Survival of *Entomophthora* spp. in Laboratory Cultures

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The prolong survival of cultures of *Entomophthora coronata* (COST.) KEVORK. [= *Conidiobolus coronatus* (COST.) TYRRELL and MACLEOD], *E. virulenta* HALL and DUNN, *E. thaxteriana* (PETCH) HALL and BELL, and *E. sphaerosperma* FRES. under air-tight condition appeared to be dependent on the hyphae, some of which were knobby and might become chlamydospores. The conidia were very short lived, and the resting spores produced by *E. virulenta* also did not survive for long periods. The conidia survived longer at 5°C than at room temperatures.

Under air-tight condition, the metabolic activity of the fungus was reduced as indicated by the production of only a few small conidia and narrow hyphae. As air became available more conidia were produced, the hyphae increased in size, became vacuolated, fragmented, and then collapsed and disintegrated.

INTRODUCTION

In the culture of certain entomophthoraceous fungi, a serious difficulty is the necessity to transfer the cultures at frequent intervals in order to maintain the fungi (see MÜLLER-KÖGLER, 1965). PRASERTPHON (1967) made an important discovery in the culture of these fungi when he found that cultures, maintained in tubes kept under air-tight condition, could be stored without subculturing for more than a year and possibly more than two years. We found that some of the cultures given to us (Y. TANADA) by Dr. PRASERTPHON were still viable after over 5 years.

We have investigated the survival and development of the entomophthoraceous fungi under air-tight condition.

MATERIALS AND METHODS

Dr. PRASERTPHON left cultures of *Entomophthora coronata* (COST.) KEVORK. [= *Conidiobolus coronatus* (COST.) TYRRELL and MACLEOD], *E. virulenta* HALL and DUNN, *E. thaxteriana* (PETCH) HALL and BELL, and *E. sphaerosperma* FRES. with Y. TANADA and they were used in this study. The cultures had been maintained on SABOURAUD's dextrose agar with 2% yeast extract (SDAY) in air-tight tubes covered with rubber snap caps (Pyrex) as described by PRASERTPHON (1967). They had been held in a glass cabinet under laboratory room conditions. Some of the cultures were over 5 years old and others had been transferred about 3 years ago.

The fungi were cultured in SDAY medium. A few tests were conducted with tube cultures, but most of the tests were with slide cultures that were prepared as follows.

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Fig. 1. Diagram of a slide culture of *Entomophthora* sp.

The hardened medium in a test tube were liquefied by heat and then cooled to about 40 °C. The fungus was added to the medium. Three drops of the medium were then placed with a capillary pipette on a sterilized glass slide and covered with a sterilized cover glass (18 mm²). Three edges of the cover glass were sealed with paraffin as shown in Fig. 1.

In tests under accessible air conditions, the slide culture was placed in a sterilized petri dish whose bottom was lined with a sterilized moistened filter paper. The filter paper was kept moist by adding water at 3-day intervals. The cultures were held at 25°C. For the tests under air-tight condition, the slide culture was placed in a sterilized tube, 3.8 × 8.8 cm, containing a piece of moistened sterilized filter paper in the bottom of the tube. The tube was covered with a sterilized rubber stopper, sealed with paraffin, and then completely immersed in water in a beaker.

The treatments under air-tight condition were as follows: (1) slide cultures transferred immediately to air-tight tubes and considered as being 0 hr at non air-tight condition; (2) slide cultures transferred from the petri dishes after 20 hr; (3) after 40 hr; (4) control slide cultures, which had access to air, were kept in a petri dish.

The growth status of the fungi was examined with the light microscope. To confirm the viability of the fungi, it was transferred to freshly-prepared SDAY medium. Portions of the fungi were also stained with Amann lactophenol-anilin blue, which clearly indicated the presence of protoplasm in the spores and hyphae. Protoplasm when live stained blue, and pink when dead.

The number of conidia produced in the slide cultures was determined at 10 × 10 magnification of the light microscope. All of the conidia in a single field were counted. Widths of a specified number of randomly selected hyphae were measured under the light microscope.

In the conidial germination studies, the conidia were collected on clean glass slides which were held at room temperatures and at 5°C. After the designated period of storage, the conidia on the slides were exposed to high humidity in petri dishes lined with moistened filter paper and held at room temperatures.

RESULTS

The old cultures of *E. coronata*, *E. virulenta*, and *E. thaxteriana*, which had been inoculated on August 23, 1966, were nonviable (Fig. 2A, C), but those prepared on February 25, 1967 (Fig. 2B, D) and later were still viable after a period of over 5 years

Fig. 2. Cultures of *Entomophthora* spp. maintained under air-tight and accessible air conditions. A and C, *E. coronata* and *E. thaxteriana* grown under air-tight condition since 1966 showing dissolution of mycelium and were nonviable; B and D, The same two fungi grown under air-tight condition since 1967 showing viable mycelium with knobby hyphae; E and G, *E. coronata* and *E. thaxteriana* grown for 14 days under available air condition showing thick hyphae. Note abundant conidia in E; F and H, The same two fungi grown under air-tight condition for 14 days showing thin hyphae.

(Table 1). In the case of *E. sphaerosperma*, the culture prepared on February 25, 1967 was dead but that of June 20, 1969 was viable. The old culture of *E. thaxteriana* had resting spores but those of the other fungus species had no conidium or resting spores. The resting spores of *E. thaxteriana* did not germinate, and since their contents did not stain intensely with Amann lactophenol-anilin blue, they appeared to be dead.

The hyphae of the nonviable cultures were shrunken, collapsed, disintegrated, and stained poorly (Fig. 2A, C). On the other hand, the viable old cultures had a few hyphae which appeared to be living and their contents stained intensely blue

Table 1. SURVIVAL OF *Entomophthora* spp. IN CULTURES MAINTAINED IN AIR-TIGHT TUBES AND HELD AT ROOM TEMPERATURES^a

Fungus species	Date of culture inoculation ^b	Date of transfer	Age of culture (days)	Viability
<i>E. coronata</i>	Jun. 20, 1969	Sep. 8, 1972	1176	viable
	Feb. 25, 1969	Dec. 8, 1972	2113	viable
	Aug. 23, 1966	Dec. 8, 1972	2299	nonviable
<i>E. virulenta</i>	Feb. 25, 1967	Sep. 8, 1972	2022	viable
	Aug. 23, 1966	Dec. 8, 1972	2299	nonviable
<i>E. thaxteriana</i>	Feb. 25, 1967	Sep. 8, 1972	2022	viable
	Aug. 23, 1966	Dec. 8, 1972	2299	nonviable
<i>E. sphaerosperma</i>	Jun. 20, 1969	Sep. 8, 1972	1176	viable
	Feb. 25, 1967	Dec. 8, 1972	2113	nonviable

^a SABOURAUD's dextrose agar with 0.2% yeast extract, was used for culturing.

^b The cultures of 1966 and 1967 were inoculated by S. PRASERTPHON and those of 1969 by Y. TANADA.

(Fig. 2B, D). Some of the mycelium of the viable old cultures, especially those of *E. coronata*, had knobby hyphae which might become chlamydospores.

When held as cultures in test tubes plugged with cotton, *E. coronata* survived for 105 days, *E. virulenta* for 121 days, *E. thaxteriana* and *E. sphaerosperma* for 102 days. Under air-tight condition and on slide cultures, all 4 species survived for 176 days even after the cultures had been exposed to 20 and 40 hr to air. However, the control cultures, which had access to air, were not viable at the end of 176 days. The survival of these fungi appeared to be not by conidia or resting spores but by the hyphae.

The morphological characteristics of these cultures after 67 days of growth were as follows. For *E. virulenta* held at air-tight condition throughout, the hyphae were very slender, filled with cytoplasm, and stained strongly blue. There were very few conidia, which were small and about 1/5 that of the average conidium. The interior of the conidia stained pink and the periphery blue. At 20 hr exposure to air, the hyphae were slender and stained blue. Most of the conidia had germinated, and the ungerminated ones stained as above. After 40 hr of exposure to air, the hyphae showed good growth, some were vacuolated, and stained strongly blue. More conidia were present than in the above 2 categories, but most of them had germinated and were empty, and others stained pink in the interior, and blue or unstained in the periphery. The control, which had access to air throughout, showed good growth but the hyphae had fragmented to small pieces, and over 50% were vacuolated and stained less intensely than the above 3 categories. There were many conidia but most of them had germinated and stained as those of 40-hr exposure. No resting spores were produced by *E. coronata*.

The cultures of *E. virulenta* and *E. thaxteriana* had hyphae resembling those of *E. coronata* at various periods of exposure to air. The conidia of *E. virulenta*, however, had changed to resting spores, and their numbers increased with the period of exposure to air. The resting spores stained pink in the interior and the periphery was unstained. None of the spores had germinated.

The cultures when held for 176 days showed the following characteristics. In *E. coronata*, the cultures, which were held under air-tight condition throughout, had well grown hyphae which were slender but not as slender as at 67 days. The hyphae

were knobby, filled with cytoplasm, and stained from light to dense blue. Some of the conidia had germinated or broken down and others stained pink in the interior and deep blue or unstained in the periphery. The 20-hr aerated culture had well grown hyphae, but some were vacuolated and stained pink. Knobs were formed on the hyphae. The 40-hr aerated culture was well developed, and had vacuolated, empty, fragmented hyphae which stained pink, light, or dense blue. Some hyphae were knobby. The hyphae of the control, which had been exposed throughout to air, were mostly vacuolated, empty or collapsed. They were broad and stained intensely pink or a light blue. A few hyphae were knobby and stained pink. The number of conidia which had germinated increased with the period of exposure to air. No resting spores were produced.

The cultures of *E. virulenta* were essentially similar to those of *E. coronata*, except that they had resting spores instead of conidia. In *E. thaxteriana*, the cultures showed a slightly more advanced stage of growth and deterioration than in *E. virulenta*. Even the cultures, maintained under air-tight condition, grew slightly and had a few well grown hyphae, some of which had fragmented and were vacuolated or empty, and others were knobby. In the control, the hyphae were collapsed and most of them empty. There were no resting spores.

E. coronata and *E. thaxteriana*, under different periods of exposure to air and after 14 days of culture, produced more conidia in cultures exposed for longer periods to air (Table 2). Over a 100 times more conidia were produced in the control cultures

Table 2. FORMATION OF CONIDIA BY *E. coronata* AND *E. thaxteriana* UNDER DIFFERENT PERIODS OF EXPOSURE TO AIR AND AFTER 14 DAYS OF CULTURE^a

Period of exposure to air (hr)	<i>E. coronata</i>			<i>E. thaxteriana</i>		
	No. of conidia	No. of fields	No. conidia per field	No. of conidia	No. of fields	No. conidia per field
0	122.3	57.0	2.15	51.0	58.0	0.88
20	259.0	54.7	4.74	170.7	56.0	3.05
40	560.3	57.3	9.77	280.0	55.7	5.03
Control (throughout)	12643.7	55.7	227.13	10991.0	56.7	193.96

^a Average of three counts.

Table 3. VARIATION IN THE WIDTHS OF HYPHAE OF *E. coronata* AND *E. thaxteriana* UNDER DIFFERENT PERIODS OF EXPOSURE TO AIR AND AFTER 14 AND 176 DAYS OF CULTURE^a

Period of exposure to air (hr)	14-day culture				176-day culture			
	<i>E. coronata</i>		<i>E. thaxteriana</i>		<i>E. coronata</i>		<i>E. thaxteriana</i>	
	Mean	Standard error	Mean	Standard error	Mean	Standard error	Mean	Standard error
0	4.63	0.80	3.60	0.86	7.33	1.99	5.48	1.28
20	5.05	0.95	3.74	0.92	7.60	2.01	6.22	1.67
40	5.81	1.12	3.79	0.92	8.52	1.84	6.56	1.27
Control (throughout)	7.10	1.64	5.93	1.16	6.08	1.59	6.32	1.64

^a Widths expressed in microns.

Table 4. GERMINATION OF ISOLATED CONIDIA OF *Entomophthora* spp. HELD AT ROOM TEMPERATURES FOR UP TO 10 DAYS

Fungus species	Per cent of germinating conidia ^a									
	Days after collection									
	1	2	3	4	5	6	7	8	9	10
<i>E. coronata</i>	89.0	85.1	76.8	62.4	8.8	0				
<i>E. virulenta</i>	89.9	83.6	80.8	62.5	5.8	0				
<i>E. thaxteriana</i>	94.4	88.0	85.4	79.3	69.3	67.0	22.9	17.2	12.0	0

^a Percentage based on 500 conidia examined at each period. The results are averages of two examinations.

Table 5. GERMINATION OF ISOLATED CONIDIA OF *Entomophthora* spp. HELD AT 5°C AND ROOM TEMPERATURES FOR 12 TO 18 DAYS AND THEN EXPOSED TO FAVORABLE TEMPERATURE AND HUMIDITY

Fungus species	Per cent of germinating conidia ^a					
	Days after collection					
	12		15		18	
	5°C	Room temp.	5°C	Room temp.	5°C	Room temp.
<i>E. coronata</i>	76.7	0	2.7	0	2.3	0
<i>E. virulenta</i>	68.4	0	2.4	0	1.8	0
<i>E. thaxteriana</i>	69.4	0	8.8	0	2.5	0

^a Percentage based on 500 conidia examined at each period. The results are averages of two examinations.

which had access to air than in cultures maintained under air-tight condition.

The widths of hyphae of *E. coronata* and *E. thaxteriana* varied with the length of exposure to air, especially after 14 days of culture (Table 3). They were narrower in cultures maintained under air-tight condition or with a short period of exposure to air than those of cultures exposed to air throughout (Fig. 2E, F, G, H). After 176 days of culture, there were some growth in the size of cultures maintained under limited air supply. In the control, the extent of development was limited because the hyphae, after attaining full growth, became deteriorated and collapsed.

The conidia of *E. coronata* and *E. virulenta*, which had been collected on glass slides and stored at room temperatures, did not germinate after 5 days when placed under favorable germinating conditions (Table 4). Those of *E. thaxteriana*, however, germinated up to 9 days of storage. When the conidia were held at 5°C, a few conidia of the three fungus species were capable of germinating after 18 days of storage (Table 5).

DISCUSSION

PRASERTPHON (1967) speculated that cultures of *Entomophthora* spp. could be stored for more than 2 years under air-tight condition at room temperatures. The cultures that he had prepared and left with us were viable after over 5 years. He believed that under the air-tight condition, the ratio of CO₂ to O₂ increased, which in turn arrested fungus development. When the culture was exposed to air, the growth was

resumed. This has been confirmed by our study. Under air-tight condition, the cultures exhibit very limited growth as indicated by the production of a few conidia and the gradual increase in the width of the hyphae. Some of the hyphae are knobby, and probably develop into chlamydospores. Under aerated condition, the fungus grows rapidly, produces abundant conidia which are larger than those produced under air-tight condition. The hyphae are also broader initially, but in a short period become vacuolated, fragmented, and collapsed.

The survival of entomophthoraceous fungi is associated with their metabolic activity, and they are generally maintained in the laboratory under refrigeration. Since they are capable of surviving for several months in insect cadavers at low temperatures and especially under dry conditions (ROCKWOOD, 1950; REMAUDIÈRE and MICHEL, 1971; KENNETH et al., 1972; WILDING, 1973), they may survive in this manner in nature. According to KENNETH et al. (1972), *Triplosporium floridanum* [Entomophthorales] survives in dried, mummified, two-spotted spider mites, *Tetranychus telarius*, as normal-appearing hyphal bodies.

The entomophthoraceous fungi, apparently survive under air-tight condition by means of their hyphae, some of which are knobby. The conidia are incapable of prolonged survival, but live longer at 5 °C than at room temperatures. In our study, only one of the species, *E. virulenta*, has produced resting spores, but they appear to be incapable of germination after 176 days and, when stained, appeared to be nonviable.

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