infested with A. flavus. These infestations undoubtedly contributed greatly to the level of A. flavus conidia encountered in the soil of the beds.

Photographs were taken in the field of various stages of A. flavus infections as well as of larvae infected by the stibellaceous fungus and the "white fungus complex." These are presented in Figures 1 through 5.

Figure 1 portrays the early stages of an A. flavus infection showing the typical darkening of the larva and a small amount of non-sporulating mycelium. This larva was brought into the laboratory where it eventually became covered with a mass of hyphae and

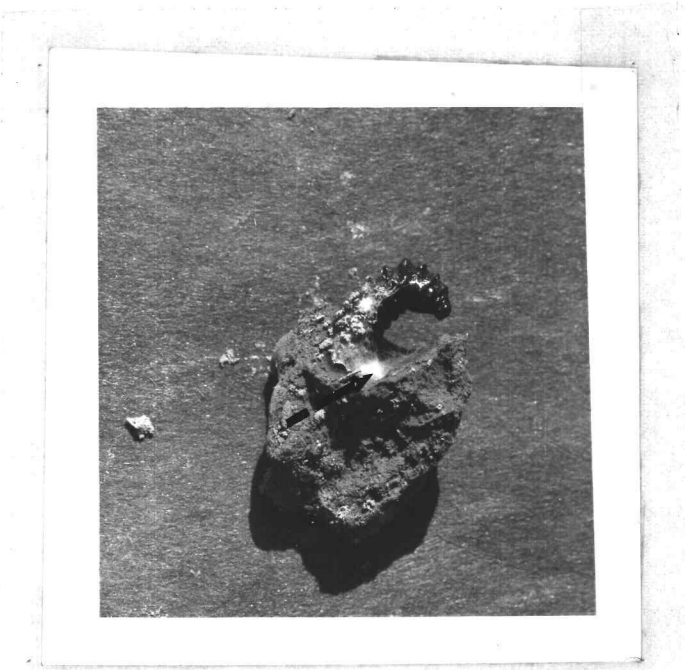

Figure 1. Early stages of an Aspergillus flavus infection. Note darkening of the larva and fungus mycelium at arrow.

Figure 2. Advanced stage of Aspergillus flavus infection. The anterior end shows typical hyphal tufts (arrow).

Figure 3. Final stage of an Aspergillus flavus infection. Note conidial mass indicated by the arrow.

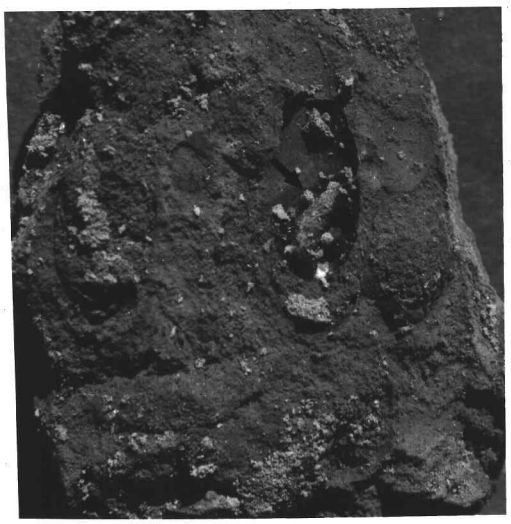

Figure 4. Final stage of an infection by the orange stilbellaceous fungus. Note synnema indicated by the arrow.

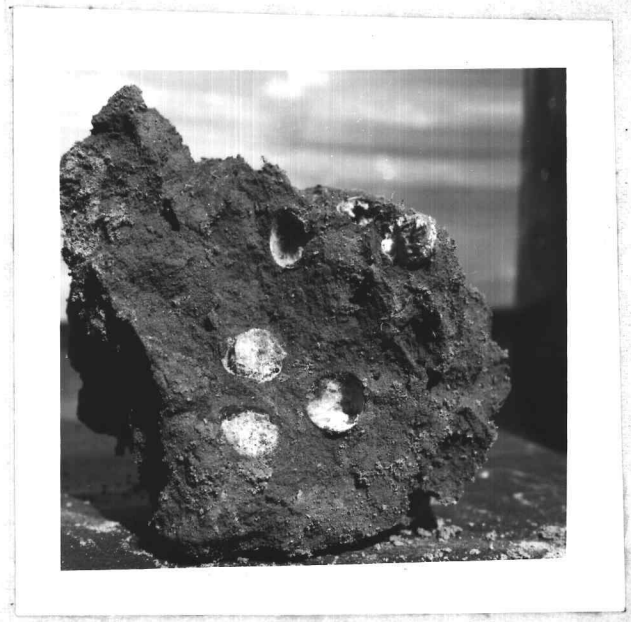

Figure 5. Alkali bee cells occupied by the "white fungus complex" consisting of Aspergillus flavus, Cunninghamella sp. and a Chaetomium sp.

conidiophores of A. flavus.

Figure 2 shows a more advanced stage of an A. flavus infection. The larva shows somewhat less darkening than that of Figure 1. The prominent tufts of hyphae at the anterior end of the larvae are typical. This was brought into the laboratory where typical sporulation occurred.

Figure 3 portrays the final stage of an A. flavus infection. Here the larva has become completely covered by the fungus mycelium which has produced a mass of sporulating hyphae at the anterior end of the larva. This was typical of the infections found in the late summer through the early spring of the following year.

Figure 4 portrays a mature infection by the orange stilbellaceous fungus. The larva was typically darkened and dehydrated. The fungus mycelium was internal and represented externally only by bright orange synnemata bearing a mass of single-celled orange conidia.

Figure 5 portrays the "white fungus complex" consisting of Aspergillus flavus, Cunninghamella sp., and a Chaetomium sp. As mentioned above A. flavus was always present in this complex and larval mortality was attributed to the presence of this fungus.

Core Samples

Soil cores yielded a quantitative measure of population trends and factors contributing to the trends. Core samples taken at eight dates distributed over one year were analyzed for the number of A. flavus infected larvae dissected from the brood cells. Results appear in Tables 1, 2, 3, 4, and 5. The Chi-square Test was used to determine significance of differences in infections among sample populations collected at the same date and to compare differences of infections among the eight different sampling dates.

Samples from the Wallace-Key bed showed such uniformly low infection levels (2 percent on 2/23/63, 4.1 percent on 5/18/63, and 0 percent on all other dates) that it was not included among the Chi-square tests.

Comparisons among the Garbe bed and the two areas of the Harris bed indicated a significant difference in the level of A. flavus infection among the beds at each date with the exception of the first date, 7/23/62. Comparison of the Harris bed I and II, indicate significant differences only on dates 7/23/62 and 2/23/63. Comparisons of the Garbe bed and the combined samples from Harris bed I and II indicate significant differences on all dates except 7/23/62, 8/23/62, 4/20/63.

One may interpret Figure 6 in the following fashion. On

Table 1. Number of healthy and diseased larvae collected from soil cores from 3 beds on different dates

Date	Bed	Healthy Larvae	% of Total	Infected Larvae	% of Total	Total
7/23/62	Garbe	51	75	17	25	68
8/3/62		56	61.5	35	38.5	91
8/25/62		110	64	61	35.7	171
2/23/63		192	75.6	62	24.4	254
3/31/63		83	72.8	31	27.2	114
4/20/63		73	81.1	17	18.9	90
5/18/63		48	65.7	25	34.2	73
6/27/63		120	61.5	75	38.5	195
7/11/63		38	50.6	37	49.3	75
7/23/62	Harris Sec I	15	55.5	12	44.4	27
8/3/62		59	77.6	17	22.4	76
8/25/62		159	85	28	15	187
2/23/63		139	89.7	16	10.3	155
3/31/68		230	95.4	11	4.6	241
4/20/63		180	90.9	18	9.1	198
5/18/63		121	89	15	11	136
6/27/63		119	86.9	18	13.1	137
7/11/63		51	92.7	4	7.3	55
7/23/62	Harris Sec II	31	81.6	7	18.4	38
8/3/62		47	71.2	19	28.8	66
8/25/62		52	76.5	16	23.5	68
2/23/63		65	80.3	16	19.8	81
3/31/63		70	94.6	4	5.4	74
4/20/63		70	83.3	14	16.6	84
5/18/63		24	96	1	4	25
6/27/63		47	31	11	18	58
7/11/63		34	97.1	1	2.9	35
7/23/62	Wallace-Key	40	93	0	0	43
8/3/62		41	87.2	0	0	47
8/25/62		51	82.3	0	0	62
2/23/63		141	97.9	3	2	144
3/31/63		82	100	0	0	82
4/20/63		107	100	0	0	107
5/18/63		93	95.9	4	4.1	97
6/27/63		61	100	0	0	61
7/11/63		15	93.8	0	0	16

Table 2. X^2 values with one degree of freedom in a test for significant differences among populations of diseased larvae in core samples taken from 3 beds on different dates

Garbe		
	7/23/62-8/3/62	3.28 n. s.
	8/3/62-8/25/62	0.5 n. s.
	8/25/62-2/23/63	0.62*
	2/23/63-3/31/63	0.164 n. s.
	3/21/62-4/20/63	2.03 n. s.
	4/20/63-5/18/63	4.58**
	5/18/63-6/27/63	0.53 n. s.
	6/27/63-7/11/63	2.62 n. s.
Harris I		
	7/23/62-8/3/62	4.35**
	8/3/62-8/25/62	2.2 n. s.
	8/25/62-2/23/63	1.53 n. s.
	2/23/63-3/31/63	5.05**
	3/21/63-4/20/63	3.71 n. s.
	4/20/68-5/18/63	0.24 n. s.
	5/18/63-6/27/63	0.14 n. s.
	6/27/63-7/11/63	1.18 n. s.

* significant at the two per cent level
** significant at the five per cent level

Harris II		
	7/23/62-8/3/62	2.11 n. s.
	8/3/62-8/25/62	1.24 n. s.
	8/25/62-2/23/63	0.28 n. s.
	2/23/63-3/31/63	7.17***
	3/31/63-4/20/63	9.02***
	4/20/63-5/18/63	2.49 n. s.
	5/18/63-6/27/63	2.91 n. s.
	6/27/63-7/11/63	5.09**

** significant at the five per cent level
*** significant at the one per cent level

Table 3. X^2 values with two degrees of freedom in a test for significant differences among populations of diseased larvae in core samples from three sites, Garbe, Harris I and Harris II

7/23/62	5.60 n.s.
8/3/62	6.22*
8/25/62	21.11**
2/23/63	12.23**
3/31/63	44.31**
4/20/63	5.99***
5/18/63	21.28**
6/27/63	28.34**
7/11/63	40.89**

* significant at the two per cent level
** significant at the one per cent level
*** significant at the five per cent level

Table 4. X^2 values with one degree of freedom in a test for significant differences between populations of diseased larvae in core samples from Harris I and II

7/23/62	5.16*
8/3/62	0.37 n.s.
8/25/62	2.43 n.s.
2/23/63	3.93*
3/31/63	0.04 n.s.
4/20/63	3.46 n.s.
5/18/63	1.19 n.s.
6/27/63	1.0 n.s.
7/11/63	0.88 n.s.

* significant at the five per cent level

Table 5. X^2 values with one degree of freedom in a test for significant differences between populations of diseased larvae in core samples from the Garbe bed and the combined Harris I and II beds

7/23/62	0.21 n.s.
8/3/62	0.16 n.s.
8/25/62	19.24*
2/23/63	9.24*
3/31/63	44.53*
4/20/63	3.14 n.s.
5/18/63	20.55*
6/27/63	27.69*
7/11/63	40.66*

* significant at the one per cent level

7/23/62 Harris I had a higher level of fungus mortality than Garbe or Harris II; however, the Garbe and Harris II were not significantly different from one another. On 8/3/62 there was no significant difference in mortality levels among the three beds. On 8/25/63 the Garbe bed had a significantly higher level of fungus caused mortality than either Harris I or Harris II which did not differ from one another significantly. On 2/23/63 the Garbe and Harris II beds had a higher level of mortality than Harris I, but did not differ significantly from each other. On 3/31/63 both Harris beds had a significantly lower level of mortality than the Garbe bed; whereas, on 4/20/63 the Harris I bed had significantly less mortality than the Garbe bed but did not differ significantly from Harris II. On 5/18/63, 7/27/63 and 7/11/63 the Garbe bed had a significantly higher level of fungus mortality than Harris I and II which did not

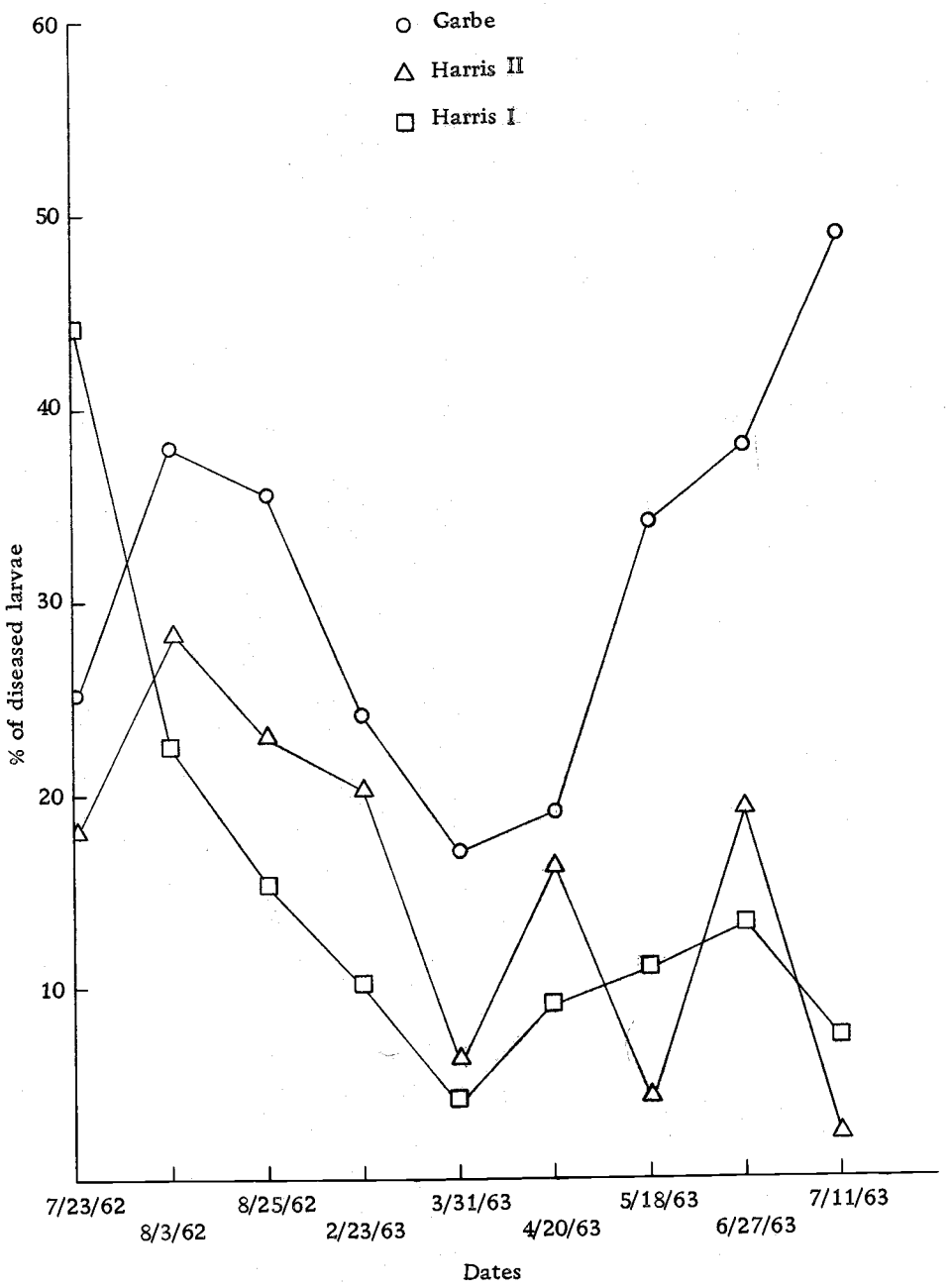

Figure 6. Fungus mortality in core samples from three sites on nine dates.

differ significantly from each other.

To summarize, the three beds showed a decline in fungus caused mortality between July 1962 and February 1963. The Garbe bed showed an increased incidence of mortality from March 1963 until the end of the sampling period. Decline in mortality occurred in both sections of the Harris bed through March 1963. There was a slight increase in mortality from April through June 1963, followed by a decline by the end of the sampling period in July, 1963.

Isolations from the Soil

Soil dilutions provided further information on the occurrance of fungi in the environment of the alkali bee. Soil samples were analyzed for A. flavus on six dates during 1963 and 1964 using standard soil dilution techniques. The 1/10,000 dilutions provided the most readable samples. The results appear in Table 6. During this study period A. flavus was not isolated from soils of the Wallace-Key bed. However, samples from both the Garbe and Harris beds always contained A. flavus. Because of the limitations on applicability of the soil dilution technique to assays for fungi, it is not clear that differences in colony counts between the Garbe and Harris beds are truly significant.

In 1962 and again in 1964, soil samples from the three beds were analyzed for the variety of fungus species present. Three plates

Table 6. Soil analyses for Aspergillus flavus only on six different dates

	Dil.	A. flavus colonies in each of two replicates	
		1	2
		number	number
6/27/63			
Garbe	1/10,000	3	5
Harris	"	6	4
Wallace-Key	"	0	0
7/19/63			
Garbe	1/10,000	8	10
Harris	"	8	7
Wallace-Key	"	0	0
7/9/64			
Garbe	1/10,000	15	11
Harris	"	10	10
Wallace-Key	"	0	0
8/10/64			
Garbe	1/10,000	10	10
Harris	"	3	4
Wallace-Key	"	0	0
8/17/64			
Garbe	1/10,000	12	8
Harris	"	13	11
Wallace-Key	"	0	0
8/31/64			
Garbe	1/10,000	5	6
Harris	"	7	5
Wallace-Key	"	0	0

of each dilution were prepared and again the 1/10,000 dilution proved to be most readable. The fungi isolated are reported in Tables 7 and 8. A. flavus was isolated from all plates of the Garbe and Harris beds but again not from the Wallace-Key bed.

The remainder of the fungi isolated were common soil saprophytes having little or no history of association with the production of insect mycoses. In the tables those forms marked with an asterisk were found in isolations from adult bees, pollen balls, and larval feces as well as from the soil. They served as index fungi to detect the presence of common soil contaminants in these materials.

Isolations from Adult Bees

Since the adult bees were exposed to surface contamination by soil inhabiting fungi through life, a study was made of the contaminating organisms. In 1964 ten adult alkali bees from each bed were studied to determine which soil fungi could be isolated from the surface of adult bees.

In 1965 this study was enlarged to include 25 adults from the Garbe and Harris beds. The Wallace-Key bed was not included in the 1965 study because of the very low population of adult bees at the time the study was carried out. Results are reported in Table 9. In 1964, A. flavus was isolated from 50 percent of the adults taken from the Garbe and Harris beds and from none of the adults taken from the

Table 7. Frequency of isolations of particular soil fungi in 1962

Fungi isolated	Bed, year and replication								
	Garbe 62			Harris 62			Wallace-Key 62		
	1	2	3	1	2	3	1	2	3
	number								
Aspergillus flavus	7	3	5	8	3	5	0	0	0
*Cladosporium herbarum	3	0	1	3	1	1	0	1	0
*Alternaria tenuis	1	3	1	1	1	1	5	2	3
*Alternaria humicola	0	1	2	1	1	0	0	3	1
Aspergillus niger	2	1	1	0	0	1	1	0	0
Aspergillus spp.	3	5	2	4	5	2	2	1	0
Penicillium spp.	4	1	2	2	3	1	1	3	1
*Fusarium sp.	1	2	0	0	1	1	0	0	1
*Mucor jansseni	1	0	1	1	0	1	0	2	1
*Cunninghamella echinulata	1	1	0	1	2	0	1	2	2
*Absidia spinosa	1	0	0	1	1	0	0	1	0
Spicaria violacea	0	1	2	0	0	1	0	0	0
*Chaetomium sp.	0	1	0	0	0	1	0	0	0
Stemphylium piriforme	1	1	0	2	1	1	2	1	3
Acremonium vitis	0	0	0	0	0	0	0	1	0
Helminthosporium sativum	0	1	0	0	1	0	0	0	0

* Designates species isolated from at least 2 substrates (feces, adult, pollen balls) other than the soil

Table 8. Frequency of isolations of particular soil fungi in 1964

Fungi isolated	Bed, year and replication								
	Garbe 64			Harris 64			Wallace-Key 64		
	1	2	3	1	2	3	1	2	3
	number								
Aspergillus flavus	6	2	7	9	7	6	0	0	0
*Cladosporium herbarum	2	1	0	0	2	1	2	1	1
*Alternaria tenuis	3	3	1	2	1	3	1	4	3
*Alternaria humicola	2	0	1	2	1	0	2	0	2
Aspergillus niger	1	3	0	1	0	2	0	1	1
Aspergillus spp.	4	1	3	1	5	3	1	0	3
Penicillium spp.	1	2	4	1	4	2	0	2	1
*Fusarium sp.	0	2	0	1	0	1	2	1	0
*Mucor jansseni	0	1	1	2	0	1	1	1	0
*Cunninghamella echinulata	1	0	0	1	1	1	1	0	1
*Absidia spinosa	1	1	1	0	1	0	1	0	0
Spicaria violacea	0	2	1	0	1	0	0	0	0
*Chaetomium sp.	0	0	1	0	1	0	0	0	0
Stemphylium piriforme	0	1	1	0	3	1	0	1	2
Acremonium vitis	0	0	0	0	0	0	0	1	0
Helminthosporium sativum	0	0	0	0	0	0	0	1	0

* Designates species isolated from at least 2 substrates (feces, adult, pollen balls) other than the soil

Table 9. Number of isolations of a particular fungus from the surface of adult Alkali Bees

Fungi Isolated	Bed and year: Garbe 64[1]	%	Garbe 65[2]	%	Harris 64[1]	%	Harris 65[2]	%	Wallace-Key 64[1]	%	Wallace-Key 65[3]	%
Aspergillus flavus	5	50	20	80	5	50	21	84	0	0		
Cladosporium herbarium	10	100	24	96	9	90	23	92	8	80		
Alternaria tenius	2	20	3	12	3	30	7	28	4	40		
Alternaria humicola	0	0	2	8	0	0	0	0	0	0		
Aspergillus niger	0	0	0	0	1	10	0	0	0	0		
Fusarium sp.	1	10	0	0	0	0	0	0	0	0		
Mucor jansseni	5	50	9	36	10	100	25	100	10	100		
Cunninghamella echinulata	1	10	1	4	3	30	4	16	2	20		
Absidia spinosa	4	40	9		6		17		5			

[1] out of ten adult bees

[2] out of 25 adult bees

[3] no collections were made from the Wallace-Key bed in 1965

Wallace-Key bed. In 1965, A. flavus was isolated from 80 and 84 percent of the cases in the Garbe and Harris beds respectively.

Of the other fungi listed only Aspergillus niger did not occur in at least three of the four aspects of the alkali bee biosphere studied. The remainder were common soil saprophytes reported in the studies on soil fungi previously described. Alternaria humicola and the Fusarium species were less common than others isolated from adult bees. The Wallace-Key bed demonstrated a smaller diversity of forms than either the Garbe or Harris beds. Only Cladosporium herbarum, Alternaria tenuis and the mucoraceous species were common. With few exceptions the fungi isolated from all beds during the two-year period of analysis remained the same from season to season.

Isolations from Pollen Balls

Fifty pollen balls from each bed were analyzed for the presence of fungi in 1965. In 1966 the study was restricted to a smaller number of balls taken from the Garbe and Harris beds because it was impossible to obtain pollen balls from the Wallace-Key bed at that time. Results of these studies are summarized in Table 10.

In 1965 A. flavus proved to be a contaminant of 18 percent of the pollen balls in the Garbe bed and 10 percent of the pollen balls in the Harris bed. It could not be isolated from pollen balls taken

Table 10. Number of pollen balls with a particular species of fungus

Bed	Garbe				Harris				Wallace-Key		
Year	65				65				65	66**	
Fungi Isolated	50*	%	13	%	50*	%	18*	%	50*	%	%
Aspergillus flavus	9	18	3	23	5	10	7	39	0	0	
Cladosporium herbarum	5	10	2	15	11	22	3	17	17	34	
Alternaria tenuis	20	40	1	8	5	10	6	33	13	26	
Alternaria humicola	11	22	2	15	7	14	5	28	9	18	
Mucor jansseni	5	10	1	8	4	8	5	28	2	4	
Cunninghamella echinulata	6	12	2	15	3	6	1	6	1	2	
Absidia spinosa	4	8	1	8	3	6	0	0	5	10	
Chaetomium sp.	6	12	0	0	5	10	1	6	0	0	
Spicaria violacia	3	6	1	8	0	0	0	0	1	2	
Stemphylium piriforme	8	16	3	23	14	28	5	28	8	16	
Penicillium sp.	3	6	1	8	0	0	0	0	1	2	
Acremonium vitis	0	0	0	0	0	0	0	0	1	2	
Helminthosporium sativum	0	0	0	0	0	0	0	0	1	2	

* Number of pollen balls studied
** No isolations made in 1966

from the Wallace-Key bed. During the 1966 study contamination with A. flavus was 23 percent and 39 percent respectively.

The pollen balls from all three beds exhibited a diversity in soil saprophytes corresponding closely to those species found in soil of the beds as given above. In general the number of species of fungi found associated with pollen was greater than the number found as surface contaminants of adult bees or in larval feces. Acremonium and Helminthosporium were unique to the Wallace-Key bed, though Helminthosporium was isolated occasionally from all beds during soil dilution studies. Spicaria violacea, which was reported from both soil and pollen of the Harris and Garbe beds, was also observed occasionally during the course of field observations where it was seen growing on pollen balls and cell linings.

Isolations from Larval Feces

Since the larvae consume pollen which is contaminated by a variety of fungi including A. flavus, a study was made of those fungi which could survive passage through the larval digestive tract and be isolated from the larval feces.

During 1965 predefecating 5th instar larvae were brought into the laboratory and allowed to defecate under sterile conditions. Fungi found in the feces are reported in Table 11. The number of larvae studied was relatively small because difficulty was encountered in

obtaining the appropriate stage in the field and because it was difficult to rear larvae through the defecation period and laboratory conditions.

Table 11. Number of isolations of a particular fungus from larvae feces

Bed No. of larvae Studied	Garbe 14	Harris 10	Wallace-Key 10
Aspergillus flavus	10	7	0
Alternaria tenuis	1	2	1
Alternaria humicola	0	1	0
Fusarium sp.	4	2	3
Chaetomium sp.	3	1	0
Cunninghamella echinulata	3	3	1
Mucor jansenii	2	1	2
Absidia spinosa	1	3	3

A. flavus was isolated from the feces of 71 percent of the larvae obtained from the Garbe bed and 70 percent of the larvae from the Harris bed. A. flavus was not isolated from the feces of larvae obtained from the Wallace-Key bed.

The number of soil saprophytic species isolated from feces of bee larvae was similar to those isolated from the surface of the adult alkali bees but was much smaller than found in isolations from soil or pollen balls. Again, the Wallace-Key bed had a lesser diversity than

either the Garbe or Harris beds. All soil saprophytes isolated were species that had been isolated from at least three of the four areas of the biosphere studied.

In Vitro Disease Induction

Aspergillus flavus was observed and isolated in the field from larvae which subsequently died. An attempt was made to determine the pathogenicity of A. flavus in the laboratory, as well as to delineate the moisture and temperature conditions which favor pathogenicity.

Data from the two experiments on in vitro disease induction by surface inoculation with conidia of A. flavus are presented in Table 12. An analysis of variance of the data appears in Table 13.[1] The experiments were designed to detect differences in larval infection between inoculated and uninoculated larvae incubated at 30, 25, and 20°C. and five different levels of relative humidity, 100, 90, 80, 50 and 30 percent. Since no infection occurred below 90 percent relative humidity, the 50 and 30 percent humidity values were eliminated from the analysis of variance.

As was expected differences between the inoculated larvae and the uninoculated controls were significant for each temperature and relative humidity at which infection took place. The data show that

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[1] The analysis was performed by the Utah State Computer Center.

Table 12. Number of healthy and diseased larvae in the various treatments of the two *in vitro* disease induction experiments

Experiment I		Diseased larvae			
Temperature	Relative Humidity	Inoculated	Control	Inoculated	Control
		Number		Percent	
30°C	100%	7	0	70	0
	90%	5	0	50	0
	80%	0	0	0	0
	50%	0	0	0	0
	30%	0	0	0	0
25°C	100%	6	1	60	10
	90%	2	0	20	0
	80%	0	0	0	0
	50%	0	0	0	0
	30%	0	0	0	0
20°C	100%	0	0	0	0
	90%	0	0	0	0
	80%	0	0	0	0
	50%	0	0	0	0
	30%	0	0	0	0
Experiment II					
30°C	100%	5	0	50	0
	90%	2	0	20	0
	80%	0	0	0	0
	50%	0	0	0	0
	30%	0	0	0	0
25°C	100%	4	0	40	0
	90%	2	0	20	0
	80%	0	0	0	0
	50%	0	0	0	0
	30%	0	0	0	0
20°C	100%	2	0	10	0
	90%	0	0	0	0
	80%	0	0	0	0
	50%	0	0	0	0
	30%	0	0	0	0

infection of the inoculated larvae was influenced by an interaction between temperature and relative humidity. Infection was greatest at 30°C. and 100 percent relative humidity. However, infections did occur in the 30° to 25°C. range at both 100 percent and 90 percent relative humidity. Only two larvae became infected at 20°C. and both were exposed to 100 percent relative humidity. During the course of the experiment one of the controls developed an A. flavus infection. This was attributed to incomplete surface sterilization.

Table 13. Analysis of variance F values for the two experiments on in vitro disease induction

Source	DF	F
Experiment N	1	1.700 (N.S.)
Temperature	2	11.192 **
Rel humidity	2	22.243 **
Treatment (inoculation)	1	54.592 **
Temp × treat	2	10.531 **
Temp × r hum	4	3.471 *
Hum × treat	2	18.748 **
T × h × trt	4	2.951 (N.S.)
Error	17	
Total	35	

* indicates significance at the 5% level

** indicates significance at the 1% level

One may conclude from the data that infection of the larvae resulting from germinating A. flavus conidia was favored particularly by temperatures in the 25° to 30°C. range and by relative humidities between 100 percent and 90 percent at the favorable temperatures. Infection was retarded by the 20°C. temperature treatment and apparently prevented by relative humidities of 80 percent and below.

During the course of the two experiments observational data concerning disease development were collected. At 30°C. disease symptoms first appeared between the fifth and eighth day. The first noticeable symptom was a slight brownish discoloration of the prepupa; the discoloration became progressively greater until the prepupa attained a glossy brownish-black cast. Between the 12th and 15th day the first non-sporulating hyphae emerged from the cervical region of the larva. Hyphae later emerged from the lateral spiracles and eventually the entire prepupa was covered with fungus mycelium. Sporulation began during the 15th to 18th day and the prepupa was covered with a mass of green conidia anteriorly and white mycelium posteriorly. At this stage the bee was represented as a collapsed dehydrated mass of integrated prepupal and fungal tissue.

Disease development was similar at 20° and 25°C. except that in the latter development was delayed approximately one day while at the former temperature, in the one case observed, initial symptoms were observed on the 8th day, hyphae emerged on the 13th day and

sporulation was completed on the 19th day.

Progress of the disease was similar under field conditions; however, it was not possible to determine the length of intervals between each stage of development.

DISCUSSION

The present study was undertaken in order to learn the identity and behavior of those fungi associated with alkali bee larvae within particular bee nesting sites along the central Oregon-Washington border. Fungus mortality in this area had, by 1962, reached a level which caused concern among the alfalfa growers over the prospects of poor pollination.

Stephen (1959) reported the presence of Aspergillus ustus and A. terreus on diseased alkali bee larvae. Initial studies reported here involving infected larvae obtained from the Washington-Oregon border beds revealed the presence of A. flavus in most of the larvae. Neither A. ustus nor A. terreus was encountered in the course of the study. Since neither is normally considered a pathogen of bees (Raper and Fennell, 1965), an explanation is needed to account for Stephen's observations. It is possible that the larvae he studied became contaminated by conidia of these soil saprophytes. Such contamination from the soil was encountered as a problem in this study. On the other hand identifications in parts of this genus are difficult (sometimes even for the specialist) a situation sometimes contributing to error of reporting.

It became clear early in the study that A. flavus was the principal fungus pathogen in two of the three alkali bee nesting sites

selected for investigation. This information stimulated questions as to the relationship of the fungus to the bee in the field, as to the expression of seasonal variation with respect to the incidence of infection, and, finally, as to whether or not the disease could be reproduced in the laboratory.

Results of a study of soil cores taken from the beds over the course of a year indicated that there was indeed variation among the beds with respect to disease incidence and that seasonal variation with respect to incidence of the disease also occurred.

In the Garbe and Harris beds studied the incidence of diseased larvae was high in late spring and summer. It declined in the winter and reached a new high the following spring and summer. These observations correlate well with results of the laboratory studies concerning the effects of temperature on disease induction in the larvae surface inoculated with conidia of A. flavus. In the inoculation experiments it became apparent that temperatures of 25-30°C. favor infection while 20°C. inhibits infection. Soil temperatures in the late spring and summer in the study beds range between 25-30°C. which is well within the range favoring infection. During the fall and winter soil temperatures drop below 20°C. which inhibits infection. That the incidence of diseased larvae in both sections of the Harris bed in 1963 did not reach the level of the previous year may be explained by a decline in the total population of that bed during the summer of 1963.

This decline was directly attributed to heavy rains during July and August which killed adult bees prematurely.

The soil core studies revealed an interesting variation among the three beds with respect to incidence of disease. The Garbe bed with one exception exhibited a significantly higher incidence of infection than either the Harris or Wallace-Key beds. With the exceptions of tests on only two dates both sections of the Harris bed were alike with respect to frequency of diseased larvae during the year. Because of this similarity the bed was treated as a single unit for the remainder of the investigations. The Wallace-Key bed produced essentially no diseased larvae during the course of the study. It is difficult to explain the low incidence of disease in this bed, since it was near (ten miles) the Garbe and Harris beds and had a high soil moisture level and should from known relationships, have provided an excellent environment for fungal growth and disease development. Studies on the presence of A. flavus in the biosphere also indicated the absence of A. flavus from the Wallace-Key bed; however, this does not fully clarify the situation since the absence of the fungus from the biosphere itself requires explanation. The distribution of fungi in the soil is influenced by the activities of the adult alkali bees. In the course of cell construction the female alkali bee burrows through cells of previous years and carries soil from a depth of 6 to 10 inches below the surface to the surface. The activities of

numerous female bees result in extensive mixing of the soil and deposition of several inches of subsoil on the surface of the bed during the course of the nesting season. It is to be expected that conidia of A. flavus developing on a variety of substrates from previous infections would in this way be mixed into the soil of the bed. Therefore, it was of interest to analyse the soil of each bed for the presence of A. flavus propagules, i. e. conidia, hyphal fragments or other viable structures of the fungus. The soil dilution technique, when applied to fungi, is a less than perfect technique since it does not give data as quantitatively precise as when applied to soil bacteria for example. In the latter case there can be essentially a 1:1 ratio between the colonies on the plate and the viable bacteria in the dilution. In the case of the fungi, however, each fungus colony on the plate does not necessarily represent a single fungal organism or even a single conidium, but rather a single propagule which may be a hyphal fragment of an actively growing mycelium, a single conidium, on some other viable unit of the fungus.

At best then the soil dilution plate technique provides a means for a rough comparison of the relative abundance of A. flavus propagules among the soils of the three beds. While it is most likely that these propagules are predominantly conidia, no attempt was made to prove this point.

Data from soils of the three beds during 1963 and 1964 indicate

the presence of A. flavus propagules in the soils of the Garbe and Harris beds and their absence from the Wallace-Key bed. Because of limitations of the dilution plate technique it is not possible to conclude that there were significant quantitative differences between the Harris and Garbe beds. Nevertheless, it is clear that A. flavus propagules could be readily and consistently isolated from the two beds in which larval mortality was high while it could not be isolated from the soil of the bed lacking fungus mortality of the larvae.

The 1964 dilution study was run in order to gain a picture of the number of individuals and variety of species represented in the soil fungal floras. A. flavus predominated from the Garbe and Harris beds and was the only fungus parasite of insects present in any of the beds. Many common soil saprophytes were isolated including: Cladosporium sp., Alternaria spp., Fusarium sp., and a variety of mucoraceous forms but none of these was nearly as common as A. flavus. This experiment also showed that there was little change in the composition of the soil fungal flora between 1962 and 1964, thus suggesting a general stability among the fungal floras of the several soils. Certain of the non-parasitic soil fungi provided valuable evidence as to the occurrence of soil contamination during studies of the fungus flora on adult bees, pollen balls, and feces passed by bee larvae when this matter was in question.

After the fungal flora of soils of the beds had been analyzed

attention was turned to fungi on the insects themselves. A. flavus was the only fungal parasite isolated from either adult bees or larvae. Its prevalence was exceeded only by Cladosporium herbarum and Mucor jansseni both of which are common soil saprophytes. A. flavus was not found on bees taken from the Wallace-Key bed.

The fungi which contaminated the surface of adult alkali bees were forms known from previous isolation studies to be present in the soil. It is most likely that they owe their presence on the insects to the burrowing activities of these bees.

Pollen balls are constructed by the female bee whose external surfaces may be contaminated and capable of spreading A. flavus. Therefore, it was logical to investigate the fungus flora of the provision. A. flavus proved to be a common contaminant of pollen balls taken from the Garbe and Harris beds, but again it was absent from the Wallace-Key bed. Other fungi isolated from the pollen balls were common soil saprophytes many of which were the same species contaminating the surface of adult bees. All were isolated from dilution plates of bed soils. The evidence indicates that the fungi contaminating pollen balls was introduced either directly from the soil as the pollen was stored in the cell or indirectly from the contaminated surface of the adult bee.

Attention concerning further perpetuation of the fungi was turned to the flora of the larval feces, where it was found that the

floras do not differ markedly from the expected as established from studies involving soil, adult bees, and pollen balls. A. flavus was isolated from approximately 70 percent of the feces studied from larvae of the Garbe and Harris beds, and again not from the Wallace-Key bed. The remainder of fungi isolated were the commonly encountered soil saprophytes of the previous studies. In general the variety of fungi isolated from larval feces was less than that from the pollen balls, suggesting some inactivation during passage through the larval digestive tract.

The fact that propagules of A. flavus could pass through the digestive tract of the larvae without infecting the larva nor themselves being rendered non-viable is a highly interesting observation. The low oxygen tensions of the digestive tract of the larva combined with unfavorable pH presumably inhibited growth of the propagules of A. flavus and thus prevented infection of the larvae. The viable propagules of A. flavus are deposited with the feces as a layer covering the bottom of the cell and thus are returned to the population of A. flavus propagules in the cell.

Previous experiments have demonstrated that propagules of A. flavus could be isolated from the soil of the Garbe and Harris beds, and further, that such propagules could be transferred from the soil to the pollen ball by the provisioning adult bee. Propagules in the pollen ball are consumed by the bee larvae and passed through the

digestive tract of the larvae where they apparently are prevented from germinating and infecting the larvae. Propagules are deposited with the feces to constitute part of the environment of the overwintering larvae in the prepupal stage.

Studies of the Wallace-Key bed reveal essentially the same soil saprophyte populations as those found in the Garbe and Harris beds. However, throughout the course of these studies, A. flavus was absent from the biosphere of the bees in this bed. There are two probable explanations for this fact. The first is the age of the bed which was not more than three years old at the time this study was initiated. The second involves the density of the bee population itself, which was always low in comparison to the Garbe and Harris beds. It is concluded therefore that time and density of insects was insufficient for A. flavus to reach detectable levels in this bed.

The Garbe bed was 10 or more years old when these studies were initiated and the bee population was high. Thus, there was the opportunity for introduction and consequent spread of the disease and disease inoculum. The older section (section I) of the Harris bed was constructed six years before the study was initiated and was started with soil cores from the Garbe bed, thus providing a source of soil inoculum. The bee population in the Harris I bed was normally high providing excellent conditions for the spread of the disease and maintenance of inoculum. The newer section of the bed (section

II) was constructed in 1958 as an extension of the older section and would, consequently, have received an inoculum of A. flavus from the soil and adults of the older section. It is not surprising, therefore, that disease incidence in the two sections differed little. It should also be pointed out that the distance between the Garbe and Harris beds was less than two miles, a distance allowing free interchange of bees between the two beds. The 10 mile distance between these beds and the Wallace-Key bed essentially precluded the interchange of bee populations among all three beds.

The question of pathogenesis remained to be answered. The experiments testing the effects of temperature and humidity on inoculation of prepupae revealed the following facts: (1) infection of the prepupal stage could take place from conidia on the surface of the prepupae; (2) temperatures of 30° to 25°C. favored infection, while lower temperatures inhibited infection; (3) relative humidities above 90 percent favored infection, if the temperature was suitable, while lower relative humidities inhibited infection even at suitable temperatures; and, (4) propagules in the digestive tract of the prepupae were not infectious under the conditions of these experiments.

Results of these laboratory experiments relate to A. flavus infections in the field. Propagules of A. flavus may be found in all facets of the biosphere including the cell in which the prepupae overwinter, as shown in studies of the Garbe and Harris beds. Incidence

of A. flavus infection increases during the higher temperature periods of spring and summer as was seen in cases from the beds. Spring and summer temperatures stabilize in the range favoring A. flavus infection of prepupae. Because of technical difficulties successful measurements of relative humidity in the cells was not accomplished. One, therefore, can only infer that the relative humidity values would be higher during the spring and summer as a result of higher ambient soil moistures. It would seem that A. flavus infection of alkali bee prepupae during the spring and summer is supported by environmental conditions favoring germination and growth of A. flavus conidia on the surface of the prepupae or in the cell containing the larva.

The field observations demonstrated that mortality due to A. flavus results not only from prepupal mortality but also from growth of A. flavus on the pollen ball either before egg eclusion or before the larva consumed the ball. Field observations indicate that the latter type of mortality occurs when moisture collects in the cells after pollen is deposited and before the cell is sealed. Such moist cells were found within a week after exposure to a heavy summer shower. Environmental factors, such as summer showers, undoubtedly contribute to fluctuating levels of infection during the course of the year. However, soil core studies reflect only infections of prepupae and do not provide data on mortality of egg and young larvae resulting from A. flavus infections.

The impact of bee mortality caused by A. flavus on the ability of the insect to meet pollination requirements is not known, as studies have not been carried out to determine optimum levels of alkali bee populations required for optimum alfalfa pollination. Such studies have not yet been made primarily because of foreseen difficulties in obtaining factual data. At present concerned growers tend to look upon any mortality as undesirable. Stephen (1959) estimated that reduction of the adult alkali bee population by bird predation "is greater than the combined losses caused by all other parasites and predators."

Control of A. flavus must be aimed at elimination of the fungus from the alkali bee biosphere. Also, control measures must be economically feasible where an individual grower can afford to manage his bee nesting site. Beds such as the Garbe site, which have unusually high rates of fungus mortality, would be more suitable for control experiments than beds where fungus mortality is present at relatively low levels and does not increase markedly during the year and from year to year. Introduction of fungicides into the sub-irrigation water supply of natural and artificial beds should be tested even though it might not prove economically feasible. Another method of control under investigation (Johansen, 1968) involves plowing sections of a bed and allowing adult bees to renest in the plowed area. It would seem that this technique would result

in an even wider dispersal of A. flavus within the biosphere of the alkali bee. However, if such plowing stimulated germination of A. flavus propagules in a nutrient poor environment, a reduction in the fungus population might result. Attempts to control the fungus through control of moisture and temperature of the beds seem unpromising since best conditions for bee development, etc., is a function of soil temperature and moisture at levels which favor A. flavus infection.

SUMMARY

1. A study of A. flavus pathogenicity on the alkali bee larvae was conducted using sample materials taken from three nesting sites along the eastern Oregon-Washington border.

2. Field observations and soil cores from the three sites indicated that A. flavus was the predominant fungus pathogen in two of the three sites studied. One site was essentially free of the pathogen.

3. Studies of soil dilution plates and fungi isolated from the surface of adult alkali bees, pollen balls, and larval feces indicated the presence of A. flavus propagules in the biosphere of the alkali bee in two of the three sites studied. The soil saprophytes found in these aspects of the biosphere did not vary significantly over a three year period. They provided a useful index of soil contamination of the alkali bee adults, pollen balls, and larval feces.

4. Studies on the effect of relative humidity and temperature on disease induction in larvae surface inoculated with A. flavus conidia revealed that a temperature range of 25° to 30°C and a relative-humidity range of 90 percent to 100 percent favored disease induction while lower temperatures and/or lower relative humidities inhibit disease induction.

5. It was concluded that the increased incidence of A. flavus

disease of larvae during the spring and summer is a result of increasing soil temperatures and relative humidities within the cells. Conversely, a reduced incidence of disease during the fall and winter results from declining temperatures and relative humidities.

6. Higher incidence of A. flavus disease in the Garbe bed was attributed to its age and higher bee population. Soil cores taken from the Garbe bed to establish the Harris bed appear to account for the prevalence of disease in this bed. The lack of A. flavus disease in the Wallace-Key bed is not clear, but its age and lower population density offer a partial explanation.

7. It is suggested that control of A. flavus within the nesting site be aimed at elimination of the fungus from the soil. Sub-irrigation of the beds with fungicide is one possible control, if costs are not prohibitive.

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