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Evaluation of *Beauveria bassiana* and *B. brongniartii* strains and four wild-type fungal species against adults of *Bactrocera oleae* and *Ceratitis capitata*

M.A. KONSTANTOPOULOU* and B.E. MAZOMENOS
Chemical Ecology and Natural Products Laboratory, Institute of Biology NCSR "Demokritos", P.O. Box 60228, 153 10 Aghia Paraskevi, Attikis, Greece
**Author for correspondence; e-mail: mkonstan@bio.demokritos.gr*

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Abstract. The virulence of two isolates of the hyphomycete fungi, *Beauveria bassiana* and *B. brongniartii*, and additional fungal species isolated from diseased *Bactrocera oleae* pupae and *Sesamia nonagrioides* larvae were assessed against adults of the olive fruit fly *B. oleae* and the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae). Contact and oral bioassays revealed that moderate to high mortality rates for the olive fruit fly occurred when the adults were exposed to conidia of *Mucor hiemalis*, *Penicillium aurantiogriseum*, *P. chrysogenum and B. bassiana* isolates. A strain of *M. hiemalis* isolated from *S. nonagrioides* larvae was the most toxic resulting in 85.2% mortality to the olive fruit fly adults. *B. brongniartii* and *B. bassiana* were the most pathogenic to the *C. capitata* adults causing 97.4 and 85.6% mortality. Metabolites collected from the *M. hiemalis* and *P. chrysogenum* isolates were toxic to adults of both species.

Key words: *Bactrocera oleae*, *Beauveria bassiana*, *Beauveria brongniartii*, *Ceratitis capitata*, *Mucor hiemalis*, Mucorales, *Penicillium chrysogenum*, toxic metabolites

Introduction

The olive fruit fly *Bactrocera oleae* (Gmelin) and Mediterranean fruit fly *Ceratitis capitata* (Wiedermann) (Diptera: Tephritidae) are two economically destructive species. *B. oleae* attacks olive fruits in the olive-growing Mediterranean countries. *C. capitata* is universally distributed; it has many generations per year and attacks a large variety of fruits.

The predominant method to control the two flies' populations has been the use of conventional pesticides. However, the continued use of pesticides has caused serious problems: environmental pollution, development of insecticide resistance, and contamination of products. This has caused concern and has led to a concerted effort to reduce the amount of pesticides that is being used to control these pests. As an alternative to chemical control, genetic methods

(sterile insect techniques), natural enemies, microbial pesticides (bacteria and fungi), natural insecticides, insect growth regulators and semiochemicals, have been evaluated as constituents of an IPM strategy for both pests (Delrio, 1992).

Entomopathogenic fungi comprise a diverse group of pathogens that have been recorded from all major taxa of arthropods (Roberts and Humber, 1981). Species of Hyphomycetes occasionally cause high infection levels in insect populations but within the last years several species have been or are being developed as biocontrol agents (Tanada and Kaya, 1993; Hajek and St. Leger, 1994). Strains of the species *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff), *Verticillium lecanii* (Zimmermen) and *Paecilomyces fumosoroseus* (Wize) are currently used for insect control (Lacey et al., 2001). In contrast, species from Entomophthorales (Zygomycetes) often cause high infection levels among insects. To our knowledge no information is available on fungal species pathogenic to the two fruit fly species. However other higher fly species have been reported to suffer high infection levels caused by fungi from Entomophthorales. *Entomophthora muscae* infects several species e.g., the onion fly *Delia antigua*, the wheat bulb fly *Delia coarctata* (Diptera: Anthomyiidae), the carrot fly *Chamaepsila rosae* (Diptera: Psilidae) (Wilding and Lauckner, 1974; Carruthers et al., 1985; Eilenberg and Philipsen, 1988); it also infects flies associated with animal production, e.g., the house fly *Musca domestica* (Diptera: Muscidae) and the lesser house fly *Fannia canicularis* (Diptera: Fanniidae) (Mullens et al., 1987; Mullens, 1989). The Hyphomycetes *B. bassiana*, *M. anisopliae*, and *Tolypocladium cylindrosporum* have been reported to be pathogenic to the house fly (Steinkraus et al., 1990; Barson et al., 1994; Watson et al., 1995, 1996). Strains of *M. anisopliae* and *Paecilomyces fumosoroseus* were pathogenic to *C. capitata* adults in laboratory bioassays (Castillo et al., 2000).

The objective of this study was: (1) to evaluate the virulence of two *Beauveria* species against adults of the two fruit flies as well as the toxicity of the fungal species isolated from field-collected diseased pupae of the olive fruit fly *B. oleae* and larvae of the corn borer *S. nonagrioides,* and (2) to determine the relationship between the virulence of the isolates from the infected insects and the toxicity of their metabolites produced in liquid medium cultures.

Materials and methods

Fungi isolated from diseased insects

Infested olive fruits were collected in autumn from olive groves in the area of Attiki, Greece. During this same period, corn plants infested with *S. nonagrioides* were collected from Thessaly, central Greece. In the laboratory

corn plants were dissected and diseased larvae bearing signs of fungal infection were surface sterilized by dipping them in a 1% sodium hydrochloride solution for 5 min. Subsequently larvae were washed three times with distilled water and each infected larva was placed on a Potato dextrose agar (PDA) plate. Plates were then incubated at 25 °C to obtain growth of mycelia occurring internally.

The collected infested olive fruits were placed in humid conditions and maintained under constant laboratory conditions, while the larvae completed their development. Usually, fully grown larvae exit the fruit to pupate. Prepupae or pupae bearing signs of fungal infection were sterilized as above, placed on PDA plates and incubated at 25 °C.

On plates where infected larvae of *S. nonagrioides* were incubated, one fungal colony developed, while four colonies, distinct in color and shape, developed on plates where diseased olive fly prepupae/pupae were incubated.

Pure cultures were sent to CABI Bioscience, Fungal and Bacteria Identification Service London, UK for identification. The fungus isolated from *S. nonagrioides* larvae was *Mucor hiemalis* Wehmer (SMU-21) (Zygomycetes: Mucorales). *M. hiemalis* Wehmer (DMU-01), *Penicillium aurantiogriseum*, Dierckx (PAU-01) and *P. chrysogenum* Thom (PCH-01) (Ascomycetes: Eurotiales) were isolated from *B. oleae* pupae.

All the species are saprophytic and not true entomopathogens, however *Mucor* and *Penicillium* species have been reported as toxic to insects (Brooks and Raun, 1965; Miczulski and Machowicz-Stafaniak, 1977; Lynch and Lewis 1978). In preliminary tests, laboratory reared *B. oleae* flies exposed to fully grown conidia of *Mucor* and *Penicillium* isolates suffered high mortality rates equal to, and in some cases higher than, flies exposed to *Beauveria* species. In view of these results the toxicity of the above isolates was studied further.

Also the virulence of the *B. bassiana* (BCIP 1333), isolated from *B. oleae* pupae, and *B. brongniartii* (BCIP 1335), isolated from *Melolontha* sp., obtained from the fungal collection of the Benaki Phytopathological Institute was evaluated against olive fly and medfly adults.

Insects

B. oleae and *C. capitata* adults used in the present work were obtained from laboratory colonies maintained in our laboratory at 25 ± 2 °C and 60–65% relative humidity (RH) and 12:12 (L:D). Adults were provided with water and a solid diet consisting of 40% sugar, 10% hydrolyzed yeast, 5% egg yolk.

Bioassays against olive fly adults

All fungal isolates were tested against adults of the olive fruit fly using contact and oral bioassays.

Contact bioassay

Ten 3-day-old flies were collected in test tubes, immobilized on ice and carefully transferred to PDA dishes (9 cm diameter) containing the six fully developed fungal colonies. The flies were allowed to walk on the fungal colonies for 5–10 min depending on fly mobility until the flies collected spores on their body. The flies were then removed from the Petri dishes and placed in small cages (10 cm × 10 cm × 10 cm). The same number of flies treated similarly but with uninoculated PDA plates was used as controls. Solid diet and water were offered to flies and kept under rearing conditions. Dead flies were counted and removed from the cages daily for 21 days. Each treatment was replicated five times.

Oral bioassays

Conidia of *M. hiemalis*, *P. aurantiogriseum*, and *P. chrysogenum*, were harvested from 20-day old PDA cultures and suspended in distilled water containing 0.05% Tween 80. Conidial suspensions of *B. bassiana* and *B. brongniartii* were harvested from 40-day-old cultures the same way. Suspensions (1 ml) of each fungus, adjusted to 10^8 conidia/ml, were mixed with 1 ml of a 20% solid diet aqueous solution making a final concentration of 10^4 conidia/ml. Small droplets (approx. 10–15 μl) of the mixture were placed on a piece of waxed paper and offered to 3-day-old flies. Insects consumed the liquid diet within 2–3 days, after which the same liquid diet without fungal conidia, was offered. Liquid diet without fungal conidia was offered to flies used as controls. Ten insects were placed in small wooden cages (10 cm × 10 cm × 10 cm) under standard rearing conditions for each of 15 repetitions for each isolate and control. Mortality was assessed at intervals of 24 h for 21 days. To confirm mycosis, the dead insects were sterilized with 80% ethanol, placed on moist filter paper in Petri dishes and incubated at 25 °C.

Collection of fungal metabolites

The two *Mucor* and *Penicillium* isolates were cultured in liquid medium, to collect their secondary metabolites. Their bioactivity was subsequently tested on the flies. Fully grown conidia from 20-day-old PDA *Mucor* and *Penicillium* cultures were transferred to 500 ml flasks containing 200 ml of Richard's medium (25 g glucose, 10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 0.02 $FeCl_3$, and 0.005 g $ZnSO_4$ in 1 l distilled water) and incubated under static conditions at 26 °C for 20 days in continuous dark. The supernatant of each culture was filtered through a Gen–purpose filter paper (Schleicher and Schuell, Germany) and the filtrate was freeze-dried. The dried residue was diluted in 50 ml of distilled water and tested for biological activity (see below).

Supernatants found to possess biological activity were purified. The lipophilic chemicals present in the active supernatants were extracted with 50 ml chloroform; the extraction was repeated three times (total volume of chloroform 150 ml). The chloroform fraction was concentrated under reduced pressure in a rotary evaporator, dissolved in 10 ml of chloroform and stored at −4 °C until use. The water layer was lyophilised and the dry material was suspended in 10 ml methanol. The methanolic solution was placed at 4 °C for 24 h, while proteins and possibly other polar compounds precipitated. The methanolic solution was filtered at low temperature (4 °C). The filtrate and the residue were collected. The filtrate was evaporated to dryness and then resuspended in 10 ml of distilled water. The residue was also suspended in 10 ml of water and stored at −4 °C.

Toxicity of the fungal metabolites against fruit fly adults

The toxicity of the metabolites produced by each of the isolates was tested on *B. oleae* and *C. capitata* flies. One milliliter, containing 200 mg of dried material collected from each supernatant, was mixed with 1 ml of liquid adult diet; the diet was placed in droplets on waxed paper and offered to 10 3-day-old flies in small wooden cages (10 cm × 10 cm × 10 cm). Fifteen repetitions for each supernatant and for each one of the three fractions of the two active supernatants were carried out. Flies were starved for 24 h prior to the feeding experiments. To ensure that flies were consuming the diet, 0.001 mg/g of a non-toxic insect dye (Waner Jenkinson Co, Inc., K7057 D + C RED 33) was added, so that abdomens of flies ingesting the diet became red.

The toxicity of the lipophilic chemicals extracted in chloroform and those soluble in methanol and the residue was also tested. 1.5 ml of the chloroform fraction, equivalent to 5 mg of dry material, was concentrated to a volume of 0.3 ml and mixed with 1 ml of liquid diet. The mixture was left at room temperature for ca 2 h until chloroform evaporation. At the same time another mixture containing the same volume of chloroform and liquid diet was prepared, treated similarly and used as control. Moreover, 1 ml, equivalent to 5 mg dried material of the methanol soluble and the residue fractions, was mixed with 1 ml of liquid diet and offered to the flies. Pure liquid diet was offered to flies used as controls. Mortality was assessed at intervals of 2 h for 48 h.

Statistical analysis

Mortality data were analyzed with one-way analysis of variance (ANOVA) of arcsine transformed corrected mortality (Abbott, 1925). Means were separated by the Tukey's studentized range honestly significant difference (HSD) (Tukey, 1953). LT50s were calculated by probit analysis (Finney, 1971). The SPSS 8.0 software was used for our statistical analysis.

Results

Toxicity of fungi to B.oleae *and* C.capitata *adults*

Contact bioassays

Olive flies were susceptible to infection by several fungal isolates tested (Table 1). *M. hiemalis* isolate (SMU-21) and *P. aurantiogriseum*, were more toxic than the other isolates, during the first 7 days of the experiment ($F = 9.224$, $p = 0.015$, $df = 6$). Fourteen or 21 days after treatment, the toxicity of all fungal isolates had increased. No significant difference on the total fly mortality among the isolates *M. hiemalis* (SMU-21), *P. chrysogenum* and *B. bassiana*, was recorded by 21 days. The greatest total mortality occurred to flies exposed to *M. hiemalis* (SMU-21) conidia, followed by those exposed to *B. bassiana* and *P. chrysogenum*. Fly mortality recorded for isolates of *B. bassiana* and *P. chrysogenum* was not significantly different from the final mortality of flies exposed to conidia of *M. hiemalis* (DMU-01) and *P. aurantiogriseum* ($F = 4.705$, $p = 0.006$, $df = 6$). *B. brongniartii*-exposed flies displayed mortality similar to, or slightly higher than that observed in the control flies.

Oral bioassays – B.oleae

Similar results were obtained when aqueous conidial suspensions were mixed with liquid diet for adults. At the concentration of 10^4 conidia/ml, the level of fly mortality was significantly different among the six isolates tested ($F = 5.264$, $p = 0.001$, $df = 6$) (Table 2). The most toxic isolate to *B. oleae* flies was *M. hiemalis* (SMU-21), which gave 85.2% total adult mortality, followed by *P. chrysogenum* and *B. bassiana* isolates. The final mortality level produced

Table 1. Percentage mortality of *Bactrocera oleae* flies after exposure for 10 min in PDA cultures of fully grown colonies of six fungi isolates (n = 50)

Isolates	Host	Contact bioassays		
		Time (days)		
		7	14	21
Beauveria bassiana	*B. oleae*	7.5b	57.8b	66.5ab
B. brongniartii	*Melolontha* spp.	8.3b	25.8c	38.6c
Mucor hiemalis (SMU-21)	*S. nonagrioides*	24.6a	71.2a	88.3a
M. hiemalis (DMU-01)	*B. oleae*	9.5b	51.7bc	58.6b
Penicillium aurantiogriseum	*B. oleae*	19.4a	53.1b	57.7b
P. chrysogenum	*B. oleae*	9.7b	63.3ab	69.4ab
Control	–	1.4c	8.7d	20.5c

Means followed by the same letter within each column are not significantly different (ANOVA; 7 days: $F = 9.224$, $p = 0.015$, df = 6; 14 days: $F = 5.324$, $p = 0.0011$, df = 6; 21 days: $F = 4.705$, $p = 0.006$, df = 6).

Table 2. Total percentage mortality of adult *Bactrocera oleae* and *Ceratitis capitata*, fed with liquid adult diet containing 10^4 conidia/ml of fungal isolates (fly mortality recorded for 21 days, n = 150)

Isolates	*Bactrocera oleae*		*Ceratitis capitata*	
	Mean	Mycosis (%)*	Mean	Mycosis (%)
Beauveria bassiana	62.6ab	78.6	85.6ab	84.7
B. brongniartii	36.5c	83.4	97.4a	86.4
Mucor hiemalis (SMU-21)	85.2a	99.8	64.2b	99.3
M. hiemalis (DMU-01)	51.4b	98.9	60.0b	99.1
Penicillium aurantiogriseum	54.8b	93.3	61.8b	93.1
P. chrysogenum	63.2ab	97.1	61.2b	94.2
Control	28.2c		22.2c	

Means followed by the same letter within each column are not significantly different (ANOVA; *B. oleae*: $F = 5.264$, $p = 0.001$, df = 6; C. *capitata*: $F = 15.106$, $p < 001$, df = 6).

* Mycosis (%) is the percentage of the incubated dead insects with fungal out growth.

by the isolate *M. hiemalis* (SMU-21) was not significantly different from *P. chrysogenum* and *B. bassiana*. The mortality level for the latter two isolates was not different from that obtained for the isolates *M. hiemalis* (MDU-01) and *P. aurantiogriseum*.

Probit analysis of the time-mortality response revealed that *M. hiemalis* (SMU-21) killed flies significantly more quickly than the other four isolates ($LT_{50} = 8.2$ days), the LT_{50} for all the other isolates tested being similar (Table 3).

Med fly

B. brongniartii and *B. bassiana* were the most pathogenic fungi to *C. capitata*. Levels of mortality due to these two species were 97.4 and 85.6%, respectively (Table 2). The two *Mucor* and the two *Penicillium* isolates induced moderate mortality rates, ranging from 60–64.2% in *C. capitata* adults. The level of toxicity of all species tested was significantly different from the control ($F = 15.106$, $p < 001$, df = 6).

Table 3. Time-mortality response of *Bactrocera oleae* adults fed with liquid diet containing 1×10^4 conidia suspension of five toxic fungal isolates (n = 150)

Isolates	Slope ± SE	LT_{50} (95% CL)	χ^2	df	*p*
Beauveria bassiana	0.071 ± 0.003	17 (15.7–18.6)	298.39	40	0.000
Mucor hiemalis (SMU-21)	0.131 ± 0.003	8.2 (6.7–9.6)	677.61	40	0.000
M. hiemalis (DMU-01)	0.098 ± 0.006	18.1 (15.6–21.7)	146.20	40	0.000
Penicillium chrysogenum	0.11 ± 0.006	21.7 (15.1–19.2)	193.71	40	0.000
P. aurantiogriseum	0.11 ± 0.004	19.1 (17.6–21.1)	225.42	40	0.000

Probit analysis of the time-mortality response, however, revealed that LT_{50} (= 10.7 days) for *B. brongniartii* was significantly shorter than that of *B. bassiana* (Table 4). The time-mortality response was equal for the *Mucor hiemalis*, *P. chrysogenum* and *B. bassiana* isolates since the 95% confidence limits for these isolates overlapped. *P. aurantiogriseum* killed flies significantly more slowly than all the other isolates.

A high percentage of the dead flies, exposed to diet inoculated with *Mucor* and *Penicillium* isolates developed mycosis, while the percentage to flies exposed to *Beauveria* species was lower. No evidence of mycosis in any control cadaver was observed (Table 2).

Biological activity of fungal metabolites

Evaluation of fungal metabolites collected after culturing four of the isolates in Richard's medium showed that toxicity of the *M. hiemalis* (SMU-21) and *P. chrysogenum* isolates is related to toxic metabolites produced by the fungi (Table 5). The flies consumed fairly high quantities of food and at least for the first 6 h no signs of toxicity were recorded. However, the metabolites produced by *M. hiemalis* (SMU-21) were very toxic, resulting in 100% mortality of *B. oleae* and *C. capitata* adults 24 h after exposure. *P. chrysogenum* also produced toxic metabolites.

Further feeding tests of the chemicals present in the three fractions (chloroform, methanol, and residue) revealed that the bioactivity of *M. hiemalis* (SMU-21) was retained only in the methanol fraction, causing high levels of mortality (Table 6). The bioactivity of *P. chrysogenum* was found in the methanol and the precipitate fractions.

Discussion

To our knowledge there are no reports on the susceptibility of *B. oleae* to entomopathogenic fungi and limited information is available for *C. capitata*. The fungal species *M. anisopliae* strain CG-260, and *P. fumosoroseus* were

Table 4. Time mortality response of *Ceratitis capitata* adults fed with liquid diet containing 1×10^4 conidia suspension of six toxic fungal isolates (n = 130)

Isolates	Slope ± SE	LT_{50} (95% CL)	χ^2	df	*p*
Beauveria brongniartii	0.28 ± 0.008	10.7 (10.1–11.4)	367.79	34	0.000
B. bassiana	0.19 ± 0.006	13.4 (12.5–16.7)	352.94	34	0.000
Mucor hiemalis (SMU-21)	0.127 ± 0.005	15.4 (14.3–16.7)	277.50	34	0.000
M. hiemalis (DMU-01)	0.129 ± 0.005	16.7 (15.8–17.7)	223.75	34	0.000
Penicillium chrysogenum	0.14 ± 0.005	16.6 (15.9–17.5)	101.04	34	0.000
P. aurantiogriseum	0.087 ± 0.005	21.4 (19.1–25.1)	208.60	34	0.000

Table 5. Percentage mortality of *Bactrocera oleae* and *Ceratitis capitata* adults fed with liquid diet containing 200 mg of the four fungi metabolites collected from Richard's medium cultures (n = 150)

Fungal (supernatant)	%Mortality (Means ± SE)			
	Hours after feeding			
	Bactrocera oleae		*Ceratitis capitata*	
	24	48	24	48
Mucor hiemalis (SMU-21)	100	100	100	100
M. hiemalis (DMU-01)	0	0	0	0
Penicillium aurantiogriseum	0	0	0	0
P. chrysogenum	61.2 ± 2.4	91.1 ± 4.3	46.7 ± 3.1	86.8 ± 2.5
Control	0	0	0	0

Table 6. Percentage mortality of *Bactrocera oleae* and *Ceratitis capitata* adults fed with liquid diet containing 5 mg of fractions of the metabolites collected from Richard's medium cultures of isolates SMU-21 and PCH-01 (n = 150)

Fractions	Means ± SE (hours after feeding)			
	Bactrocera oleae		*Ceratitis capitata*	
	24	48	24	48
Mucor hiemalis (SMU-21)				
Chloroform	3.2 ± 0.4	5.8 ± 1.3	2.1 ± 0.6	7.2 ± 2.8
Methanol	84.5 ± 5.2	100	79.3 ± 3.1	93.7 ± 4.7
Precipitate	0	0	0	0
Penicillium chrysogenum				
Chloroform	6.2 ± 2.3	9.3 ± 5.2	6.1 ± 1.4	9.4 ± 4.3
Methanol	15.1 ± 2.8	82.5 ± 6.3	12.3 ± 4.7	79.3 ± 5.6
Precipitate	16.3 ± 2.4	85.0 ± 5.2	21.3 ± 3.8	82.4 ± 4.7
Control				
Liquid diet	0	0	0	0
Liquid diet + Chloroform	3 ± 0.7	7 ± 2.1	5.8 ± 0.4	6.9 ± 0.7

reported to be pathogenic to *C. capitata* adults (Castillo et al., 2000). In the same study, a lower level of toxicity was shown for the fungal species *Aspergillus ochraceous*, *B. bassiana* and *P. chrysogenum*. Strains of the two fungi *B. bassiana* and *P. chrysogenum* were also included in our study. Although there are differences in the methods of treatment, strains used and the time of evaluation, the levels of mortality are comparable. Castillo et al. (2000) reported mortality levels of 8 and 30% for *P. chrysogenum* and *B. bassiana* 10 days after exposure at concentrations 10^4 conidia/fly, whereas in our experiments the mortality levels at the same concentration, 10 days after feeding, were 7.8 and 31.2% respectively.

M. hiemalis (SMU-21) was the most toxic fungus to *B. oleae* adults. The *M. hiemalis* (DMU-01) isolate from *B. oleae* pupae also showed moderate toxicity, but was less toxic than that of (SMU-21). These results and the results obtained from the feeding experiments with the metabolites produced by the two *Mucor* isolates in liquid cultures, suggest that these two isolates might be different strains.

In general *M. hiemalis* is normally a harmless saprophytic species and has been found in injured insects. However, Heitor (1962) isolated a strain of *M. hiemalis* with entomotoxic properties from larvae of *Mamestra brassicae* L. (Lepidoptera: Noctuidae).

Differences in the susceptibility of the two fruit fly species to the fungal isolates were observed only for the *B. brongniartii* isolate. This isolate showed high virulence to *C. capitata* flies while virulence to *B. oleae* flies was very low. A strain of *B. brongniartii* was found to be the least pathogenic in screening tests, where several entomopathogenic fungal species were evaluated against adults of the house fly (Barson et al., 1994).

The level of mycosis was generally high for all fungal species, however mycosis of cadavers inoculated with *Beauveria* species was lower. Under our experimental conditions the two *Beauveria* species grew much more slowly than the other fungal species and this might be one of the reasons that mycosis observed in these cadavers was lower. Ferron (1977) reported humidity-independent infection of *Acanthoscelides obtectus* Say by *B. bassiana*, but the development of the fungus on the cadavers was only possible at humidity near saturation. Moreover delay in mycosis, or lack, has been reported for cadavers of several insect species inoculated with pathogenic fungi, despite having been killed by the fungus (Shimazu, 1994; Shimazu et al., 2002).

Many entomopathogenic fungi of the Hyphomycetes are known to produce toxic insect secondary metabolites in nutrient-rich media. Some of these metabolites from Hyphomycetes have been isolated and identified (Krasnoff et al., 1991; Amini et al., 1999; Abendstein and Strasser, 2000; Bandani et al., 2000) and their chemical structures have been elucidated (e.g. oosporein, beauvericin and beauveriolides). Studies have shown that quantities of these metabolites produced *in vivo* are usually much less than those secreted in nutrient media (Strasser, 2000).

Little information is available on toxin production in the Mucorales; Reiss (1993) screening the toxigenicity of representatives of 15 species of Mucorales including *M. hiemalis* against larvae of the brine shrimp *Artemia salina* concluded that species of Mucorales have only a weak toxigenicity. Aqueous extracts from *M. hiemalis*, had zero toxicity to brine shrimp larvae. In our study the methanol-soluble secondary metabolites produced by *M. hiemalis* (SMU-21) were highly toxic to the adults of the two fruit flies. Toxic symptoms appeared as soon as the flies consumed very small quantities of the offered diet.

Usually after a brief exposure to the food flies flew away, remained motionless and finally died in the same position and site.

Concerning the genus *Penicillium* it is known to include many toxigenic species and the range of mycotoxin classes produced is much broader than that of any other genus. *P. chrysogenum* and *P. notatum* are the two species producing high quantities of the antibiotic penicillin (Sweeney and Dobson, 1998; Demain and Fang, 2000); these toxins have not been reported as toxic to insects.

The toxicity of the metabolites produced by *P. chrysogenum* was less than that of isolate SMU-21. However 91.1% of *B. oleae* and 86.8% of *C. capitata* flies died within 48 h after exposure.

Data showing that toxic metabolites are produced by *P. chrysogenum* have been reported also by Castillo et al. (2000). The metabolites were extracted from the fungal mycelia developed on PDA in dichloromethane. The toxicity of these metabolites was low, with 44% of the flies dying 6 days after eating them. Comparing the results of these studies with our results, it seems possible that the dichloromethane extracts contained lower quantities of the toxic metabolites or different metabolites than those collected from the Richard's medium cultures. Another important parameter might lie in differences in the strains used in the two studies.

This study demonstrated that both fruit fly species are susceptible to different species and isolates of toxic fungi, and that *B. oleae* and *C. capitata* adults show quite different degree of susceptibility. *M. hiemalis* (SMU-21) was more virulent to *B. oleae* flies than the other toxic fungi whereas *C. capitata* flies were more susceptible to *B. brongniartii* followed by *B. bassiana*. Our study also shows that the toxicity of *M. hiemalis* (SMU-21) and *P. chrysogenum* resides in their produced metabolites. The high toxicity of the metabolites produced by the two species justifies further research for the isolation and identification of these potent toxins.

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References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18: 265–267.

Abendstein, D. and H. Strasser, 2000. Consideration on toxic metabolites produced by *Beauveria brongniartii*. In: S. Kelley (ed), *Integrated Control of Soil Pests Subgroup "Melolontha". IOBC/WRP Bulletin*, Dijon. pp. 99–105.

Amini, B., L. Ibrahim and T.M. Butt, 1999. Antifeedant properties of destruxins and their use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocont. Sci. Technol.* 9: 487–498.

Bandani, A.R., B.P.S. Khambay, J. Faull, R. Newton, M. Deadman and T.M. Butt, 2000. Production of efrapectins by *Tolypocladium* species (Deuteromycotina: Hyphomycetes) and evaluation of their insecticidal and antimicrobial properties. *Mycol. Res.* 104: 537–544.

Barson, G., N. Renn, A.F. Bywater, 1994. Laboratory evaluation of six species of entomopathogenic fungi for the control of the house fly (*Musca domestica* L.) a pest of intensive animal units. *J. Invertebr. Pathol.* 64: 107–113.

Brooks, D.L. and E.S. Raun, 1965. Entomogenous fungi from corn insects in Iowa. *J. Invertebr. Pathol.* 7: 79–81.

Castillo, M.A., P. Moya, E. Hernandez, E. Primo-Yufera, 2000. Susceptibility of *Ceratitis capitata* Wiedermann (Diptera: Tephritidae) to entomopathogenic fungi and their extracts. *Biol. Control* 19: 274–282.

Carruthers, R.I., D.L. Haynes and D.M. MacLeod, 1985. *Entomophthora muscae* (Entomophthorales: Etnomophthoraceae) in the onion fly, *Delia antiqua* (Diptera: Anthonyiidae). *J. Invertebr. Pathol.* 45: 81–93.

Delrio, G., 1992. Integrated control in olive groves. In: Biological Control and Integrated Crop Protection: Towards Environmentally Safer Agriculture. *Proc. Int. Conf. IOBC.WPRS*, Veldhoven, Netherlands, 8–13 September 1991, pp. 67–76.

Demain, A.L. and A. Fang, 2000. The natural functions of secondary metabolites. *Adv. Biochem. Engin./Biotechnol.* 69: 1–39.

Eilenberg, J. and H. Philipsen, 1988. The occurrence of Entomophthorales on the carrot fly (*Psila rosae* F.) in field during two successive seasons. *Entomophaga* 33: 135–144.

Ferron, P., 1977. Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* (Fungi Imperfecti, Moniliales) in imagines of *Acanthoscelides obtectus* (Col.: Bruchidae). *Entomophaga* 22: 393–396.

Finney, D.J., 1971. Probit analysis. Cambridge University Press, Cambridge.

Hajek, A.E. and R.J. St. Leger, 1994. Interactions between fungal pathogens and insect's hosts. *Annu. Rev. Entomol.* 39: 293–322.

Heitor, F., 1962. Parasitisme de blessure par le champignon *Mucor hiemalis* Wehmer chez les insecte. *Ann. Epiphyties* 13: 179–205.

Krasnoff, S.B., S. St. Gupta, R.J. Leger and J.A.A. Renwick, 1991. Antifungal and insecticidal properties of the efrapeptins: metabolites of the fungus *Tolypocladium niveum*. *J. Invertebr. Pathol.* 58: 180–188.

Lacey, L.A., R. Frutos, H.K. Kaya and P. Vails, 2001. Insect pathogens as biological control agents: do they have a future? *Biol. Control* 21: 230–248.

Lynch, R.E. and L.C. Lewis, 1978. Fungi associated with eggs and first instar larvae of the European corn borer. *J. Invertebr. Pathol.* 32: 6–11.

Miczulski, B. and Z. Machowicz-Stefaniak, 1977. Fungi associated with the cereal leaf beetle *Oulema gallaeciana* (Coleoptera: Chrysomelidae). *J. Invertebr. Pathol.* 29: 386–387.

Mullens, B.A., 1989. Cross-transmission of *Entomophthora muscae* (Zygomycetes: Entomophthoraceae) among naturally infected muscoid fly (Diptera: Muscidae) hosts. *J. Invertebr. Pathol.* 53: 272–275.

Mullens, B.A., J.L. Rodriguez and J.A. Meyer, 1987. An epizootiological study of *Entomophthora muscae* in muscoid fly populations on southern California poultry facilities with emphasis on *Musca domestica*. *Hilgardia* 55(3): 1–41.

Reiss, J., 1993. Biotoxic activity in Mucorales. *Mycopathologia* 121: 123–127.

Roberts, D.W. and R.A. Humber, 1981. Entomopathogenic fungi. In: G.T. Cole and W.B. Kendrick (eds), *Biology of Conidial Fungi*. Vol. 2. Academic Press, New York. pp. 201–236.

Shimazu, M., 1994. Potential of the cerambycid-parasitic type of *Beauveria brongniartii* (Deuteromycotina: Hyphomycetes) for microbial control of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* 29: 127–130.

Shimazu M., B. Zhang and Y. Liu, 2002. Fungal pathogens of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and their virulences. *Bull. FFPRI* 1(382): 123–130.

Steinkraus, D.C., C.J. Geden, D.A Rurz and J.P. Kramer, 1990. First report of the natural occurrence of *Beauveria bassiana* (Moniliales: Moniliaceae) in *Musca domestica* (Diptera: Muscidae). *J. Med. Entomol.* 27: 309–312.

Strasser, H., A. Vey and T. Butt, 2000. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? *Biocont. Sci. Technol.* 10: 717–735.

Sweeney, M.J. and A.D.W. Dobson, 1998. Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *Internat. J. Food Microbiol.* 43: 141–158.

Tanada, Y. and H.K. Kaya, 1993. *Insect Pathology*. Academic Press, San Diego, California.

Tukey, J.W., 1953. Some selected quick and easy methods for statistical analysis. *Trans. N.Y. Acad. Sci. (Ser 2)* 16: 88–97.

Watson, D.W., C.J. Geden, S.J. Long and D.A. Rutz, 1995. Efficacy of *Beauveria bassiana* for controlling the house fly and stable fly (Diptera: Muscidae). *Biol. Control* 5: 405–411.

Watson, D.W., D.A. Rutz and S.J. Long, 1996. *Beauveria bassiana* and sawdust bedding for the management of the house fly *Musca domestica* (Diptera: Muscidae) in calf hutches. *Biol. Control* 7: 221–227.

Wilding, N. and F.B. Lauckner, 1974. *Entomophthora* infecting wheat bulb fly at Rothamsted, Hertfordshire, 1967–1971. *Ann. Appl. Biol.* 76: 161–170.