STUDIES ON TWO NEW ENTOMOGENOUS FUNGI (STILBACEAE : *Hymenostilbe*) AND THE BIOLOGY AND DISTRIBUTION OF THEIR HOST

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PETER JOHN WIGLEY

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C O N T E N T S

I N T R O D U C T I O N

Species of the sand weevil Cecyropa, occur on the foreshore all around the New Zealand coast, and extend for several miles inland in pastures of consolidated sand country. The adults vary widely in size and in the pattern of the mottled grey and brown cryptic colouration of the elytra and pronotum. Larvae of Cecyropa are external feeders on plant roots at depths of up to eighteen inches. In the Manawatu there are two species of Cecyropa: a larger species (C. maritima) confined to the unstablized sand dune area, and a smaller species (C. setigera) occurring in both the unstabilized dunes and in pastures of the consolidated sand country.

The stimulus for the present study was provided by reports indicating that adults of Cecyropa (presumably C. setigera) had caused damage, sometimes severe, to the seedling stages of crops grown in the Manawatu sand country (Graham and Hopkins 1965, May 1966). Consequently a study was initiated into the life history and ecology of Cecyropa setigera in pastures of this area. The study initially took the form of a sampling programme designed to recover larvae from the field and was supported by breeding studies in the laboratory. However, due to difficulties associated with the recovery of larvae and adults in sufficient numbers, and the distance of the study area from the University, the emphasis of the study was swung to an investigation of two previously undescribed natural enemies of C. setigera. These were two entomogenous

fungi, host specific on the immature stages of the sand weevil C. setigera in pastures of the Manawatu sand country.

Thus the areas of study can be defined as follows:

PART 1. The Host.

Aspects of the bionomics of C. setigera and a consideration of factors affecting spatial distribution.

PART 2. The Two Entomogenous Fungi.

(a) the morphdogy and taxonomy of two entomogenous fungi (Hymenostilbe sp.) pathogenic to the immature stages of C. setigera.

(b) the two fungi in artificial culture.

(c) the infectivity and etiology of the two fungi.

PART ONE

THE HOST

CHAPTER 1.

BIONOMICS AND DISTRIBUTION OF CECYROPA SETIGERA

INTRODUCTION

The genus Cecyropa Pascoe (1875) is an adelognathous member of the Otiorrhynchinae, one of 15 sub-families that comprise the family Curculionidae. The genus is endemic to New Zealand and comprises 18 species one of which was described by Pascoe, one by Sharp and the remaining 16 by Broun (1880-1921). There is scant reference in the literature to species of Cecyropa apart from the original descriptions. Hudson (1934, 1950) refers briefly to Cecyropa and illustrates Cecyropa lineifera Broun in the former work. The most recent references were those by Somerfield (1966) who dealt briefly with the distribution of an adult Cecyropa in the sand dunes at Piha, and May (1966) who briefly described aspects of the biology of Cecyropa discors Broun and provided a valuable key to the larvae of C. discors and other common soil inhabiting weevils.

Damage caused by Cecyropa species has only been recorded for the adults and the effect of larvae on pasture growth is unknown. Adults of Cecyropa have damaged foliage of onion and radish at Wanganui, and turnip seedlings at Foxton (May 1966). May also stated that adults caused damage to lucerne crops at Palmerston North but as Cecyropa species are restricted to sand country areas she was probably referring to crops of lucerne at Himatangi, 20 miles towards the coast from Palmerston North, where Dale (pers. comm.) witnessed severe

damage to a crop of seedling lucerne probably caused by adults of Cecyropa.

The identity of Cecyropa species in the Manawatu has caused some confusion. At the beginning of this study a comprehensive series of adults of Cecyropa taken from both dunes and pasture was sent to Dr. G. Kuschel (D.S.I.R., Nelson) for identification. Dr. Kuschel identified the smaller species as C. discors and the larger as Cecyropa maritima Broun and remarked that these were probably the only species of Cecyropa in this area. This study on the smaller weevil was thus carried out under the impression that the correct identificat-ionwas C. discors. Larvae taken in pasture were readily identified and separated from the other weevil species using May's description and key for C. discors and the morphology of larvae corresponded closely to that described and figured in her paper. However, shortly before this thesis was completed, Dr. Kuschel informed the author that following a study of the New Zealand species of Cecyropa he had ~~concluded that he had~~ concluded that he had mistakenly identified the smaller species and that C. discors was correctly identified as Cecyropa setigera Broun. He further mentioned that C. setigera occurred on both coasts south of Gisborne, C. discors north of Gisborne, while C. maritima was found right around the New Zealand coasts. This amendment thus casts doubt on the identity of the larvae described by May. Unfortunately the author has no knowledge of the source, or correct identification, of the adults from which May obtained larvae for her description. In view of the close similarity of larvae of C. setigera to those described as

C. discors by May it is probable that either her specimens were incorrectly identified and were actually C. setigera, or that larvae of C. setigera are morphologically very similar to those of C. discors.

This chapter firstly presents data accumulated on the bionomics of Cecyropa setigera and then considers factors affecting the spatial distribution of larvae within sand country pastures. Brief descriptive notes are included to expand May's description and to point out differences between larvae of C. setigera and C. discors where they occur.

A. MATERIALS AND METHODS.

The data in this chapter are obtained from three sources:

1. A sampling programme in a paddock 1½ miles inland from Himatangi Beach.

The sampling programme provided data on larval bionomics and distribution. A full account of the sampling sites, sampling technique and extraction processes is contained in Appendix 1. Briefly, the method consisted of taking a number of four inch cores to a depth of 14 inches. These cores were transferred to the laboratory where they were wet sieved and larvae extracted from the residue by a combined process of flotation and differential wetting. Head capsule widths of all larvae were measured using an eyepiece scale in a stereoscopic microscope.

2. Collection of adults in the field.

Adults were difficult to detect amongst sand and debris because of their small size and cryptic colouration. After several methods were unsuccessfully attempted (Berlese funnel, wet sieving, and dry sieving beneath pasture) two methods of collection were adopted.

(a) Using bait

Carrots were cut in half and placed on the ground amongst a mature crop of lucerne. Cecyropa weevils emerged after dusk and climbed onto the carrots. Two hours after dusk, four or five weevils of C. setigera could generally be picked off each carrot slice.

(b) Dry sieving in the sand dunes

In the dunes adults of both C. setigera and C. maritima congregate under the spreading leaves of the flatweed 'catsear' (Hypocheoris radicata) and are also found in considerable numbers beneath sowthistle (Sonchus oleraceus). Spade sized samples taken to a depth of three inches and incorporating these plants were sieved through a 12 gauge garden sieve. The residue of leaves, plant roots and other debris was transferred to plastic bags and carefully hand sorted in the laboratory. (Searching for adult weevils in the field proved too time consuming).

3. Breeding methods in the laboratory.

The methods of this section are presented in the text.

B. THE EGG

1. Descriptive notes and egg development.

May (1966) described the eggs of C. discors as sub-spherical, pearly-white turning grey as they mature and easily desiccated. Egg dimensions were given as 0.8 x 0.6 mm while hatching took eleven days in February, 21 days in April and 32 days in June.

Observations on the eggs of C. setigera conform closely to May's description but a difference in egg width was noted.

The mean egg dimensions of C. setigera were 0.78 x 0.51 mm with a range of 0.69 - 0.87 x 0.46 - 0.55 mm. The distribution of egg lengths and widths is shown in Table 1.

TABLE 1

Frequency distribution of egg length and width.

Width			Length		
Class (mm)		Frequency	Class (mm)		Frequency
0.449	-	0	0.692	-	1
0.462	-	3	0.718	-	5
0.475	-	3	0.744	-	9
0.487	-	16	0.770	-	20
0.500	-	15	0.796	-	18
0.513	-	22	0.820	-	13
0.526	-	7	0.846	-	2
0.538	-	3	0.872	-	2
0.551	-	1	0.897	-	0

The eggs of C. setigera were thus of the same length as those of C. discors but narrower. As May gave no indication of size range it is difficult to assess the significance of this size difference since such a difference could be a manifestation of a population difference within a species or could reflect a more fundamental difference between two species, ~~or could reflect a more fundamental difference between two species~~.

Eclosion from the egg in C. setigera was witnessed on one occasion. The mature embryo was clearly visible through the chorion and began moving on exposure to the heat of the microscopelight. After ten minutes the larva was very active, arching the head back and forth within the egg and scraping the mandibles against the interior upper surface of the egg. The mandibles broke through the chorion after 15 - 20 minutes

and by further wriggling and tearing, the larva managed to create an irregular rent and escape from the egg. There was no evidence of an egg burster being employed and thus the mode of hatching in Cecyropa corresponds to that of other Otiorrhynchid larvae (Van Emden, 1952).

The effect of incubation temperature on the egg was not investigated but it can be noted that for three eggs in which the date of laying was accurately known, hatching took place at 11, 11, and 12 days at temperatures ranging from 66 - 72°F.

C. THE LARVA

No difference could be found between May's description of larvae of C. discors and the morphology of C. setigera. Accordingly her descriptive notes are presented below.

> "The larva has a pale yellow head and is lightly sclerotized on the lobes of the last three segments giving it a reddish tinge and making detection extremely difficult in the light sand. It is distinguished by the unusual type of setae on these segments and by having three or four epipleural setae instead of the more usual two."

It was found that the most useful characters for the separation of Cecyropa from the other weevil larvae were the three or four epipleural setae and the thick blunt-ended setae of the last three segments. The use of May's key presented no problems in the separation of Cecyropa from all other weevil larvae encountered in the soils of the sand country.

1. Rearing individual larvae in the laboratory.

Although a number of attempts were made and several methods tried, only one larva was reared through from an egg to an adult in the laboratory. The following account describes the technique employed.

Discs of carrot approximately 3/16 inch thick were placed

on damp filter paper in a petri dish. Single larvae were placed in small holes made in the tissue of the carrot. (Unless a hole was made the larvae could not obtain sufficient purchase to penetrate the carrot). After twenty four hours the larvae burrowed further into the tissue of the carrot and could be left unattended for five to six days. Following this period larvae were dissected from the carrot, measured and replaced in a fresh carrot disc. This method proved satisfactory for studies on the growth of larvae but because of the labour involved and the mortality on dissection, it was not suitable for rearing large numbers of larvae in the laboratory.

2. Number of instars.

In an ecological study it is desirable to have some method of determining the age of the insect. Such an assessment is usually made in soft-bodied insects by measuring the changes in head capsule width that accompany each moult. As there was no information on this aspect for any species of Cecyropa, an investigation was undertaken to determine the number of instars in Cecyropa setigera.

Live larvae were measured after they had been anaesthetized with CO2 and all measurements were taken at the widest point across the head capsule. Measurements were made using a stereoscopic microscope with an eyepiece scale and all head capsule widths are expressed in as eyepiece divisions (100 divs. = 2.56 mm). An assessment of the number of instars, and the head capsule widths characteristic of each, was made from measurements of larvae recovered from the sampling programme and also from larvae reared in the laboratory.

The head capsule widths of larvae collected in the field during the sampling programme and extracted from soil cores in the laboratory, were measured immediately after death. The frequency distribution of head capsule widths for 493 larvae is illustrated in Fig. 1. On the basis of these results the measurements can be grouped into six classes that may be regarded as corresponding to six instars.

Instar	Class Interval			Mean
one	11.0	to	13.25	12.08
two	13.5	to	15.75	14.57
three	16.0	to	18.75	17.14
four	19.0	to	22.75	20.88
five	23.0	to	26.75	24.44
six	27.0	to	32.25	28.69

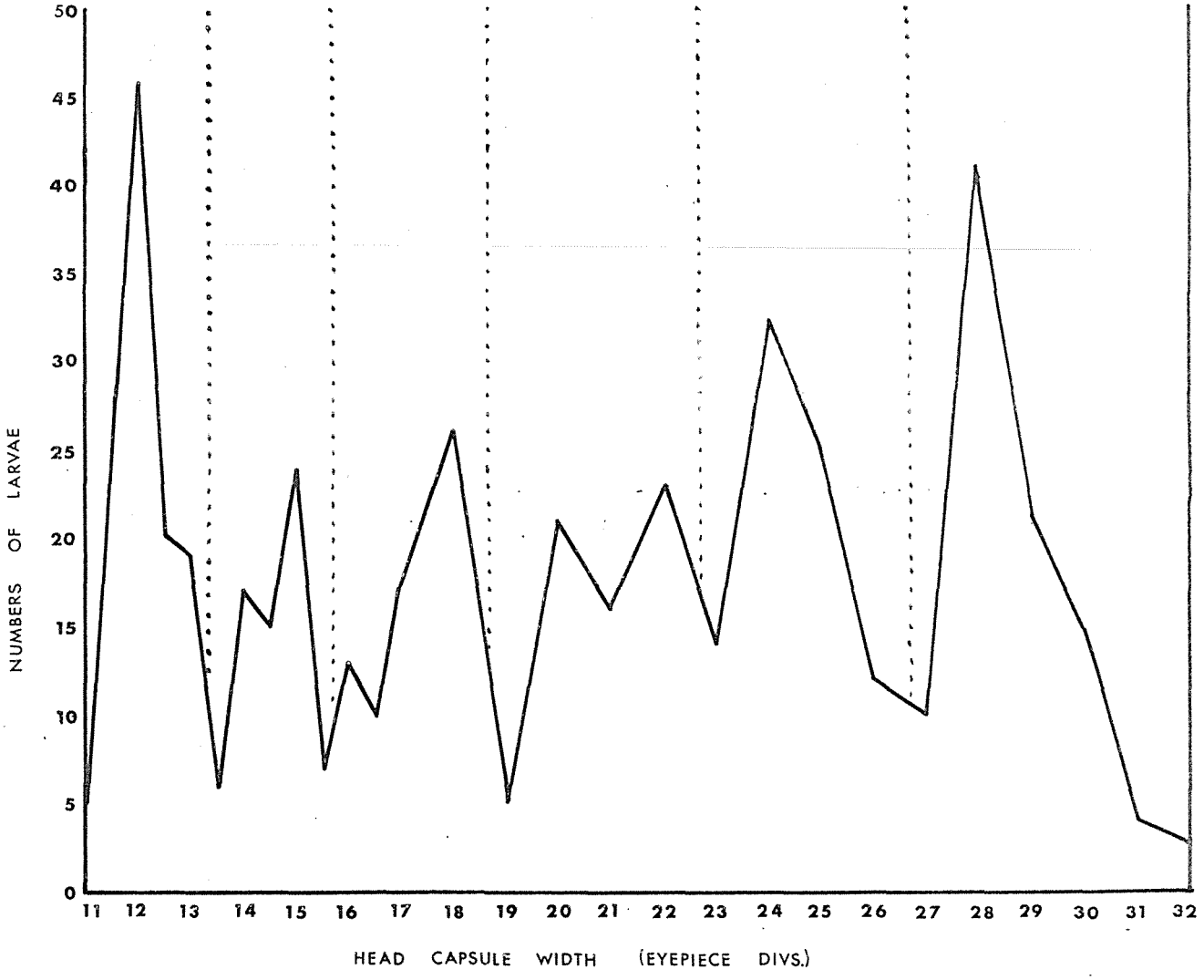
The division into six instars is supported by changes that took place in the head capsule widths of larvae reared in the laboratory. Larvae recovered alive from the field were reared on carrot slivers in the laboratory until one ecdysis had taken place. The following changes in individual head capsule widths occurred.

Before ecdysis		after ecdysis
12	-	15
12	-	15.5
16	-	21
16	-	20.5
22	-	26
22	-	25

Before ecdysis		After ecdysis
25	-	28.5
25	-	pupa
26	-	28.5
28	-	pupa
29	-	pupa
31	-	pupa

In each case the increase in size of the head capsule was sufficient to elevate the larva from its original instar class to the one immediately above. In no instance did the increase in size accompanying an ecdysis elevate a larva through more than one of the proposed instar classes.

Figure 1



Distribution of head capsule widths of 493 larvae recovered from the field

..... proposed instar divisions

Further evidence substantiating the validity of the proposed instar classes was provided by the changes in head capsule widths of a larva reared from an egg to an adult. The egg was laid between the 1st and 5th of April and hatched between the 13th and 17th of April. The egg dimensions were 0.75 x 0.49 mm. After hatching the larva was reared on carrot slivers and displayed the following changes in head capsule widths.

17th April	-	11.5	-	1st instar
24th April	-	14.0	-	2nd instar
1st May	-	17.5	-	3rd instar
8th May	-	22.0	-	4th instar
17th May-7th June	-	25.0	-	5th instar
12th June		pupated		

On the basis of these measurements it is evident that the larva passed through five instars before pupation and that the changes in head capsule widths fell within the first five age classes proposed above.

From a consideration of all results it is suggested that larvae of C. setigera generally pass through six instars in the field before pupation. Pupation can however, occur after five instars as evidenced by the pupation of laboatory reared larvae but the large number of sixth instar larvae encountered in the field suggests that such an occurrence is not the norm. It is also apparent from the variability in the head capsule widths of various instars that there is some degree of overlapping in the various instars. However, it is suggested that the six instar classes proposed above are ~~the most~~ suitable divisions for determining the age structure of larval populations of C. setigera as they occur in the field.

3. Duration of larval development.

The only evidence relating to the duration of larval development was obtained from the single larva reared from egg to adult in the laboratory. In this case the first four instars occupied approximately seven days each but the development of the final instar took 28 days before pupation occurred. Thus larval development in the laboratory in this one example occupied eight weeks at 66 to 72°F. As such temperatures occur in the soil only during the summer period, it is likely that larval development may be somewhat slower in the field at other seasons.

D. THE PUPA

Although the ecdysis of the prepupa has not been observed it was noted that one or two weeks before pupation the final instar larvae assumed an opaque white appearance and became slightly compressed dorsally and ventrally. In the laboratory prepupae also ceased feeding during this period. The pupa of C. setigera could not be distinguished from that of C. discors and accordingly May's (1966) descriptive notes are presented below.

> "The pupa, like the adult weevil, is proportionately broad. It is clothed with fine, rather long, pale bristles which, on the terminal segments, are longer than the horn-like pseudocerci. Secondary pterothecae are lacking and thecae of the mandibular cusps are inconspicuous. In the teneral adult, these deciduous cusps are small and straight and the scar, resulting from their loss can be easily overlooked."

May also mentioned that pupation took 28days in October. The pupal durations of three individuals of Cecyropa setigera were found to be 13, 15, and 16 days at 66 - 72°F.

The periods of occurrence of pupae in the field are not

known with any accuracy since this stage, being delicate, was easily broken during sieving. Only eight pupae were recovered from the 412 cores of sampling programme. Pupae were observed in the field, however, during spade searches and it was noted that numbers peaked during early November to early December. Numbers then fell off until March when they were again more frequently encountered with numbers apparently decreasing in April and early May. Pupae were not found from May to late October. It must be emphasized here that these are subjective observations and consequently only a limited weight can be placed on their significance.

E. THE ADULT

Members of the Otiorrhynchinae are charaterized by deciduous mandibular cusps that break off shortly after emergence to leave an oval scar on each mandible. In Cecyropa setigera the mandibular cusps of the teneral adult are small and straight and the scar resulting from their loss is difficult to detect. May (1966) describes similar mandibular cusps for C. discors.

In Cecyropa setigera the adult male can be distinguished from the female by the concavity of the first and second ventrites and the presence of two widely separated tubercules. In the female the tubercules are absent and the ventrites are convex.

1. Adult emergence

Although an adult emergence was not observed the following events were noted. Four days before emergence the sclerotization of mandibles and cusps, compound eyes and tarsal tips was visible through the pupal cuticle. Twenty

four hours before emergence the sclerotization had spread to other parts of the body which now appeared light brown while the mandibles, cusps and tarsi were a dark brownish-black. Twenty four hours after emergence the teneral adult was a golden brown colour which gradually darkened over the next 10 days to the mottled grey, brown and black colouration of the typical adult. The mandibular cusps broke off on the first feeding, which took place at seven days.

2. Oviposition

(a) Obtaining eggs in the laboratory

Van Emden (1952) states that generally adelognathous weevils readily lay eggs in a petri dish lined with damp filter paper, but May (1966) found that females of C. discors would not deposit eggs in such a container until a small quantity of sand had been sprinkled beneath the filter paper. In this study it was found that although May's method was satisfactory for up to two pairs of weevils, the confinement of more than this number to a petri dish inhibited oviposition.

The most satisfactory method for obtaining large numbers of eggs in the laboratory was as follows. Two inches of sieved (52 mesh) damp sand was placed in a clear plastic lunch box (approx. 6x4x4") and 20 males and 20 females added. Carrot pieces were added as food and the perforated lid replaced Fresh carrot was added as needed. After four weeks' incubation at room temperatures, the contents of the lunch box were wet sieved through 52 mesh gauze. Eggs and adults were washed into a petri dish, removed and counted using a stereoscopic microscope.

(b) Rate of oviposition and fecundity

No records were made of oviposition rates under field conditions. In the laboratory the average oviposition rate, as established from breeding experiments above, varied from one egg per female every three days to one egg every ten days. However, results from a study (see below) on the oviposition rates of two individual females indicate that the figures above may be considerably lower than normal.

Two weeks after emergence, two females reared in the laboratory from field collected pupae (collected, 30th November 1967) were paired with two field collected adult males. The two pairs were confined to two petri dishes lined with damp filter paper with a sprinkle of sand beneath. The two cultures were examined weekly by washing the contents of the two petri dishes onto a 52 mesh sieve. Following this process fresh filter paper and sand were added, adults replaced and fresh carrot given as food supply. Copulation was first noted ten days after the addition of males (i.e. approximately 24 days after emergence). One female began laying eggs at five weeks after emergence and laid the following number of eggs weekly (beginning at fifth week): 4, 7, 8, 4, 6, 4, 3, 4, 2. Eggs were deposited singly amongst the sand grains. Both male and female died from a fungus infection in the 16th week after female emergence. The second female began laying at the seventh week and laid the following number of eggs weekly: 4, 7, 7, 6, 2, 1, 2, 0, 1, 0, 0, 0. This female died, also from a fungus infection, 21 weeks after emergence. The first female thus layed 42 eggs and the second female 30 eggs.

These figures indicate that, under laboratory conditions,

there is a peak in oviposition rates about eight to ten weeks after female emergence. This peak is followed by a gradual decline.

(c) Depth of Oviposition.

During attempts to obtain eggs in the laboratory an opportunity was taken to observe the depth at which females laid their eggs.

Twenty males and twenty females were placed in a narrow container with sliding glass walls (2" apart and 10" long and 10" deep). The container was filled with damp sand, 52 mesh sieved, to a depth of eight inches. Carrot was supplied as food and the container topped with gauze. At three weeks, when the contents of the container were examined, the top inch of the soil had dried out in a sharply defined layer, but the remainder of the sand was still damp. The soil was removed in three layers and passed through a 52 mesh sieve. The top inch of the soil contained two eggs and 24 adults. At the one to three inch depth there were 29 eggs, two first instar larvae and seven adults while in the bottom five inches there were no eggs and only one adult.

These results thus support May's (1966) contention that in the field females burrow down to the damp sand to deposit their eggs.

(d) Oviposition periods in the field.

Oviposition periods in the field were assessed indirectly by counting the number of first instar larvae present at various times of the year. Unfortunately, due to a change in the extraction technique, results from the first four samples from December 1966 till 3rd March 1967 are invalid for assessing proportions of first instar larvae. In addition, samples five, six and

seven are based on 16 cores, a number which gives only a crude estimate of population composition over that period of the year. Results are presented in Table 2 below.. (Table 1, Appendix 1 presents total numbers of larvae in different instars recovered from samples 1-11)

TABLE 2.

Percentage of first instar larvae present in samples five to eleven.

	5/4/67 s.5 16cores	6/5/67 s.6 16cores	17/6/67 s.7 16cores	10/8/67 s.8 80cores	24/9/67 s.9 80cores	14/12/67 s.10 80cores	4/5/69 s.11 60cores
Percentage of 1st instar larvae	25	3	0	0	50	17	33

A peak in numbers of first instar larvae occurred in late october. This is probably a reflection of the increase in soil temperatures that occured in early September (Table 5). This increase in temperature probably provided the stimulus for the arousal of adults from their winter quiescence to begin oviposition. Oviposition diminished in late November as evidenced by the smaller proportion of first instar larvae, and from subjective observations it is thought that it was also low during January to March when the soil was very dry. During late March and early April, oviposition appeared to increase again, probably due to the increasing rainfall of that period, and finally tailed off into May and June. It is probable that oviposition was either absent or occured very infrequently in the months late June to mid-August, a period during which the adults were in a quiescent state.

It must be emphasized that this is an indirect method of assessing oviposition periods and that it is subject to the possibility that the early spring peak of first instar larvae may originate from eggs that had undergone a winter diapause which was broken by the rise in spring temperatures. However, since adults collected from the field during winter began laying eggs after being exposed to the warm temperatures of the laboratory, it is probable that at least part of this early peak of first instar larvae is due to a spring oviposition.

3. Adult Nutrition

It was found that adults were polyphagous in the laboratory. They consumed the following foods in rough order of preference: 'catsear', carrot, cabbage, lettuce, 'Hawksbeard', spinach, radish seedlings, tomato, lucerne seedlings, white clover stems and leaves, bread, ryegrass, young and mature lucerne leaves. The main preference appeared to be one for succulence rather than any one particular food plant. The white clover, lucerne leaves and ryegrass were eaten only with reluctance. As adults in the field are found clustered beneath the flatweeds 'catsear', 'hawkbit', and 'hawksbeard', it is probable that these plants are the main adult food supply, especially in summer when they are the only common succulent plants which survive the summer dryness.

4. Adult Behaviour

Otiorrhynchid adults are typically nocturnal insects, being most active just after dusk (Van Emden, 1952). In the laboratory adults of C. setigera crawled about foliage and up the walls of the container if they were kept in a dark

cupboard. On being exposed to bright light however, the majority immediately climbed down and burrowed into the sand, leaving only an occasional individual on the surface. The few weevils remaining on the surface were generally actively feeding on carrot.

During the day adults in the field typically bury themselves just beneath the surface of the sand, usually beneath or among the spreading leaves of flatweeds. They emerge to feed at night. The nocturnal habits of Cecyropa setigera were the subject of a brief investigation in the field on the 14th April, 1967.

This investigation consisted of recording weevil activity in a lucerne crop over one night. Activity was gauged in two ways: (i) 17 carrots were split longitudinally in half and placed on the ground at the base of lucerne plants. The number of weevils visible on the flat surfaces of the carrots were counted at intervals during the night. (ii) Two hundred sweeps with a net were made through the foliage of the lucerne crop at each of the observation periods. Different transects of the paddock (which was uniformly flat) were taken at each period. The contents of the net were placed in plastic bags and weevils removed by hand sorting in the laboratory. Air temperatures were recorded during the night. Dusk began falling at 6 p.m. and it was fully dark by 7 p.m. First light was noted at 5.15 a.m. and it was fully light at 6.15 a.m.

Results are summarized in Table 3 below.

TABLE 3. Number of weevils taken at intervals over one night

Time	Nos. on carrots	Nos. from sweeps	Air temp. °F.
5pm.	22	0	50
6.15	38	7	49
7.15	6	44	45
8.30	8	79	43
9.30	12	58	42
10.30	22	59	41
11.30pm.	18	54	40
12.30am.	21	36	39
1.45	18	20	39
2.30	22	12	38
4.30	33	3	41
5.30	37	2	33
5.45	33	not taken	34
6.15	11	0	32
6.45	2	not taken	31
7.00am.	1	0	31

From a study of this table and from observations during the night, the following course of events is suggested. Firstly, the weevils emerged at dusk, possibly on the stimulus of a decrease in soil temperatures. The emergence was reflected in the build up of numbers on the carrot at ground level. The weevils then climbed on the lucerne plants and migrated upwards. This is reflected by the decrease in the numbers of the carrot and an increase in the number of weevils taken from the sweeps of the lucerne foliage. This situation apparently remained more or less static until 12.30 a.m. when a number of weevils began to descend. This descent continued through the night as evidenced by the decrease in numbers on the foliage. Finally with the stimulus of the brightening sky the adults

burrowed down into the surface sand or hid beneath flatweeds. This is reflected in the decrease in numbers on carrots over the period 4.30 to 7 a.m. The sudden drop in temperature from 4.30 to 5.30 am. apparently had little to do with the regulation of weevil activity since the weevil numbers on carrots did not drop appreciable until the period 5.45 - 6.15 a period at which the light intensity was rapidly increasing. It is of interest to note that whereas copulation was observed at intervals all through the night, most couples broke their association at the onset of dawn, although one couple was observed in copulo at 6.45 am. when temperatures were one degree below freezing!

From these observations it is evident that the major period of activity for adults of Cecyropa setigera occurs during the night. It is regretable that, despite an assiduous search by torchlight, no adults were actually observed on the foliage of the lucerne plants. It is possible that the adults ascended to feed on the young lucerne shoots but this is difficult to reconcile with the reluctance of adults to feed on either lucerne shoots or mature leaves in the laboratory, and it should be noted that more observations are needed to verify the suggested pattern of nocturnal behaviour.

5. Natural enemies of Cecyropa setigera.

(a) Eggs

Eggs were attacked in the laboratory by two fungi, Fusarium sp, and Metarrhizium anisopliae.

(b) Larvae

(i) Fungi. In the field large numbers of dead

larvae were found that had been attacked by the two Hymenostible fungi discussed in Part II of this thesis. Metarrhizium anisopliae was observed on dead larvae in the field.

(ii) Nematodes. The following nematodes were recovered from field collected dead larvae of C. setigera and three other insect larvae.

TABLE 4. Species of Nematodes recovered from dead larvae of C. setigera and three other insects.

Date	Sample	Larval instar	Nematodes
10th Aug.	8	4th	Mermithid & Neoaplectana sp. (juveniles)
24th Oct.	9	6th	Neoaplectana sp. (females)
"	9	6th	Juvenile Rhabditid*
"	9	6th	Neoaplectana sp. (females)
"	9	4th	Juvenile Rhabditid*
"	9	6th	Rhabditid (female)*
"	9	5th	Alloionema ? juveniles*
"	9	Larva Costelytra zealandica.	Rhabditid females*
"	9	Larva Phlyctinus callosus.	Mermithid
"	"	Larva Graphognathus leucoloma.	Rhabditid*

* these were probably saprophytic upon hosts already dead.

(c) Pupae.

(i) Fungi. Pupae attacked by Metarrhizium anisopliae and the two Hymenostilbe fungi were recovered from the field.

(d) Adults.

(i) Fungi. Adults in the laboratory were attacked by both Metarrhizium anisopliae and Beauvaria sp.

(ii) An hymenopterous parasite. Of 41 adults collected on the night of March 25th, 1968, 30 were subject to parasitism from an hymenopterous parasite. The first hymenopteran adults emerged on the 30th March and emergence continued

till 11th April. Specimens were sent to Mr. E. Valentine, D.S.I.R. for identification but were damaged and Mr. Valentine could only suggest that they appeared to be in the subfamily Euporinae (Braconidae) and very near, if not in fact, the genus Perilitus.

F. SPATIAL DISTRIBUTION OF THE LARVAL POPULATION

Both vertical and horizontal distributions of larvae were investigated during the course of the sampling series in pasture near Himatangi.

1. Description of the Habitat.

The map reference of the field sampling site is given in Appendix 1 together with the materials and methods employed in sampling and extraction of larvae. The sampling site was situated on a pasture-covered sand plain which was characterized by small undulations. The difference between the highest and lowest elevation of these undulations was about four feet. In the winter of 1967 the water table rose to flood the low portions of the paddock for several days, but the sandy soils of the hummocks remained comparatively dry. 1/

The pasture cover varied with the elevation and the season. In the low elevations, subject to winter saturation, Yorkshire fog and cocksfoot predominated but at intermediate elevations this gave way to crested dogstail, ryegrass, subclover, some danthonia, and the flatweeds, catsear (*Hypochoeris radicata*) hawksbeard (*Crepis capillaris*) and hawkbit (*Leontodon taraxicoides*).

1/ Where subsequent mentions are made of low elevations they refer to the lowest portions of the paddock that become very wet in winter; medium elevations are those approximately two feet above the low elevations, while high elevations refer to the dry upper levels of hummocks approximately two feet above those of the intermediate levels.

At the highest and driest elevations, the plant cover was predominately a mixture of moss, Danthonia, crested dogstail, some ryegrass and a large number of flatweeds. Over the summer period the sandy soil dried out severely, particularly at the intermediate and upper levels where the flatweeds which possessed a very deep rooting system, were the only common green plants.

The soils of the sampling site are classifed as Hokio strongly mottled sand (Cowie and Smith 1958) and are part of the Hokio - Waitarere association consisting of Hokio soils on the sand plains and Waitarere sand in the dunes. (Cowie, Fitzgerald and Owens, 1967) Cowie and Smith describe the Hokio strongly mottled sand as a "weakly gleyed soil developed on low rises on the sand plains of the younger dune complex. The water table is lower than Hokio sand and during summer there is insufficient soil moisture to maintain high quality pasture growth." A typical profile of this soil is (after Cowie and Smith 1958):

0 - 2 inches - black to very dark brown sand, very friable, weakly developed fine granular structure, boundary abrupt.

2 - 5 inches - brown to light brown sand, extremely friable, loose.

5 - 10 inches - light grey, loose, single-grained sand with abundant, distinct, medium yellowish-brown mottles.

10 inches and below - grey, compact sand with a few distinct dark red mottles.

2. Changes in the soil environment over the year.

Both soil moisture and soil temperature were measured over the year. Soil temperatures were taken at the surface and at three, and ten inch depths using an ordinary laboratory mercury thermometer pushed several inches into the wall of a hole dug in the sand. Soil temperature changes at monthly intervals are presented in Table 5 . Soil moisture levels were measured at five depths: $\frac{1}{2}$, 3, 5, 10 and 14 inches. Samples for soil moisture determinations were taken with glass tubes (3 x $\frac{3}{4}$") that were scraped against the wall of the hole at the required depth until they were full of sand. Moisture levels were assessed in the laboratory by measuring the weight loss of these samples after drying for 24 hours at 110°C. Soil moisture levels are expressed as a percentage loss of weight resulting from the water loss on drying. Soil moisture levels over ten samples, taken at various times of the year and at each of the three elevations; low, medium and high, are presented in Table 6 .

3. Vertical Distribution of Larvae in the Soil

The vertical distribution of larvae in the soil was assessed from the combined results of the first ten samples. In each of the ten samples 16 cores were removed in five layers at depths of 0 - 2, 2 - 4, 6 - 10, and 10 - 14 inches. These were sieved separately and results noted for individual depths. Table 7 presents the combined results for the distribution of different larval instars over ten samples.

TABLE. 5 Soil temperatures of Hokio strongly mottled sand at four depths over twelve months (taken in the first week of each month)

Months (1967)	Depth (inches) 0	3	10
January	28°C.	22°C.	21°C.
February	24	22	22
March	20	19	19
April	18	17	16.5
May	18	15	14
June	11	9	9
July	8	7	7
August	8	7.5	8
September	14	13	13
October	17	15	14
November	21	16	15
December	33	21	20

TABLE 6 Moisture content (% loss in weight on drying) of Hokio strongly mottled sand at low, medium and high elevations over ten sampling periods at five depths.

	Sample 1 23/12/66			Sample 2 15/1/67			Sample 3 6/2/67		
	Low.	Med.	High	Low.	Med.	High	Low.	Med.	High
A	21.1	2.7	1.2	21.0	8.5	4.8	27.1	9.9	2.1
B	9.7	1.5	1.3	6.6	3.4	1.4	9.3	7.4	0.5
C	7.1	1.4	1.0	6.3	3.9	0.8	17.7	5.9	1.3
D	11.7	2.5	0.9	14.6	3.6	3.3	19.0	6.8	1.9
E	15.3	3.2	0.9	13.0	10.3	2.8	17.0	12.5	3.5

	Sample 4 2/3/67			Sample 5 5/4/67			Sample 6 6/5/67		
	Low.	Med.	High	Low.	Med.	High	Low.	Med.	High
A	29.2	11.9	3.2	14.3	6.4	3.3	10.6	10.2	1.9
B	24.6	2.4	1.1	7.3	1.4	1.2	4.4	5.1	1.9
C	11.3	4.7	1.1	5.7	1.7	0.8	4.0	4.7	1.8
D	11.6	4.6	2.4	7.7	2.1	2.6	4.2	4.2	1.8
E	14.4	5.8	3.7	9.6	5.0	2.9	4.9	4.1	2.5

	Sample 7 17/6/67			Sample 8 16/8/67			Sample 9 24/10/67		
	Low.	Med.	High	Low.	Med.	High	Low.	Med.	High
A	19.2	6.9	8.1	25.6	18.4	7.1	26.3	7.4	1.8
B	11.4	3.7	2.8	20.2	9.2	1.7	14.5	5.5	1.0
C	5.2	6.3	2.1	8.9	8.9	2.3	11.4	6.8	0.7
D	5.6	4.2	3.5	7.3	6.8	6.3	14.1	6.2	1.0
E	7.8	4.5	3.5	10.7	8.1	7.5	16.4	8.0	3.3

	Sample 10 4/4/68		
	Low.	Med.	High
A	27.6	6.2	2.3
B	23.6	2.5	0.5
C	14.2	2.7	0.6
D	7.0	2.2	0.9
E	8.3	4.1	1.7

Level A - 0 to 2"
Level B - 2 to 4"
Level C - 4 to 6"
Level D - 6 to 10"
Level E - 10 to 14"

TABLE 7 The distribution of larval instars at five successive depths.

Depth (inches)	Instars 1	2	3	4	5	6	Total no. of larvae.
0 - 2	11	9	2	2	3	0	27
2 - 4	1	3	11	10	13	2	40
4 - 6	0	2	8	13	9	21	53
6 - 10	0	0	1	2	13	7	23
10 - 14	0	0	1	0	5	6	12

Two features are evident from a study of this table. Firstly, most larvae are found in the two to six inch zone. Secondly, first instar larvae are found almost exclusively in the 0 - 2 inch level and there is a general trend for larvae to move downwards with increasing age.

From an examination of the soil moisture and temperature characteristics of the various depths (Tables 5.6) it can be seen that the four to six inch level, where most larvae occur, is characterized, at all periods tested, by generally having the lowest soil moisture level of any of the other depths from 0 - 10 inches. Larvae at this level would thus have the least chance of dying from asphyxiation due to saturation of the soil with surface moisture from heavy rain or in the medium and high elevations from the rising water table of winter. Also soil temperatures in this region do not display the seasonal variability characteristic of the upper level, while plant roots occur commonly down to the ten inch depth. Also the soil is generally of a loose single grained structure at the 4 - 6 inch depth permitting easy larval movement.

The restriction of first instar larvae to the upper soil horizon further supports the laboratory observations that females actively seek out damp sand to deposit their eggs, since over all periods of the year the soil moisture content in the 0 - 2 inch zone is higher than that of all other levels down to ten inches. This is due to the high organic matter content of this horizon which retains a large amount of moisture. Thus in being laid in the upper horizon, the eggs stand the least chance of being subject to desiccation while in addition they are in a zone where there is a plentiful supply of roots and rootlets as a food source. The tendency for the final instar larvae to burrow deeper into the soil probably represents the fact that there is generally some moisture (from the water table) at this level over all periods of the year. Thus the immobile pupae would stand the least chance of being subject to dessication if they inhabited the lower levels.

It is thus suggested that the general preference shown for this level is a reflection of the relatively stable soil environment in this zone connected with adequate food supplies and a soil structure that permits easy larval movement to find new food sources or to avoid unfavourable conditions.

4. Horizontal distribution of larvae in the field.

The stimulus for this study of the major factors affecting the horizontal distribution of larvae in the sand plain pasture was provided firstly by the realization that the small numbers of larvae collected in samples five, six and seven were inadequate to reflect larval population changes and secondly by an examination of larval distribution characteristics, which revealed that

larvae were exhibiting a clumped or contagious distribution. Clumped distributions are of the negative binomial type where the intensity of clumping can be expressed in terms of the dispersion parameter 'K' (Southwood, 1966). A usual value of K, indicating moderate clumping is two. Values above eight indicate a distribution approaching randomness while the smaller the value of K, the greater the amount of clumping. The K values of samples one to seven, as estimated by $k = \frac{\bar{x}^2}{s^2 - \bar{x}}$ were as follows (in order): 1.74, 0.36, 0.31, 0.71, 0.23, 0.54, 0.67. It was obvious that there was a considerable degree of clumping in the larval populations of C.setigera. Accordingly, in sample eight the number of cores was increased from 16 to 80 to increase the numbers of larvae taken and as it had been noted that the larval population appeared to be at its greatest density in the higher elevations of the paddock, each of the 64 additional cores was subjectively scored for elevation; either low, medium or high, as defined earlier. The method of sampling for the 64 additional cores is explained in Appendix 1 , but briefly it consisted of taking a series of transects across the paddock along which samples were taken every 15 yards.

The mean numbers of larvae per core in sample eight, taken at each of the three elevations, low, medium and high were (in order): 0, 0.36, 1.32. A single factor analysis of variance on the population density at three levels is presented below in Table 8 . A log x + 1 transformation was used.

TABLE 8 Analysis of variance on the number of larvae at three elevations (Log x + 1 transformation)

Analysis of Variance

Source	d.f.	S.S.	M.S.	F test
Elevations	2	0.69	0.345	7.97**
Error	61	2.64	0.043	
Total	63	3.33		

There was a highly significant difference ($P<0.01$) between the larval population densities at the three elevation categories. On a comparison of the means it was found that the population at the high elevation was significantly greater ($P<0.05$) than that at either the medium or the low elevations but that the density at the medium elevation was not significantly different from that at the low elevation.

It is suggested that the complete absence of larvae in samples recovered from the low elevation was directly due to the effects of winter flooding as at the time of this sample the soil in this level was saturated and two weeks previously had been under several inches of water. Survival of larvae under such conditions is unlikely. High moisture contents also probably had a similar effect on the populations at the intermediate levels but it must be noted that there is the possibility that the lower larval density at this elevation could be an indirect reflection of the effect of water content on the distribution of preferred host plants.

These observations on the preference of larvae for regions of higher elevation were further strengthened by samples nine and ten. In sample nine the mean larval densities/core (in order of increasing elevations) were 0, 0.77 and 2.19 while in sample ten the mean larval densities/core of the intermediate and higher elevations were 0.37, and 0.77. (In sample ten, cores were not taken from the low elevation because of the previously demonstrated absence of larvae.)

Although these results cast light on the gross distribution characteristics of the larvae they did not satisfactorily explain the high degree of clumping exhibited. In samples one to ten, all cores had been scored for percentage plant cover. However on analysis this was found to be an inadequate method for assessing the preference of larvae for various plant species. No correlations were evident and Whittaker and Fairbanks' Index of Association (Southwood, 1966) failed to reveal any clear associations between larvae of C.setigera and any particular plant. However from observational data it was noted that larvae and adults appeared to be grouped under the flatweeds:catsear, hawkbit and hawksbeard. Accordingly on the 15th of April samples were taken from a different sampling site (Appendix 1) 6½ miles inland from Himatangi beach. This site was chosen because from previous spade observations it was thought that larval populations were high in this particular paddock.

The method of investigation involved taking 30 cores in which one edge of the four inch diameter soil corer was placed over the centre of a large flatweed or group of flatweeds and a core taken to a depth of 12 inches. The remaining 30 cores were taken in the same area but at sites which were at least 18 inches distant from the nearest flatweed. A comparison of the numbers of larvae of C.setigera and other insects under flatweeds and not under flatweeds is presented in Table 9 below.

TABLE 9 A comparison of the numbers of larvae of C.setigera and larvae of other insects under flatweeds and not under flatweeds.

Species	Under flatweeds		Not under flatweeds	
	No.	No./core	No.	No./core
C. setigera	126	4.2	29	0.97
Graphognath us leucoloma Boheman	280	9.3	123	4.1
Phlyctinus callosus Boheman	173	5.8	9	0.3
Listroderes sp.	0	0	4	0.13
Grassgrubs	17	0.56	6	0.2
Phycocus lobatus Broun (adults)	122	4.07	21	0.7
Desiantha maculata Boheman	11	0.33	3	0.08

(A complete list of Coleoptera recovered in all of the eleven samples is presented in Appendix 1B)

From this table it is evident that larval populations of Cecyropa setigera display a marked clumping under flatweeds. (It was also observed that the distribution of dead larvae infected with the Hymenostilbe fungi followed a very similar pattern of clumping.)

The association of the larvae of Cecyropa setigera with flatweeds illuminates the observation of an increase in the density of larvae at the higher elevations of the sand plain. It has been shown previously that larvae do not inhabit the lower elevations of the sand plain pasture. This is probably due to the saturated and sometimes flooded conditions over winter and early spring. Thus the larvae are confined to the medium and high elevations. However, these elevations dry out severely over summer and larvae and adults must find some source of food and moisture to survive. Flatweeds, the most common of which is catsear, provide these requirements. The extensive and deep root systems of these plants enable them to penetrate the sand to sufficient depths to reach the moisture. Thus larvae, in being clumped under these plants, are supplied not only with food in the form of roots but also with moisture absorbed through the roots from the water below. In addition, flatweeds supply shelter to adults of C.setigera which burrow into the sand beneath the spreading leaves, and from the laboratory observations, it is suggested that they also serve as a source of food for the adult. The close association of larvae with the flatweeds of the sand plain of pastures is thus the basis for the strong degree of clumping evident in the distribution of the larval populations of C.setigera.

G. GENERAL DISCUSSION

In habits and biology Cecyropa setigera has proved similar to other Otiorrhynchid weevils. From a comparison of the biology of C. discors with that of C.setigera it is evident that the two weevils display a close similarity and that the correct identification of the larvae described by May could in fact be C.setigera.

The area of major interest in this study was found in the investigation of factors affecting the spatial distribution of Cecyropa setigera in pastures of the sand plain. The vertical and horizontal distributions characteristic of the larval population of C.setigera can be regarded as a response to the dominating environmental influence of changing soil moisture levels at various times of the year.

The vertical distribution of C.setigera in the soil reflects the varying capacities of different soil horizons to provide conditions most suitable for the survival of the different stages in the life cycle. The adults of C.setigera deposit their eggs in the top two inches of soil, a region in which moisture retention is greater than at any other depth, because of the large quantities of raw organic matter present in this horizon. In view of the clumped distribution of both adults and larvae and the necessity for an easily available food source for first instar larvae, it is probable that oviposition takes place beneath the flatweeds. After hatching the larvae exhibit a downward movement through the soil with increasing age, to the more stable but drier environment of the four to six inch zone. Both food and moisture are available from the deep and extensive root systems of the flatweeds. The greater proportion of final instar larvae at the deep levels probably represents a selection of this environmentally stable yet moist zone for pupation.

The clumped horizontal distribution of C.setigera is a reflection of the changing soil moisture conditions of the sand plain which by winter saturation of the low lying areas and summer drought of the higher elevations, favour the close association of larvae with the deep rooted flatweeds. It is also evident that the distribution of other soil inhabiting Coleoptera is similarly affected.

As weevil species are the most numerous of the injurious Coleoptera in pastures of the sand plains the demonstration of this association with pasture flatweeds is probably the most important single point discovered in the sampling programme. Large areas of the Manawatu sand country are characterized by open pastures containing large numbers of flatweeds. These pastures thus supply a multiplicity of suitable sites for the maintenance of high weevil populations and as farmers generally select the poorer weedy pastures for cropping the chances of damage to the seedling stages of crops becomes correspondingly high. The association demonstrated thus opens the possibility of reducing weevil numbers in pastures by controlling the plant host. This control could be effected by improved pasture management techniques or by the employment of weedicides and in being an indirect cultural method would diminish the need for the application of insecticides with their attendant residue problems.

PART TWO

THE TWO ENTOMOGENOUS FUNGI

CHAPTER 2

MORPHOLOGY OF THE TWO FUNGI ON THE HOST

INTRODUCTION

During sampling studies on the larval population of *Cecyropa setigera* dead larvae were frequently encountered that displayed an appearance indicative of a fungous disease. Attempts to establish the identity of this fungus proved unsuccessful and it was concluded that it was a new record for New Zealand or a new species. At a later date larvae were found which bore evidence of a second fungous disease. This particular fungus was readily identified and belonged in the genus *Hymenostilbe*. Later it was found that the fungus first discovered also belonged in this genus.

Generally, after death from a fungous infection, insects assume a dried mummy-like appearance. This 'mummy' is known as an endosclerotium and results from a replacement of the soft body tissues of the host with fungal mycelium. Endosclerotia often contain resting spores (gemmae) that enable the fungus to survive periods of adverse environmental conditions. Following colonisation of the dead insects the fungus emerges through the integument to produce fruiting bodies and spores.

The two Hymenostilbe fungi followed this general pattern of development and formed endosclerotia that were packed with hyphae and gemmae. However the two fungi also produced finger-like outgrowths of aggregated hyphae on which spores were borne. These structures are known as synnemata and are characteristic of the family Stilbaceae of which the genus Hymenostilbe is a member. The synnemata of the two fungi provided a basis for their identification, in that the synnemata of one were a reddish brown colour while those of the other were white.

The two Hymenostilbe fungi both produced two distinct types of conidia. The morphology of these two distinct conidial states was similar in both fungi. Production of one conidial state occurred on both endosclerotia and synnemata but the other developed only on the synnemata. The first mentioned conidial state consisted of balls of buff coloured hyphae (Figs 7 and 8) that enclosed a number of oblong conidia; this conidial state was given the name 'conidial state A' on the basis of a previous description of an almost identical structure by Mathieson (1949). The other conidial state, borne only on the synnemata, was characterized by the production of phialides and pip-shaped conidia (phialospores) (Figs 9 to 12). This conidial state was called the 'phialospore conidial state' and was the conidial state employed in classifying the two fungi in the genus Hymenostilbe.

To avoid confusion both fungi were given a tentative varietal[1] rank; the variety producing red synnemata was named Hymenostilbe R (red) while the other variety producing white synnemata, was named Hymenostilbe W (white).

In this chapter there is first an account of the morphology of these two fungi and of their conidial states. This is followed by a discussion of their taxonomic status.

1/ Variety is defined under the Botanical Code Art. 4 as 'a subdivision of a species below the rank of subspecies and above the rank of form.' A variety is delimited on morphological characteristics.

A. MATERIALS AND METHODS

1. Recovery of Specimens

Infected larvae and pupae were obtained during the sampling programme. Details of site location, sampling methods, pattern and timing are described (see Appendix 1). The laboratory method for extracting infected specimens from sand samples was identical to the extraction method for separating live larvae. However, the separation of Hymenostilbe infected larvae and pupae was not quantitative due to the entanglement of synnemata in the organic material underlying the salt solution/kerosene interface (see Appendix 1).

2. Production of Asexual Spore Stages

Specimens recovered from the kerosene were first drained for a few seconds on slightly damp filter paper and then transferred to petri dishes (five inch diameter) lined with several layers of damp filter paper. Five to ten specimens were placed in each petri dish and incubated at 24°C. The filter paper was kept moist to maintain high humidity.

3. Examination of Endosclerotia, Synnemata and Asexual Spore Stages

These stages were studied using three techniques:

(a) Temporary and permanent whole mounts

Discrete, fragmented and hand-sectioned material was mounted and stained in lactophenol acid fuchsin and lactophenol cotton blue. Slides of value were preserved by sealing the edges of the coverslip with nail polish.

(b) Paraffin sections of endosclerotia

The specimens were fixed in Carnoy's fluid, dehydrated, cleared in Terpiniol and embedded in paraffin, using a vacuum embedding oven. Sections were cut on a rotary microtome at seven and ten u and stained variously with Haemotoxylin/Eosin, Periodic Acid-Schiff, and by using Gomori's silver methamine technique for staining fungi in tissue sections.

(c) Direct observation

Specimens were observed through a stereoscopic microscope.

4. Dimensions

All measurements of spores, hyphae, gemmae, phialides and conidiophores were taken from the slide preparations above using a Leitz microscope fitted with a Leitz eyepiece micrometer (model OKNOR). Spores were measured only if both apices could be sharply defined in the same plane of focus. Measurements of endosclerotia, and of synnematal length and width, were taken from fresh specimens using a stereoscopic microscope fitted with an eyepiece scale.

5. Drawings

Drawings were made with the aid of an Olympus camera lucida fitted to an Olympus compound microscope.

6. Photographs

An adaptation of the Olympus compound microscope camera (model P.M.6) enabled photographs to be taken down the eyepiece of a stereoscopic microscope (at magnifications ranging from six to forty times). Photographs of slide preparations were taken using the camera attachment of a Leitz 'Ortholux' compound microscope.

B. MORPHOLOGY OF ENDOSCLEROTIA AND SYNNEMATA

1. Hymenostilbe R

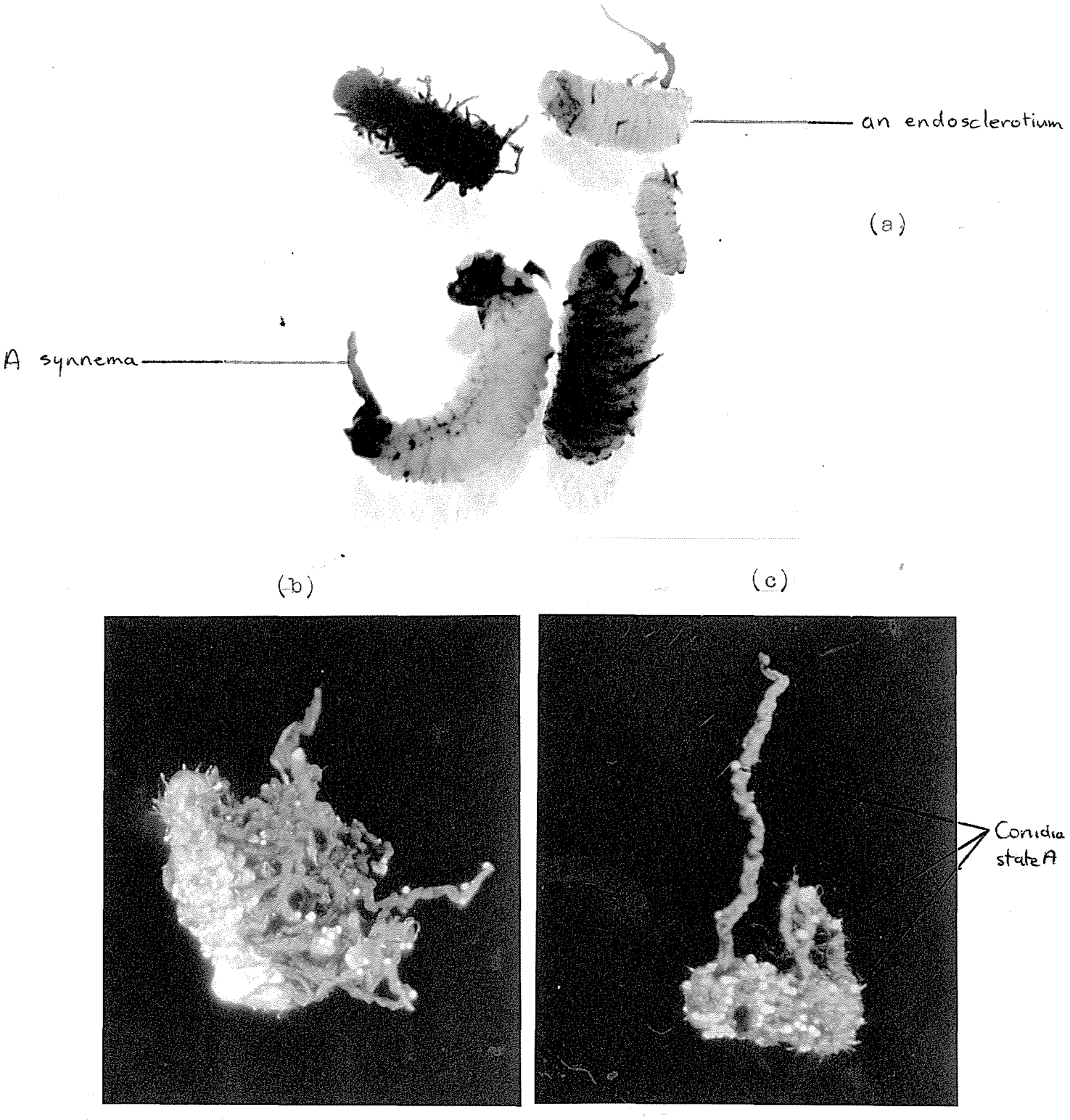
Endosclerotia (Fig. 2a, b, c) in the early stages of formation retain the external form of the larva but the body becomes an opaque white (or light pink/buff - 3ga*), and firm to the touch. Internally the body is packed with a yellowish white mycelium composed of fine hyphae and gemmae. The gemmae (Fig. 3) are both globose, and oval with flattened ends. They occur both singly and in groups of two or three. They may be terminal or intercalary to the hyphae. They are 5 - 20 u long and 5 - 15 u wide while the hyphae are 1 - 5 u wide. (Most gemmae are between 8 - 13 u long and 8 - 10 u wide.) As the endosclerotia age they shrink slightly and become quite hard, commonly assuming a yellow/buff shade (between 2ga and 3ga). Very old and dehydrated endosclerotia usually retain a semblance of larval form but such specimens are often fragmented and devoid of setae while their colours range from dark brown/black (3,4,5 pl,3,4,5 ni) through to a light yellow/buff (2ea). Most commonly, old endosclerotia are a buff shade (approximately 3ic).

The size of the endosclerotia depends on the age of the host at the time of infection. Dimensions of the larval endosclerotia vary from 1.2 x 0.6mm at the first instar, to 4.2 x 1.8mm at the final instar. Pupal endosclerotia are approximately 4.4 x 1.8mm.

Synnemata (Fig. 2a, b, c) first appear seven to fourteen days after the insect dies. They initially appear as red/brown (6ng) spots beneath or on the integument, enlarging over the

* Ostwald Colour Standards. Ostwald, W. 1931. 'The Ostwald Colour Album' and 'Colour Science'. Part I. Winsor and Newton Ltd., London.

Figure 2

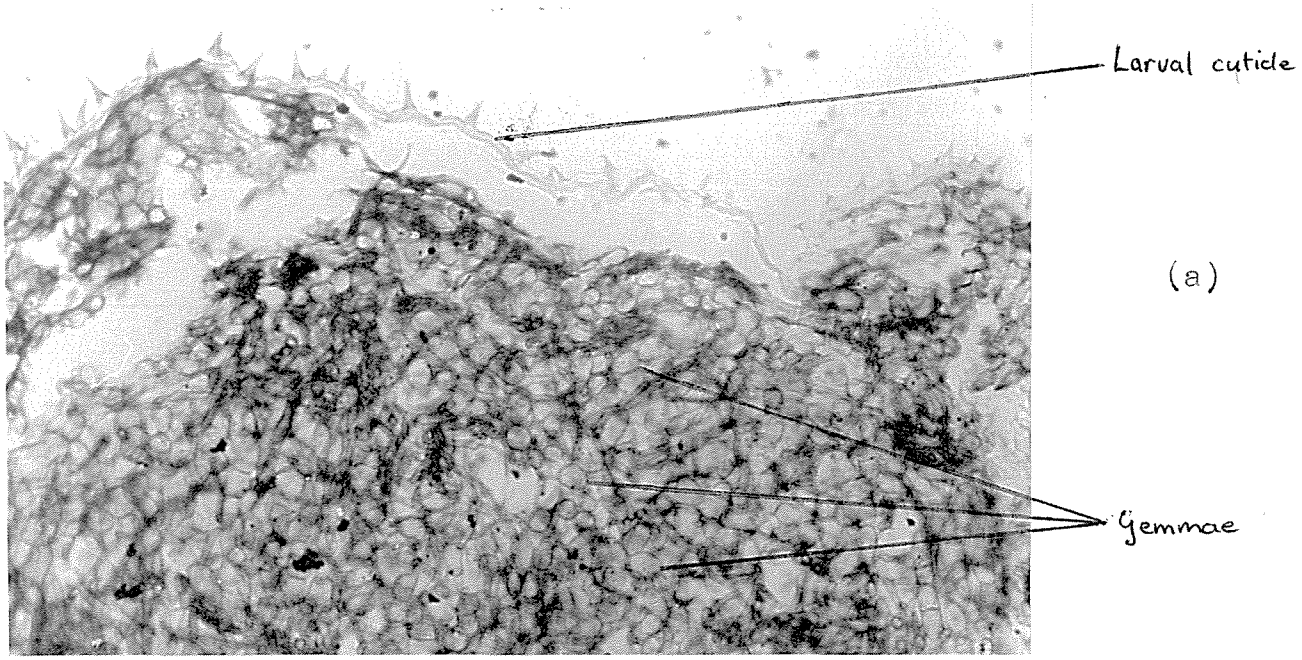


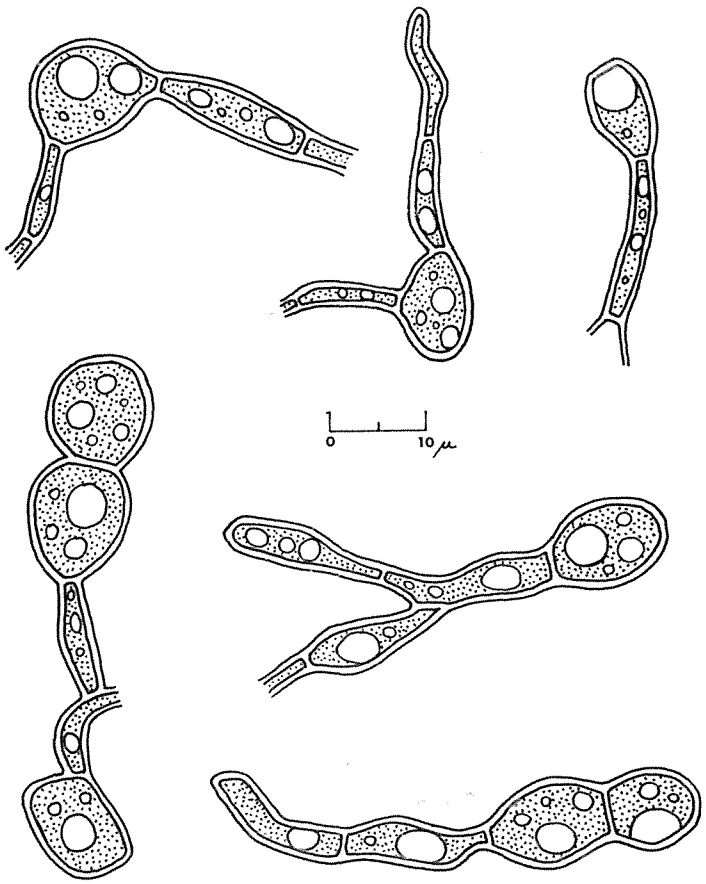
Hymenostilbe R Endosclerotia and synnemata.

(a) immediately after sieving and extraction from soil samples

(b),(c) after incubation under high humidity (note conidial state A produced on the endosclerotia and synnemata)

Figure 3





Hymenostilbe R. Gemmae.
(a) section through an endosclerotium
(b) individual gemmae

next three to four days to produce small firm cushions of red/brown mycelium (between 6pi and 6ng) that are a pale pink/brown (near 5 and 6ea) at the growing tip. These mycelial cushions elongate to produce the synnemata.

In the field mature synnemata are invariably gnarled and contorted and sometimes bear a short lateral branch. The synnemata are most frequently 1.0 - 1.5mm long although specimens with synnemata up to 2.6mm long have been observed. The width of the synnemata is most usually from 0.05 - 0.1mm with a range of 0.03 - 0.20mm. The great majority of the specimens recovered in the sampling programme were naked but this was due to the vigorous extraction process, as specimens observed directly in the field often bore conidial state A both on synnemata and endosclerotia.

The colour of synnemata in the field varies markedly depending on the soil moisture. Mature synnemata are generally a dull red/brown (6pg to 6pe, 6pc, and 6ne) but on very old and dry specimens they are usually black/brown (5,6,7pl), a colour similar to that of the shrivelled endosclerotium from which they arise. Actively growing synnemata can be distinguished by the pink colour of the growing tip, and also in the laboratory by the shiny red/brown (6pi to 6ng) of the synnematal shaft.

The number and position of the synnemata varies greatly on field recovered specimens (Fig.2 a,b,c). Some endosclerotia bear only two to three synnemata, these generally arising from the mouth, the anus and/or terminal lobes. Most commonly ten to twenty synnemata are produced, although endosclerotia with over a hundred synnemata have been observed.

Synnemata produced from the endosclerotium under incubation at high humidity in the laboratory are more uniform in shape and size than those produced in the field, and branching has not been observed. Such synnemata are terete, and often bear clumps of conidiophores of conidial state A (Fig. 7) on their tips. They very occasionally bear the alternative phialospore conidial state (Fig. 9) with or without conidial state A.

Internally the synnemata consist of a tightly packed central core of whitish, longitudinally arranged hyphae 2 - 5 u wide and from 10 - 20 u between septa. These hyphae are covered by a thin, tightly woven, differentiated outer layer of distorted, thick-walled, brown coloured, and closely septate hyphae (5 - 10 u wide and 8 - 15 u between the septa). These outer hyphae run in a more or less longitudinal manner but are irregular in the basal areas and at any thickened or branched regions.

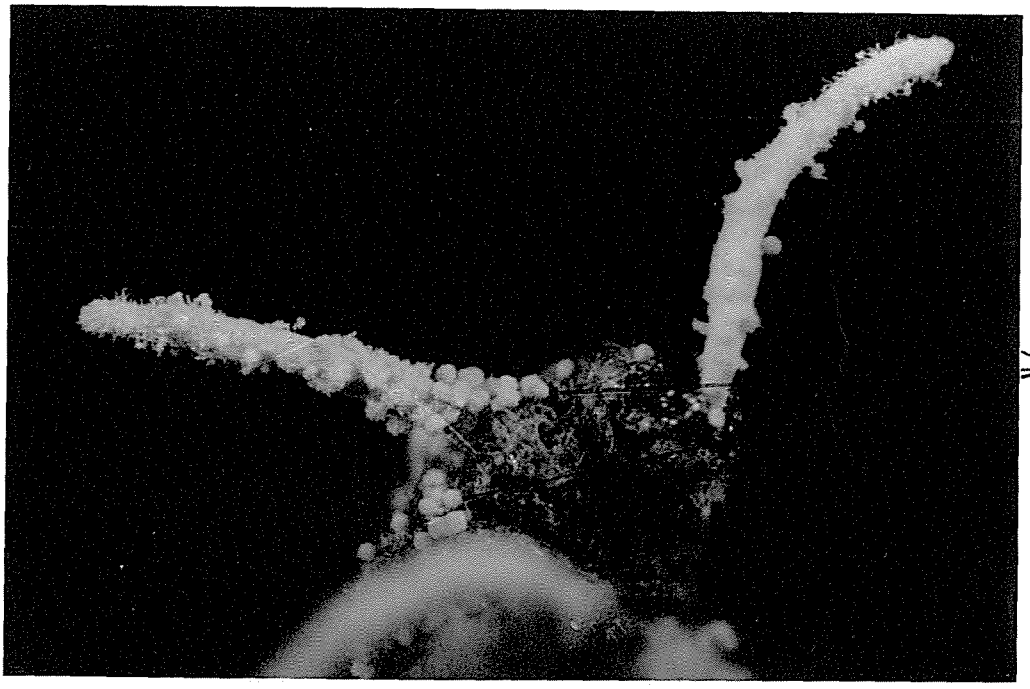
2. Hymenostilbe W

In the field, endosclerotia of H.W can be readily observed against the background of sand due to the possession of a number of flexible, white to yellowish buff (3ga) synnemata. The synnemata are seldom branched and grow away from the endosclerotium in a contorted manner. They are from 1 - 8mm in length and from 0.1 - 0.25mm wide. Many of the synnemata bear conidial state A (Figs. 7 & 11) and in addition often bear the alternative phialospore conidial state (Fig. 11). Generally 5 - 20 synnemata are produced from an endosclerotium. Occasionally one or two short (1 - 2mm), thick (0.20mm)

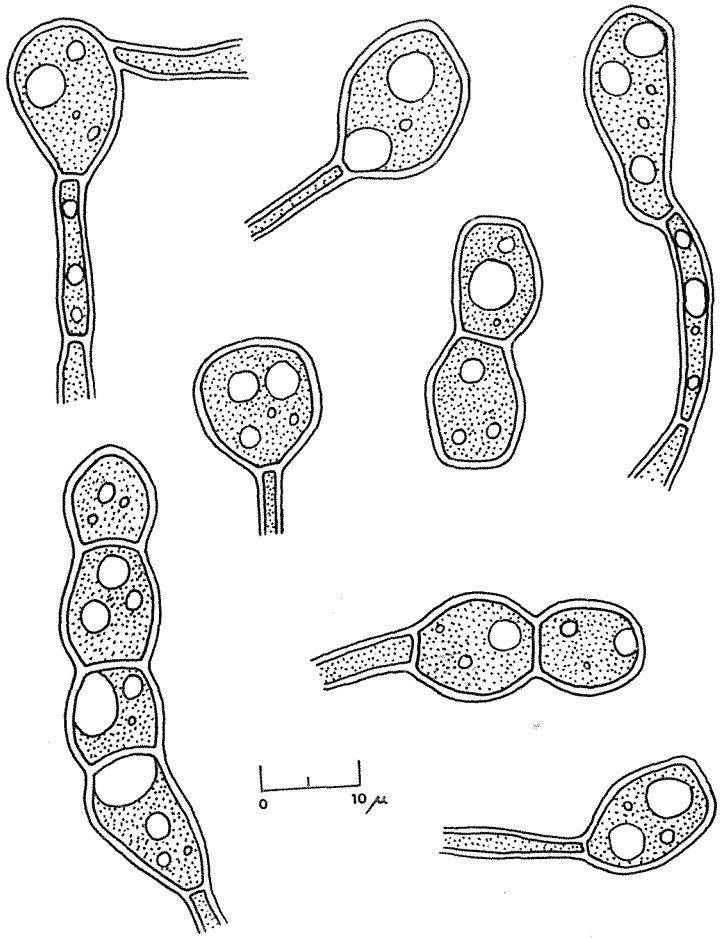
synnemata are produced from the same endosclerotium, particularly from the anal region. These are gnarled and contorted, a dark brown (4pl) to olive/brown (3pn) colour and bear a dense covering of the phialospore conidial state on their surface. On the basis of laboratory observations it is suggested that such synnemata are produced during marginal soil moisture levels. Endosclerotia collected from sand during the sampling programme were free of conidial state A due to the vigorous extraction process, but on incubation at high humidity a dense covering of conidial state A was produced over the surface of the endosclerotia and their synnemata. Internally the gemmae and hyphae (Fig. 5) are very similar to those of H.R, although endosclerotia of H.W have a greater proportion of flat ended gemmae that reach up to 25 u in length.

Larvae infected with H.W and incubated under high humidity conditions in the laboratory first produce outward manifestations of the disease two to five days after death, when hyphae emerge through the integument and form a dense covering of conidial state A. Three to seven days later synnematal initials appear as fascicles of hyaline hyphae that arise from the body of the endosclerotium amongst the conidiophores of conidial state A. Many of these fascicular hyphae produce the early stages of conidial state A at their tips (Fig. 5). As elongation proceeds a sterile, loosely aggregated growing tip becomes apparent, behind which the early stages of conidial state A are produced from the outer mycelium while the internal hyphae aggregate longitudinally to form the shaft of the synnemata. Under moist conditions the initials rapidly elongate

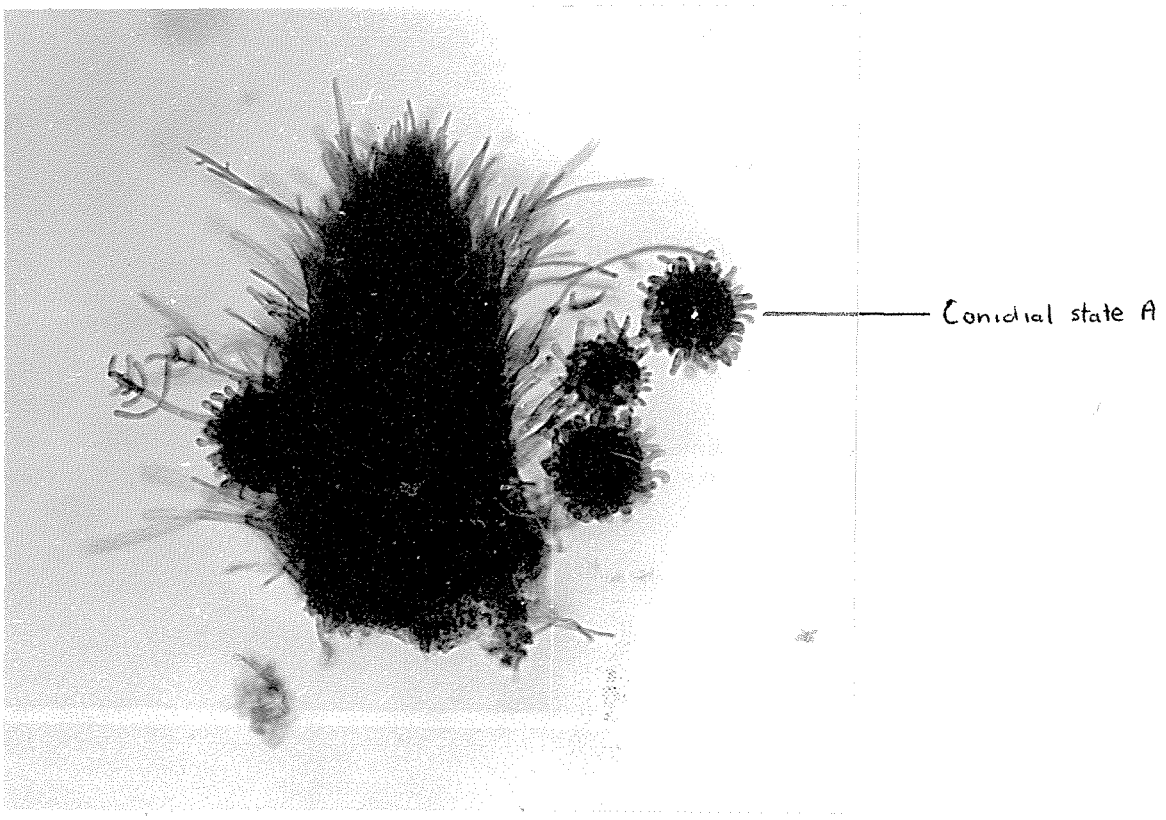
Figure 4



Hymenostilbe W. Endosclerotia and synnemata producing both conidial state A and the phialospore conidial state.



Hymenostilbe W. Gemmae.



Hymenostilbe W. Synnematal initial and conidial state A

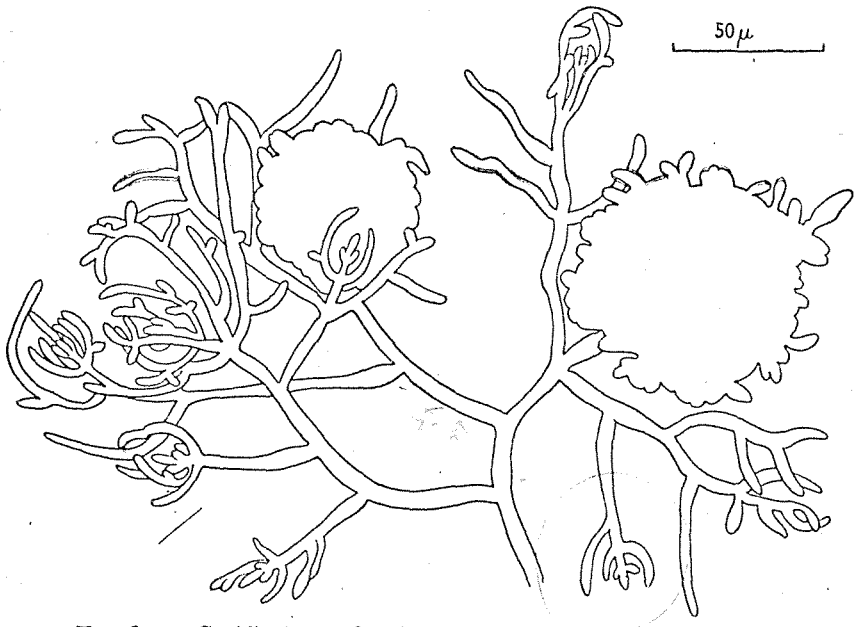
to form terete, white synnemata that are often slightly attenuated upwards (Fig. 4). Internally the synnemata consist of a tightly packed core of longitudinal hyphae 2 - 5 u wide and from 10 - 25 u between the septa. This is covered by an outer mantle of loosely aggregated, fertile hyphae (4 - 8 u wide) that bear a dense hymenium of phialides and spores of the phialospore conidial state, interspersed with conidiophores of conidial state A (Figs. 4, 11). The hymenium is of a velvety appearance and is visible between the scattered balls of conidial state A that are produced behind the sterile growing tip. Under continuous high humidity conditions the synnemata and conidia are white but if the specimens are allowed to dry, the colour turns to a deep yellow buff shade (between 3ea and 3ga) and longitudinal growth stops.

C. MORPHOLOGY OF THE TWO CONIDIAL STATES OF HYMENOSTILBE R AND HYMENOSTILBE W

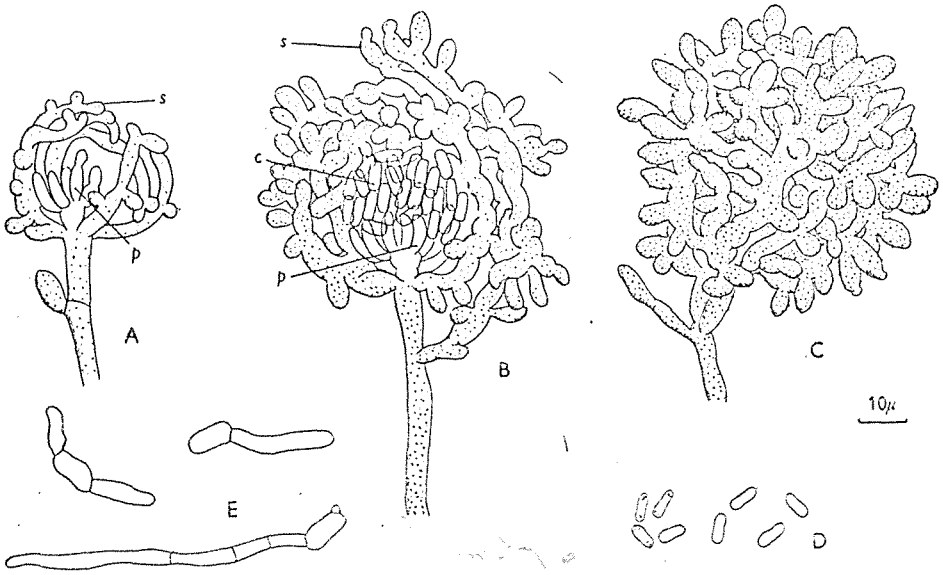
1. Conidial State A of both Hymenostilbe R and Hymenostilbe W

The conidial state A is practically identical in both Hymenostilbe R and Hymenostilbe W (Figs. 7, 9b, 11). It is a conidial structure described only once previously as an unnamed alternative conidial state for Cordyceps aphodii Mathieson. In her description Mathieson (1949) merely proposed its designation as Status conidialis A as she could not discover an affinity to other conidial or vegetative structures. A search by the author of subsequent references to the genus Cordyceps and its conidial states, Hymenostilbe, and various other entomogenous stilbaceous fungi, failed to reveal further information.

Figure 6



Text-fig. 2. Conidiophores of conidial stage A, of *C. aphodii*. The mature conidiophores are only shown in outline.



Text-fig. 3. Structure of conidiophores of conidial stage A of *C. aphodii*, and conidia. A, young conidiophore; B, mature conidiophore in optical section; C external appearance of mature conidiophore; D, conidia; E, germinating conidia. *c*, conidia; *p*, phialides; *s*, sterile hyphae.

Conidial state A of Cordyceps aphodii

It was subsequently decided not to pursue the matter experimentally as an investigation into the speciation and systematic affinities of this conidial state would be outside the scope of this thesis.

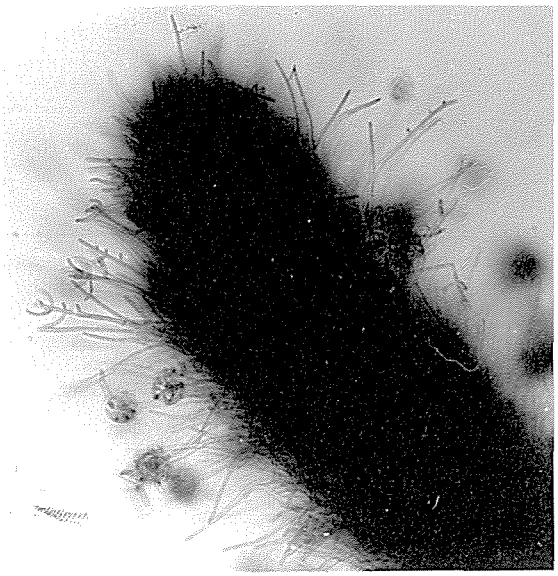
The conidial state A of Cordyceps aphodii (Fig. 6) is morphologically very similar to the conidial state A of both H.R and H.W (Figs. 7,8,9,11). As Mathieson's description of this conidial state applies almost equally well to H.R and H.W it is presented below in only a slightly modified form.

The spherical conidiophores of conidial state A are the most obvious and dominant form of sporulation in both H.R and H.W. This state may begin to develop from synnemata when they are very small. It is not restricted to the synnemata and may develop from anywhere on the surface of the endosclerotia.

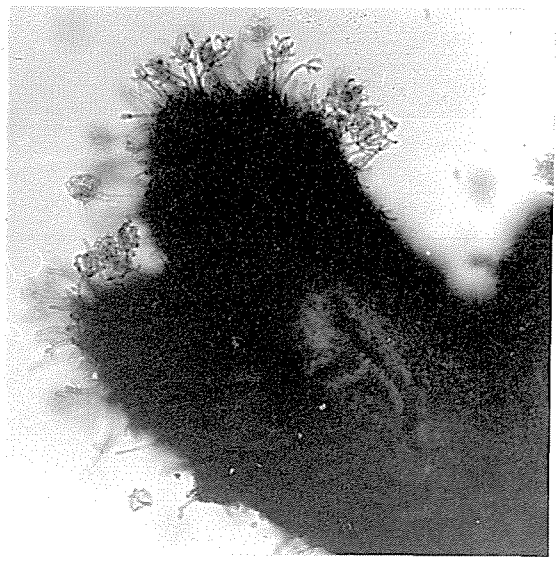
At first, unbranched colourless hyphae grow out from the surface of the synnemata or endosclerotia (Figs. 7a, 7b). At the extreme tip of these hyphae several very short, blunt phialides arise, which are hyaline, non-septate and about 6.25 u long. At the same time, immediately behind these, and for a short distance back along the branch, a number of laterals develop and, becoming sickle-shaped, curve around the apex, at the same time producing many short branches on their convex side. Colourless spores are budded off from the phialides until they completely fill the cavity enclosed by the outer sterile hyphae. The spores are oval, smooth walled unicellular bodies (Fig. 8) and measure approximately 10 x 3.5 u. Spore dimensions of H.R and H.W are similar (see Tables 10 & 11). The sterile hyphae are 5 u wide and markedly tuberculate. At first

Figure 7

(a)



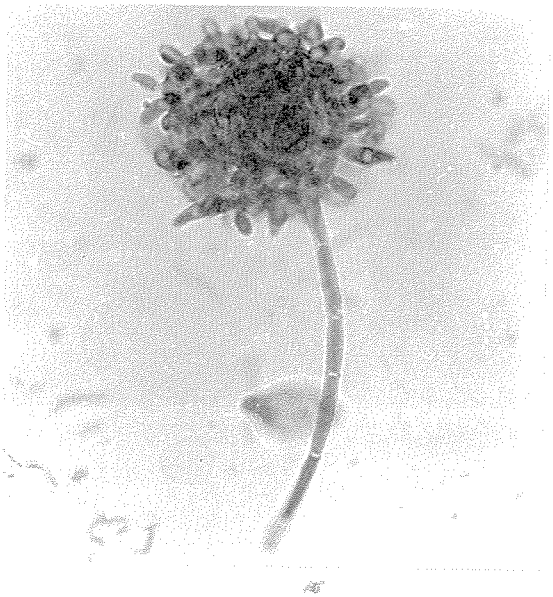
(b)



Developing conidiophores of conidial state A
(a) on a synnema of Hymenostilbe W
(b) on a synnema of Hymenostilbe K

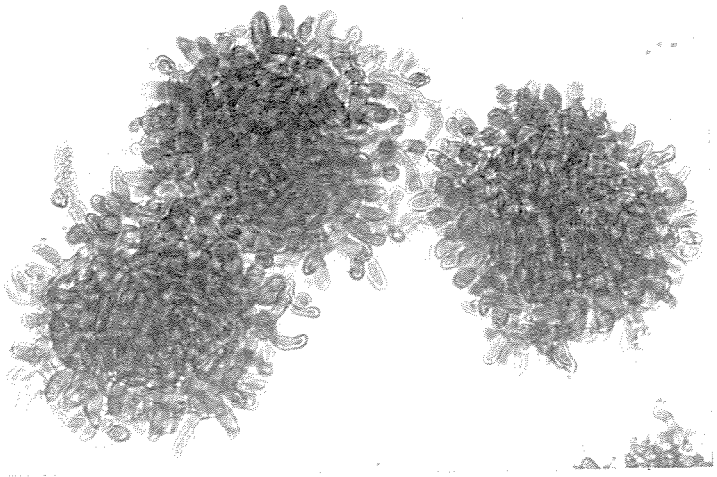
(c)

(d)

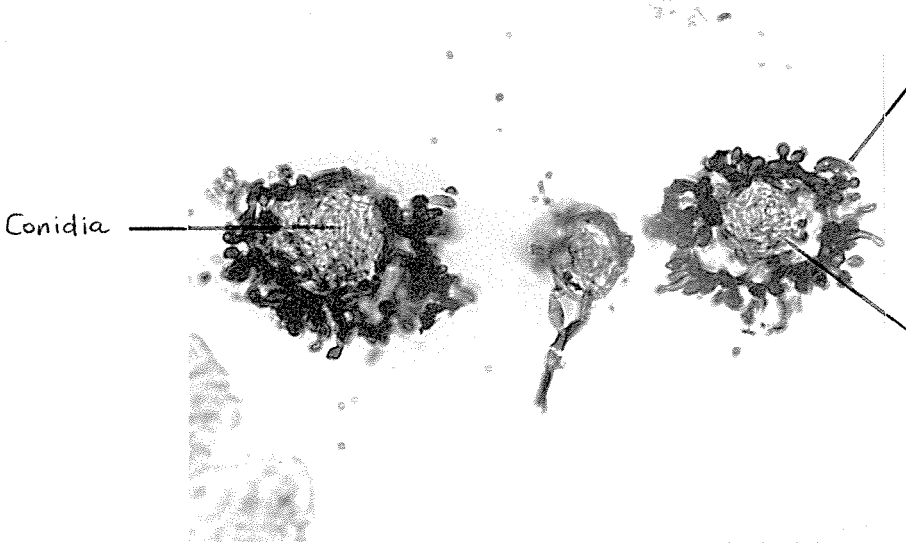


Conidiophores of conidial state A
(c) Hymenostilbe W
(d) Hymenostilbe K

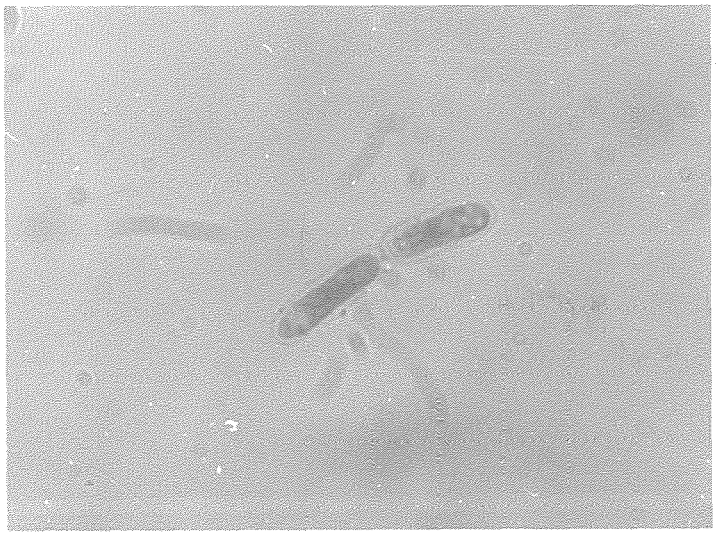
Figure 8



Mature conidiophores



A section through two mature conidiophores and one immature conidiophore



Spores (conidia) of conidial state A

Hymenostilbe R . Conidial state A .

TABLE 10 Spore dimensions of conidial state A from H.R

Measurements in u ($\pm$ 0.1 u)

30 spores

Spore length (u)	Spore width (u)
11.1	3.9
11.1	4.1
11.0	4.1
10.2	4.0
10.6	3.7
11.4	3.3
11.5	3.8
10.7	3.7
11.1	3.2
10.3	3.7
11.2	3.2
11.9	3.5
10.4	3.4
10.4	3.5
10.5	3.3
10.3	3.3
10.7	3.6
9.9	3.4
9.9	3.4
9.9	3.4
11.2	3.8
10.0	3.3
11.2	3.2
10.5	3.8
11.1	3.8
11.5	3.3
9.9	3.2
10.3	3.7
10.8	4.1
11.3	3.7

Length: Mean = 10.73 $\pm$ 0.10u (S.E.); Range: 9.9-11.9u

Width: Mean = 3.58 $\pm$ 0.05u (S.E.); Range: 3.2-4.1u

TABLE II Spore dimensions of conidial state A from H.W

Measurements in u (± 0.1u)

30 spores

Spore length (u)	Spore width (u)
10.1	3.7
9.3	3.5
9.5	3.4
10.8	3.3
10.2	3.6
9.4	3.6
11.4	3.2
8.7	3.9
10.1	3.9
9.5	3.9
9.8	3.8
11.1	3.5
9.5	3.2
10.7	3.2
9.7	3.8
10.5	3.6
9.5	3.4
9.5	3.2
10.7	3.4
10.9	3.3
9.7	3.7
10.3	3.3
10.3	3.5
9.9	3.3
10.6	3.4
9.4	3.9
10.0	3.2
11.1	3.3
10.4	3.4
9.8	3.3

Length: Mean = 10.08 ± 0.12u (S.E.); Range: 8.7-11.4u

Width: Mean = 3.49 ± 0.04u (S.E.); Range: 3.2-3.9u

they are colourless but at maturity become buff coloured.

When the conidiophores are mature, i.e. the spherical heads are full of spores and have changed colour, the stalks wither and the conidiophores may remain adhering to each other by the roughness of the sterile hyphae. Although in the field synnemata and endosclerotia are sparsely covered with conidiophores a new crop will begin to develop following two to three days incubation in the laboratory.

An examination of Mathieson's illustrations (Fig. 6) and a comparison with Figs. 7, 8, 9&11 reveals the overall morphological similarity of these three conidial states. However two important differences arise between Mathieson's description and that for H.R and H.W. These are:

(i) The conidia of conidial state A of C. aphodii are smaller:- 5.0 - 7.5 x 2.0 - 2.5 u.

(ii) In C. aphodii the conidiophores develop on the ends of much branched hyphae. In H.R they develop on an unbranched stalk, although in H.W occasional branched stalks have also been observed.

Although similarities are exhibited in the three conidial states, it is suggested that developmental ontogeny may reveal a stable means of delimitation.

2. The Phialospore Conidial State

(a) Hymenostilbe R

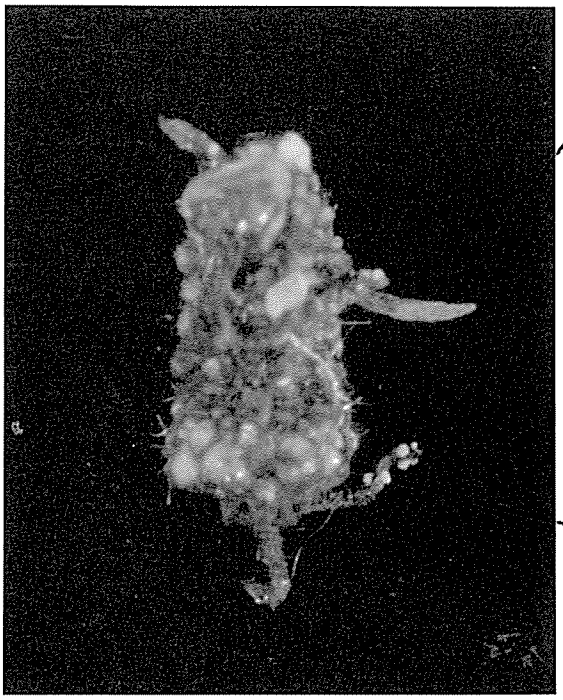
This conidial state has been observed on synnemata produced from endosclerotia on only four occasions. In each case the synnema bearing these conidia wholly developed from the endosclerotium following laboratory incubation. The

phialospore conidial state has never been observed on synnemata of field recovered specimens although, as noted previously, this could be due to the vigorous extraction process. In view of the rarity of this conidial state and its obvious taxonomic significance, one of the main aims of physiological and nutritional studies (Chapter 3) was the production of phialides and conidia on synnemata produced in artificial culture. The development of this conidial state in culture proved difficult and it was only at the very end of this study that consistent production was achieved. The description of the conidial state below is taken from synnemata and conidia produced from endosclerotia on incubation and from colonies in artificial culture.

The phialospore conidial state of H.R develops on synnemata alone or in conjunction with conidial state A (Fig.9a,b). The synnemata bearing phialides and conidia are terete and are a shiny red/brown colour (6ng) in the lower region (Fig.9a). On the upper half or third of the synnemata the colour changes to a pinkish white shade with a velvety appearance due to the production of phialides and spores on the outside of the synnematal shaft.

Phialides (Fig.10 Nos. 9-16) are produced only from the outer longitudinal hyphae of the synnemata. They are scattered and arise either as lateral sessile or occasionally terminal cells of the outer hyphae or much less frequently as terminal cells of short one or two celled lateral branches. The phialides are flexuous to irregularly bent or straight, smooth walled, and may have occasional warty protuberances. They are 2.5 - 5.0 x 7 - 15 u, often subcylindric and attenuate either

Figure 9



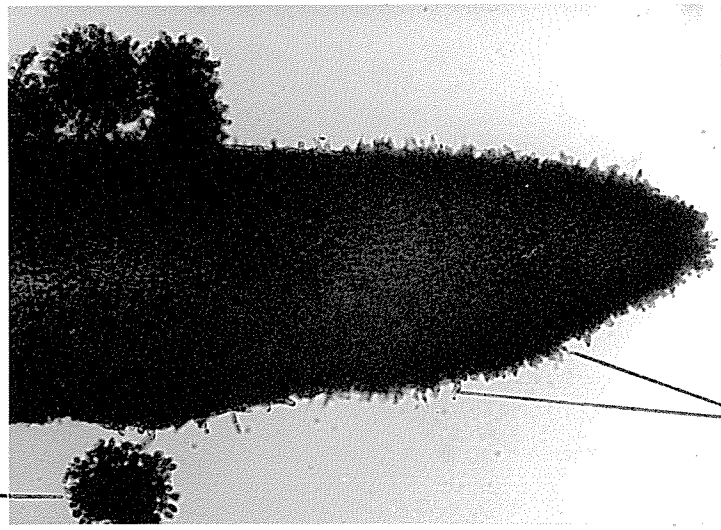
A synnema bearing the phialospore conidial state.

Hymenostilbe R

An endosclerotium producing a synnema bearing the phialospore conidial state

(a)

Conidial state A



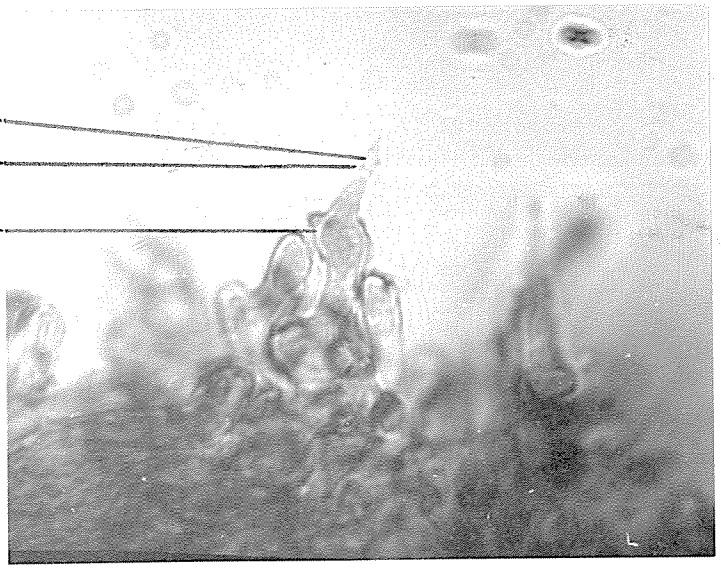
(b)

Hymenostilbe R

A synnema producing both conidial state A and the phialospore conidial state

phialides

Conidial state A



phialospore

collarette

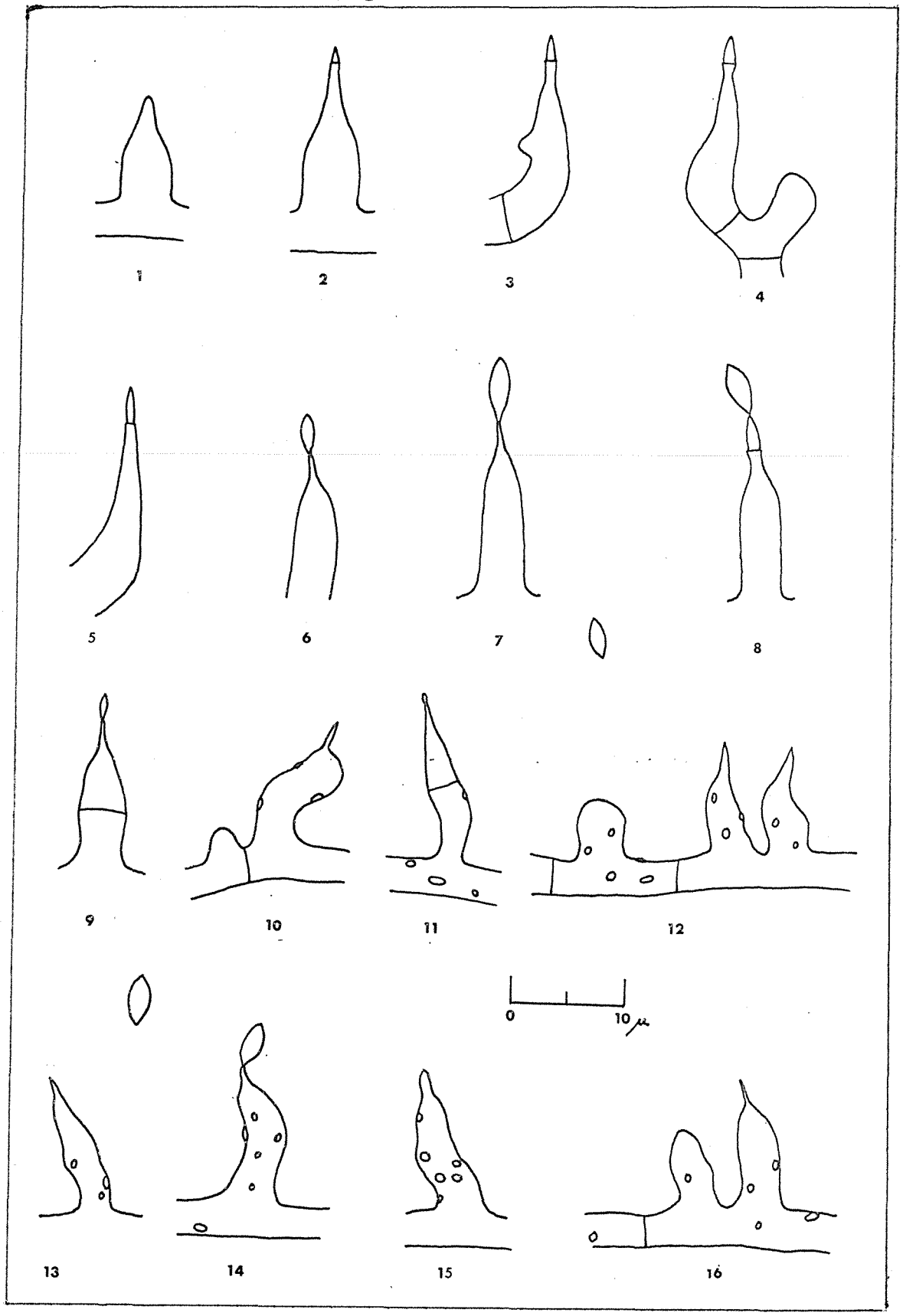
phialide

(c)

Hymenostilbe R

A phialide producing a partially extruded phialospore

Figure 10



Hymenostilbe R 1 - 8, Conidial (phialospore) ontogeny in artificial culture; 9 - 16, naturally occurring phialides and conidia.

gently or abruptly to single, short, acute or acuminate sterigmata which are 2 - 5 u long. The apex of the phialide occasionally bears a partially extruded phialospore (Fig.10 Nos 1-5) that enables the distinction of an evanescent and very inconspicuous collarette (Fig.9c).

The conidia (phialospores) are produced singly and are broadly fusiform, hyaline, smooth walled, and 1.2 - 1.8 x 3.5 - 5.0 u (Table 12).

(b) Hymenostilbe W

Synnemata bearing the phialospore conidial state were readily found on endosclerotia of field material. Production of synnemata bearing the phialospore conidial state was also readily induced in the laboratory (Fig.4) when endosclerotia were subjected to incubation under conditions of high humidity.

On field material the phialides and conidia are more commonly localized on the central regions of synnemata as a loose hymenial layer. On synnemata produced following laboratory incubation, a dense hymenium of tightly packed phialides and conidia is found. Also synnemata from either source often bear conidial state A (Fig.11) particularly in the basal regions where phialide production is generally sparse or absent. Phialides and conidia form on nearly all synnemata but occasionally synnemata are produced that bear only a dense covering of conidial state A. The following description relates to synnemata produced from endosclerotia after incubation under high humidity in the laboratory.

Phialides (Fig.12) are borne only on the loosely interwoven, echinulate, outer hyphae of synnemata. They form a

TABLE 12 Spore dimensions of the phialospore conidial state from H.R

Measurements in u (± 0.1u)

17 spores*

Spore length (u)	Spore width (u)
4.5	1.7
4.4	1.5
4.5	1.6
4.7	1.6
4.2	1.5
3.6	1.3
4.3	1.6
4.5	1.5
5.0	1.5
5.0	1.5
4.1	1.4
4.6	1.5
4.6	1.8
3.6	1.6
4.3	1.4
3.4	1.2
3.7	1.6

Length: mean = 4.29 ± 0.11u (S.E.); Range: 3.4-5.0u

Width: mean = 1.51 ± 0.03u (S.E.); Range: 1.2-1.8u

*Total number of measurable spores produced on synnemata from endosclerotia

TABLE 13 Spore dimensions of the phialospore conidial state from H.W

Measurements in u (± 0.1u)

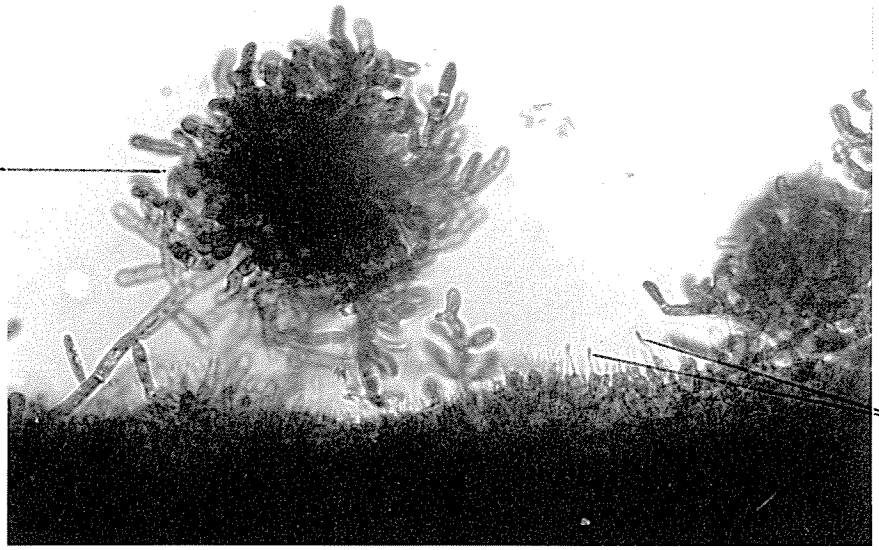
30 spores

Spore length (u)	Spore width (u)
4.6	1.5
4.5	1.4
5.1	1.8
5.4	1.4
4.4	1.3
4.6	1.9
4.6	1.5
4.7	1.6
5.3	1.4
5.7	1.4
4.6	1.4
4.6	1.4
4.6	1.4
4.0	1.7
4.1	1.7
5.3	1.5
3.6	1.1
5.8	1.2
5.1	1.4
6.3	1.5
4.1	1.7
5.1	1.8
5.4	1.2
4.3	1.5
4.0	1.8
5.9	1.6
5.3	1.3
5.6	1.2
5.1	1.8
4.9	2.0

Length: mean = 4.85 ± 0.10u (S.E.); Range: 3.0-6.3u

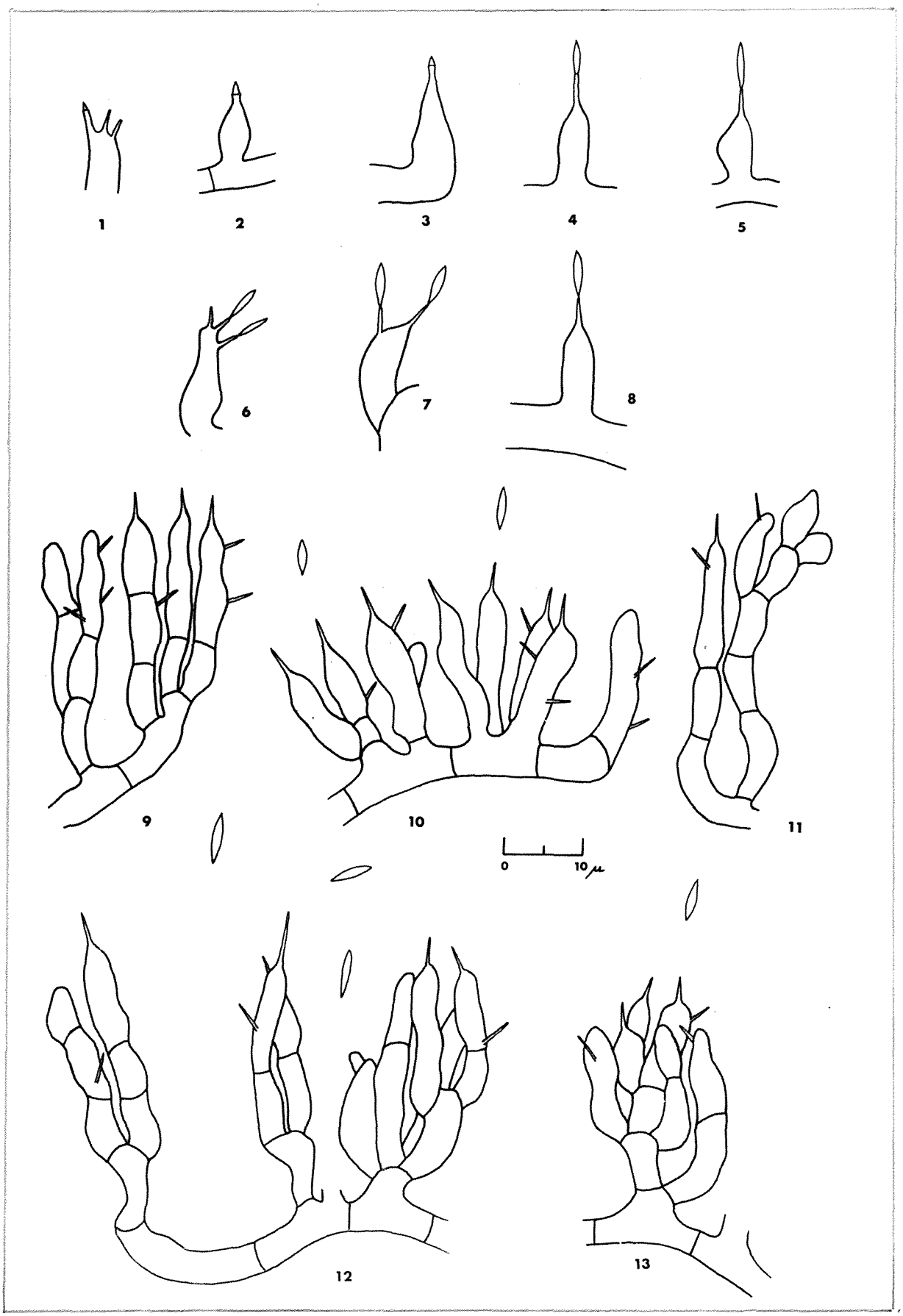
Width: mean = 1.50 ± 0.07u (S.E.); Range: 1.1-2.0u

Figure 11



Hymenostilbe W Conidial state A and the phialospore conidial state produced on the same synnema

Figure 12



Hymenostilbe W 1 - 8, Conidial (phialospore) ontogeny in artificial culture; 9 - 13, naturally occurring phialides and conidia.

dense pallisade layer and may be sessile or arise at the terminations of the outer longitudinal hyphae. More frequently they are borne on 1 - 5 celled lateral branches as terminal or lateral, sessile or short stalked phialides which occur singly, as pairs, or in clusters of up to five (Fig. 12 Nos 9-13). The phialides are generally subcylindric to ventricose, sinuous, or with one or two constrictions. They are smooth walled with occasional warty protuberances and are 10 - 20 x 2 - 5 u. Phialides generally taper abruptly into short acute sterigmata 3 - 7 u long. Sometimes up to four sterigmata can arise, either apically or laterally, from a single phialide, and in addition from the upper cells of the lateral branches. The apices of the sterigmata are generally acute. Occasionally a partially extruded phialospore enables the distinction of an evanescent, and extremely inconspicuous collarette (Fig. 12 Nos 1-4).

The conidia (phialospores) are produced singly and are fusiform, hyaline, smooth walled and 1.1 - 2.0 x 3.0 - 6.0 u (Table 13).

3. Conclusion

The morphological features of the phialospore conidial state (as described above) indicate that the two varieties (H.R and H.W) must be assigned to the genus Hymenostilbe. On the basis of observations on laboratory incubated specimens, field recovered specimens, and artificial cultures, the differences between H.R and H.W that are least prone to environmentally induced variability are:

(a) Single sterigmata in H.R as against multiple sterigmata in H.W.

(b) Phialides very rarely arising from one or two celled lateral branches in H.R whereas phialides are commonly produced on one to five celled lateral branches in H.W.

(c) The reddish brown colour of synnemata in H.R contrasts the white or yellowish buff colour in H.W.

(d) There is a very rare production of the phialospore conidial state on synnemata of H.R whereas synnemata of H.W often bear the phialospore conidial state.

On the basis of the above characteristics H.R and H.W are readily identified both in the field and the laboratory.

D. TAXONOMY OF HYMENOSTILBE R AND HYMENOSTILBE W

1. A Review of Systematics and Speciation in the Deuteromycetes

The genus Hymenostilbe Petch (1931) is a member of the form-class Deuteromycetes (Fungi Imperfecti), a large group of fungi characterized by an apparent absence of a sexual phase and which reproduce asexually by the production of conidia. In their general structure and development the Deuteromycetes resemble the Ascomycetes and to a lesser extent the Basidiomycetes and their sexual phases when discovered fall into these two classes. In cases where the sexual state of an imperfect fungus is described, and placed in a perfect class, it is proper to retain the existing classification of the asexual conidial state in the Deuteromycetes. (This double binomial system was sanctioned in the Botanical Rules, Art. 59, 1950 - cited Ainsworth, 1962.) Ainsworth and Bisby (1966) speculate that up

to one-third of the Deuteromycetes have a known perfect stage, another third may have an unnamed perfect stage, while the rest probably have no perfect state at all.

Classification within the Deuteromycetes follows a system propounded in a series of volumes by Saccardo (1882-1931). In the Saccardian system the Fungi Imperfecti are first divided into three form-orders on the basis of the type of fructification. Following this criterion Hymenostilbe is placed in the Moniliales, a large group of fungi which produce spores freely on the mycelium or on conidiophores arising from it. Within the Moniliales four form-families are delimited on the basis of conidiophore grouping and colour. Accordingly Hymenostilbe belongs in the Stilbaceae characterized by the production of upright aggregations of hyphae (synnemata) from which conidia are produced. Within form-families form-genera are based on such characters as type of conidiophores and colour, shape, and septation of conidia. Form-species are based largely upon the host on which they are found, and the dimensions of the conidia.

Today the Saccardian system is regarded primarily as a useful method of indexing fungi as research has increasingly revealed the environmental instability of the delimiting characters and their unsuitability for demonstrating systematic affinities. Accordingly modern taxonomists have been searching for an alternative basis for the classification of the Deuteromycetes. Recently the approach of Hughes (1953) based on developmental morphology of the conidium has attracted considerable attention. Hughes grouped member species into eight sections on the basis of spore and sporophore ontogeny. Under

this scheme Hymenostilbe Petch would be placed in Section IV, characterized by the production of phialospores (see Fig. 10 Nos 1-8 for phialospore ontogeny in H.R). Phialospores are defined as 'spores abstricted in basiseptal[1] succession from the mouth of phialides[2] (which may or may not produce an evident collarette) and may be grouped into false heads or forming chains' (Tubaki 1966). Phialides with multiple sterigmata as in H.W (see Fig. 12 Nos. 1-8 for phialospore ontogeny in H.W) are termed polyphialides by Hughes (1953, 1968).

Since the development of this scheme modifications have been proposed (Tubaki 1958, 1963) and amongst them it is of particular interest to note that Subramanian (1962 - cited Tubaki 1966) proposed the term 'spiculospore' as another section to accommodate fungi in which the spore is produced at the tip of a pointed structure as in Akanthomyces Lebert (1858) and Hirsutella Patouillard (1892). If this suggestion is adopted Hymenostilbe should be included in this new section.

It is now generally accepted that Hughes' scheme of classification holds great promise of providing a new basis for the systematics of the Deuteromycetes but as yet the system only encompasses the Moniliales and a great number of developmental studies are needed to determine the distribution of fungi amongst these sections. Thus the status of the major systematic delimitations in the Deuteromycetes may be regarded as in a state of flux.

1/ Development in the direction of the base; thus the apical member of the chain is the oldest (Hawker 1966).

2/ Terminal portion of a conidiophore or of its branches from which the conidia are abstricted and from which they are not cut off by a cross-wall until they are fully mature, the mature phialide remaining constant in length (Hawker 1966).

This confusion is further compounded by the critical approach of present day taxonomists to the criteria previously employed in the delimitation of both form-species and form-genera. The early taxonomists of the 19th and 20th century frequently described species on the basis of minute differences in either physiology or morphology. As they were without the present day appreciation of specific variability, and the repeated demonstrations of environmental instability in many of the Saccardian characters, it is not surprising that a considerable degree of synonomy exists in both species and genera. This state of nomenclatural confusion has stimulated the formulation of the 'modern' concept of the fungal species, major exponents of which were Snyder and Hansen. These two workers in a classic series of monographs (1940, 1941, 1945, 1954) reduced over 1,000 members of the genus Fusarium to only nine species. In their papers Snyder and Hansen reversed the 19th and early 20th century concept of species being narrow and relatively fixed entities to propose a scheme recognizing that - "species were based on similarities amongst the different individuals that make up the species. If species were based on differences then every individual becomes a species."

Briefly Snyder and Hansen's method consisted of assembling in pure culture a large number of monosporous isolates from widely differing sources and establishing the limits of genetic and phenotypic variability in their various morphological characters. In this way they distinguished stable morphological features common to all members of a species but which served to distinguish all these individuals from all

members of other species. Snyder and Hansen stressed the use of morphological characters for delimitation of species and higher categories, holding that a species should be easily identifiable whether alive or dead, regardless of the substrate on which the fungus is growing. To accommodate the need for the identification of intraspecific groups of individuals that exhibit some characteristic of pathogenic or biological significance, these workers further advocated the use of the term formae speciales. In this manner Snyder and Tousson (1965) maintained that the system offered a taxonomically sound method of naming fungi which could also be adapted to discriminate strains of economic importance.

Although Snyder and Hansen's ideas as applied to Fusarium have been subject to criticism (Miller 1946, Ainsworth 1962) their proposals have gained general favour and in succeeding years have undergone revision and extension (Ainsworth 1962, Nelson 1965). The present concept of a species as it now stands calls for the fungus to be:

(a) delimited strictly on stable morphological criteria

(b) clearly distinct amongst closely related species

(c) fully representative of the biologic species as it occurs in the field.

2. The genus Hymenostilbe

The genus Hymenostilbe Petch was erected in 1931, with H. muscarium as the type species. Subsequently eleven additional members have been added by Petch (1931, 1932, 1937, 1942, 1948), Mains (1950) and Kobayasi (1941). All species of Hymenostilbe are recorded as arising from the bodies of insects or spiders,

and the genus has a wide distribution being recorded from both North and South America, Britain, Japan, Ceylon and Australia. Most are described as the conidial stages of various species of the entomogenous Ascomycete Cordyceps.

The salient characters of the genus as defined by Petch in 1931 are; cylindric to clavate phialides borne in a hymenial layer on a synnema, usually very short sterigmata about 0.5 u long, and conidia produced singly or very rarely catenulate. Since the original generic description there has been only one revision of the genus by Mains (1950) who dealt with the entomogenous species of the stilbaceous genera, Akanthomyces, Hymenostilbe and Insecticola of North America. In this paper Mains expanded Petch's original concept of the genus to incorporate details of the manner in which phialides arise from the hyphae of the synnema;

".....phialides in a hymenial layer covering the synnema, produced as terminal cells of short lateral branches from the longitudinal hyphae of the synnema, or as lateral cells or buds directly from the longitudinal hyphae or less frequently as terminal cells of the longitudinal hyphae....."

Within the genus both Mains and Petch delimited species on the characters of shape and size of phialides, sterigmata and conidia. As none of the present Hymenostilbe species has been isolated in pure culture an assessment of the stability of such characters was not available. Furthermore there are no data relating to proof of pathogenicity, or cross-inoculations to different hosts, which means that the influence of host type on morphology, and the degree of host specificity remain entirely unknown. This unfortunate taxonomic situation is further complicated by an uncertainty in delimitation from two form-genera of close affinity, Akanthomyces and Hirsutella.

These genera are separated from Hymenostilbe mainly on the nature of the conidia; dry and catenulate in Akanthomyces, in mucus droplets in Hirsutella, and dry and single in Hymenostilbe. The separation of Akanthomyces from Hymenostilbe is indistinct by present standards since in his original description Petch allowed the possibility that Hymenostilbe conidia could be occasionally catenulate. In fact both Petch (1933) and Mains (1950) speculated that these two genera could be combined, although Petch later concluded the genera were distinct (1944) on the basis that catenulation of conidia was commonly used as a character in the separation of form-genera. Mains (1950), however, expressed doubt as to whether the spore production of phialides in Hymenostilbe was actually limited to one conidium and he speculated that several conidia which do not adhere are produced in succession and consequently spores are found singly on the phialides. As Mains wrote "....If this (i.e. sequential conidial production) is so, then the distinction between the genera is slight." Sequential conidial production occurs in Hymenostilbe R (Fig. 10 No. 8). Thus it is probable that synonymy of Akanthomyces and Hymenostilbe is warranted under the form-genus Akanthomyces proposed in 1858 by Lebert (cited Mains 1950).

The separation of these two genera from Hirsutella is also anomalous. Mains (1951) states that in some species not all conidia are enveloped in the mucus droplets characteristic of Hirsutella, thus some individuals could lie between Akanthomyces/Hymenostilbe and Hirsutella, separable only by the long sterigmata of the latter. Unfortunately sterigmatal lengths overlap in several species of Hirsutella and Hymenostilbe.

It is thus evident that under present taxonomic concepts a critical examination is needed of the generic and specific characters delimiting these genera of entomogenous fungi.

5. Classification of Hymenostilbe R and Hymenostilbe W

There is little doubt that under the present taxonomic situation the two varieties described in this study (H.R and H.W) would be acceptable as valid new species of the form genus Hymenostilbe. There have been no endemic species of Hymenostilbe described in New Zealand and to the author's knowledge no records of their occurrence, other than as an unnamed conidial state of the endemic species Cordyceps novae-zealandica Dingley. In her description Dingley (1953) stated that the spores of this conidial state were "oval to fusiform, 4 - 6 u long x 2 - 5 u wide, hyaline, smooth and catenulated from phialides 10 - 12 u long, phialides borne in terminal clusters on aerial mycelium on the endosclerotium - Hymenostilbe spp." Neither H.R nor H.W resemble this conidial state, which on the basis of catenulate conidia, is more accurately placed in Akanthomyces.

Cordyceps aphodii Mathieson (1949) would appear to be the fungus of closest affinity to H.R and H.W. This species is pathogenic on the larva of the cockchafer beetle Aphodius howitti Hope in South Eastern Australia, and has two conidial states: one is an unnamed Hymenostilbe and the other is conidial state A. In dimensions and shape the conidia of the Hymenostilbe state of this fungus are similar to those of both H.R and H.W, but on phialide characteristics the fungi are clearly delimited. The conidial state A of H.R and H.W is almost identical to conidial state A of Cordyceps aphodii. To the author's knowledge

the description of conidial state A in this thesis is the first record of its presence in New Zealand and the only report of its existence, apart from the occurrence on C. aphodii. In this study, however, the three conidial states A of C. aphodii, H.W and H.R were treated as being conspecific, as the questions of affinities and species delimitation were outside the realms of this investigation. It is of interest to note that Dingley doubts the existence of a well differentiated sporophore in conidial state A (in conversation) and suggests that the 'conidia' may in fact be arthrospores formed by fragmentation of the constituent hyphae of the conidiophore. If this is so, under Hughes' (1953) scheme of classification, the conidial states A of C. aphodii, H.R and H.W belong in section VII, characterized by arthrospore production.

It is fortunate that of the very few Cordyceps studied in artificial culture Mathieson's observations on C. aphodii are some of the most detailed and comprehensive. Although she was unable to produce phialides, or phialospores of the hymenostilbe conidial state in culture she established beyond reasonable doubt the connection between the two conidial states and the perfect stage. This has only been achieved in very few species of Cordyceps and usually in the remaining 200 odd members of this genus, proof of association rests merely on the simultaneous production of perfect and conidial states on the same stroma, or arising from the same endosclerotium (Kobayasi 1941). In both H.R and H.W experimental proof has been obtained of a connection between the two conidial states (Chapter 3). Apart from Mathieson's description of C. aphodii this represents the

first record of an alternative conidial state in the form-genus Hymenostilbe.

Thus as H.R and H.W are clearly separable from the unnamed Hymenostilbe conidial state of C.aphodii, and are delimited from all other species in the genus Hymenostilbe on phialide and phialospore features, the possession of an alternative conidial state, and by the habit of host specificity on the immature stages of the endemic weevil C.setigera, it is proposed that both H.R and H.W can be regarded as new species of the genus Hymenostilbe.

4. Conclusion

A definite need is indicated for a critical re-examination of the stability of characters used in the delimitation of form-genera and form-species in the stilbaceous entomogenous fungi. This could be embodied in future descriptions of new species in which proof of pathogenicity and host range studies should be included. Under the present taxonomic situation both H.R and H.W could be presented as new species of Hymenostilbe pending a possible synonymy of this genus with Akanthomyces. Both H.R and H.W display a close relationship with Cordyceps aphodii and it is suggested that if the ability to form the perfect state has not been entirely lost the two fungi will form perithecia characteristic of this genus.

CHAPTER 3

A STUDY OF HYMENOSTILBE R AND HYMENOSTILBE W IN ARTIFICIAL CULTURE

INTRODUCTION

Growth in artificial culture was first achieved for H.R when a colony grew on Lab. PDA from a conidiophore of conidial state A. Subsequent exploratory isolations were made to Lab. PCA and Lab. carrot agar but cultures on all three media failed to sporulate or to produce synnemata, and growth rates were poor. The first isolations of H.W to Lab. PDA, Lab. PCA, and Lab. carrot agar, although similarly slow growing, proved morphologically distinct from colonies of H.R. They produced conidial state A over the colony surface and in addition white synnemata were eventually formed on cultures in Lab. PDA, although such synnemata were sterile. This evidence of cultural diversity, plus an obvious morphological discrimination of H.R and H.W in the field, reinforced the belief that there were two separate fungi attacking larvae of Cecyropa setigera. Accordingly a study of the characteristics of the two fungi in artificial culture was initiated with the following aims.

1. To define a medium, or media, which would support a substantial vegetative growth.

2. To define a medium, or media, on which production of the two conidial states could be achieved.

3. To provide cultural evidence supporting the taxonomic discrimination of two distinct fungi.

4. To demonstrate experimentally for both fungi a connection between the phialospore conidial state and conidial state A.

This chapter contains a sequential series of experiments in which it was attempted to fulfil the aims above.

A. A BRIEF REVIEW OF ENTOMOGENOUS FUNGI IN ARTIFICIAL CULTURE

Although the large majority of entomogenous fungi have never been isolated to artificial culture a few species have been cultured for many decades. This is mainly a reflection of the relative economic potential of the few entomogenous fungi that are virulent to a wide host range. Investigations into the mass production of the green muscardine fungus Metarrhizium anisopliae (Metch.) Sorokin were initiated as early as 1880 when Metchnikoff in Russia (cited Martignoni, 1964) employed a semi-liquid beer mash medium to produce large quantities of spores for field dissemination. The success of this method stimulated experimental studies into the large scale production of other virulent entomogenous fungi and by the 1920's this had been achieved for several species, most notably Aschersonia spp. and the white muscardine fungus Beauvaria bassiana (Bals.) Viull. In these studies, complex natural formulations were used as growth media. They incorporated such raw materials as cornmeal mush, sweet potato, oats, bran, rice, sawdust, dog dung, swordfish and herrings (Martignoni 1964). With the exception of Sawyer (1929), (who studied the growth of two species of Entomophthora on over 40 different media), the investigators of this period made little effort to examine critically the nutritional requirements of these fungi.

Since this early period of mass culture there have been relatively few studies on the nutrition and growth of entomogenous fungi in artificial culture. Most studies involve cultures on solid media of conventional formulations and

relate to taxonomic problems, e.g. MacLeod (1954a), Brown and Smith (1957), or to specific studies of physiology and morphogenesis, e.g. Shanor (1936), Basith and Madelin (1968). Recently the reawakened interest in biological control has again stimulated investigations into the mass production of the classical entomogenous fungi, Entomophthora spp., Beauvaria bassiana, and Metarrhizium anisopliae, and these fungi have been recently studied in some detail using modern techniques, e.g. Wolf (1951), Huber (1958), Schaerffenberg (1964), Samsinakova (1966). However the vast majority of entomogenous fungi still remain uncultured.

MacLeod (1959a) attributed this lack, in part, to difficulties in establishing and maintaining these fungi in pure cultures. He further speculated that as a large number of entomogenous fungi are apparently host specific the failure of some of them to grow in culture may be due to the absence of essential nutrients only available from their natural hosts. In order to obtain precise data on this subject he initiated an investigation into the nutrition, metabolism and sporulation of the host specific, stilbaceous, entomogenous fungus Hirsutella gigantea. In a series of experiments employing modern quantitative techniques of shake culture, MacLeod (1959a, b, 1960) determined the amino acid nutrition of the fungus and later Lougheed (1961, 1963), using solid media, determined the conditions necessary for the production of synnemata, and later described their morphogenesis. These experiments represent one of the most comprehensive studies of the nutrition and physiology of any entomogenous fungus.

It is fortuitous that such an investigation has been carried out on a member of the genus Hirsutella as this genus is probably of close affinity to H.R and H.W (see discussion, Chapter 3). Accordingly, although MacLeod's methods were more sophisticated, his experiments were borne in mind during this present study.

B. JUSTIFICATION OF METHODS

The cultural investigations in this study were carried out on solid media. Although the use of solid media in the culture of fungi is a well known technique its extension to nutritional and physiological studies is open to criticism (Cochrane 1958, Lilly 1965). The objections revolve around two major points: firstly, cultures on solid media are heterogeneous; secondly, there are generally inadequacies in the measurement of growth rates on solid media.

Most critical studies on the nutrition and physiology of fungi take place in a homogenous shaken liquid culture. Such a method enables the precise definition of media constituents and ensures a uniform aeration and dispersal of nutrients to all cells of the inoculum. In solid culture all media constituents cannot be easily defined, since agar is a complex polysaccharide that has been shown to contain small amounts of trace elements and organic compounds (Lilly 1965). Furthermore Cochrane points out that on solid media the cells of the developing culture are of two types and are exposed to two different environments, namely, submerged mycelium in anaerobic conditions and aerial mycelium in aerobic conditions. Thus

under these circumstances it is possible that the growth response of a fungus may not be wholly explainable in terms of the experimental variable. However under the aims and conditions of these experiments it is felt that the approach adopted was both valid and adequate. Firstly adequate controls were employed to assess the effects of agar constituents on the variability of growth. Secondly this is a study designed to assess tne relative effects of varying nutritional treatments on growth and sporulation and thirdly the aims of studying simultaneously growth, sporulation and synnematal production could only be achieved on solid media.

The second objection concerns the difficulties involved in measuring growth rates on solid media. Most fungi on solid media grow in two dimensions, i.e. across the surface of the medium and an increase in colony diameter is generally taken as the measurement of growth. This measurement has often been shown to be inadequate for many purposes and the onus to prove its validity rests on tne experimenter (Cochrane 1958). In H.R and H.W the three dimensional growth form of the cultures precluded diameter as a means of assessing growth rates but enabled instead the adoption of mean dry weight of colonies as a measure of growth. Mean dry weight is generally accepted as being the best parameter for the measurement of growth but it is not generally applicable to cultures on solid media because of difficulties inherent in the removal of agar from fungal mycelium. In this study a method of removing the agar was devised that included immersing the colonies in boiling water. This allowed a satisfactory estimation of tne relative dry weights of colonies on the various media.

A third possible objection concerns the carry-over of nutrients, with the mycelial inoculum, to the experimental media. In order to minimise the amount of nutrient medium carried over, only the smallest discs of mycelium which could be satisfactorily handled were employed.

C. GENERAL PROCEDURE

1. Preparation of Inoculum

Colonies of each variety, grown for at least 20 days on laboratory PDA, were cut from the agar and aseptically transferred to previously sterilized McCartney bottles containing 15 mls distilled water and 15-20 glass beads. One colony only was transferred to each McCartney bottle. Violent agitation of the McCartney bottles fragmented the colony and produced a dense suspension of dispersed mycelium. This suspension was then aseptically pipetted (in 1.5 ml aliquots) on to the surface of agar plates containing 1.5% agar, 1.5% yeast extract and 3% dextrose. The suspension was evenly distributed by gently rotating the plate until the whole agar surface was covered. Following incubation for 12 days at 24°C each strain produced a large number of moist, mound-like colonies of densely aggregated surface mycelium. This mycelium was approximately ¼ mm thick and in some places united to form a continuous layer. The prepared inoculum was stored in a refrigerator at 3°C, and used within a 14 day period.

2. Inoculation

Using a binocular microscope with a graduated eyepiece scale, and a set of fine needles, small measured discs were

cut from the surface mycelium and gently detached from the underlying agar. These mycelial discs were then transferred to the experimental media and placed the same way up on the agar surface. It was found necessary to exercise care in placing the discs gently, but directly on to the agar surface using only the needle tip. If a groove or furrow was made on the medium fragments of mycelium adhering to the needle would, on incubation, produce an outgrowth of mycelium along the line of needle contact. This increased individual variability within the experiment.

The inoculations were carried out in a closed room sterilized with ultra-violet light, and normal aseptic technique was employed.

3. Inoculum Size

The inoculum size for H.R and H.W was different as the two strains produced slightly different growth forms in flooded inoculum preparations. In the case of H.R the colonies were moist, grey/white, and somewhat rubbery. The average colony size was such that a disc of 1.0 mm diameter was found convenient. In the case of H.W the colonies were a pinkish/buff colour crumbly and smaller than H.R. Accordingly a disc diameter of 0.75 mm was used.

4. Sterilization of Media

All media were sterilized by autoclaving for 15 minutes at 15 lbs pressure.

5. Media Constituents

All media constituents are expressed percentage concentrations (W/V). Commercial media were prepared according to instructions

on the labels while the composition of laboratory media is given in Appendix 2.

6. Method of Assessing Growth

Oven dry weight was employed as the measure of growth. The method took advantage of the densely aggregated growth form of the colonies. At the end of the incubation period individual colonies were cut free from the medium as discrete lumps bearing only a minimum of adhering agar. (A scalpel with a laterally curved blade proved ideal for this purpose.) Each colony was then transferred to individual test tubes one third filled with water. The agar remaining on the colonies was melted by immersing these test tubes in a boiling water bath until the water in the test tubes had boiled for at least five minutes. The colonies were then collected on filter paper in a Buchner funnel by vacuum filtration. Care was taken to prevent the water in the test tubes from cooling to the solidification point of agar. After filtration colonies were transferred to lidless two inch diameter glass petri dishes and dried for 24 hours at 90°C. The petri dishes containing the colonies were then transferred to desiccators[1] and individual colonies weighed as soon as possible on a Sartorius balance (model 2600) with an accuracy of 1×10^{-5} gm. With few exceptions each colony remained a single lump during the entire cutting, boiling and drying procedure and there was negligible loss of mycelium.

7. Other Features Recorded

Prior to the above process certain morphological features

1/ A preliminary experiment had established that during a 48 hour period water resorption was prevented by the use of desiccators prior to weighing.

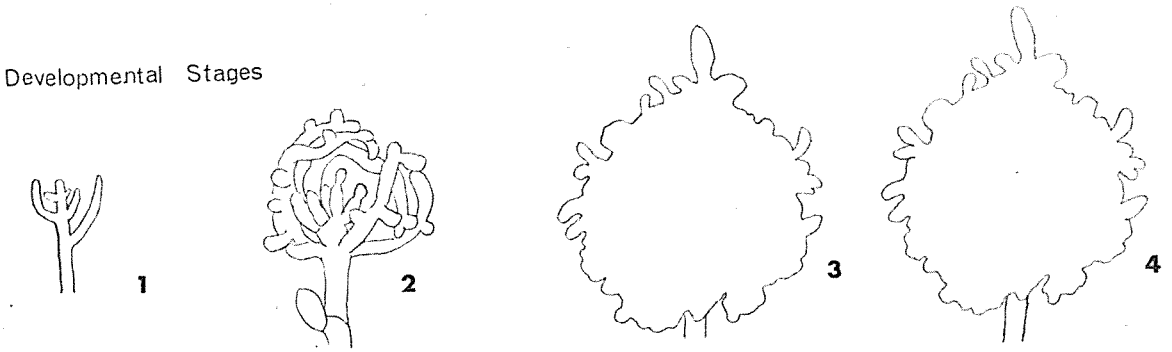
of the colonies were recorded. Such features are presented in the results where pertinent.

(a) Relative production of conidiophores of conidial state A

This was assessed in three ways:

(i) Percentage area of the colony surface covered by conidial state A.

(ii) The developmental stages present on a colony; the development of a conidiophore was divided into four phases and a record made of the number of stages present on the colony surface.



after Mathieson (

(iii) Dominant stage of conidiophore maturity; this was recorded by placing the relevant developmental stage in parenthesis e.g. 1,2,(3),4 - all four developmental stages were present on the colony surface but the majority of the conidiophores were in developmental stage three.

(b) Sporulation index

In order to present the relative sporulation of conidial state A in a condensed yet significant manner, a sporulation index was prepared. The index was constructed by multiplying the dominant stage of conidiophore maturity by the percentage area of the colony covered by conidial state A as a whole. This index reflects the relative suitability of various media

for early initiation and rapid growth to maturity of conidial state A.

(c) Relative density and length of aerial mycelium

Density and length were divided into five categories with the density and length on lab. PDA taken as three in both H.R and H.W.

(d) Colony shape

Rough profile sketches were made and trends in colony shape noted.

(e) Colour of aerial mycelium and/or colour of colony mycelium

Colony mycelium was defined as that mycelium comprising the vegetative mass of the colony, being densely aggregated, moist, and composed of fine hyphae with some gemmae. Aerial mycelium was the loose mat of ramifying hyphae overlying the colony mycelium. All colours were assessed under an Olympus stereoscopic microscope, with 20 times oculars and 0.7 times magnification. An Olympus epi-illuminator was used as a single light source against a white stage background. Colours were designated a colour code from the Ostwald Colour Album (1931).

(f) Length and number of synnemata

Synnemata were measured using an Olympus stereoscopic microscope with an eyepiece scale.

D. EXPERIMENTS

EXPERIMENT I: Growth, sporulation and morphology of H.R and H.W at 20 days on various media

This first experiment was concerned with a survey of available solid media to investigate their influence on growth, sporulation and morphology of H.R and H.W. A wide

selection of media was employed, containing varying amounts and types of organic nitrogen and carbohydrates. In addition Oxoid Czapek Dox agar containing only an inorganic nitrogen source, and water agar, containing neither inorganic nor organic nitrogen were employed.

Materials and Methods

Twenty one media were prepared in 100 ml lots, and sterilized in the autoclave. Each lot was poured into five disposable plastic petri dishes. Two inoculations of H.R and two of H.W were made in each plate. The compositions of laboratory prepared media are presented in Appendix 2. Results were recorded following 20 days' incubation at 24°C. Contaminated cultures were discarded.

Results

Tables 14 and 15 summarise the morphological characteristics and the mean dry weights of H.R and H.W on the 21 media.

(a) Colony growth

Dry weights (mg) of the colonies grown on the different media are presented and analysed as a single factor analysis of variance in Appendix 2 (Tables 1 and 2).

Hymenostilbe R

(i) Lab. PDA supported maximal vegetative growth ($P<0.01$).

(ii) Difco Sabouraud Dextrose agar and Difco Brain Heart Infusion agar ranked second and third as media supporting growth but did not produce yields significantly different ($P<0.05$) from each other.

TABLE 14

Morphological Characteristics and Mean Dry Weight of Hymenostilbe R at 20 days on Various Media

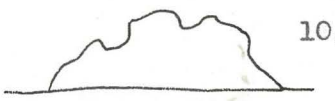
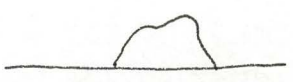
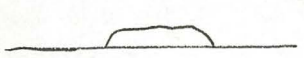
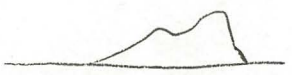
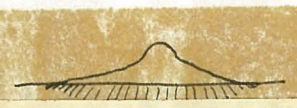
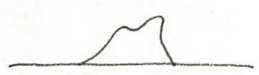
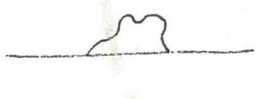
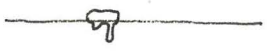
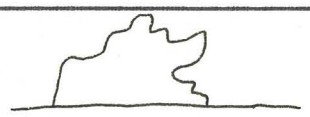
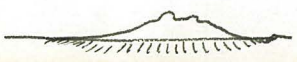
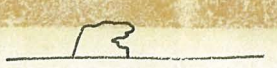
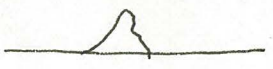
Medium	pH	Colour of Colony Mycelium (c.m.) and Aerial Mycelium (a.m.)	Aerial Mycelium Length	Aerial Mycelium Density	Conidial State A Develop.	Conidial State A % Area	Colony Profile	Colony Mean Diameter (mm)	Colony Mean Dry Weight (mg)
Lab. PDA	5.8	Patchy light pink (5ea) a.m. amongst hyaline a.m.	3	3	-	-		10.2	21.93
Difco Sabouraud Dextrose Agar	5.6	Hyaline a.m. around orange (4-5ia) central a.m.	4	5	-	-		6.3	7.69
Difco Brain Heart Infusion Agar	7.4	Moist grey c.m.; sparse covering of hyaline a.m. in centre	5	2	-	-		8.6	6.65
Lab. Carrot Agar	6.5	Hyaline a.m. around mixed green buff (3ne)/orange buff central a.m.	3	4	-	-		5.2	4.56
Difco Mycobiotic Agar	6.5	Overall covering of hyaline a.m.	4	5	-	-		5.1	4.09
Difco Sabouraud Maltose Agar	5.6	Hyaline a.m. around central buff (3ea) a.m.	3	3	-	-		5.9	3.84
Difco Beef Lactose Agar	6.8	Dense hyaline a.m. on upper colony. Lower colony naked, grey c.m.	4	2	-	-		6.4	2.62
Lab. Cornmeal Agar	5.4	Wide circle of submerged white hyphae; central colony raised buff (4ea) a.m.	3	2	-	-		7.1	2.37
Difco PDA	5.6	Hyaline a.m. around central brown a.m. (4le)	3	3	-	-		4.9	2.21
Lab. PCA	6.3	Submerged hyphae around ring of hyaline a.m., around central brown (4lg) a.m.	3	2	-	-		6.4	2.21
Oxoid Plate Count Agar (T.G.Y.A.)	7.0	Central a.m. brick red (6ia). Sparse hyaline a.m. on rim of grey, moist, c.m.	3	2	-	-		5.8	2.14
Oxoid Milk Agar	7.2	Mixed hyaline & pink/buff (4ea) a.m.	4	4	-	-		4.8	1.69
Oxoid PDA	5.6	c.m. khaki/brown (3pi) a.m. hyaline, overall	1	3	-	-		4.0	1.55
Oxoid Tryptone Soya Agar	7.3	c.m. buff (3ea). Straggling hyaline a.m. on upper colony	5	2	-	-		5.1	1.44
Difco Malt Agar	5.5	Narrow ring of hyaline a.m. around central brown (4le) c.m.	2	3	-	-		4.0	1.40
Difco Prune Agar	5.6	Ring of submerged hyphae around central dark brown/ buff (3ie) c.m.	2	3	-	-		3.4	0.82
Oxoid Nutrient Agar	7.4	Moist grey c.m. Straggly hyaline a.m. on upper colony only	5	2	-	-		3.2	0.50
Oxoid Czapek Dox Agar	6.8	Yellowish submerged hyphae around moist brown (4pg) c.m.	1	1	-	-		4.2	0.36
Oxoid Tomato Juice Agar	6.1	a.m. patchy hyaline. c.m. dark red brown (6pi)	3	2	-	-		1.9	0.25
Lab. Starch Agar	5.2	Pinkish ring of submerged hyphae around light brown (5ie) c.m.	1	1	-	-		1.8	0.20
Lab. Water Agar	5.8	c.m. light buff (3ic). Synnematal-like, red (7pe) 'taproots' growing into agar	0	0	-	-		1.2	0.05

TABLE 15

Morphological Characteristics and Mean Dry Weight of Hymenostilbe W at 20 days on Various Media

Medium	pH	Colour of Colony Mycelium (c.m.) and Aerial Mycelium (a.m.)	Aerial Mycelium Length	Aerial Mycelium Density	Conidial State A Develop.	Conidial State A % Area	Colony Profile	Colony Mean Diameter (mm)	Colony Mean Dry Weight (mg)
Lab. PDA	5.8	c.m. light pink/red brown (6 & 7ie) a.m. tufty	3	3	1,2,(3),(4)	50%		5.8	6.74
Lab. Carrot Agar	6.5	c.m. light pink/buff (4gc) Patchy a.m.	2	2	1,2,(3)	40%		6.4	4.32
Difco Sabouraud Maltose Agar	5.6	c.m. light grey/buff (between 2 & 3ea) Darker in upper central portion (3gc)			1,2,(3),4	15%		5.9	3.56
Difco Sabouraud Dextrose Agar	5.6	c.m. deep orange/buff (between 4ia & 4ga)	4	5	-	-		4.9	3.28
Difco PDA	5.6	c.m. pink (6ie)	2	4	1,2,(3),4	40%		4.4	2.92
Difco Brain Heart Infusion Agar	7.4	c.m. very light pink/off white, moist. Virtually no a.m.	1	1	-	-		6.2	2.73
Difco Mycobiotic Agar	6.5	c.m. pink/buff (5ga), moist	2	2	-	-		4.6	2.58
Oxoid Tomato Juice Agar	6.1	c.m. yellow/buff (3ga), moist. a.m. tufty	2	1	-	-		5.0	1.64
Oxoid PDA	5.6	c.m. khaki/buff brown (3pi)	1	3	1,(2)	10%		3.7	1.48
Lab. Cornmeal Agar	5.4	Circle of white, submerged mycelium with central raised portion. Covered with buff (4ea) a.m.	1	2	1,2,(3),(4)	25%		4.0	1.35
Difco Malt Agar	5.5	c.m. buff/pink (between 5 & 6ie)	3	4	-	-		3.8	0.97
Difco Beef Lactose Agar	6.8	c.m. light pink/buff (4ea) Patchy a.m.	2	2	-	-		3.3	0.64
Oxoid Milk Agar	7.2	c.m. grey/buff (3gc) Straggly a.m.	5	2	-	-		3.9	0.62
Oxoid Tryptone Soya Agar	7.3	c.m. grey/buff (3gc) Straggly a.m.	4	2	-	-		2.9	0.45
Oxoid Plate Count Agar (T.G.Y.E.)	7.0	c.m. very dark brown (5pn) a.m. very sparse	1	1	-	-		2.6	0.44
Lab. PCA	6.3	c.m. dark brown (between 5pl & 6pl) where visible beneath conid. state A (3ga)	1	1	1,2,3,(4)	95%		1.8	0.37
Oxoid Nutrient Agar	7.4	c.m. grey/light buff (between 4ea & 4ca)	1	1	-	-		2.6	0.35
Difco Prune Agar	5.6	c.m. dark red/brown (between 6pi & 6ng)	1	2	-	-		1.8	0.24
Oxoid Czapek Dox Agar	6.8	c.m. buff/grey (3gc)	1	2	1,(2),(3),4	70%		1.3	0.12
Lab. Starch Agar	5.2	c.m. very dark brown (5pl) Surrounded ring of yellow exudate (3pc)	1	1	(1)	5%		1.0	0.06
Lab. Water Agar	5.8	c.m. light buff (3ic)	1	1	1,2,(3),4	90%		1.0	0.02

Hymenostilbe W

(i) Lab. PDA supported maximal vegetative growth ($P<0.05$).

(ii) There was no significant difference in colony mean dry weight between lab. carrot, Difco Sabouraud Maltose, Difco Sabouraud Dextrose, Difco Brain Heart Infusion and Difco Mycobiotic agars. These media ranked (in order) second to fifth in their ability to support vegetative growth.

(b) Relative production of conidial state A

Hymenostilbe R

Conidial state A did not appear on colonies of H.R during the 20 day incubation period.

Hymenostilbe W

Conidial state A was present at 20 days on colonies grown on 10 different media. Details of relative area covered, and stages of maturity present, and dominant, can be read from Table 15. The sporulation indices and the mean dry weights of colonies on the 10 media producing conidial state A are given in Table 16 below.

TABLE 16 Relative production of conidial state A by H.W on ten media

Medium	Sporulation Index	Colony mean dry weight (mg)
Lab. PCA	380	0.37
Lab. Water	270	0.02
Oxoid Czapek-Dox	175	0.12
Lab. PDA	175	6.74
Lab. Carrot	120	4.32
Difco PDA	120	2.92
Lab. Cornmeal	88	1.35
Difco Sabouraud Maltose	45	3.56
Oxoid PDA	20	2.92
Lab. Starch	5	0.06

(c) Colony morphology (Fig. 13)

(i) A general description of colony development and morphology on lab. PDA is given below.

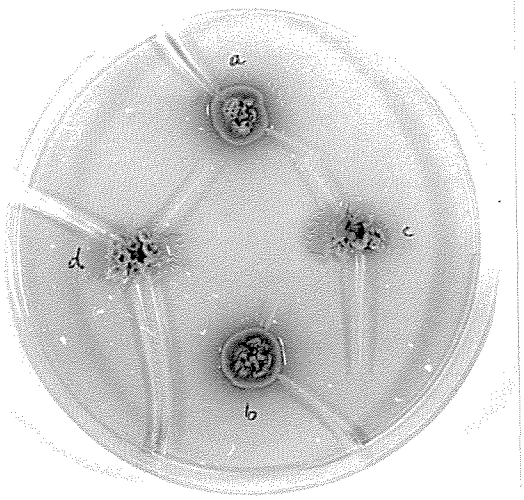
Hymenostilbe R

The colony first developed from the inoculum disc, at two to four days, as a mound of moist grey mycelium, often with a pinkish tinge. Growth continued both vertically and laterally and a narrow ring of submerged assimilative hyphae became apparent as it grew down into the agar. On the upper surface of the colony a covering of ramifying aerial hyphae appeared. At 20 days the colony consisted of a diffuse base of fine assimilative hyphae (1 - 5 u wide), a mound of fine, densely packed hyphae interspersed with swollen hyphae and occasional gemmae, and finally a mat of ramifying aerial hyphae. The vegetative mound was a greyish pink colour and the aerial hyphae were pigmented pink (5ea) in central patches, but were generally hyaline with a translucent white appearance. The whole colony formed a tough, leathery, irregular mound.

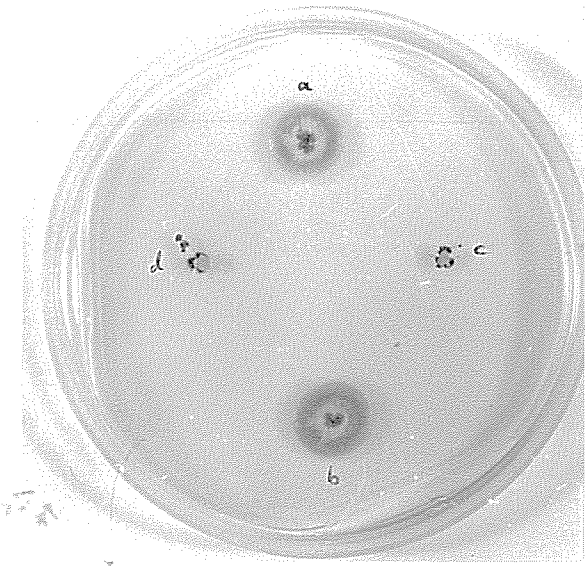
Hymenostilbe W

Overall development was similar to H.R but some differences were apparent. The first moundlike growth at two to four days was usually a dark red/brown colour, although occasionally a pinkish/grey. The colonies were fragile at 20 days, in the sense that mycelial segments were readily broken off with a needle (a feat difficult with the tougner H.R colonies). At 20 days the colonies of H.W were greatly convoluted while aerial hyphae were short and not dense enough

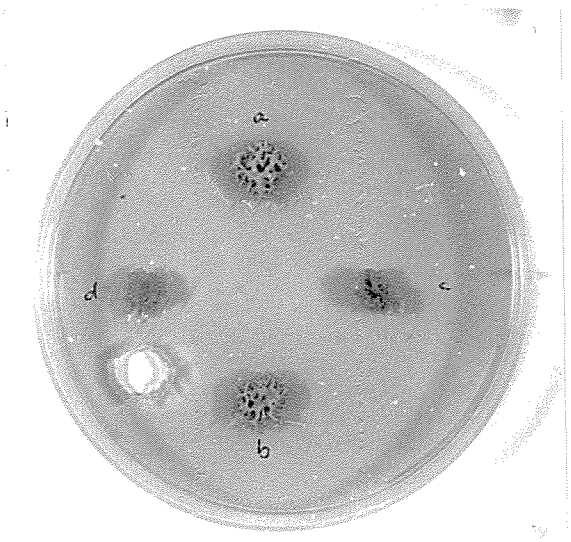
Figure 13



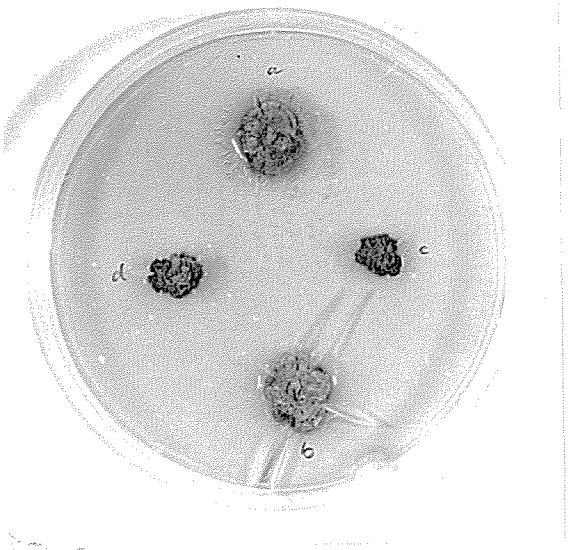
Difco Sabouraud Maltose Agar



Difco Brain Heart Infusion Agar



Lab. PCA



Lab. PDA

Cultural characters of Hymenostilbe R and Hymenostilbe W on various media at 20 days (a & b H.R, c & d H.W)

to obscure the red/brown to pink mycelium of the vegetative mound below. After eight to twelve days conidial state A formed amongst the aerial hyphae on the upper central parts of the colony and matured to a buff yellow shade (3ga).

(ii) For both H.R and H.W a general trend was noted for the colour of the vegetative mound to increase in intensity on media supporting progressively lower rates of growth. Particular attention is drawn to the features of H.R and H.W on lab. starch agar. On this medium H.W produced a yellow ring in the agar surrounding the dark brown colony. H.R did not produce such a ring in the agar while the colony mycelium was a light brown.

(iii) For both H.R and H.W a general tendency was noted (see colony profile columns - Tables 14 & 15) for colonies on media supporting lower rates of growth to produce a greater proportion of submerged assimilative hyphae.

(iv) The growth of H.R on water agar is worthy of note. On this medium H.R showed no evidence of an increase in diameter or in thickness of the mycelial disc due to growth, but did produce red 'taproots' growing down into the agar. In appearance these were very like the synnemata produced from endosclerotia.

Discussion

From an examination of media constituents it was apparent that growth of H.R and H.W was enhanced on media rich in both organic nitrogen and dextrose. On media containing low concentrations of these nutrients growth was suppressed. In the complete absence of organic nitrogen and dextrose (water agar) growth was almost negligible and could be explained by a

carry-over of nutrients with the original inoculum. Similarly growth on an inorganic nitrogen source (the sucrose-nitrate Czapek-dox agar) was also strictly confined, and it is suggested that neither H.R nor H.W can utilize inorganic nitrogen. (The seven-fold increase in dry weight of both H.R and H.W on Czapek-dox agar over that on water agar is probably the result of the additional sucrose in this medium supplementing nutrients contained in the inoculum.) The inability of H.R and H.W to grow on an inorganic nitrogen source was not unexpected since MacLeod (1954b) found a similar situation in Hirsutella gigantea and speculated that other host specific entomogenous fungi would probably exhibit such a characteristic.

Although conidial state A was not produced on colonies of H.R it was apparent that there was generally an inverse relationship between vegetative growth and sporulation in H.W. This is in line with the factors generally taken to control the initiation of sporulation of fungi in general, that is, a switch from a primarily vegetative phase of growth to a sporulating phase that is initiated by an exhaustion of nutrients (Cochrane 1958). The occurrence of conidial state A on lab. PDA, which supported a substantial growth, does not invalidate this idea since it is possible that growth restriction was taking place in these colonies due to some factor such as an accumulation of metabolites in the medium.

Morphologically the colonies of H.R especially, and H.W to a lesser extent, were most similar to colonies of Cordyceps aphodii on lab. PDA as described by Mathieson (1949). This fact, together with the production of conidial state A from colonies of H.W, further suggests a close link between these fungi.

Conclusion

Although this media survey provided valuable descriptions of colony morphology on various media it did not realize the production of synnemata or conidial state A for H.R, while growth of both H.R and H.W was not improved over that of lab. PDA. The similarity of the general nutritional requirements of both H.R and H.W to those of H. gigantea served as a stimulus for further experiments into their nutrition and physiology.

EXPERIMENT II: Growth and sporulation of H.R and H.W on a basal dextrose medium (1%) containing various natural supplements

Introduction

In an effort to resolve the shortcomings of the previous experiment it was decided to undertake a more comprehensive study of the nutritional requirements of H.R and H.W. The results of the previous experiment indicated the importance of organic nitrogen and dextrose in the nutrition of these fungi. Accordingly eight natural supplements were tested on a basal dextrose medium to ascertain the best source of organic nitrogen and/or growth factors for vegetative growth and sporulation of the two fungi.

Materials and Methods

Eight natural supplements at a concentration of 1.5% were added to a medium of 1% dextrose[1] and 1.5% agar[2]. The medium was prepared in 110 cc lots in 250 ml flasks as set out in General Procedure. Five plates were poured for each medium

1/ May & Baker Ltd., Dagenham, England.

2/ Davis Gelatine N.Z. Ltd., Christchurch.

and two inoculations of H.R and two of H.W were made in each petri dish. Hydrogen ion concentration was recorded but not controlled. Plates were incubated for sixteen days at 24°C.

Results

(a) Colony growth

Analyses of variance on the original data are presented in Appendix 2 (Tables 3 and 4). The pH of the various media and the mean dry weights of both H.R and H.W are presented below.

TABLE 17 The pH of media and the colony mean dry weights of H.R and H.W at sixteen days

Supplement	Colony mean dry weight (mg) H.R	H.W	pH
Difco Yeast Extract	12.35	8.55	6.9
Difco Bacto Tryptone	8.21	5.70	6.9
Oxoid Mycological Peptone	6.11	2.64	5.2
Difco Proteose Peptone	5.68	2.88	6.8
Oxoid Soya Peptone	4.21	1.98	5.9
Oxoid Liver Broth	3.94	3.10	6.2
Difco Bacto Peptone	3.40	0.49	6.9
Difco Neopeptone	1.75	0.74	6.8

For both H.R and H.W Difco Yeast Extract was superior ($P<0.05$) to all other natural supplements tested as a source of organic nitrogen and/or growth factors. For both H.R and H.W Difco Bacto Tryptone ranked second as a medium for growth.

(b) Relative production of conidial state A

(i) Conidial state A did not develop on colonies of H.R.

(ii) Colonies of H.W produced conidial state A on two media, namely, Difco Yeast Extract (stages 1,2,3 and 40% surface area) and Oxoid Liver Broth (stages 1 and 2 and 15% surface area).

Discussion

The superiority of yeast extract for growth and sporulation was not unexpected as it is commonly known to be a growth stimulant and is often added to various mediaas a source of readily available amino acids and B vitamins (Oxoid and Difco manuals). In addition MacLeod (1959a) found that yeast extract and tryptone produced a superior growth in Hirsutella gigantea when compared to other sources of organic nitrogen, and MacLeod used the former nutrient as a source of organic nitrogen in his early studies on the nutrition of this fungus in shake culture.

Sporulation of conidial state A in H.W occurred only on yeast extract and liver broth, both recognized as potent sources of growth factors. This would suggest that a specific nutrient may be involved in initiation of sporulation. However Hawker (1966) maintained that general evidence pointed to the fact that, providing a sufficient concentration of nutrients was available, sporulation would take place on any nitrogen source that supported vegetative growth. In view of the limited incubation period the present evidence for H.R and H.W cannot be regarded as being contrary to that hypothesis.

Conclusion

This experiment succeeded in indicating a superior source of organic nitrogen for growth of both fungi and for sporulation

in H.W but it failed to induce sporulation in H.R. At this stage it was thought that this suppression may have been due to the concentration of nutrients used or to environmental factors such as hydrogen ion concentration or temperature.

EXPERIMENT III: Growth, sporulation and morphology of H.R and H.W on a dextrose (1%) - yeast extract (3%) medium at varying hydrogen ion concentrations

Introduction

This experiment was initiated with the aims of further defining optimum cultural conditions for H.R and H.W and in the hope that sporulation of H.R would be induced.

The effects of hydrogen ion concentration on growth and sporulation of entomogenous fungi receive scant reference in the literature. Müller-Kögler (1965) noted this lack of knowledge and expressed the need for obtaining such information, particularly as it relates to the growth and sporulation of host specific entomogenous fungi. MacLeod (1959a) in his studies on H. gigantea merely noted that H. gigantea grew best in a liquid medium at pH six, but did not further explain how he arrived at this conclusion.

The effect of hydrogen ion concentration on fungi in general is however well documented, and has been the subject of recent reviews by Cochrane (1958), Lilly (1965), and Hawker (1966). Generally most fungi grow within the range pH 4 - 8. All three authors stress the importance of noting that the mechanism of pH action on fungal growth varies at different hydrogen ion concentrations. Cochrane points out that one part of the pH curve may reflect the effect of a low pH on enzyme

activity say, while another part of the curve may affect metal solubility or the entry into the cell of organic acids or vitamins. This complexity of action is further compounded in artificial culture where media constituents may also vary with pH, e.g. weakly ionized constituents may dissociate and themselves affect growth of the fungus. The interpretation of results should therefore be treated with caution.

Materials and Methods

The buffer used was a disodium hydrogen phosphate/citric acid buffer adjusted to pH 6.0 (252.6 mls of 0.2M $Na_2 HPO_4$ - 146.4 mls of 0.1M citric acid - Dawson et al, 1959). The pH of the media was adjusted by the addition of 1N HCl or 1N NaOH. A Beckman glass electrode pH meter was used to determine hydrogen ion concentrations.

A standard medium (120 mls of 3% Difco Yeast Extract, 1% dextrose and 1.5% agar) was added to fourteen 250 ml flasks. A quantity of buffer (6 mls) was added to thirteen of the flasks following which all flasks, together with 100 mls of 1N HCl and 100 mls of 1N NaOH were autoclaved for 15 minutes at 15 lbs pressure. The pH was not stabilized before autoclaving since the exposure of acid media to high temperatures causes acid hydrolysis of media constituents and may completely destroy the gelling properties of the agar (Difco and Oxoid Manuals). Following sterilization the media were held in a water bath at 70°C. This high holding temperature prevented solidification of the media during the process of pH adjustment.

The pH of the media was adjusted by aseptically adding drops of acid or alkali to a 10 ml sample drawn from each

flask, until the required pH had been attained. The sample was then discarded and the number of drops of acid or alkali to be added to the 110 mls of medium was calculated. A control was employed in which neither acid, alkali, nor buffer was added to the medium. The control medium had a pH of 6.8.

Five plates were poured immediately after the pH of each medium was adjusted. Care was taken to leave approximately 10 mls of agar in the bottom of each flask. This was used as a further sample to determine the exact pH of each medium.

Each plate was inoculated with two inoculum discs of H.R and two of H.W and incubated for 20 days at 24°C.

Results

(a) Colony growth

The mean dry weights of H.R and H.W on media at various hydrogen ion concentrations are recorded graphically in Fig. 14 while analyses of variance on the original data are presented in Tables 5 & 6 (Appendix 2).

Hymenostilbe R

(i) With the exception of the control there was no significant difference ($P<0.05$) between yields on media in the range pH 5.5 - 7.4. Yields on media within this pH range were greater than yields on media at other pH values.

(ii) The yield from the control of pH 6.8 was not significantly different ($P<0.05$) than the yield at pH values 5.5, 5.8, 6.35 and 6.9 but was significantly greater than yields on media at all other pH values ($P<0.05$).

(iii) There was no growth at pH 5.0 and below.

Hymenostilbe W

(i) With the exception of the control there was no significant difference ($P<0.05$) between yields on media in the range pH 5.5 - 7.4.

(ii) The yield from the control at pH 6.8 was not significantly different ($P<0.05$) from the yield at pH 5.5 or at pH 7.4, but was significantly different from yields at all other pH values ($P<0.05$).

(iii) There was a significant difference ($P<0.05$) between yields at pH 6.8 (control) and at pH 6.7 and pH 6.9.

(iv) There was a significant difference ($P<0.05$) between yields on media at pH 7.4 and 7.9.

(v) There was no growth at pH 4.0 and below.

(b) Relative sporulation of conidial state A

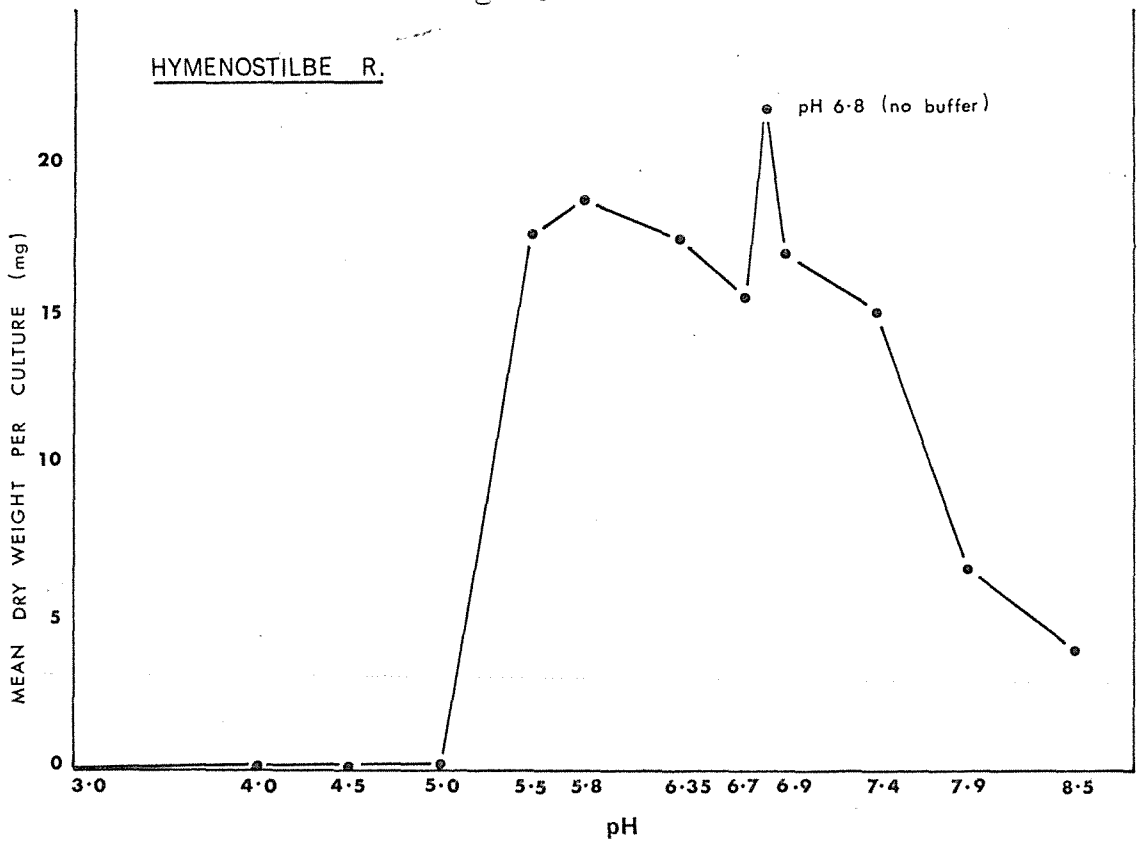
(i) Conidial state A was not produced on colonies of H.R.

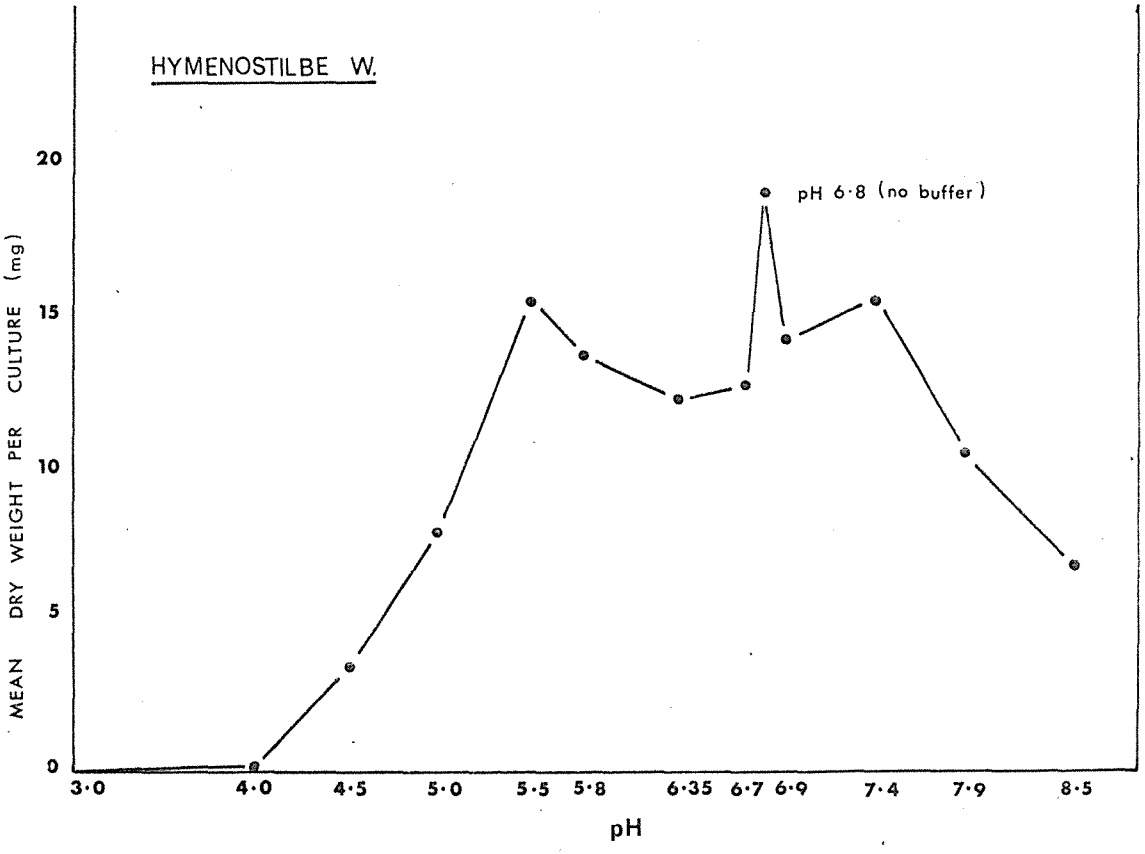
(ii) Conidial state A developed on colonies of H.W on media at pH values ranging from 4.5 to 7.4. The pH values of media and their indices of sporulation are presented below in Table 18.

TABLE 18 The effect of media pH on production of conidial state A

pH	Sporulation Index
4.5	30
5.0	75
5.5	75
5.8	30
6.35	1
6.7	1
6.8 (control)	75
7.4	1

Figure 14





Yield of Hymenostilbe R and Hymenostilbe W at 20 days on a yeast extract (3%) - dextrose (1%) medium at varying hydrogen ion concentrations

Under the conditions of this experiment development of conidial state A was maximal on media at pH values 5.0, 5.5 and 6.8 (control).

(c) Colony morphology (Fig.15)

(i) Shape

On media at hydrogen ion concentrations of up to pH 6.0, H.R colonies were a typical irregular mound. On media above pH 6.0 colonies became increasingly fluted and fragile, until at pH 7.9 and 8.5 the colonies had deeply fluted, irregular, sides and were quite fragile, similar to normal H.W colonies.

The morphology of H.W colonies exhibited a similar response to a changing pH. Normally convoluted colonies with fluted sides developed on media with pH values of up to 6.9. On media at pH 6.9 and above the convolutions grew more open and developed into spaciously scalloped lobes. These colonies were exceptionally fragile.

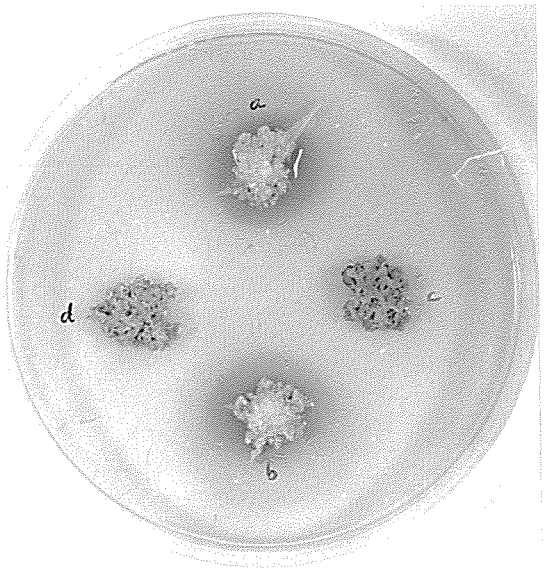
The morphology of H.R and H.W on the control medium was little different from that on media at pH 6.7 and pH 6.9.

Discussion

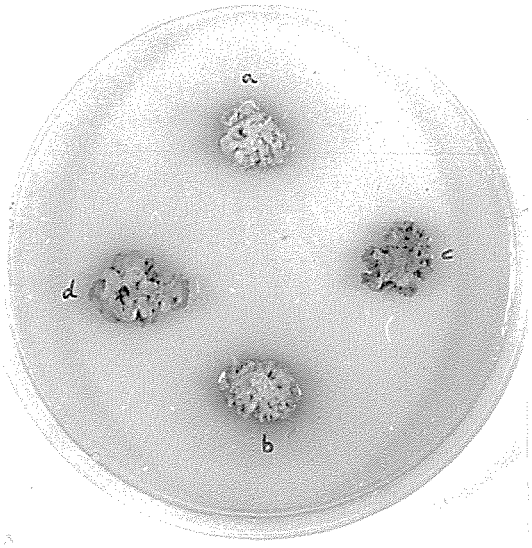
The results indicate that for both H.R and H.W, on a 3% yeast extract and a 1% dextrose solid medium, hydrogen ion concentration has little effect on growth in the range pH 5.5 - 7.4. This encompasses the range of the haemolymph hydrogen ion concentration for larvae of Cecyropa setigera (pH 7.0 to 7.2 - determined with pH papers).

It is also probable that the buffer used had a depressant effect on growth despite the fact that the yield on several pH values (pH 5.5, 5.8, 6.35, and 6.9 in H.R, and pH 5.5, and 7.4

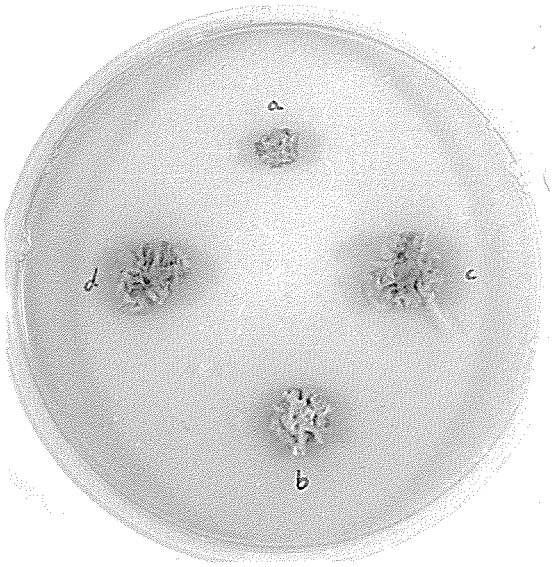
Figure 15



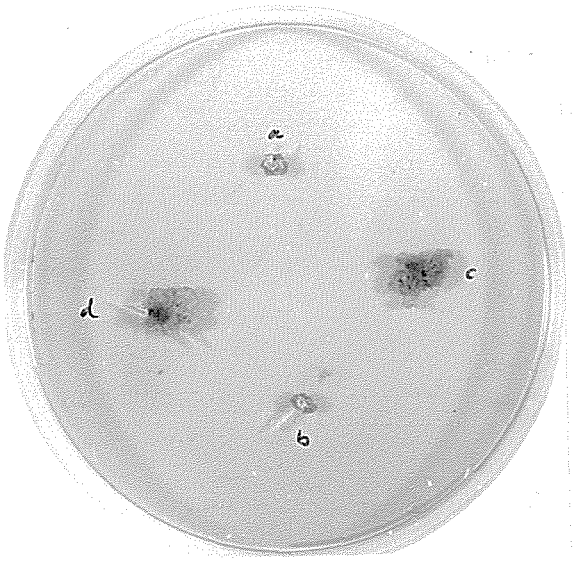
pH 6.9



pH 7.4



pH 7.9



pH 8.5

Cultural characters of Hymenostilbe R and Hymenostilbe W at 20 days on a yeast extract - dextrose medium at varying hydrogen ion concentrations (a & b H.R, c & d H.W)

in H.W) was not significantly different from that of the control (pH 6.8). Against this apparent non-significant difference however it can be argued that as different metabolic functions in the cell are affected differently by a varying pH the yield of the control should most properly be compared with that of pH values immediately adjacent to it. On this basis, although the yield on pH 6.9 in H.R does not quite attain a significant difference ($P<0.05$), yields at pH 6.7 in H.R, and pH 6.7 and 6.9 in H.W are all significantly different from that of the control. This evidence thus more strongly supports the contention that some agent in the buffer depressed growth. This agent was possibly the phosphate ion to which many fungi are sensitive (Cochrane 1958).

Cochrane (1958) and Hawker (1966) also note that the pH range for sporulation is often less than that for growth. Although this also was apparently the situation for the production of conidial state A in H.W little weight can be attached to the present results in view of the inhibitory action of the buffer on both growth and sporulation.

In both fungi a morphological change in colony structure was noted. The reason underlying this phenomenon is unknown.

The occurrence of a double pH optimum (as in H.W) is not uncommon in fungi and has been the subject of detailed study by Fries (1945, 1956 - cited Cochrane 1958). Fries examined this phenomenon in Coprinus spp. and concluded that the central minimum merely reflected the pH dependable unavailability of one or more inorganic elements. It is probable that a similar pH dependent availability is reflected in the curve of H.W although its underlying nature remains a matter for conjecture.

Conclusion

This experiment failed to elucidate the effect of pH on sporulation in H.R and H.W. However, as a substantial growth took place between pH 5.5 and 7.4 it is reasonable to assume that sporulation also takes place between these pH values, although over a narrower range. As sporulation of conidial state A took place on the unadjusted control medium at pH 6.8, it was assumed that this was a suitable pH for sporulation and all other experiments were conducted on media at this hydrogen ion concentration.

EXPERIMENT IV: The growth response of H.R and H.W at 20 days on a yeast extract (3.0%) - dextrose (1%) medium at varying incubation temperatures

Introduction

Temperature is a fundamental feature of the environment and as such affects the basic metabolic processes of the fungal cell. Consequently temperature has a profound influence on the spore germination, growth, and reproduction of all fungi.

A reflection of this profound influence is found in the fact that there is more information on the effects of temperature on the growth and sporulation of entomogenous fungi than on any other single factor of the environment. Müller-Kögler (1965) has compiled a table summarising work on the temperature requirements of entomogenous fungi up to 1964. From this table it is apparent that most entomogenous fungi grow best at a temperature of 20-26°C. However there is once again very little information on the temperature requirements of entomogenous stilbaceous fungi. MacLeod (1959a,b, 1960) carried out his experiments at 23°C

but did not mention any investigations to justify use of this temperature, while similarly Mathieson (1949) used 24°C as an incubation temperature for cultures of Cordyceps aphodii without mentioning experimental vindication.

Most fungi grow over the range 0 - 37°C but sporulation occurs over a narrower temperature range than that required for vegetative growth. The optimum temperature for growth and sporulation is frequently expressed as a 'cardinal point' but several workers have warned that this concept must be treated with caution. The growth of a fungus is the result of an interaction of various enzymatically controlled reactions, each of which may have different optima, and which in turn are themselves affected by other metabolic processes (Deverall 1966). The view of a simple temperature dependence is thus misleading. An example of this is afforded by Fries (1953 - cited Cochrane 1958) who found that the growth of one fungus was poor at high temperatures because of the failure of methionine biosynthesis to keep pace with other processes. Thus Cochrane (1958) states that temperature optima are only valid under specified conditions and that there is no single optimum temperature for growth. Nonetheless, he maintained that mycologists would continue to employ the concept of a cardinal point on the grounds that it was 'empirically useful and that gross differences in apparent optima provide a basis for ecological studies'.

With these comments in mind a study was made of the apparent temperature requirements for growth and sporulation of H.R and H.W.

Materials and Methods

Eight hundred millilitres of a medium containing 3% Difco yeast extract, 1% dextrose, and 1.5% agar was prepared, sterilized and poured into 40 petri dishes. Each petri dish was inoculated with two inoculum discs of H.R and two of H.W. Five inoculated petri dishes were incubated for 20 days at each of the following eight temperatures: 16,20,24,26,28,30,33, and 36°C. Incubators were not available for temperatures below 16°C.

Results

(a) Colony growth

The mean dry weights of H.R and H.W at various temperatures are recorded graphically in Fig. , while analyses of variance on the original data are presented in Appendix 2 (Tables 7 & 8).

Hymenostilbe R

(i) There was no significant difference ($P<0.01$) between yields at incubation temperatures from 24-33°C.

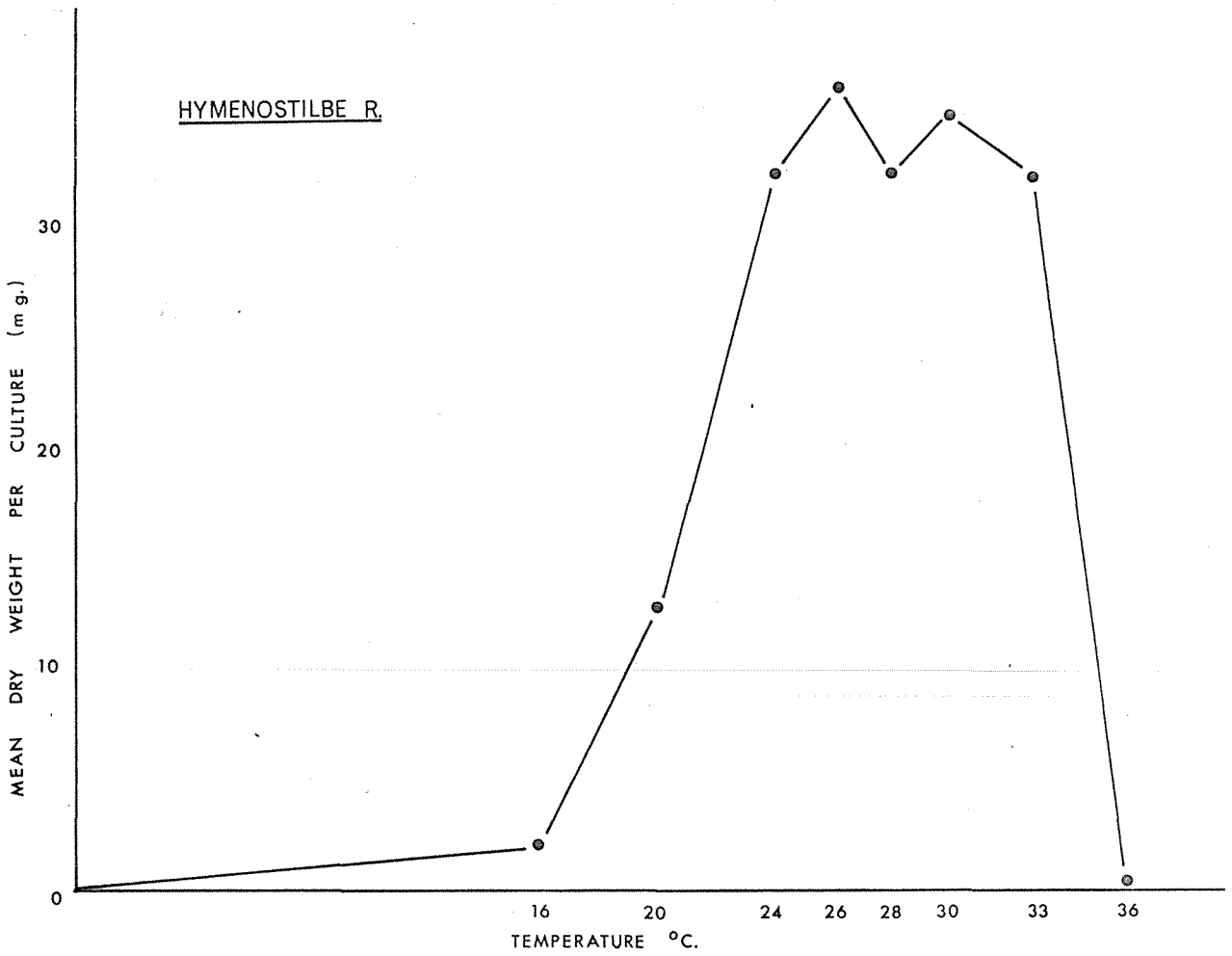
(ii) Yields at 20 and 36°C were significantly below ($P<0.01$) those in the range 24-33°C and the yields at 16°C were less than those at 20°C ($P<0.01$).

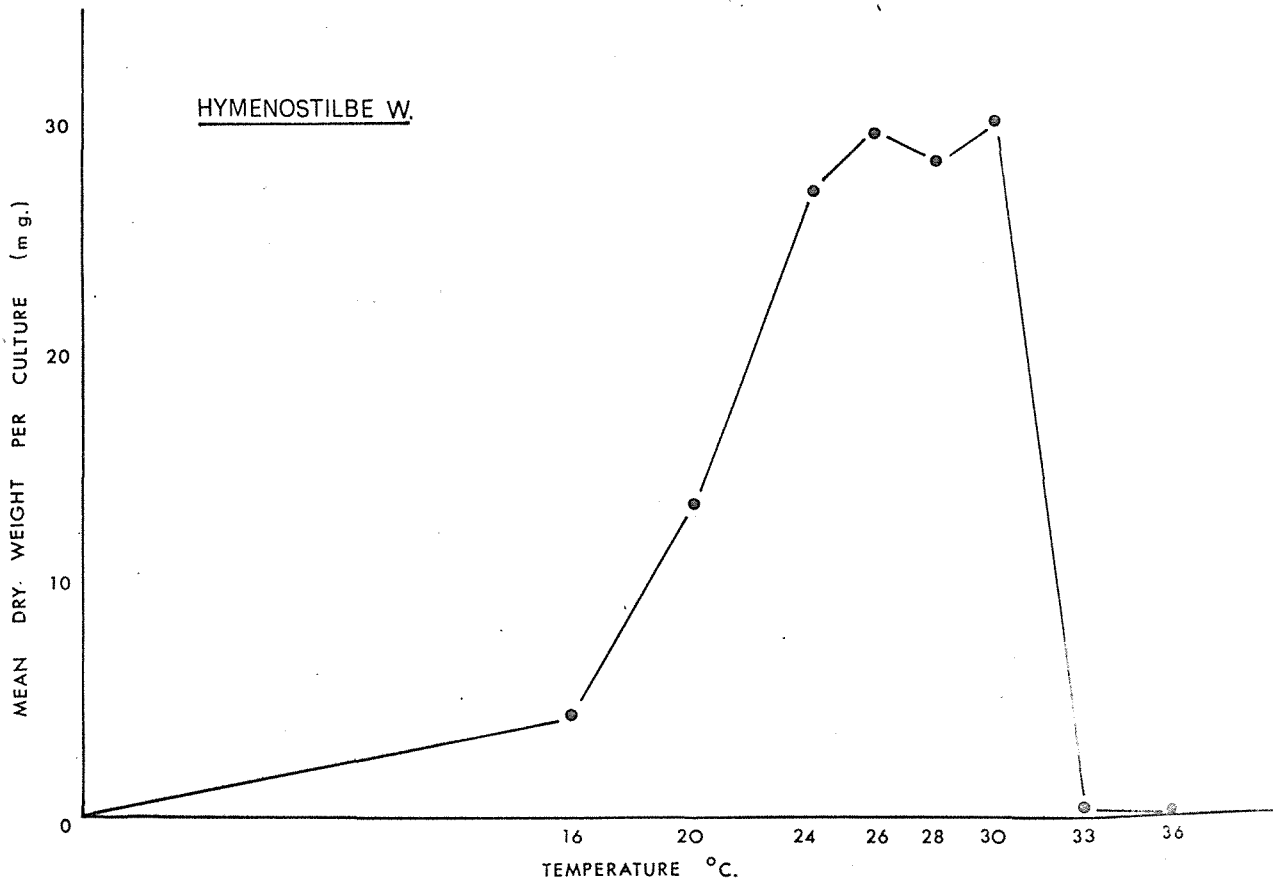
Hymenostilbe W

(i) There was no significant difference ($P<0.01$) between yields at incubation temperatures ranging from 24-30°C.

(ii) Yields at 20, 33 and 36°C were significantly below ($P<0.01$) those in the range 24-30°C and the yield at 16°C was lower than that at 20°C ($P<0.01$).

Figure 16





Yield of Hymenostilbe R and Hymenostilbe W at 20 days on a yeast extract (3%) - dextrose (1%) medium at different incubation temperatures

(b) Relative sporulation of conidial state A

(i) Conidial state A was not produced on colonies of H.R at any of the incubation temperatures tested.

(ii) H.W produced conidial state A at temperatures ranging from 20 to 30°C. The temperatures at which sporulation occurred and the corresponding sporulation indices are given below in Table 19.

TABLE 19 The effect of incubation temperatures on the production of conidial state A

Temperature °C	20	24	26	28	30
Sporulation Index	10	100	63	63	20

(iii) Sporulation was maximal at 24°C.

Discussion

Both H.R and H.W had a similar growth response to varying temperatures. This was characterized by a lag in growth to 16°C followed by a period of more or less linear increase to a broad optimum range and terminated by a sharp decrease to zero growth at temperatures above the maximum. Such a growth curve is typical of the response of most fungi to different incubation temperatures, although the initial lag period at low temperatures is not generally so evident.

H.W is also typical in that conidial state A was produced over a narrower temperature range than that required for vegetative growth.

Conclusion

24°C is a suitable incubation temperature for growth of both fungi and a suitable temperature for sporulation in H.W.

EXPERIMENT V: Growth, sporulation and morphology of H.R and H.W on varying concentrations of yeast extract and dextrose at 20 days

Introduction

The preceding four experiments had established that yeast extract was a superior source of organic nitrogen and/or growth factors. A suitable hydrogen ion concentration and incubation temperature for growth and sporulation had similarly been defined. Accordingly it followed that the next step in formulating optimal media for H.R and H.W was to test the effects of varying concentrations of yeast extract and dextrose on growth and sporulation.

Materials and Methods

The experiment was set out in a 5 x 5 factorial design, and Difco Yeast Extract was added to the media at concentrations of 0, 0.75, 1.5, 3.0 and 6.0%, while dextrose was added at concentrations of 0, 1, 3, 6 and 9%. One hundred and ten millilitres of each nutrient combination were prepared in 250 ml flasks and agar added at 1.5%. The media were stabilized at pH 6.8 by the addition of 1N HCl or 1N NaOH. Media were sterilized, poured and inoculated with two mycelial discs of H.R and two of H.W. Five plates were poured for each nutrient combination. The inoculated plates were randomised within the incubator, and results given are for a random selection of three plates from each of the 25 treatments.

The cultures were incubated for 20 days at 24°C. Contaminated plates were discarded.

(a) Colony Growth

The original data and the analyses of variance for H.R and H.W are set out in Appendix 2 (Tables 9 & 10).

Hymenostilbe R

The mean dry weights (mg) of cultures on the various nutrient combinations after 20 days incubation are presented in Table 20 and depicted graphically in Fig. 17.

TABLE 20 The effect of varying concentrations of yeast extract and dextrose on the colony mean dry weight (mg) of H.R at 20 days

Yeast Extract (%)	Dextrose (%) 0	1	3	6	9
0	0.06	0.63	0.70	0.55	0.27
0.75	1.44	14.03	18.63	17.19	14.41
1.5	3.66	22.22	31.66	29.41	23.67
3.0	7.67	22.49	46.09	49.47	42.97
6.0	16.00	36.83	40.31	37.88	16.57

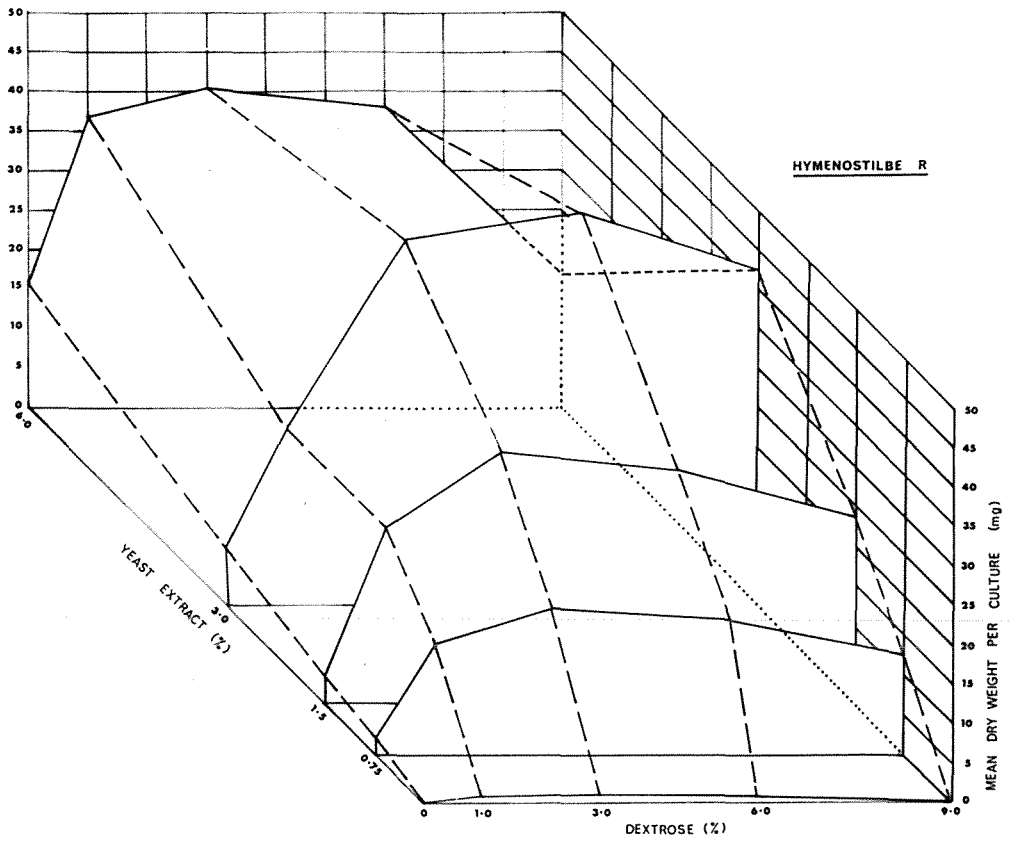
$D_{0.05} = 8.48$

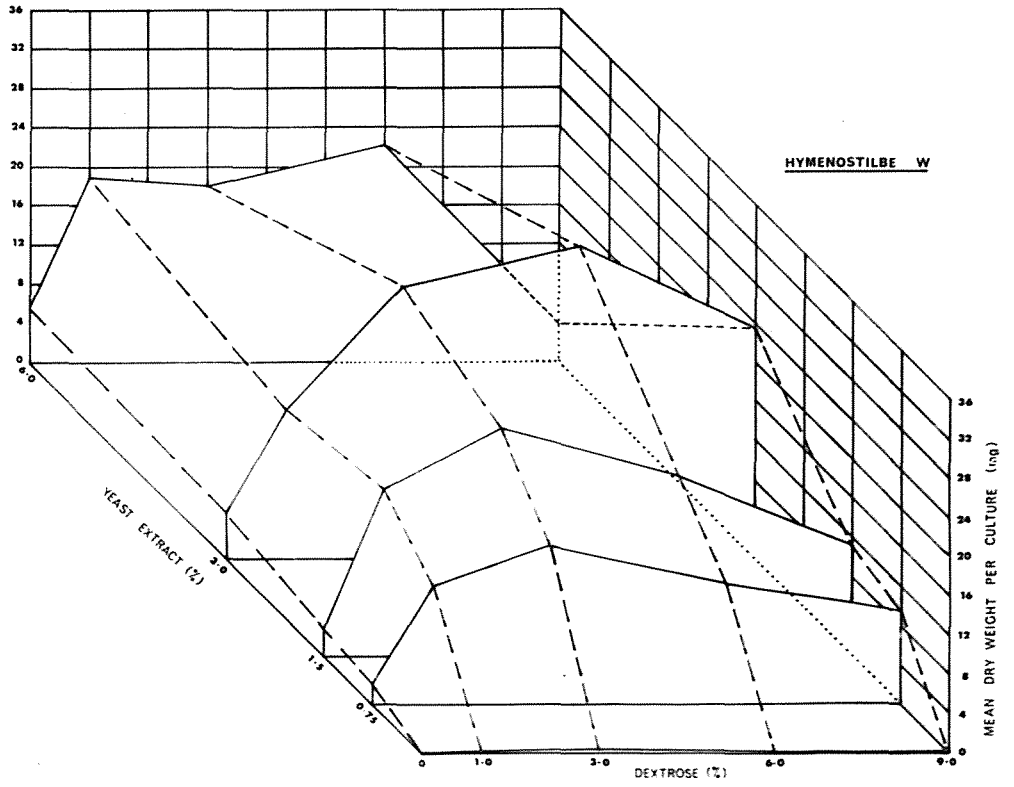
$D_{0.01} = 9.80$

(i) Yield was maximal at 3% yeast extract and 6% dextrose.

(ii) This yield was not significantly different ($P<0.05$) from the yield at 3% yeast extract combined with 3% and 9% dextrose but was significantly greater ($P<0.01$) than yields at all other combinations of yeast extract and dextrose.

Figure 17





Yield of Hymenostilbe R and Hymenostilbe W at 20 days on a medium containing different concentrations of yeast extract and dextrose

Hymenostilbe W

The mean dry weights (mg) of cultures on the various nutrient combinations after 20 days incubation are presented below in Table 21 and depicted graphically in Fig. 17 .

TABLE 21 The effect of varying concentrations of yeast extract and dextrose on the colony mean dry weight (mg) of H.W at 20 days

Yeast Extract (%)	Dextrose (%) 0	1	3	6	9
0	0.02	0.11	0.14	0.05	0.06
0.75	2.22	12.16	16.06	12.67	9.42
1.5	3.33	17.13	23.52	18.31	11.10
3.0	4.76	15.31	27.86	31.88	23.45
6.0	5.66	18.93	18.00	22.09	4.09

DO.05 = 5.27

DO.01 = 5.98

(i) Yield was maximal at 3% yeast extract and 6% dextrose.

(ii) This yield was not significantly different ($P<0.05$) from the yield at 3% yeast extract and 3% dextrose, but was significantly greater ($P<0.01$) than yields at all other combinations of yeast extract and dextrose.

(b) Sporulation of conidial state A

(i) Conidial state A did not develop on colonies of H.R.

(ii) Conidial state A of H.W developed at 20 days on 10 different nutrient combinations. The combinations of yeast extract and dextrose at which sporulation occurred are given

below in Table 22 together with the corresponding sporulation indices.

TABLE 22 The effect of varying yeast extract and dextrose levels on the production of conidial state A by H.W at 20 days

Yeast Extract (%)	Dextrose (%)	Sporulation Index	Colony mean dry weight (mg)
0	0	380	0.02
0	1	120	0.11
0	3	40	0.14
0	6	45	0.05
0	9	10	0.06
0.75	0	45	2.22
0.75	1	60	12.16
1.5	0	60	3.33
1.5	1	175	17.13
3.0	1	75	15.31

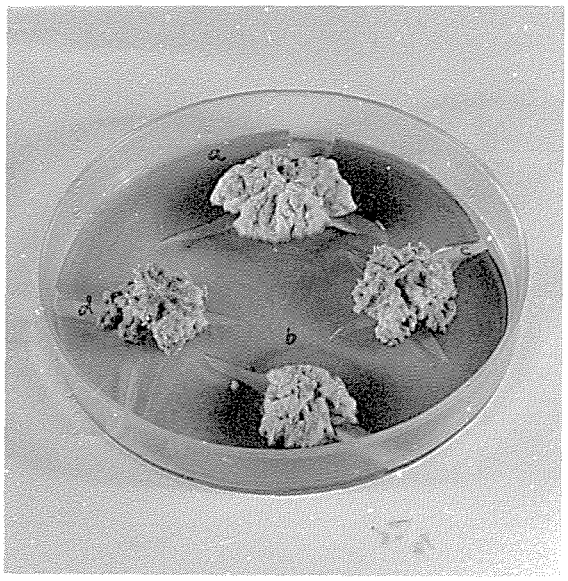
(i) Sporulation was maximal at 0% yeast extract and 0% dextrose, a medium which supported minimal vegetative growth.

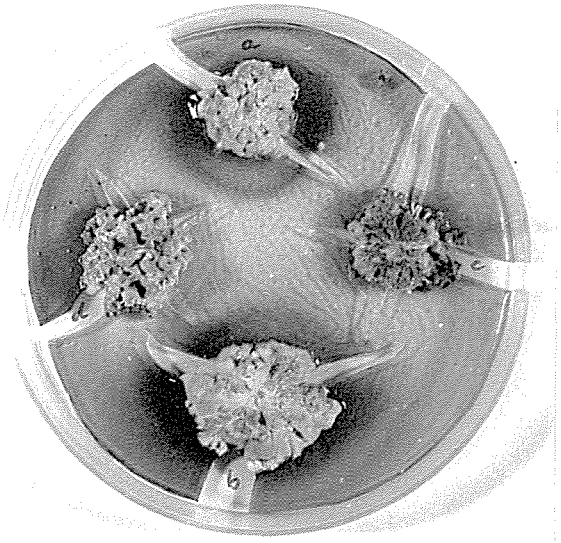
(ii) The medium supporting the second greatest sporulation was 1.5% yeast extract and 1% dextrose. This medium supported a substantial vegetative growth.

(iii) Dextrose levels above 1% inhibited sporulation.

(iv) Yeast extract levels above 1.5% inhibited sporulation.

Figure 18





Cultural characters of Hymenostilbe R and Hymenostilbe W at 28 days on a 3% yeast extract and 6% dextrose medium (The same culture from two views, a & b H.R, c & d H.W)

(c) Production of synnemata

(i) Synnemata did not develop on colonies of H.R.

(ii) Synnemata developed on colonies of H.W on two different nutrient combinations. These were 1.5% and 3.0% yeast extract combined with 1% dextrose.

(d) Colony morphology

The morphology of colonies of H.R and H.W on the medium supporting maximal growth (3% yeast extract and 6% dextrose) is illustrated in Fig. 18 .

Discussion

This experiment succeeded in defining the optimal nutrient concentrations for vegetative growth of H.R and H.W in solid culture at 20 days. It also succeeded in defining a suitable medium for production of synnemata and conidial state A on cultures of H.W. However, the experiment suffered from the limitation that observations were taken at only one incubation period. Thus generalizations on the effects of various nutrient concentrations on growth and sporulation were treated with caution since this experiment was, in effect, only a temporal slice through 25 different growth rates on 25 different media. Consequently little real knowledge had been gained on the factors affecting growth and sporulation in culture by this one observation. This is well illustrated by the apparent inhibition of growth of H.R and H.W at very high yeast extract and dextrose concentrations. From observations taken during the course of the experiment (and supported by MacLeod, 1959a) it was hypothesized that this 'inhibition' of growth was in reality the reflection of a 'lag phase' at these high nutrient

concentrations. MacLeod found that growth of Hirsutella gigantea was at first inhibited by high yeast extract concentrations but after an initial lag period the fungus adapted and underwent a period of rapid growth. It was thought that a similar situation had arisen in H.R and H.W but that it was not identified by the single observation period.

If the existence of such a lag phase in growth is accepted, then the lack of sporulation at high dextrose and yeast extract concentrations can be explained in terms of an inhibition due to a delayed active phase of colony growth. Such an inhibition, rather than one due solely to high dextrose or yeast extract concentrations, corresponds with the present concepts of factors affecting sporulation.

As concurrent observations had revealed that production of synnemata and conidial state A by H.R required very long incubation periods, a final experiment was designed primarily to investigate factors involved in the production of synnemata and conidial state A by H.W.

EXPERIMENT VI: Growth, sporulation and synnematal production of H.R and H.W on varying concentrations of yeast extract and dextrose at different incubation periods

This experiment sought to establish whether or not there was a link between growth rates on various media and production of conidial state A. Further, production of synnemata was examined in the hope of elucidating factors controlling their development.

Synnematal production was assessed using three sets of data, as suggested by Lougheed (1961). Lougheed considered that

the process of synnematal formation could be considered as consisting of two phases, an initiation phase followed by an elongation phase due to growth. To show the effects of nutrients on each phase, and to illustrate the overall effect of a nutrient, he advocated the following measurements:-

(a) The total length of the synnemata, to measure the overall effectiveness of each treatment.

(b) The number of synnemata, to measure the initiation phase.

(c) The mean length of synnemata, to measure the growth phase.

Materials and Methods

The experiment was set out in a 3 x 3 x 6 factorial design. Difco Yeast Extract was added to media at concentrations of 0.75, 1.5, and 3.0% while dextrose was added at 1.0, 2.47 and 6.0%. The nine media were prepared in 450 ml lots contained in one litre flasks and agar was added at 1.5%. Media pH was not adjusted as it varied from only 6.7 - 6.9. Media were sterilized, poured, and inoculated with one disc of H.R and one of H.W as set out in General Procedure. The plates were incubated at 24°C. At the end of each of the six incubation periods (5, 8, 12, 16, 20, and 28 days) results were recorded from three plates chosen at random from each of the nine treatments. Synnemata were measured (using a stereoscopic microscope with an eyepiece scale) after they had been picked off the colony surface and laid flat on the agar. Contaminated cultures were discarded.

Results

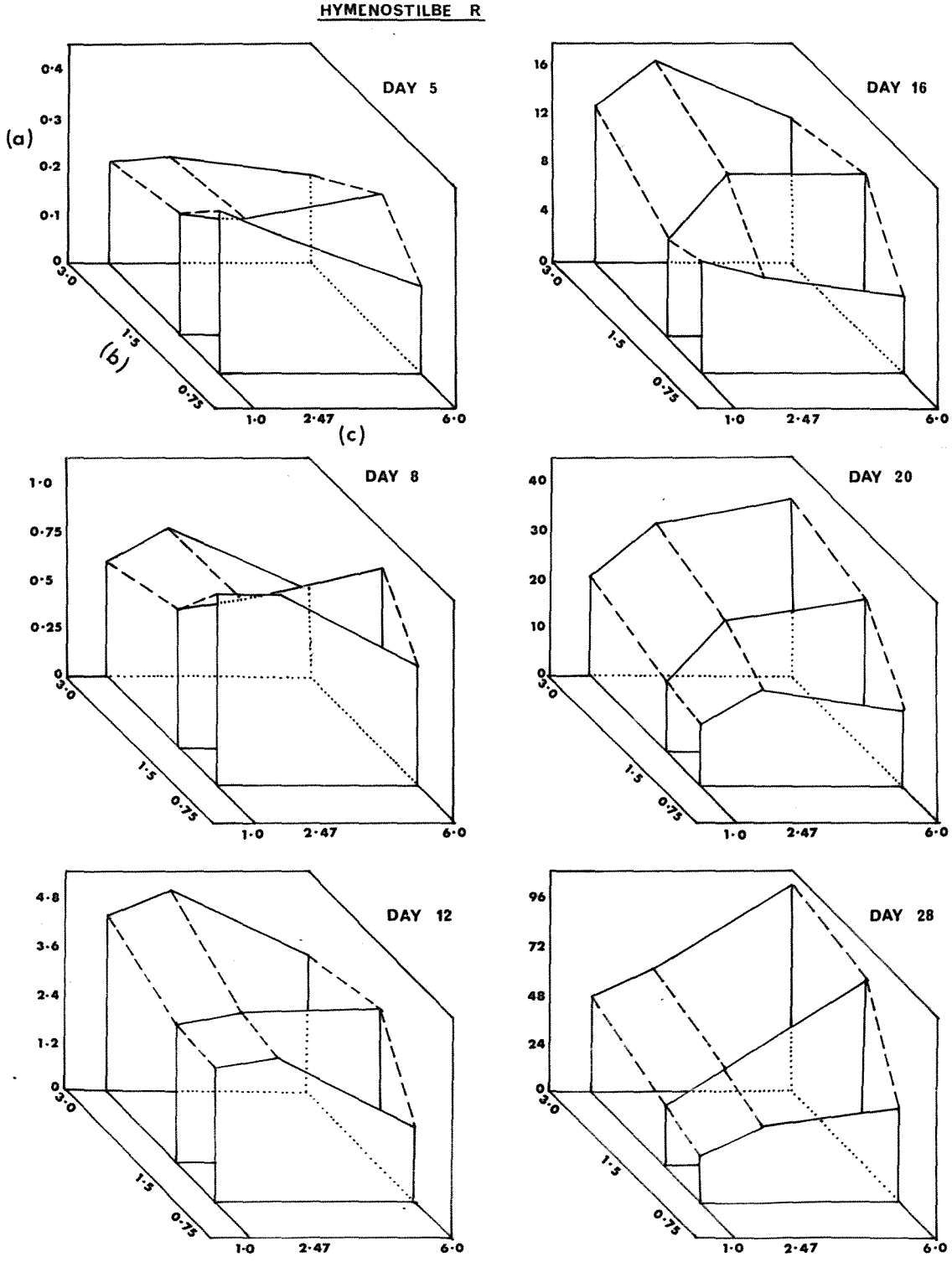
(a) Colony growth

Analyses of variance on the original data are presented in Appendix 2 (Tables 11 & 12). Transformation of data was considered but rejected after a graph of individual variances against means showed a broad scatter.

Hymenostilbe R

Results are depicted in Fig. 19. The existence of three two-way interactions and one three-way interaction, all of which are highly significant makes interpretation complicated. However the results can be viewed in the following manner. For both yeast extract and dextrose at any one incubation period, the yield on a particular nutrient combination is different from the yield that would be expected if each of the nutrients had acted on growth independently. At each incubation period there is a pattern of nine such results. The existence of a three-way interaction between incubation periods, yeast extract and dextrose concentrations means that the very nature of this pattern of nine results varies with the incubation period considered. It is this final effect that is the significant feature of this experiment. The effect can be followed through in Fig. 19 where a sequential comparison of the yield patterns for the various incubation periods indicates a trend for maximal yields to shift from low yeast extract and dextrose concentrations at the early incubation periods, through to high yeast extract and dextrose concentrations at extended incubation periods. An examination of F tests for the two two-way interactions of yeast extract and dextrose levels with

Figure 19



Growth response of Hymenostilbe R to varying concentrations of yeast extract and dextrose at different incubation periods
(a) mean dry weight per culture
(b) yeast extract (%)
(c) dextrose (%)

incubation periods (10%.9 for Y.E. conc. x Inc. P. and 223.0 for Dextrose conc. x Inc. P.) indicates that dextrose levels exerted the strongest influence on this trend during incubation. The relative effects of yeast extract and dextrose levels are perhaps more clearly shown in Tables 23 and 24 below.

TABLE 23 Mean dry weights (mg) of H.R at six incubation periods and at three dextrose concentrations (each averaged over three yeast extract concentrations)

Dextrose Concentration	Days 5	8	12	16	20	28
1.0%	0.27*	0.77	3.75	10.68	15.65	32.73
2.47%	0.25	0.83*	4.12*	12.64*	25.75	48.33
6.0%	0.22	0.67	3.02	10.77	27.17*	78.58*

*Maximum yield at each incubation period

It can be seen that at first growth was inhibited by high dextrose concentrations but that with increasing incubation periods this effect faded until there was a clear stimulation of growth at 28 days on the highest dextrose level.

TABLE 24 Mean dry weights (mg) of H.R at six incubation periods and at three yeast extract concentrations (each averaged over three dextrose concentrations)

Yeast Extract Concentration	Days 5	8	12	16	20	28
0.75%	0.264*	0.85*	2.93	8.42	15.68	35.30
1.5%	0.260	0.81	3.76	11.85	23.62	55.24
3.0%	0.204	0.61	4.20*	13.84*	29.27*	69.10*

*Maximum yield at each incubation period

From the table above it can be seen that the differential effect of yeast extract levels on growth at varying incubation periods is not as marked as that of dextrose levels. Yield was at first inhibited by high yeast extract concentrations but from the third incubation period on, growth was maximal at the highest yeast extract concentration.

Hymenostilbe W

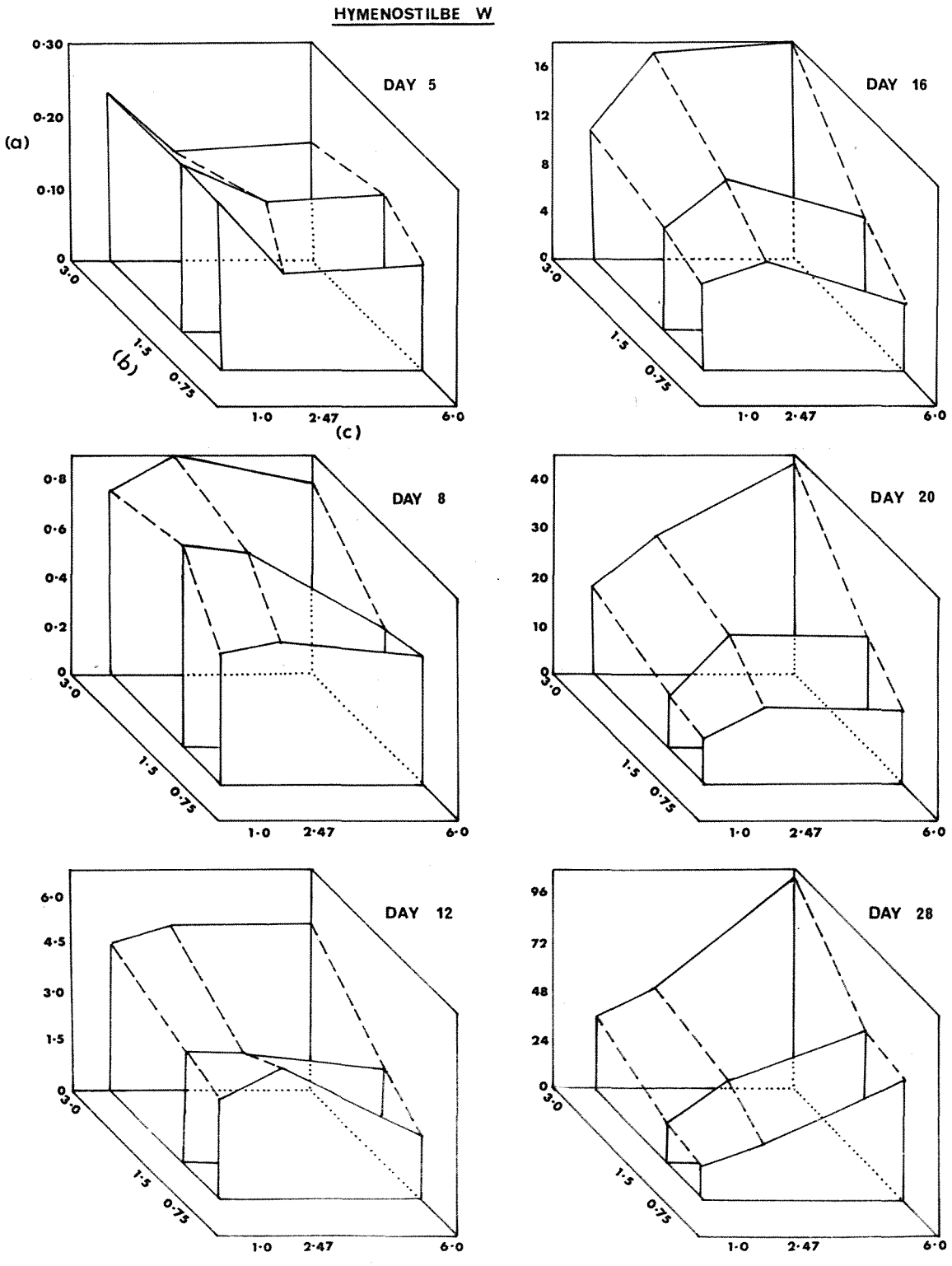
The remarks concerning interpretation of results for H.R apply equally well to H.W. An examination of Fig. 20 shows that the differential effects of yeast extract and dextrose concentrations on yield of H.W at different incubation periods were similar to those of H.R. The differential effects of dextrose levels on yield were however greater than those of yeast extract, as shown by the difference in the F tests for the two two-way interactions (49.9 for Y.E. conc. x Inc. P. and 154.75 for dextrose conc. x Inc. P.). The relative effects of yeast extract and dextrose levels are perhaps more clearly shown in Tables 25 and 26 below.

TABLE 25 Mean dry weights (mg) of H.W at six incubation periods and at three dextrose concentrations (each averaged over three yeast extract concentrations)

Dextrose Concentration	Days 5	8	12	16	20	28
1.0%	0.23*	0.71	3.71	9.20	13.05	23.90
2.47%	0.15	0.76*	4.12*	12.85*	22.26	39.32
6.0%	0.16	0.60	3.27	10.09	27.00*	76.23*

*Maximum yield at each incubation period

Figure 20



Growth response of Hymenostilbe W to varying concentrations of yeast extract and dextrose at different incubation periods
(a) mean dry weight per culture
(b) yeast extract (%)
(c) dextrose (%)

Growth was at first inhibited by high dextrose levels but with increasing incubation periods this effect faded until growth was maximal at 28 days on the highest dextrose concentration.

TABLE 26 Mean dry weights (mg) of H.W at six incubation periods and at three yeast extract concentrations (each averaged over three dextrose concentrations)

Yeast Extract Concentration	Days 5	8	12	16	20	28
0.75%	0.17	0.49	2.98	7.11	13.43	33.58
1.5%	0.20*	0.70	3.24	10.14	19.19	42.08
3.0%	0.18	0.81*	4.9*	15.44*	30.00*	63.79*

*Maximum yield at each incubation period

Inhibition of growth at high yeast extract concentrations occurred only at the five day incubation period.

(b) Relative sporulation of conidial state A

(i) Conidial state A was not produced on cultures of H.R.

(ii) Conidial state A was first recorded from cultures of H.W at 12 days. The table below summarises the rates of development on the different media during the six incubation periods. Figures given are the sporulation indices.

TABLE 27 The effect of varying concentrations of yeast extract and dextrose on sporulation of conidial state A at different incubation periods

Dextrose (%)	Yeast Extract (%)	Days 5	8	12	16	20	28
1.0	0.75	-	-	2.5	75	240	360
1.0	1.5	-	-	5	100	120	320
1.0	3.0	-	-	5	80	150	320
2.47	0.75	-	-	-	20	30	210
2.47	1.5	-	-	-	30	37	320
2.47	3.0	-	-	-	20	75	280
6.0	0.75	-	-	-	-	-	10
6.0	1.5	-	-	-	-	4	88
6.0	3.0	-	-	-	-	-	60

Study of the above table indicates that:

(i) The onset of sporulation was inhibited by dextrose concentrations above 1%.

(ii) This inhibition diminished progressively with increasing incubation period.

(iii) The effect of various yeast extract concentrations on the initiation of conidial state A was relatively small.

(c) Production of synnemata

Synnemata first appeared after 20 days incubation on a medium of 1% dextrose and 1.5% yeast extract. After 28 days incubation synnemata were produced from colonies of H.W on eight nutrient combinations. Results are summarised below in Table 28.

TABLE 28 The effect of varying yeast extract and dextrose concentrations on synnematal production at 28 days

Media		Synnemata				Colony
Yeast Extract (%)	Dextrose (%)	Total Length	No.	Mean Length ± S.E.	Range	Mean Dry Weight (mg)
0.75	1.0	116[¤]	8	14.5[¤] ± 8.6	7[*] - 32	14.86
0.75	2.47	524	26	20.1 ± 2.3	7 - 37	27.80
0.75	6.0					36.00
1.5	1.0	3072	119	25.8 ± 2.2	5 - 75	20.84
1.5	2.47	3739	283	13.2 ± 0.1	4 - 46	40.00
1.5	6.0	1502	92	16.0 ± 0.6	4 - 47	65.08
3.0	1.0	2047	80	25.6 ± 1.8	4 - 61	36.00
3.0	2.47	2789	173	16.2 ± 0.5	4 - 48	49.82
3.0	6.0	1021	113	9.0 ± 0.1	4 - 20	105.53

[¤]eyepiece divisions (1mm = 39 eyepiece divs.)

Study of the above table indicates that:

(i) Overall synnematal production was maximal on 1.5% yeast extract and 2.47% dextrose.

(ii) Synnemata were of the greatest length on 1.5% and 3.0% yeast extract combined with 1.0% dextrose.

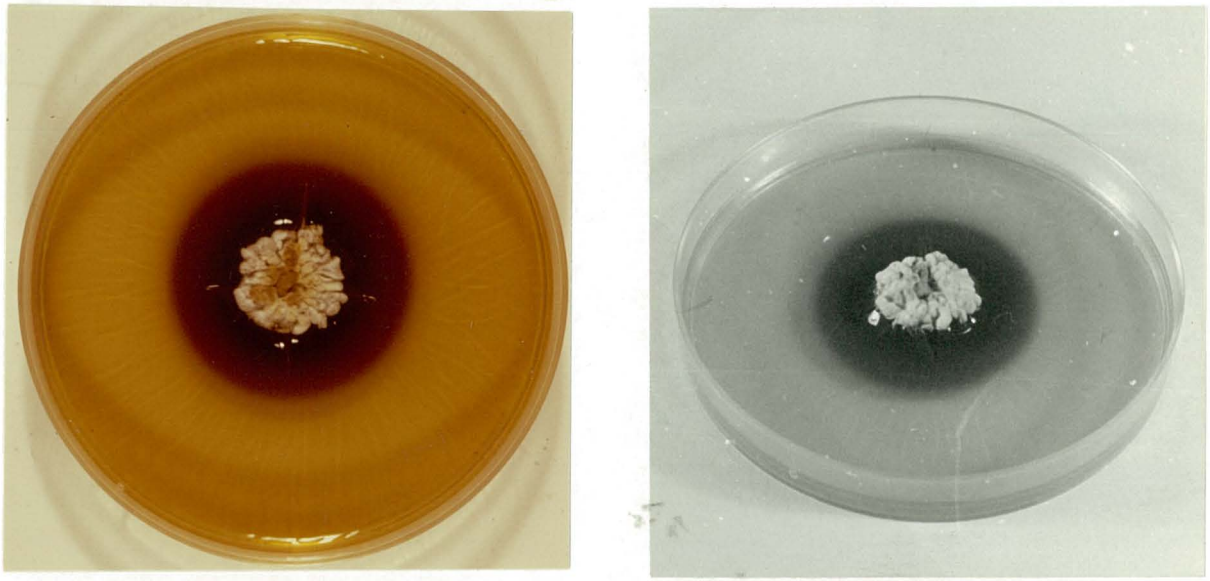
(iii) The greatest number of synnemata were produced on 1.5% yeast extract and 2.47% dextrose.

(iv) A yeast extract concentration of 0.75% inhibited synnematal production.

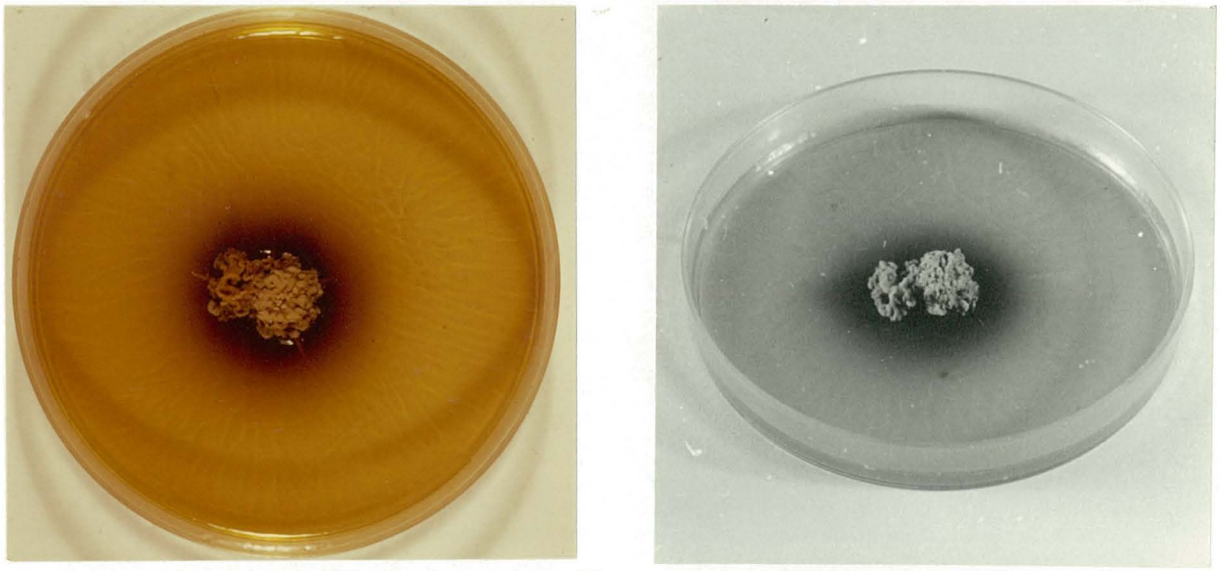
(d) Colony morphology

The morphology of colonies of H.R and H.W on a yeast extract (3%) - dextrose (1%) medium at 28 days is illustrated in Fig. 21 .

Figure 21



Hymenostilbe R



Hymenostilbe W

Cultural characters of Hymenostilbe R and Hymenostilbe W at 28 days on a 3% yeast extract and 1% dextrose medium (Two views of the same culture in each case)

Discussion

This experiment demonstrated that growth and sporulation responses to the various nutrient concentrations tested could be explained mainly in terms of a changing reaction, at different incubation periods, to various dextrose concentrations. The possession of a 'lag period' for growth, as hypothesized in the previous experiment, was confirmed and it was apparent that high concentrations of dextrose exerted a stronger effect on this lag period than did high concentrations of yeast extract. It is of interest to note that whereas most fungi grow mainly in a two dimensional manner across the surface of the agar, H.R and H.W exhibited a strong three dimensional colony growth form. The data available from this experiment permitted the formulation of an hypothesis explaining the kinetics of growth for such a three dimensional growth form on solid media.

Firstly, it is a commonly accepted principle for fungi that growth is primarily limited by an exhaustion of the carbon source and that nitrogen is utilized at a rate depending on the available carbon (Cochrane 1958). Secondly, the dynamics of growth in fungal cultures are related to the number of hyphal tips available for nutrient absorption rather than being a function of the total number of cells present, as in cultures of bacteria and yeast (Cochrane 1958). In the cultures of H.R and H.W, colony growth took a three dimensional form (roughly conical or hemispherical), while the assimilative hyphae at the base of the colonies were in a two dimensional plane. In a structure of this nature the volumetric size increases at a greater rate (approx. 2x) than the increase in basal area. Thus, since growth

is essentially volumetric it is reasonable to assume that on a favourable medium for growth the nutrient requirements of the colony will outstrip the ability of the assimilative hyphae to extend into new areas of the medium and absorb the required nutrients. Consequently at a certain stage the absorption of nutrients by the assimilative hyphae will become a factor limiting the rate of colony growth. The nutrient concentration of the medium will have a profound effect on the stage of colony growth at which this limitation takes place.

In this experiment growth was at first enhanced by a low concentration of yeast extract and dextrose (it is suggested that such concentrations are inherently the most favourable for growth[1]). The postulated limitation of colony growth rates first occurred during the eight to twelve day incubation period when the growth rates of colonies on low dextrose levels were surpassed by those of colonies on the intermediate dextrose levels. In a similar manner the superiority of the intermediate dextrose levels declined with further incubation periods until finally at 20 and 28 days the lag effect of high nutrient concentrations was overcome and colony growth became maximal on the greatest nutrient concentration.

Although this hypothesis adequately explains the upward shift in the optimum concentrations of nutrients with incubation periods it must be remembered that this study has not investigated the influence of other factors on colony growth such as metabolic exudates, or the differential availability of

1/ If, as is widely accepted, the lag in growth at higher nutrient concentrations represents a period of adaptation to an initially unfavourable nutrient milieu, it can be assumed that low concentrations of yeast extract and dextrose are probably near the true optimum for the growth of single cells.

supplementary growth factors which may be present in the yeast extract. Although it is probable that such factors exert some effect on the growth response it is suggested that the hypothesis put forward explains to a large degree the pattern of growth for the three dimensional colony form of H.R and H.W on solid media.

If this hypothesis is accepted then it will also explain to a certain extent the production of conidial state A in terms of presently accepted theories of sporulation mechanisms.

Hawker (1966), in a review of factors affecting sporulation considered that in young colonies up to a period of maximum growth, internal changes take place that predispose the fungus to reproduction. These changes may be inhibited or speeded up by external conditions during that stage. After a peak is passed growth declines and if external conditions are favourable spore initials develop and later mature. Cochrane (1958) states that it is widely accepted that the concentration of the carbon source has a major effect on sporulation mainly through its influence on vegetative growth, sporulation being depressed at high rates of growth.

Production of conidial state A in H.W is quite clearly inhibited at high dextrose concentrations but this inhibition decreases with increasing incubation periods. If the postulated view of the kinetics of colony growth IS accepted then production of conidial state A can be quite closely associated with a limitation of growth probably linked to a decreased relative absorption of dextrose and other nutrients. Such a view is supported by the fact that sporulation occurs on the upper extremities of the colonies and usually begins on the

centre of the vegetative mound. Such an area is furthest away from the assimilative hyphae and it suggested that a shortage of nutrients, predisposing the switch to a sporulating phase, would be first apparent in this region.

The factors controlling synnematal production in H.W appear to be at variance with those affecting production of conidial state A. This is not surprising as many instances have been recorded in which alternative conidial states, or perfect phases, have different nutritional and environmental requirements (Cochrane 1958, Hawker 1966). Such a situation has survival value in that it would spread propagation over a variety of environmental conditions.

From the results obtained it is apparent that a low yeast extract concentration suppressed synnematal production, a situation also recorded by Lougheed (1961) for synnemata of Hirsutella gigantea. This suppression mainly took the form of an inhibition of initiation sites. Yeast extract levels above 0.75% had little effect on synnematal production, while dextrose levels seemed to exert their effect mainly by modifying the number of initiation points, and slightly delaying initiation (as evidenced by the shorter synnemata at high dextrose levels). There appeared to be little correlation of synnematal production with growth rates.

It would thus seem probable that the conclusions of Taber and Vining (1959) for Isaria cretacea apply to the nutritional requirements of synnematal production in H.W on a yeast extract medium; that is, under some circumstances the concentration of nutrients can be a critical factor for synnematal production but that generally production of synnemata takes place over a variety of nutritional conditions.

EXPERIMENT VII: Methods for the production of synnemata and conidial state A by H.R. and some observations on the production of synnemata by H.W

Introduction

During the course of the previous experiments it was found that production of synnemata and conidial state A did not occur in cultures of H.R following incubation for 28 days. There was still the possibility however that the production of synnemata and conidial state A could be induced following an extended incubation period. Accordingly an approach of 'studied neglect' was adopted whereby cultures on a variety of media and from a variety of sources were incubated for several months at 24°C. This approach resulted in the production of both conidial state A and synnemata bearing the phialospore conidial state in cultures of H.R. The following account concerns their development on various media (Section A) while Section B contains two observations on the synnemata of H.W.

A. Production of synnemata and conidial state A on cultures of H.R

1. On a lab. PDA slope at 10 weeks

The first indication that synnemata and conidial state A could be produced from colonies of H.R occurred when a very irregular dense accumulation of dark brown hyphae appeared amongst the aerial mycelium of a colony grown from a conidiophore of conidial state A. This accumulation of hyphae produced a number of conidiophores of conidial state A and although it could not be considered a synnema it indicated that production could be achieved in artificial culture.

2. After seven to ten weeks on Difco Mycobiotic Agar

The colonies on this medium were produced from two discs of

the standard inoculum of both H.R and H.W. After seven weeks incubation a number of red/brown (6pi) synnemata that were a light pink at the growing tip were produced from the surface of the colony (Fig.22). Most of these synnemata were sterile but an occasional one would produce scattered phialides and conidia of the phialospore conidial state. The synnemata closely resembled those produced from endosclerotia after incubation in the laboratory under conditions of high humidity but conidial state A was not produced, neither on the synnemata, nor on the colony surface. It is of interest to note that although colonies of H.W produced a dense covering of conidial state A on this medium synnemata were not produced, even at the extended incubation period.

3. After eight to twelve weeks on media at various yeast extract and dextrose concentrations

(a) inoculation by flooding

The cultures used were surplus products of the inoculum preparations used for earlier experiments. Details of their method of production are explained in General Procedures - 'preparation of inoculum'.

Synnemata first appeared at eight weeks on a 1.5% yeast extract and 3% dextrose medium that was beginning to dry up. They were produced along the curled edges of the culture. As the colony dried out further, synnemata were produced in the central portion of the colony. They were a dark red brown (6pg) and regular in shape, but not fertile.

Synnemata were also produced after ten weeks on a 3% yeast extract and 6% dextrose medium inoculated by the same technique

Figure 22

Synnemata produced from a colony of Hymenostilbe R at ten weeks on Difco Mycobiotic Agar

(Fig.23a). However these synnemata were distorted, often accumulated in irregular clumps, and were produced in patches over the colony surface. All were infertile until 15 weeks, at which time an occasional conidiophore of conidial state A was produced from a few synnematal tips. The phialospore conidial state was not observed.

(b) inoculation using standard inoculum discs

These cultures were the product of an abandoned experiment set up to test various concentrations of yeast extract and dextrose. Three inoculations of H.R and three of H.W were made in each petri dish as set out in General Procedure.

Synnemata were produced after ten weeks, mainly from the partially submerged mycelium at the periphery of the colonies (Fig. 23b). Synnemata appeared first, and were produced most numerously, on a medium containing 0.75% yeast extract and 3% dextrose. Both low (1%) and high (6%) dextrose concentrations inhibited synnematal production while 0.75% yeast extract was superior to 1.5% yeast extract. At twelve to fifteen weeks some of the synnemata produced conidial state A from their tips while three synnemata were detected that had produced the phialospore conidial state.

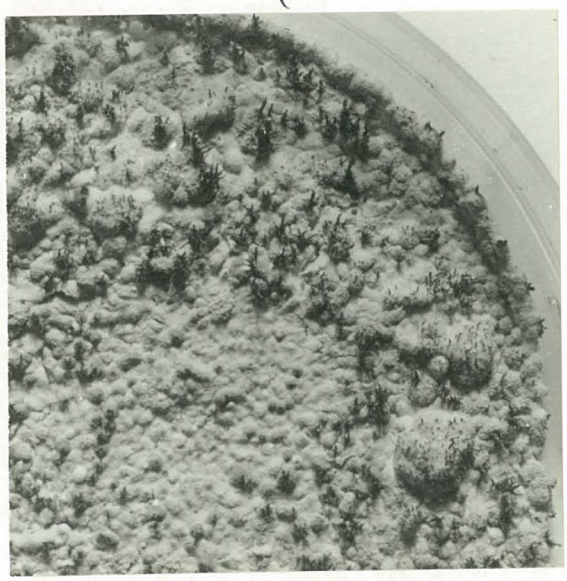
Production of synnemata was markedly stimulated by the presence of an unidentified contaminant fungus (Fig.24a).

4. Transfer of colonies to lab. PCA and lab. water agar

In this experiment the 15 week cultures on 0.75% yeast extract and 3% dextrose, described immediately above in 3(b), were transferred to lab. water agar and to lab. PCA. After six days incubation conidial state A appeared on the lower slopes of the colony and on the tips of some synnemata. In

Figure 23

(a)



Hymenostilbe R Synnemata on a 3% yeast extract 6% dextrose medium at ten weeks (inoculated by flooding)

(b)

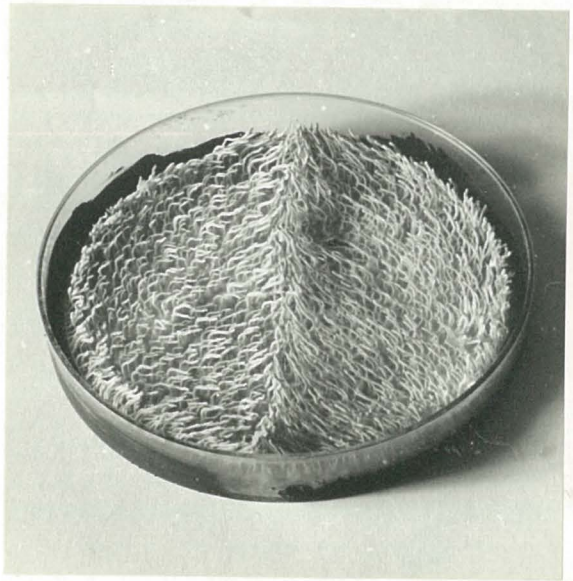
Synnemata on colonies of Hymenostilbe R and Hymenostilbe W at ten weeks on a 0.75% yeast extract and 3% dextrose medium (the three colonies on the right are H.R, those on the left H.W)

Figure 24

(a)

Stimulation of synnematal production in Hymenostilbe R by the presence of a contaminant Fungus (Hymenostilbe R on the right)

(b)



A phototrophic response displayed by synnemata of Hymenostilbe W

addition, the synnemata at this stage had resumed growth and become a velvety pink at their tips. On microscopic examination this velvety pink appearance was seen to be due to the production of a dense hymenium of phialides and conidia of the phialospore conidial state. Further incubation up to fourteen days (when the observations were terminated) intensified both conidial state A and phialospore production while a large number of synnematal initials developed on the upper slopes of the colony. It was noted that while lab. water agar favoured the production of conidial state A the greatest synnematal growth and production of the phialospore conidial state occurred on lab. PCA.

B. Observations on the production of synnemata by H.W

During the above investigations into synnematal production by H.R observations were also made on the development of synnemata in cultures of H.W.

1. After 28 days on a 1.5% yeast extract and 3.0% dextrose medium inoculated by flooding

Inoculation was as set out in General Procedure - preparation of inoculum. At 28 days a large number of white synnemata were produced from the vegetative mat. These synnemata bore a dense hymenium of phialides and conidia. Phialides and conidia were also produced amongst the mycelium of the colony proper; that is, not on the synnemata. The synnemata were up to 8mm long and few conidiophores of conidial state A were produced either on the synnemata or colony surface.

2. Phototropism

Synnemata of H.W exhibited a phototropic response. A petri dish culture inoculated by flooding (as in General

Procedure - preparation of inoculum) was blackened by painting, except for a window strip 5mm wide that bisected the lid. The culture was incubated at room temperatures and exposed to normal conditions of daylight and darkness. After 20 days synnemata were formed and at 30 days could be seen curving towards the light (Fig.24b). Both length of synnemata and density of phialospore production were stimulated by light. Synnemata furthest from the window strip were not as large nor aggregated so tightly as those exposed to full light. Further, they were often sterile.

Discussion

The above descriptions represent the only instances in which synnemata of H.R have been produced in pure culture. Difco Mycobiotic agar allowed the development of synnemata most closely resembling those produced from endosclerotia following incubation under high humidity in the laboratory, while production of the phialospore conidial state and conidial state A was best achieved by a transference of colonies to lab. water agar or lab. PCA.

The available data allows little comment on the factors controlling synnematal production in H.R, except that the transference of cultures to a dilute medium, such as water agar or lab. PCA, has often been shown to stimulate sporulation in other fungi (Cochrane 1958), while Hejmanek and Hejmankova-Uhrova (1956 - cited Turian 1966) stated that synnematal production was stimulated by the desiccation of cultures and an accumulation of metabolites. Although the first factor has been demonstrated and the second probably operates a causal

relationship has not been proven, a criticism Lougheed (1961) similarly made of the work of Hejmanek and Hejmankova-Uhrova.

The stimulation of synnematal production by a contaminant fungus has also been recorded by Taber (1959), although he did not comment on the underlying mechanism.

The observations on synnematal production in H.W also correspond to other observations in the literature. Lougheed (1961) found that inoculation by flooding was a superior method of synnematal production and speculated that the large number of synnemata formed was a response to the very quick absorption of nutrients by the vegetative mat which entirely covered the surface of the medium. Similarly the stimulation of sporulation by light has often been noted by other workers and the photo-tropic response of vegetative structures such as synnemata frequently demonstrated (Cochrane 1958, Hawker 1966).

E. GENERAL DISCUSSION

Each of the four aims expressed in the introduction to this chapter has been fulfilled for both H.R and H.W. Firstly, media have been described which supported a substantial vegetative growth. Secondly, methods have been described for the production of both conidial state A, and the phialospore conidial state. Thirdly, the constant morphological differences exhibited between the two fungi under a variety of experimental conditions support the proposal that H.R and H.W are two species of entomogenous fungi. Fourthly, for both H.R and H.W the production of the phialospore conidial state and the conidial state A on the same cultures, each derived from inoculum grown from a conidiophore

of conidial state A, clearly demonstrates that both fungi possess two conidial states. The data accrued during the experimental series allowed the formulation of an hypothesis relating to growth and sporulation in H.W which, it is hoped, will provide the basis for a more refined study.

Few attempts have been made to study critically the nutrition of entomogenous fungi. The accumulation of nutritional information is desirable, not only for those fungi pathogenic on a wide variety of hosts, for which most investigations have taken place (such as Beauvaria bassiana or Metarrhizium anisopliae), but also for host specific fungi which are often difficult to grow in pure culture. Such information would not only permit detailed morphological and taxonomic studies but would also facilitate investigations on pathogenicity and host range, and perhaps could lead ultimately to mass production for field utilization.

The present state of knowledge on the nutrition of host specific entomogenous stilbaceous fungi stems almost entirely from the investigations of MacLeod (1954, 1959a,b, 1961) on the nutrition of Hirsutella gigantea. It is noticeable, however, that MacLeod makes no attempt to correlate his nutritional data with the ability of the insect host to supply nutrients to the fungus.

In a host specific entomogenous fungus the entire existence of the pathogen is restricted to an initial incubation period within the haemolymph, followed by saprophytic colonisation of the dead host, and finally sporulation. It is likely that such fungi are highly adapted to conditions found within

the body tissues of the particular insect attacked. In this respect it is suggested that the haemolymph if of primary importance to the nutritional adaptation of the pathogen, since the composition of the cells of the various tissues of the insect body closely resembles the composition of the haemolymph (Florkin and Jeuniaux 1964) and it is in this medium that the incubation phase, leading to the death of the insect, takes place. Thus the nutrition of host specific entomogenous fungi should show a close connection to the nutrient composition of the haemolymph.

The main feature distinguishing insect haemolymph from the blood of all other animals is the very high concentration of free amino acids (15% of the total nitrogen and 20-30 times that of human blood) and the considerable amount of residual nitrogen in peptide form. Proteins are present in much the same concentrations as in mammalian plasma but whereas in mammals the chief carbohydrate is the reducing sugar glucose, in insect haemolymph the chief carbohydrate is the non-reducing sugar trehalose (Wigglesworth 1966). Organic acids and organic phosphates are also present in considerable quantities. In the words of Florkin and Jeuniaux (1964) "the haemolymph of insects appears therefore with the characteristics of a fluid tissue, with its own metabolism, revealing a composition more similar to that of the intracellular fluid than to that of the blood of vertebrates." Utilizing this knowledge of the composition of insect haemolymph, the available data on the nutritional requirements of various entomogenous fungi correlates well with their mode of pathogenicity. Pathogens

such as M. anisopliae and B. bassiana have simple nutritional requirements and can utilize inorganic nitrogen. Such an unspecialized nutrition tallies well with their ability to attack a wide variety of hosts and assume a saprophytic existence in the soil (Madelin 1963). However, host specific fungi such as H.R, H.W and H. gigantea are more exacting in their nutritional requirements and need a source of organic nitrogen for growth. Of the organic nitrogen sources tested, yeast extract, a superior source of amino acids and growth factors, supported maximal vegetative growth in H.R, H.W and H. gigantea. Furthermore, MacLeod, in additional investigations, found that growth of H. gigantea was even further stimulated by a mixture of amino acids in particular proportions and that an unidentified growth factor, thought possibly to be a peptide, was necessary for substantial growth. Such nutritional characteristics correspond closely to those expected of fungi adapted to a parasitic mode of existence in tissues containing high concentrations of free amino acids and peptides. The stimulation in the growth response of H. gigantea to a particular mixture of amino acids probably represents a high degree of adaptation, and may be connected to host specificity since the relative proportions of various amino acids and other haemolymph constituents varies markedly, even between species. It is therefore suggested that an analysis of the haemolymph of the host would provide valuable information on the nutritional requirements of host specific entomogenous fungi.

It is also suggested that the role of lipids in the nutrition of entomogenous fungi should be investigated.

Haemolymph contains a substantial quantity of organic acids and there are also large quantities of fat stored in the various parts of the insect body. The importance of lipids as a source of energy for entomogenous fungi was demonstrated by Schaerffenberg (1964) for Metarrhizium anisopliae and Beauvaria bassiana when he found that the addition of fats to artificial media resulted in a stimulation of growth. It is probable that utilization of lipids by other entomogenous fungi will be or similar significance. In this respect it is of interest to note the large number of fat droplets present in the gemmae of H.R and H.W (Figs. 3 & 5). These fat droplets represent stored energy and following the demonstration by Taber (1960) of the utilization of endogenous nutrients in the production of synnemata, it is probable that they may play a large role in the formation of synnemata from endosclerotia. The ability to store and utilize lipids may thus be of considerable significance in the production of synnemata and spores in the field, and in the continued survival of endosclerotia under adverse environmental conditions.

Conclusion

The nutrition of H.R and H.W is similar to that of H. gigantea. It is suggested that other host specific entomogenous fungi will display similar fundamental nutritional characteristics. The particular amino acid composition of the haemolymph of various insect species may represent a partial basis for the habit of host specificity that is widely displayed in entomogenous fungi.

CHAPTER 4

INFECTIVITY AND ETIOLOGY OF HYMENOSTILBE R AND HYMENOSTILBE W

INTRODUCTION

This chapter is divided into two sections. In section A there is consideration of the pathogenicity of H.R and H.W to larvae of Cecyropa setigera and a brief description of experiments relating to the ability of H.R and H.W to infect larvae of other insect species. Section B. deals with the development of H.R and H.W within the larva (etiology) and factors affecting the dynamic relationship of the pathogen and host in the field.

A. INFECTIVITY TESTS

Introduction

Fungi are classified as being entomogenous if they are found constantly associated with an insect or insect cadaver. However the mere observation of a fungus in, or on its supposed host does not prove that it is a pathogenic organism capable of inciting a diseased state. For unequivocal proof of such a causal relationship between a fungus (or any microorganism) and a particular disease, it is generally accepted that a set of conditions known as 'Kochs Postulates' should be fulfilled.

These may be expressed as follows:

1. The microorganism must be present in every case of the disease.

2. The microorganism must be isolated to pure culture.

3. The microorganism in pure culture must, when inoculated into a susceptible host, give rise to the disease.

4. The same microorganism must be present in, and recoverable from, the experimentally diseased animal.

The successful completion of this set of requirements guarantees virtually conclusive evidence that a particular fungus is pathogenic to the host considered.

Although the recent emphasis on insect pathology as a distinct discipline (Steinhaus 1949, 1963) has stimulated the wider application of critical techniques, there is still a complete lack of pathogenicity or cross-infectivity tests for the majority of entomogenous fungi. With the exception of the few entomogenous fungi possessing a wide host range, this lack can be largely attributed to three factors. Firstly, work on the majority of entomogenous fungi has been carried out by mycologists whose interests have been primarily taxonomic. Secondly, these fungi are often difficult to grow in pure culture and a suitable supply of the insect host is usually difficult to procure. Thirdly, even if the fungus is grown in pure culture and host insects are supplied it is often difficult to induce the disease experimentally.

In the case of the genus Hymenostilbe, literature is confined to the taxonomic papers of Petch et al. (Chapter 2) and there have been no infectivity tests or isolations

to artificial culture. Mathieson (1949) attempted to fulfil Koch's postulates for the closely related[1] Cordyceps aphodii but although she used over 1000 larvae, results were inconclusive because of a heavy handling mortality. In this study attempts were made to prove Koch's postulates for H.R and H.W on Cecyropa larvae, and to cross infect other insect hosts.

Experiments

1. Koch's Postulates

Although the first two requirements of constant association and isolation to artificial culture were achieved for both H.R and H.W, the fulfilment of Koch's postulates was frustrated by difficulties associated with the third step involving infection of the host with the fungus in pure culture. This was mainly due to problems of obtaining a suitable supply of Cecyropa larvae. Field collected larvae were very difficult to obtain, especially over the winter months when this phase of the study was being conducted. In addition such larvae could not be guaranteed pathogen free as could those reared in the laboratory. Production of larvae from eggs laid in the laboratory was thwarted however, firstly by the parasitism of adults by an ichneumon parasite (which caused 72% mortality) and secondly, by an attack of white muscardine fungus Beauvaria bassiana which caused the death of the remaining weevils. By the time a breeding colony had been re-established this study was in its final stage. However the tests that were accomplished are outlined below.

(a) Direct exposure of the larvae to cultures of H.R and H.W produced from an inoculation by flooding

Eight first and second instar larvae of Cecyropa setigera were obtained from eggs laid and hatched in the laboratory. Three larvae were confined to a ten week old culture of H.R on a 3% yeast extract and 6% dextrose medium, and another three to a six week old culture of H.W on a 1.5% dextrose and 3% yeast extract medium. The two larvae remaining were confined to a medium of 1% dextrose and 3% yeast extract over which a sheet of sterile damp filter paper had been laid (to simulate the presence of a mycelial mat). The cultures of H.R and H.W consisted of a mat of vegetative mycelium completely cover-

[1] See Chapter 2 where the relationship of H.R and H.W to C. Aphodii is discussed in detail.

ing the medium (see figs 23a, 24b, Ch.3) and although occasional distorted synnemata were present in the culture of H.R these were infertile and there were no spores of either conidial state produced on the mycelium. However in H.W a large number of synnemata, carrying both the phialospore conidial state and conidial state A were produced on the cultures.

After 24 hours the larvae in all three petri dishes had burrowed down into the agar and were tunnelling just below the surface. They emerged after several hours but again burrowed down through the mycelium (and over the edge of the filter paper in the control) into the agar. The larvae were removed after 48 hours and transferred to three sterile petri dishes containing several layers of sterile damp filter paper.

All the larvae died three days after removal, with the symptoms of a bacterial infection. It was speculated that damage to the larvae may have occurred during handling and that this could have induced the disease. The rich medium through which the larvae were burrowing was ideal for bacterial development and could have aggravated an incipient infection.

(b) Contamination of the larval environment with H.R and H.W

As the previous experiment exhausted larval stocks an attempt was made to circumvent handling by confining a number of adults to environments contaminated by the fungi. Field observations and experiments involving the confinement of adults to cultures, as in the experiment above, indicated that adults were immune to infection by H.R and H.W Accordingly it was hoped that the weevils would breed and produce eggs from which larvae would hatch and become infected.

The method involved placing 30 grams of sieved air dry sand (52 mesh) into each of three small screw-top jars (1¼ inches in diameter and 2 inches tall). Two mls of distilled water was added to each jar, and they were then capped with aluminium foil and autoclaved for 15 minutes at 15 lb pressure. The bottom inch of a thoroughly scrubbed carrot was placed into each jar while 20 adult males and 20 adult females, collected in mid August, were also added. Colonies of H.R and H.W grown for 15 weeks on 1.5% yeast extract and 1.5% dextrose

medium were separately fragmented in two MacCartney bottles containing 10 mls of sterile water and glass beads. Two mls of the resulting mycelial suspension of H.R was added to one jar, and two mls of the spore and mycelial suspension of H.W to another. The third jar to which two mls of sterile water was added, but neither H.R nor H.W, was employed as a control. Fresh carrot was added weekly and the jars were capped with foil.

Colonies were inspected after six weeks when the contents of the jar were wet-sieved through a 52 mesh gauze. In the control jar there were 16 eggs but no larvae. In the jar containing H.R there were 13 eggs and no larvae, while in the jar containing H.W there were eight eggs and two larvae, neither of which subsequently became infected.

This experiment would possibly have proved successful if a longer incubation period had been maintained; time did not allow this.

(c) Discussion on proof of pathogenicity

Koch's first and second postulates were fulfilled for both H.R and H.W, but the failure to artificially infect larvae meant that postulates three and four were not accomplished. Nevertheless hyphal bodies, the incubative phase of H.R and H.W, were found in the haemolymph of live larvae on nine separate occasions. Following death, three of the larvae produced H.R only, while the other six larvae produced H.W only. The presence of hyphal bodies in live larvae would be difficult to explain unless the two fungi were pathogenic. To this extent both H.R and H.W can be regarded as pathogenic to larvae of Cecyropa setigera.

2. Cross-infectivity Tests

Although the larvae of eight species of weevils and numerous other beetles were encountered during the sampling programme, often in large numbers, none was found to be infected with either H.R or H.W; Cecyropa setigera was the only host on which these two fungi were found. To investigate the basis of this host specificity four common insects were tested for susceptibility to an injected inoculum.

Method

The susceptibility of the larvae of the following insects was tested:

Pericoptus sp. (five larvae)

Wiseana sp. (Two larvae)
Costelytra zealandica (20 larvae)
Graphognathus leucoloma (80 larvae)

All larvae were injected with a mycelial suspension of either H.R or H.W in a sterile insect saline medium. (A preliminary experiment had established the viability of mycelium in this fluid.) An 'Agla' micrometer syringe with a size Luer 27G hypodermic needle was sterilized and mounted horizontally on a swivel arm. The smaller larvae were injected under observation through a stereoscopic microscope and were held by a pair of screw tightening forceps with two pieces of foam rubber glued to the inside of the two prongs. A dose of 0.005 ml of the suspension was injected into the white fringe weevil and grass grub larvae but 0.01 ml was injected into the larger Pericoptus and Wiseana larvae. The needle wounds were cauterized with a hot needle. Larvae were placed in individual containers together with two inches of sterile damp sand.

Results

Three Pericoptus larvae died after seven days with the symptoms of a bacterial infection. Two lived for six weeks, when on dissection no trace of a fungal infection could be found. Small brown bodies were found in the haemolymph.

The two Wiseana larvae died within 24 hours. The cause of death was unknown.

Eighteen grass-grub larvae died at three days with symptoms of a bacterial infection. Two lived for six weeks when they were dissected. No trace of fungus infection was found but a number of brown bodies were present in the haemolymph. The largest was 0.25mm in diameter.

Fifty-eight white fringe weevil larvae died seven days after injection with the symptoms of a bacterial infection. Four died from one to six weeks later with similar symptoms. At six weeks ten living larvae were dissected. Brown bodies similar to those already mentioned above were present in the haemolymph and could also be seen through the integument of the remaining eight live larvae. The latter eight larvae were still alive four months after injection.

Discussion

The small brown bodies formed in the haemolymph of both the grass grubs and white fringe weevil larvae proved to be melanized masses of cells that had encapsululated the mycelial fragments. In a few of these masses an occasional hypha could be seen projecting. According to Stephens (1963) encapsulation is the main haemolymph defence mechanism against the presence of foreign bodies that are too large for ingestion by phagocytic haemocytes. Encapsulation is accomplished by an agglomeration of leucocytes and lymphocytes about the foreign matter to form a nodule of cells that occasionally develops a brownish-black pigment (Stephens 1963, Jones 1964). The foreign body is rendered harmless by inactivation or death.

It is suggested that such a defence reaction took place to inactivate mycelial cells of both H.R and H.W in the haemolymph of grass grub, white fringe weevil, and Pericoptus larvae. Although an experiment involving the injection of the various spore stages would be preferable it is suggested that the inability of H.R and H.W to infect at least these three larvae in the field was due not only to the uninvestigated effects of the integumental barrier but also to the cellular defence mechnaisms of these insects.

3. General Conclusion

Although the need for further experimentation has been indicated the available evidence does not contradict the suggestion that both H.R and H.W are host specific pathogens of the immature stages of Cecyropa setigera.

B. THE ETIOLOGY OF HYMENOSTILBE R AND HYMENOSTILBE W

Introduction

Etiology is the study of the causes of a disease (Ainsworth and Bisby 1966) or more particularly, the study of the pathogen within the context of its developmental relationship to the host. For most of the pathogenic entomogenous fungi a general developmental pattern is apparent. Usually after penetration of the host's outer boundary the pathogen establishes an infection and proliferates within the body, either as free cells in the blood, or less frequently as penetrant filamentous hyphae. During this process the pathogen exerts an influence on the host that evokes the condition of disease. When the

insect finally succumbs to the disease the fungus saprophytically colonises the dead tissues. On completion of saprophytic colonisation, and if external conditions are favourable, the fungus sporulates and thus the potentiality for infection is renewed.

Hymenostilbe R and Hymenostilbe W deviate little from this general pattern and the different stages of their disease cycle in larvae of Cecyropa setigera are discussed below.

1. Materials and Methods

The methods of Chapter 2 were employed. These largely involved study of paraffin sections, whole mounts of fragmented tissue, and direct observation of diseased and healthy larvae both in the field and in the laboratory.

2. The Disease Cycle

(a) Penetration and infection

The two pathogens, H.R and H.W, exist in the soils of the Manawatu sand country in three forms:

(1) the infected but live larva.
(2) the non-sporulating cadaver.
(3) the sporulating endosclerotium with, or without, synnemata.

Infection from the first two categories is unlikely and it is probable that in both H.R and H.W the sporulating endosclerotium is the sole source of infective units. These could either be conidial state A or the phialospore conidial state while in addition, there is the possibility that gemmae and synnematal cells may cause infection.

Although nothing is known of the ability of gemmae and synnematal cells to infect larvae, one piece of evidence suggests that spores are effective agents of infection, at least in H.R.

During early work on rearing techniques, six larvae were hatched on damp filter paper from eggs laid in the laboratory. They were subsequently transferred to a two inch diameter plastic pill-box, half filled with non-sterile sand from the sampling site at Himatangi. (The sand in the pill-box had previously been passed through a 52 mesh gauze.) The larvae were fed weekly on carrot slivers. After six weeks incubation two of the six larvae were dead from an infection of H.R and were producing synnemata and spores of conidial state A.

Since the mesh of the sieve was small enough to exclude all endosclerotia, but was large enough to let either conidial state A or the phialospore conidial state pass through, this indicates that an infection can occur in the absence of endosclerotia, and would suggest that a spore stage of H.R was capable of infecting larvae. Unfortunately there is no comparable evidence regarding the infectivity of the conidial states of H.W, as attempts to induce infection failed (Section A of this chapter).

The mode of entry of the two pathogens into the host is unknown.

(b) Incubation, symptom expression and death

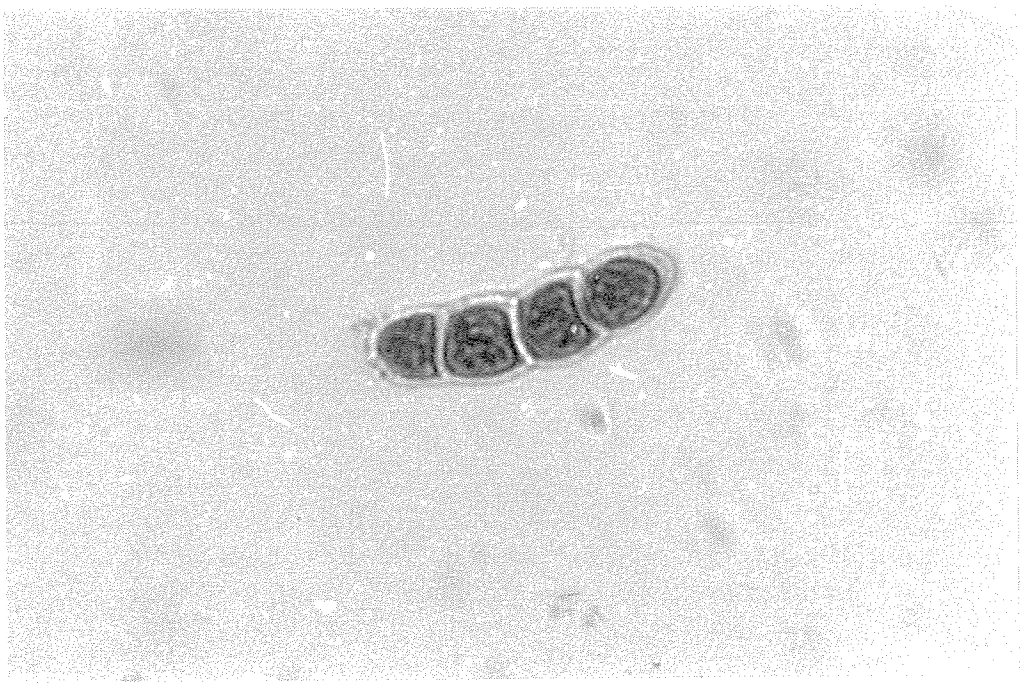
The following cycle of events has only been fully validated for H.W but as larvae infected by H.R produced similar external symptoms it is likely that internal events follow much the same course.

Following penetration and infection the fungus proliferates in the haemolymph in the form of hyphal bodies (Fig. 25). The hyphal bodies occur as single cells, or in chains of up to six cells. They were most frequently observed as being one, two, or three septate. The cells of the hyphal bodies are 8-15 u between septa and 8-12 u wide. The multicelled chains arise from the single cells by an abjunctive process and then separate to produce further free cells.

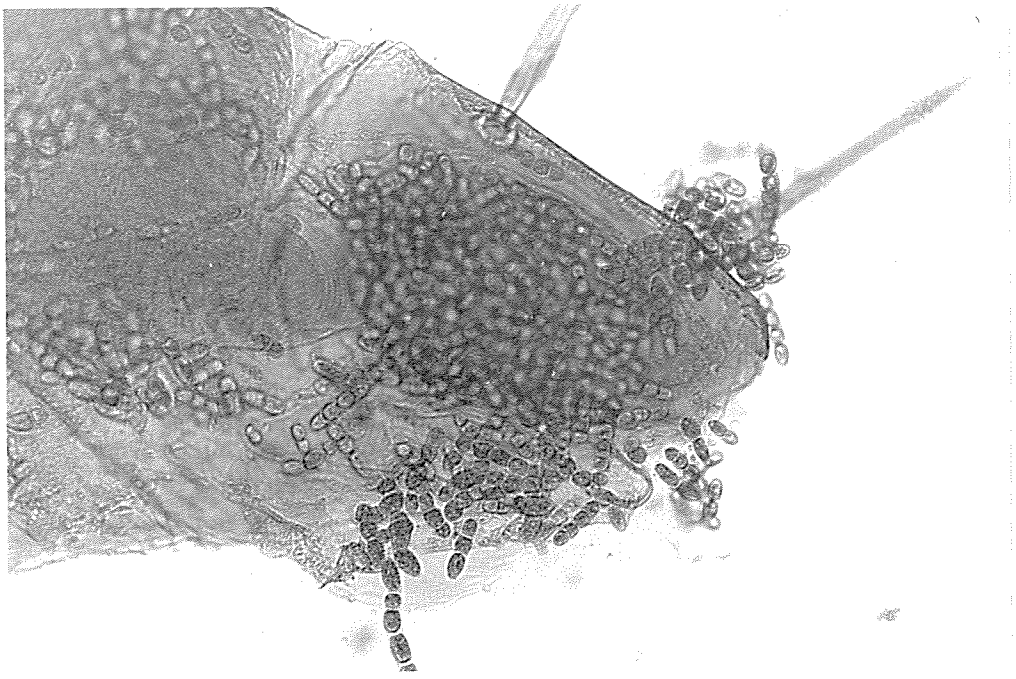
In this manner the hyphal bodies accumulate in the haemolymph and the larvae assume an opaque white appearance and tend to remain quiescent, lying fully extended and slightly flattened out on the surface of a petri dish. In contrast, healthy larvae have a creamy white appearance, are active and generally assume a curled position on their sides when still. They rarely lie in a fully extended prone position. As the infection progresses the larvae display a marked decrease in irritability and as death approaches they became increasingly torpid. At death the body is soft, but retains a needle imprint for several seconds. Forty-eight hours after death the body becomes firm to the touch and on further incubation produces synnemata and conidial state A, as described earlier in Chapter Two.

On the day before death, hyphal bodies pack the haemolymph (fig. 25) greatly decreasing its circulation. They generally do not begin to germinate until the insect is dead although occasional cells with peg-like hyphae 1-5 u long have been observed in a moribund larva. The actual cause of death is unknown but is probably due to the mechanical impediment of

Figure 25



A hyphal body of Hymenostilbe W



Non-germinating densely packed hyphal bodies of Hymenostilbe W found within the haemolymph of a moribund larva

circulation by hyphal bodies and the accumulation of fungal metabolites in the haemolymph, as suggested by Schaerffenberg (1959).

(c) Saprophytic colonisation of the dead tissues

Twenty-four hours after death the hyphal bodies in the haemolymph produce fine hyphae that begin to penetrate muscle and fat tissue (Fig. 26). At 72 hours such hyphae permeate the tissues of the larva (Fig, 26). Finally saprophytic colonisation culminates in the entire replacement of the soft tissues of the host by a hard compact mass of hyphae and gemmae. The hyphal bodies producing the penetrant hyphae now appear as typical gemmae of the endosclerotium.

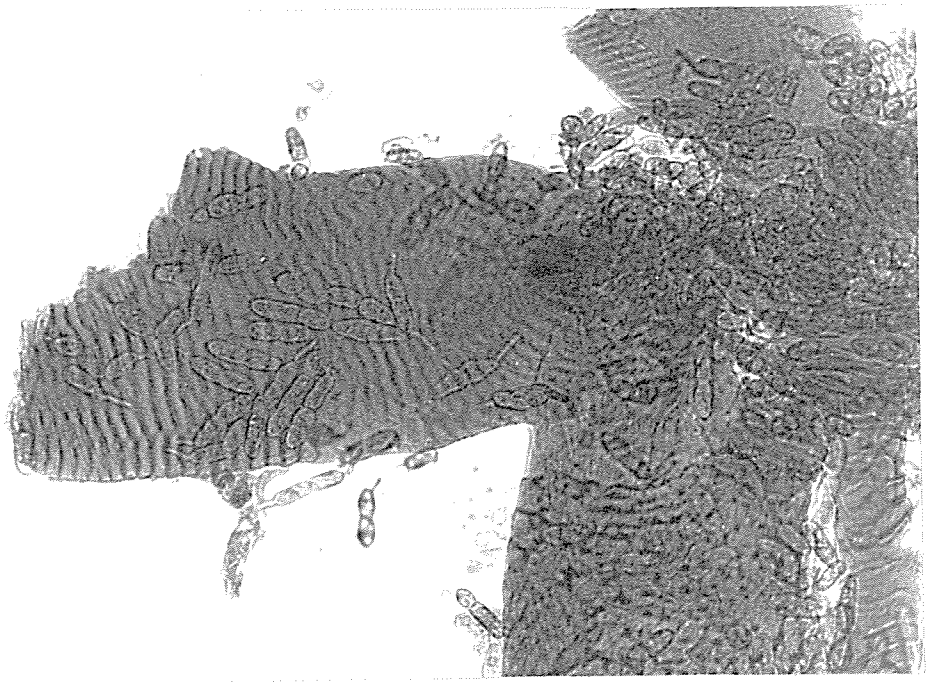
(d) Emergence, sporulation, dissemination and survival

In both H.R and H.W under high humidity in the laboratory hyphae emerge through the integument seven to fourteen days after death to form both conidial state A and synnemata. It is probable that in the field where soil moistures and temperatures fluctuate both seasonally and diurnally, sporulation and production of synnemata does not occur with the same rapidity.

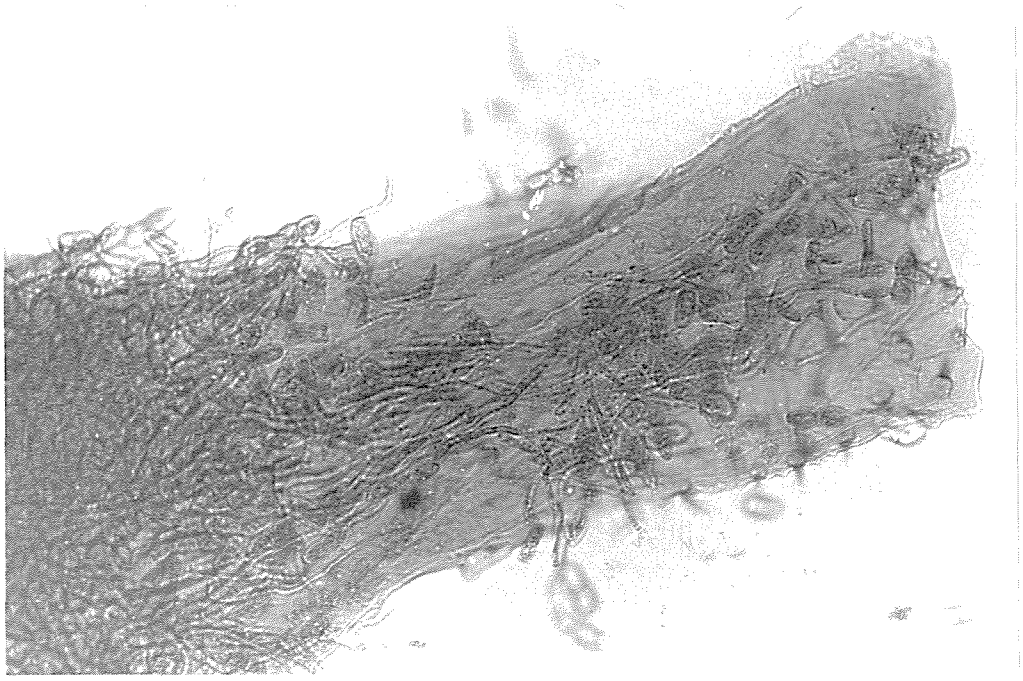
The dissemination of the phialospore conidial state probably occurs by way of soil moisture which permeates rapidly through sandy soils, but the way in which conidial state A is disseminated is more uncertain since its large diameter (75 u) would limit distribution through all but the largest pores of the sandy soil. It is possible that the conidia of conidial state A may break free of the enclosing sterile hyphae and then permeate freely but it has been noted that the small oblong conidia are bound together in a large (50 u diameter) persistent spore ball in the centre of the conidiophore, a condition which would similarly limit passage through the soil pores.

The longevity of conidia of either conidial state in the soil is unknown and it is probable that the long term survival of the pathogens depends largely on endosclerotia, which are packed with thickwalled gemmae. The major survival problem facing the pathogens occurs over the summer period when the sand dries out at the three to ten inch depth at which larvae and endosclerotia are found. The ability of endosclerotia to survive long periods of desiccation was confirmed in the laboratory by the incubation, under conditions of high humidity, of three specimens that had been air dried for three months. All three

Figure 26



Germinating hyphal bodies of Hymenostilbe W penetrating muscle tissue 24 hours after death of the larva



Germinating hyphal bodies of Hymenostilbe W 72 hours after death of the larva

specimens revived to produce new synnemata and conidiophores of conidial state A. Other endosclerotia recovered from the field in a desiccated state have revived under conditions of high humidity to produce conidial state A. It was noted however that the older endosclerotia frequently produced superparasitic fungi following incubation under high humidity.

(e) Factors affecting infection and spread of H.R and H.W

The dominant factors affecting the infection and spread of entomogenous fungi are environmental features, the population density of the host, and the density of the pathogen.

In this study three environmental factors were recorded, the soil temperature, the soil pH, and the soil moisture.

The monthly soil temperature fluctuations at 3-10" depths at Himatangi (Table 5 Chapter 1) are such as to check development of H.R and H.W over the winter months but permit a slow to moderate vegetative growth over spring and autumn and a substantial growth over summer. However, infectivity may not follow a similar course since several workers have established that the temperatures most favourable for vegetative growth need not necessarily be those most favourable for infection, (Madelin, 1963). In view of the relatively narrow temperature range ($7^{o}C$) over spring, summer, and autumn it is possible that temperature may not have a large effect on the infectivity of H.R and H.W over these periods.

The pH of the soils tested varied from pH 5.4 - 7.3, a range unlikely to affect adversely the development of H.R and H.W.

Soil moisture has a strong influence on the development of soil-borne mycoses (Hurpin and Vago, 1958) and is

considered to be the key environmental factor affecting infection and spread of H.R and H.W in the Manawatu sand country. Soil moisture conditions over the year affect the behaviour of larvae and probably the infectivity of the pathogens. Infectivity of the pathogens is most likely to be maximal when soil is moist but warm. Soil moisture levels at the 3 - 10" level at Himatangi reach their peak during the winter and early spring months with the increased rainfall and rise of the water table. The water table recedes during October, November and December and the soils begin to dry out, until over the months late December to early April there is little moisture in the soil. Sporadic rains during this summer period do little to alleviate the dryness of the 3 - 10" level since such moisture is largely retained in the organic matter of the upper soil horizon. The soil moisture level increases once again with the mid and late autumn rains. Since the temperature is thought to be suitable over spring, summer and autumn infectivity of the pathogens is thus most likely to be maximal over the months October to December, and April to late May.

The changing soil moisture levels also affect the density and distribution of the larval population. Oviposition probably occurs early in October, after the cold winter months (Chapter 1), and the larval population density peaks during late October, November and early December, at a time at which conditions are suitable for infection. Following the dry summer period, when both oviposition and infection are proably inhibited, there is a second build up of the larval population

during late March and April. The chances of infection are again high during this period. The extreme dryness of the summer months also causes larvae to exhibit a clumped distribution about the roots of flatweeds that survive dessication by virtue of their deep rooting system. As endosclerotia are similarly clumped around the flatweeds and are found intermingled with live larvae, the larvae are thus exposed to an environment containing very high inoculum levels. Such conditions enhance the chances of infection by the pathogens.

Soil moisture levels can thus be seen as effecting the concentration of both pathogen and host into the same limited environment and to some extent synchronising periods of probable maxiumum infectivity with periods of maximum host density. For these reasons it is suggested that soil moisture is the most important single environmental factor affecting the relationship of the host and pathogens in the Manawatu sand country.

Discussion

Although there is considerable information (Madelin 1960, 1963, 1966) on the etiology of the more common entomogenous fungi, e.g. Beauvaria sp., Metarrhizium anisopliae, Aspergillus sp., Paecilomyces (Isaria) farinosa, there have been no studies on Hymenostilbe spp., or Akanthomyces spp. Also information is scanty on Hirsutella spp. and Cordyceps spp.. Mathieson's (1949) description of the etiology of C. aphodii on Aphodius howitti is one of the most detailed studies available on any species of Cordyceps.

In their etiological characteristics H.R and H.W closely

resemble C. aphodii which, after penetration of the insect (in an undiscovered manner) reproduces in the haemolymph by budding hyphal bodies and follows a subsequent course of development almost identical to that described for H.W. The capacity to assume a yeast-like budding phase in the haemolymph is a characteristic common to many entomogenous fungi and can be regard d as an exhibition of the phenomenon of dimorphism.

Amongst fungi in general dimorphism refers to an environmentally controlled (in this case within an environment of haemolymph) reversible interconversion of yeast and mycelial phases. In a recent review of dimorphism Romano (1966) states that 'the generalization has been made that those fungi that are capable of causing an infection in deep organs of a man and higher animals are characterized by the capacity to develop the yeast form and that the yeast form is that which is characteristically found in infected tissues'. It is suggested that this generalization could be extended to include the majority of entomogenous fungi, including H.W and probably H.R.

The development of a yeast-like form in the invasive stage has also invited speculation as to the relationship of dimorphism to pathogenicity. Ainsworth (1958) has reasoned that in deep infections of the higher animals the live host is an unfavourable environment for growth, when host defence mechanisms are considered, and that a yeast-like phase of growth may be a response to adverse conditions. Madelin (1963) extended the basis of this idea to embrace hyphomycetous entomogenous fungi and generalized that such fungi are

"probably pathogenic chiefly because they are able to tolerate those chemical and physical conditions which are presented by live insect bodies, and thereby are able to escape from their competitors. It is significant that they attain their climax of development - sporulation - only after the death of their hosts."

In presenting this argument Madelin has inferred that the optimum conditions for development of the pathogen, at the expense of the host, occur only after death and that the expression of such a relationship is evidenced by sporulation, - the 'climax' of development.

It can, however, be argued that for host specific fungal pathogens the reverse is true. It seems logical to assume that the climax of pathogenic development is actually that condition when the rate of reproduction of the pathogenic organism at the expense of the host is maximal. Such a condition is most likely to occur in the build-up of hyphal bodies in the haemolymph of the live insect. The haemolymph of an insect is a complex liquid medium serving mainly to transport nutrient materials to tissues and waste products to excretory organs. Such a nutrient environment is thus likely to be suitable for rapid growth of the pathogen, particularly to host specific pathogens that are probably highly adapted to their host. Also rapid reproduction in the early phases of infection and incubation is particularly necessary to defeat the defence mechanisms of the haemolymph. (These primarily involve encapsulation, since hyphal bodies are generally too large for phagocytosis.) Free-floating hyphal bodies that reproduce by some form of

budding represent the best way of accomplishing rapid growth. Such a growth form allows the development of the greatest number of cells with the least synthesis of protoplasm and takes place in an exponential manner, depending on the total number of cells present, rather than on the number of hyphal tips, as in a filamentous growth form. This growth form also serves to pack all parts of the haemocoele with fungal cells and at death maximises the rate of subsequent exploitation, by allowing the less efficient filamentous saprophytic colonisation to be initiated from a multiplicity of points thus decreasing the chance of competition from saprophytic fungi and bacteria. Sporulation, termed the 'climax' of development by Madelin, is more aptly expressed as the fulfilment of pathogenic development, since under present concepts the switch to sporulation is initiated primarily by an exhaustion of nutrients, an exhaustion not likely to occur during the period of most rapid growth.

It is of interest to note that whereas in systemic infections of large animals the yeast phase of pathogenic fungi actively invades host tissues (Ainsworth 1958), in the case of entomogenous fungi penetration of body tissues does not generally occur until after death of the insect. Madelin (1963) speculated that penetration of the internal organs of an insect is beyond the capabilities of isolated cells floating free in the haemolymph, and that hyphal bodies probably require an anchoring point to be able to thrust or digest enzymatically into a discrete tissue. Although the lack of a firm substrate may partially explain the non-germination of hyphal bodies of H.W in live Cecyropa larvae it must be noted that in regions

such as the pleural and terminal lobes, hyphal bodies accumulate in large numbers and are not disturbed by the circulation of the haemolymph. Under these conditions the accumulation of cells is probably sufficiently dense to provide a base for the thrusting action of penetrant hyphae. It is thus probable some factor in the haemolymph additionally affects the germinating capacity of hyphal bodies in live larvae. This factor may be modified either by the death of the insect or by an accumulation of metabolites that reach the required level only when the concentration of hyphal bodies is sufficient to cause death or immobilisation of the larva.

The further events in H.R and H.W of saprophytic colonization of the cadaver are similar to other insects.

Epizootiological studies on fungal pathogens of insects are even rarer than etiological investigations, particularly for soil-borne insects and fungi. There has been no consideration of the epizootiology of species of Hymenostilbe, or Akanthomyces, although Allison (1949) discussed the natural control of the clover weevil Sitona cylindricollis Fabr. by Hirsutella sp., and other Hirsutella spp. have been recorded as attacking populations of citrus scale in Florida (Muma et al, 1961) and spruce budworm in Canada (MacLeod 1959). Literature is similarly lacking for species of Cordyceps, although Hocking (1966) and Mathieson (1949) speculated that C. barnsii and C. aphodii respectively were actively involved in natural control of their respective host populations.

From this present investigation it is suggested that both H.R and H.W can be regarded as enzootic diseases of Cecyropa setigera. Tanada (1963) describes an enzootic disease as one

which is of low incidence and continually present in the population. He goes on to state that an enzootic disease may oscillate at intervals between an enzootic and epizootic phase depending on a complex of interacting factors. It is suggested that the primary factors underlying the relationship of H.R and H.W to Cecyropa setigera are the intermingled, clumped distribution of pathogen and host, the ability of endosclerotia to survive desiccation for extended periods, and the dominant environmental factor of soil moisture levels. Assuming a survival of endosclerotia for a period of up to one year, which is deemed not improbable by Madelin (1963), it is suggested that the mortality of Cecyropa larvae over the spring, summer and autumn months due to H.R and H.W is almost entirely dependent on two factors, namely the soil moisture level, which is a reflection of the rainfall pattern, and the density of the larval population during these periods of moist soil conditions.

SUMMARY

1. Aspects of the bionomics of Cecyropa setigera are presented and compared where relevant to May's (1966) descriptive notes on C. discors.

2. Evidence is presented showing that C.setigera usually has six larval instars but that individuals can pupate after five. The head capsule widths of various instars were determined.

3. Aspects of oviposition, nutrition and behaviour are noted and discussed. A list of natural enemies is presented.

4. Factors affecting vertical distribution of larvae in the soil are considered. It is shown that first instar larvae are restricted to the upper soil horizon and that larvae move deeper into the soil with increasing age. It is suggested that the downward movement is connected with the environmental stability at the lower depths. The greatest number of larvae occur at the 4 - 6" depth.

5. The horizontal distribution of larvae is shown to be clumped. An increase in larval population density occurs at the higher elevations of the undulating sand plain. The absence of larvae in the lower regions of the pasture is thought to be connected to the saturation of soil in these areas due to the rise of the water table in winter.

6. There is a close association of C.setigera with pasture flatweeds. Over the summer period flatweeds are the only common green plant in the higher areas of the sand plain pastures. As such they present a source of food and moisture to the larvae of C.setigera without which they are unlikely to survive. This association is the basis of the clumped distribution discovered.

7. Two entomogenous fungi were found on dead larvae and pupae of C.setigera in the field. Both fungi possessed two distinct conidial states. The morphology of these two conidial states was similar in both fungi. One conidial state (conidial state A) could not be placed in a genus but the other conidial state, produced on synnemata in both fungi, could be placed in the genus Hymenostilbe. One fungus was named HYmenostilbe R the other Hymenostilbe W.

8. An account is given of the morphology of the endosclerotia, synnemata, and conidial states of the two fungi. The conidial state A was similar in both fungi but H.R and H.W could be clearly separated on synnemata and phialide characteristics.

9. Taxonomy of the genus Hymenostilbe is discussed. An unsatisfactory delimitation of Hymenostilbe from form genera of close affinity is indicated. Both H.R and H.W display a close association with Cordyceps aphodii. It is suggested that H.R and H.W could be presented as new species of the genus Hymenostilbe.

10. Both H.R and H.W were isolated to artificial culture. The effect on growth and cultural characteristics of the following variables was tested: media type, source of organic nitrogen, temperature, pH, different nutrient levels of the optimal source of organic nitrogen, and the effects of varying concentrations of this nitrogen source at different incubation periods.

11. Media and methods are described whereby conidial state A, synnemata and the phialospore conidial state were produced on colonies of H.R and H.W in artificial culture. Cultural evidence has been presented whereby it can be stated that both H.R and H.W possess two conidial states. The constant difference in cultural characters between H.R and H.W supports their taxonomic discrimination.

12. H.R and H.W display a close correlation in their nutritional requirements with Hirsutella gigantea, another host specific entomogenous fungi. A suggestion was advanced that the nutritional requirements of H.R , H.W and H.gigantea reflect a close adaptation to the composition of the body tissues of the insect host. The high amino-acid content of the haemolymph was thought to be of particular significance.

13. Attempts to fulfill Koch's postulates to prove the pathogenicity of H.R and H.W to larvae of Cecyropa setigera were unsuccessful.

14. Cross infection to other insect hosts was attempted using an injected inoculum but none of the deaths could be attributed either of the two fungi. Encapsulation of mycelial fragments was noted in Pericoptus sp., Costelytra zealandia, and Graphognath us leucoloma.

15. The etiological events (following penetration in an undiscovered manner) were followed in H.W. The fungus proliferated in the haemolymph in the form of hyphal bodies, that germinated on death to produce penetrant filamentous hyphae.

16. It is considered that the primary factors affecting the relationship of pathogen and host in the field are the intermingled, clumped distribution of larvae and pathogens, the survival ability of endosclerotia and the dominant influence of soil moisture levels.

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APPENDIX I

A. THE SAMPLING PROGRAMME

1. The Locality of Sampling Sites.

The sampling programme was conducted in two areas. The first locality (A) from which the first ten samples were taken was 1½ miles inland along the Himatangi Beach - Himatangi road (324773).[1/] The second locality (B) from which sample eleven was taken was 6½ miles inland from Himatangi Beach along the Himatangi - Palmerston North road (309850).[1/]

The soil of locality A is classified as Hokio strongly mottled sand while locality B was situated on soils of the Himatangi - Foxton Association (Soil Bureau Bulletins 16 and 27).

In area A two plots were established. One plot was on a flat area of the pasture immediately adjacent to the road. The second plot was established 150 yards to the North and was chosen to include a small hillock approximately four feet high. Each plot measured 45 x 21 feet and was divided into eight sub-plots. Each sub-plot was further divided into sixteen sub-units. One core was taken at random from one of the 16 sub-units in each of the eight plots every sampling period (randomized numbers were assigned to the grid pattern and used to determine position of the core). Thus in each sampling trip, eight cores were taken in a stratified random manner from within each of the two plots, making sixteen cores in all. The number of cores in samples eight to ten was extended to include a further 64 cores taken every fifteen paces in several transects across the paddock.

1/ Map references from 'Soil Map of the Manawatu-Rangitikei Sand Country, North Island, New Zealand. (Cowie et al 1967 - Soil Bureau Bull 27)

Each transect was approximately 150 yards long and 40 yards apart.

2. Taking the Cores.

Soil cores were taken using a four inch diameter golf-hole borer. Each of the 16 cores of the two plots was taken at five separate levels at 2", 4", 6", 10" and 14" but in the open field cores of samples eight to ten, (i.e. the additional 64 cores) single cores were taken to a depth of 14 inches. All cores or sections of cores were transferred to plastic bags, labelled and tightly closed with a wire pipe cleaner. They were subsequently removed to the laboratory.

3. The extraction process:

The method used was an adaptation of Salt and Hollick's (1944) method for the extraction of wireworms in pasture. It consisted of 4 distinct steps: (a) a sieving process to remove a large proportion of the sand (b) flotation of the residue to remove the sand from the plant and animal material (c) different wetting to separate plant material from the insects (d) a search of the kerosene/salt solution interface using a stereoscopic microscope on a swinging arm.

All soils cores, or sub-cores from different depths, were sieved separately. Two garden sieves were used, one fitting on top of the other. The upper sieve had a 6 gauge mesh while the bottom sieve possessed a 32 gauge mesh (brass wire). The bottom sieve was sealed around the inner bottom edge with fibre glass to prevent larvae being lodged between the wood and the wire mesh.

The soil was placed on the upper mesh of the two sieves and washed through to the sieve below using a jet of water from a garden hose. This process separated the coarse organic matter from the sample. The top sieve was then removed and the bottom sieve containing water, sand and organic material transferred to a 44 gallon drum full of water. Here the sieve was rotated gently while semi-submerged in the water. Water was not allowed to slop over the edge of the sieve or any material to float out.

This process of gentle rotation caused the sand to wash through the mesh leaving behind the organic matter and insects. The organic material was then brought to a point at the edge of the sieve by rotating the sieve on an angle and then tipped out into plastic bowls approximately 6" in diameter and 5" deep. The residue was washed from the sieve into the bowl using a small quantity of a 20% $MgSO_4$ (W/V) salt solution.

All samples were subjected to this process. The bowls containing the organic matter residue were removed to the lab and filled to within 1½" from the top with the salt solution above. The high specific gravity of the salt solution floated both insects and plant material up to the surface. At this stage a ½ - 1" depth of kerosene was poured into the bowl. This kerosene lay in a layer on the top of the salt solution. The whole mixture was now stirred vigorously for approximately 15 seconds. Arthropod cuticle is wetted by kerosene whereas plant material lacking the lipoid arthropod cuticle is not. Consequently, following this process the insects were now lying on top of the salt solution free of the organic material.

Following this process of differential wetting the contents of the bowl were examined through a stereoscopic microscope and insects picked off the surface of the salt solution/kerosene interface using a fine pair of tweezers. Specimens were placed into Carnoy's fluid which dispersed the kerosene and at the same time acted as a fixative. Head capsule widths of larvae were measured at this stage before transferrence to 75% alcohol for storage.

This method proved satisfactory for the quantitative recovery of Cecyropa larvae from sand samples but was not suitable for the quantitative recovery of endosclerotia of H.R and H.W since the synnemata became entangled in the organic matter beneath the salt solution/kerosene interface. In a brief trial to assess accuracy of the method, preserved larvae were mixed in with previously sieved sand samples to which a quantity of sieved organic matter had been added. The sand samples were of a size equivalent to a normal sand core taken from the field to a depth of 14 inches. Two trials were conducted in which 20 first instar larvae, 20 third and fourth instar larvae and 20 fourth and fifth instar larvae were added. In the first trial 14, 19, 19 larvae were recovered and in the second 16, 18, 20 were recovered. This gave an overall accuracy of 87% and 90% in the two trials.

TABLE I Frequency distribution of larval head capsule widths over eleven samples

H.C.W.*	Sample Number 1	2	3	4	5	6	7	8	9	10	11	Sum
11									1	1	4	6
11.5				1	1				12	2	12	28
12					1	1			27	1	16	46
12.5					1				8		11	20
13			1	1					2	4	10	18
13.5				1	1				1	1	2	6
14		1		2	2	1			6	1	4	17
14.5									2		13	15
15		1				2		1	6	3	10	24
15.5	1	1		1		2					2	7
16	1	1				2			1	1	7	13
16.5									3		7	10
17	2	1			1	2		1	2		8	17
18	3	1	2		1	3		3	1	2	10	26
19		1						2			2	5
20	2		1			2	3	4		1	8	21
21		4				1	2	4			5	16
22	2	4				4		3	1	3	6	23
23	2	1				2		3	1	2	3	14
24	1	3			2	1	5	4	3	5	8	32
25	4	4		2	1	2	2	2	2	3	3	25
26	1	1		2	1	1	1	2	1	1	1	12
27	3	1						1	2	2	1	10
28	6	6	2	5		2	1	2	10	6	1	41
29	4	2	1	1		1		3	4	4	1	21
30	1							2	4	3		10
31	2	1		1				2	1			8
32		1		1								2
Sum	37	35	7	18	12	29	14	39	101	46	154	493

* All head capsule widths in micrometer eyepiece divisions (100 div. equals 2.56 mm).

B. A COMPLETE LIST OF THE COLEOPTERA RECOVERED FROM SAMPLES ONE TO ELEVEN (23rd DECEMBER - 4th APRIL 1968).

ANTHICIDAE:

Anthicus flavitarsis Broun

Anthicus sp.

ANTHRIBIDAE:

Anthribus brouni Sharp

CARABIDAE:

Hypharpax sp.

Clivina rugithorax Putzeys

CURCULIONIDAE:

Peristoreus sp.

Listroderes obliquus Klug.

Hyperodes bonariensis Kusch

Phlyctinus callosus Boheman

Listroderes delaiguei Germain

Asynonychus cervinus Boheman

Desiantha maculata Blackburn

Irenemus compressus Brown

Cecyropa setigera Broun

APIONIDAE

Apion ulicis Forst

SCOLYTIDAE :

Hylastes ater Pake

Dendrotrupes vestitus Broun

ELATERIDAE :

Conoderus exsul Sharp

LATHRIDIIDAE:

Aridius dualis Broun

Melanophthalma sp.

PSELAPHIDAE:

Pselaphophus sp.

SCARABAEIDAE:

Costelytra zealandica White

Pyronota setosa Given

Phycochus lobatus Broun

Aphodius pseudolividus Balth.

STAPHYLINIDAE:

Oxytelus sp. (Oxytelinae)

Conosomus sp. (TACHYPORINAE)

Atheta sp. (ALEOCHARINAE)

STAPHYLININAE (larvae)

TENEBRIONIDAE:

Actizeta sp. nov.

Enneboeus sp.

APPENDIX 2

COMPOSITION AND PREPARATION OF LABORATORY CULTURE MEDIA

Distilled water was used to prepare all media and the volume of preparations adjusted as required before autoclaving. Unless otherwise stated, media were autoclaved at 15 p.s.i. for 20 minutes immediately after preparation and then stored in 250-ml flasks or 5oz and 10oz McCartney bottles.

(a) Potato-Dextrose Agar (Lab PDA)

agar	12gm
potatoes (sliced and peeled)	200gm
dextrose	10gm
water	1000ml

The sliced potatoes were cooked gently for one hour in 500ml of water and the filtrate obtained after straining the mixture through cheesecloth. Agar was melted in 500ml of water, dextrose added and then the two solutions combined.

(b) Carrot Agar

agar	12gm
carrot root juice	200ml
water	800ml

Four large carrots were put through a Braun juice extractor and a filtered extract of carrot obtained. This, plus dissolved agar, was added to the water.

(c) Cornmeal Agar

agar	17gm
cornmeal	20gm
water	1000ml

The cornmeal was cooked for one hour by placing a flask containing the cornmeal suspended in 500ml of water, in a simmering water bath. The cooked cornmeal was filtered through cheesecloth and the filtrate added to 500ml of water in which the agar was dissolved.

(d) Potato-Carrot Agar (Lab PCA)

agar	12gm
carrot	20gm
potato	20gm
water	1000ml

The potato and carrot were cooked gently for an hour in 500ml of water and the procedure for Lab PDA followed except that no dextrose was added.

(e) Starch Agar

oxoid nutrient agar	28gm
starch	20gm
water	1000ml

The starch was added to the oxoid nutrient agar before autoclaving.

(f) Water Agar

agar	12gm
water	1000ml

The agar was dissolved in hot water before autoclaving.

TABLE 1 Dry weight (mg) per colony of Hymenostilbe R at 20 days on various media

Media	Yield dry weight/colony (mg)										Treatment Sum	No.	Mean
Lab. PDA	19.89	24.76	20.16	22.18	23.53	21.05					131.57	6	21.93
Difco Sab. Dextrose	6.94	7.69	7.74	7.35	7.59	8.83					46.14	6	7.69
Difco Br. Heart Inf.	9.67	5.00	8.00	4.74	5.83						33.24	5	6.65
Lab. Carrot	5.27	4.16	4.49	4.60	4.26						22.78	5	4.65
Difco Mycobiotic	3.79	4.26	4.39	3.86	4.50	3.80	3.94	4.41	3.57	4.40	40.92	10	4.09
Difco Sab. Maltose	3.27	3.98	3.88	3.88	3.64	4.38					23.03	6	3.84
Difco Beef Lactose	3.23	2.10	2.42	2.53	2.82						13.10	5	2.62
Lab. Cornmeal	1.62	3.10	3.15	1.62							9.49	4	2.37
Difco PDA	2.44	2.00	1.98	2.24	1.90	2.67					13.23	6	2.21
Lab. PCA	2.12	2.11	2.36	2.43	2.30	1.96					13.28	6	2.21
Oxoid T.G.Y.E.	2.11	1.75	3.78	1.14	1.54	2.54					12.86	6	2.14
Oxoid Milk	1.74	3.00	1.37	1.56	1.08	1.38					10.13	6	1.69
Oxoid PDA	1.64	1.64	1.41	1.51							6.20	4	1.55
Oxoid Tryptone Soya	1.08	2.00	1.68	0.89							5.65	4	1.44
Difco Malt	1.35	1.30	1.46	1.52	1.23	1.72	1.24				9.82	7	1.40
Difco Prune	0.82	0.87	0.72	0.89							3.30	4	0.82
Oxoid Nutrient	0.48	0.54	0.64	0.85	0.31	0.20					3.02	6	0.50
Oxoid Czapek Dox	0.37	0.36	0.41	0.41	0.26	0.35					2.16	6	0.36
Oxoid Tomato Juice	0.37	0.38	0.41	0.11	0.11	0.11					1.49	6	0.25
Lab. Starch	0.20	0.30	0.10	0.20	0.20	0.25	0.15	0.20			1.60	8	0.20
Lab. Water	(0.05)*	(0.05)	(0.05)	(0.05)							0.20	4	0.05
											403.21	120	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Media	20	2,659	133	208**
Error	96	61	0.64	
Total	116	2,720		

Comparison between means.

All mean differences less than 1.39 are not significant (P0.05).
All mean differences greater than 2.07 are significant (P0.05).
The significance of mean differences at intermediate values must be determined individually.

*Individual colony weights could not be determined accurately because of the limitations of the balance. In such cases error was minimised by weighing all colonies together and entering the average individual weight (bracketed) in the body of the table.

TABLE 2 Dry weight (mg) per colony of Hymenostilbe W at 20 days on various media

Media	Yield dry weight/colony (mg)										Treatment Sum	No.	Mean
Lab. PDA	8.79	6.63	4.52	4.06	8.19	8.24					40.43	6	6.74
Lab. Carrot	4.31	4.31	3.44	4.05	5.61						21.72	5	4.32
Difco Sab. Maltose	3.16	2.97	3.78	3.62	4.15	3.69					21.37	6	3.56
Difco Sab. Dextrose	4.92	3.26	2.55	3.00	2.65						16.38	5	3.28
Difco PDA	2.78	3.64	2.93	3.36	1.91						14.62	5	2.92
Difco Br. Heart Inf.	2.15	3.51	3.30	2.69	2.00						13.65	5	2.73
Difco Mycobiotic	2.67	2.95	2.91	2.46	2.83	2.36	3.14	2.12	2.40	2.00	25.84	10	2.58
Oxoid Tomato Juice	1.63	1.80	2.00	1.53	1.76	1.15	1.61				11.48	7	1.64
Oxoid PDA	1.61	1.38	1.45								4.44	3	1.48
Lab. Cornmeal	1.67	0.97	1.00	1.76							5.40	4	1.35
Difco Malt	1.15	1.08	0.91	0.81	1.04	1.00	0.92	0.84			7.75	8	0.97
Difco Beef Lactose	1.10	0.76	0.40	0.63	0.49	0.46					3.84	6	0.64
Oxoid Milk	0.67	0.67	0.70	0.38	0.57	0.74	0.61	0.61			4.95	8	0.62
Oxoid Tryptone Soya	0.20	0.50	0.66								1.36	3	0.45
Oxoid T.G.Y.E.	0.48	0.67	0.59	0.27	0.20						2.21	5	0.44
Lab. PCA	(0.37)	(0.37)	(0.37)	(0.37)	(0.37)	(0.37)	(0.37)				2.59	7	0.37
Oxoid Nutrient	0.50	0.22	0.22	0.47	0.32						1.73	5	0.35
Difco Prune	(0.24)	(0.24)	(0.24)	(0.24)							0.96	4	0.24
Oxoid Czapek Dox	(0.12)	(0.12)	(0.12)	(0.12)	(0.12)	(0.12)					0.72	6	0.12
Lab. Starch	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)			0.48	8	0.06
Lab. Water	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)				0.14	7	0.02
											201.82	123	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Media	20	294	14.7	12.89**
Error	75	102	1.14	
Total	95	396		

Comparison between means.

All mean differences less than 1.88 are not significant (P0.05).
All mean differences greater than 2.61 are significant (P0.05).
The significance of mean differences at intermediate values must be determined individually.

TABLE 3 Dry weight (mg) per colony of Hymenostilbe R at 16 days on a basal dextrose (1%) medium containing various natural supplements (1.5%)

Supplements	Yield: Dry weight/colony (mg)								Treatment: Sum	No.	Mean
Difco Yeast Extract	11.07	10.20	13.49	15.49	15.07	11.70	9.43		86.45	7	12.35
Difco Bacto Tryptone	6.94	6.21	9.09	10.08	8.26	7.85	9.03		57.46	7	8.21
Oxoid Mycological Peptone	5.91	5.69	6.49	5.10	6.26	6.19	7.41	5.82	48.87	8	6.11
Difco Proteose Peptone	7.04	4.71	6.33	4.72	4.72	6.54			34.06	6	5.68
Oxoid Soya Peptone	4.77	3.99	4.17	4.36	4.00	3.32	4.21	4.87	33.69	8	4.21
Oxoid Liver Broth	3.09	4.72	4.31	4.91	3.98	2.62			23.63	6	3.94
Difco Bacto Peptone	2.83	3.10	3.49	4.50	3.40	3.08			20.40	6	3.40
Difco Neopeptone	1.19	1.79	1.07	1.19	2.86	2.52	1.60		12.22	7	1.75
									316.78	55	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Supplement	7	531	75.9	48.4**
Error	47	73	1.55	
Total	54	604		

Comparison between means.

All mean differences less than 1.98 are not significant (P0.05).
All mean differences greater than 2.28 are significant (P0.05).
The significance of mean differences at intermediate values must be determined individually.

TABLE 4 Dry weight (mg) per colony of Hymenostilbe W at 16 days on a basal dextrose (1%) medium containing various natural supplements (1.5%)

Supplements	Yield: Dry weight/colony (mg)								Treatment: Sum	No.	Mean
Difco Yeast Extract	7.97	7.42	8.86	9.61	9.32	7.76	8.88		59.82	7	8.55
Difco Bacto Tryptone	9.02	6.00	4.13	5.45	5.24	4.62	5.46		39.92	7	5.70
Oxoid Liver Broth	3.51	3.12	3.26	2.83	2.71	3.20			18.63	6	3.10
Difco Proteose Peptone	2.73	3.63	2.30	3.18	2.80	2.32	3.28	2.84	23.08	8	2.88
Oxoid Mycological Peptone	2.85	2.78	2.55	2.78	2.45	2.66	2.39		18.46	7	2.64
Oxoid Soya Peptone	2.61	2.20	1.43	1.68	1.78	2.20			11.90	6	1.98
Oxoid Neopeptone	0.62	0.72	0.75	0.69	0.70	0.81	0.80	0.86	5.95	8	0.74
Difco Bacto Peptone	0.44	0.60	0.55	0.55	0.33				2.47	5	0.49
									180.23	54	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Supplements	7	275	39.3	20.8**
Error	46	87	1.89	
Total	53	362		

Comparison between means.

All mean differences less than 2.19 are not significant (P0.05).
All mean differences greater than 2.52 are significant (P0.05).
The significance of mean differences at intermediate values must be determined individually.

TABLE 5

Dry weight (mg) per colony of Hymenostilbe R at 20 days on a yeast extract (3%) - Dextrose (1%) medium at different hydrogen ion concentrations

pH	Yield: Dry weight/colony (mg)										Treatment: Sum	No.	Mean
3.0	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)				0.49	7	0.07
4.0	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)			0.54	8	0.07
4.5	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)			0.46	8	0.06
5.0	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)					0.30	5	0.06
5.5	15.83	16.66	21.49	17.65	19.89	17.48	16.00	15.65			140.65	8	17.58
5.8	26.37	18.63	17.34	12.55	12.30	22.05	18.89	20.98			149.11	8	18.64
6.35	14.94	18.37	19.28	16.76	14.48	20.16	17.27				121.26	7	17.32
6.7	16.05	13.34	12.42	13.15	13.56	24.22					92.74	6	15.46
6.8(control)	27.70	18.52	24.66	18.33	18.19	23.10					130.50	6	21.75
6.9	19.18	17.56	17.78	18.24	15.00	12.81	17.49				118.06	7	16.87
7.4	19.09	16.86	14.96	14.86	13.16	13.11	16.69	11.22			119.95	8	14.99
7.9	7.87	9.49	6.06	7.45	3.52	4.84					39.32	6	6.56
8.5	3.70	5.27	2.80	3.99	5.13	4.81	2.38	3.57	4.63	3.25	39.53	10	3.96
											952.91	94	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
pH	12	6,062	505.17	57.34**
Error	57	502	8.81	
Total	69	6,564		

Comparison between means.

All mean differences less than 4.87 are not significant (P0.05).
All mean differences greater than 6.21 are significant (P0.05).
The significance of mean differences at intermediate values must be determined individually.

TABLE 6 Dry weight (mg) per colony of Hymenostilbe W at 20 days on a yeast extract (3%) - Dextrose (1%) medium at different hydrogen ion concentrations

pH	Yield: Dry weight/colony (mg)								Treatment: Sum	No.	Mean
3.0	(0.05)	(0.05)	(0.05)	(0.05)	(0.05)	(0.05)	(0.05)		0.35	7	0.05
4.0	(0.12)	(0.12)	(0.12)	(0.12)	(0.12)	(0.12)			0.72	6	0.12
4.5	4.00	3.11	1.64	2.92	5.03	3.80			20.50	6	3.42
5.0	11.28	5.91	8.53	6.41	7.02	8.42			47.57	6	7.91
5.5	19.06	10.34	12.34	14.66	18.94	14.44	17.71		107.49	7	15.36
5.8	14.71	9.52	18.33	15.69	13.08	12.44	15.22	9.51	108.50	8	13.57
6.35	13.71	14.13	12.21	10.89	10.50	11.72			73.16	6	12.19
6.7	14.35	9.13	11.08	15.57	11.51	14.26			75.90	6	12.65
6.8(control)	15.43	25.72	16.41	20.24	16.77	18.77			113.34	6	18.89
6.9	17.34	13.53	14.89	12.75	12.56	9.90	18.08	13.27	112.32	8	14.04
7.4	14.80	14.58	16.24	15.87	15.19	18.19	13.00	16.02	123.89	8	15.49
7.9	9.74	11.55	10.40	9.78					41.47	4	10.37
8.5	7.33	6.24	5.38	6.76	6.58	7.88	6.84	7.44	54.45	8	6.81
									879.66	86	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
pH	12	2,862	238.5	43.1**
Error	62	342	5.52	
Total	74	3,204		

Comparison between means.

All mean differences less than 4.06 are not significant (P0.05).
All mean differences greater than 5.25 are significant (P0.05).
The significance of mean differences at intermediate values must be determined individually.

TABLE 7 Dry weight (mg) per colony of Hymenostilbe R at 20 days on a yeast extract (3%) - Dextrose (1%) medium at varying incubation temperatures

Temperature	Yield Dry weight/colony (mg)						Treatment Sum	Treatment Mean
16°C	1.81	1.48	1.93	1.89	1.65	2.14	10.90	1.82
20°C	14.42	10.36	13.02	14.42	13.72	10.44	76.38	12.73
24°C	30.61	37.52	35.00	32.50	34.14	24.19	193.96	32.32
26°C	40.37	31.19	41.85	29.65	32.94	41.23	217.23	36.20
28°C	33.71	31.19	26.38	32.34	38.67	31.19	193.48	32.25
30°C	44.42	43.85	28.90	37.14	23.45	32.17	210.47	35.08
33°C	23.47	34.74	31.81	35.61	31.40	38.40	195.43	32.26
36°C	1.15	0.82	0.05	0.06	0.07	0.04	2.19	0.36
	189.96	191.15	178.45	183.61	176.04	180.34	1,100.04	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Temperature	7	9,905	1,981	94.47**
Error	40	839	20.97	
Total	47	10,744		

Comparison between means.

D0.05 = 8.46
D0.01 = 10.08

TABLE 8

Dry weight (mg) per colony of Hymenostilbe W at 20 days on a yeast extract (3%) - Dextrose (1%) medium at varying incubation temperatures

Temperature	Yield Dry weight/colony (mg)						Treatment Sum	Treatment Mean
16°C	3.25	3.34	4.53	4.94	4.98	4.48	25.46	4.24
20°C	11.91	16.51	7.45	13.12	12.61	18.50	80.10	13.35
24°C	29.10	32.16	23.13	25.21	21.84	31.66	163.10	27.18
26°C	25.32	40.30	26.43	26.87	24.84	35.22	178.98	29.83
28°C	28.14	29.63	25.86	31.13	28.45	29.05	172.26	28.71
30°C	32.70	27.59	33.87	25.94	37.19	30.11	187.40	31.23
33°C	0.48	0.30	0.26	0.12	0.16	0.14	1.46	0.24
36°C	(0.03)	(0.03)	(0.03)	(0.03)	(0.03)	(0.03)	0.18	0.03
	130.93	149.86	121.56	127.36	130.10	149.13	808.94	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Temperature	7	8,115	1,159.3	84.44**
Error	35	481	13.74	
Total	42	8,596		

Comparison between means.

$D_{0.05} = 6.83$
$D_{0.01} = 8.14$

TABLE 9

Dry weight (mg) per colony of Hymenostilbe R at 20 days on a medium containing varying concentrations of yeast extract and dextrose

Factors: yeast extract concentrations (5) x dextrose concentrations (5); 6 culture replications

Yeast Extract	Dextrose	Yield: Dry weight/colony (mg)						Treatment: Sum	Treatment: Mean
0%	0%	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	0.36	0.06
	1%	0.63	0.48	0.68	0.70	0.66	0.63	3.78	0.63
	3%	0.70	0.58	0.62	0.83	0.69	0.78	4.20	0.70
	6%	0.57	0.41	0.62	0.53	0.60	0.55	3.28	0.55
	9%	0.27	0.20	0.33	0.27	0.27	0.27	1.61	0.27
0.75%	0%	2.20	1.86	2.00	1.72	1.83	2.03	11.64	1.44
	1%	12.58	11.87	15.30	16.84	15.75	11.82	84.16	14.03
	3%	17.22	19.00	20.39	16.43	19.76	19.00	111.80	18.63
	6%	17.37	17.86	17.60	15.93	16.87	17.51	103.14	17.19
	9%	12.21	18.33	11.16	16.06	14.21	14.50	86.47	14.41
1.5%	0%	3.66	4.15	3.68	4.24	2.85	3.38	21.96	3.66
	1%	22.00	24.94	21.28	18.94	24.00	22.16	133.32	22.22
	3%	24.16	28.12	36.14	43.96	29.44	28.18	190.00	31.66
	6%	31.36	29.39	31.10	24.17	33.58	26.86	176.46	29.41
	9%	24.96	19.09	28.84	26.23	19.29	23.59	142.00	23.67
3.0%	0%	6.29	7.32	8.03	6.31	10.49	7.58	46.02	7.67
	1%	26.37	18.96	25.62	21.38	22.51	20.10	134.94	22.49
	3%	44.43	40.54	46.45	47.55	49.00	48.54	276.51	46.09
	6%	49.27	60.07	56.92	39.20	45.38	46.00	296.84	49.47
	9%	53.15	41.58	37.10	48.26	34.74	42.99	257.82	42.97
6.0%	0%	21.50	15.50	15.52	13.78	16.48	13.22	96.00	16.00
	1%	40.52	36.39	35.85	30.05	44.59	33.59	220.99	36.83
	3%	42.75	54.53	32.24	41.40	34.82	36.10	241.84	40.31
	6%	40.73	30.86	39.77	40.16	35.02	40.73	227.28	37.88
	9%	27.57	18.12	7.45	14.53	21.00	10.76	99.43	16.57
		522.53	500.21	494.75	489.53	493.90	470.93	2,971.85	

Continued.....

TABLE 9 Continued.....

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Yeast Extract (Y.E.)	4	21,357	5,339	324.56**
Dextrose (D.)	4	9,117	2,279	138.54**
Interaction (D.xY.E.)	16	6,145	384	23.34**
Error	120	1,974	16.45	
Total	144	38,593		

Comparison between means.

Both dextrose and yeast extract	DO.05 = 2.94 DO.01 = 3.53
Dextrose x yeast extract	DO.05 = 8.48 DO.01 = 9.80

TABLE 10

Dry weight (mg) per colony of Hymenostilbe W at 20 days on a medium containing varying concentrations of yeast extract and dextrose

Factors: yeast extract concentrations (5) x dextrose concentrations (5); 6 replications

Yeast Extract	Dextrose	Yield: Dry weight/colony (mg)						Treatment: Sum	Treatment: Mean
0%	0%	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	0.12	0.02
	1%	(0.11)	(0.11)	(0.11)	(0.11)	(0.11)	(0.11)	0.66	0.11
	3%	(0.14)	(0.14)	(0.14)	(0.14)	(0.14)	(0.14)	0.84	0.14
	6%	(0.05)	(0.05)	(0.05)	(0.05)	(0.05)	(0.05)	0.30	0.05
	9%	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	(0.06)	0.36	0.06
0.75%	0%	2.05	2.51	1.88	2.45	2.00	2.44	13.33	2.22
	1%	10.91	13.55	10.80	14.46	11.07	12.21	73.00	12.16
	3%	17.00	14.69	16.70	12.60	19.39	16.00	96.38	16.06
	6%	13.51	12.07	13.21	12.15	14.59	10.51	76.04	12.67
	9%	12.76	6.23	6.92	5.64	17.07	7.83	56.45	9.42
1.5%	0%	3.28	4.00	3.23	2.41	3.70	3.36	19.98	3.33
	1%	16.41	14.18	16.60	19.19	20.28	16.14	102.80	17.13
	3%	27.26	21.11	19.16	22.70	24.42	26.48	141.13	23.52
	6%	17.71	16.56	22.69	15.45	18.33	19.12	109.86	18.31
	9%	13.55	10.29	15.07	6.50	11.65	9.55	66.61	11.10
3.0%	0%	5.68	5.19	4.02	5.51	4.53	3.81	28.56	4.76
	1%	15.45	15.30	13.24	12.54	18.00	17.33	91.86	15.31
	3%	25.75	29.70	28.45	28.82	23.90	30.56	167.18	27.86
	6%	35.38	31.39	31.27	36.29	23.47	33.47	191.27	31.88
	9%	23.46	22.14	23.43	18.52	22.15	31.00	140.70	23.45
6.0%	0%	6.55	4.65	5.00	6.44	4.97	6.35	33.96	5.66
	1%	26.00	15.04	15.34	17.28	18.92	21.00	113.58	18.93
	3%	14.33	20.88	19.32	22.68	16.89	13.89	107.99	18.00
	6%	23.11	25.27	18.50	20.09	21.50	24.07	132.54	22.09
	9%	3.11	4.10	4.97	4.90	5.20	2.26	24.54	4.09
		313.64	289.23	290.18	287.00	302.23	307.76	1,790.04	

Continued.....

TABLE 10 Continued.....

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Yeast Extract (Y.E.)	4	6,885	1,721	223.5**
Dextrose (D.)	4	4,044	1,011	131.3**
Interaction (D.xY.E.)	16	2,455	153.4	19.92**
Error	100	770	7.70	
Total	124	14,154		

Comparison between means.

Both dextrose and yeast extract	DO.05 = 2.00 DO.01 = 2.42
Dextrose x yeast extract	DO.05 = 5.27 DO.01 = 5.98

TABLE II

Growth response of Hymenostilbe R to varying concentrations of yeast extract and dextrose at different incubation periods

Factors: incubation periods (6) x dextrose concentrations (3) x yeast extract concentrations (3); 3 replications

Days	Dextrose	Yeast Extract	Yield: Dry weight/colony (mg)			Treatment: Sum	Treatment: Mean
		0.75%	0.32	0.35	0.33	1.00	0.33
	1%	1.5%	0.31	0.21	0.23	0.75	0.25
		3.0%	0.23	0.16	0.23	0.62	0.21
		0.75%	0.28	0.24	0.33	0.85	0.28
5	2.47%	1.5%	0.23	0.29	0.20	0.72	0.24
		3.0%	0.24	0.23	0.20	0.67	0.22
		0.75%	0.18	0.15	0.20	0.53	0.18
	6%	1.5%	0.30	0.28	0.29	0.87	0.29
		3.0%	0.19	0.15	0.21	0.55	0.18
		0.75%	0.98	0.84	1.12	2.94	0.98
	1%	1.5%	0.72	0.67	0.85	2.24	0.72
		3.0%	0.65	0.51	0.63	1.79	0.60
		0.75%	0.86	1.14	0.91	2.91	0.97
8	2.47%	1.5%	0.82	0.74	0.74	2.30	0.77
		3.0%	0.80	0.72	0.76	2.28	0.76
		0.75%	0.68	0.59	0.56	1.83	0.61
	6.0%	1.5%	0.97	0.97	0.86	2.77	0.92
		3.0%	0.52	0.49	0.38	1.39	0.46
		0.75%	3.62	3.31	3.11	10.04	3.35
	1%	1.5%	3.26	3.44	3.92	10.62	3.54
		3.0%	3.32	5.14	4.65	13.11	4.37
		0.75%	4.21	3.41	3.45	11.07	3.69
12	2.47%	1.5%	3.70	3.50	4.10	11.30	3.77
		3.0%	5.51	4.57	4.63	14.71	4.90
		0.75%	1.93	1.53	1.81	5.27	1.76
	6%	1.5%	4.28	3.59	4.08	11.95	3.98
		3.0%	3.55	3.03	3.42	10.00	3.33
		0.75%	10.34	9.98	10.37	30.69	10.23
	1%	1.5%	9.09	8.57	7.84	25.50	8.50
		3.0%	14.59	10.44	15.03	40.06	13.35
		0.75%	7.91	8.56	7.29	23.76	7.92
16	2.47%	1.5%	13.79	14.29	12.36	40.44	13.48
		3.0%	16.15	19.03	14.42	49.60	16.53

Continued.....

TABLE 11 Continued.....

		0.75%	6.99	7.57	6.74	21.30	7.10
	6%	1.5%	14.24	12.69	13.74	40.67	13.56
		3.0%	13.56	11.90	9.47	34.93	11.64
		0.75%	12.79	13.50	11.41	37.70	12.57
	1%	1.5%	13.19	15.21	12.99	41.39	13.80
		3.0%	23.98	20.24	17.54	61.76	20.59
		0.75%	21.45	19.17	18.09	58.71	19.57
20	2.47%	1.5%	26.52	25.84	27.59	79.95	26.65
		3.0%	32.62	29.70	30.83	93.15	31.05
		0.75%	14.36	16.09	14.32	44.77	14.92
	6%	1.5%	32.92	30.86	27.49	91.27	30.42
		3.0%	31.49	38.20	38.84	108.53	36.18
		0.75%	22.70	22.97	23.83	69.50	23.17
	1%	1.5%	31.09	27.45	26.77	85.31	28.44
		3.0%	46.34	45.99	47.50	139.83	46.61
		0.75%	35.66	36.45	40.39	112.59	37.53
28	2.47%	1.5%	45.37	45.78	50.62	141.77	47.26
		3.0%	63.53	56.73	60.37	180.63	60.21
		0.75%	50.61	42.25	42.83	135.69	45.23
	6%	1.5%	89.81	90.97	89.80	270.08	90.03
		3.0%	109.26	91.71	100.45	301.42	100.47
			853.01	811.98	821.09	2,486.08	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	Ftest
Days	5	56,282	11,256.0	3,092.3**
Dextrose	2	2,398	1,199.0	329.4**
Yeast Extract (Y.E.)	2	2,195	1,097.5	301.5**
Days x Dextrose	10	8,118	811.8	223.0**
Days x Y.E.	10	3,890	389.0	106.9**
Dextrose x Y.E.	4	611	152.7	41.95**
Days x Dextrose x Y.E.	20	1,424	121.2	33.29**
Error	108	393	3.64	
Total	161	75,311		

Comparison between means.

Days	DO.05 = 1.48
	DO.01 = 1.74
Dextrose and Yeast Extract	DO.05 = 0.83
	DO.01 = 1.03
Days x Dextrose and Days x Y.E.	DO.05 = 3.22
	DO.01 = 3.69
Yeast Extract x Dextrose	DO.05 = 2.01
	DO.01 = 2.35
Days x Dextrose x Yeast Extract	DO.05 = 6.31
	DO.01 = 6.60

TABLE 12

Growth response of Hymenostilbe W to varying concentrations of yeast extract and dextrose at different incubation periods

Factors: incubation periods (6) x dextrose concentrations (3) x yeast extract concentrations (3); 3 replications

Days	Dextrose	Yeast Extract	Yield: Dry weight/colony (mg)			Treatment: Sum	Treatment: Mean
		0.75%	0.24	0.21	0.23	0.68	0.23
	1%	1.5%	0.20	0.24	0.25	0.69	0.23
		3.0%	0.20	0.22	0.26	0.68	0.23
		0.75%	0.12	0.17	0.10	0.39	0.13
5	2.47%	1.5%	0.18	0.19	0.18	0.55	0.18
		3.0%	0.15	0.14	0.16	0.45	0.15
		0.75%	0.17	0.14	0.14	0.45	0.15
	6%	1.5%	0.19	0.23	0.15	0.57	0.19
		3.0%	0.15	0.17	0.15	0.47	0.16
		0.75%	0.56	0.48	0.57	1.61	0.54
	1%	1.5%	0.86	0.78	0.84	2.48	0.83
		3.0%	0.77	0.61	0.90	2.28	0.76
		0.75%	0.61	0.65	0.48	1.74	0.58
8	2.47%	1.5%	0.85	0.79	0.76	2.39	0.80
		3.0%	0.94	0.86	0.89	2.69	0.90
		0.75%	0.49	0.51	0.56	1.56	0.52
	6%	1.5%	0.46	0.42	0.55	1.43	0.48
		3.0%	1.10	0.73	0.54	2.37	0.79
		0.75%	3.06	3.21	3.09	9.36	3.12
	1%	1.5%	3.48	3.98	3.05	10.51	3.50
		3.0%	5.01	3.83	4.80	13.64	4.55
		0.75%	4.04	3.97	4.04	12.05	4.02
12	2.47%	1.5%	3.62	3.52	2.86	10.00	3.33
		3.0%	4.33	5.15	5.57	15.05	5.02
		0.75%	1.90	1.85	1.66	5.41	1.80
	6%	1.5%	2.89	2.50	3.30	8.69	2.90
		3.0%	4.49	5.65	5.20	15.34	5.10
		0.75%	7.12	7.30	6.70	21.12	7.04
	1%	1.5%	9.42	7.60	8.66	25.68	8.56
		3.0%	10.72	10.93	11.83	33.48	11.16
		0.75%	8.07	8.72	9.91	26.70	8.90
16	2.47%	1.5%	11.32	11.72	14.22	37.26	12.42
		3.0%	17.83	18.18	15.82	51.83	17.28
		0.75%	5.87	5.33	4.95	16.15	5.38
	6%	1.5%	10.06	10.08	8.15	28.29	9.43
		3.0%	16.00	19.00	18.66	53.66	17.88

Continued.....

TABLE 12 Continued.....

		0.75%	10.77	8.07	9.83	28.67	9.56
	1%	1.5%	10.02	12.15	10.88	33.05	11.02
		3.0%	16.17	18.01	21.40	55.58	18.53
		0.75%	13.75	15.84	17.52	47.11	15.70
20	2.47%	1.5%	19.79	25.26	26.72	71.77	23.92
		3.0%	32.39	27.45	24.63	84.47	28.16
		0.75%	13.98	15.62	15.46	45.06	15.02
	6%	1.5%	28.46	15.07	24.38	67.91	22.64
		3.0%	46.02	41.11	42.90	130.03	43.34
		0.75%	15.77	14.70	14.11	44.58	14.86
	1%	1.5%	21.53	17.70	23.30	62.53	20.84
		3.0%	36.93	37.15	33.92	108.00	36.00
		0.75%	28.23	25.59	29.58	83.40	27.80
28	2.47%	1.5%	41.97	38.04	40.96	120.97	40.00
		3.0%	52.37	48.81	48.29	149.47	49.82
		0.75%	55.18	53.69	65.36	174.23	58.08
	6%	1.5%	65.10	68.37	61.76	195.23	65.08
		3.0%	119.58	92.60	104.42	316.60	105.53
			765.47	715.29	755.60	2,236.36	

Analysis of Variance.

Source	d.f.	S.S.	M.S.	F test
Days	5	42,819	8,563.8	1,712.8**
Dextrose	2	3,453	1,726.3	253.1**
Yeast Extract (Y.E.)	2	2,582	1,291.1	189.3**
Days x Dextrose	10	10,554	1,055.4	154.75**
Days x Y.E.	10	3,401	340.1	49.87**
Dextrose x Y.E.	4	590	147.6	21.64**
Days x Dextrose x Y.E.	20	879	43.95	6.44**
Error	108	736	6.82	
Total	161	65,014		

Comparison between means.

Days	DO.05 = 2.07
	DO.01 = 2.44
Dextrose and Yeast Extract	DO.05 = 1.20
	DO.01 = 1.50
Days x Dextrose and Days x Y.E.	DO.05 = 4.41
	DO.01 = 5.05
Yeast Extract x Dextrose	DO.05 = 2.77
	DO.01 = 3.24
Days x Dextrose x Yeast Extract	DO.05 = 7.85
	DO.01 = 9.06