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(71) Applicant *(for all designated States except US)*: **BIOTELLIGA HOLDINGS LIMITED** [NZ/NZ]; PWC Tower, 188 Quay Street, Auckland (NZ).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FORD, Stephen Reynold** [NZ/NZ]; 47 Chamberlain Road, Bombay, Franklin, Auckland (NZ). **GLARE, Travis Robert** [AU/NZ]; 30 Millstream Drive, Lincoln 7608 (NZ).

(74) Agents: **MANSELL, John, B.** et al.; A J Park, PO Box 949, Wellington 6140 (NZ).

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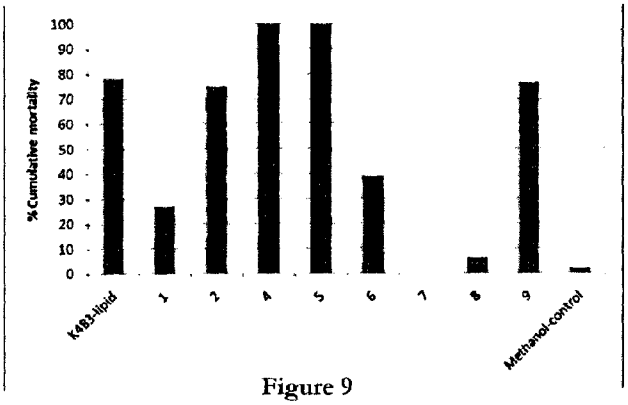

Figure 9

(57) **Abstract**: The present invention provides insecticidal lipids and lipid fractions from fungi of the phylum Ascomycota, together with compositions comprising such lipids and lipid fractions and methods of preparing same. Methods for the biological control of insects, such as phytopathogenic insects, using the lipids and lipid fractions or compositions comprising said lipids optionally together with one or more insecticidal or entomopathogenic agents including entomopathogenic fungi, are also provided.

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INSECTICIDAL AGENTS AND USES THEREOF

FIELD OF THE INVENTION

This invention relates generally to the field of biology, more particularly certain embodiments concern lipids prepared from filamentous fungi, compositions comprising said lipids, and the use of such lipids and compositions as biological control agents. Methods for the control of insects, including phytopathogenic insects, using the lipids and compositions comprising the lipids are also provided.

BACKGROUND OF THE INVENTION

The ability to control insects and insect populations is of significant importance to human and animal health, agriculture, and a wide range of economic activities. For example, insects are vectors for a number of important human diseases: mosquitos are vectors for malaria, West Nile disease, and Dengue fever, ticks are vectors for rickettsial disease such as typhus, African tick bite fever, and Lyme disease, while fleas are the vector for plague. Similarly, plant disease or loss caused by insect pests and pathogens (collectively "phytopathogens"), is a significant economic cost to plant-based agriculture and industries. Losses may arise through spoilage of produce both pre and post harvest, loss of plants themselves, or through reduction in growth and production abilities.

Traditionally, large scale control of insects, such as plant pests and pathogens, has been pursued through the application of chemical insecticides through physical methods (e.g., trapping, picking, barriers) may also be employed. The use of chemicals is subject to a number of disadvantages. Insects, such as plant pests and pathogens, can and have developed tolerance to chemicals to over time, producing resistant populations. Indeed, resistance to pesticides is the greatest challenge to the viability of plant-based agriculture and industries such as the horticultural industry.

The problem is particularly illustrated with reference to a number of economically important phytopathogenic insects. Populations of western flower thrips worldwide are reported to be resistant to most groups of pesticides including the following examples; acephate, abamectin, chlorpyrifos, endosulfan, methomyl, methiocarb, omethoate, pyrazophos and tau-fluvalinate. Populations of onion thrips in New Zealand have developed resistance to deltamethrin, and local populations have been reported to be resistance to diazinon and dichlorvos. Onion thrips in the United States have been reported to be resistant to many pesticides (Grossman, 1994). Greenhouse whitefly has reportedly developed resistance to organochlorine, organophosphate, carbamate and pyrethroid insecticides (e.g. Georghiou 1981,

Anis & Brennan 1982, Elhag & Horn 1983, Wardlow 1985, and Hommes 1986). Resistance has also been reported in newer insecticides, buprofezin and teflubenzuron (Gorman et al. 2000).

Chemical residues may also pose environmental hazards, and raise health concerns. The revival of interest in biological control such as microbial insecticides over the last 20 years has come directly from public pressure in response to concerns about chemical toxicities. Biological control presents an alternative means of controlling plant pathogens which is potentially more effective and specific than current methods, as well as reducing dependence on chemicals. Such biological control methods are perceived as a "natural" alternative to chemical insecticides with the advantage of greater public acceptance, reduced environmental contamination, and increased sustainability.

Mechanisms of biological control are diverse. One mechanism which has been demonstrated to be effective is the use of antagonistic microorganisms such as bacteria to control phytopathogenic insects. For example, the large scale production of *Bacillus thuringiensis* enabled the use of this bacterio-insecticide to control painted apple moth in Auckland, New Zealand.

There are, however, few examples of the successful application of biological control agents (BCAs), and to date BCAs have not met with significant grower acceptance and may have been perceived to be uneconomic.

There is thus a need for agents and methods for effectively controlling insects, including phytopathogenic insects, particularly agents that act faster, have increased efficacy in controlling insects, require less frequent or less intensive application, have lower cost, or have lower resulting toxicity than the currently-available insecticides.

It is therefore an object of the present invention to go some way to meeting this need, to provide one or more agents and methods useful in the control of insects and insect populations, including phytopathogenic insects, or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

The present invention provides insecticidal lipids and methods for preparing them. The dissimilarity of these lipids to known insecticides indicates the existence of a new class of insecticidal agents.

Accordingly, in a first aspect, the invention relates to one or more insecticidal lipids or one or more insecticidal lipid fractions from a fungus of the phylum Ascomycota.

In certain embodiments, one or more of the insecticidal lipids is a non-acidic lipid. In certain embodiments, one or more of the insecticidal lipid fractions is a non-acidic fraction.

In certain exemplary embodiments one or more of the insecticidal lipids or one or more of the insecticidal lipid fractions are insecticidal.

In a second aspect, the invention relates to an isolated, purified or substantially pure insecticidal lipid from a fungus of the phylum Ascomycota.

In one exemplary embodiment, the invention relates to an isolated, purified or substantially pure insecticidal non-acidic lipid from a fungus of the phylum Ascomycota.

In a third aspect the invention relates to a composition comprising one or more insecticidal lipids or one or more insecticidal lipid fractions from a fungus of the phylum Ascomycota.

In a fourth aspect the invention relates to an isolated, purified or substantially pure insecticidal lipid fraction from a fungus of the phylum Ascomycota.

In one exemplary embodiment, the invention relates to an isolated, purified or substantially pure insecticidal non-acidic lipid fraction from a fungus of the phylum Ascomycota.

The invention provides compositions and formulations that comprise one or more of the lipids disclosed herein, together with at least one agriculturally acceptable carrier, including compositions and formulations comprising one or more lipids of the invention and one or more fungi. Such compositions may be a cell extract, cell suspension, cell homogenate, cell lysate, cell supernatant, cell filtrate, or cell pellet of a cell that produce such lipids. In certain exemplary embodiments, the composition comprises a non-acidic lipid fraction from a fungus of the phylum Ascomycota. In certain exemplary embodiments, the composition is enriched in one or more lipids or lipid fractions of the invention, for example by purification or addition. In other exemplary embodiments, the composition comprises one or more added lipids or lipid fractions of the invention. For example, in one embodiment the composition is a cell extract, cell suspension, cell homogenate, cell lysate, cell supernatant, cell filtrate, or cell pellet as described above to which has been added one or more lipids or one or more lipid fractions of the invention.

In a further aspect, the invention provides a method of preparing a lipid or lipid fraction having insecticidal activity against an insect, such as for example a sucking insect, or a coleopteran, dipteran, or lepidopteran insect. The method generally involves isolating one or more of the lipids or lipid fractions described herein from a suitable culture of cells, such as a culture of one or more fungi of the phylum Ascomycota, for example a culture of *Beauvaria bassiana* strain K4B3 cells. In certain embodiments the cells are or have been grown under conditions capable of supporting mycelial growth. Such lipids may be isolated from the cell culture or supernatant or from spore suspensions derived from the cell culture and used in the

native form, or may be otherwise purified or concentrated as appropriate for the particular application.

A method of controlling an insect population is also provided by the invention. The method generally involves contacting the population with an insecticidally-effective amount of a lipid as described herein including a non-acidic lipid or with a lipid fraction as described herein including a non-acidic lipid fraction. Such methods may be used to kill or reduce the numbers of target insects in a given area, or may be prophylactically applied to an environmental area to prevent infestation by a susceptible insect.

In still a further aspect, the invention provides a method for producing a biological control composition, the method comprising:

providing a culture of one or more fungi of the phylum Ascomycota,

maintaining the culture under conditions suitable for production of at least one lipid or lipid fraction of the invention; and

i) combining the at least one lipid or lipid fraction of the invention with a carrier, or

ii) combining the at least one lipid or lipid fraction of the invention with one or more entomopathogenic fungi described herein, or

iii) separating the at least one lipid or lipid fraction of the invention from the fungi, or

iv) at least partially purifying or isolating the at least one lipid or lipid fraction of the invention, or

v) any combination of two or more of (i) to (iv).

The invention further relates to the use of a lipid or lipid fraction of the invention or a composition of the invention for the control one or more insects, such as one or more phytopathogenic insects.

In a further aspect, the present invention provides a method of controlling one or more insects, the method comprising contacting the one or more insects with a lipid or lipid fraction of the invention or a functional variant thereof.

The present invention further relates to a method for controlling one or more insects, such as one or more phytopathogenic insects, the method comprising applying to a plant or its surroundings a lipid or lipid fraction of the invention or a functional variant thereof, optionally together with at least one entomopathogenic fungus as described herein.

In another aspect, the present invention provides a method of reversing, wholly or in part, the resistance of an insect to one or more insecticides or one or more entomopathogenic agents, the method comprising contacting the insect with a lipid or lipid fraction of the invention.

Optionally, the method comprises contacting the insect with a lipid or lipid fraction of the invention together with one or more insecticides or one or more entomopathogenic agents, or any combination thereof.

In various embodiments, the one or more insecticides or one or more entomopathogenic agents administered is the same as that to which the insect is or is predicted to be or become resistant.

In a further aspect, the invention provides a method of controlling one or more insects which have been contacted with one or more lipids or lipid fractions of the invention with an amount of an insecticide or entomopathogenic agent effective to control said one or more insects.

The one or more insecticides or one or more entomopathogenic agents may be administered prior to, concurrently with, or after administration of the lipid or lipid fraction of the invention. Accordingly, administration of the one or more lipids or lipid fractions of the invention and the one or more insecticides or entomopathogenic agents may be simultaneous, sequential, or separate.

In another aspect, the present invention provides a method of reversing, wholly or in part, the resistance of an insect to one or more insecticides or to one or more entomopathogenic agents, the method comprising contacting the one or more insects with one or more lipids or lipid fractions of the invention and an entomopathogenic fungi of the invention, optionally together with another insecticide or entomopathogenic agent.

In one embodiment, the method comprises contacting the one or more insects with an entomopathogenic fungi together with one or more lipids of the invention.

The following embodiments may relate to any of the aspects herein.

In one embodiment, the at least one fungus is of the class Sordariomycetes, including one or more fungi of the following subclasses:

Hypocreomycetidae, such as one or more fungi of the order Coronophorales, Hypocreales, Melanosporales, or Microascales; Sordariomycetidae, including one or more fungi of the order Boliniales, Calosphaeriales, Chaetosphaeriales, Coniochaetales, Diaporthales, Magnaporthales, Ophiostomatales, Sordariales; Xylariomycetidae, including fungi of the order Xylariales and fungi of the order Koralionastetales, Lulworthiales, Meliolales, Phyllachorales, and Trichosphaeriales.

In one embodiment, the one or more fungi is of the order Hypocreales, such as for example one or more fungi of the family Bionectriaceae, Clavicipitaceae, Hypocreaceae, Nectriaceae, Niessliaceae, or Ophiocordycipitaceae.

In one embodiment, the one or more fungi is of the family Clavicipitaceae, including one or more fungi from the following genera:

Aciculosporium, Ascopolyporus, Atkinsonella, Atricordycep, Balansia, Berkelella, Cavimalum, Cepsiclava, Claviceps, Cordycepioideus, Cordyceps, Dussiella, Epichloë, Epicrea, Helminthascus, Heteroepichloë, Hyperdermium, Hypocrella, Konradia, Loculistroma, Metacordyceps, Moelleriella, Mycomalmus, Myriogenospora, Neobarya, Neoclaviceps, Neocordyceps, Parepichloë, Phytocordyceps, Podocrella, Regiocrella, Romanoa, Shimizuomyces, Sphaerocordyceps, Stereocrea, Torrubiella, Wakefieldiomyces, Akanthomyces, Aschersonia, Beauveria, Chaunopycnis, Corallocytostroma, Culicinomyces, Drechmeria, Ephelis, Gibellula, Haptocillium, Harposporium, Hirsutella, Hymenostilbe, Isaria, Lecanicillium, Mariannaea, Metarhizium, Microhilum, Neomunkia, Neotyphodium, Nomuraea, Paecilomyces, Pochonia, Polycephalomyces, Pseudogibellula, Simplicillium, Sorosporella, Tolypocladium or Ustilaginoidea.

For example, one or more fungi is of the genus Beauveria, including for example one or more strains of *Beauveria bassiana*, *Beauveria brongniartii*, *Beauveria felina*, or *Beauveria globulifera*.

In another embodiment the one or more fungi is of the family Hypocreaceae including one or more fungi from the following genera:

Aphysiostroma, Cladobotryum, Gliocladium, Hypocrea, Hypocreopsis, Hypomyces, Mycogone, Podostroma, Protocrea, Rogersonia, Sarawakus, Sepedonium, Sphaerostilbella, Sporophagomyces, Stephanoma or Trichoderma.

In one embodiment, the one or more fungi is of the genus *Trichoderma* including one or more of the following:

Trichoderma aggressivum, Trichoderma asperellum, Trichoderma atroviride Trichoderma aureoviride, Trichoderma austrokoningii, Trichoderma brevicompactum, Trichoderma candidum, Trichoderma caribbaeum var. aequatoriale, Trichoderma caribbaeum var. caribbaeum, Trichoderma catoptron, Trichoderma cremeum, Trichoderma ceramicum, Trichoderma cerinum, Trichoderma chlorosporum, Trichoderma chromospermum, Trichoderma cinnamomeum, Trichoderma citrinoviride, richoderma crassum, Trichoderma cremeum, Trichoderma dingleyeae, Trichoderma dorotheae, Trichoderma effusum, Trichoderma erinaceum, Trichoderma estonicum, Trichoderma fertile, Trichoderma gelatinosus, Trichoderma ghanense, Trichoderma hamatum, Trichoderma harzianum, Trichoderma helicum, Trichoderma intricatum, Trichoderma konilangbra, Trichoderma koningii, Trichoderma koningiopsis, Trichoderma longibrachiatum, Trichoderma longipile, Trichoderma minutisporum, Trichoderma

oblongisporum, Trichoderma ovalisporum, Trichoderma petersenii, Trichoderma phyllostahydis, Trichoderma piluliferum, Trichoderma pleuroticola, Trichoderma pleurotum, Trichoderma polysporum, Trichoderma pseudokoningii, Trichoderma pubescens, Trichoderma reesei, Trichoderma rogersonii, Trichoderma rossicum, Trichoderma saturnisporum, Trichoderma sinensis, Trichoderma sinuosum, Trichoderma sp. MA 3642, Trichoderma sp. PPRI 3559, Trichoderma spirale, Trichoderma stramineum, Trichoderma strigosum, Trichoderma stromaticum, Trichoderma surrotundum, Trichoderma taiwanense, Trichoderma thailandicum, Trichoderma thelephoricolum, Trichoderma theobromicola, Trichoderma tomentosum, Trichoderma velutinum, Trichoderma virens, Trichoderma viride, Trichoderma viridescens; or one or more Hypocrea species, including *Hypocrea phyllostachydis*.

In various embodiments, the one or more fungi is one or more strains selected from *Beauveria bassiana* K4B3 NMIA No. V08/025855 or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V1 (NMIA No. NM05/44593) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V2 (NMIA Accession No. NM05/44594) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V4 (NMIA Accession No. NM06/00007) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B1 (NMIA Accession No. NM05/44595) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B2 (NMIA Accession No. NM06/00010) or a strain having the identifying characteristics thereof; *Lecanicillium longisporum* strain KT4L1 (NMIA Accession No. NM06/00009) or a strain having the identifying characteristics thereof; and *Paecilomyces fumosoroseus* strain K4P1 (NMIA Accession No. NM06/00008) or a strain having the identifying characteristics thereof,

In various embodiments, the one or more insecticidal lipids is a non-acidic lipid. In some embodiments, the one or more insecticidal lipids is substantially free of acidic lipids, or is substantially free of free fatty acids.

In various embodiments, the one or more insecticidal lipid fractions is a non-acidic lipid fraction. In some embodiments, the one or more insecticidal lipid fractions is substantially free of acidic lipids, or is substantially free of free fatty acids.

In various embodiments, the one or more insecticidal lipid fractions comprises is a non-acidic lipid fraction. In some embodiments, the one or more insecticidal lipid fractions is substantially free of acidic lipids, or is substantially free of free fatty acids.

In various embodiments, the one or more insecticidal lipid fractions comprises at least about 10%, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % by weight non-acidic lipids.

In various embodiments, the w/w ratio of non-acidic lipids to acidic lipids in the lipid fraction is at least about 5:4, 4:3, 3:2, 2:1, 5:3, 5:2, 3:1, 4:1, or 5:1.

In one embodiment, the lipid is a polar lipid.

In various embodiments, the one or more insecticidal lipid fractions comprises at least about 10%, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % by weight polar lipids.

In various embodiments, the w/w ratio of polar lipids to non-polar lipids in the lipid fraction is at least about 5:4, 4:3, 3:2, 2:1, 5:3, 5:2, 3:1, 4:1, or 5:1.

In various embodiments, the lipid fraction is or has the identifying characteristics of fraction 1, fraction 5, or fraction 6 as shown in Figure 4 or as described in Example 2 herein.

In various embodiments, the lipid fraction is or has the identifying characteristics of fraction 2, fraction 4, or fraction 5 as shown in Figure 7 or as described in Example 2 herein.

In various embodiments, the lipid fraction is or has the identifying characteristics of fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein.

In various embodiments, the lipid fraction is or has the identifying characteristics of fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein.

In various embodiments, the lipid fraction is or has the identifying characteristics of fraction 2, fraction 3, fraction 4b, fraction 5a, fraction 5b, or fraction 6 as shown in Figure 10 or as described in Example 2 herein.

In various embodiments, the identifying characteristic of fraction 2, fraction 3, fraction 4b, fraction 5a, fraction 5b, or fraction 6 is a MALDI-TOF mass spectrometry (MS) profile as shown in any one of Figure 12a to 12f.

In one embodiment, the lipid fraction is or has the identifying characteristics of fraction 3 as shown in Figure 10 or as described in Example 2 herein. In one embodiment, the identifying characteristic is an NMR spectrum as shown in Figure 13.

In one embodiment, the lipid is or the lipid fraction comprises or consists of one or more of the following:

a. a lipid having an approximate mass at m/z 86 by MS/MS, or

b. a lipid having an approximate mass at m/z 147 by MS/MS, or

c. a lipid having an approximate mass at m/z 445 by MS/MS, or
d. a lipid having an approximate mass at m/z 656 by MS/MS, or
e. a lipid having an approximate mass at m/z 454 by MS/MS, or
f. a lipid having an approximate mass at m/z 751 by MS/MS, or
g. a lipid having an approximate mass at m/z 393 by MS/MS, or
h. a lipid having an approximate mass at m/z 751 by MS/MS, or
i. a lipid having an approximate mass at m/z 693 by MS/MS, or
j. a lipid having an approximate mass at m/z 695 by MS/MS, or
k. a lipid having an approximate mass at m/z 717 by MS/MS, or
l. a lipid having an approximate mass at m/z 531 by MS/MS, or
m. a lipid having a mass at m/z 86.02 by MS/MS, or
n. a lipid having a mass at m/z 146.97 by MS/MS, or
o. a lipid having a mass at m/z 444.82 by MS/MS, or
p. a lipid having a mass at m/z 655.94 by MS/MS, or
q. a lipid having a mass at m/z 454.19 by MS/MS, or
r. a lipid having a mass at m/z 750.66 by MS/MS, or
s. a lipid having a mass at m/z 393.39 by MS/MS, or
t. a lipid having a mass at m/z 750.67 by MS/MS, or
u. a lipid having a mass at m/z 692.69 by MS/MS, or
v. a lipid having a mass at m/z 694.72 by MS/MS, or
w. a lipid having a mass at m/z 716.71 by MS/MS, or
x. a lipid having a mass at m/z 531.34 by MS/MS.

In one embodiment, the lipid is or the lipid fraction comprises a lipid having a mass at m/z 86.02 by MS. In one embodiment, the lipid is or the lipid fraction comprises a lipid having a mass at m/z 146.97 by MS.

In various embodiments, particularly of compositions comprising or derived from a culture of one or more fungi of the phylum Ascomycota, the composition is enriched in one or more lipids or lipid fractions of the invention. In certain embodiments, the enrichment is an enrichment of at least about 1%, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % relative to the amount present in the composition without enrichment, such as the culture or growth media without enrichment. In other embodiments, the enrichment is at least about two-fold, three-fold, four-fold, five-fold, ten-fold, twenty-fold, 50-fold, or 100-fold relative to the amount present in the composition without enrichment.

In certain embodiments, the enrichment is by addition. In other embodiments, the enrichment is by culturing the fungus under conditions conducive to increased production of the one or more lipids or lipid fractions (for example as evidenced by assay of growth media for the presence of one of the identifying characteristics of a lipid fraction as specifically described herein), relative to that produced under normal growth conditions.

In various embodiments the composition or isolate obtained or obtainable from a fungus of the phylum Ascomycota comprises at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % by weight of a lipid, preferably a non-acidic lipid, and useful ranges may be selected between any of these values (for example, about 1 to about 99, about 5 to about 99, about 10 to about 99, about 15 to about 99, about 20 to about 99, about 25 to about 99, about 30 to about 99, about 35 to about 99, about 40 to about 99, about 45 to about 99, about 50 to about 99, about 55 to about 99, about 60 to about 99, about 65 to about 99, about 70 to about 99, about 75 to about 99, about 80 to about 99, about 85 to about 99, or about 90 to about 99 % by weight).

It should be understood that any compositions or isolates useful herein include compositions and isolates obtained or obtainable from a culture comprising one or more fungi of the phylum Ascomycota, and may be obtained from a culture in which one or more fungi of the phylum Ascomycota is or was present but has since been removed.

In one embodiment a composition useful herein comprises at least about , 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mg/mL of a lipid, preferably a non-acidic lipid, and useful ranges may be selected between any of these values (for example, about 0.01 to about 1.0, about 0.01 to about 10, about 0.01 to about 20, about 0.01 to about 30, about 0.01 to about 40, about 0.01 to about 50, about 0.01 to about 60, about 0.01 to about 70, about 0.01 to about 80, about 0.01 to about 90, about 0.01 to about 100, about 0.1 to about 1.0, about 0.1 to about 10, about 0.1 to about 20, about 0.1 to about 30, about 0.1 to about 40, about 0.1 to about 50, about 0.1 to about 60, about 0.1 to about 70, about 0.1 to about 80, about 0.1 to about 90, about 0.1 to about 100, about 0.7 to about 1.0, about 0.7 to about 10, about 0.7 to about 20, about 0.7 to about 30, about 0.7 to about 40, about 0.7 to about 50, about 0.7 to about 60, about 0.7 to about 70, about 0.7 to about 80, about 0.7 to about 90, or about 0.7 to about 100 mg/mL).

Exemplary fungal cells that produce one or more lipids of the invention include *Beauvaria bassiana* strain K4B3 on deposit at National Measurement Institute of Australia (NMIA) under Accession No. V08/025855 deposited 23 September 2008, or a culture having the

identifying characteristics thereof; *Beauvaria bassiana* strain AM2, *Beauvaria bassiana* strain F480, *Trichoderma* sp, including *Trichoderma* isolate 1328, and *Metarhizium* sp, or strains having the identifying characteristics of any one thereof.

In one embodiment, the invention relates to a method of preparing an insecticidal lipid or lipid fraction, the method comprising

providing an organic solvent extraction of a culture of one or more fungi of the phylum Ascomycota,

at least partially separating one or more non-acidic lipids from one or more acidic lipids, and

recovering the one or more non-acidic lipids or lipid fractions.

In one embodiment, the invention relates to a method of preparing an insecticidal lipid or lipid fraction, the method comprising

providing an organic solvent extraction of a culture of one or more fungi of the phylum Ascomycota,

at least partially separating one or more polar lipids from one or more non-polar lipids, and

recovering the one or more polar lipids or polar lipid fractions.

In certain embodiments, the method of preparing a lipid or lipid fraction having insecticidal activity from a fungus of the phylum Ascomycota is essentially as herein described.

In one exemplary embodiment, the organic solvent is or comprises chloroform. For example, the organic solvent comprises methanol and chloroform.

In various embodiments, the organic solvent is an alkanol including a short chain alkyl alcohol, such as but not limited to methanol, ethanol, propanol, iso-propanol, or butanol, or is chloroform.

In various embodiments, the organic solvent is an agriculturally acceptable carrier, including a carrier as described herein.

In various embodiments, the separation is by chromatography, including anion exchange chromatography and thin layer chromatography. In one exemplary embodiment, the anion exchange chromatography is with DEAE-Sephadex.

The composition of the invention may be formulated as a powder, dust, pellet, granule, spray, emulsion, colloid, solution, or such like, and may be preparable by such conventional means as desiccation, lyophilization, homogenization, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of cells comprising the lipid. In some embodiments

of exemplary compositions that contain at least one such insecticidal lipid, the lipid is present in a concentration of from about 1% to about 99% by weight.

Preferably such compositions are obtainable from one or more cultures of the *B. bassiana* cells described herein. An exemplary insecticidal lipid formulation may be prepared by a process comprising the steps of culturing a suitable *Beauvaria bassiana* strain K4B3 cell under conditions effective to produce mycelia, providing at least partially purified mycelia, and obtaining one or more lipids from the mycelia.

In a further embodiment, the invention provides methods for preparing an insecticidal lipid composition. In exemplary embodiments, such lipids may be formulated for use as an insecticidal agent, and may be used to control insect populations in an environment, including agricultural environs and the like. In some embodiments, the formulations can be used to kill an insect or insect population, either by topical application, or by ingestion of the lipid composition by the insect. In other embodiments, the formulations can be used to antagonise an insect or insect population, again either by topical application or by ingestion of the peptide composition by the insect(s). In certain instances, it may be desirable to formulate the lipids of the present invention for application to the soil, on or near plants, trees, shrubs, and the like, near live plants, livestock, domiciles, farm equipment, buildings, and the like.

In various embodiments of a composition comprising one or more lipids of the invention and one or more fungi, the one or more fungi is in a reproductively viable form and amount.

In one embodiment the invention provides a composition comprising one or more lipids as described herein and spores obtainable from a least one fungi together with at least one carrier.

Preferably, said composition is a biological control composition, more preferably said biological control composition is an insecticidal composition.

Preferably, said biological control composition comprises at least one agriculturally acceptable carrier.

Preferably, said at least one carrier is an agriculturally acceptable carriers, more preferably is selected from the group consisting of a filler stimulant, an anti-caking agent, a wetting agent, an emulsifier, and an antioxidant, more preferably said composition comprises at least one of each of a filler stimulant, an anti-caking agent, a wetting agent, an emulsifier, and an antioxidant.

Preferably, said filler stimulant is a carbohydrate source, such as a disaccharide including, for example, sucrose, fructose, glucose, or dextrose, said anti-caking agent is selected

from talc, silicon dioxide, calcium silicate, or kaelin clay, said wetting agent is skimmed milk powder, said emulsifier is a soy-based emulsifier such as lecithin or a vegetable-based emulsifier such as monodiglyceride, and said antioxidant is sodium glutamate or citric acid.

In various embodiments, the composition is a stable composition capable of supporting reproductive viability of the fungi or capable of retaining insecticidal efficacy for a period greater than about two weeks, preferably greater than about one month, about two months, about three months, about four months, about five months, more preferably greater than about six months.

In certain embodiments, the composition comprises a single strain of fungus. In one exemplary embodiment, the fungus is *Beauveria bassiana* strain K4B3 (NMIA No. V08/025855 deposited 23 September 2008).

Alternatively, the composition comprises multiple strains of said fungi. In one embodiment, the composition is a biological control composition that comprises one or more lipids of the invention, together with, in a reproductively viable form and amount one or more strains selected from *Beauveria bassiana* K4B3 NMIA No. V08/025855 or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V1 (NMIA No. NM05/44593) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V2 (NMIA Accession No. NM05/44594) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V4 (NMIA Accession No. NM06/00007) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B1 (NMIA Accession No. NM05/44595) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B2 (NMIA Accession No. NM06/00010) or a strain having the identifying characteristics thereof; *Lecanicillium longisporum* strain KT4L1 (NMIA Accession No. NM06/00009) or a strain having the identifying characteristics thereof; and *Paecilomyces fumosoroseus* strain K4P1 (NMIA Accession No. NM06/00008) or a strain having the identifying characteristics thereof, and at least one agriculturally acceptable carrier.

In one embodiment the method for producing a biological control composition comprises:

providing a culture of *Beauveria bassiana* K4B3 V08/025855,

maintaining the culture under conditions suitable for production of at least one lipid or lipid fraction of the invention; and

i) combining the at least one lipid or lipid fraction of the invention with a carrier, or

ii) combining the at least one lipid or lipid fraction of the invention with one or more entomopathogenic fungi described herein, or

iii) separating the at least one lipid or lipid fraction of the invention from the *Beauveria bassiana* K4B3 V08/025855, or

iv) at least partially purifying or isolating the at least one lipid of the invention from the *Beauveria bassiana* K4B3 V08/025855, or

v) any combination of two or more of (i) to (iv).

In certain embodiments, the method may additionally comprise after the maintaining step one or cell-lysis steps.

In various embodiments the separation is by centrifugation or by filtration.

In various embodiments, the separation is effective to remove greater than about 50% of the fungi, for example *Beauveria bassiana* K4B3 V08/025855, greater than about 55%, greater than about 60%, greater than about 65%, greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85%, greater than about 90%, greater than about 95%, greater than about 99%, or about 100% of the fungi, for example about 100% of the *Beauveria bassiana* K4B3 V08/025855.

Accordingly, in one particularly contemplated embodiment, the method comprises providing a culture of *Beauveria bassiana* K4B3 V08/025855, maintaining the culture under conditions suitable for production of at least one secreted lipid or lipid fraction of the invention, and separating the at least one secreted lipid or lipid fraction of the invention from the *Beauveria bassiana* K4B3 V08/025855.

Preferably, the carrier is an agriculturally acceptable carrier, preferably the at least one carrier is selected from the group consisting of a filler stimulant, an anti-caking agent, a wetting agent, an emulsifier, and an antioxidant, more preferably said composition comprises at least one of each of a filler stimulant, an anti-caking agent, a wetting agent, an emulsifier, and an antioxidant.

In various embodiments the phytopathogenic insect is of the order *Hemiptera*.

Preferably, said one or more phytopathogenic insects is selected from the group consisting of mosquito, moths including diamond back moth, Thrips (*Thysanoptera*), Aphids, Psyllids, Scale or Whitefly (*Hemiptera*).

In one example, the lipid or lipid fraction of the invention or functional variant thereof may be present in a composition as described herein.

In one embodiment, the composition comprises two or more lipids of the invention.

In various embodiments, the composition comprises at least one lipid of the invention, together with:

i) at least one beauvericin;

ii) at least one bassianolide;

iii) at least one entomopathogenic fungi

iv) any two or more of (i) to (iii) above.

In one embodiment, lipids or compositions of the invention are applied directly to the plant or its surroundings. For example, a composition of the invention is admixed with a solvent or emulsified (for example with water) and applied as described herein.

In one embodiment, the present invention provides a method for controlling one or more insects, such as one or more phytopathogenic insects, the method comprising applying to a plant or its surroundings a composition of the present invention.

In various embodiments, the lipid or composition of the invention is applied prophylactically, for example before a plant is infected by or exposed to the phytopathogen. In other embodiments, the composition is applied when infection is establish or the pathogen is present, for example when a plant is infected by or exposed to a phytopathogen, or when a phytopathogen is present on or in the plant or its surroundings.

Preferably, the composition is admixed with water to a final concentration of lipid of about 0.5gm/L to about 10gm/L prior to application, and more preferably to a final concentration of about 1gm/L.

Preferably, a desiccation protection agent, such as Deep Fried™, Fortune™, or Fortune Plus™, is admixed to a final concentration of about 1ml/L prior to application.

For compositions comprising one or more entomopathogenic fungi, an exemplary concentration range is from about 1×10^2 to about 1×10^{12} spores per ml, from about 1×10^2 to about 1×10^{11} spores per ml, from about 1×10^2 to about 1×10^{10} spores per ml, from about 1×10^2 to about 1×10^9 spores per ml, from about 1×10^3 to about 1×10^9 spores per ml, from about 1×10^4 to about 1×10^9 spores per ml, preferably from about 1×10^5 to about 5×10^8, and more preferably about 1×10^6 to about 2×10^8 spores per ml. In certain embodiments, the composition comprises at least 10^7 spores per millilitre at application, at least about 5×10^7 spores per millilitre at application, or at least 10^8 spores per ml at application.

In various embodiments when one or more fungi are present in the composition, the compositions of the invention may be applied at a rate of from about 1×10^8 to about 1×10^{15} infectious units (IU) per hectare, from about 1×10^9 to about 1×10^{15} IU per hectare, from about 1×10^{10} to about 1×10^{15} IU per hectare, from about 1×10^{11} to about 1×10^{15} IU per hectare, preferably from about 1×10^{10} to about 1×10^{14} IU per hectare, more preferably from about 5×10^{10} to about 1×10^{14} IU per hectare, more preferably about 1×10^{11} to about 5×10^{11} IU per hectare.

In one embodiment, the infectious unit is a spore, such as an endospore, and the composition is applied at a rate of from about 1 x 10^8 to about 1 x 10^{15} spores per hectare, from about 1 x 10^9 to about 1 x 10^{15} spores per hectare, from about 1 x 10^{10} to about 1 x 10^{15} spores per hectare, from about 1 x 10^{11} to about 1 x 10^{15} spores per hectare, preferably from about 1 x 10^{10} to about 1 x 10^{14} spores per hectare, more preferably from about 5 x 10^{10} to about 1 x 10^{14} spores per hectare, more preferably about 1 x 10^{11} to about 5 x 10^{11} spores per hectare.

Conveniently, such a rate of application can be achieved by formulating said composition at about 10^8 spores per millilitre or more, and applying said composition at a rate of about 1L per hectare. As discussed herein, such an application rate can be conveniently achieved by dissolution of the composition in a larger volume of agriculturally acceptable solvent, for example, water.

Preferably, the composition is admixed with water prior to application. In one embodiment, the composition is admixed with water and applied in at least about 100L water/Ha, in at least about 150L/Ha, in at least about 200L/Ha, in at least about 250L/Ha, in at least about 300L/Ha, in at least about 350L/Ha, in at least about 400L/Ha, in at least about 450L/Ha, or in at least about 500L/Ha. In a preferred embodiment, the composition is admixed with water to a final concentration of about 1 x 10^{11} to about 5 x 10^{11} spores per 500L water prior to application and applied at a rate of 500L/hectare.

Preferably, said application is by spraying.

Preferably, a composition comprising *Beauveria bassiana* strain K4B3 (NMIA Accession No. V08/025855) or a culture having the identifying characteristics thereof is applied at a rate of from about 1 x 10^{10} to about 1 x 10^{15} spores per hectare, preferably from about 1 x 10^{12} to about 1 x 10^{14} spores per hectare, more preferably from about 5 x 10^{12} to about 1 x 10^{14} spores per hectare, more preferably about 1-3 x 10^{13} spores per hectare.

Conveniently, such a rate of application can be achieved by formulating said composition at about 10^7 spores per milligram or more, and applying said composition at a rate of about 1kg per hectare. As discussed herein, such an application rate can be conveniently achieved by dissolution of the composition in a larger volume of agriculturally acceptable solvent, for example, water.

The invention is applicable to any plant or its surroundings. Exemplary plants are in certain embodiments monocotyledonous or dicotyledonous plants such as alfalfa, barley, canola, corn, cotton, flax, kapok, peanut, potato, oat, rice, rye, sorghum, soybean, sugarbeet, sugarcane, sunflower, tobacco, tomato, wheat, turf grass, pasture grass, berry, fruit, legume, vegetable, ornamental plants, shrubs, cactuses, succulents, and trees.

In further illustrative embodiments, the plant may be any plant, including plants selected from the order Solanales, including plants from the following families: Convolvulaceae, Hydroleaceae, Montiniaceae, Solanaceae, and Sphenocleaceae, and plants from the order Asparagales, including plants from the following families: Amaryllidaceae, Asparagaceae, Asteliaceae, Blandfordiaceae, Boryaceae, Doryanthaceae, Hypoxidaceae, Iridaceae, Ixioliriaceae, Lanariaceae, Orchidaceae, Tecophilaeaceae, Xanthorrhoeaceae, and Xeronemataceae.

To those skilled in the art to which the invention relates, many changes in construction and differing embodiments and applications of the invention will suggest themselves without departing from the scope of the invention as defined in the appended claims. The disclosures and the descriptions herein are purely illustrative and are not intended to be in any sense limiting.

In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.

It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9 and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5 and 3.1 to 4.7) and, therefore, all sub-ranges of all ranges expressly disclosed herein are hereby expressly disclosed. These are only examples of what is specifically intended and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application in a similar manner.

DESCRIPTION OF FIGURES

Figure 1 shows thin layer chromatography (TLC) of centrifuged material. Two different sample loads and a lipid standard (right-hand lane) were analysed.

Figure 2 shows TLC of acidic/non-acidic fractions, compared to the starting material. The acidic (A) fraction mainly shows a strong fatty acid band, whereas the non-acidic (NA) fraction contains a multitude of other lipid bands.

Figure 3 shows bioassay results of the acidic/non-acidic fractions.

Figure 4 shows TLC of the six chromatography fractions. The right hand panel shows a repeat of fractions 1, 5 and 6 using conditions optimised to show all lipids.

Figure 5 shows bioassay results of the six chromatography fractions.

Figure 6 shows TLC of the 11 methanol chromatography fractions.

Figure 7 shows bioassay results of the chromatography fractions.

Figure 8 shows analytical TLC of the fractions from preparative TLC plate 1.

Figure 9 shows bioassay results of the preparative TLC fractions (from plate 1).

Figure 10 shows analytical TLC of the fractions from preparative TLC plate 2.

Figure 11 shows bioassay results of the preparative TLC fractions (from plate 2).

Figure 12 shows MALDI-TOF MS of Fractions 2, 3, 4b, 5a, 5b (top to bottom).

Figure 13 shows low mass range MS spectrum of fraction 3.

Figure 14 shows bioassay results against aphids.

Figure 15 shows bioassay of chloroform extractions from K4B3 mycelia culture (replicate tubes) against aphids.

Figure 16 shows bioassay results against aphids.

Figure 17 shows bioassay results against diamondback moth.

Figure 18 shows % cumulative mortality of green peach aphid at 20 hours (at 20°C) in the bioassay of total lipids extracted from *Beauveria bassiana* strains AM2, F480, and K4B3 as described in Example 5 herein. Samples 1-9 are various fractions of K4B3 lipids.

Figure 19 shows % mortality of green peach aphid at 21 hours (at 20°C) in the bioassay of various lipid fractions from *Beauveria bassiana* strain K4B3 (*Beauveria*), *Trichoderma* and *Metarhizium* as described in Example 5 herein. K4B3-lipid1 and K4B3-lipid2 are repeat isolations from the same batch culture, with methanol as negative control.

Figure 20 shows % cumulative mortality of green peach aphid in the bioassay of figure 19 and as described herein in Example 5.

Figure 21 shows % cumulative mortality of Diamondback moth larvae in the bioassay of various lipid fractions from *Beauveria bassiana* strain K4B3 (*Beauveria*), *Trichoderma* and *Metarhizium* as described herein in Example 5.

Figure 22 shows % mortality of green peach aphid at 21 hours (at 20°C) in the bioassay of various lipid fractions from *Beauveria bassiana* strain K4B3 (FS, and Beaublast), and *Trichoderma* as described herein in Example 5. Fractions tested include methanol-extracted (-MeOH) and chloroform extracted (-Chloro) fractions, with methanol and water as negative controls.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is in part directed to one or more lipids isolated from various strains of Ascomycota fungi, including *Beauveria spp.* and *Trichoderma spp.*, wherein the one or more lipids have efficacy against insects, such as phytopathogenic insects, and the use of such lipids in controlling insects such as phytopathogenic insects.

Definitions

The term "biological control agent" (BCA) as used herein refers to an agent that is or is derived from a biological agent which acts as an antagonist of one or more organisms, typically one or more pathogens such as one or more phytopathogens, such as a phytopathogenic insect, or is able to control one or more one or more pathogens such as one or more phytopathogens. Antagonism may take a number of forms. In one form, the biological control agent may simply act as a repellent. In another form, the biological control agent may render the environment unfavourable for the pathogen. In a further, preferred form, the biological control agent may parasitise, incapacitate, render infertile, impede the growth of, impede the spread or distribution of, and/or kill the pathogen. Accordingly, the antagonistic mechanisms include but are not limited to antibiosis, parasitism, immobilisation, infertility, and toxicity. Therefore, agents which are derived from or which act as antagonists of one or more pathogenic insects can be said to have entomopathogenic efficacy or insecticidal activity. Furthermore, a biological agent that is an antagonist of an insect, including a phytopathogenic insect, can be said to be an entomopathogenic agent.

As used herein, a "biological control composition" is a composition comprising or including at least one biological control agent that is an antagonist of one or more pathogens, such as one or more phytopathogens. Such control agents include, but are not limited to, agents that act as repellents, agents that render the environment unfavourable for the pathogen, and agents that incapacitate, render infertile, and/or kill the pathogen.

Accordingly, as used herein an "anti-phytopathogenic composition" is a composition which comprises or includes at least one agent that is an antagonist of one or more phytopathogens. Such a composition is herein considered to have anti-phytopathogenic efficacy.

The term "comprising" as used in this specification means "consisting at least in part of". When interpreting each statement in this specification that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner.

The term "control" or "controlling" as used herein generally comprehends preventing, reducing, or eradicating infection by one or more pathogens such as infection by one or more

phytopathogens, or inhibiting the rate and extent of such infection, such as reducing a phytopathogen population in or on a plant or its surroundings, wherein such prevention or reduction in the infection(s) or population(s) is statistically significant with respect to untreated infection(s) or population(s). Curative treatment is also contemplated. Preferably, such control is achieved by increased mortality amongst the pathogen population.

The phrases "entomopathogenic activity" and "entomopathogenic efficacy" are used interchangeably herein and refer to the ability of certain agents, such as certain microorganisms or agents derived from certain microorganims, to antagonise one or more pathogenic insects, such as one or more phytopathogenic insects.

In various embodiments, said entomopathogenic efficacy is the ability to parasitise and incapacitate, render infertile, impede the growth of, or kill one or more insects, such as phytopathogenic insects, preferably within 14 days of contact with the insect, more preferably within 7 days, more preferably still the ability to kill one or more phytopathogenic insects within 7 days. Alternatively, said entomopathogenic efficacy is the ability to support or promote the growth of one more entomopathogenic microorganisms, such as one or more entomopathogenic fungi.

Accordingly, as used herein an "entomopathogenic composition" is typically a composition which comprises or includes at least one agent that is an antagonist of one or more pathogenic insects. Such a composition is herein considered to have entomopathogenic efficacy.

The phrases "insecticidal activity" and "insecticidal efficacy" are used interchangeably herein and refer to the ability of certain agents, such as those derived from certain microorganisms, to incapacitate, render infertile, impede the growth of, or kill one or more insects, such as one or more phytopathogenic insects.

In various embodiments, said insecticidal efficacy is the ability to incapacitate, render infertile, impede the growth of, or kill one or more insects, such as phytopathogenic insects, preferably within 14 days of contact with the insect, more preferably within 7 days, more preferably still the ability to kill one or more insects within 7 days.

In certain embodiments, the entomopathogenic activity is insecticidal acivity. For example, certain embodiments of the lipid or lipid fractions of the invention are insecticidal.

Accordingly, as used herein an "insecticidal composition" is typically a composition which comprises or includes at least one agent that is capable of incapacitating, of rendering infertile, of impeding the growth of, or of killing one or more insects. Such a composition is herein considered to have insecticidal efficacy.

The term "functional variant" as used herein in reference to one or more lipids or lipid fractions, for example in respect of one or more lipid fractions as exemplified herein in the Examples, refers to a lipid or lipid fraction different from the specifically identified entity, for example wherein one or more groups, such as one or more fatty acid groups is deleted, substituted, or added, but which possesses at least in part one or more of the biological activities of the specifically-identified entity, such as an ability to elicit one or more biological effects elicited by the specifically-identified lipid or lipid fraction. Functional variants may be from the same or from other species and may encompass homologues, paralogues and orthologues.

In the present case, the functional variant will preferably retain at least a portion of the insecticidal activity of the specifically-identified lipid or lipid fraction.

Methods and assays to determine one of more biological effects elicited by the lipids or lipid fractions of the invention, such as insecticidal efficacy, are well known in the art, and such methods and assays can be used to identify or verify one or more functional variants of one or more of the lipids or lipid fractions of the invention. For example, an assay of the ability of a lipid of the invention to kill or otherwise antagonise the growth of a target insect, such as those described herein in the Examples, is amenable to identifying one or more functional variants of the lipid.

The term "identifying characteristic" as used herein contemplates one or more attributes possessed by and determinable in the reference entity, including for example, a physicochemical characteristic such as the presence or absence of particular peaks on a spectrometry profile, the presence or absence of compounds or fractions having a given mobility or elution profile in a chromatography assay, such as the presence or absence of bands having a given mobility on TLC.

The term "lipid", as used herein, encompasses highly reduced carbon-rich substances that are insoluble in water and comprise one or more fatty acids, carboxylic acids with hydrocarbon chains typically ranging from 4 to 36 carbon atoms in length. Lipids include triacylglycerols, phospholipids including glycerophospholipids and sphingolipids, glycolipids, and sterols.

The term "lipid fraction", as used herein, encompasses a composition comprising one or more lipids, free fatty acids, or both, wherein the fraction comprises or consists of a subset of total lipids present in the unfractionated source material. Typically, lipid fractions will have a determinable and identifiable composition, for example a characteristic chromatography profile or mass spectragraphic profile. Examples of lipid fractions are presented herein.

The term "plant" as used herein encompasses not only whole plants, but extends to plant parts, cuttings as well as plant products including roots, leaves, flowers, seeds, stems, callus tissue, nuts and fruit, bulbs, tubers, corms, grains, cuttings, root stock, or scions, and includes any plant material whether pre-planting, during growth, and at or post harvest. Plants that may benefit from the application of the present invention cover a broad range of agricultural and horticultural crops. The compositions of the present invention are also especially suitable for application in organic production systems.

When used in respect of an insecticidal agent, such as an insecticidal lipid or an entomopathogenic fungal strain, the phrases "retaining insecticidal efficacy" or "retaining entomopathogenic efficacy" and grammatical equivalents and derivatives thereof is intended to mean that the agent still has useful insecticidal or entomopathogenic activity. Preferably, the retained activity is at least about 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100% of the original activity, and useful ranges may be selected between any of these values (for example, from about 35 to about 100%, from about 50 to about 100%, from about 60 to about 100%, from about 70 to about 100%, from about 80 to about 100%, and from about 90 to about 100%). For example, preferred lipid functional variants or fractions of the present invention should retain insecticidal activity, that is, retain at least about 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100% of the insecticidal activity of the specified parent lipid or lipid fraction. Accordingly, a functional variant of one of the lipid fractions described herein, such as a variant of the lipid fractions exemplified in the examples should retain at least about 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100% of the insecticidal activity of the respective lipid fraction. Similarly, preferred compositions of the invention are capable of supporting the maintenance of useful entomopathogenic activity of the entomopathogenic agent(s) they comprise, and can be said to retain entomopathogenic activity, ideally until applied using the methods contemplated herein.

As used herein, the term "stable" when used in relation to a composition of the invention means a composition capable of supporting insecticidal or entomopathogenic efficacy for several weeks, preferably about one, about two, about three, about four, preferably about five, more preferably about six months, or longer. For example, when used in relation to a composition additionally comprising one or more entomopathogenic fungi, the term "stable" refers to a composition capable of supporting reproductive viability of the entomopathogenic fungi for several weeks, preferably about one, about two, about three, about four, preferably about five, more preferably about six months or longer.

A "strain having the identifying characteristics of [a specified strain]", or a "culture having the identifying characteristics of [a specified culture]" including a homologue or mutant of the specified strain, is closely related to (i.e., shares a common ancestor with) or is derived from the specified strain, but will usually differ from the specified strain in one or more genotypic or phenotypic characteristics. Mutants are generally identifiable through assessment of genetic differences. Homologues are identifiable through assessment of the degree of genetic, biochemical and morphological difference and use of taxonomic methods, including for example analyses such as cladistics. However, a strain having the identifying characteristics of [a specified strain], including a homologue or mutant of the specified strain will retain entomopathogenic efficacy, will be distinguishable from other strains, and will be identifiable as a homologue or mutant of the parent strain using the techniques described herein.

The term "surroundings" when used in reference to a plant subject to the fungi, methods and compositions of the present invention includes soil, water, leaf litter, and/or growth media adjacent to or around the plant or the roots, tubers or the like thereof, adjacent plants, cuttings of said plant, supports, water to be administered to the plant, and coatings including seed coatings. It further includes storage, packaging or processing materials such as protective coatings, boxes and wrappers, and planting, maintenance or harvesting equipment.

Control of phytopathogens

The present invention recognises that the horticultural sectors of many countries, including for example that of the United States of America, of New Zealand, and many states of Europe, are faced with the problem of increasing insecticide resistance amongst phytopathogenic insect pests. This is compounded under some regulatory regimes by a reduction in the availability of new chemical insecticides due to regulatory barriers.

The use of insecticidal lipids derived from fungi as biological control agents presents a solution to this problem. Effective biological control agents can be selected according their ability to incapacitate or kill a target phytopathogenic insect or insect population. Under conducive conditions, phytopathogenic insects such as aphids, thrips and whitefly may infect plants and their surroundings including soil, leaf litter, adjacent plants, supports, and the like. Insecticidal lipids derived from fungi and agents derived therefrom may be applied so as to incapacitate and/or kill the phytopathogenic insect, thereby preventing or limiting the disease-causing capability of the pathogen. The effectiveness of these insecticidal lipids derived from fungi in the field is frequently in turn dependent on their ability to retain insecticidal efficacy in varying climatic conditions, such as interrupted wet periods and desiccation. The effectiveness

of agents derived from entomopathogenic fungi such as the lipids described herein does not typically require a maintenance of viability, but rather a maintenance of insecticidal efficacy.

A lipid or lipid fraction of the invention, effective against insects, such as phytopathogenic insects, and therefore suitable for use in accordance with the invention, is identified as one which is effective at reducing the population of the target insect species by a statistically significant amount with respect to the control treatment against which the lipids of the invention or functional variants thereof is compared. Such lipids or lipid fractions can be considered as having insecticidal efficacy. As described herein, the reduction in the population of the target insect may be by various antagonistic mechanisms. For example, the lipid may incapacitate, render infertile, inhibit the growth or development of, and/or preferably kill the phytopathogenic insect, or may support or promote the growth and entomopathogenic efficacy of one or more entomopathogens also present, such as an entomopathogenic fungi present in a composition together with the lipid or lipid fraction of the invention (whether separately, simultaneously, or sequentially). As such, the lipids or lipid fractions of the invention may enable or support the ability of the entomopathogen such as an entomopathogenic fungi to parasitise, incapacitate, render infertile, and/or preferably kill the phytopathogenic insect. The lipids or lipid fractions of the invention may also reduce the population of the target insect by rendering the environment, for example the plant to which the one or more fungi is applied or its surroundings, unfavourable for the phytopathogenic insect. In this embodiment, the lipid or lipid fraction may be considered to be acting as a repellent, and reducing the effective population of the target insect in the vicinity of the plant or its surroundings.

In one embodiment the lipid is a functional variant as defined herein.

Preferably, suitable lipids or lipid fractions of the invention or functional variants thereof exhibit about 5% insecticidal efficacy, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, more preferably at least about 50% insecticidal efficacy expressed as a percentage reduction of the population of the relevant insect species compared to the control treatment. By way of illustration, the methodology described herein was employed to identify *Beauveria* lipid fractions isolate effective against a variety of target insects, whereas procedures analogous to those described herein can be employed in relation to other fungi and insect species.

Although insecticidal efficacy is a principal requisite for a lipid or lipid fraction to be considered suitable for use as a biological control agent, the lipid or lipid fraction may have additional characteristics to be suitable for use as a biological control agent.

For example, the lipid or lipid fraction should be able to be stored in effective form for a reasonable period, ultimately so as to allow it to be applied to the target plant or its surroundings in a form and concentration that is effective as a biological control agent.

Those skilled in the art will recognise that the lipids or lipid fractions and compositions of the invention may comprise or the methods of the invention may use one or more functional variants of one or more of the lipids or lipid fractions of the invention including those exemplified herein. Combinations of lipids or lipid fractions and functional variants thereof are also useful herein.

Methods for isolating lipids

Exemplary methods to produce and isolate one or more of the lipids or lipid fractions of the invention are described herein. These include isolation of one or more lipids or lipid fractions from a culture of one or more fungi of the phylum Ascomycota, such as for example a *Beauveria* species including for example *B. bassiana* K4B3 or a *Trichoderma* species.

Alternatively, the lipids or lipid fractions of the invention, including functional variants thereof, may be prepared using lipid synthesis methods well known in the art.

Compositions comprising entomopathogenic fungi

The importation of entomopathogenic fungi is frequently problematic, costly, and impractical if not impossible under certain regulatory regimes. For example, entomopathogenic fungi available outside a given country may not be available to horticulturalists within that country because of regulatory and legislative preclusions. The present invention therefore recognises there are distinct advantages to identifying and preparing agents that have entomopathogenic efficacy from such fungi, or are able to support or promote the growth of entomopathogenic fungi (or other entomopathogens) and so may be able to help such entomopathogens flourish under a wide variety of environmental conditions, or both.

Isolates of said fungi may conveniently be obtained from the environment, including, for example, from plants, their surroundings, and from pathogens of said plants. In certain embodiments, isolates of said fungi may be obtained from the target insect, or from the plant species (or surroundings) to which the biological control agent comprising said fungi or a composition comprising said fungi will subsequently be applied.

Methods to determine growth of said fungi under different conditions, including different temperatures and on different media or other substrates, are well known in the art. Likewise, methods to determine a positive impact on growth of fungi of a lipid of the invention, such as an increase in virulence or entomopathogenic efficacy of said fungi in the presence of the lipid(s), compared to that observed in the absence of said lipid(s) are also well known in the art.

Examples of methods to determine the ability of a lipid of the invention to influence growth of fungi at various temperatures or on various plants, environments, or target organisms, are described herein.

Although entomopathogenic efficacy is a principal requisite for an isolate to be considered suitable for use as a biological control agent, the fungal isolate should have additional characteristics to be suitable for use as a biological control agent.

For example, the fungi should be able to be stored in a viable form for a reasonable period, ultimately so as to allow it to be applied to the target plant or its surroundings in a form and concentration that is effective as a biological control agent.

The fungi should also be able to achieve infection threshold when applied to a plant or its surroundings for it to be suitable for use as a biological control agent. As used herein, infection threshold refers to the concentration of fungi required for the fungi to become established on the target plant or its surroundings so as to then have entomopathogenic efficacy. As will be appreciated, in order to achieve infection threshold, some isolates of fungi may require application at such a high rate as to be impractical or unviable. Furthermore, some fungal isolates may not be able to achieve infection threshold irrespective of the concentration or rate at which they are applied. Suitable entomopathogenic fungi are able to achieve infection threshold when applied at a rate of not less that 10^{10} spores per hectare, or applied at a concentration not less than 10^{7} spores per milligram of composition when said composition is applied at a rate of about 1kg/1000L/hectare.

Methods to determine infection threshold are well known in the art, and examples of such methods are presented herein. In certain embodiments, infection threshold can be determined directly, for example by analysing one or more samples obtained from a target plant, its surroundings, and/or a pathogen of said plant, and determining the presence or amount of fungus on or in said sample. In other embodiments, infection threshold can be determined indirectly, for example by observing a reduction in the population of one or more phytopathogenic insects. Combinations of such methods are also envisaged.

Beauveria bassiana is a soil born fungi that attacks both immature and adult insects including, for example, grasshoppers, aphids, thrips, moths, and several other species. Typically, *B. bassiana* can be isolated from insect cadavers, such as aphids, borers, and thrips, and may also be isolated from soil. The exemplary entomopathogenic *Beauveria bassiana* strain K4B3 may be used in certain embodiments of the invention, and was deposited in the National Measurement Institute of Australia (NMIA, formerly the Australian Government Analytical Laboratories (AGAL)), 1 Suakin Street, Pymble, New South Wales, Australia on 23 September 2008

according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number V08/025855.

Accordingly, in one aspect the present invention provides methods and compositions comprising reliant on one or more lipids of the invention, or one or more polynucleotides encoding them, together with a reproductively viable form and amount of *B. bassiana* strain K4B3, NMIA No. V08/025855, or *Beauveria* having the identifying characteristics of strain K4B3, NMIA No. V08/025855.

B. bassiana strain K4B3 is a particularly effective biological control agent, being capable of surviving interrupted wet periods, desiccation, and colonising, incapacitating and killing phytopathogenic insects such as, but not limited to, aphids, caterpillars, whitefly, moths, Varroa mite, cicada, and thrips in the field.

In other embodiments of the present invention, *B. bassiana* K4B3 may be used to prepare a composition comprising one or more lipids as identified herein.

In one embodiment, the method comprises maintaining a culture of *Beauveria bassiana* K4B3 V08/025855 under conditions suitable for production of at least one lipid; and separating the at least one lipid from the *Beauveria bassiana* K4B3 V08/025855.

In one embodiment, the composition comprises two or more lipids as described herein. Notably, none of these lipids has significant identity or homology to known insecticidal agents for *Beauveria*, such as beauvericin, beauvericin-F, and bassianolide. In one embodiment, the composition is a synergistic composition comprising two or more lipids as described herein.

In another embodiment, the composition additionally comprises less than about $1mgL^{-1}$ beauvericin, less than about $0.5mgL^{-1}$ beauvericin, less than about $0.1mgL^{-1}$ beauvericin, less than about $0.05mgL^{-1}$ beauvericin, less than about $0.01mgL^{-1}$ beauvericin, less than about $0.005mgL^{-1}$ beauvericin, less than about $0.001mgL^{-1}$ beauvericin, less than about $0.0005mgL^{-1}$ beauvericin, or less than about $0.0001mgL^{-1}$ beauvericin.

In another embodiment, the composition additionally comprises less than about $1mgL^{-1}$ beauvericin-F, less than about $0.5mgL^{-1}$ beauvericin-F, less than about $0.1mgL^{-1}$ beauvericin-F, less than about $0.05mgL^{-1}$ beauvericin-F, less than about $0.01mgL^{-1}$ beauvericin-F, less than about $0.005mgL^{-1}$ beauvericin-F, less than about $0.001mgL^{-1}$ beauvericin-F, less than about $0.0005mgL^{-1}$ beauvericin-F, or less than about $0.0001mgL^{-1}$ beauvericin-F.

In another embodiment, the composition additionally comprises less than about $1mgL^{-1}$ of a bassianolide, less than about $0.5mgL^{-1}$ of a bassianolide, less than about $0.1mgL^{-1}$ of a bassianolide, less than about $0.05mgL^{-1}$ of a bassianolide, less than about $0.01mgL^{-1}$ of a bassianolide, less than about $0.005mgL^{-1}$ of a bassianolide, less than about $0.001mgL^{-1}$ of a

bassianolide, less than about 0.0005mgL^{-1} of a bassianolide, or less than about 0.0001mgL^{-1} of a bassianolide.

In various embodiments, the composition is a synergistic composition comprising one or more lipids as described herein, together with one or more beauvericin, such as beauvericin-A, beauvericin-D, beauvericin-E, or beauvericin-F, or with one or more bassianolide, or any combination thereof.

For example, the composition additionally comprises more than about 0.1mgL^{-1} of a beauvericin, more than about 0.5mgL^{-1} of a beauvericin, more than about 1mgL^{-1} of a beauvericin, more than about 5mgL^{-1} of a beauvericin, more than about 10mgL^{-1} of a beauvericin, more than about 50mgL^{-1} of a beauvericin, or more than about 100mgL^{-1} of a beauvericin.

In another example, the composition additionally comprises more than about 0.1mgL^{-1} of a bassianolide, more than about 0.5mgL^{-1} of a bassianolide, more than about 1mgL^{-1} of a bassianolide, more than about 5mgL^{-1} of a bassianolide, more than about 10mgL^{-1} of a bassianolide, more than about 50mgL^{-1} of a bassianolide, or more than about 100mgL^{-1} of a bassianolide.

In another embodiment, the composition is a synergistic composition comprising one or more lipids as described herein and one or more entomopathogenic fungi as described herein, together with one or more beauvericin, such as beauvericin-A, beauvericin-D, beauvericin-E, or beauvericin-F, or with one or more bassianolide, or any combination thereof.

Beauveria bassiana strain K4B3 of the invention may be used singly, or in combination with other entomopathogenic fungi described herein. Examples of other entomopathogenic fungi are described in more detail below.

Beauveria bassiana strain K4B1 was isolated from a borer larva within a pine forest in Bombay, New Zealand. This *B. bassiana* isolate has been deposited in the National Measurement Institute of Australia, 1 Suakin Street, Pymble, New South Wales, Australia on 16 March 2005 according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number NM05/44595.

Beauveria bassiana isolate K4B1 shows a preference for thrips adults, and is also pathogenic to thrip juveniles and pupae, aphids and whitefly. The conidia of K4B1 form cream aggregations.

Beauveria bassiana isolate K4B2 was isolated from a Lepidoptera caterpillar on a sunflower in the Aka Aka flats, New Zealand. This *B. bassiana* isolate has been deposited in the National Measurement Institute of Australia on 3 March 2006 according to the Budapest Treaty

for the purposes of patent procedure. The isolate has been accorded the deposit number NM06/00010.

Beauveria bassiana isolate K4B2 exhibits a preference for caterpillars, including soybean looper caterpillar and white butterfly and army worm caterpillar. This isolate is also pathogenic to thrip juveniles, adults, and pupae, aphids and whitefly. The conidia of K4B2 form yellow dusty aggregations.

NMIA No. V08/025855, NMIA No. NM05/44595, NMIA No. NM06/00010 and other suitable isolates of *B. bassiana* may be used in combination with one or more lipids of the invention or functional variants or fragments thereof, and are particularly effective biological control agents, being capable of surviving interrupted wet periods, desiccation, and colonising, incapacitating and killing phytopathogenic insects such as, but not limited to, aphids, caterpillars, whitefly, moths, Varroa mite and thrips in the field. The degree of killing of whitefly, thrips and aphids by these isolates of *B. bassiana* is generally as good as the commonly used insecticides as described above. Resistance to these insecticides has developed; in these and other instances, compositions comprising or methods utilising *B. bassiana* isolates provide an effective alternative for insect control. This potent activity in the control of plant disease coupled with the absence of any observations of plant pathogenicity induced by *B. bassiana* demonstrate that isolates of these species have desirable attributes for use as a biological control agent.

Trichoderma species have not previously been ascribed any insecticidal activities or effects. *Trichoderma* cultures are typically fast growing at 25-30°C, but will not grow at 35°C. Colonies are transparent at first on media such as cornmeal dextrose agar (CMD) or white on richer media such as potato dextrose agar (PDA). Mycelium are not typically obvious on CMD, conidia typically form within one week in compact or loose tufts in shades of green or yellow or less frequently white. A yellow pigment may be secreted into the agar, especially on PDA. Some species produce a characteristic sweet or 'coconut' odor.

Conidiophores are highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae. Main branches of the *conidiophores* produce lateral side branches that may be paired or not, the longest branches distant from the tip and often phialides arising directly from the main axis near the tip. The branches may rebranch, with the secondary branches often paired and longest secondary branches being closest to the main axis. All primary and secondary branches arise at or near 90° with respect to the main axis. The typical *Trichoderma* conidiophore, with paired branches assumes a pyramidal aspect. Typically the conidiophore terminates in one or a few phialides. In some species (e.g. *T. polysporum*) the main branches are terminated by long,

simple or branched, hooked, straight or sinuous, septate, thin-walled, sterile or terminally fertile elongations. The main axis may be the same width as the base of the phialide or it may be much wider.

Phialides are typically enlarged in the middle but may be cylindrical or nearly subglobose. Phialides may be held in whorls, at an angle of 90° with respect to other members of the whorl, or they may be variously penicillate (gliocladium-like). Phialides may be densely clustered on wide main axis (e.g. *T. polysporum*, *T. hamatum*) or they may be solitary (e.g. *T. longibrachiatum*).

Conidia typically appear dry but in some species they may be held in drops of clear green or yellow liquid (e.g. *T. virens*, *T. flavofuscum*). Conidia of most species are ellipsoidal, 3-5 x 2-4 µm (L/W = > 1.3); globose conidia (L/W < 1.3) are rare. Conidia are typically smooth but tuberculate to finely warted conidia are known in a few species.

Synanamorphs are formed by some species that also have typical *Trichoderma* pustules. Synanamorphs are recognized by their solitary conidiophores that are verticillately branched and that bear conidia in a drop of clear green liquid at the tip of each phialide.

Chlamydospores may be produced by all species, but not all species produce chlamydospores on CMD at 20°C within 10 days. Chlamydospores are typically unicellular subglobose and terminate short hyphae; they may also be formed within hyphal cells. Chlamydospores of some species are multicellular (e.g. *T. stromaticum*).

Lecanicillium muscarium is an entomopathogenic fungi with a broad host range including homopteran insects and other arthropod groups. *L. muscarium* is considered a species complex, which includes isolates of varied morphological and biochemical characteristics. Typically, *L. muscarium* can be isolated from insect cadavers, such as aphids, thrips, whitefly, and mealy bugs, and may also be isolated from soil.

Lecanicillium muscarium strain K4V1 was isolated from whitefly in a greenhouse tomato crop in Pukekohe, New Zealand. This *L. muscarium* isolate has been deposited in the National Measurement Institute of Australia on 16 March 2005 according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number NM05/44593.

K4V1 has the additional identifying characteristics – 60% Conidia 1.0x1.0 micron on whitefly scale, 30% Conidia 2.0x1.0 micron on thrip juveniles (nymphs), 10% Conidia 2.5x1.3 micron on thrip pupae. Underside of mycelium thallus sparsely creased, Mycelium thallus removes from the agar very easily.

L. muscarium strain K4V2 was isolated from whitefly in a cucumber greenhouse in Ruakaka, New Zealand. This *L. muscarium* isolate has been deposited in the National Measurement Institute of Australia on 16 March 2005 according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number NM05/44594.

K4V2 has the additional identifying characteristics – 50% Conidia 2.0 x 1.5μm, 30% Conidia 2.0 x 1.0μm, 20% Conidia 1.0 x 1.0μm, pathogenic to Whitefly adults, while Blastospores pathogenic to aphids. Underside of mycelium thallus frequently creased, Mycelium thallus difficult to remove from agar surface.

L. muscarium strain K4V4 was isolated from isolated from an outdoor organic tamarillo crop. This *L. muscarium* isolate has been deposited in the National Measurement Institute of Australia on 3 March 2006 according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number NM06/00007.

K4V4 has the additional identifying characteristics – 50% Conidia 1.0 x 0.5μm, pathogenic to whitefly scale and adults, very aggressive at low humidity 65-75%, high temp 28-32°. Generally v.1 > 75%. 50% Condidia 0.5 x 0.5μm. Underside of mycelium thallus sparsely creased, Mycelium thallus diffuses custard yellow to light orange pigment in media.

NMIA No. NM05/44593, NMIA No. NM05/44594, NMIA No. NM06/00007 and other suitable isolates of *L. muscarium* may be used in combination with one or more lipids or functional variants of the invention, and are particularly effective biological control agents, being capable of surviving interrupted wet periods, desiccation, and colonising, incapacitating and killing phytopathogenic insects such as, but not limited to, aphids, whitefly, mealy bugs, Varroa mite, and thrips, in the field.

Lecanicillium longisporum is an entomopathogenic fungi that is particularly pathogenic to aphids. *Lecanicillium longisporum* strain KT4L1 was isolated from aphids in Barley grass Banker plants in Franklin, Auckland, New Zealand. This *L. longisporum* isolate has been deposited in the National Measurement Institute of Australia on 3 March 2006 according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number NM06/00009.

The isolate KT4L1 has the following identifying characteristics: 100% Condidia 6.0 x 2.1 μm, Mycelium thallus is offwhite to yellow growing very roughly which could be described as lumpy in consistency. Mycelium thallus diffuses light red brown colour into agar.

NMIA No. NM06/00009 and other suitable isolates of *L. longisporum* may be used in combination with one or more lipids or functional variants of the invention, and are particularly

effective biological control agents, being capable of surviving interrupted wet periods, desiccation, and colonising, incapacitating and killing phytopathogenic insects such as aphids, in the field.

Paecilomyces fumosoroseus is an entomopathogenic fungi found in infected and dead insects, and in some soils. *P. fumosoroseus* typically infects whiteflies, thrips, aphids, and caterpillars.

The K4P1 strain of *Paecilomyces fumosoroseus* was isolated from Diamond Back Moth caterpillar present on cabbage in Runciman, New Zealand. This *P. fumosoroseus* isolate has been deposited in the National Measurement Institute of Australia on 3 March 2006 according to the Budapest Treaty for the purposes of patent procedure. The isolate has been accorded the deposit number NM06/00008.

NMIA No. NM06/00008 and other suitable isolates of *P. fumosoroseus* may be used in combination with one or more lipids or functional variants of the invention, and are particularly effective biological control agents, being capable of surviving interrupted wet periods, desiccation, and colonising, incapacitating and killing phytopathogenic insects such as, but not limited to, whitefly, Varroa mite, and Lepidoptera caterpillar in the field.

As discussed above, many plant pathogenic insects have developed resistance to a number of insecticides; in these and other instances, compositions of the invention, optionally comprising or administered together with one or more fungal isolates such as those described above provide an effective alternative for insect control. This potent activity in the control of plant disease coupled with the absence of any observations of plant pathogenicity induced by these agents demonstrate that lipids of the invention, and when present the fungal isolates of these species, have desirable attributes for use as a biological control agent.

The present invention provides a composition which comprises one or more lipids or lipid fractions of the invention or functional variants thereof, together with one or more entomopathogenic fungi and at least one carrier.

The composition may include multiple strains of entomopathogenic fungi, and in certain embodiments, multiple strains may be utilised to target a number of phytopathogenic species, or a number of different developmental stages of a single phytopathogen, or indeed a combination of same. For example, the pupal form of a phytopathogenic insect may be targeted with one fungal strain, while the adult form of the phytopathogenic insect may be targeted with another fungal strain, wherein both strains are included in a composition of the invention. In other embodiments, three strains or less will be preferred, and frequently a single strain will be preferred.

Suitably, the composition comprises fungi selected from the group consisting of *Lecanicillium muscarium* strain K4V1 (NMIA Accession No. NM05/44593) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V2 (NMIA Accession No. NM05/44594) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V4 (NMIA Accession No. NM06/00007) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B1 (NMIA Accession No. NM05/44595) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B2 (NMIA Accession No. NM06/00010) or a strain having the identifying characteristics thereof; *Lecanicillium longisporum* strain KT4L1 (NMIA Accession No. NM06/00009) or a strain having the identifying characteristics thereof; and *Paecilomyces fumosoroseus* strain K4P1 (NMIA Accession No. NM06/00008) or a strain having the identifying characteristics thereof.

Particularly contemplated are compositions comprising one or more lipids of the invention or functional variants thereof and *Lecanicillium muscarium* strain K4V1 (NM05/44593) or a strain having the identifying characteristics thereof, compositions comprising one or more lipids of the invention or functional variants thereof and *Lecanicillium muscarium* strain K4V2 (NM05/44594) or a strain having the identifying characteristics thereof, and compositions comprising one or more lipids of the invention or functional variants thereof and both *Lecanicillium muscarium* strain K4V1 (NM05/44593) or a strain having the identifying characteristics thereof, compositions comprising one or more lipids of the invention or functional variants thereof and *Lecanicillium muscarium* strain K4V2 (NM05/44594) or a strain having the identifying characteristics thereof.

Examples of compositions comprising entomopathogenic fungi are well known in the art, and include those described in, for example, WO95/10597 (published as PCT/US94/11542) to Mycotech Corporation, WO2003/043417 (published as PCT/US2002/037218) to The United States of America as represented by The Secretary of Agriculture, US Patent No. 4,530,834 to McCabe et al., and US Patent Application No. 10/657,982 (published as US 2004/0047841) to Wright et al., each incorporated by reference herein in its entirety.

To be suitable for application to a plant or its surroundings, said at least one carrier is an agriculturally acceptable carrier, more preferably is selected from the group consisting of a filler stimulant, an anti-caking agent, a wetting agent, an emulsifier, and an antioxidant, more preferably said composition comprises at least one of each of a filler stimulant, an anti-caking agent, a wetting agent, an emulsifier, and an antioxidant. Preferably, said filler stimulant is a carbohydrate source, such as a disaccharide including, for example, sucrose, fructose, glucose, or dextrose, said anti-caking agent is selected from talc, silicon dioxide, calcium silicate, or kaelin

clay, said wetting agent is skimmed milk powder, said emulsifier is a soy-based emulsifier such as lecithin or a vegetable-based emulsifier such as monodiglyceride, and said antioxidant is sodium glutamate or citric acid. However, other examples well known in the art may be substituted, provided the ability of the composition to support insecticidal or entomopathogenic efficacy, and fungal viability where necessary, is maintained.

Preferably, said composition is a biological control composition. The concentration of the insecticidal lipid or lipid fraction of the invention present in the composition that is required to be effective as biological control agents may vary depending on the end use, physiological condition of the plant; type (including insect species), concentration and degree of pathogen infection; temperature, season, humidity, stage in the growing season and the age of plant; number and type of conventional insecticides or other treatments (including fungicides) being applied; and plant treatments (such as deleafing and pruning) may all be taken into account in formulating the composition.

Insecticidal compositions

Lipid Compositions and Methods of Use

The inventors contemplate that the lipid compositions disclosed herein will find particular utility as BCA compositions for topical and/or systemic application to field crops, grasses, fruits and vegetables, lawns, trees, and/or ornamental plants. Alternatively, the lipids disclosed herein may be formulated as a spray, dust, powder, or other aqueous, atomized or aerosol for killing an insect, or controlling an insect population. The lipid compositions disclosed herein may be used prophylactically, or alternatively, may be administered to an environment once target insects have been identified in the particular environment to be treated. The lipid compositions may comprise an individual lipid or may contain various combinations of the lipids disclosed herein.

Regardless of the method of application, the amount of the active lipid component(s) is applied at an insecticidally-effective amount, which will vary depending on such factors as, for example, the specific target insects to be controlled, the specific environment, location, plant, crop, or agricultural site to be treated, the environmental conditions, and the method, rate, concentration, stability, and quantity of application of the insecticidally-active lipid composition. The formulations may also vary with respect to climatic conditions, environmental considerations, and/or frequency of application and/or severity of insect infestation.

The compositions described may be made by formulating the one or more lipids, functional variants or functional fragments thereof, optionally together with the fungal cell and/or spore suspension, with the desired agriculturally-acceptable carrier. The compositions

may be formulated prior to administration in an appropriate means such as lyophilized, freeze-dried, desiccated, or in an aqueous carrier, medium or suitable diluent, such as saline or other buffer. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral), or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally-acceptable carrier" covers all adjuvants, inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in insecticide formulation technology; these are well known to those skilled in insecticide formulation. The formulations may be mixed with one or more solid or liquid adjuvants and prepared by various means, e.g., by homogeneously mixing, blending and/or grinding the insecticidal composition with suitable adjuvants using conventional formulation techniques.

The compositions may include one or more fungal strains, may include one or more bacterial species, or both. Exemplary bacterial species include those such as *B. thuringiensis*, *B. megaterium*, *B. subtilis*, *B. cereus*, *E. coli*, *Salmonella* spp., *Agrobacterium* spp., or *Pseudomonas* spp.

Oil Flowable Suspensions

In one exemplary embodiment, the bioinsecticide composition comprises an oil flowable suspension of one or more lipids of the invention, functional variants or functional fragments thereof, optionally together with one or more fungal cells, including one or more fungal cells which expresses one or more of the novel proteins disclosed herein.

Water-Dispersible Granules

In another important exemplary embodiment, the bioinsecticide composition comprises a water dispersible granule. This granule comprises one or more lipids of the invention, functional variants or functional fragments thereof, optionally together with one or more fungal cells, including one or more fungal cells which produces a lipid or lipid fraction of the invention disclosed herein.

Powders, Dusts, and Spore Formulations

In a third important exemplary embodiment, the bioinsecticide composition comprises a wettable powder, dust, spore formulation, cell pellet, or colloidal concentrate. This powder comprises one or more lipids of the invention, functional variants or functional fragments thereof, optionally together with one or more fungal cells, including one or more fungal cells which produces a lipid or lipid fraction of the invention disclosed herein. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or

alternatively, dissolve in a controlled-release, sustained-release, or other time-dependent manner. Such compositions may be applied to, or ingested by, the target insect, and as such, may be used to control the numbers of insects, or the spread of such insects in a given environment.

Aqueous Suspensions and Fungal Cell Filtrates or Lysates

In a fourth important exemplary embodiment, the bioinsecticide composition comprises an aqueous suspension of one or more lipids of the invention, functional variants or functional fragments thereof, optionally together with one or more fungal cells, including one or more fungal cells capable of producing a lipid or lipid fraction of the invention disclosed herein. Such aqueous suspensions may be provided as a concentrated stock solution which is diluted prior to application, or alternatively, as a diluted solution ready-to-apply.

When the insecticidal compositions comprise intact cells producing the lipid of interest, such cells may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

Alternatively, the novel insecticidal lipids may be prepared by native or recombinant expression systems in vitro and isolated for subsequent field application. Such lipids or lipid fractions may be either in crude cell lysates, suspensions, colloids, etc., or alternatively may be purified, refined, buffered, and/or further processed, before formulating in an active biocidal formulation. Likewise, under certain circumstances, it may be desirable to isolate lipids or spores from cultures producing the lipid and apply solutions, suspensions, or colloidal preparations of such lipids or spores as the active bioinsecticidal composition.

Multifunctional Formulations

In certain embodiments, for example those when the control of multiple insect species is desired, the insecticidal formulations described herein may also further comprise one or more chemical pesticides, (such as chemical pesticides, nematocides, fungicides, virucides, microbicides, amoebicides, insecticides, etc.), and/or one or more lipids or lipid fractions having the same, or different insecticidal activities or insecticidal specificities, as the insecticidal lipids or lipid fractions identified herein. The insecticidal lipids or lipid fractions may also be used in conjunction with other treatments such as fertilizers, weed killers, cryoprotectants, surfactants,

detergents, insecticidal soaps, dormant oils, polymers, and/or time-release or biodegradable carrier formulations that permit long-term dosing of a target area following a single application of the formulation. Likewise the formulations may be prepared into edible "baits" or fashioned into insect "traps" to permit feeding or ingestion by a target insect of the insecticidal formulation.

The insecticidal compositions of the invention may also be used in consecutive or simultaneous application to an environmental site singly or in combination with one or more additional insecticides, pesticides, chemicals, fertilizers, or other compounds.

Compositions comprising fungi

For use as a biological control agent, when present in the composition the entomopathogenic fungi of the invention should be in a reproductively viable form. The term reproductively viable as used herein includes mycelial and spore forms of the fungi. For example, for most purposes, fungal strains are desirably incorporated into the composition in the form of spores (conidia or blastospores). Spores are obtainable from all the fungal strains described herein, and may be produced using known art techniques. Compositions of the invention comprising one or more lipids of the invention, functional variants or functional fragments thereof, optionally together with one or more fungal cells, including one or more spores obtained from fungal strains described herein form a further aspect of the invention. The concentration of the fungal spores in the composition will depend on the utility to which the composition is to be put. An exemplary concentration range is from about 1×10^6 to 1×10^{12} spores per ml, preferably from about 1×10^7 to 2×10^{10}, and more preferably 1×10^7 to 1×10^8 spores per ml.

In theory one infective unit should be sufficient to infect a host but in actual situations a minimum number of infective units are required to initiate an infection. The concept of lethal dose (LD) regularly used with chemical pesticides is inappropriate for those microbial pesticides in which certain elements of entomopathogenic activity are reliant on colonisation of the plant or its surroundings by an entomopathogenic fungi. Concepts of infective dose (ID) or infective concentration (IC) are more precise or applicable. ID or IC refer to the actual number of infective units needed to initiate infection or the number of infective units exposed to the pathogen to cause death. Therefore, the number of infective units applied in the field or greenhouse against a pahtogen will affect the degree of control. It is important to apply the desired concentration of the anti-phytopathogenic fungi, properly placed and at the right time, to obtain good control of the pest: this is known as the "infection threshold".

It will be apparent that the concentration of fungal spores in a composition formulated for application may be less than that in a composition formulated for, for example, storage. The

Applicants have determined that with the entomopathogenic fungi described herein, infection threshold occurs at about 10^7 spores per ml of sprayable solution, when applied at a rate of about 1L per hectare. Accordingly, in one example, a composition formulated for application will preferably have a concentration of at least about 10^7 spores per ml. In another example, a composition formulated for storage (for example, a composition such as a wettable powder capable of formulation into a composition suitable for application) will preferably have a concentration of about 10^{10} spores per gram. It will be apparent that the spore concentration of a composition formulated for storage and subsequent formulation into a composition suitable for application must be adequate to allow said composition for application to also be sufficiently concentrated so as to be able to be applied to reach infection threshold.

In certain embodiments, the composition is a stable composition capable of supporting insecticidal efficacy (for example, of one or more lipids) for a period greater than about two weeks, preferably greater than about one month, about two months, about three months, about four months, about five months, more preferably greater than about six months. To be suitable for use in embodiments utilising one or more entomopathogens, such as an entomopathogenic fungi as described herein, the composition preferably is able to support reproductive viability of the entomopathogen or entomopathogenic efficacy for a period greater than about six months.

Using conventional solid substrate and liquid fermentation technologies well known in the art, the insecticidal lipids of the invention can be produced in sufficient amounts to allow use as biological control agents. For example, lipids and lipid fractions of the invention may be produced in sufficient quantity using these growing techniques, and exemplary techniques are presented herein in the Examples. Growth is generally effected under aerobic conditions at any temperature satisfactory for growth of the organism. For example, for *B. bassiana*, a temperature range of from 10 to 32°C, preferably 25 to 30°C, and most preferably 23°C, is preferred. The pH of the growth medium is slightly acid to neutral, that is, about 5.0 to 7.0, and most preferably 5.5. Incubation time is sufficient for the isolate to reach a stationary growth phase, about 21 days when incubated at 23°C, and will occur in normal photoperiod.

Spores from selected strains can be produced in bulk for field application using nutrient film, submerged culture, and rice substrate growing techniques. The spores may be harvested by methods well known in the art, for example, by conventional filtering or sedimentary methodologies (eg. centrifugation) or harvested dry using a cyclone system. Spores can be used immediately or stored, chilled at 0° to 6°C, preferably 2°C, for as long as they remain reproductively viable. It is however generally preferred that when not incorporated into a composition of the invention, use occurs within two weeks of harvesting.

Similarly, when required, the one or more lipids produced by *B. bassiana* K4B3 may be separated from the *B. bassiana* K4B3 by methods well known in the art, for example, by fractionation, filtering or sedimentary methodologies (eg. centrifugation), whether in combination with one or more cell-lysis steps (for example, for intracellular lipids) or not (for example, for lipids that are secreted into the growth media).

The composition of the invention may also include one or more carriers, preferably one or more agriculturally acceptable carriers. In one embodiment the carrier, such as an agriculturally acceptable carrier, can be solid or liquid. Carriers useful herein include any substance typically used to formulate agricultural composition.

In one embodiment the agriculturally acceptable carrier maybe selected from the group comprising fillers, solvents, excipients, surfactants, suspending agents, speaders/stickers (adhesives), antifoaming agents, dispersants, wetting agents, drift reducing agents, auxiliaries, adjuvants or a mixture thereof.

Compositions of the invention may be formulated as, for example, concentrates, solutions, sprays, aerosols, immersion baths, dips, emulsions, wettable powders, soluble powders, suspension concentrates, dusts, granules, water dispersible granules, microcapsules, pastes, gels and other formulation types by well-established procedures.

These procedures include mixing and/or milling of the active ingredients with agriculturally acceptable carrier substances, such as fillers, solvents, excipients, surfactants, suspending agents, speaders/stickers (adhesives), antifoaming agents, dispersants, wetting agents, drift reducing agents, auxiliaries and adjuvants.

In one embodiment solid carriers include but are not limited to mineral earths such as silicic acids, silica gels, silicates, talc, kaolin, attapulgus clay, limestone, lime, chalk, bole, loess, clay, dolomite, diatomaceous earth, aluminas calcium sulfate, magnesium sulfate, magnesium oxide, ground plastics, fertilizers such as ammonium sulfate, ammonium phosphate, ammonium nitrate, and ureas, and vegetable products such as grain meals, bark meal, wood meal, and nutshell meal, cellulosic powders and the like. As solid carriers for granules the following are suitable: crushed or fractionated natural rocks such as calcite, marble, pumice, sepiolite and dolomite; synthetic granules of inorganic or organic meals; granules of organic material such as sawdust, coconut shells, corn cobs, corn husks or tobacco stalks; kieselguhr, tricalcium phosphate, powdered cork, or absorbent carbon black; water soluble polymers, resins, waxes; or solid fertilizers. Such solid compositions may, if desired, contain one or more compatible wetting, dispersing, emulsifying or colouring agents which, when solid, may also serve as a diluent.

In one embodiment the carrier may also be liquid, for example, water; alcohols, particularly butanol or glycol, as well as their ethers or esters, particularly methylglycol acetate; ketones, particularly acetone, cyclohexanone, methylethyl ketone, methylisobutylketone, or isophorone; petroleum fractions such as paraffinic or aromatic hydrocarbons, particularly xylenes or alkyl naphthalenes; mineral or vegetable oils; aliphatic chlorinated hydrocarbons, particularly trichloroethane or methylene chloride; aromatic chlorinated hydrocarbons, particularly chlorobenzenes; water-soluble or strongly polar solvents such as dimethylformamide, dimethyl sulfoxide, or N-methylpyrrolidone; liquefied gases; or the like or a mixture thereof.

In one embodiment surfactants include nonionic surfactants, anionic surfactants, cationic surfactants and/or amphoteric surfactants and promote the ability of aggregates to remain in solution during spraying.

Spreaders/stickers promote the ability of the compositions of the invention to adhere to plant surfaces. Examples of surfactants, spreaders/stickers include but are not limited to Tween and Triton (Rhom and Hass Company), Deep Fried™, Fortune®, Pulse, C. Daxoil®, Codacide oil®, D-C. Tate®, Supamet Oil, Bond®, Penetrant, Glowelt® and Freeway, Citowett®, Fortune Plus™, Fortune Plus Lite, Fruimec, Fruimec lite, alkali metal, alkaline earth metal and ammonium salts of aromatic sulfonic acids, e.g., ligninsulfonic acid, phenolsulfonic acid, naphthalenesulfonic acid and dibutylnaphthalenesulfonic acid, and of fatty acids, alkyl and alkylaryl sulfonates, and alkyl, lauryl ether and fatty alcohol sulfates, and salts of sulfated hexadecanols, heptadecanols, and octadecanols, salts of fatty alcohol glycol ethers, condensation products of sulfonated naphthalene and naphthalene derivatives with formaldehyde, condensation products of naphthalene or naphthalenesulfonic acids with phenol and formaldehyde, polyoxyethylene octylphenol ethers, ethoxylated isooctylphenol, ethoxylated octylphenol and ethoxylated nonylphenol, alkylphenol polyglycol ethers, tributylphenyl polyglycol ethers, alkylaryl polyether alcohols, isotridecyl alcohol, fatty alcohol ethylene oxide condensates, ethoxylated castor oil, polyoxyethylene alkyl ethers, ethoxylated polyoxypropylene, lauryl alcohol polyglycol ether acetal, sorbitol esters, lignin-sulfite waste liquors and methyl cellulose. Where selected for inclusion, one or more agricultural surfactants, such as Tween are desirably included in the composition according to known protocols.

Wetting agents reduce surface tension of water in the composition and thus increase the surface area over which a given amount of the composition may be applied. Examples of wetting agents include but are not limited to salts of polyacrylic acids, salts of lignosulfonic acids, salts of phenolsulfonic or naphthalenesulfonic acids, polycondensates of ethylene oxide

with fatty alcohols or fatty acids or fatty esters or fatty amines, substituted phenols (particularly alkylphenols or arylphenols), salts of sulfosuccinic acid esters, taurine derivatives (particularly alkyltaurates), phosphoric esters of alcohols or of polycondensates of ethylene oxide with phenols, esters of fatty acids with polyols, or sulfate, sulfonate or phosphate functional derivatives of the above compounds.

In one embodiment the preferred method of applying the compound or composition of the invention is to spray a dilute or concentrated solution by handgun or commercial airblast.

As described above, the compositions of the present invention may be used alone or in combination with one or more other agricultural agents, including pesticides, insecticides, acaracides, fungicides or bactericides (provided such fungicides or bactericides are not detrimental or toxic to any fungi or bacteria present in the composition), herbicides, antibiotics, antimicrobials, nemacides, rodenticides, entomopathogens, pheromones, attractants, plant growth regulators, plant hormones, insect growth regulators, chemosterilants, microbial pest control agents, repellents, viruses, phagostimulents, plant nutrients, plant fertilisers and biological controls. When used in combination with other agricultural agents the administration of the two agents may be separate, simultaneous or sequential. Specific examples of these agricultural agents are known to those skilled in the art, and many are readily commercially available.

Examples of plant nutrients include but are not limited to nitrogen, magnesium, calcium, boron, potassium, copper, iron, phosphorus, manganese, molybdenum, cobalt, boron, copper, silicon, selenium, nickel, aluminum, chromium and zinc.

Examples of antibiotics include but are not limited to oxytetracyline and streptomycin.

Examples of fungicides include but are not limited to the following classes of fungicides: carboxamides, benzimidazoles, triazoles, hydroxypyridines, dicarboxamides, phenylamides, thiadiazoles, carbamates, cyano-oximes, cinnamic acid derivatives, morpholines, imidazoles, beta-methoxy acrylates and pyridines/pyrimidines.

Further examples of fungicides include but are not limited to natural fungicides, organic fungicides, sulphur-based fungicides, copper/calcium fungicides and elicitors of plant host defences.

Examples of natural fungicides include but are not limited to whole milk, whey, fatty acids or esterified fatty acids.

Examples of organic fungicides include but are not limited to any fungicide which passes an organic certification standard such as biocontrol agents, natural products, elicitors (some of may also be classed as natural products), and sulphur and copper fungicides (limited to restricted use).

An example of a sulphur-based fungicide is Kumulus™ DF (BASF, Germany).

An example of a copper fungicide is Kocide® 2000 DF (Griffin Corporation, USA).

Examples of elicitors include but are not limited to chitosan, Bion™, BABA (DL-3-amino-n-butanoic acid, β-aminobutyric acid) and Milsana™ (Western Farm Service, Inc., USA).

In some embodiments non-organic fungicides may be employed. Examples of non-organic fungicides include but are not limited to Bravo™ (for control of PM on cucurbits); Supershield™ (Yates, NZ) (for control of Botrytis and PM on roses); Topas® 200EW (for control of PM on grapes and cucurbits); Flint™ (for control of PM on apples and cucurbits); Amistar® WG (for control of rust and PM on cereals); and Captan™, Dithane™, Euparen™, Rovral™, Scala™, Shirlan™, Switch™ and Teldor™ (for control of Botrytis on grapes).

Examples of pesticides include but are not limited to azoxystrobin, bitertanol, carboxin, Cu_2O, cymoxanil, cyproconazole, cyprodinil, dichlofluamid, difenoconazole, diniconazole, epoxiconazole, fenpiclonil, fludioxonil, fluquiconazole, flusilazole, flutriafol, furalaxyl, guazatin, hexaconazole, hymexazol, imazalil, imibenconazole, ipconazole, kresoxim-methyl, mancozeb, metalaxyl, R-metalaxyl, metconazole, oxadixyl, pefurazoate, penconazole, pencycuron, prochloraz, propiconazole, pyroquilone, SSF-109, spiroxamin, tebuconazole, thiabendazole, tolifluamid, triazoxide, triadimefon, triadimenol, triflumizole, triticonazole and uniconazole.

An example of a biological control agent other than a fungal strain described herein is the BotryZen™ biological control agent comprising *Ulocladium oudemansii*.

The compositions may also comprise a broad range of additives such as stablisers and penetrants used to enhance the active ingredients and so-called 'stressing' additives to improve spore vigor, germination and survivability such as potassium chloride, glycerol, sodium chloride and glucose. Additives may also include compositions which assist in maintaining microorganism viability in long term storage, for example unrefined corn oil and so called invert emulsions containing a mixture of oils and waxes on the outside and water, sodium alginate and conidia on the inside.

As will be appreciated by those skilled in the art, it is important that any additives used are present in amounts that do not interfere with the effectiveness of the biological control agents.

Examples of suitable compositions including carriers, preservations, surfactants and wetting agents, spreaders, and nutrients are provided in US 5780023, incorporated herein in its entirety by reference.

The Applicants have also determined that many commonly used fungicides do not adversely affect the entomopathogenic fungi when present, described herein. The compositions of the invention comprising said fungi may therefore also include such fungicides. Alternatively, the compositions may be used separately but in conjunction with such fungicides in control programmes.

The invention also provides a method of producing a composition comprising one or more lipids or lipid fractions of the invention or one or more functional variants thereof and one or more entomopathogenic fungi described herein, said method comprising providing a reproductively viable form of said entomopathogenic fungi, and combining said reproductively viable form of said entomopathogenic fungi with one or more lipids or lipid fractions of the invention or functional variants thereof and at least one agriculturally acceptable carrier.

The compositions may be prepared in a number of forms. One preparation comprises a powdered form of a composition of the invention which may be dusted on to a plant or its surroundings. In a further form, the composition is mixed with a diluent such as water to form a spray, foam, gel or dip and applied appropriately using known protocols. In a presently preferred embodiment, a composition formulated as described above is mixed with water using a pressurised sprayer at about 1gm/L, or about 1 to 3 kg/ha in no less than 1000L water per ha. Preferably, Deep Fried™ or Fortune Plus™ is added to the composition as a UV and desiccation protection agent at about 1ml/L. Compositions comprising *L. muscarium*, *L. longisporum*, or *P. fumosoroseus* can be applied in a similar manner.

Compositions formulated for other methods of application such as injection, rubbing or brushing, may also be used, as indeed may any known art method. Indirect applications of the composition to the plant surroundings or environment such as soil, water, or as seed coatings are potentially possible.

As discussed above, the concentration at which the compositions of the invention, including those comprising entomopathogenic fungi as described herein are to be applied so as to be effective biological control agents may vary depending on the end use, physiological condition of the plant; type (including insect species), concentration and degree of pathogen infection; temperature, season, humidity, stage in the growing season and the age of plant; number and type of conventional insecticides or other treatments (including fungicides) being applied; and plant treatments (such as leaf plucking and pruning).

Other application techniques, including dusting, sprinkling, soil soaking, soil injection, seed coating, seedling coating, foliar spraying, aerating, misting, atomizing, fumigating, aerosolizing, and the like, are also feasible and may be required under certain circumstances such

as e.g., insects that cause root or stalk infestation, or for application to delicate vegetation or ornamental plants. These application procedures are also well-known to those of skill in the art.

The insecticidal compositions of the present invention may also be formulated for preventative or prophylactic application to an area, and may in certain circumstances be applied to pets, livestock, animal bedding, or in and around farm equipment, barns, domiciles, or agricultural or industrial facilities, and the like.

The concentration of insecticidal composition which is used for environmental, systemic, topical, or foliar application will vary widely depending upon the nature of the particular formulation, means of application, environmental conditions, and degree of biocidal activity. Typically, the bioinsecticidal composition will be present in the applied formulation at a concentration of at least about 1% by weight and may be up to and including about 99% by weight. Dry formulations of the lipid compositions may be from about 1% to about 99% or more by weight of the lipid or lipid fraction composition, while liquid formulations may generally comprise from about 1% to about 99% or more of the active ingredient by weight. As such, a variety of formulations are preparable, including those formulations that comprise from about 5% to about 95% or more by weight of the insecticidal lipid, including those formulations that comprise from about 10% to about 90% or more by weight of the insecticidal lipid. Naturally, compositions comprising from about 15% to about 85% or more by weight of the insecticidal lipid, and formulations comprising from about 20% to about 80% or more by weight of the insecticidal lipid are also considered to fall within the scope of the present disclosure.

In the case of compositions in which intact fungal cells that contain the insecticidal lipid are included, preparations will generally contain from about 10^4 to about 10^8 cells/mg, although in certain embodiments it may be desirable to utilize formulations comprising from about 10^2 to about 10^4 cells/mg, or when more concentrated formulations are desired, compositions comprising from about 10^8 to about 10^{10} or 10^{11} cells/mg may also be formulated. Alternatively, cell pastes, spore concentrates, or lipid or lipid fraction suspension concentrates may be prepared that contain the equivalent of from about 10^{12} to 10^{13} cells/mg of the active lipid, and such concentrates may be diluted prior to application.

The insecticidal formulation described above may be administered to a particular plant or target area in one or more applications as needed, with a typical field application rate per hectare ranging on the order of from about 50 g/hectare to about 500 g/hectare of active ingredient, or alternatively, from about 500 g/hectare to about 1000 g/hectare may be utilized. In certain instances, it may even be desirable to apply the insecticidal formulation to a target area at an application rate of from about 1000 g/hectare to about 5000 g/hectare or more of active

ingredient. In fact, all application rates in the range of from about 50 g of active lipid per hectare to about 10,000 g/hectare are contemplated to be useful in the management, control, and killing, of target insect pests using such insecticidal formulations. As such, rates of about 100 g/hectare, about 200 g/hectare, about 300 g/hectare, about 400 g/hectare, about 500 g/hectare, about 600 g/hectare, about 700 g/hectare, about 800 g/hectare, about 900 g/hectare, about 1 kg/hectare, about 1.1 kg/hectare, about 1.2 kg/hectare, about 1.3 kg/hectare, about 1.4 kg/hectare, about 1.5 kg/hectare, about 1.6 kg/hectare, about 1.7 kg/hectare, about 1.8 kg/hectare, about 1.9 kg/hectare, about 2.0 kg/hectare, about 2.5 kg/hectare, about 3.0 kg/hectare, about 3.5 kg/hectare, about 4.0 kg/hectare, about 4.5 kg/hectare, about 6.0 kg/hectare, about 7.0 kg/hectare, about 8.0 kg/hectare, about 8.5 kg/hectare, about 9.0 kg/hectare, and even up to and including about 10.0 kg/hectare or greater of active lipid may be utilized in certain agricultural, industrial, and domestic applications of the pesticidal formulations described hereinabove.

For example, in certain applications, a composition comprising a fungus as described herein may be applied at a rate of from about 1×10^{10} to about 1×10^{15} spores per hectare, preferably from about 1×10^{12} to about 1×10^{14} spores per hectare, more preferably from about 5×10^{12} to about 1×10^{14} spores per hectare, more preferably about $1\text{-}3 \times 10^{13}$ spores per hectare.

In a further aspect the present invention provides a method for controlling one or more phytopathogenic insects, the method comprising applying to a plant or its surroundings a lipid or lipid fraction as described herein.

In one embodiment, the application is of one or more lipid fractions as exemplified herein together with one or more other entomopathogenic fungi as described herein.

Preferably, said one or more other fungi is selected from the group consisting of *B. bassiana* strain K4B3 (NMIA Accession No. No. V08/025855) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V1 (NMIA Accession No. NM05/44593) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V2 (NMIA Accession No. NM05/44594) or a strain having the identifying characteristics thereof; *Lecanicillium muscarium* strain K4V4 (NMIA Accession No. NM06/00007) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B1 (NMIA Accession No. NM05/44595) or a strain having the identifying characteristics thereof; *Beauveria bassiana* strain K4B2 (NMIA Accession No. NM06/00010) or a strain having the identifying characteristics thereof; *Lecanicillium longisporum* strain KT4L1 (NMIA Accession No. NM06/00009) or a strain having the identifying characteristics thereof; and *Paecilomyces fumosoroseus* strain K4P1 (NMIA Accession No. NM06/00008) or a strain having the identifying characteristics thereof.

Again, multiple lipids of the invention, whether or not in combination with multiple strains of the entomopathogenic fungi with activity against one or more phytopathogenic insect species, may be employed in the control process.

Young seedlings are typically most susceptible to damage from insect pests. Therefore, application of the compositions of the invention to freshly planted out crops, prior to emergence, is contemplated, as is application on emergence.

Repeated applications at the same or different times in a crop cycle are also contemplated. The insecticidal lipids of the invention, compositions comprising the insecticidal lipids of the invention may be applied either earlier or later in the season. This may be over flowering or during fruiting. The insecticidal lipids of the invention or compositions comprising the insecticidal lipids of the invention may also be applied immediately prior to harvest, or after harvest to rapidly colonise necrotic or senescing leaves, fruit, stems, machine harvested stalks and the like to prevent insect colonisation. The insecticidal lipids of the invention or compositions of the invention may also be applied to dormant plants in winter to slow insect growth on dormant tissues.

Application may be at a time before or after bud burst and before and after harvest. However, treatment preferably occurs between flowering and harvest. To increase efficacy, multiple applications (for example, 2 to 6 applications over the stages of flowering through fruiting) of the insecticidal lipids of the invention or a composition of the invention is preferred.

Reapplication of the insecticidal lipids of the invention or composition should also be considered after rain. Using insect infectivity prediction models or infection analysis data, application of the BCA can also be timed to account for insect infection risk periods.

In various exemplary embodiments, the lipids or lipid fractions of the present invention and compositions comprising such lipids or lipid fractions are not deleterious to the plants or plant surroundings to which they are applied at dosage rates capable of achieving insecticidal efficacy.

In the presently preferred embodiments, the insecticidal lipids of the invention or a composition comprising same is applied in a solution, for example as described above, using a pressurised sprayer. The plant parts should be lightly sprayed until just before run off. Applications may be made to any part of the plant and/or its surroundings, for example to the whole plant canopy, to the area in the canopy where the flowers and developing fruit are concentrated, or to the plant stem and/or soil, water or growth media adjacent to or surrounding the roots, tubers or the like.

Preferably the composition is stable. As used herein, the term "stable" refers to a composition capable of supporting insecticidal efficacy for several weeks, preferably about one, about two, about three, about four, preferably about five, more preferably about six months, or longer. Preferably, the composition is stable without a requirement for storage under special conditions, such as, for example, refrigeration or freezing.

The applied compositions control phytopathogenic insects. Phytopathogenic insects are responsible for many of the pre- and post-harvest diseases which attack plant parts and reduce growth rate, flowering, fruiting, production and may cause death of afflicted plants. As used herein, phytopathogenic insects include insects which are themselves plant pathogens, and insects which may act as a vector for other plant pathogens, for example, phytopathogenic fungi and bacteria. It will be appreciated that by controlling host insects which act as vectors for other phytopathogens, the incidence and/or severity of plant disease can be minimised.

Examples of the major phytopathogenic insects afflicting a number of important horticultural crops grown in New Zealand are presented in Table 1 below.

Table 1. Major Insect Pests

Crop	No. of Growers	Planted area (ha)	Major Pest
Cherries		550	Aphids
Potatoes	321	10,611	Aphids, whitefly
Tomatoes (indoor)	390	167	Whitefly, caterpillars
Brassicas	227	3,746	Whitefly, caterpillars
Squash	181	6,560	Whitefly, aphids
Tamarillos	175	270	Whitefly, aphids
Strawberries	125	361	Aphids, thrips
Cucumber (indoor)		55	Aphids, thrips, whitefly
Onions	150	5,488	Thrips
Tomatoes (outdoor)	80	609	Whitefly, caterpillars, thrips
Capsicum	142	87	Thrips, aphids, whitefly, caterpillars
Lettuce	252	1,287	Aphids, thrips
Pumpkin	125	1,033	Whitefly, aphids

Control of *Hemiptera*, such as whitefly, thrips, aphids, and caterpillars including in the crops outlined above using the lipids and lipid fractions, the compositions, and the methods of the present invention is particularly contemplated. Control of Varroa mite using lipids or lipid fractions of the invention, either alone or together with one or more *B. bassiana* fungal strains, or with *L. muscarium,* or *Paecilomyces fumosoroseus* and compositions of the present invention comprising same are also particularly contemplated.

The methods of the invention have particular application to plants and plant products, either pre- or post-harvest. For example, the composition of the invention may be applied to stored products of the type listed above including fruits, vegetables, cut flowers and seeds. Suitable application techniques encompass those identified above, particularly spraying.

The composition can potentially be used to treat or pretreat soils or seeds, as opposed to direct application to a plant. The composition may find use in plant processing materials such as protective coatings, boxes and wrappers.

Also encompassed by the present invention are plants, plant products, soils and seeds treated directly with a lipid of the invention or a composition of the invention.

The invention consists in the foregoing and also envisages constructions of which the following gives examples only and in no way limit the scope thereof.

EXAMPLE 1 – BIOASSAYS OF INSECT CONTROL

This example describes the development of a robust bioassay to determine the insecticidal efficacy of various lipids or lipid fractions.

The target insect assay was developed and assessed using the criteria 1) availability, 2) susceptibility and 3) ease of use.

The target insect *Myzus persicae* (green peach aphid, order *Hemiptera*), reared on cabbage plants in a constant temperature room, was used in all experiments. To inoculate aphids with lipid fraction samples, aphids were transferred to a piece of cabbage leaf on the surface of a 1% water agar plate using 0.05% Tween 80 as a wetting agent between leaf and agar. Aphids of mixed age were used, usually between 30-50/Petri dish.

A hand-held Paasche airbrush was modified to take micro volumes and used to atomise 300 µl of test or control solutions. Subsequently, plates with treated aphids were maintained at 20°C, 12h light:12h dark and checked daily. Dead were removed. Counts of aphids inoculated were made directly after spraying to avoid including newborn aphids in % mortality, but no effort was made to remove neonate nymphs during incubation.

EXAMPLE 2 – IDENTIFICATION OF INSECTICIDAL LIPIDS

This example describes the identification and preparation of a range of insecticidal lipid fractions from *Beauvaria bassiana* K4B3.

Methods

Sample

Organic solvent-extracted K4B3 mycelia samples were supplied and used for these studies.

Analytical TLC

Analytical TLC was performed on Merck high performance thin layer chromatography (HPTLC) plates developed using the solvent systems described by Macala *et al.* (1983). Plates were visualised by dipping in copper sulphate – phosphoric acid and heating at 170° for 15-30 min.

SAX acidic/non acidic fractionation

Waters AccellPlus QMA solid phase extraction cartridges were used for anion exchange separation of acidic lipids from non-acidics. Cartridges were first conditioned with chloroform-methanol-0.8M sodium acetate (30:60:8) and then washed with chloroform-methanol-water (30:60:8). Samples were loaded in the latter solvent mixture, which was also used to elute the non-retained (non-acidic) fraction. The retained acidic lipids were eluted with chloroform-methanol-0.8M sodium acetate (30:60:8).

WAX acidic/non acidic fractionation

For larger scale separations of acidic lipids from non-acidics, the procedure described above was followed, except that columns were self-packed with DEAE-Sephadex A50 (Pharmacia) that had been conditioned with chloroform-methanol-0.8M sodium acetate (30:60:8) and then equilibrated with chloroform-methanol-water (30:60:8).

Normal phase chromatography

Normal phase chromatography was performed on Silica gel 60 (BDH, 120 mesh) columns self-packed in chloroform. Samples were loaded in chloroform, and were eluted with chloroform (~7 bed volumes), chloroform-acetone (9:1, ~7 bed volumes) and finally methanol (~7 bed volumes).

Preparative TLC

Preparative TLC was performed on 20 x 20 cm silica gel plates (Merck, 2 mm thickness) developed with chloroform-methanol-acetic acid-formic acid-water (35:15:6:2:1)1 diluted with chloroform in a ratio of either 2:1 or 3:1. Bands were located by dipping about 1 cm of each vertical edge of developed plates in a solution of iodine in chloroform. The intervening regions were then scraped from the plates and separated components were eluted from the silica gel with methanol.

MALDI-TOF/TOF mass spectrometry

A Bruker Daltonics Ultraflex III MALDI-TOF/TOF was used for mass determination and MS/MS fragmentation of compounds. Alpha-cyano-hydroxycinnamic acid was used as

MALDI matrix. Samples were mixed with matrix and spotted on a Bruker Anchorchip target plate. Calibration was performed using the Bruker peptide calibration mix.

NMR

A Varian 500 MHz NMR was used for analysis of fraction 3 of the methanol-eluted fraction from normal phase chromatography.

Results and Discussion

Centrifugal fractionation

The starting material was centrifuged. The centrifuged particulate material and the filtered supernatant were tested in the bioassay. All bioactivity was found to be contained in the particulate material. The material had a fat-like consistency and was poorly soluble in water. Microscopy analysis of the material revealed no clear defining or identifiable structure.

Thin layer chromatography (TLC) was performed to test for the presence of lipids. Figure 1 shows the developed TLC plate. The sample clearly contained an abundance of components that migrated similar to the lipid standards. A strong fatty acid component was particularly evident.

Acidic/non-acidic fractionation

The lipids were separated into acidic and non-acidic fractions using SAX SPE (strong anion exchange solid phase extraction). Both fractions were run on TLC (Figure 2) and tested in the bioassay. Results showed that although only incomplete separation of acidics from non-acidics had been achieved, stronger activity was evident in the non-acidic fraction (Figure 3).

Normal phase chromatography

The non-acidic sample was fractionated according to polarity using normal phase column chromatography. Six fractions were collected, run on TLC (Figure 4) and tested in the bioassay (Figure 5). Strongest activity was found in fraction 5, although fraction 1 also demonstrated activity two days after inoculation. This strongly suggested bioactivity from multiple compounds.

Fraction 1 was eluted with chloroform and contained mainly low polarity components, while fraction 5 was eluted with methanol and contained a high proportion of highly polar material.

The samples prepared as above comprised an appreciable amount of acidic lipids (Figure 4). Therefore, a new acidic/non-acidic fractionation technique using an alternative anion exchange chromatography media (DEAE-Sephadex) was performed, which effectively removed essentially all acidic lipids (as shown by lane 2 in Figure 6).

A new normal phase chromatography fractionation was performed, with collection of smaller sub-fractions, focusing on the fractions eluting with methanol. Eleven fractions were collected and analysed using TLC (Figure 6). Some fractions (4-5 and 7-11) were combined for testing in the bioassay (Figure 7). Fractions 2 and 4-5 showed the highest bioactivity.

Preparative TLC

To further purify the active compound(s), preparative TLC was performed, which allowed collection of a relatively large amount of each separated band. Nine bands were collected from the preparative TLC plate and analysed by analytical TLC (Figure 8), showing successful fractionation. Unfortunately fraction 3 was lost during rotary evaporation.

Eight fractions were analysed in the bioassay (Figure 9). Fractions 4 and 5 showed the highest activity, followed by fractions 2 and 9. Notably, fraction 3 showed a strong band in TLC that was also present as a minor component of fractions 4 and 5, and despite no direct bioassay data was included in subsequent analyses.

A new preparative TLC was performed, with conditions optimised to maximise separation in the polar region (previous fractions 2-5).

The new separation provided 6 fractions: 2, 3, 4b, 5a, 5b and 6 (with the numbering of these fractions being consistent with the previous separation). The samples were examined using analytical TLC (Figure 10), which revealed that better separation of the components than had been achieved in the previous preparative TLC. Strongest activity in the bioassay was observed in fractions 3, 4b, 5a and 5b (Figure 11). The bioassay results for these fractions were highly consistent with the previous results (Figure 9)

Mass spectrometry

Fractions 2, 3, 4b, 5a and 5b were analysed using MALDI-TOF mass spectrometry (MS) (Figure 12). A number of interesting peaks were observed and selected for MS/MS (fragmentation) analysis (Table 2).

Table 2. Peaks selected for MS/MS.

1	Fraction	2	m/z
3	2	4	444.82
		5	655.94
6	3	7	454.19
8	4b	9	750.66
10	5a	11	393.39
		12	750.67

13	5b	14	692.69
		15	694.72
		16	716.71
		17	531.34

Fraction 3 was selected as the first of these fractions for subsequent investigation, as it showed as the purest compound both on TLC and in MS. Furthermore, this fraction contained the lowest absolute amount of material (by weight) as submitted for bioassay analysis (approximately 50% of the amount in the other fractions), yet showed bioactivity at the same level as fractions 4b, 5a and 5b.

NMR analysis of Fraction 3 provided evidence for a low molecular mass compound. Indeed, in the low mass range, MS analysis (Figure 13) showed candidate masses at m/z 86.02 and 146.97. Hence it was concluded that Fraction 3 contains more than one compound to which bioactivity could be attributed.

Improved preparation protocol

Insight into the character of the active compound allowed us to provide an initial suggestion for improvement of the compound preparation protocol. This protocol was applied at the laboratory scale (50 ml) and tested in the bioassay. Activity of this optimised preparation was confirmed to be very high.

Additional fractionation

The fatty residue in the methanol extracted samples was tested by bioassay to establish whether this solid fraction contained active compounds. Against aphids, it was found that this solid fraction from the K4B3-MeOH sample was more active than the liquid portion (Figure 14). This bioassay also demonstrated little active after filtering hyphae from another culture of *B. bassiana* K4B3.

Chloroform extraction from K4B3 mycelia culture

Chloroform extraction is used to separate lipids from other fractions. 50 ml of bioreactor produced K4B3 mycelia culture was centrifuged to collect the mycelium, which was homogenised and filtered using 40 ml of 1:2 methanol:chloroform. Nine ml of 0.02% $CaCl_2$ was added and mixed. When the phases had separated, the top phase was removed and the chloroform left to evaporate overnight at room temperature. One ml of methanol was used to resuspend the dried residue. Three hundred µl of this solution was assayed against aphids (Figure 15) and shown to be highly active.

HP20 sample

Methanol extraction of the culture filtrate of K4B3 mycelia culture (HP20 sample) was found to lack activity against aphids (Figure 16), but was active against larvae of the diamondback moth (Figure 17). The HP20 sample did affect leaves over the 4-5 days of bioassay, suggesting some phytotoxic component(s) may be present.

Conclusions

These experiments suggest that there are multiple active insecticidal lipids produced by filamentous fungi, such as *Beauveria bassiana* K4B3. Lipid fractions having improved bioactivity, including improved insecticidal activity, have been produced using the preparative methods provided by the present invention.

EXAMPLE 3 – BIOASSAYS OF INSECT CONTROL

This example describes the development of an expanded range of bioassays to determine the insecticidal efficacy of various lipids or lipid fractions.

Several target insect assays are developed and assessed using the criteria 1) availability, 2) susceptibility and 3) ease of use. Four insect systems are tested: whiteflies, diamond back moth, mealworm, and mosquito.

1. *Whiteflies nymphs (Hemiptera)*

Whitefly is a major target species, currently lacking suitable control agents. Whitefly nymphs were obtained from Bioforce Ltd (Auckland, New Zealand). Samples of K4B3 lipid fractions and control broth are used to inoculate groups of approximately 100 nymphs through a Potter Tower.

2. *Mealworm (Tenebrio molitor) larvae (Coleoptera)*

Tenebrio larvae are obtained from biosuppliers. Ten larvae are sprayed with K4B3 lipid sample or water control and monitored for 2 weeks.

3. *Diamondback moth-DBM (Plutella xylostella) larvae (Lepidoptera)*

Diamondback moth is a major pest of brassica crops around the world and an insect which has become resistant to most control chemicals. A culture is obtained from Lincoln University for testing with the K4B3 lipid samples.

The standard method uses a mini-version of the Potter Tower. A hand-held A320 airbrush is modified to take micro volumes to atomise lipid samples or control solutions. Larvae are maintained on small cabbage leaves held on the surface of a water agar plate (water + 1% agar) using 0.05% Tween 80. Between 5-20 larvae of all sizes (1st to prepupal) are used in each experiment. In one test, larvae are also dipped in drops of solution. Droplet feeding is attempted to determine if toxicity is topical or ingestion.

Thirty-eight 2^{nd}-6^{th} instar larvae are inoculated in the K4B3 lipid fractions and 19 in the control. Larvae are maintained at 20°C in 16hL:8hD after inoculation.

4. Mosquito larvae

Bioassays are conducted in which varying amounts of K4B3 lipid fractions are added to bottles of approximately 12.5ml of water containing larvae of *Culex perviligans*. Larvae are nearing pupation and some may pupate during the experiment.

EXAMPLE 4 –TOXICITY OF *Beauveria bassiana* K4B3 LIPID IN A MAMMALIAN MODEL

Introduction

This example describes an assessment of the toxicity of the K4B3 lipid in a mammalian model.

Methods

K4B3 lipid fractions are isolated as described above in Example 2.

Testing is conducted in mice according to OECD Guideline 425 (Acute Oral Toxicity - Up-and-down Procedure). Since this material is not expected to be highly toxic, the Limit Test with a single dose level of 2,000 mg/kg by oral intubation is chosen. This dose is the highest recommended by the OECD for evaluation of acute toxicity, except under exceptional circumstances.

A single 2,000 mg/kg dose of K4B3 lipid is administered by oral intubation to five female Swiss mice, as follows.

Test Conditions

Food is withdrawn from one of the mice at approximately 4 pm and its body weight is measured. Next morning, the mouse is weighed again and the weight of K4B3 lipid required to provide a dose of 2,000 mg/kg is calculated. This amount is weighed, and diluted with 150 µl of water. The whole volume is administered to the mouse by gavage.

After dosing, the mouse is allowed immediate access to food. It is observed intensively for 60 minutes after dosing and then at several intervals throughout the day of dosing and subsequent days, as specified in the OECD Guideline for the Testing of Chemicals, Revised Draft Guideline 425, October 2000. A second mouse is dosed with K4B4 polypetide 48 hours after the first, again at a dose of 2,000 mg/kg body weight. The third, fourth, and fifth mice are subsequently dosed at 48 hour intervals, all at 2,000 mg/kg.

The mice are housed individually with water and food *ad lib* (except for the overnight fast before dosing). Mice are observed daily and body weight measured for 2 weeks following

administration. Body weights are recorded 1 day, 1 week, and 2 weeks after dosing, after which the animals are killed by carbon dioxide inhalation and subjected to post-mortem examination.

Results

Results showing no toxic effects after administration of the K4B3 complex, with mice remaining in good health throughout the observation period, feeding shortly after dosing, and behaving normally during the day of dosing and throughout the experiment, are indicative of no toxicity.

Body weights. Results showing unchanged mean body weights of the mice at various time intervals throughout the experiment, such as those shown in Table 3 below, are indicative of no toxicity.

Table 3. Body Weights of Mice Receiving K4B3 Lipid

	Weight before food withdrawal (g)	Weight at dosing (g)	Weight 1 day after dosing (g)	Weight 7 days after dosing (g)	Weight 14 days after dosing (g)
Mean	25.0	22.6	24.4	25.3	25.4
Mouse 1	25.4	23.1	25.6	26.5	25.6
2	25.5	23.2	24.4	25.8	27.7
3	27.1	24.1	25.3	25.6	26.1
4	23.0	20.8	22.7	23.8	23.1
5	24.0	21.6	24.0	24.6	24.7

After an overnight fast, mice typically lose an average of 2.4 grams in body weight. In circumstances where this loss is largely regained by the next day after access to food is restored following dosing, and in circumstances where the mice maintain their weight throughout the two-week observation period after dosing, no toxicity is indicated.

Post-mortem findings. Results showing no abnormalities are observed in the mice at necropsy, and results such as those shown in Table 4 below indicating that the weights of the livers, kidneys, spleens, hearts, lungs and intestine (pylorus to anus) of the mice are within their normal range, are indicative of no toxicity.

Table 4. Relative Organ Weights of Mice Receiving K4B3 Lipid

	Relative organ weight (g/100g body weight)					
	Liver	**Kidneys**	**Spleen**	**Heart**	**Lungs**	**Intestine**
Mean	5.17	1.39	0.50	0.548	0.874	10.49
Mouse 1	4.96	1.35	0.50	0.512	0.836	10.17
2	5.83	1.43	0.690	0.534	0.903	10.77
3	5.55	1.33	0.475	0.498	0.858	9.79
4	4.73	1.39	0.394	0.589	0.931	10.93
5	4.79	1.43	0.441	0.607	0.842	10.79

Discussion

Results showing oral administration of K4B3 polypetide to mice at a dose of 2,000 mg/kg causes no discernable adverse effects, wherein no deaths occur, no abnormalities are noted at necropsy, organ weights are within the normal range, and the behavior of the mice is entirely normal are indicative of low acute toxicity.

Such results are indicative that K4B3 lipid exhibits low acute oral toxicity, with an LD_{50} greater than 2,000 mg/kg body weight. Such a result indicates that the K4B3 lipid would be classified in the lowest hazard category under the New Zealand Hazardous Substances and New Organisms (HSNO) Act 1996.

EXAMPLE 5 – ASSESSMENT OF TOTAL LIPIDS FROM FUNGI FOR TOXICITY TO APHIDS AND DIAMONDBACK MOTH

Introduction

This example describes the assessment of the toxicity to aphids and diamondback moth of lipids from various filamentous fungi.

Methods

Four fungi were used to prepare lipid extracts:

1. *Beauveria bassiana* strain AM2.
2. *Beauveria bassiana* strain F480.
3. *Trichoderma* sp. isolate 1328.
4. *Metarhizium* sp.

All fungi were grown for ~3 days at room temperature in 100 ml of PDB, shaking at 140-160 rpm. 50 ml of fungal culture was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pelleted mycelium added to 20 ml of 1:2 methanol:chloroform. The mycelium was hand homogenised and then filtered through Whatman's no. 1 filter paper. A further 20 mls of 1:2 methanol:chloroform was used to wash the filter. Nine ml of 0.02% $CaCl_2$ was added and the mixture left to separate for 2 hrs or more. The top layer was discarded and the chloroform/lipid solution was left to evaporate overnight. Once dry, 1 ml of 100% methanol was used to resuspend the lipids.

Lipid extract (50 ml) from Beauveria bassiana strain K4B3 prepared as described above was also used.

Myzus persicae (green peach aphid) or Diamondback moth larvae, reared on cabbage plants in a constant temperature room, were used. Aphids of mixed age were used, usually between 30-50/Petri dish. DBM larvae of 3-5th instar were used.

To inoculate aphids or larvae with extracts, insects were transferred to a piece of cabbage leaf on the surface of a 1% water agar plate using 0.05% Tween 80 as a wetting agent between leaf and agar.

A hand-held Paasche airbrush was modified to take micro volumes and used to atomise 300 µl of test or control solutions. Subsequently, plates with treated insects were maintained at 20°C, 12h light:12h dark and checked daily. Dead were removed. Counts of aphids inoculated were made directly after spraying to avoid including newborn aphids in % mortality, but no effort was made to remove neonate nymphs during incubation.

Results

Generally, 50 ml of the *Beauveria*, and *Metarhizium* cultures yielded approximately 3 ml equivalent of wet hyphae. 50 ml of the *Trichoderma* cultures yielded 15 ml wet hyphae equivalent.

The bioassay of total lipids extracted from *Beauveria bassiana* strains AM2, F480, and K4B3 shows these lipids have extremely potent insecticidal activity. As can be seen in Figure 18, the application total lipids from each strain resulted in almost 100% mortality after only 21 hours. Similarly, lipid fractions 4 and 5 from *Beauveria bassiana* strain K4B3 elicited 100% mortality among the target aphids.

These results were also observed with total lipids from other filamentous fungi. Lipids from *Trichoderma* and *Metarhizium*, like those from *Beauveria bassiana,* show potent insecticidal activity. As shown in Figure 19, close to 100% mortality among aphids exposed to

lipids from *Trichoderma* and *Beauveria bassiana* strain K4B3 was observed after only 21 hours, with almost 50% mortality observed among those aphids exposed to lipids from *Metarhizum* at the same time point. Baseline mortality was established by the methanol negative control.

This bioassay was continued for a total of 3 days, and the % cumulative mortality among green peach aphid is shown in Figure 20. This data suggests the insecticidal activity exhibited by these lipids acts rapidly, with some additional killing over a longer timeframe.

The bioassay of various lipid fractions from *Beauveria bassiana* strain K4B3 (*Beauveria*), *Trichoderma* and *Metarhizium* against Diamondback moth larvae suggests that certain of these lipids, though less effective than against aphids, still exhibit some insecticidal activity against moth larvae. As shown in Figure 21, over 30% cumulative mortality was observed at 4 to 5 days with lipids from *Beauveria bassiana* strain K4B3 and *Metarhizium.*

Figure 22 shows % mortality of green peach aphid at 21 hours (at 20°C) in the bioassay of various lipid fractions from *Beauveria bassiana* strain K4B3, and *Trichoderma* as described herein in Example 5. Fractions tested include methanol-extracted (-MeOH) and chloroform extracted (-Chloro) fractions, with methanol and water as negative controls.

A bioassay of various lipid fractions from *Trichoderma* and from *Beauveria bassiana* against aphids shows that chloroform- and methanol-extracted lipid fractions had potent insecticidal activity. As shown in Figure 22, close to 100% mortality among aphids exposed to chloroform- and methanol-extracted lipids from *Trichoderma* and *Beauveria bassiana* strain K4B3 was observed after only 21 hours exposure. FS-MeOH and Beaublast-MeOH are different extractions from the K4B3 product.

Discussion

This example indicates that insecticidal lipids have been prepared from a range of filamentous fungi, and the production of such lipids is not limited to the *Beauveria bassiana* K4B3 strain. Extracted lipids have potent insecticidal activity, frequently killing close to 100% of target insects within 24 hours exposure.

INDUSTRIAL APPLICATION

As will be evident from the above description, the present invention provides insecticidal lipids or lipid fractions from filamentous fungi together with compositions comprising said lipids or lipid fractions useful for the control of insects, such as phytopathogenic insects. The use of such lipids and lipid fractions in methods to control insects, such as phytopathogenic insects, are also provided. The lipids or lipid fractions and compositions of the invention have utility in a wide range of agricultural and horticultural applications.

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CLAIMS:

1. One or more isolated, purified, or substantially pure insecticidal lipids or lipid fractions from a fungus of the phylum Ascomycota.
2. The insecticidal lipid or lipid fraction of claim 1 wherein the lipid or lipid fraction is or comprises or consists of one or more non-acidic lipids or non-acidic lipid fractions.
3. The lipid fraction of claim 1 or claim 2 wherein the lipid fraction is substantially free of acidic lipids.
4. The lipid fraction of any one of claims 1 to 3 wherein the lipid fraction is substantially free of free fatty acids.
5. The lipid fraction of any one of claims 1 to 4 wherein the lipid fraction is or has the identifying characteristics of any one or more of the following:
    a. fraction 1, fraction 5, or fraction 6 as shown in Figure 4 or as described in Example 2 herein;
    b. fraction 2, fraction 4, or fraction 5 as shown in Figure 7 or as described in Example 2 herein;
    c. fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein;
    d. fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein;
    e. fraction 2, fraction 3, fraction 4b, fraction 5a, fraction 5b, or fraction 6 as shown in Figure 10 or as described in Example 2 herein.
6. The lipid fraction of any one of claims 1 to 5 wherein the identifying characteristic of fraction 2, fraction 3, fraction 4b, fraction 5a, fraction 5b, or fraction 6 is a MALDI-TOF mass spectrometry (MS) profile as shown in any one of Figures 12a to 12f.
7. The lipid fraction according to any one of claims 1 to 6 wherein the lipid fraction is or has the identifying characteristics of fraction 3 as shown in Figure 10 or as described in Example 2 herein.
8. The lipid fraction of claim 7 wherein the identifying characteristic is an NMR spectrum as shown in Figure 13.
9. The lipid according to any one of claims 1 to 8 wherein the lipid is one of the following:

a. a lipid having an approximate mass at m/z 86 by MS/MS, or
b. a lipid having an approximate mass at m/z 147 by MS/MS, or
c. a lipid having an approximate mass at m/z 445 by MS/MS, or
d. a lipid having an approximate mass at m/z 656 by MS/MS, or
e. a lipid having an approximate mass at m/z 454 by MS/MS, or
f. a lipid having an approximate mass at m/z 751 by MS/MS, or
g. a lipid having an approximate mass at m/z 393 by MS/MS, or
h. a lipid having an approximate mass at m/z 751 by MS/MS, or
i. a lipid having an approximate mass at m/z 693 by MS/MS, or
j. a lipid having an approximate mass at m/z 695 by MS/MS, or
k. a lipid having an approximate mass at m/z 717 by MS/MS, or
l. a lipid having an approximate mass at m/z 531 by MS/MS, or
m. a lipid having a mass at m/z 86.02 by MS/MS, or
n. a lipid having a mass at m/z 146.97 by MS/MS, or
o. a lipid having a mass at m/z 444.82 by MS/MS, or
p. a lipid having a mass at m/z 655.94 by MS/MS, or
q. a lipid having a mass at m/z 454.19 by MS/MS, or
r. a lipid having a mass at m/z 750.66 by MS/MS, or
s. a lipid having a mass at m/z 393.39 by MS/MS, or
t. a lipid having a mass at m/z 750.67 by MS/MS, or
u. a lipid having a mass at m/z 692.69 by MS/MS, or
v. a lipid having a mass at m/z 694.72 by MS/MS, or
w. a lipid having a mass at m/z 716.71 by MS/MS, or
x. a lipid having a mass at m/z 531.34 by MS/MS.

10. The lipid fraction according to any one of claims 1 to 8 wherein the lipid fraction comprises or consists of one or more of the following:

a. a lipid having an approximate mass at m/z 86 by MS/MS, or
b. a lipid having an approximate mass at m/z 147 by MS/MS, or
c. a lipid having an approximate mass at m/z 445 by MS/MS, or
d. a lipid having an approximate mass at m/z 656 by MS/MS, or
e. a lipid having an approximate mass at m/z 454 by MS/MS, or
f. a lipid having an approximate mass at m/z 751 by MS/MS, or
g. a lipid having an approximate mass at m/z 393 by MS/MS, or

h. a lipid having an approximate mass at m/z 751 by MS/MS, or
i. a lipid having an approximate mass at m/z 693 by MS/MS, or
j. a lipid having an approximate mass at m/z 695 by MS/MS, or
k. a lipid having an approximate mass at m/z 717 by MS/MS, or
l. a lipid having an approximate mass at m/z 531 by MS/MS, or
m. a lipid having a mass at m/z 86.02 by MS/MS, or
n. a lipid having a mass at m/z 146.97 by MS/MS, or
o. a lipid having a mass at m/z 444.82 by MS/MS, or
p. a lipid having a mass at m/z 655.94 by MS/MS, or
q. a lipid having a mass at m/z 454.19 by MS/MS, or
r. a lipid having a mass at m/z 750.66 by MS/MS, or
s. a lipid having a mass at m/z 393.39 by MS/MS, or
t. a lipid having a mass at m/z 750.67 by MS/MS, or
u. a lipid having a mass at m/z 692.69 by MS/MS, or
v. a lipid having a mass at m/z 694.72 by MS/MS, or
w. a lipid having a mass at m/z 716.71 by MS/MS, or
x. a lipid having a mass at m/z 531.34 by MS/MS.

11. The lipid or lipid fraction according to any one of claims 1 to 10 wherein the fungus of the phylum Ascomycota is selected from the group comprising *Beauvaria bassiana* strain K4B3 on deposit at National Measurement Institute of Australia (NMIA) under Accession No. V08/025855 deposited 23 September 2008, or a strain having the identifying characteristics thereof; *Beauvaria bassiana* strain AM2 or a strain having the identifying characteristics thereof; *Beauvaria bassiana* strain F480 or a strain having the identifying characteristics thereof; one or more *Trichoderma* sp, including *Trichoderma* isolate 1328 or a strain having the identifying characteristics thereof; and one or more *Metarhizium* sp.

12. A composition which comprises one or more insecticidal lipids or one or more insecticidal lipid fractions from a fungus of the phylum Ascomycota together with at least one carrier.

13. A composition to which has been added one or more insecticidal lipids or one or more insecticidal lipid fractions from a fungus of the phylum Ascomycota together with at least one carrier.

14. The composition of claim 12 or 13 wherein the composition is or comprises a culture, cell extract, cell suspension, cell homogenate, cell lysate, cell supernatant, cell filtrate, cell pellet, or growth media from a culture of a fungus of the phylum Ascomycota.

15. The composition of any one of claims 12 to 14 additionally comprising spores obtainable from a fungus of the phylum Ascomycota.

16. The composition of claim 15 comprising spores obtainable from a fungi selected from the group comprising *Beauvaria bassiana* strain K4B3 on deposit at National Measurement Institute of Australia (NMIA) under Accession No. V08/025855 deposited 23 September 2008, or a strain having the identifying characteristics thereof; *Beauvaria bassiana* strain AM2 or a strain having the identifying characteristics thereof; *Beauvaria bassiana* strain F480 or a strain having the identifying characteristics thereof; one or more *Trichoderma* sp, including *Trichoderma* isolate 1328 or a strain having the identifying characteristics thereof; and one or more *Metarhizium* sp.

17. The composition of claim 16 comprising spores obtainable from *Beauveria bassiana* strain K4B3.

18. The composition according to any one of claims 12 to 17 which comprises one or more lipids or lipid fractions from a fungi selected from the group comprising *Beauvaria bassiana* strain K4B3 on deposit at National Measurement Institute of Australia (NMIA) under Accession No. V08/025855 deposited 23 September 2008, or a strain having the identifying characteristics thereof; *Beauvaria bassiana* strain AM2 or a strain having the identifying characteristics thereof; *Beauvaria bassiana* strain F480 or a strain having the identifying characteristics thereof; one or more *Trichoderma* sp, including *Trichoderma* isolate 1328 or a strain having the identifying characteristics thereof; and one or more *Metarhizium* sp.

19. The composition according to claim 18 which comprises one or more lipids or lipid fractions from *Beauveria bassiana* strain K4B3.

20. The composition according to any one of claims 12 to 19 which comprises one or more lipid fractions wherein the lipid fraction is or has the identifying characteristics of any one or more of the following:

    a. fraction 1, fraction 5, or fraction 6 as shown in Figure 4 or as described in Example 2 herein;

b. fraction 2, fraction 4, or fraction 5 as shown in Figure 7 or as described in Example 2 herein;

c. fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein;

d. fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein;

e. fraction 2, fraction 3, fraction 4b, fraction 5a, fraction 5b, or fraction 6 as shown in Figure 10 or as described in Example 2 herein.

21. The composition according to any one of claims 12 to 20 wherein the composition is enriched in a non-acid lipid.

22. The composition according to claim 21 wherein the enrichment is of one or more lipids present in one or more lipid fractions being or having the identifying characteristics of any one or more of the following:

a. fraction 1, fraction 5, or fraction 6 as shown in Figure 4 or as described in Example 2 herein;

b. fraction 2, fraction 4, or fraction 5 as shown in Figure 7 or as described in Example 2 herein;

c. fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein;

d. fraction 2, fraction 3, fraction 4, fraction 5, or fraction 9 as shown in Figure 8 or as described in Example 2 herein;

e. fraction 2, fraction 3, fraction 4b, fraction 5a, fraction 5b, or fraction 6 as shown in Figure 10 or as described in Example 2 herein.

23. The composition according to claim 21 wherein the enrichment is of one or more of the following:

a. a lipid having an approximate mass at m/z 86 by MS/MS, or

b. a lipid having an approximate mass at m/z 147 by MS/MS, or

c. a lipid having an approximate mass at m/z 445 by MS/MS, or

d. a lipid having an approximate mass at m/z 656 by MS/MS, or

e. a lipid having an approximate mass at m/z 454 by MS/MS, or

f. a lipid having an approximate mass at m/z 751 by MS/MS, or

g. a lipid having an approximate mass at m/z 393 by MS/MS, or

h. a lipid having an approximate mass at m/z 751 by MS/MS, or

i. a lipid having an approximate mass at m/z 693 by MS/MS, or
j. a lipid having an approximate mass at m/z 695 by MS/MS, or
k. a lipid having an approximate mass at m/z 717 by MS/MS, or
l. a lipid having an approximate mass at m/z 531 by MS/MS, or
m. a lipid having a mass at m/z 86.02 by MS/MS, or
n. a lipid having a mass at m/z 146.97 by MS/MS, or
o. a lipid having a mass at m/z 444.82 by MS/MS, or
p. a lipid having a mass at m/z 655.94 by MS/MS, or
q. a lipid having a mass at m/z 454.19 by MS/MS, or
r. a lipid having a mass at m/z 750.66 by MS/MS, or
s. a lipid having a mass at m/z 393.39 by MS/MS, or
t. a lipid having a mass at m/z 750.67 by MS/MS, or
u. a lipid having a mass at m/z 692.69 by MS/MS, or
v. a lipid having a mass at m/z 694.72 by MS/MS, or
w. a lipid having a mass at m/z 716.71 by MS/MS, or
x. a lipid having a mass at m/z 531.34 by MS/MS.

24. The composition according to any one of claims 21 to 23 wherein the enrichment an enrichment of at least about 1%, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % relative to the amount present in the composition without enrichment.

25. A method for controlling one or more insects, the method comprising contacting the one or more insects with a lipid or lipid fraction of any one of claims 1 to 11 or a composition according to any one of claims 12 to 24.

26. A method for controlling one or more insects, the method comprising applying to a plant or its surroundings a lipid or lipid fraction of any one of claims 1 to 11 or a composition according to any one of claims 12 to 24.

27. A method for producing a biological control composition, the method comprising:

providing a culture of one or more fungi of the phylum Ascomycota,

maintaining the culture under conditions suitable for production of at least one lipid or lipid fraction; and

i.combining the at least one lipid or lipid fraction with a carrier, or

ii.combining the at least one lipid or lipid fraction with one or more entomopathogenic fungi or a culture thereof, or

iii.separating the at least one lipid or lipid fraction from the fungi, or

iv.at least partially purifying or isolating the at least one lipid of the invention, or

v.any combination of two or more of (i) to (iv).

28. The method of claim 27 wherein the lipid of lipid fraction is, comprises, or consists of a lipid or lipid fraction as claimed in any one of claims 2 to 11.

29. A method of preparing an insecticidal lipid or lipid fraction, the method comprising

providing an organic solvent extraction of a culture of one or more fungi of the phylum Ascomycota,

at least partially separating one or more non-acidic lipids from one or more acidic lipids, and

recovering the one or more non-acidic lipids or lipid fractions.

30. A method of preparing an insecticidal lipid or lipid fraction, the method comprising

providing an organic solvent extraction of a culture of one or more fungi of the phylum Ascomycota,

at least partially separating one or more polar lipids from one or more non-polar lipids, and

recovering the one or more polar lipids or polar lipid fractions.

31. The method of claim 29 or 30 wherein the organic solvent is an alkanol including a short chain alkyl alcohol, methanol, ethanol, propanol, iso-propanol, or butanol, or is chloroform.

32. The method of any one of claims 29 to 31 wherein the recovered lipids or lipid fractions is, comprises, or consists of a lipid or lipid fraction as claimed in any one of claims 2 to 11.

33. A method of reversing, wholly or in part, the resistance of an insect to one or more insecticides or one or more entomopathogenic agents, the method comprising contacting the insect with a lipid or lipid fraction of any one of claims 1 to 11 or a composition according to any one of claims 12 to 24.

34. The method of claim 33 comprising contacting the insect with the lipid or lipid fraction together with one or more insecticides or one or more entomopathogenic agents, or any combination thereof.

35. The method of claim 33 wherein the one or more insecticides or one or more entomopathogenic agents administered is the same as that to which the insect is or is predicted to be or become resistant.

36. A method of controlling one or more insects which have been contacted with a lipid or lipid fraction of any one of claims 1 to 11 or a composition according to any one of claims 12 to 24, the method comprising contacting the one or more insects with an amount of an insecticide or entomopathogenic agent effective to control said one or more insects.

37. The method of claim 36 wherein the one or more insecticides or one or more entomopathogenic agents is administered prior to, concurrently with, or after administration of the lipid or lipid fraction.

38. The method of any one of claims 33 to 37 wherein the composition comprises an entomopathogenic fungi, optionally together with another insecticide or entomopathogenic agent.

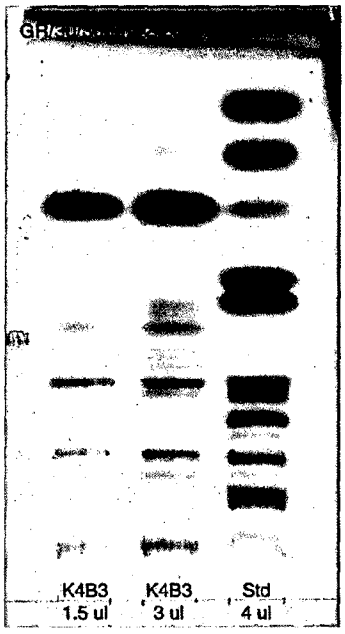


Figure 1

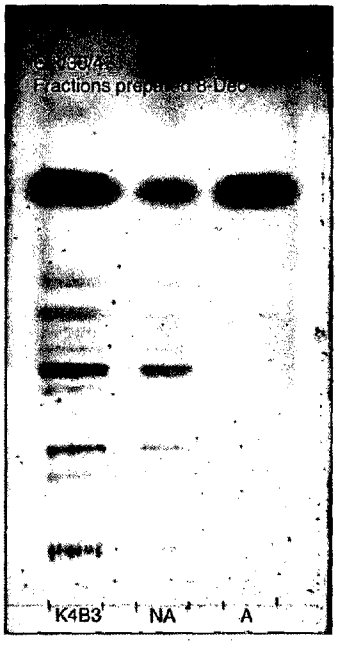
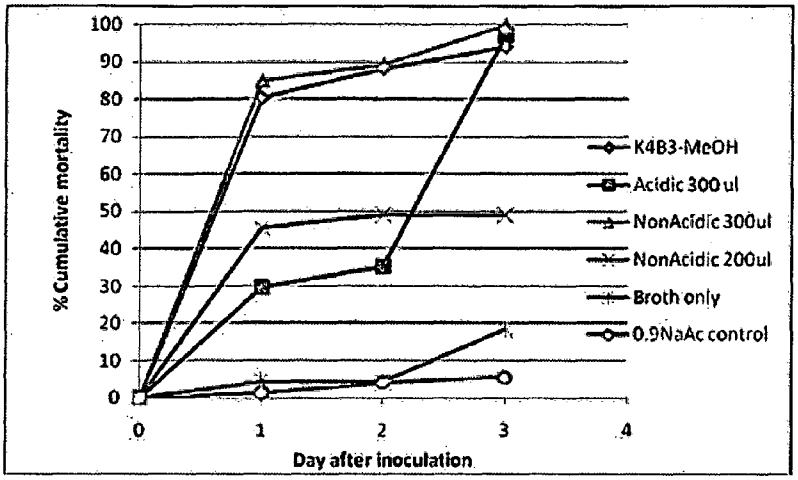


Figure 2



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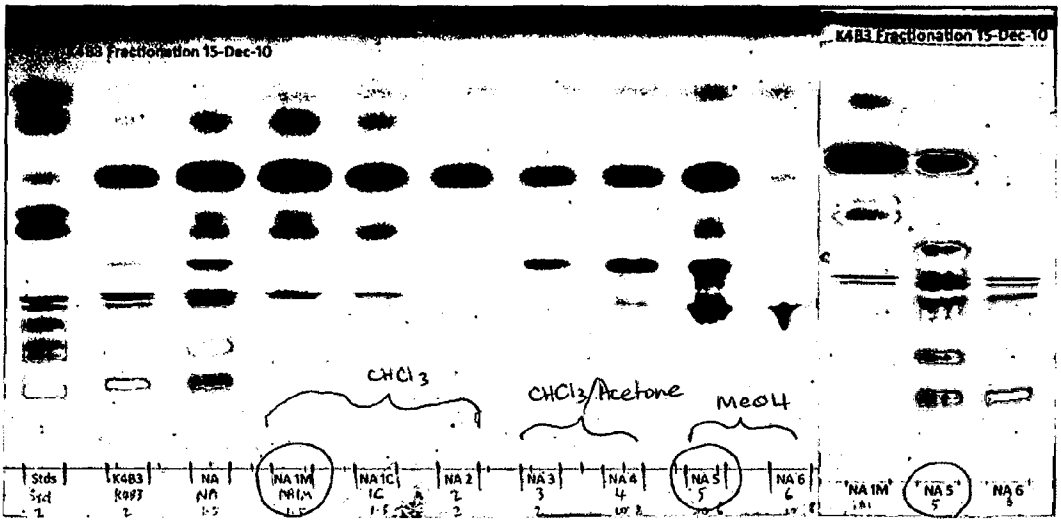
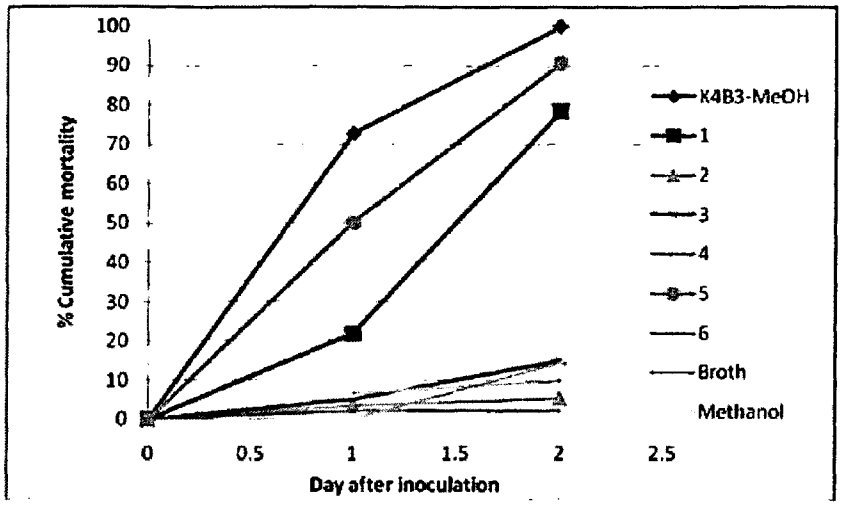


Figure 4



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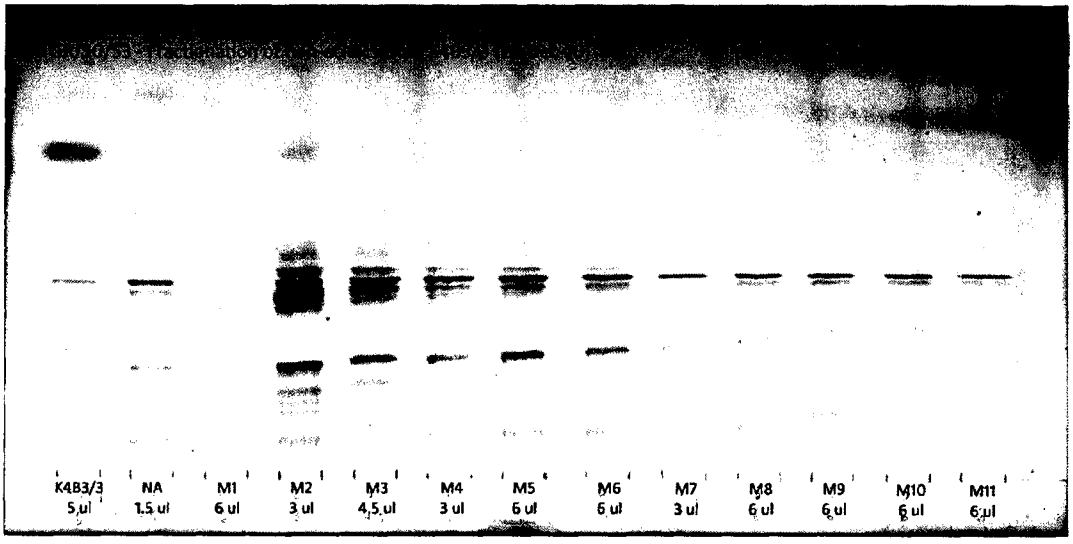

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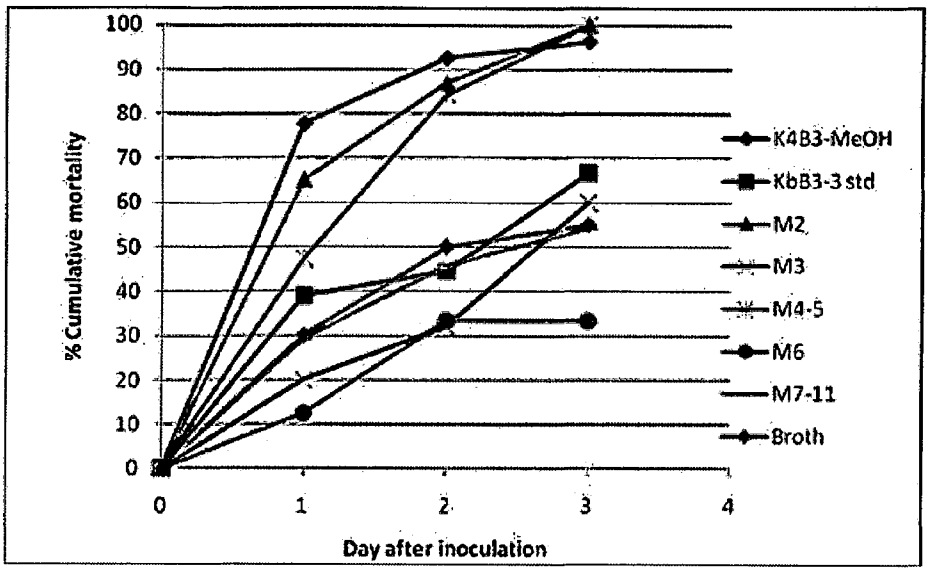

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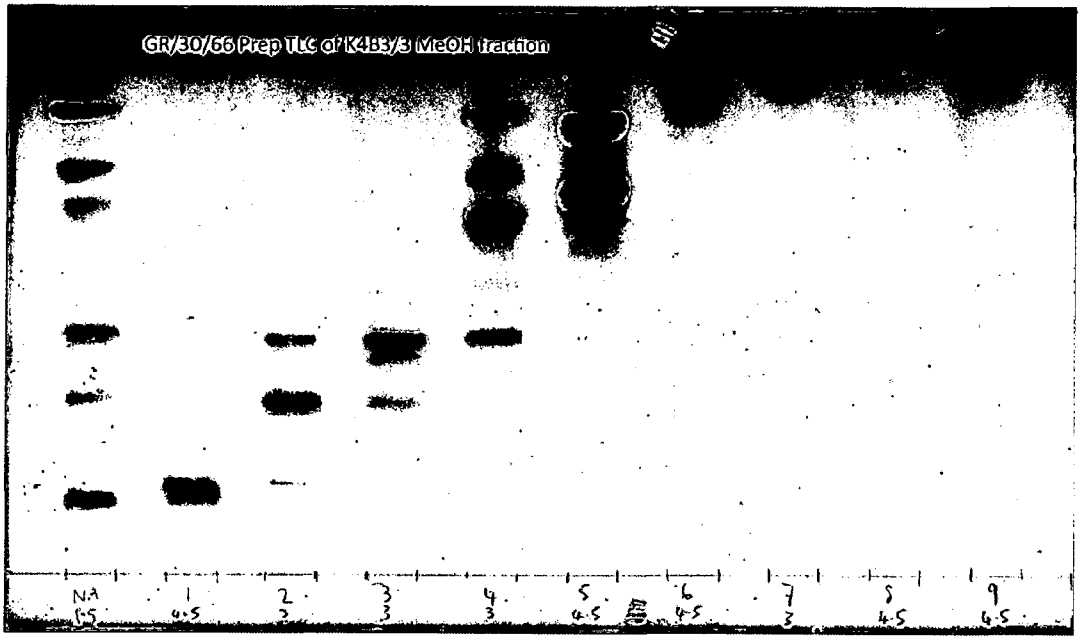

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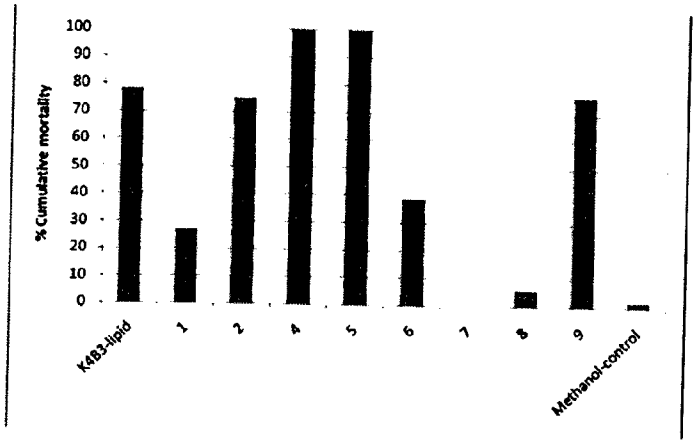

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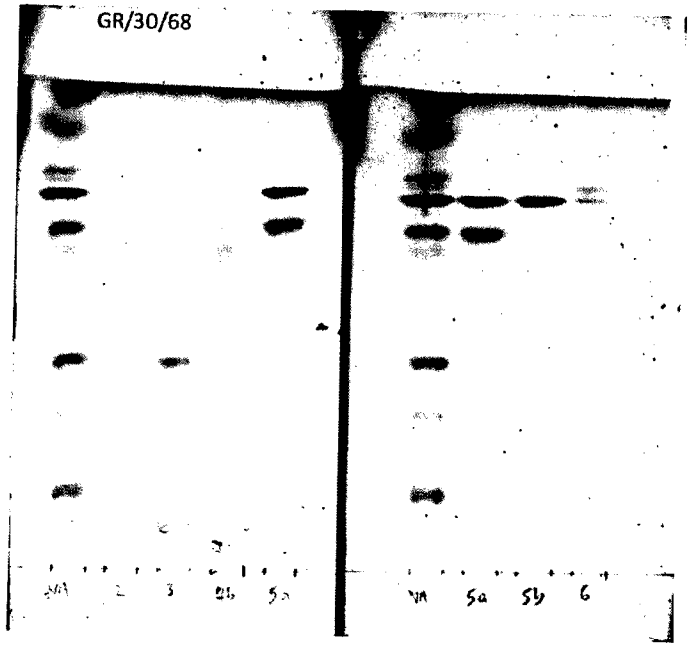
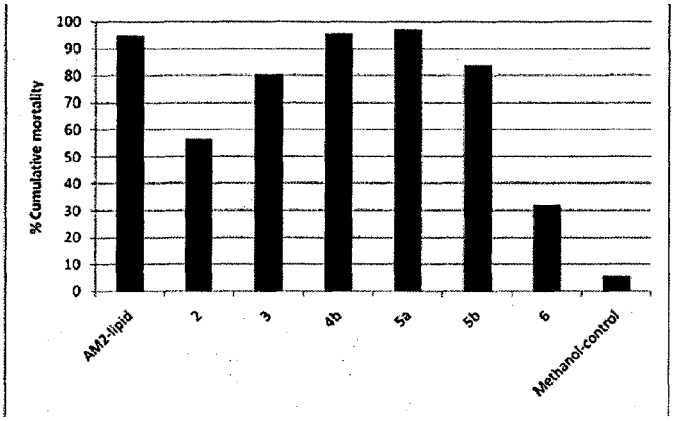


Figure 10



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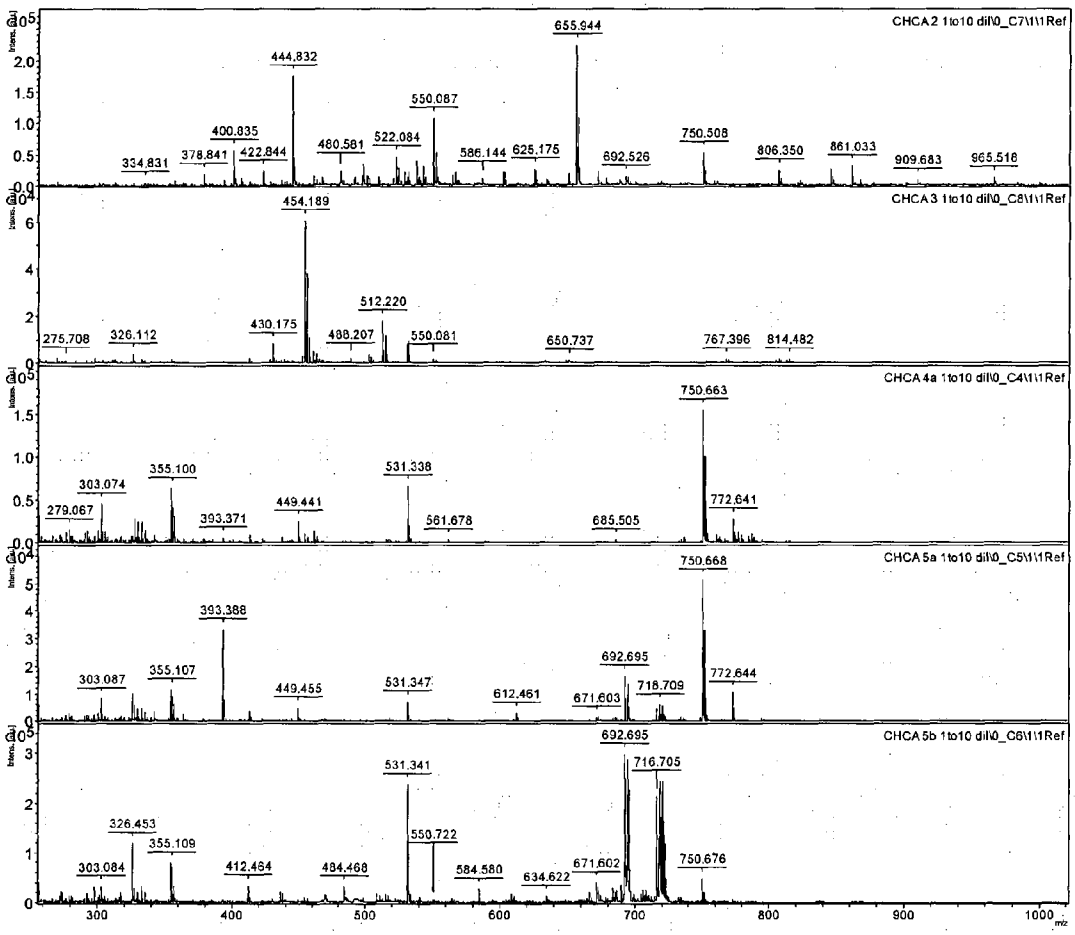

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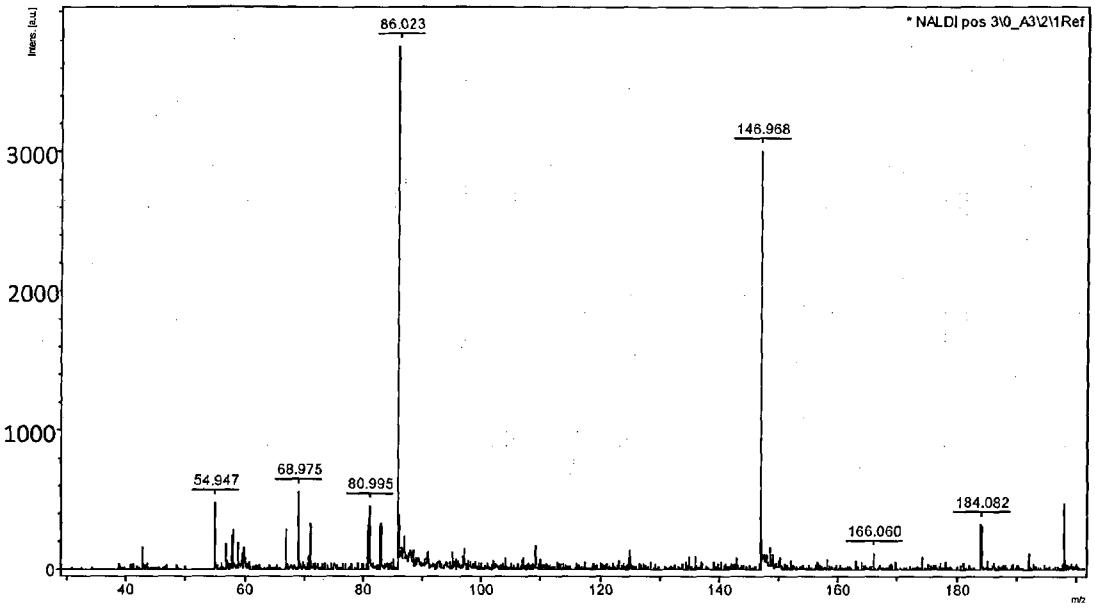

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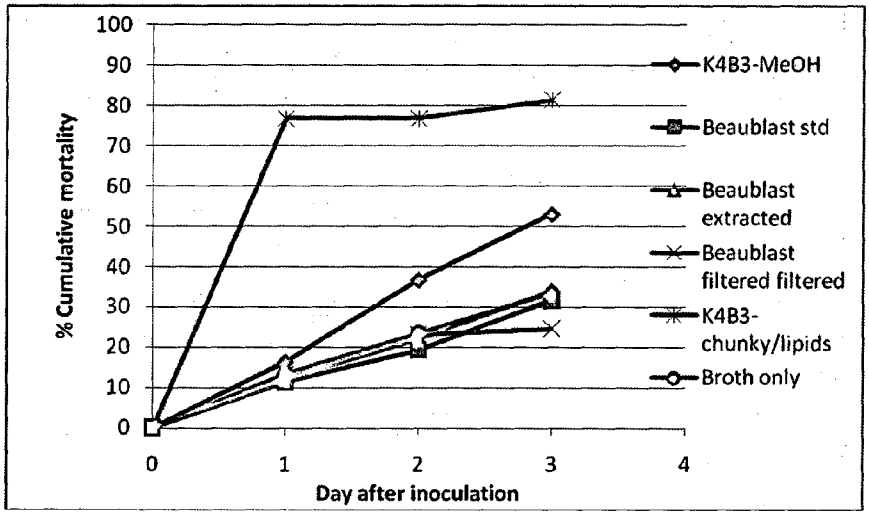

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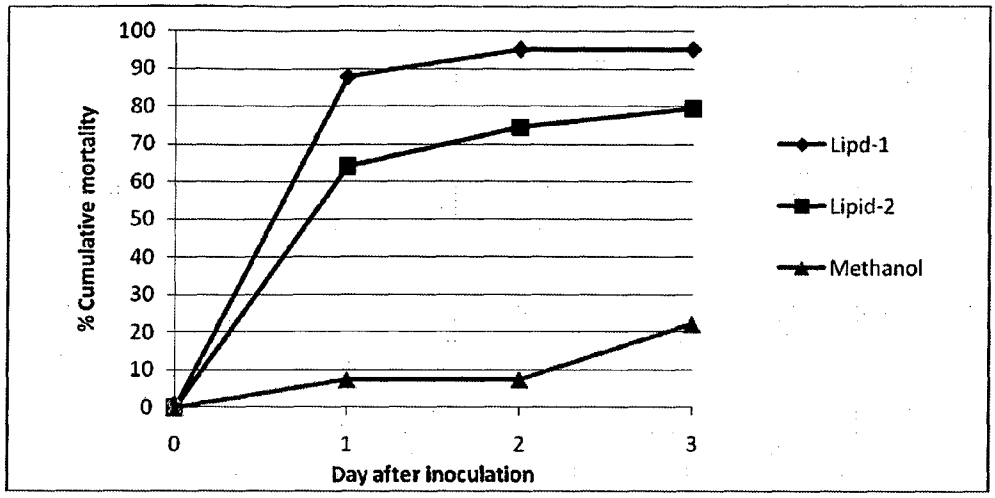

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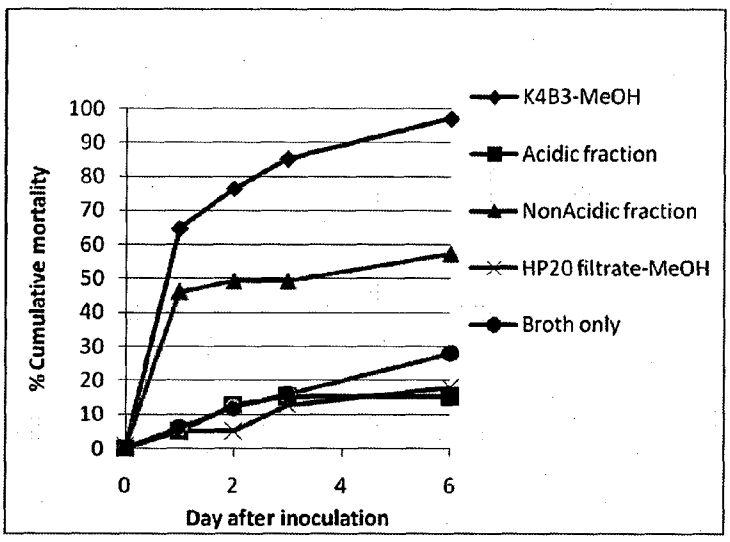

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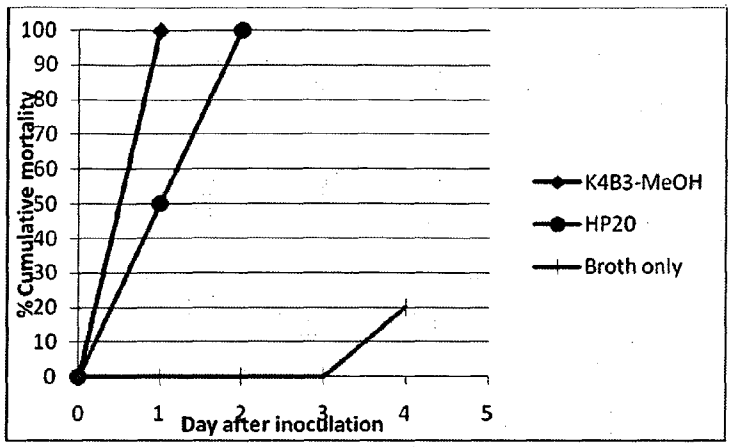

Figure 17

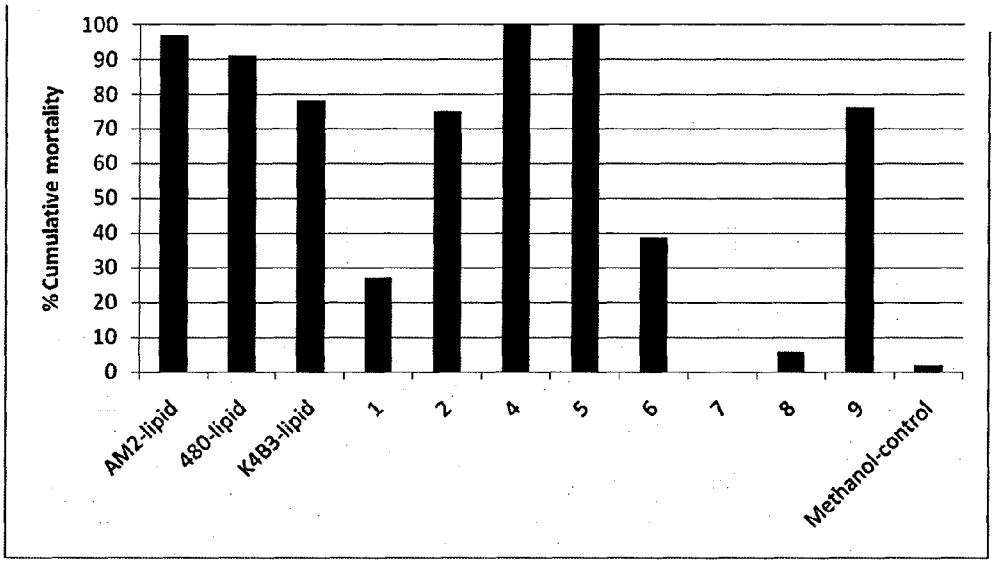

Figure 18

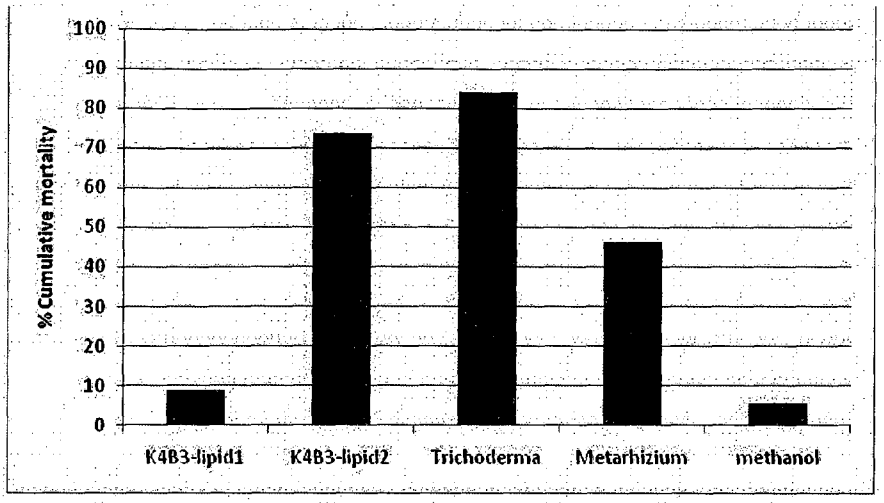

Figure 19

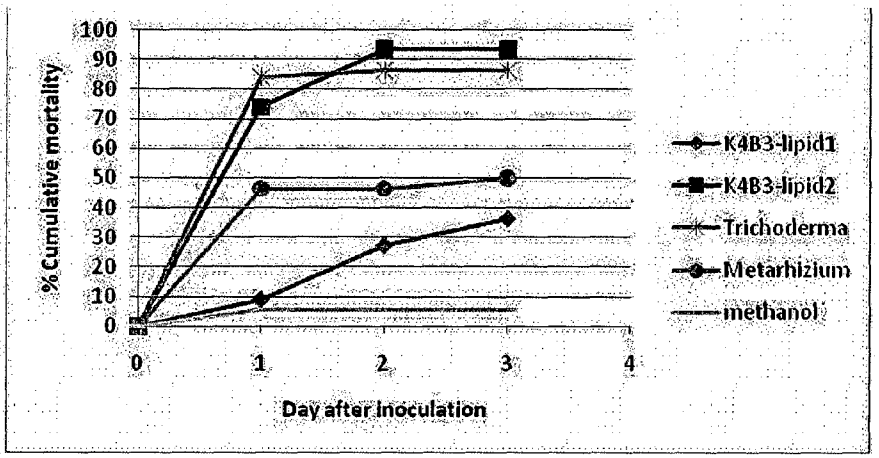

Figure 20

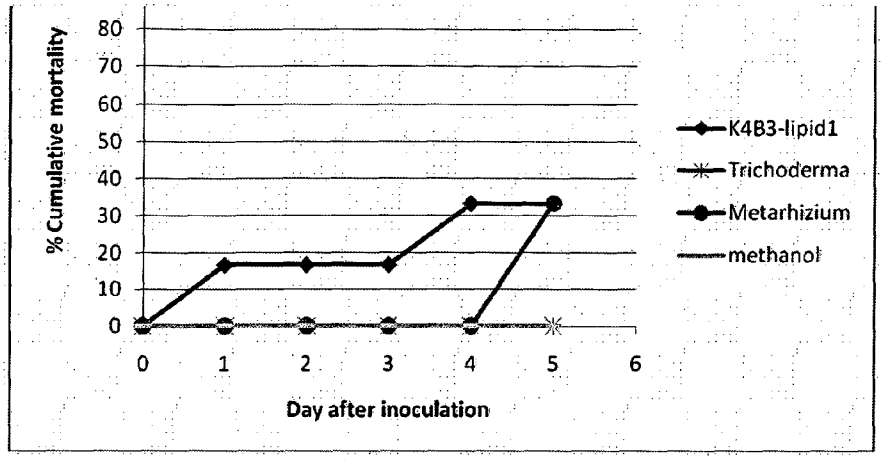

Figure 21

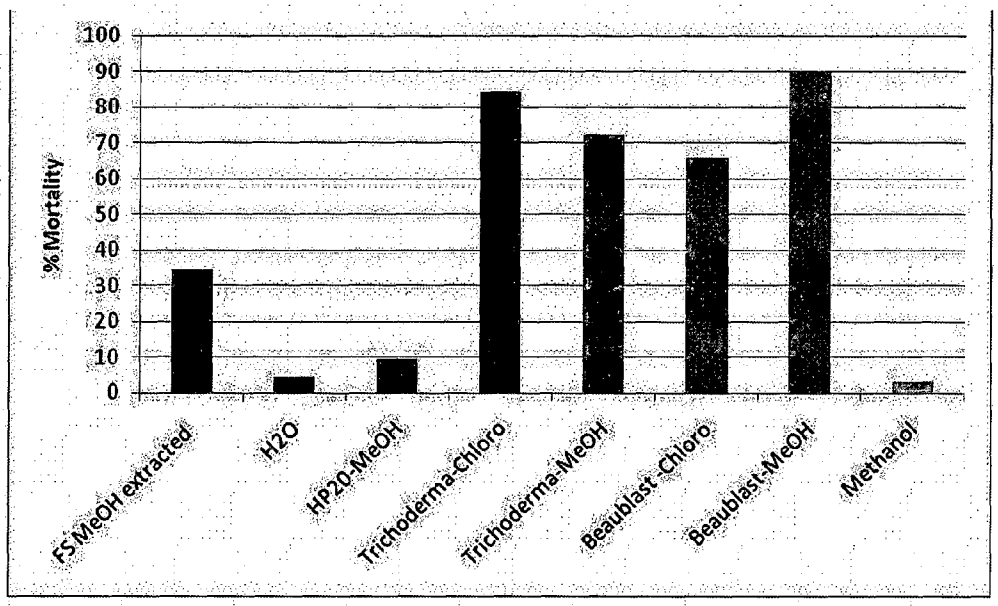

Figure 22