BioControl **47:** 685–696, 2002.

Increased mortality of *Acanthoscelides obtectus* by alkane-grown *Beauveria bassiana*

R. CRESPO[1], M.P. JUÁREZ[1,*], G.M. DAL BELLO[2], S. PADÍN[3], G. CALDERÓN FERNÁNDEZ[1] and N. PEDRINI[1]
[1]*Instituto de Investigaciones Bioquímicas de La Plata, Conicet, Facultad de Ciencias Médicas, UNLP, La Plata, Argentina;* [2]*Centro de Investigaciones en Fitopatología, Facultad de Ciencias Agrarias y Forestales, UNLP, La Plata, Argentina;* [3]*Cátedra de Terapéutica Vegetal, Facultad de Ciencias Agrarias y Forestales, UNLP, La Plata, Argentina*
**Author for correspondence; e-mail: mjuarez@isis.unlp.edu.ar*

Received 11 January 2001; accepted in revised form 21 October 2001

Abstract. The effect of alkane-growth induction of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes), on the ability to kill the bean weevil *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae), was tested. Adult insects were sprayed with an 0.01% Tween 20 aqueous suspension of 4×10^6 conidia/ml. The performance of fungi grown in complete agar medium containing glucose as carbon source (FS_0) was compared to that of alkane-grown fungi (FS_1) with n-hexadecane as the only carbon source. Mortality increased ($p < 0.05$) from $22 \pm 4.5\%$ to $44 \pm 11.4\%$ at day 7, and from $26 \pm 5.5\%$ to $60 \pm 7.1\%$ 14 days after treatment with FS_0 or FS_1 respectively. The insect epicuticular hydrocarbons were analysed by capillary gas chromatography (CGC); major components were saturated hydrocarbons, 27 to 29 carbons in length. A variety of methyl-branched isomers of C27 were the prevailing structures, and nC27 was the major straight chain component. Whole insect hydrocarbons were qualitatively identical to those of the epicuticular surface. Oleic, linoleic and palmitic acids accounted for almost 88% of the fungal fatty acids, irrespective of the carbon source used for growth; however, the unsaturated/saturated ratio diminished markedly from 4.32 (FS_0) to 2.47 (FS_1). These results indicate that alkane supplementation of culture media might be a tool to improve the virulence of some mycoinsecticides.

Key words: alkane-growth, Bruchidae, Coleoptera, Deuteromycotina, entomopathogenic fungi, fatty acids, hydrocarbons, insect epicuticle, storage bruchid

Abbreviations: CGC – capillary gas chromatography; CMA – complete media agar; DMA – defficient media agar; FAME – fatty acid methyl ester; FID – flame ionization detector; FS_0 – fungal strain grown on CMA; FS_1 – fungal strain grown on hydrocarbon-enriched DMA; HPTLC – high performance thin layer chromatography; KI – Kovats indices; TLC – thin layer chromatography

Introduction

Storage of grains requires continuous protection against insects, mites and fungi, which can cause hydrolysis, oxidation and hence a general decreased level of certain nutrients. The common bean, *Phaseolus vulgaris* L. is one of the most widely cultivated grain legumes (Kapeya et al., 1993). A wide range of insect pests cause significant damage to beans both in the field and during storage. Among bruchid species that infest beans, one of the most important is the bean weevil *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae), producing both quantitative and qualitative damage to grain (Giga et al., 1990). Quantitative damage is due to grain weight loss caused by insect feeding (Steffan, 1963; Golebiowska, 1969). Qualitative damage is due to product alterations such as reduction of nutritional and aesthetic value, and alteration of industrial (baking) characteristics. In Brazil and Colombia, losses due to bruchids have been estimated to be about 13% and 7.4%, respectively, whereas in Africa, post-harvest losses of 73% have been reported in Kenya, 30% in Tanzania, and 23% in Uganda (Karel and Autrique, 1989). The use of chemical pesticides for storage pest control is very limited because of the strict requirements imposed for their safe use on or near food. Prolonged exposure to chemical pesticides can lead to serious problems such as insecticide resistance (Pacheco et al., 1990; Sartori et al., 1990). Non-chemical methods are attractive since they do not leave chemical residues in the commodity nor do they cause resistance in insects. A promising strategy to minimize the adverse effects of regularly used insecticides is the use of entomopathogenic fungi and other microbial control agents. The potential of using fungal pathogens to control insects is well documented (Roberts and Hajek, 1992), and the use of fungi as control agents against storage pests has been recently reviewed (Adane, 1994; Adane et al., 1996; Padín et al., 1997; Hidalgo et al., 1998; Moino et al., 1998).

The mode of action of entomopathogenic fungi involves attachment of fungal spores to the cuticle, followed by spore germination and appressorium formation. Penetration is then initiated, involving both mechanical and enzymatic mechanisms (Fargues, 1984). The ability of the fungus to fully degrade the epicuticular hydrocarbon components of its insect host, utilizing them as exogenous carbon source, was recently shown (Napolitano and Juárez, 1997). When fungi were grown on a hydrocarbon-enriched medium as the sole carbon source, the hydrocarbon degrading activity was noticeably increased (Crespo et al., 2000).

The aim of this study was to determine the relevance of inducible hydrocarbon-degrading pathways in relation to virulence of mycoinsecticides and alterations in lipid composition of the fungus when grown on alkane-enriched media.

Materials and methods

Fungal strain culture and development conditions

The *B. bassiana* strain used in this study (USDA-ARS culture collection, Ithaca, New York, ARSEF 5500 (Humber, 1998) was initially isolated in 1993 from dead larva of *Diatraea saccharalis* Fab. (Lepidoptera: Pyralidae) collected in a corn field near Oliveros, Santa Fe, Argentina. The fungus was cultured on complete media agar (CMA)[1] plates containing 0.4 g KH_2P0_4, 1.4 g Na_2HP0_4, 0.6 g $mgS0_4 \cdot 7H_20$, 1.0 g KCl, 0.7 g $NH_4N0_3 \cdot 7H_20$, 10 g glucose, 5 g yeast extract, 15 g agar in 1 l of distilled water. Plates were incubated at 27 $\pm$ 2 °C for 14 days with alternating light (3500 lux) under a 12 h/12 h-light:dark cycle plus the addition of NUV light (365 nm) to induce sporulation) (FS_0). Fungal suspensions were prepared by adding 0.01% Tween 20 aqueous solution into the plates and rubbing the sporulating colonies with a sterile bent needle. After filtering through a nylon sieve, the suspension was adjusted to 4×10^6 spores ml^{-1} with sterile water.

Fungal growth on synthetic hydrocarbon-enriched media

Media agar plates with the same composition as the CMA, but deficient in glucose and yeast extract (DMA) and containing n-hexadecane (500 μl of a 10% hexane solution, w/v), were employed. The hydrocarbon was layered onto the surface media and evaporated. The fungal cultures maintained on CMA (FS_0) were inoculated on the DMA plates, and incubated at 27 $\pm$ 2 °C for 12–14 days (FS_1).

Insect rearing

Adults of *A. obtectus* were mass produced in glass jars (250 ml) covered with a piece of fine cloth, starting with insects found in grains pre-tested to be free of pesticides. Insects were reared on a diet of wheat flour, wheat kernels or bean grains, successively. Grains belonging to commercial varieties of durum wheat and butter bean were used. The cultures were maintained in an environmental chamber under a 12 h/12 h light: dark photoperiod at 27 $\pm$ 2 °C and 70 $\pm$ 5% r.h. All experiments were carried out under the same environmental conditions used to maintain the cultures. Adult insects, two weeks after eclosion, were used for bioassays.

Chemicals

Solvents were obtained from Carlo Erba (Milan, Italy) and Merck Ltd. (Darmstadt, Germany). Silica gel and other chemicals were from Aldrich (Milwaukee, Wisconsin, USA). Hydrocarbons and fatty acid standards were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Solvents were redistilled prior to use. High performance thin layer chromatography (HPTLC) silica plates were from Merck Ltd.

Bioassays

Laboratory – reared adult insects (15 days old), randomly collected from rearing jars, were used for this study. To test the virulence of each fungal culture, three Petri dishes containing filter paper (9 cm diameter) were assembled and twenty insects were placed in each Petri dish. Using a perfume sprayer each of five replicates of *A. obtectus* were sprayed directly with 1 ml of fungal suspension (in 0.01% Tween 20 in distilled water) containing 4×10^6 spores ml^{-1} of *B. bassiana*. Controls were sprayed with 0.01% Tween 20 in distilled water. Each treated replicate was kept for 24 hours without food at 27 ± 2 °C and $70\% \pm 5\%$ r.h. After 24 h, insects from each replicate were transferred to a 100 ml-plastic cup containing 50 g undamaged bean grains. Each cup was sealed using muslin cloth and a perforated lid. Before starting the assay, the beans were stored at -15 °C for 10 days to ensure all treatments were initially insect-free. All the insects were maintained under controlled conditions as described above. Observations of each of the flasks were done at 7 and 14 days after exposure to the fungus, by emptying the contents of each flask onto a white paper to identify dead individuals. Dead adults were submerged in 95% ethanol for 1 min, washed in sterile distilled water for 5 min, dried, and then placed in plates on moistened filter paper. Cadavers were kept at 27 ± 2 °C for 3–5 days in the dark, and those that showed hyphal growth characteristic of the entomopathogenic fungi were recorded as infected.

Insect hydrocarbon analysis

After several washes with re-distilled water, epicuticular hydrocarbons from *A. obtectus* were extracted by immersion in hexane (5 min, 6 ml/gr) and partitioned against distilled water (5:1, v/v) (Juárez et al., 2000). Total hydrocarbons were similarly extracted after a 24 hr-immersion period in hexane. Solvent was concentrated under vacuum and the extract was passed through a minicolumn (2.5 cm $\times$ 0.5 cm) of Biosil (Bio-Rad Lab, Richmond, CA); hydrocarbons were eluted with n-hexane, as previously described (Napolitano

and Juárez, 1997). The eluent was concentrated and analysed on a Hewlett-Packard 6890 gas chromatograph with a flame ionization detector (FID). A 30 m × 0.32 mm I.D. 0.5 mm stationary phase DB-5 (J & W Scientific, Folson, CA, USA) capillary column was used in splitless mode, with an initial temperature of 50 °C for 1 min, and a temperature program from 50 to 200 °C at a rate of 50 °C/min, then at 3 °C/min up to 300 °C, and a final holding time of 15 min at 300 °C. To check for the presence of unsaturated components, the hydrocarbon extract was spotted on a silica gel TLC plate impregnated with 20% $AgNO_3$, using hexane: diethyl ether (80:20, v/v) as the developing solvent. Appropriate saturated and unsaturated hydrocarbon standards were spotted on separate lanes for comparison. Each hydrocarbon fraction was scraped from the TLC plate and analysed by capillary gas chromatography (CGC) as described above. Hydrocarbon chain lengths were estimated by calculating their Kovats indices (KI) (Kovats, 1965), and by comparison of their retention time with that of appropriate standards.

Analysis of fungal lipids and fatty acids

For the analysis of lipids, whole fungal cells from FS_0 and FS_1 cultures were extracted with chloroform: methanol (2:1, v/v) and partitioned with distilled water (5:1 v/v) according to Folch method (Folch et al., 1957). Afterwards, organic layers were reduced in volume under vacuum or N_2 stream and stored at −18 °C. In order to determine the total amount of lipids, the chloroform layer was dried to constant dried weight. Analysis by HPTLC was performed on silica gel plates developed in petroleum ether:diethyl ether:acetic acid (80:20:1, v/v). Plates were charred after immersion in H_2SO_4 (10% in ethanol). For individual fatty acid analyses, fungal lipids were extracted as described above. The chloroform extract was evaporated under N_2 and resuspended in 1 ml toluene. Transesterification was performed at 100 °C with BF_3-methanol for 1 hour. The resulting fatty acid methyl esters (FAME) were extracted with chloroform and partitioned against distilled water (3:2 v/v). CGC analyses of the FAME were performed on a Hewlett-Packard 6890 gas chromatograph. Samples were analysed on a 30 m × 0.32 mm I.D. 0.5 mm stationary phase Omega Wax 250 (Alltech Associates, Arlington Heights, Il, USA) capillary column. The temperature of the oven was programmed from 175 to 220 °C at a rate of 3 °C/min, then held at 220 °C for 15 min.

Table 1. Mean percentage mortality of *A. obtectus* adults treated with *B. bassiana* (ARSEF 5500)

Fungal incubation condition[b]	Percentage mortality[a]	
	Day 7	Day 14
FS_0	22 ± 4.5	26 ± 5.5
FS_1	44 ± 11.4	60 ± 7.1

[a]Values are means of 5 replicates ± standard deviation (SD). Means differed significantly by one-tailed *t*-test, $p < 0.05$, within each column. FS_1 values were also significantly different when compared at day 7 and day 14, whereas FS_0 values showed no significant difference for the same period.

[b]Fungal suspensions containing 4×10^6 conidia/ml of Bb5500 grown in complete agar medium (FS_0), and in hexadecane-enriched medium (FS_1), as described in Materials and methods. Mortality was not observed for control insects (sprayed with 0.01% Tween 20 in water).

Results

Bioassays

Observations of cadavers indicated that the mycelium appears externally within 24–48 h after placement on damp filter paper. Dead insects were collected at 7 and 14 days after exposure to the fungus, as described in Materials and Methods. Post-mortem mycelial and conidial growth demonstrated that the insects had died due to pathogen infection. Large differences in bioinsecticide efficacy were evident for fungi grown on CMA (FS_0) as compared to that grown on hydrocarbon-enriched-DMA agar media (FS_1). Higher kill was obtained from fungi grown on hydrocarbon-enriched media. FS_0 and FS_1 differed significantly (by one-tailed *t*-test, $p < 0.05$) when cultures were compared at 7 or 14 days post-treatment, percentage mortality ranged from $22 \pm 4.5\%$ to $44 \pm 11.4\%$, and from $26 \pm 5.5\%$ to $60 \pm 7.1\%$ respectively (Table 1). Significant temporal differences in mortality were detected for the alkane-grown culture FS_1 ($p < 0.05$, in Table 1), whereas percentage mortality for FS_0 did not change significantly between 7 and 14 days post-treatment.

Hydrocarbon analysis of Acanthoscelides obtectus

A series of saturated n-alkanes ranging from 25 to 30 carbons in length was detected by gas chromatographic analysis of the epicuticular hydrocarbons of *A. obtectus* (Table 2). Absence of unsaturated components was determined after $AgNO_3$-TLC fractionation, as described in Materials and Methods, and confirmed by mass spectrometric analysis (Juárez and Cafferata, unpublished). The major components were methyl-branched hydrocarbons with 27

Table 2. Percentage composition of the hydrocarbons from the bean weevil *A. obtectus*

CGC peak[a]	Hydrocarbon[b]	Kovats indices	Hydrocarbon percentage[c] Epicuticle X	Epicuticle SD	Whole insect X	Whole insect SD
1	n-C25	2500	1.54	0.39	1.20	0.02
2	Monomethylbranched-C25	2529	1.07	0.06	1.83	0.08
3	Monomethyl- plus dimethylbranched-C25	2539	3.33	0.23	5.86	0.18
4	n-C27	2700	14.65	0.28	9.42	0.44
5	Monomethyl- plus dimethylbranched-C27	2741	16.24	0.83	16.19	0.39
6	(Sub)terminally branched monomethyl-C27	2751	1.62	0.53	3.17	0.06
7	Dimethylbranched-C27	2770	24.30	1.73	22.76	0.08
8	Dimethyl- plus trimethylbranched-C27	2780	12.28	1.66	14.82	0.37
9	Trimethylbranched-C27	2790	3.75	0.15	4.34	0.04
10	n-C28	2800	1.09	0.16	0.79	0.06
11	Terminally branched dimethyl-C27	2808	2.97	0.16	3.85	0.05
12	Internally branched monomethyl-C28	2834	2.23	0.09	2.26	0.02
13	Dimethylbranched-C28	2859	1.61	0.10	1.45	0.04
14	n-C29	2900	5.29	0.51	3.65	0.31
15	Internally branched monomethyl-C29	2928	4.18	0.18	4.57	0.08
16	Monomethylbranched-C29	2955	2.66	0.11	4.17	0.83
17	Monomethyl- plus dimethylbranched-C29	2964	1.20	0.21	1.16	0.53
	Branched/normal		3.43		5.65	

[a]Numbers correspond to peaks from Figure 2.
[b]Straight chain hydrocarbons were identified by comparison of their retention times with that of the corresponding standards. Metehylbranching was estimated based on its elution pattern, according to Carolson et al. (1998).

and 29 carbons in the straight chain backbone, according to their retention indices (KI values). Branching patterns were estimated following the protocol described by Carlson et al. (1998) to assign methyl-branched structures based on their elution pattern (Table 2). The relative amounts of major hydrocarbon components both of the epicuticle and whole insect (mostly from hemolymph and integument) are detailed in Table 2. No qualitative differences were detected, as evidenced by identical KI values. The major quantitative change is observed in the branched to straight chain ratio increasing from 3.43 in the epicuticle to 5.65 for whole insect extracts, probably due to a significant percentage of branched hydrocarbons in the hemolymph.

Table 3. Fatty acid composition of *B. bassiana* (*Bb* 5500) grown on CMA (FS_0) and on n-hexadecane-enriched DMA (FS_1)

Fatty acid	FS_0		FS_1	
	X[a]	SD	X[b]	SD
14:0	tr	–	0.5	0.13
15:0	tr	–	0.5	0.04
16:0	12.4	1.67	21.8	4.35
17:0	0.8	0.34	2.1	0.18
17:1	0.5	0.07	0.5	0.02
18:0	5.5	3.18	5.2	1.02
18:1	60.6	5.18	39.1	2.01
18:2	14.6	1.15	26.2	3.65
18:3	1.1	1.02	1.6	0.10
20:1	2.2	1.17	1.5	0.37
Unsaturated/ saturated	4.32		2.47	

Lipid content is 24.7 ± 3.2 mg/gr Bb (w/w).
[a]Numbers represents the mean value of n = 4.
[b]Numbers represents the mean value of n = 3.
tr = traces.

Beauveria bassiana *lipids and fatty acids*

HPTLC analysis of the total fungal lipids showed that for Bb5500 grown in FS_0 culture conditions, the major components were sterols; they also contained phospholipids, free fatty acids, diacylglycerols, monoacylglycerols and hydrocarbons (data not shown). The amount of lipids was 24.7 ± 3.2 mg/gr of spores (w/w) (Table 3). The lipid pattern remained essentially unchanged for FS_1 cultures. The analysis of *B. bassiana* fatty acids in FS_0 cultures showed a rather simple profile with three major fatty acids: oleic acid (60.6%), followed by linoleic acid (14.69%) and palmitic acid (12.40%); minor components ranged from 14 to 20 carbons (Table 3). This pattern was modified for FS_1 cultures, and although oleic acid (39.19%) remained the major component, there was a significant increase in the relative amount of linoleic acid (26.28%), and also of palmitic acid (21.8%). Thus, the unsaturated/saturated ratio varied from 4.32 for FS_0 to 2.47 for FS_1 (Table 3). Trace amounts of fatty acids with chain lengths over 20 carbons were also detected.

Discussion

Increased use of mycoinsecticides will depend on the development of more virulent strains with the capacity to kill their hosts quickly. Evidence is herein presented correlating hydrocarbon utilization as the sole carbon source for fungal growth and increased virulence. The aggressiveness of a fungal strain is controlled by numerous genes (Taborsky, 1992). Nevertheless, the genes responsible for the expression of cuticular degrading enzymes that might be accounting for the increased host mortality here reported, or for an enhanced speed of kill (Juárez M.P. and Crespo R, unpublished), could be cloned and their reinsertion into genome of another strain or species has the potential to increase the efficiency of a mycoinsecticide. A similar approach has been claimed for oil spill removal employing microorganisms having plasmids expressing hydrocarbon-degrading pathways (Chakrabarty, 1981).

Results reported here show that the virulence of a fungal strain can be increased by a 14-day incubation period in a hydrocarbon-enriched medium (Table 1). A second subculture on alkane-supplemented media did not enhance virulence any more (data not shown). Strains of *B. bassiana* and *Metarhizium anisopliae* were shown to be able to utilize hydrocarbons as the only carbon source for their growth, the preferred substrates being the cuticular hydrocarbons of their host (Napolitano and Juárez, 1997). n-Heptacosane, n-pentacosane, n-octacosane, and methyl-branched nonacosane, with chain lengths similar to the major hydrocarbon components of *A. obtectus*, were easily degraded and incorporated into the fungal lipids and were also released as β-oxidation products (Crespo et al., 2000; Napolitano and Juárez, 1997). A shorter chain length hydrocarbon, hexadecane, which has the advantage of easier handling, lower cost, and is similarly degraded by fungi, was selected for this study.

The surface of insects is covered by a waxy layer which plays a key role in survival of the insect by providing protection from dessication, as well as serving as a barrier against microorganisms, abrasion and chemicals (Blomquist et al., 1987). Epicuticular hydrocarbons of *A. obtectus* comprise a mixture of 17 saturated components ranging between 25 and 31 total carbons (Table 2). Major components are methyl-branched, providing the necessary fluidity to the surface coating. The cuticle of other storage pests, such as *Sitophilus* sp. weevils, have more complex structures, with the presence of large amounts of olefins, alkenes, alkadienes and alkatrienes, which, in addition, had higher molecular weight (Baker et al., 1984). In the cuticle of the red flour beetle *Tribolium castaneum*, two groups of hydrocarbons were reported; one, unsaturated and of low molecular weight, was associated with defensive secretions; the other, associated with the cuticular lipids, comprised a mixture of normal and branched chains, quite similar to those here reported for the

bean weevil (Baker et al., 1978). The alkane-grown fungal strain (FS_1) did not show an enhanced ability to kill either *Tribolium* sp. or *Sithophilus* sp. insects (Padín S. and G.M. Dal Bello, unpublished). Among a variety of factors involved in the specificity of entomopathogenic fungi-host cuticle interaction, the composition of the host cuticular components might stimulate or inhibit fungal growth.

Deuteromycetes are known to require an external carbon source for their growth, which is usually supplied as glucose and yeast extract in standard culture media (Smith and Grula, 1981). Utilization of n-hexadecane as carbon source for fungal growth did not alter the lipid profile of Bb5500 after a period of growth induction (data not shown). Fungal lipids accounted for 24.7 ($\pm$ 3.2) mg per gram of cells (w/w),with a quite simple fatty acid profile (Table 3), similar to that reported for other entomopathogenic fungi (Boucias et al., 1984; Juárez et al., 2000). The most significant change in fatty acid composition between alkane-grown and glucose-grown cultures is a decrease in the unsaturated/saturated ratio from 4.32 to 2.47, mainly due to a 60% gain in the relative amount of palmitic acid (Table 3). This increase is probably due to the sixteen carbon fatty acid produced by hexadecane oxidation, and its direct incorporation into fungal cells, rather than complete β-oxidation steps as proposed for very long chains (Crespo et al., 2000).

A high increase of host mortality by hydrocarbon degrading fungal enzyme induction, obtained by a simple modification in the culture conditions of this microorganism, suggests a possible approach for mycoinsecticide improvement.

Acknowledgement

This work was supported in part by the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Argentina.

References

Adane, K., D. Moore and S.A. Archer, 1996. Preliminary studies on the use of *Beauveria bassiana* to control *Sitophilus zeamais* (Coleoptera: Curculionidae) in the laboratory. *J. Stored Prod. Res.* 32: 105–113.

Adane, K., 1994. Microbial control of storage pests using the entomopathogenic fungus, *Beauveria bassiana* with special references to *Sitophilus zeamais* and *Callosobruchus chinensis*. M.Sc. Thesis, University of London. 93 pp.

Baker, J.E., D.R. Sukkestad, S.M. Woo and D.R. Nelson, 1978. Cuticular hydrocarbons of *Tribolium castaneum*: effects of the food additive Tricalcium Phosphate. *Insect. Biochem.* 8: 159–167.

Baker, J.E., S.M. Woo, D.R. Nelson and C.L. Fatland, 1984. Olefins as major components of epicuticular lipids of three *Sitophilus* weevils. *Comp. Biochem. Physiol.* 77B: 877–884.

Blomquist, G.J., D.R. Nelson and M. de Renobales, 1987. Insect cuticular lipids. *Arch. Insect Biochem. Physiol.* 6: 227–265.

Boucias, D.G., D.L. Brasaemle and J.L. Nation, 1984. Lipid composition of the entomopathogenic fungi *Nomuraea rileyi*. *J. Invertebr. Pathol.* 43: 254–258.

Carlson, D.R., U.R. Bernier and B.C. Sutton, 1998. Elution pattern from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* 24: 1845–1865.

Chakrabarty, A.M., 1981. Microorganisms having multiple compatible degradative energy-generating plasmids and preparation thereof. US Patent 4,259,444.

Crespo, R., M.P. Juárez and L.F.R. Cafferatta, 2000. Biochemistry of the interaction between entomopathogenous fungi and their insect host-like hydrocarbons. *Mycologia* 92: 528–536.

Fargues, J., 1984. Adhesion of the fungal spore to the insect cuticle in relation to pathogenicity. In: D.W. Roberts and J.R. Aist (eds), *Infection Processes of Fungi*. The Rockefeller Foundation Conf. Report, New York. pp. 90–110.

Folch, J., M. Lees and G.H. Sloane-Stanley, 1957. A simple method for isolation and purification of animal lipids. *J. Biol. Chem.* 226: 497–509.

Giga, D., S. Nchimbi and S. Slumpa, 1990. Bruchid Research in Southern Africa. In: J.B. Smithson (ed), *Progress in Improvement of Common Beans in Eastern and Southern Africa. Proceedings of the Nineth SUA/CRSP and SADC/CIAT Bean Research Workshop*, Morogoro, Tanzania. pp. 228–236.

Golebiowska, Z., 1969. The feeding and fecundity of *Sitophilus granarius* L., *Sitophilus oryzae* L. and *Rhyzopertha dominica* F. in wheat grain. *J. Stored Prod. Res.* 5: 143–155.

Hidalgo, E., D. Moore and G. Le Patourel, 1998. The effect of different formulations of *Beauveria bassiana* on *Sitophilus zeamais* in stored maize. *J. Stored Prod. Res.* 34: 171–179.

Humber, R.A., 1998. *Collection of Entomopathogenic Fungal Cultures*. USDA-ARS (Suppl. to the 1992 Catalog), Ithaca, NY, USA.

Juárez, M.P., R. Crespo, G. Calderón, F.R. Lecuona and L.F.R. Cafferatta, 2000. Characterization and carbon metabolism in fungi pathogenic to *Triatoma infestans*, a Chagas Disease vector. *J. Invertebr. Pathol.* 76: 198–207.

Kapeya, E.H., D.C. Munthali, G.K.C. Nyirenda and J. Logan, 1993. The incidence of bean fly on bean cultivars and the effect of different bean fly levels on seed yield losses. In: D.C. Munthali, J.D.T. Kumwenda and F. Kisyombe (eds), *Proceedings of the Conference on Agricultural Research for Development*, June 7–11, Mangochi, Malawi. pp. 297–304.

Karel, A.K. and A. Autrique, 1989. Insect Pests and other pests in Africa. In: H.F. Schartz and M.A. Pastor-Corrales (eds), *Bean Production and Problems in the Tropics*, 2nd edn. CIAT Cali, Colombia. pp. 455–504.

Kovats, E., 1965. Gas chromatographic comparison of organic substances in the retentionindex system. *Adv. Chromatogr.* 1: 229–247.

Moino, A. Jr, S.B. Alves and R.M. Pereira, 1998. Efficacy of *Beauveria bassiana* (Balsamo) Vuillemin isolates for control of stored-grain pests. *J. Appl. Entomol.* 122: 301–305.

Napolitano, R. and M.P. Juárez, 1997. Entomopathogenous fungi degrade epicuticular hydrocarbons of *Triatoma infestans*. *Arch. Biochem. Biophys.* 344: 208–214.

Pacheco, I.A., M.R. Sartori and R.W.D. Taylor, 1990. Levantamento de resistência de insetos-pragas de graos armazenados a fosfina, no Estado de Sao Paulo. *Col. ITAL* 20: 144–154.

Padín, S.B., G.M. Dal Bello and A.L. Vasicek, 1997. Pathogenicity of *Beauveria bassiana* for adults of *Tribolium castaneum* (Col.: Tenebrionidae) in stored grains. *Entomophaga* 42: 569–574.

Roberts, D.W. and A.E. Hajek, 1992. Entomopathogenic fungi as bioinsecticides. In: G.J. Leatham (ed), *Frontiers in Industrial Mycology*. Chapman and Hall, New York. pp. 145–159.

Sartori, M.R., I.A. Pacheco, M. Iaderosa and R.W.D. Taylor, 1990. Ocorrência e especificidade de resistência ao inseticida malathion em insetos-pragas de graos armazenados, no Estado de Sao Paulo. *Col. ITAL* 20: 194–209.

Smith, R.J. and E.A. Grula, 1981. Nutritional requirements for conidial germination and hyphal growth of *Beauveria bassiana*. *J. Invertebr. Pathol.* 37: 222–230.

Steffan, J.R., 1963. Tribu des calandrini. Les calandres des grains (*Sitophilus*). In: A.S. Balachowsky (ed), *Entomoloquie appliquèe a l'agriculture*, Tome I, seconde volume. Masson et Cie, Paris. pp. 1070–1099.

Taborsky, V., 1992. *Small-scale Processing of Microbial Pesticides*. FAO Agricultural Services Bulletin No. 96. Food and Agriculture Organization of the United Nations, Rome.