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ZOOPHTHORA PHYTONOMI (ZYGOMYCETES: ENTOMOPHTHORACEAE) A NEW RECORD IN IRAQ

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ABSTRACT

Morphological and phonological studies of fungal pathogen infecting alfalfa weevil *Hypera postica* (Gyllenhal) indicating that infection has been shown to develop along two distinct physiological lines, each culminating in the production of either conidial or resting spores, in host cadavers which are morphologically distinct. The percent of infection and epizootic development appeared to be dependent on host density. Farther evidence to entail proper correlation between conidia and resting spores suggest that these two forms of spores are stages in the development of one pathogen.

INTRODUCTION

Taxonomy

The class zygomycetes of the subdivision zygomycotina is comprised of three, or possibly four orders. One of them, the Entomophthorales, is characterized by the presence of sexual spores, believed to be sporangia, that have evolved to function as single conidia and which are forcibly discharged at maturity (Benjamin 1979; Heseltine & Ellis 1973). Batko's classification, as well as other schemes derived from his work by Ben Zeev and Kenneth (1982), recognize three families in this order: *Entomophthoraceae* Warming 1884; *Basidiobolaceae* Engler & Glig 1924, and *Ancylistaceae* Ubrizy & Voro 1966.

Several systems of classification were devised for the arthropod-attacking species of the family *Entomophthoraceae*, allocating them to genera based on morphological and cytological characters (Lakon 1919; Nowakowski 1883; Thaxter 1888). However, most authorities agree that the most comprehensive, distinctive classification was that developed in several steps by Batko (1964a, b, c; 1966a, b; batko & Weiser 1965). Batko (1964b) restricted *Entomophthora* to a few obviously related species, and proposed three new genera including *Zoophthora* (Batko 1964). The last step in Batko's classification was to rearrange the species previously classified in the genus Zoophthora (Batko 1964c; 1966a) into four groups of subgeneric level: Z. (*Zoophthora*), Z. (*Erynia*), Z. (*Pandora*), and Z. (*Furia*).

Later, taxonomic criteria for Entomophthorales classification were revised by Ben Zeev and Kenneth (1982). The major feature-criterion considered in this classification were: vegetative nuclei, conidial nuclei number, structure and function of primary conidia, production of secondary conidia, rhizoid structure, and the morphology of primary conidia.

However, another classification system for *Entomophthoraceae* was developed by Remaudiera and Hennebert (1980), and Remaudiera and Keller (1980), which was also derived from Batko's system. The classification scheme of Remaudiera and co-worker was based mainly on the external morphology of the primary conidia, giving less importance to the nuclear number in the conidia.

Zoophthora Phytonomi

The recognition of two genera, Zoophthora Bakto (1964) and Erynia Nowakoski (1881), separated principally by formation of secondary conidia, by Remaudiera and Hennebert (1980), had raised a problem in synonymy. Humber (1981a, b; 1982), Ben Zeev (1980), and Ben Zeev and Kenneth (1981a, b; 1982) find no basis for the separate and simultaneous use of both *Zoophthora* and *Erynia*. A possible resolution to this synonymy problem would be to take Erynia Nowakowski as the correct name of the genus.

The genus Erynia Nowakowski (Entomophthorales: *Entomophthoraceae*) is limited to species having uninucleate, bitunicate primary conidia born on (digitately and apically) branched conidiophores and forcibly discharged by the evection of the basal papilla. However later publications referred to the fungal pathogen as *Zoophthora phytonomi* (Parr et al. 1993; Giles & Obrycki 1997; Kuhar 1999).

Phenology

Erynia epizootic behavior among alfalfa weevil (AW) populations have been reported to be depend on critical biological and physiological factors. These factors govern the initiation, progression, and determination of an epizootic under field conditions and its effect on prevalence of parasitism by *B. curculionis* and *B. anurus*, both of which attack the larval stage that markedly affect AW populations.(Puttler et al. 1961; Hagan & Manglitz 1967; Dysart & Day 1976; Berbert & Gibson 1976; Gonzales et al. 1980; Millstein, et. al. 1982; Norden, et. al. 1983; Oloumi et al. 1993; Parr et al. 1993; Giles et al. 1994; Berberet & Bisges 1998).

MATERIALS AND METHODS

1. Microscopic study

Alfalfa weevil larvae, displaying tan-brown color symptoms typical of the "type a" syndrome as described by Ben Zeev and Kenneth (1982), were field collected and placed in 0.5 1 cartons, then brought to the laboratory. Those cadavers which had attached to stems or leaves by rhizoids, and which had discharged, or were about to discharge conidia, were assayed for the presence of the pathogen using wet mount preparation. This involved placing larval tissue on a glass slide in a drop of cotton blue (aniline-blue in lacto-phenol). The tissue was then smeared, covered with coverslip, and examined under a compound microscope at 320x magnification. A similar series of wet mounts were prepared, but a synthetic orecin stain was used (2.0g synthetic orecin, 50.0 ml of 85% lactic acid, and 50.0 ml glacial acetic acid).

Spores from dead cadavers exhibiting "type a" symptoms were also examined using third instar field-collected larvae, and in a manner similar to that described by Millstein et. al. (1983) in which larvae were selected on the basis of rhizoid attachment, and the presence of emerged conidiophores. This was necessary to ensure that only those larvae likely to discharge conidia with 24 hours would be used in the experiment. Spore-containing cadavers, singly, were attached ventrally to the insides of filter paper-lined 10x1.5 cm glass petri dish lids. These lids were then placed inside humidity chambers (100% R. H.) in such a way that the cadavers were suspended above the water. At intervals, a glass slide was placed on the porcelain support beneath the cadavers to collect any discharged conidia. The conidia were then stained and prepared as wet mount slides as described previously, and examined under compound microscope at 320x magnification.

Blackened cadavers, typical of larvae containing resting spores referred to by Ben Zeev and Kenneth as "type b" syndrome, were field collected and examined in the laboratory. Cadavers were placed singly in a hand held tissue homogenizer with 1.0 ml. sterile distilled water (SDW), then ground until spores were completely dispersed. Spores were then flushed out

and cleaned by three alternate centrifugation and SDW washes, then prepared as wet mounts, covered with slips, and examined under compound microscope at 320x magnification.

Representative photographs of the above preparations were obtained using a Wild MPS 51 camera mounted on Zeiss Universal Compound microscope.

2. Field study

A study area was established in a three-hectare alfalfa field. The actual sampling area (plot) in the field was restricted to one hectare (100x100 m.), which was subdivided into nine (33x33 m.) subplot of equal size, resulting in 3x3 subplot grid.

To determine the disease mortality and epizootic development of fungal pathogen, samples consisting of 10 alfalfa stems from each subplot were taken twice weekly throughout AW activity season. These stems were then brought to the laboratory, and inspected for dead larvae which had discharged or were about to discharge conidia, or black cadavers thought to contain resting spores. Such cadavers were counted and examined. Likewise, all alive AW larvae were removed from the stem, counted and then placed individually in ventilated, 1 oz. cups with fresh alfalfa foliage. Larvae were provided with fresh alfalfa and examined daily for disease symptoms.

Larvae exhibiting tan to brown color disease symptoms were placed singly on water agar (15 g/l water) in 3.5xl cm. sterile disposable petri dishes, held at 20 $\pm$ 1 C and 14:10 (L:D) photoperiod, and observed daily for the presence of conidial spores. All black larvae were placed singly in petri plates and examined under binocular microscope (60x) to determine if they had produced internal resting spores.

RESULT AND DISCUSSION

1. Microscopic study

Infection of fungal pathogen has been shown to develop along either of two distinct physiological lines, each culminating in the production of different morphological types of spores, and in host cadavers which are morphologically distinct.

Conidial stage

Specimens infected with conidial stage of the pathogen are light tan to creamy-brown in color, and are usually attached to the central area of leaf, or curled across the leaf edge, or coiled around the stem (Fig. 1A). Usually these cadavers are covered with a dense growth of mycelia. The conidiophores appear palisade-like and are white to beige in color; almost white when first develop, and darkening with time. They may cover all but the ventral portion of the body.

Mycelium

In wet mount observation, mycelia appear to be coenocytic, and divided into large branched hyphal bodies. The hyphae are uniform in size, hyaline, and extensively branched, and the contents are finely granular in appearance with vacuoles of different sizes. Nuclei are easily distinguished being large (5 u in dia.) ovoid, granular in appearance, and without prominent nucleolus. Nuclei are numerous, and are frequently evenly spaced in linear sequence (Fig. 1C).

Rhizoids

Upon death, some of the hyphae in the thoracic region aggregate and push out from the ventral surface of the larval body where come in contact with solid surface fan out into funnel-like structure which serve to anchor the larva securely to leaf surface (Fig. 1A). The appearance of rhizoids is indicative of the completion of vegetative growth within host, and this face is soon followed by development of conidiophores, or by the development of resting spores.

Conidial spores

Primary conidia are elongated and oval with round apexes. They appear slightly constricted above the conspicuous collar of the blunt triangular papilla marking the ring of attachment to the conidiophores (Fig. 1D). Conidia are uniform in size, hyaline, uninucleate, and bitunicate (with a separable outer wall layer except over the basal papilla). The cytoplasm is evenly and finely granular, and non-vacuolated in newly formed spores. A single nucleus, oval, 3 to 4 u in dia., and centrally located, is always present (Fig. 1E). Conidial spores show high affinity to cotton blue stain (Fig. 1D), thus the nucleus is not readily distinguishable when using this stain.

Resting stage

Specimens containing this stage of the pathogen are black, shriveled, and usually coiled on upper portion of stems and branches, or laying flat on leaf (Fig. 1B), and filled with a brownish-black fluid. Additionally, the integument of the host may be seen in various stages of disintegration, and in time may become no more than an empty leathery integument containing a black fluid which is packed with resting spores. The same symptoms found is blackened prepupae inside their cocoons (Fig. 1B), whereas tan-brown symptoms of conidial stage infection were not found in the cocoon stage. The black fluid contains rough-walled spores, black in gross observation, yet yellowish-brown to dark brown when viewed singly under a compound microscope at (320x) magnification (Fig. 1F).

Resting spores

Resting spores are usually spherical or globose in shape, averaging 34 u in diameter, with two thickened cell wall layers. The outer wall is up to 5 u thick; the episodes is thin, and difficult to separate from endospore, variously ornamented, and is budded terminally from parental hyphae. The contents of the spore consist of granular particles and oil globules as observed under 320x magnification (Fig. 1F).

2. Field study

The epizootic progression pattern and host density are similar in their step like incremental progress and percent of infection (Fig. 2). The epizootic initiation was in range of 340 DD, although it is difficult to predict if the disease progression is necessarily depend on DD. However, comparison of host densities versus the size of epizootic showed no obvious correlation in many instances (Nordin et. al. 1983). However, in this instance, the percent of infection and epizootic development appeared to be dependent on host density. This relationship has not been well documented by several research, probably due to different techniques used to determine the incidence of the disease in the field. Unfortunately, most workers have calculated the percent incidence of mortality at given time from the number of diseased larvae that showered or appeared ready to exhibit a conidial shower in the field. This method does not give the actual percent of infection and mortality of the pathogen, but rather over estimates disease incidence as diseased larvae could be accumulated and counted more than once at separate sampling intervals, and yet not have showered because of improper environmental conditions in the field. On the other hand, our method is based on rearing

population aliquots in the laboratory, in addition to observing and recording diseased larvae in the field.

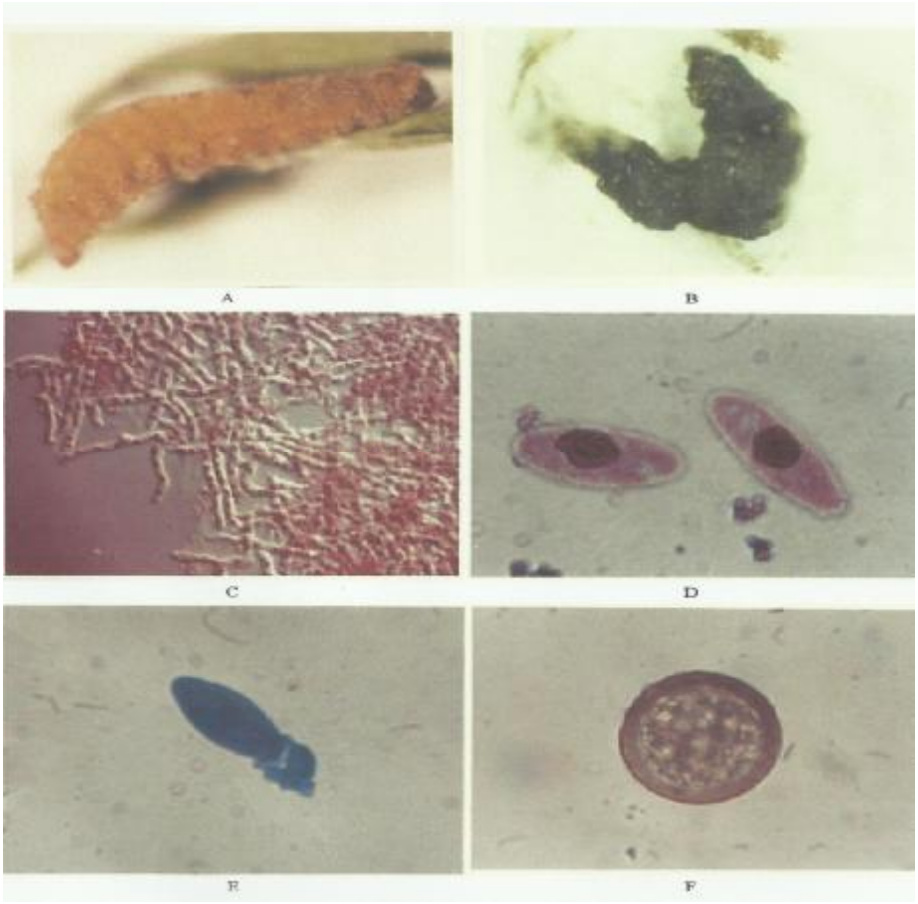

Figure1. *Erynia phytonomi*, A: conidial stage, B: resting stage, C: mycelium, D: Conidial spores, E: conidial spore (cotton blue), F: resting spore

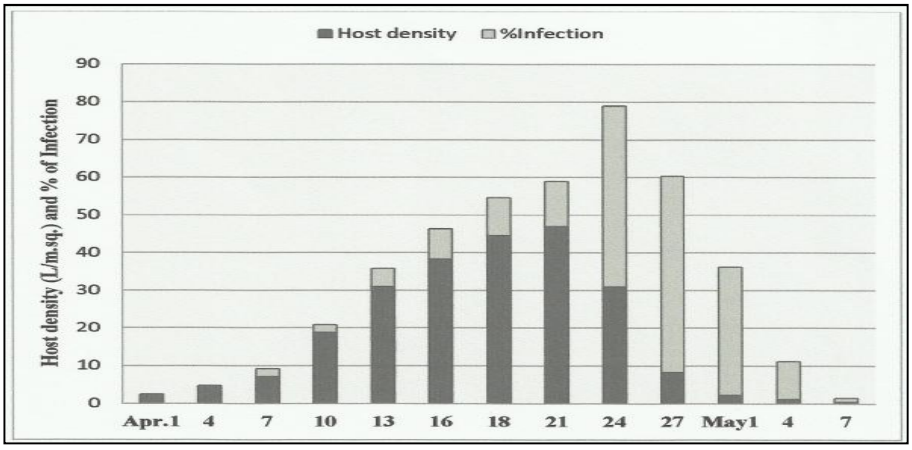

Figure 2. Population of alfalfa weevil density and percent of *Erynia phytonomi* infection.

In order to provide farther evidence to entail proper correlation between conidia and resting spores, and support previous suggestion that these two forms of spores are stages in the development of one pathogen, we monitored the epizootic of pathogen regardless of spore type produced. Based on data presented in figure 3, all larval instars are equally susceptible, and produced similar epizootic patterns. This is unlikely to be observed if the small and large larvae are infected by different pathogens, and forming different types of spores.

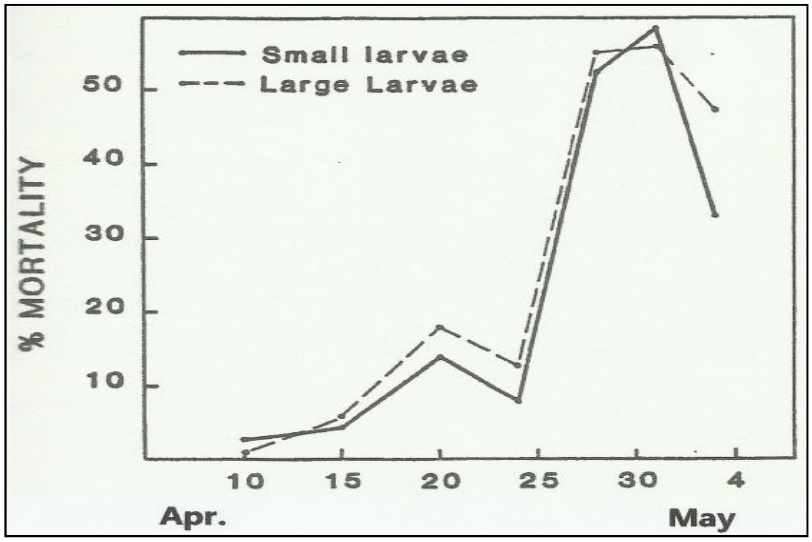

Figure 3. Mortality of alfalfa weevil in different larval instars

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الدراسة الفطرية والتطورية للفطر الممرض الذي يصيب سوسة الجت *PHYTONOMI*

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الخلاصة

الدراسة الفطرية والتطورية للفطر الممرض الذي يصيب سوسة الجت *PHYTONOMI* كدليل لاصابة تظهر تطور خطين تطوريين لتنتج اما سبورات الكوتيدية او باقي السبورات في العائل والتي تتميز مظهرها. ان نسبة الاصابة وتاثيرها في العائل تعتمد على كثافة العائل. علاوة على ان الدليل او الترابط بين الكيدتيديا وباقي السبورات يعتمد انها شكلين من السبورات ومراحل في تطور فطر واحد.