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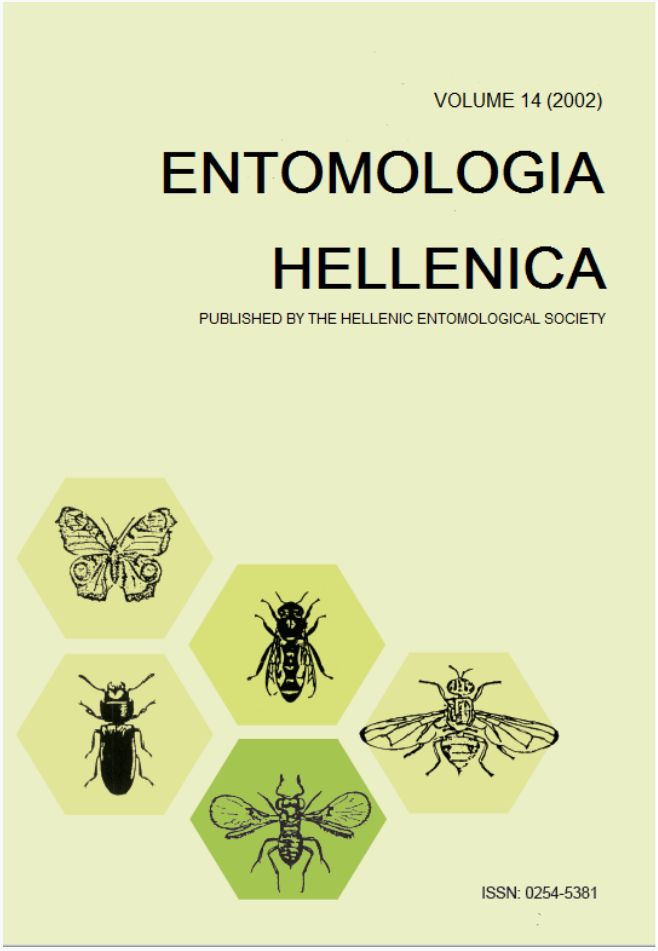

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Entomopathogens of *Anacridium aegyptium* L. in Crete[1]

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ABSTRACT

The entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuil. was recorded for the first time on *Anacridium aegyptium* L. in Crete. The insects were fed on pieces of leaf subjected to a serial dilution of spores over three to four orders of magnitute. Comparative studies on the virulence of *B. bassiana* (I 91612 local isolate) and *Metarhizium anisopliae var. acridum* (IMI 330189 standard isolate of IIBC) showed that *M. anisopliae var. acridum* was more virulent than *B. bassiana* at a conidial concentration lower or equal to 10^6 per ml while they were similarly virulent on first stage nymphs at 10^7 conidia per ml.

Introduction

A three year study (1990-1992) was started on locusts in Crete aiming to study Cretan acridofauna, including species composition and their seasonal abundance on main crops, harmful species and native biological agents. Grapes are the second crop in order of importance of cretan agriculture. Crop losses dued to locust *Anacridium aegyptium* had been noticed on certain locations in Crete in the past (Roditakis 1990).

Anacridium aegyptium L. is the most abundant (55%) species of the acridofauna of cretan vineyards and it is also an insect of secondary importance for grape vines in Crete (Roditakis 1990, Kollaros 1993). This species is very common in all Greece as it is also in other countries of tropical and subtropical regions (Willemse 1985). The population density is very low (5-10 adults/ha) in central Crete not allowing this species to be generally harmful on grapevines. There are only two restricted grapevine areas in Crete, a nothern one in Kalessa and a southern one in Pompia, where we have noticed a higher population density (20-40 ind./ha) in winter that reaches much higher levels during some summers. The reasons of these sporadic outbreaks remain unknown. By contrast it is always harmful on field vegetables in Chandras, Sitia (East Crete) so the local Agricultural Advisory Services suggests extensive control measures based on insecticides.

It is known that microbial agents such as the entomopathogenic fungi cause epizootics affecting population size in the field. The main factors favoring epizootics are temperature and humidity conditions (>92-93%) (Walstad et al. 1970, Burges and Hussey 1971, Ferron 1978) (wet and rainy weather, irrigation etc) and microenviroment (Moore 1973, Krammer 1980, Doberski 1981). The pathogens are generally highly heterogenous species but there exist host-adapted genotypes (Riba et Miergejewska 1986, Hajek and Leger 1994) and geographically distinct populations which show high degree of virulence (Fargues et al 1992). One of them, *Metarhizium anisopliae var. acridum* has recently been used extensively against very hazardous locusts such as the desert locust *Schistocerca gregaria* (Prior 1990).

The use of pathogens for pest control could remove some problems associated with insecticides such as hazards to enviromental pollution and tox-

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icity to beneficial fauna. The current research aims at discovering local pathogens of this locust in order to use them in IPM programmes either on vines or on vegetables.

Materials and Methods

The adults of *A. aegyptium* were collected with sweep nets in the field and reared in the laboratory in small wooden cages (30 cm × 35 cm × 35 cm) in a rearing room under natural illumination and 24±1°C. Fresh vine leaves and oat flakes were offered as food. The cages were kept clean from faeces and remainders of food daily. The leaves provided were rinsed with chlorinated tap water. The females deposited their eggs on small transparent plastic boxes filled with sterilized lightly moistured sea sand. When one or two egg pods were laid per plastic box, the box was removed and placed in a separate wooden cage.

Every insect suspected of being ill, was removed and placed in separate transparent plastic box (12 cm diameter and 8 cm height). After its death, it was sectioned, spread, pinned and air dried. Some of the insects were kept on lightly moistured cotton, until fungal hyphae erupted from the cadaver. Following this, a one-conidium culture was initiated on potato dextrose agar (PDA). The pathogens isolated were sent to the International Institute of Biological Control – Insect Pathology Laboratory (IIBC) for identification.

The pathogens isolated were tested both on adults and on first stage nymphs in the laboratory. We used conidia concentrations 2.5, 5, 10 and 20 × 10^6 per ml on adults and 2 × 10^3, 2 × 10^4, 2 × 10^5, 2 × 10^6 and 2 × 10^7 per ml conidia on first stage nymphs.

The conidia were harvested from Petri dishes using sterile distilled water agitated by a bent glass rod. The conidia suspension was cleared of hyphal debris by filtering through coarse-mashed muslin. After that, the coagulated conidia were separated by a sonicator. Four Petri dishes (9 cm diam) were enough in order to obtain 100 ml conidia suspension of 20 × 10^6 conidia/ml concentration.

The tests for pathogenicity of isolated fungi were carried out by administering small pieces (5cm × 5cm) of lettuce leaves immersed in a certain conidium concentation for 10 seconds. One or two small drops of Tween 80 was added as emulsifier in each conidia concentration before immersion of leaf. The treated pieces of lettuce leaves were offered as a food the first two days followed by untreated ones during the next period. The treated individuals were kept on small separate transparent plastic boxes (10 cm diam, 5.5 cm height).

Four groups of 12 adults each (48 in total/treatment) were used came from a colony reared in the laboratory during summer. In order to achieve the appropriate number of adults for laboratory tests, we reared the locusts for 3-4 generations under laboratory conditions and natural illumination from May to September. These adults of *A. aegyptium* were subjected to reproductive diapause in automn. Adults of *A. aegyptium* entered to reproductive diapause consumed fewer food (1/5-1/9 of food consumed by non diapause adults) (personal observations) and we took it into account during laboratory tests. The chi square was used for statitistcal analysis of percentage mortality by Statgraphic statistical programme.

Four groups of 16 first stage nymphs each (64 in total per treatment) were also used. The pathogenicity of local pathogens isolated was compared with an IMI 330189 *Metarhizium anisopliae var. acridum* isolate provided by IIBC. The previous publications on the biological control of locusts and grasshoppers using IMI 330189 referred as *M. flavoviride* Games & Rozsypal (Drivers et al. 2000). The first stage nymphs used were very voracious and ate the food offered. They consumed much food (fresh vine leaves) (0.18 gr/24h) (Kollaros 1993) than the non diapause adults consumed 0.5-0.9 gr/24h (fresh vine leaves) (personal observations). Lt_{50} was calculated by a computer programme provided by the IOBC laboratory.

Dead insects were kept on separate Petri dishes with a lightly moistened filter paper on the bottom and closed firmly all around by parafilm. There they remained until fungal hyphae were projecting from the cadaver. This was interpreted as confirmation of the pathogen-related death.

TABLE 1. Mortality of adults of *Anacridium aegyptium* fed on pieces of lettuce leaves treated with different conidia concentrations of fungi *Beauveria bassiana* (total number of adults per treatment 48).

Treatments conidia/ml	Dead insects on 10th day	Corrected mortality %*
2.5 × 10^6	48	100
5.0 × 10^6	40	80
10.0 × 10^6	36	70
20.0 × 10^6	36	70
Control	8	0

* = Abbott's formula
$x^2 = 13.2$

TABLE 2. Mortality (%) of first stage nymphs of *Anacridium aegyptium* fed on pieces of lettuce leaves treated with different conidia concentrations of *Beauveria bassiana* and *Metarhizium anisopliae var. acridum* (total insects per treatment 64).

I. *Beauveria bassiana*						
	Days post inuculation**					
	5 days		10 days		20 days	
Conidia/ml	No of dead insects	Mortality (%)*	No of dead insects	Corrected mortality (%)*	No of dead insects	Corrected mortality (%)*
Control	0	0.0	4	0.0	4	0.0
2×10^3	0	0.0	4	0.0	5	1.9
2×10^4	0	0.0	12	13.6	12	13.6
2×10^5	4	6.2	16	20.2	20	26.8
2×10^6	4	6.2	12	13.6	14	17.9
2×10^7	4	6.2	56	86.0	60	93.3
II. *Metarhizium anisopliae var. acridum*						
Control	0	0.0	0	0.0	0	0.0
2×10^3	0	0.0	0	0.0	12	18.7
2×10^4	8	12.5	24	37.5	36	56.2
2×10^5	8	12.5	48	75.0	64	100.0
2×10^6	12	18.7	60	93.7	60	93.7

* Abbot's formula **Feeding on leaves with conidia
$X^2 = 4.82$

Koch's postulates were satisfied by the reculture of fungus from the infected insects.

Results

Five dead adults from 48 specimens collected was firstly became reddish and when put on lightly moistened filter paper on a glass Petri dish, a rich white mycelium covered all the body in 5-8 days. Firstly, the mycelium covered head and antennae and later the thorax, abdomen and legs. The fungus isolated was *Beauveria bassiana* (Bals.) Vuill. This fungus proved virulent on adults (Table 1) and first stage nymphs (Table 2) as well. According to the data presented in Table 1, conidia concentrations from 2.5 to 20 $\times$ 10^6 per ml caused high mortality ranging from 70-100%. The mortality of first stage nymphs was 86 and 93.3% at 2 $\times$ 10^7 conidia concentration after 10 and 20 days after treatment respectively (Table 2).

The LT_{50} (Lethal time for 50% individuals) was 7 days at 2 $\times$ 10^7 conidia concentration for both fungi but it was longer at lower concentrations (Table 3). The LT_{50} of *M. flavoviride* was generally shorter than that of *B. bassiana* at the lower concentration of 2 $\times$ 10^6 and 2 $\times$ 10^5 per ml.

We observed also *Aspergillus flavus* Link in a few dead speciments in laboratory colonies where adults were crowded.

TABLE 3. The lethal time for 50% (LT_{50}) of first stage nymphs of *Anacridium aegyptium* at several conidia concentrations of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae var. acridum*.

	LT_{50} in days	
Conidia concentrations	*Beauveria bassiana*	*Metarhizium anisopliae var. acridum*
2×10^4	>20	>20
2×10^5	>20	16
2×10^6	>20	8
2×10^7	7	7

Discussion

The entomopathogenic fungus *B. bassiana*, known as muscardine disease is an entomopathogen in many locust species (Dresner 1949, Moore and Erlandson 1988). This fungus was also isolated from grape berry moth larvae in Crete in 1985 (IMI 294185). *A. aegyptium* inhabits several parts of Greece and this the first record of *B. bassiana* on this locust in Greece. Even if this locust is of secondary importance in agriculture there are some other locusts very harmful on cereals and vegetables on which it could be a usefull biological control agent. There are three regions in Crete where *A. aegyptium* is harmful, at Kalessa (North Crete) Pompia (South Crete) where it attacks grape vines and at Chandras (East Crete) where it attacks vegetables.

In Chandras, Sitia (East Crete) *A. aegyptium* is very harmful and the local agricultural authorities take extensive control measures based on insecticides every year. Concerning vines this locust should be under consideration in IPM programs on grape vines in South Crete.

Entomopathogenic fungi usually penetrate the cuticle of insects and then cause mycosis which spreads through haemolymph to all parts of the body. Oral ingestion of fungal conidia could infect the insect either from gut wall either from buccal cavity. There are very few reports that propose infection through gut wall after ingestion of fungal spores (reviewed by Dillon and Charnley 1991). Dillon and Charnley (1995) noted that the germination of conidia of *Metarhizium* was inhibited by bacteria in the gut of desert locust due to antimicrobial phenols associated with *Pantoea agglomerans*. Gut infection seems to occur often in aquatic insects (Sweeney 1979, Knight 1980). *B. bassiana* can infect *Aedes aegyptii* Rock larvae through the gut (Miranpuri and Khachatourians 1991). However there are also reports in which conidia germination in buccal cavity. Siebeneicher et al. (1992) showed that *Solenopsis invicta* Buren workers possessed germination *B. bassiana* conidia in their buccal cavities. Shabel (1976) demonstrated istologically that *Metarhizium anisopliae* conidia invade through the buccal cavity of the weevil *Hylobius pales* Herbst. We isolated *B. bassiana* from the hypopharynx and crop of *A. aegyptium* showed symptoms of illness. Under low humidity conditions, we observed a rich mycelium with conidiophores only inside the head capsule causing a whitish discoloration of the head of dead insects. Dead insects kept under high humidity conditions on Petri dishes showed visible mycelium growth firstly on antennae and head and later on rest parts of the its body. The observations displayed above show that buccal cavity of this locust could be initially the place of conidia germination, following with penetration of neigbour parts of its body.

A. flavus has often been isolated from insects and mites (Moore and Erladson 1988, Pelagati et al. 1988, Wicklow and Dowed 1989). This fungus was isolated only from insects reared in our laboratory. It was often isolated from our rearing units where adults were crowded. *A. flavus* infections are fairly common in crowded insects due to stress and it might be the cause of its occurrence in our laboratory colonies. It is known that the insecticidal activity of *A. flavus* is mainly based on the aflatoxin complex. The aflatoxin complex is toxic and carcinogenetic to vertebrates so it is unlikely to be used in microbial control. Following that, the present study was focused on the local strain of *B. bassiana*.

The ability of entomopathogenic fungi to control pests is affected by abiotic and biotic factors e.g. microbial antagonists, host suitability, pathogen virulence, inoculum thresholds, sunlight, pesticides, temperature and humidity. Many of the above constraints could be overcome with formulation and application strategy (Zimmermann 1994). Recently an oil formulation of *Metarhizium anisopliae var. acridum* was excellent against *Schistocerca gregaria* under low humidity condition (Bateman et al. 1993). The addition of UV protectants can also increase significantly their persistence in the field (Inglis et al. 1995).

B. bassiana can be easily mass produced and formulated in high conidia concentrations (Hall and Papierok 1982, Auld 1991). Its mass production, formulation and application methods can also be impoved considerably (Goetel and Roberts 1991, Bateman 1991). The virulence of *B. bassiana* compared to that of *M. flavoviride* was lower at lower concentrations ($<10^7$) while it was virulent as well at 2×10^6 conidia concentration for both fungi. Considering that indigenous pathogens have been suggested for use because of the risk of unknown effects from non indigenous pathogens on the local fauna (Goettel and Roberts 1991, Prior 1990, 1991) the use of local strain *B. bassiana* as a biological control agent in an IPM programmes on vine pests should be considered.

Acnowledgements

I would like to thank Dr. Chris Prior of the International Institute of Biological Control Ascot London U.K. for the determination of species of entomopathogenic fungi and for providing the strain of *Metarhizium anisopliae var. acridum* for comparative studies.

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KEYWORDS: *Anacridium aegyptium, Beauveria bassiana, Metarhizium anisopliae var. acridum,* virulence, grape vines.

Εντομοπαθογόνα του *Anacridium aegyptium* L. στην Κρήτη

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ΠΕΡΙΛΗΨΗ

Στα πλαίσια ενός τριετούς ερευνητικού προγράμματος για τις ακρίδες στην Κρήτη ερευνήθηκε η ύπαρξη ιθαγενών εντομοπαθογόνων μικροοργανισμών για την βιολογική καταπολέμησή τους. Απομονώθηκε για πρώτη φορά ο μύκητας *Beauveria bassiana* (Bals) Vuill., αξιολογήθηκε η αποτελεσματικότητά του στα ακμαία και νύμφες πρώτης ηλικίας της ακρίδας *Anacridium aegyptium* L., είδος βλαβερό σε αμπέλια και λαχανικά, και συγκρίθηκε με το στέλεχος του εντομοπαθογόνου μύκητα *Metarhizium anisopliae var. acridum* (IMI 330189). Τα πειράματα έδειξαν ότι το ιθαγενές στέλεχος του μύκητα *B. bassiana* που απομονώθηκε είχε εξαιρετική αποτελεσματικότητα σε ακμαία και νύμφες πρώτης ηλικίας της ακρίδας *A. aegyptium*, δεν υστερεί σε αποτελεσματικότητα από το μύκητα *M. flavoviride* και ενδείκνυται για το βιολογικό έλεγχο και προγράμματα ολοκληρωμένης διαχείρισης.